Vet. Res. (2009) 40:23
DOI: 10.1051/vetres/2009006

www.vetres.org

Original article

Rapid and widely disseminated acute phase protein response after experimental bacterial infection of pigs

Kerstin Skovgaard[1]*, Shila Mortensen[1], Mette Boye[1], Karin T. Poulsen[1], Fiona M. Campbell[2,3], P. David Eckersall[2], Peter M.H. Heegaard[1]

[1] National Veterinary Institute, Technical University of Denmark, 27 Bülowsvej, DK-1790 Copenhagen V, Denmark
[2] Faculty of Veterinary Medicine, University of Glasgow, Glasgow, United Kingdom
[3] Present address: The Rowett Research Institute, Greenburn Road, Bucksburn, Aberdeen, United Kingdom

(Received 4 July 2008; accepted 20 February 2009)

Abstract – The acute phase protein response is a well-described generalized early host response to tissue injury, inflammation and infection, observed as pronounced changes in the concentrations of a number of circulating serum proteins. The biological function of this response and its interplay with other parts of innate host defence reactions remain somewhat elusive. In order to gain new insight into this early host defence response in the context of bacterial infection we studied gene expression changes in peripheral lymphoid tissues as compared to hepatic expression changes, 14–18 h after lung infection in pigs. The lung infection was established with the pig specific respiratory pathogen *Actinobacillus pleuropneumoniae*. Quantitative real-time PCR based expression analysis were performed on samples from liver, tracheobronchial lymph node, tonsils, spleen and on blood leukocytes, supplemented with measurements of interleukin-6 and selected acute phase proteins in serum. C-reactive protein and serum amyloid A were clearly induced 14–18 h after infection. Extrahepatic expression of acute phase proteins was found to be dramatically altered as a result of the lung infection with an extrahepatic acute phase protein response occurring concomitantly with the hepatic response. This suggests that the acute phase protein response is a more disseminated systemic response than previously thought. The current study provides to our knowledge the first example of porcine extrahepatic expression and regulation of C-reactive protein, haptoglobin, fibrinogen, pig major acute phase protein, and transferrin in peripheral lymphoid tissues.

acute phase protein / systemic response / innate defence / gene expression / pig

1. INTRODUCTION

The acute phase response is a well-orchestrated series of early host response events playing an important role in innate immunity of all vertebrates, where it is believed to help restore homeostasis after e.g. infection or other events involving some degree of tissue destruction [5]. Altered hepatic synthesis of a wide spectrum of acute phase proteins (APP), is one of the main outcomes of the acute phase response. APP are defined as any serum protein whose plasma concentration increases (positive APP) or decreases (negative APP) more than 25% after infection, inflammation, trauma, and some types of cancer [27].

APP have been known since the discovery of C-reactive protein (CRP) in 1941 [1], however, although widely used as markers of infection and inflammation in human and veterinary clinical medicine [39], little is known of the significance of the APP response and its interplay with other types of innate defence responses. Some APP have functions directly associated with host defences e.g. by promoting wound repair (fibrinogen, FIB), enhancing phagocytosis (CRP), binding of hemoglobin and reducing oxidative

* Corresponding author: kesk@vet.dtu.dk

damage (haptoglobin). However, the role of other APP as e.g. serum amyloid A (SAA), which is a very dominant APP in most species, is less evident or speculative [8, 49]. The liver is the major producer of APP [14]. Extrahepatic expression of APP has been demonstrated in mice undergoing lipopolysaccharide induced systemic [23, 35], and local (lung [46]) inflammation. In addition, non-hepatic production of SAA, haptoglobin (Hp) and α1-acid glycoprotein (AGP) has been described in the mammary gland and in lung tissue of mice, primates, and cattle during various infections [10, 34, 48]. Knowledge on the extrahepatic production and regulation of APP in porcine peripheral lymphoid tissues is scarce. In a recent study, Hiss et al. [19] demonstrated an increased number of Hp-positive cells (epithelial cells and immigrated leukocytes) in lungs from pigs with bronchopneumonia, supporting a role for Hp as a hemoglobin scavenger during extravasation of erythrocytes into the lungs. Naranjo et al. [36] demonstrated elevation of SAA mRNA and decrease of albumin (ALB) and apolipoprotein A-I (APOA1) protein in lymph nodes of European wild boars after natural infection with *Mycobacterium bovis*.

The objective of the present study was to gain new insight into the nature and regulation of the APP response shortly after establishment of a localized bacterial infection with specific emphasis on the role played by non-hepatic tissues different from the infection locus. As also noted by Kalmovarin et al. [23] extrahepatic expression of APP could serve as an efficient rapid response to local infection and would add to the biological importance of the APP response. Thus we hypothesized that the APP response would occur in a disseminated manner, mobilizing different tissues simultaneously with the liver to produce APP. To study this, pigs were experimentally infected with *Actinobacillus pleuropneumoniae*. This Gram negative bacterium causes an acute and very rapidly evolving pleuropneumonia in pigs, reliably reproduced by an endotracheal/intratracheal infection model [25]. As pleuropneumonia caused by *A. pleuropneumoniae* is highly relevant in intensive pig production worldwide, some aspects of the accompanying host responses have previously been published, including cytokine, APP and cellular responses in serum and blood [17, 21], in lung tissue [2] and in lung macrophages [20]. Porcine APP for which correlations between serum concentrations and disease have been studied [21, 28], include CRP, SAA, Hp, FIB, AGP, and pig major acute phase protein (pigMAP) also known as inter-alpha trypsin inhibitor heavy chain 4 (ITIH4) as positive APP and ALB, APOA1, transthyretin (TTR), and transferrin (TF) as negative APP [7, 39, 43].

We report here for the first time a detailed study of the changes in expression of APP in several porcine tissues after experimental *A. pleuropneumoniae* infection. Expression patterns of innate immune factors including a selection of the APP and the key pro-inflammatory mediator interleukin-6 (IL-6) were compared between tonsils, spleen, trancheobronchial lymph nodes, blood leukocytes, and liver. In addition, protein serum concentrations of selected APP and IL-6 were determined. It was demonstrated that APP expression and acute phase regulation were disseminated to a wide range of tissues throughout the body as early as 14–18 h after experimental lung infection.

2. MATERIALS AND METHODS

2.1. Experimental infections, tissue collection and RNA extraction

Twelve 8–10-week-old castrates of Danish Landrace/Yorkshire/Duroc crosses from a high health herd, free from *A. pleuropneumoniae*, (pigs Nos. 25–36) were inoculated intranasally with *A. pleuropneumoniae* serotype 5b, isolate L20 as described previously by Skovgaard et al. [42]. Five non-inoculated pigs from the same herd constituted the non-inoculated control group (pigs Nos. 20–24). The low number of animals in the control group was accepted, as this group was believed to behave in a more homogeneous way than the inoculated group. All animal procedures were approved by the Danish Animal Experiments Inspectorate.

Cultivation of bacteria as well as inoculation of the pigs were performed as previously described [25]. Animals were sacrificed 14–18 h after inoculation and necropsied immediately. Severity of lