Whole Blood Gene Expression Profiling in Preclinical and Clinical Cattle Infected with Atypical Bovine Spongiform Encephalopathy

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Abstract

Prion diseases, such as bovine spongiform encephalopathies (BSE), are transmissible neurodegenerative disorders affecting humans and a wide variety of mammals. Variant Creutzfeldt-Jakob disease (vCJD), a prion disease in humans, has been linked to exposure to BSE prions. This classical BSE (cBSE) is now rapidly disappearing as a result of appropriate measures to control animal feeding. Besides cBSE, two atypical forms (named H- and L-type BSE) have recently been described in Europe, Japan, and North America. Here we describe the first wide-spectrum microarray analysis in whole blood of atypical BSE-infected cattle. Transcriptome changes in infected animals were analyzed prior to and after the onset of clinical signs. The microarray analysis revealed gene expression changes in blood prior to the appearance of the clinical signs and during the progression of the disease. A set of 32 differentially expressed genes was found to be in common between clinical and preclinical stages and showed a very similar expression pattern in the two phases. A 22-gene signature showed an oscillating pattern of expression, being differentially expressed in the preclinical stage and then going back to control levels in the symptomatic phase. One gene, SEL1L3, was downregulated during the progression of the disease. Most of the studies performed up to date utilized various tissues, which are not suitable for a rapid analysis of infected animals and patients. Our findings suggest the intriguing possibility to take advantage of whole blood RNA transcriptional profiling for the preclinical identification of prion infection. Further, this study highlighted several pathways, such as immune response and metabolism that may play an important role in peripheral prion pathogenesis. Finally, the gene expression changes identified in the present study may be further investigated as a fingerprint for monitoring the progression of disease and for developing targeted therapeutic interventions.
Introduction
Transmissible spongiform encephalopathies (TSEs), or prion diseases, are a group of fatal neurodegenerative disorders, which affect humans and a wide variety of animals. They include Creutzfeldt–Jakob disease (CJD), Gerstmann–Sträussler–Scheinker syndrome (GSS) and fatal familial insomnia (FFI) in humans [1], scrapie in goats and sheep [2], chronic wasting disease (CWD) in cervids [3] and bovine spongiform encephalopathy (BSE) in cattle [4]. The etiological agent of TSEs is an abnormally folded isoform (PrPSc) of the cellular prion protein (PrPC), which accumulates in the nervous and lymphoreticular systems during the progression of the disease [5]. PrPSc accumulation, neuronal loss, spongiosis and astrogliosis are common hallmarks of prion diseases [6]. Despite the fact that the pathological features of these diseases are well characterized, the molecular mechanisms and the signaling pathways underlying TSEs are largely unknown.

The appearance of BSE in the United Kingdom (UK) in 1986 [7] led to an increased interest in these diseases, especially because of its epidemic nature in the UK. Foodborne transmission of BSE prions to humans was observed in the 1990s with the appearance of a new variant form of CJD (vCJD) [8]. It has been shown experimentally that BSE prions have strain characteristics identical to those of prion isolates from human cases of vCJD [9]. So far, 229 cases of vCJD have been reported around the world [10]. In recent years, two atypical forms of BSE have been identified in several European countries [11], Japan [12, 13], the United States [14] and Canada [15]. The two atypical BSE strains are denoted as H-type BSE and L-type BSE (also named bovine amyloidotic spongiform encephalopathy, BASE) [16, 17]. The “H” and “L” identify the higher and lower electrophoretic mobility of the unglycosylated protease resistant PrPSc fragment, respectively [18]. So far, both atypical subtypes have been identified only in cattle that were at least eight years old [19]. In view of that, it has been postulated that, unlike classical BSE (cBSE), cases of atypical BSE may have risen spontaneously, although transmission through feed or the environment cannot be ruled out. Indeed, histopathological as well as immunohistochemical analyses showed that atypical forms of BSE can be experimentally transmitted to mice [20–22] as well as to cattle. Moreover, they differ from cBSE and from each other in terms of clinical features [23–25] and biochemical properties [26–28]. Interestingly, some recent studies showed that H- and L-type BSE prions may acquire cBSE–like properties during propagation in animals expressing homologous bovine prion protein [29] or during inter-species transmission [17, 30], respectively. These findings support the view that the epidemic BSE agent could have originated from atypical cattle prions. While cBSE cases are now rapidly disappearing as a result of appropriate measures to control animal feeding, more insight into atypical BSE would be necessary in order to carry out risk assessment and to adopt appropriate control measures.

Given the infective nature of prions, the identification of specific molecular signatures may be helpful for the development of preclinical diagnostic tests in order to prevent horizontal transmission of the disease and potentially to develop targeted therapies in humans. High-throughput genomic techniques, such as DNA microarrays and RNA-seq, are the most frequently used methodologies for the identification of differentially expressed genes [31]. Gene expression approaches were first applied for studying scrapie [32, 33], while for BSE, and particularly for atypical BSE, they have appeared only recently in the literature [34–36]. Rodent models have been widely employed for large-scale studies of prion diseases [37, 38]; however, it is of the utmost importance to extend these studies to the ruminant species naturally affected by these diseases. In particular, most analyses in cattle have been performed using central nervous system (CNS) tissues from infected animals. Such studies are certainly of relevance but are not particularly suitable for diagnostic purposes. Also, the large majority of these genomic...
studies have been focused on the cBSE infection, while very few data are available about the involvement of peripheral tissues in atypical BSE infected cattle [36]. Peripheral blood is a readily accessible source of biological information on disease status and it is a suitable tissue for prospective rapid diagnostic tests in animals and patients. The objective of the present study was to identify molecular patterns in whole blood of atypical BSE-infected cattle in both clinical and preclinical stages of the disease. Transcriptional changes were analyzed using microarray technology and data were validated by Reverse Transcriptase quantitative PCR (RT-qPCR).

Materials and Methods
All procedures involving animals were approved by the Home Office of the UK government according to the Animal (Scientific Procedures) Act 1986 and in conformity with the institutional guidelines of the Istituto Zooprofilattico Sperimentale del Piemonte Liguria e Valle d’Aosta, Turin, Italy (IZSPLV), that were in compliance with national (D.L. no. 116, G.U. suppl. 40, Feb. 18, 1992, Circular No.8, G.U., 14 July 1994) and international regulations (EEC Council Directive 86/609, OJ L 358, 1 Dec.12, 1987). All the experimental protocols proposed were reviewed and approved by the IZSPLV Animal Care and Use Committee (IACUC).

Blood Samples
Blood samples from 8 BSE-infected cattle (4 with H-type and 4 with L-type BSE) and 2 non-infected controls were provided by the Biological Archive Group at the Animal and Plant Health Agency, United Kingdom. All procedures involving animals were approved by the Home Office of the UK government according to the Animal (Scientific Procedures) Act 1986. The calves were born by crossing Aberdeen Angus with females imported from Denmark (Danish Holstein, Danish milking red). The inoculation details have been reported previously [23]. Briefly, experimental cattle were intracerebrally inoculated with 1 ml of a 10% brain homogenate of either L-type or H-type BSE at 10–11 months of age [23]. All infected cattle used in this study were females. The negative controls were age and sex-matched with the infected group. For each animal, the blood sampling was performed at 2 different time points after inoculation, corresponding to the preclinical (6-months post infection) and the clinical (from 22 to 26 months post infection) stage of the disease. In this way we obtained 16 samples, 8 in the preclinical and 8 in the clinical stage. The estimated clinical onset after infection was based on the presence of changes in behavior, unexpected startle responses, and difficulty in rising [23]. Neurological examination and behavioral observations were conducted routinely until the culling of the animals. TSE infection was confirmed by post-mortem immunohistochemistry on brain sections of the animals [23]. Detailed information on the husbandry procedures and the pathological signs have been described in a previous study published by Konold et al. [23]. Finally, blood samples from 6 sex-matched Aberdeen Angus from a different herd were added to the study and used as additional negative controls to obtain a sample size comparable to the one of the infected animals (8 samples).

RNA Isolation
500 μL of fresh blood were stabilized in 1.3 mL RNA later® Solution and immediately frozen at -20°C. Samples were sent in dry ice to IZSTO (Turin, Italy), where the RNA was isolated according to the RiboPure®-Blood Kit manufacturer’s instructions (Ambion®). DNase I treatment (Ambion®) was included in the RNA extraction protocol to reduce DNA contamination. Purified RNA was eluted in 50 μL elution solution and the final concentration, as well as the absence of protein, was determined using a Thermo Scientific™ NanoDrop 2000 spectrophotometer. Since the RNA concentration was too low to proceed with the subsequent analysis,
the RNA samples were concentrated using a Labconco CentriVap concentrator. The new concentration was assessed using a Thermo Scientific™ NanoDrop 2000 spectrophotometer and the integrity of the RNA was determined by capillary electrophoresis (Agilent 2100 Bioanalyzer, Agilent Technologies, Santa Clara, USA).

**Microarray Hybridization, Statistical Analysis and Data Mining**

24 RNA samples were used for the microarray analysis: 8 preclinical (P1, P2, P4, P5, P7, P8, EP9 and P10), 8 clinical (S1, S2, S3, S4, S7, S8, S9 and S10), and 8 control (c.2, c.3, c.P3, c.5, c.S5, C.P6, C.S6 and c.9) samples. 120 ng of each total RNA were used as template for the synthesis of biotin-labeled cRNA according to the standard one-cycle amplification and labeling protocol developed by Affymetrix (Santa Clara, CA). cRNA was then fragmented and hybridized on GeneChip® Bovine Genome Array, which contains over 24128 probe sets. The microarrays were washed, stained (Affymetrix fluidic station 450 DX) and scanned (Affymetrix scanner 3000 7G). Cell intensity values from the raw array data were computed using the Affymetrix GeneChip® Operating Software (GCOS). Microarray quality control and statistical analysis were performed in the software system R using the Bioconductor package OneChannelGUI [39–41]. The LIMMA algorithm was used to compute a linear model fit [42]. Data filtering and normalization was carried out using GC-Robust multi-array analysis (GCRMA) from imported Affymetrix data (.CEL) files. After the assessment and inspection of microarray quality controls (RNA degradation plot, RLE and NUSE plots) we identified one low quality control sample (cS5) and excluded it from the analysis (S1 Appendix). Gene probes with a p value ≤0.05 and fold-change ≥2 were considered to be differentially expressed. Differentially expressed probe sets were functionally classified using David Bioinformatics tool [43, 44] on the Affymetrix bovine background. Heat maps were generated using the heatmap.2 function from the gplots library in the R statistical environment [45, 46]. Probe set data were hierarchically clustered with complete linkage using the Euclidean metric.

**RT-qPCR**

To confirm the microarray results, we performed RT-qPCR using SYBR® green assay (Bio-Rad Laboratories, Inc.) for a selected number of target genes. The RT-qPCR analysis was performed on 22 samples (from 7 control, 8 preclinical and 7 clinical animals). For each sample, 250 ng of total RNA were used as template for the cDNA synthesis with the SuperScript® III Reverse Transcriptase and the Oligo (dT)20 Primer (Life Technologies). PCR primers were designed using the online tool Primer-BLAST [47] provided by NCBI. Whenever possible, the primer pairs were designed in order to span an exon-exon junction, thus preventing the amplification of genomic DNA. The primer sequences were as follows: for GNLY 5'-AGC CCG ATG AGA ATA CCG TT and 5' - CGA TGT CCT CAG CAG TGG TA; for CD40L 5’ - ACA ACC TCT GTT CTC CAG TG and 5’ - GCT GTT TCC CGT TTT CGA GG; for PDK4 5’ - TGG TGT TCC CCT GAG AGT CA and 5’ - GCT AAG TGG GGG AAG TAG GT; for XIST 5’ - GTG GCA AGG ACC AGA ATG GA and 5’ - TCC GAC CCC AGT ATT AGC CA; for IDO1 5’ - ATT GGT GGA GTC CCT GCA GAC and 5’ - CTG CAG GGT AGC ATT GCC TT; for RPL12 5’ - AGG TCG TGC GTG GTG GC and 5’ - ACT GTC ACT GCT TGC AGC C; for GAPDH 5’ - AGG TCG GAG TGA GAT TC and 5’ - ATG GCG ACG ATG TCC ACT TT. The RT-qPCR reactions
were carried out by denaturing at 95°C for 15s, annealing at 60°C for 1 min and extension at 55°C for 1 min for 45 cycles. Melt curve analysis and gel electrophoresis of amplification products were performed for each primer pair to confirm the production of a single PCR amplicon. The amplification was performed using a CFX96™ Real-Time PCR Detection System (Bio-Rad Laboratories, Inc.). All the RT-qPCR reactions were run in triplicate and included the following controls: no template (NTC) and minus-reverse transcriptase (RT-) negative controls. The normalization accuracy was improved by geometric averaging of multiple reference genes [48] and using two inter-run calibrators to reduce inter-run variation. We decided to use a normalization factor based on three reference genes (GAPDH, RPL12 and ACTB) since it has been shown in the literature that this is the minimal number required for a reliable normalization [49]. Stability of the selected reference genes was determined by calculating their geNorm M value (M) and the coefficient of variation (CV) on the normalized relative quantities [50]. M and CV values were then compared against empirically determined thresholds for acceptable stability (~ 1 and ~ 0.5 for M and CV values respectively) [50] (S2 Appendix). The statistical analysis and the fold change calculation were carried out using qBasePlus 1.1 software [50].

Results
Identification of Differentially Expressed Genes (DEGs) in the Blood of Atypical BSE-Infected Cattle

To investigate if gene expression alterations were present in blood from atypical BSE-infected cattle (clinical and preclinical), we performed microarray experiments using Affymetrix GeneChip® Bovine Genome Array. Since the goal of this project was to identify a common pattern of DEGs in atypical BSE infection, we defined the 4 H- and the 4 L- type inoculated-cattle as one single group of 8 animals named as atypical infected cattle. This approach allowed us to increase the sample size to improve the statistics and thus obtain more reliable results. The distribution of signal intensities, relative log expression (RLE) and normalized unscaled standard error (NUSE) plots were examined in order to avoid procedural failures and the presence of degraded RNA samples. After the assessment of microarray quality controls we identified one control sample (cS5) as an outlier and excluded it from the analysis (see S1 Appendix). Statistical analysis was performed on microarray results using the oneChannelGUI Bioconductor package [39]. The raw microarray data were deposited in the Gene Expression Omnibus (GEO) repository and assigned the accession number GSE69048. The data sets supporting the results of this article are available in the Gene Expression Omnibus (GEO) repository: http://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?token=mzgxuaagdwwftcz&acc=GSE69048.

Statistical comparison between the infected animals (clinical and preclinical) and the control group (IvsCtrl) revealed a total of 101 differentially regulated probe sets (p value lower than 0.05 and changes in expression higher than 2-fold) as shown in Table 1. Some of these probe sets encoded for the same gene. Gene annotation, performed using DAVID Bioinformatics Resources [43, 44], identified a subset of 93 genes with known functions. The most relevant functional groups are reported in Table 2.

To evaluate to what extent gene expression alterations in blood were related to the preclinical or clinical stage of the disease, two distinct statistical analyses were performed comparing each group (8 samples) with the control one: clinical versus control (CvsCtrl) and preclinical versus control (PvsCtrl). In the clinical stage, a total of 207 probe sets showed significant alteration in expression levels compared to the control group. Among these, 87 were up-regulated while 120 had a reduction in expression. Interestingly, a pronounced alteration in the gene expression profile was also found in the preclinical stage, with a total number of 113 differentially expressed probe sets (55 genes were up-regulated while 58 were down-regulated). Two
Table 1. Differentially expressed genes found in infected animals versus control group by the microarray analysisa.

| Probe ID | Gene symbol | Gene name                                                                 | P value  | FC       |
|----------|-------------|---------------------------------------------------------------------------|----------|----------|
| Bt.6653.2.1_at | ALKBH4   | alkB, alkylation repair homolog 4 (E. coli)                              | 1.35E-07 | -2.31687 |
| Bt.8586.1.S1_at | LOC512150 | myeloid-associated differentiation marker-like                           | 4.17E-05 | 4.192159 |
| Bt.21996.1.S1_at | IGHE     | Immunoglobulin heavy constant epsilon                                    | 4.77E-05 | -5.1041  |
| Bt.16101.1.S1_s_at | GNLY /// LOC100300483 | granulysin // antimicrobial peptide NK-lysin-like                        | 5.7E-05  | -4.17735 |
| Bt.28383.1.S1_at | GNLY     | granulysin                                                                | 8.98E-05 | -4.83354 |
| Bt.9265.2.S1_at | BATF     | basic leucine zipper transcription factor, ATF-like                       | 0.000112 | -2.57096 |
| Bt.14153.1.S1_at | NEB      | nebulin                                                                   | 0.000145 | -4.89569 |
| Bt.16101.1.S1_at | GNLY     | granulysin                                                                | 0.000212 | -4.81011 |
| Bt.12986.1.S1_at | MAD2L1   | MAD2 mitotic arrest deficient-like 1 (yeast)                              | 0.000235 | -2.72219 |
| Bt.9265.1.A1_at | BATF     | basic leucine zipper transcription factor, ATF-like                       | 0.000236 | -2.55269 |
| Bt.23123.1.S1_at | BHLHE40  | basic helix-loop-helix family, member e40                                | 0.000575 | -2.36861 |
| Bt.26326.1.A1_at | MTBP     | Mdm2, transformed 3T3 cell double minute 2, p53 binding protein (mouse) binding protein, 104kDa | 0.000623 | 2.784763 |
| Bt.22526.1.S1_at | HSPB8    | heat shock 22kDa protein 8                                                 | 0.000856 | -2.39332 |
| Bt.16916.3.S1_at | KLF11    | Kruppel-like factor 11                                                   | 0.000929 | 4.455542 |
| Bt.24923.2.S1_a_at | SEL1L3  | sel-1 suppressor of lin-12-like 3 (C. elegans)                           | 0.001057 | -3.34168 |
| Bt.8804.1.S1_at | NELL2    | NEL-like 2 (chicken)                                                     | 0.00117  | -2.34981 |
| Bt.28654.1.S1_at | LOC100850906 /// USP42 | ubiquitin carboxyl-terminal hydrolase 42-like /// ubiquitin specific peptidase 42 | 0.00132  | 3.549622 |
| Bt.18321.1.A1_at | GNB4     | guanine nucleotide binding protein (G protein), beta polypeptide 4        | 0.001338 | 8.267761 |
| Bt.24630.2.S1_at | 41527    | septin 10                                                                 | 0.001368 | -3.33002 |
| Bt.21975.1.S1_at | PRF1     | perforin 1 (pore forming protein)                                        | 0.001549 | -2.00589 |
| Bt.20330.1.S1_at | PRSS23   | protease, serine, 23                                                     | 0.001702 | -3.42322 |
| Bt.24929.1.A1_at | RG9MTD3  | RNA (guanine-9-) methyltransferase domain containing 3                   | 0.001896 | 2.420786 |
| Bt.6968.1.S1_at | IPCEF1   | Interaction protein for cytohesin exchange factors 1                     | 0.001963 | 2.424841 |
| Bt.9675.1.S1_at | LOC100847724 | extracellular peptidase inhibitor-like                                      | 0.002117 | -3.52792 |
| Bt.21979.1.S1_at | CXCR6    | chemokine (C-X-C motif) receptor 6                                        | 0.002164 | -2.20884 |
| Bt.9504.1.A1_at | CCL4     | chemokine (C-C motif) ligand 4                                             | 0.002853 | -2.65024 |

(Continued)
Table 1. (Continued)

| Probe ID   | Gene symbol | Gene name                                                        | P value  | FC     |
|------------|-------------|-----------------------------------------------------------------|----------|--------|
| Bt.28040.1 | LOC781494   | myeloid-associated differentiation marker-like                 | 0.002999 | -3.59576 |
| S1_at      |             |                                                                 |          |        |
| Bt.23505.1 | PDK4        | pyruvate dehydrogenase kinase, isozyme 4                        | 0.003176 | 3.186119 |
| S1_at      |             |                                                                 |          |        |
| Bt.24236.1 | DLC1        | deleted in liver cancer 1                                        | 0.003559 | 2.297004 |
| S1_at      |             |                                                                 |          |        |
| Bt.26636.1 | NKG7        | natural killer cell group 7 sequence                             | 0.003806 | -2.16912 |
| S1_at      |             |                                                                 |          |        |
| Bt.6147.1  | METTL12     | methyltransferase like 12                                        | 0.00446  | -2.15129 |
| S1_at      |             |                                                                 |          |        |
| Bt.49.1.S1 | CD40LG      | CD40 ligand                                                      | 0.004517 | -3.23666 |
| S1_at      |             |                                                                 |          |        |
| Bt.26259.1 | ZNF462      | zinc finger protein 462                                         | 0.004806 | 3.969328 |
| A1_at      |             |                                                                 |          |        |
| Bt.15915.1 | LRRC70      | leucine rich repeat containing 70                               | 0.005099 | 2.154164 |
| S1_at      |             |                                                                 |          |        |
| Bt.17280.1 | PLEKHH1     | pleckstrin homology domain-containing family H member 1-like    | 0.005117 | 2.356958 |
| S1_at      |             |                                                                 |          |        |
| Bt.29009.1 | RYR3        | ryanodine receptor 3                                             | 0.00525  | -5.46377 |
| A1_at      |             |                                                                 |          |        |
| Bt.2129.1  | LOC100850064| versican core protein-like                                       | 0.005936 | 2.001974 |
| S1_at      |             |                                                                 |          |        |
| Bt.22415.2 | LOC512863   | sialic acid-binding Ig-like lectin 14-like                      | 0.006306 | 2.230891 |
| A1_at      |             |                                                                 |          |        |
| Bt.19308.1 | BACH2       | BTB and CNC homology 1, basic leucine zipper transcription factor 2 | 0.006597 | 3.175266 |
| S1_at      |             |                                                                 |          |        |
| Bt.16861.1 | LOC515128   | major facilitator superfamily domain-containing protein 4-like  | 0.006682 | 2.319431 |
| A1_at      |             |                                                                 |          |        |
| Bt.22301.1 | ATP6V0A4    | ATPase, H+ transporting, lysosomal V0 subunit a4                 | 0.006816 | -2.30662 |
| S1_at      |             |                                                                 |          |        |
| Bt.15705.1 | DSTN        | destrin (actin depolymerizing factor)                            | 0.006907 | 2.216088 |
| S1_at      |             |                                                                 |          |        |
| Bt.28637.1 | LOC100848843| myeloid-associated differentiation marker-like                  | 0.007707 | 2.740488 |
| S1_at      |             |                                                                 |          |        |
| Bt.24983.1 | DYN1C2H1    | dynein, cytoplasmic 2, heavy chain 1                            | 0.008144 | -2.08508 |
| A1_at      |             |                                                                 |          |        |
| Bt.24543.1 | KCTD1       | potassium channel tetramerisation domain containing 1           | 0.01017  | 2.673429 |
| A1_at      |             |                                                                 |          |        |
| Bt.16916.1 | KLF11       | Kruppel-like factor 11                                           | 0.01016  | 2.110118 |
| S1_at      |             |                                                                 |          |        |
| Bt.11259.1 | IFI27       | putative ISG12(a) protein                                       | 0.010909 | 3.109152 |
| S1_at      |             |                                                                 |          |        |
| Bt.17081.2 | LMO2        | LIM domain only 2 (rhombotin-like 1)                            | 0.011572 | -2.37451 |
| S1_at      |             |                                                                 |          |        |
| Bt.22214.1 | CD180       | CD180 molecule                                                   | 0.011606 | -2.04421 |
| S1_at      |             |                                                                 |          |        |
| Bt.7145.1  | GZMB /// LOC100125946 | granzyme B (granzyme 2, cytotoxic T-lymphocyte-associated serine esterase 1) /// uncharacterized LOC100125946 | 0.015009 | -3.15552 |
| S1_at      |             |                                                                 |          |        |
| Bt.20540.1 | CD79B       | CD79b molecule, immunoglobulin-associated beta                  | 0.015089 | -3.58646 |
| S1_at      |             |                                                                 |          |        |
| Bt.20257.2 | BCNT2       | Bucentaur-2                                                      | 0.015853 | -2.28942 |
| S1_at      |             |                                                                 |          |        |
| Bt.155.1.S1| IL8         | interleukin 8                                                   | 0.016    | 2.249822 |

(Continued)
## Table 1. (Continued)

| Probe ID | Gene symbol | Gene name                                                                 | P value | FC     |
|----------|-------------|---------------------------------------------------------------------------|---------|--------|
| Bt.24112.1. A1_at | CXHXorf57    | chromosome X open reading frame, human CXorf57                           | 0.016027 | -2.76004 |
| Bt.18253.1. A1_at | KIAA1324L    | KIAA1324-like ortholog                                                      | 0.016067 | 2.359824 |
| Bt.27043.2. S1_at | FCER1A       | Fc fragment of IgE, high affinity l, receptor for; alpha polypeptide       | 0.016226 | -2.06287 |
| Bt.13469.1. S1_at | SDSL         | serine dehydratase-like                                                    | 0.016296 | -2.5064 |
| Bt.9262.1. A1_at | SPIB         | Spi-B transcription factor (Spi-1/PU.1 related)                            | 0.016742 | -3.49334 |
| Bt.9974.1. S1_at | CCL3         | chemokine (C-C motif) ligand 3                                             | 0.01705 | -2.10999 |
| Bt.19014.1. A1_at | NDUFS3       | NADH dehydrogenase (ubiquinone) Fe-S protein 3, 30kDa (NADH-coenzyme Q reductase) | 0.017446 | 4.152097 |
| Bt.2899.1. S2_at | FOS          | FBJ murine osteosarcoma viral oncogene homolog                             | 0.017668 | 2.486687 |
| Bt.6438.1. A1_at | TGFB2        | transforming growth factor, beta 2                                         | 0.019127 | 3.436742 |
| Bt.3352.1. A1_at | ASIP         | agouti signaling protein                                                  | 0.019681 | -2.09284 |
| Bt.9163.2. S1_at | P2RY10       | purinergic receptor P2Y, G-protein coupled, 10                             | 0.019736 | -2.0468 |
| Bt.27760.1. S1_at | BoLA // BOLA-A | major histocompatibility complex, class I, A // major histocompatibility complex, class I, A | 0.02069 | -3.40943 |
| Bt.3805.1. S1_at | BOLA-N // JSP.1 // LOC100125916 | MHC class I antigen // MHC Class I JSP.1 // uncharacterized protein 100125016 | 0.021303 | 3.897296 |
| Bt.17019.1. A1_at | FOXP1        | Forkhead box P1                                                           | 0.021388 | 2.003436 |
| Bt.20640.1. A1_at | C22H3orf64   | chromosome 22 open reading frame, human C3orf64                           | 0.021803 | 2.039162 |
| Bt.411.1.S1_at | NRG1         | neuregulin 1                                                              | 0.021883 | 2.757968 |
| Bt.11847.1. A1_at | XIST         | X (inactive)-specific transcript                                           | 0.023013 | -21.2975 |
| Bt.24923.1. S1_at | SEL1L3       | sel-1 suppressor of lin-12-like 3 (C. elegans)                            | 0.023619 | -3.25125 |
| Bt.13637.1. A1_at | XIST         | X (inactive)-specific transcript                                           | 0.024218 | -34.6725 |
| Bt.29820.1. S1_s_at | BOLA        | MHC class I heavy chain                                                    | 0.024899 | -6.08329 |
| Bt.23911.1. A1_at | XIST         | X (inactive)-specific transcript                                           | 0.025193 | -29.733 |
| Bt.22139.1. S1_at | COBLL1       | COBL-like 1                                                                | 0.026423 | -2.76573 |
| Bt.22854.1. S1_at | CA2          | carbonic anhydrase II                                                      | 0.026826 | -2.33612 |
| Bt.13330.1. S1_at | PDK4         | pyruvate dehydrogenase kinase, isozyme 4                                  | 0.02815 | 2.399512 |
| Bt.16070.2. S1_at | LOC786352    | apolipoprotein L, 1-like                                                  | 0.028855 | -2.31442 |
| Bt.17473.2. S1_at | RPE          | ribulose-5-phosphate-3-epimerase                                          | 0.030357 | 2.209343 |
| Bt.16966.1. S1_at | CXCL10       | chemokine (C-X-C motif) ligand 10                                          | 0.030459 | -3.06647 |

(Continued)
heat maps representing the differentially expressed probe sets in preclinical and clinical groups are shown in Fig 1. The complete probe set lists with the relative p values and fold changes can be found in S1 and S2 Tables.

A gene enrichment analysis was performed to identify the most enriched GO terms in the clinical and preclinical groups (Fig 2). DEGs specific of the clinical group were clustered in functional categories related to cytokine-cytokine receptor interaction, regulation of leukocyte activation, inflammatory response, autoimmune thyroid disease, chemokine activity, B cell

Table 1. (Continued)

| Probe ID  | Gene symbol | Gene name                                             | P value   | FC      |
|-----------|-------------|-------------------------------------------------------|-----------|---------|
| Bt.23172.1.1 | BAX         | BCL2-associated X protein                             | 0.030653  | -2.28799|
| S1_at     |             |                                                       |           |         |
| Bt.4609.1.1 | LOC100847474| uncharacterized LOC100847474                         | 0.031831  | -2.619  |
| S1_at     |             |                                                       |           |         |
| Bt.20687.1.1 | CSH12orf35  | chromosome 5 open reading frame, human C12orf35      | 0.032384  | 2.091363|
| A1_at     |             |                                                       |           |         |
| Bt.36512.1.1 | C15H11orf96 | chromosome 15 open reading frame, human C11orf96     | 0.035164  | 2.092887|
| A1_at     |             |                                                       |           |         |
| Bt.5408.1.1 | UCHL1       | ubiquitin carboxyl-terminal esterase L1 (ubiquitin thiolesterase) | 0.035211  | 2.097092|
| A1_at     |             |                                                       |           |         |
| Bt.7015.1.1 | EED         | embryonic ectoderm development                       | 0.036537  | -2.08921|
| S1_at     |             |                                                       |           |         |
| Bt.14010.1.1 | PTGR1      | prostaglandin reductase 1                            | 0.03669   | -2.19439|
| S1_at     |             |                                                       |           |         |
| Bt.2899.1.1 | FOS         | FBJ murine osteosarcoma viral oncogene homolog        | 0.03784   | 2.465631|
| S1_at     |             |                                                       |           |         |
| Bt.1075.1.1 | ZNF24       | zinc finger protein 24                                | 0.038258  | 2.707935|
| S1_at     |             |                                                       |           |         |
| Bt.20494.1.1 | RYBP       | RING1 and YY1 binding protein                         | 0.038552  | 2.157803|
| S1_at     |             |                                                       |           |         |
| Bt.27261.2.1 | LOC100847574| multidrug resistance-associated protein 4-like       | 0.040322  | 3.296289|
| S1_at     |             |                                                       |           |         |
| Bt.23094.1.1 | AKR1C4 ///< LOC506594 | aldo-keto reductase family 1, member C4 (chlordecone reductase; 3-alpha hydroxysteroid dehydrogenase, type I; dihydrodiol dehydrogenase 4) // prostaglandin F synthase 1-like | 0.041102  | -2.13672|
| A1_at     |             |                                                       |           |         |
| Bt.20540.3.1 | CD79B       | CD79b molecule, immunoglobulin-associated beta        | 0.04229   | -2.06338|
| S1_at     |             |                                                       |           |         |
| Bt.24372.1.1 | ZRSR2Y     | zinc finger (CCCH type), RNA-binding motif and serine/arginine rich 2 | 0.042712  | 3.786694|
| S1_at     |             |                                                       |           |         |
| Bt.7122.1.1 | HELZ        | helicase with zinc finger                             | 0.043553  | 2.564275|
| S1_at     |             |                                                       |           |         |
| Bt.213.1.S1_at | CD163L1       | CD163 molecule-like 1                                 | 0.047294  | 2.499982|
| S1_at     |             |                                                       |           |         |
| Bt.20216.2.1 | RPIA        | ribose 5-phosphate isomerase A                        | 0.048256  | -2.06759|
| S1_at     |             |                                                       |           |         |
| Bt.27759.2.1 | IDO1        | indoleamine 2,3-dioxygenase 1                         | 0.048525  | -3.72942|
| S1_at     |             |                                                       |           |         |
| Bt.29815.1.1 | BOLA        | MHC class I heavy chain                               | 0.048538  | -3.12234|
| S1_x_at   |             |                                                       |           |         |
| Bt.15705.1.1 | DSTN        | destrin (actin depolymerizing factor)                 | 0.048589  | 2.055416|
| S2_at     |             |                                                       |           |         |
| Bt.16933.2.1 | LOC100298891| aTP-binding cassette, sub-family C (CFTR/MRP), member 4-like | 0.049066  | 2.747912|
| A1_at     |             |                                                       |           |         |

*FC = fold change.

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Table 2. Functional classification of differentially expressed genes in blood of infected cattle versus control groupa.

| Pathway                          | Probe name | Gene symbol | Gene name                                                                 | P value   | Fold Enrichment |
|---------------------------------|------------|-------------|---------------------------------------------------------------------------|-----------|-----------------|
| **Autoimmune thyroid disease**  | BT.21975.1. S1_AT | PRF1        | perforin 1 (pore forming protein)                                         | 1.01E-04  | 19.14728682     |
|                                 | BT.29815.1. S1_X_AT | BOLA        | MHC class I heavy chain                                                   |           |                 |
|                                 | BT.29820.1. S1_S_AT | BOLA        | MHC class I heavy chain                                                   |           |                 |
|                                 | BT.3805.1. S1_AT | BOLA-N /// JSP.1 /// LOC100125916 | MHC class I antigen /// MHC Class I JSP.1 /// uncharacterized protein 100125016 |           |                 |
|                                 | BT.49.1. S1_AT | CD40LG      | CD40 ligand                                                              |           |                 |
|                                 | BT.7145.1. S1_AT | GZMB /// LOC100125946 | granzyme B (granzyme 2, cytotoxic T-lymphocyte-associated serine esterase 1) /// uncharacterized LOC100125946 |           |                 |
| **Extracellular region**        | BT.155.1. S1_AT | IL8         | interleukin 8                                                             | 2.22E-04  | 3.036995516     |
|                                 | BT.16070.2. S1_AT | LOC786352   | apolipoprotein L, 1-like                                                  |           |                 |
|                                 | BT.16966.1. S1_AT | CXCL10      | chemokine (C-X-C motif) ligand 10                                          |           |                 |
|                                 | BT.20330.1. S1_AT | PRSS23      | protease, serine, 23                                                      |           |                 |
|                                 | BT.20640.1. A1_AT | C22H3orf64  | chromosome 22 open reading frame, human C3orf64                           |           |                 |
|                                 | BT.2129.1. S1_AT | LOC100850064 | versican core protein-like                                                |           |                 |
|                                 | BT.213.1. S1_AT | CD163L1     | CD163 molecule-like 1                                                     |           |                 |
|                                 | BT.29009.1. A1_AT | RYR3        | ryanodine receptor 3                                                      |           |                 |
|                                 | BT.49.1. S1_AT | CD40LG      | CD40 ligand                                                              |           |                 |
|                                 | BT.6438.1. A1_AT | TGFB2       | transforming growth factor, beta 2                                        |           |                 |
|                                 | BT.8804.1. S1_AT | NELL2       | NEL-like 2 (chicken)                                                      |           |                 |
|                                 | BT.9504.1. A1_AT | CCL4        | chemokine (C-C motif) ligand 4                                            |           |                 |
|                                 | BT.9974.1. S1_AT | CCL3        | chemokine (C-C motif) ligand 3                                            |           |                 |
|                                 | BT.3352.1. S1_AT | ASIP        | agouti signaling protein                                                  |           |                 |
| **Immune response**             | BT.155.1. S1_AT | IL8         | interleukin 8                                                             | 4.91E-04  | 5.451442755     |
|                                 | BT.16966.1. S1_AT | CXCL10      | chemokine (C-X-C motif) ligand 10                                          |           |                 |
|                                 | BT.27760.1. S1_AT | BoLA /// BOLA-A | major histocompatibility complex, class I, A /// major histocompatibility complex, class I, A |           |                 |
|                                 | BT.29815.1. S1_X_AT | BOLA        | MHC class I heavy chain                                                   |           |                 |
|                                 | BT.29820.1. S1_S_AT | BOLA        | MHC class I heavy chain                                                   |           |                 |

(Continued)
| Pathway                                | Probe name | Gene symbol | Gene name                                                                 | P value | Fold Enrichment |
|----------------------------------------|------------|-------------|---------------------------------------------------------------------------|---------|-----------------|
| Chemokine activity                     | BT.155.1.  | IL8         | interleukin 8                                                             | 5.42E-04| 24.18039216     |
|                                        | S1_AT      |             |                                                                           |         |                 |
|                                        | BT.16966.1.| CXCL10      | chemokine (C-X-C motif) ligand 10                                          |         |                 |
|                                        | S1_AT      |             |                                                                           |         |                 |
|                                        | BT.9504.1. | CCL4        | chemokine (C-C motif) ligand 4                                             |         |                 |
|                                        | A1_AT      |             |                                                                           |         |                 |
|                                        | BT.9974.1. | CCL3        | chemokine (C-C motif) ligand 3                                            |         |                 |
|                                        | S1_AT      |             |                                                                           |         |                 |
| Locomotory behavior                    | BT.155.1.  | IL8         | interleukin 8                                                             | 0.00138806| 9.982954545    |
|                                        | S1_AT      |             |                                                                           |         |                 |
|                                        | BT.16966.1.| CXCL10      | chemokine (C-X-C motif) ligand 10                                          |         |                 |
|                                        | S1_AT      |             |                                                                           |         |                 |
|                                        | BT.5408.1. | UCHL1       | ubiquitin carboxyl-terminal esterase L1 (ubiquitin thiolesterase)         |         |                 |
|                                        | A1_AT      |             |                                                                           |         |                 |
|                                        | BT.9504.1. | CCL4        | chemokine (C-C motif) ligand 4                                            |         |                 |
|                                        | A1_AT      |             |                                                                           |         |                 |
|                                        | BT.9974.1. | CCL3        | chemokine (C-C motif) ligand 3                                            |         |                 |
|                                        | S1_AT      |             |                                                                           |         |                 |
| Inflammatory response                  | BT.155.1.  | IL8         | interleukin 8                                                             | 0.001604508| 9.598994755    |
|                                        | S1_AT      |             |                                                                           |         |                 |
|                                        | BT.16966.1.| CXCL10      | chemokine (C-X-C motif) ligand 10                                          |         |                 |
|                                        | S1_AT      |             |                                                                           |         |                 |
|                                        | BT.49.1.   | UCHL1       | ubiquitin carboxyl-terminal esterase L1 (ubiquitin thiolesterase)         |         |                 |
|                                        | S1_AT      |             |                                                                           |         |                 |
|                                        | BT.9504.1. | CCL4        | chemokine (C-C motif) ligand 4                                            |         |                 |
|                                        | A1_AT      |             |                                                                           |         |                 |
|                                        | BT.9974.1. | CCL3        | chemokine (C-C motif) ligand 3                                            |         |                 |
|                                        | S1_AT      |             |                                                                           |         |                 |
| Antigen processing and presentation via | BT.27760.1.| BoLA ///    | major histocompatibility complex, class I, A /// major histocompatibility | 0.002359977| 39.93181818    |
| MHC class I                            | S1_AT      | BOLA-A      | complex, class I, A                                                        |         |                 |
|                                        |            |             |                                                                           |         |                 |
|                                        | BT.29820.1.| BOLA        | MHC class I heavy chain                                                   |         |                 |
|                                        | S1_S_AT    |             |                                                                           |         |                 |
|                                        | BT.3805.1. | BOLA-N ///  | MHC class I antigen /// MHC Class I                                        |         |                 |
|                                        | S1_AT      | JSP.1 ///   |                                                                           |         |                 |
|                                        |            | LOC100125916| JSP.1 /// uncharacterized protein                                          |         |                 |
|                                        |            |             |                                                                           |         |                 |
|                                        | BT.29815.1.| BOLA        | MHC class I heavy chain                                                   |         |                 |
|                                        | S1_X_AT    |             |                                                                           |         |                 |
| B cell proliferation                   | BT.23172.1.| BAX         | BCL2-associated X protein                                                  | 0.033775277898717| 57.0454545454545|
|                                        | S1_AT      |             |                                                                           |         |                 |
|                                        | BT.49.1.   | CD40LG      | CD40 ligand                                                               |         |                 |
|                                        | S1_AT      |             |                                                                           |         |                 |
| Cell adhesion molecules (CAMs)         | BT.2129.1. | LOC100850064| versican core protein-like                                                | 0.038771205| 5.105943152    |
|                                        | S1_AT      |             |                                                                           |         |                 |

(Continued)
proliferation and differentiation, regulation of apoptosis, kinase inhibitor activity, and membrane raft (Fig 2B). The preclinical stage was characterized by enrichment in gene clusters related to chemokine signaling pathway, extracellular region, secreted protein, immune response, pyridoxal phosphate binding, transcription, myeloid-associated differentiation marker, B cell proliferation, extracellular matrix, RNA metabolic process, MHC class I, Lammin G and response to wounding (Fig 2A).

When comparing the differentially regulated probe sets identified in the preclinical and clinical groups, it was found that 35 differentially expressed probe sets (corresponding to 32 DEGs) were common between the two stages of disease (Fig 3), leaving 172 DEGs specific to clinical and 78 genes specific to preclinical animals. Remarkably, all of the 32 common DEGs displayed a very similar pattern of expression in the clinical and preclinical groups, as shown in Fig 3B. These genes are listed in bold in S1 and S2 Tables.

To further dissect gene expression alterations during the progression of the disease, we performed a statistical analysis to identify specific changes between the clinical and preclinical stages (CvsP). Indeed, we found 235 DEGs, which were significantly enriched in pathways related to immune response (regulation of B cell proliferation, leucocyte activation, ISG15-protein conjugation and chemokine signaling were among the most significant). The list of the most relevant enriched probe sets can be found in S3 Table.

We used a Venn diagram to compare the DEGs found in PvsCtrl, CvsCtrl and CvsP analyses that were previously performed and then we examined the expression levels of common DEGs (Fig 4). Venn diagram revealed the presence of one DEG in common between PvsCtrl, CvsCtrl and CvsP comparisons, while 22 genes were differentially expressed in PvsCtrl and CvsP but not in CvsCtrl comparisons (Fig 4A). We found that these 22 DEGs had an opposite fold change sign in PvsCtrl and CvsP, thus indicating an oscillatory pattern of expression (see Fig 4B, 4C and Table 3). In particular, 9 out of 22 DEGs were up-regulated in the preclinical phase and then went back roughly to the expression level of the controls in the clinical stage (Fig 4B). The remaining 13 out of 22 DEGs were down-regulated in the preclinical phase and then went back almost to control levels in the clinical phase (Fig 4C). The only gene in common between the three comparisons, namely Sel-1 Suppressor Of Lin-12-Like 3 (SEL1L3), was

### Table 2. (Continued)

| Pathway                | Probe name | Gene symbol | Gene name                                      | P value   | Fold Enrichment |
|------------------------|------------|-------------|------------------------------------------------|-----------|-----------------|
| BT.29820.1. S1_AT     | BOLA       | MHC class I heavy chain                     |          |               |
| BT.3805.1. S1_AT      | BOLA-N /// JSP.1 /// LOC100125916 | MHC class I antigen /// MHC Class I JSP.1 /// uncharacterized protein 100125016 |          |               |
| BT.49.1. S1_AT        | CD40LG     | CD40 ligand                                      |          |               |
| BT.29815.1. S1_AT     | BOLA       | MHC class I heavy chain                        |          |               |

* The gene enrichment analysis was performed using DAVID bioinformatics tool 6.7 (NIAID/NIH, USA). Only genes with a known GO and belonging to the most relevant functional categories are represented in the list.

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Fig 1. Heat maps representing the DEGs found in clinical and preclinical cattle with atypical BSE. Two heat maps were generated using the `heatmap.2` function from the `gplots` library in R statistical environment. DEGs were hierarchically clustered with complete linkage using the Euclidean metric. The heat maps represent the most significant DEGs (p value ≤ 0.05 and fold change ≥ 2) in clinical (A) and preclinical (B) animals compared to the control group. Animals are reported in the x-axis while the differentially expressed probes are in the y-axis.

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progressively down-regulated during the preclinical and the clinical phase of the infection, as shown in Fig 4D.

Validation of Microarray Results by RT-qPCR

To confirm the microarray results, RT-qPCR analysis was performed using the SYBR® green assay. A normalization factor based on three reference genes (glyceraldehyde-3-phosphate dehydrogenase, GAPDH; ribosomal protein L12, RPL12; actin, beta, ACTB) was used for the analysis. The stability of the selected reference genes was determined by calculating their geNorm M value (M) and the coefficient of variation (CV) on the normalized relative quantities (S2 Appendix file) [50]. 9 DEGs related to different functional categories were chosen for the validation: GNLY (granulysin), CD40L (CD40 ligand), PDK4 (pyruvate dehydrogenase lipoamide kinase isozyme 4), IDO1 (indoleamine 2, 3-dioxigenase 1), HBA2 (hemoglobin, alpha 2), XIST (X-inactive specific transcript), GNB4 (guanine nucleotide binding protein beta polypeptide 4), BOLA (MHC class I heavy chain), and SEL1L3 (Table 4).

These genes were selected on the basis of their fold changes, p values and relevance in the literature. Moreover, because we performed several statistical analyses of the microarray data, we chose genes that appeared as differentially expressed with the highest frequency in different resulting datasets. Despite microarray p value for HBA2 was not significant, this gene was selected for the RT-qPCR validation since a previous work published by our group highlighted its involvement in prion pathogenesis [51]. The RT-qPCR analysis confirmed the microarray results for six out of nine genes selected (XIST, CD40L, GNLY, PDK4, HBA2 and SEL1L3), which are represented in Table 5 and Fig 5: CD40L, XIST, SEL1L3 and GNLY downregulation was confirmed in both preclinical and clinical groups, while HBA2 was
significantly down-regulated only in preclinical animals. Significant PDK4 upregulation was found in the clinical stage, but not in the preclinical one. These results were in line with the microarray data (Table 5).

Discussion

Whole blood is the most suitable tissue for a prospective rapid diagnostic test since it minimizes sample handling artifacts and reduces sample variability due to fractionation. The present study revealed a substantial gene expression alteration in whole blood from atypical BSE-infected cattle, which could be investigated in future experiments and, if confirmed, could be exploited as a signature for the disease. One of the major caveats in using peripheral blood is that its cellular components may change dramatically during infections or inflammation. The animals used in the present study did not show any apparent side pathology, they were monitored daily by the husbandry staff and their blood was examined for serum aspartate aminotransferase (AST), creatine kinase (CK) and manganese [23]. Nonetheless, possible interference due to hidden pathologies or to inter-individual variations in hematocrit and white blood
DEGs in common between CvsP, PvsCtrl and CvsCtrl comparisons. (A) Venn diagram revealed the presence of 22 DEGs in common between CvsP and PvsCtrl comparisons. One DEG (SEL1L3, Sel-1 Suppressor Of Lin-12-Like 3) was common in the three comparisons. (B-C) The normalized expression values of the 22 common DEGs for CvsP and PvsCtrl comparisons are represented by the histograms. As indicated by the schematic figures on the right, these 22 DEGs showed an oscillatory pattern of expression: (B) 9/22 were upregulated in PvsCtrl and then downregulated in CvsP comparisons, while (C) 13/22 were downregulated in PvsCtrl and upregulated in CvsP comparison, respectively. (D) SEL1L3, the only gene found in common among the
cell count may affect interpretation of expression data and should be considered as an important variable in future studies. In light of this, the present results should be read as a first exploration of whole blood transcriptomics during a prion infection. To our knowledge, this is the first microarray study of whole blood from BSE-infected cattle. Indeed, in a study published by Panelli et al. [36], fractionated white blood cells were analyzed to detect gene expression changes in L-type infected animals. Very few DEGs are common between the two studies. However, this discrepancy may be explained by different infection methods, microarray platforms, statistical analysis stringency and p value cut-off. Also, the white cells used in the study of Panelli et al. were isolated from 1 year post-infection animals, while in our study we used whole blood from preclinical and clinical infected cattle (around 6 months and 22–26 post-infection respectively).

In the present study, 4 statistical comparisons were performed: infected (preclinical and clinical) versus control (IvsCtrl), preclinical versus control (PvsCtrl), clinical versus control (CvsCtrl) and clinical versus preclinical (CvsP) comparisons. Since our goal was to find a common pattern among all the atypical BSE-infected cattle, we defined them as a single group of infected (H-type and L-type) animals. Indeed, as published by Konold and colleagues [23], these animals shared a very similar phenotype in terms of behavioral and clinical signs. Also, another study by Priemer and colleagues indicated a large similarity at level of PrPSc.

### Table 3. List of DEGs characterized by an up-down/down-up pattern of expression. a

| Probe Name | Gene Symbol | Gene Name | Fold Change in PvsCtrl | Fold Change in CvsP |
|------------|-------------|-----------|-----------------------|---------------------|
| Bt.611.1.S2_at | CXCL2 | chemokine (C-X-C motif) ligand 2 | 20.96797 | -35.4276 |
| Bt.5021.1.S1_at | FBN1 | fibrillin 1 | 5.660874 | -4.47422 |
| Bt.18013.1.A1_at | RERE | arginine-glutamic acid dipeptide (RE) repeats | 3.399468 | -2.66838 |
| Bt.18234.1.S1_at | IGSF6 | immunoglobulin superfamily, member 6 | -3.28943 | 2.811553 |
| Bt.15334.2.A1_at | STAT3 | Signal transducer and activator of transcription 3 | -3.23829 | 2.678539 |
| Bt.20833.3.A1_at | NHERF2 | NHL repeat containing 2 | 2.825469 | -2.43499 |
| Bt.27161.1.A1_at | C16H1orf114 | chromosome 16 open reading frame, human C1orf114 | 2.782753 | -2.03538 |
| Bt.17789.1.A1_at | ST8SIA4 | ST8 alpha-N-acetylated neuraminidase alpha-2,8-sialyltransferase 4 | -2.77122 | 5.521504 |
| Bt.21221.1.S1_at | LST1 | leukocyte specific transcript 1 | -2.55548 | 2.531317 |
| Bt.3780.1.S1_at | C10orf13 | chromosome 10 open reading frame, human C5orf13 | 2.480665 | -2.2476 |
| Bt.9841.1.S1_at | MIR21 | microRNA mir-21 | -2.45176 | 3.249144 |
| Bt.18474.2.A1_at | SHOC2 | soc-2 suppressor of clear homolog (C. elegans) | -2.4061 | 3.30272 |
| Bt.21868.2.S1_at | IGSF6 | immunoglobulin superfamily, member 6 | -2.34146 | 2.567908 |
| Bt.27421.1.S1_at | RHOGEF2 | Rho/Rac guanine nucleotide exchange factor (GEF) 2 | -2.26254 | 2.6399 |
| Bt.16088.1.A1_at | MYOSC | myosin VC | 2.260998 | -2.64645 |
| Bt.1711.1.A1_at | LOC615263 | uncharacterized LOC615263 | -2.20341 | 2.511818 |
| Bt.22390.1.S1_at | HPS3 | Hermansky-Pudlak syndrome 3 | 2.146785 | -2.13026 |
| Bt.1847.1.S1_at | LOC789748 | Sialic acid-binding Ig-like lectin 14-like | -2.12509 | 2.458681 |
| Bt.2334.1.S1_at | CD68 | CD68 molecule | -2.11618 | 2.156622 |
| Bt.26430.1.S1_at | ZNF1B | zinc finger protein 184 | 2.099151 | -2.31926 |
| Bt.1736.1.A1_at | SOCS1 | suppressor of cytokine signaling 1 | -2.06053 | 2.221149 |
| Bt.5878.2.S1_at | SDS | serine dehydratase | -2.02673 | 2.367767 |

a For each gene the fold changes found in PvsCtrl (preclinical versus control) and CvsP (clinical versus preclinical) comparisons are reported.
anatomical distribution for the atypical strains, with only slight differences in the overall intensities between H- and L-type [27]. Nonetheless, even if H- and L-type BSE are reported to share many similarities, they constitute two distinct BSE variants which are characterized by a different electrophoretic mobility of PrPSc unglycosylated moiety after proteinase K (PK) digestion.

Table 4. Genes analyzed by RT-qPCR.a.

| Gene     | Primer sequence | Amplicon length (bp) | Accession number |
|----------|-----------------|----------------------|-----------------|
| ACTB*    | F: GGA CTT CGA GCA GGA GAT GG | 148 | NM_173979.3   |
|          | R: TTC CAT GCC CAG GAA GGA AG  |     |                |
| GAPDH*   | F: AGG TCG GAG TGA ACG GAT TC | 85  | NM_001034034.2 |
|          | R: ATG GCC ACG ATG TCC ACT TT  |     |                |
| RPL12*   | F: AGG GTC TGA GGA TTA CAG TGA AA | 83  | NM_205797.1   |
|          | R: GAT CAG GGC AGA AGA AGG     |     |                |
| CD40L    | F: ACA ACC TCT GTT CTC CAG TG | 82  | NM_174624.2   |
|          | R: GTC GTT TCC GTT TTT CGA GG  |     |                |
| XIST     | F: GTC GCA AGG ACC AGA ATG GA | 112 | NR_001464.2   |
|          | R: TCC GAC CCC AGT ATT AGC CA  |     |                |
| GNLY     | F: AGC CCG ATG AGA AGA CGT TT | 120 | NM_001075143.1|
|          | R: CCA TGT CCT CAG CGA TGG TA  |     |                |
| PDK4     | F: TGG TGT TCC CCT GAG AGT CA | 109 | NM_001101883.1|
|          | R: GTA ACC AAA ACC AGC CAG CG  |     |                |
| HBA2     | F: ACA AGG GCA ATG TCA AGG CC | 124 | NM_001077422.3|
|          | R: TGG TGG TGG GGG AAG TAG GT  |     |                |
| GNB4     | F: AGA TCG TGC AGG TGT TCT TG | 96  | NM_001099033.1|
|          | R: CTG TCC CAA GAC CCT GTT G   |     |                |
| IDO1     | F: ATT GGT GGA GTG CTC CCT GCA GAC | 150 | NM_001101866.2|
|          | R: CTG CAG GGT AGT ATT GCC TT  |     |                |
| BOLA     | F: CTC GTA GTA GCT GTC GTG GC  | 96  | NM_001038518.1|
|          | R: ACT GTC ACT GCT TGC AGC C   |     |                |
| SEL1L3   | F: TGA AGG AGT GGTTC GCC TG    | 79  | NM_001206556.2|
|          | R: TTC AAA TCC TGG CCA GTG CT  |     |                |

a Primers (F, forward; R, reverse) used for gene amplification, amplicon size, and GenBank® accession numbers for the bovine cDNA sequences used for primer design. All primers were designed according to the genome sequence of Bos taurus.

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Table 5. Differential expression of selected genes quantified by microarray and RT-qPCR analysis a.

| Gene name                  | Gene symbol | Microarray fold change | RT-qPCR ratio/FC |
|----------------------------|-------------|------------------------|------------------|
|                            |             | preclinical clinical   | preclinical clinical |
| Granulysin                 | GNLY        | -6.318/-3.698          | 0.322/-3.106     |
| X-inactive specific transcript | XIST     | -33.240/-22.612        | 0.003/-333.333  |
| Pyruvate dehydrogenase kinase 4 | PDK4    | 1.752/(ns) 5.793       | 0.665/-1.503 (ns) |
| CD40 ligand                | CD40L       | -2.786/-3.761          | 0.667/-1.499     |
| Haemoglobin, alpha 2       | HBA2        | -3.866/(ns) -2.259 (ns) | 0.269/-3.717     |
| Suppressor Of Lin-12-Like Protein 3 | SEL1L3 | -2.16/-5.17           | 0.504/-1.984     |

a For an easier interpretation, the differential expression of the downregulated genes measured by RT-qPCR is reported both as original ratio and as fold change calculated as -1/ratio. Ns = not significant

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digestion [16, 18]. Statistical comparison between the 4 H-type and the 4 L-type infected animals was carried out in preliminary analyses (see S4 Table), but only a limited number of DEGs was found. Among them, only 15 had a p value lower than 0.01 and only 16 showed a fold change higher than 3, indicating that, at least in terms of number of DEGs, these two groups did not display large differences. For these reasons, we decided to focus our attention on finding a common gene pattern among all the atypical BSE-infected cattle and therefore we pooled the two groups. Due to the high inter-animal variability, which is expected for outbreed animals, further studies in larger animal cohorts would be required to investigate in detail the strain-specific gene expression changes occurring during the progression of the disease. Still, the HvsL analysis can be used as a sort of internal control in this study.

Another aspect to be taken into account when reading the present results is that additional negative control cattle, aged from 12 to 37 months and derived from a different herd compared to the Konold’s study groups [23], were introduced in the analyses. The addition of these controls was useful to balance the samples from infected animals and allowed a preliminary exploration of the differentially expressed transcripts. However, age-related and environmental variability may have affected in some degree the data and need to be considered for their correct interpretation. Despite some limitations, since several statistical analyses were performed (including the CvsP analysis, in which all the animals derived from the same herd) a cross comparison of all them, as we did with the Venn Diagram, may be very useful in order to define a set of genes which could be a good starting point for further validation experiments in the future. In the first statistical analysis we performed (IvsC), we found that among 101 DEGs, 93 had known functions and were involved in several biological processes and molecular pathways, such as autoimmune thyroiditis, chemokine and cytokine activity, regulation of the secretion pathway, the immune system and antigen presentation[52]. Previous studies on CNS tissues from BSE-infected animals also showed the involvement of many of these pathways in prion pathogenesis [4, 35, 52]. This similarity between brain and blood may not be surprising, since it has been shown in the literature that blood transcriptome analyses identify genes that

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**Fig 5. Validation of microarray data by RT-qPCR.** Differential expression of selected genes in blood from preclinical (A) and clinical (B) atypical BSE-infected cattle. Ganulysin (GNLY), X-inactive specific transcript (XIST), pyruvate dehydrogenase kinase 4 (PDK4), CD40 ligand (CD40L), haemoglobin, alpha 2 (HBA2) and Sel-1 Suppressor Of Lin-12-Like 3 protein (SEL1L3). Gene expression (ratio) values are represented as relative to RNA levels in control animals. Ns = not significant; *P value ≤ 0.05.

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are relevant to the pathological processes occurring in the CNS [53]. Indeed, measuring disease-related gene expression in peripheral blood may be a useful proxy measure for gene expression in the CNS [53, 54].

To characterize the gene expression profile in the preclinical and clinical stages, we performed the PvsCtrl and the CvsCtrl statistical comparisons. We found that 113 probe sets were differentially regulated in the preclinical stage of the disease, while 207 probe sets had an altered expression in the clinical phase. Importantly, the present results indicated that, at least in blood, a consistent gene expression alteration is present from the early stages of the disease. This finding is in agreement with microarray analysis carried out by Tang et al., which revealed the highest degree of differential gene regulation in brains of cBSE-infected cattle at 21 months post infection, which is prior to the detection of infectivity [4]. Also, Tortosa and colleagues found a significant number of DEGs at early stages of the disease in the CNS from cBSE-infected transgenic mice [52].

Venn diagram analysis revealed that 32 DEGs were in common between the clinical and preclinical groups and, remarkably, they had a very similar pattern of expression in both stages of the disease. Since these genes are altered in both phases, it would be very interesting to confirm their differential expression in future experiments with additional negative controls, and eventually in blood from human patients.

Based on GO enrichment analysis, we found that immunity and inflammation processes were strongly involved during the progression of the disease stages. Interestingly, we found that antigen processing and presentation via MHC (major histocompatibility complex) molecules and the autoimmune thyroiditis pathway were significantly altered in atypical BSE-challenged animals. The majority of MHC class I molecule coding-genes were down-regulated in infected cattle (three out of four probes) and, also, MHC class II molecule coding transcripts were found to be down-regulated during the progression of the clinical signs (four out of four probes were down-regulated in the CvsP comparison). The involvement of MHC transcripts in prion pathogenesis is supported by another microarray study published by Kaniya and colleagues in 2009 [55]. In line with the trend found by the microarray analysis, the RT-qPCR validation experiments indicated a downregulation for MHC class I heavy chain (BOLA), even though the results failed to reach the statistical significance (data not shown).

Regarding the autoimmune thyroiditis pathway, it is well known in the literature that Hashimoto’s encephalitis, together with the associated thyroiditis, is a differential diagnosis for CJD, since the two pathologies share a very similar clinical symptomatology [56]. As hypothesized previously by Prusiner and colleagues, the clinical and neuropathological similarities between CJD and Hashimoto’s thyroiditis raise the possibility that protein misprocessing may underlie both neurodegenerative and autoimmune diseases [57].

Finally, a fourth statistical analysis was performed to identify any specific changes between the clinical and the preclinical stages of disease (CvsP). Indeed, we found that the last phases of the disease are accompanied by the overactivation of several genes involved in the immune defense response. In particular, the shift from the preclinical towards the clinical stage was characterized by the upregulation of genes involved in B cell proliferation and the ISG15 (IFN-induced 15-kDa protein) conjugation system. ISG15 is a ubiquitin-like molecule that is tightly regulated by specific innate immunity signaling pathways [58]. Interestingly, it has been shown in the literature that this protein is over-activated in the spinal cord of amyotrophic lateral sclerosis mice models [59] and it has been indicated as a general marker for both acute and chronic neuronal injuries [60].

To further analyze the data, we compared the list of DEGs found in PvsCtrl, CvsP and CvsCtrl and found 22 genes with an oscillatory pattern of expression, being differentially expressed in the preclinical stage and then going back roughly to the control level in the clinical stage.
Interestingly, some of the oscillatory DEGs are involved in regulation of transcription, thus suggesting that the gene expression during atypical BSE infection is tightly regulated. Venn diagram analysis revealed that one gene, *SEL1L3*, was down-regulated in all the comparisons (PvsCtrl, CvsCtrl, CvsP). *SEL1L3* codes for a transmembrane protein whose function is unknown. Interestingly, an important paralog of *SEL1L3, SEL1L*, is involved in the retrotranslocation of misfolded proteins from the lumen of the endoplasmic reticulum to the cytosol, where they are degraded by the proteasome in an ubiquitin-dependent manner [61]. Therefore, we could hypothesize that its down-regulation in prion infected animals would lead to a reduced degradation of PrPSc, thus supporting the progression of the disease. We validated this gene by RT-qPCR, confirming its downregulation in both the preclinical and clinical stages of the disease. Further investigation on the function of *SEL1L3L* would be of great interest since this gene may play an important role in prion disease and maybe other neurodegenerative illnesses.

Besides *SEL1L3*, five other genes were validated by RT-qPCR; here we will briefly discuss how these genes may be involved in prion pathogenesis and in host response to prion infection. *GNLY* and *CD40L* were found to be down-regulated in both preclinical and clinical stages. GNLY is a powerful antimicrobial protein contained within the granules of cytotoxic T lymphocyte and natural killer cells. This gene was found to be downregulated also in a microarray study performed on the medulla oblongata from sheep with preclinical natural scrapie [62]. Thus, it may be a good candidate as an early biomarker for atypical BSE but also for other prion diseases.

*CD40–CD40L* interactions mediate a broad variety of immune and inflammatory responses and have been implicated in the pathogenesis of Alzheimer’s disease (AD) [63, 64]. Although the importance of *CD40L* in prion disease progression has not yet been clarified [66–68], its downregulation in blood during both preclinical and clinical stages of atypical BSE-infection suggests that prion infection has an impact on the host immune system response and that immune tolerance may be an active process induced by prions.

Two other downregulated genes were validated by RT-qPCR, namely *HBA2* and *XIST*. Concerning *HBA2*, we found a downregulation in preclinical atypical BSE-infected cattle. Haemoglobins are iron-containing proteins that transport oxygen in the blood of most vertebrates. Beside blood, *HBA* and *HBB* are also expressed in mesencephalic dopaminergic neurons and glial cells [65] and are down regulated in AD, PD and other neurodegenerative diseases [66]. Haemoglobin genes expression alteration during preclinical scrapie was also found in the spleen and CNS of infected animals [67, 68], as well as in the brains of nonhuman primates infected with BSE [51]. These findings suggest an involvement of these genes in the host response to general neurodegenerative processes. Besides changes in transcript levels, it has been found that both HbA and HbB protein distribution is altered in mitochondrial fractions from PD degenerating brain [69]. Moreover, HbA is also expressed in endothelial cells, where it regulates the nitric oxide signaling [70]. Even though a clear mechanism linking these molecules to neurodegeneration has not yet been described, taken together these findings strongly suggest a central role for haemoglobin in neurodegenerative processes.

A marked downregulation in *XIST* expression was found in our infected animals. *XIST* is a gene located on X chromosomes which codes for a long non-coding RNA (LncRNA) involved in X-chromosome dosage control [71, 72]. LncRNAs are emerging as useful biomarkers for neurodegenerative diseases such as AD [73] and other disease processes [74], and they can be easily detected in blood and urine from patients. In addition, we cannot exclude the possibility that the alteration in *XIST* expression may have some role in gender-dependent response to prion infection [75].

RT-qPCR experiments confirmed the upregulation of *PDK4* in clinically affected animals. *PDK4* encodes for a mitochondrial protein involved in glucose metabolism through the
inhibition of pyruvate dehydrogenase complex, which leads to a reduction in pyruvate conversion to acetyl-CoA [76]. In the literature, a key role has been suggested for acetyl-CoA fueling for the survival of cholinergic neurons in the course of neurodegenerative diseases [77]. PDK4 overactivation can lead to a switch from glucose catabolism to fatty acid utilization [78], thus increasing the production of ketone bodies. Notably, it has been shown in the literature that these molecules are able to cross the blood brain barrier. We could speculate that in prion infection (or at least in atypical BSE infection) the concentration of ketone bodies would rise in blood, as a consequence of PDK4 upregulation, and act in the brain as neuroprotective molecules [79, 80]. This would be an attempt by the organism to prevent the neurodegeneration induced by prions.

Conclusions

In conclusion, the present study has led to the identification of several gene expression changes in whole blood from clinical and preclinical atypical BSE cattle, which upon further investigation and validation in blood from human patients, might represent a molecular fingerprint to characterize this disease. By comparing our results with other studies on various animal prion diseases, we observed that some of the most significantly altered DEGs we found in blood were found differentially expressed also in brain tissue from BSE-infected cattle; this observation indicates that whole blood transcriptome analyses may serve as a proxy measure for the changes occurring in the CNS of infected animals. Furthermore, our study underlines the importance of utilizing whole blood, without any additional manipulation, as a source tissue as it is an easily accessible body fluid. In addition, the transcription regulation activated in atypical BSE infections is similar to some extent to the one observed in the literature for cBSE, even though the clinical characteristics and biochemical properties are very different. Thus, this gene expression profile may be investigated in other BSE infections to identify a common molecular fingerprint.

Overall, our study confirmed the differential expression of 6 genes (XIST, CD40L, GNLY, PDK4, HBA2 and SEL1L3), which may play several roles in atypical BSE pathogenesis and, possibly, in other prion infections. Indeed, they are involved in multiple pathways such as immune response, inflammation, and glucose catabolism. Even though further studies are required to investigate the specific involvement of all the identified genes in prion diseases, our data indicate an important role for immune system regulation in the prion pathogenesis of atypical BSE and maybe in BSE as well as in other prion diseases in general.

Supporting Information

S1 Appendix. Post hybridization quality assessment. (A) Normalized unscaled standard error (NUSE), (B) relative log expression (RLE) and (C) raw signal intensity plots are used to check for technical problems and to spot outlier samples after GCRMA normalization. Box plots centered higher than normal (typically above 1.1 in the NUSE plot) and/or having a larger spread in the RLE plots represent arrays with quality problems. One outlier was easily identified by post hybridization quality assessment (black arrow in panel A and B, control sample cS5).

(TIF)

S2 Appendix. Reference gene expression stability. Stability of the selected reference genes was determined by calculating their geNorm M value (M) (A) and the coefficient of variation (CV) (B) on the normalized relative quantities (CNRQ). The dashed green lines in panel A and B indicate the maximum acceptable threshold for M and CV values, respectively. These
thresholds have been empirically determined by previous experiments performed by Hellemans et al. (see ref. 42).

(TIF)

S1 Table. Differentially expressed genes found in preclinical animals versus controls (PvsCtrl) by the microarray analysis. FC = fold change. Common DEGs between preclinical and clinical animals are highlighted in bold.

(XLSX)

S2 Table. Differentially expressed genes found in clinical animals versus controls (CvsCtrl) by the microarray analysis. FC = fold change. Common DEGs between preclinical and clinical animals are highlighted in bold.

(XLSX)

S3 Table. Functional classification of differentially expressed genes found in blood of clinical versus preclinical animals. The gene enrichment analysis was performed using DAVID bioinformatics tool 6.7 (NIAID/NIH, USA). Only genes with a known GO are represented in the list. The DEGs which fell in more than one category for simplicity are presented under a single functional heading. FC and P values refer to the microarray analysis.

(XLSX)

S4 Table. List of DEGs found in HvsL statistical comparison. FC = fold change.

(XLSX)

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Author Contributions

Conceived and designed the experiments: GL MB C. Casalone C. Corona MC. Performed the experiments: EX HK MNC EVC. Analyzed the data: PV. Contributed reagents/materials/analysis tools: MS. Wrote the paper: EX MB GL MS.

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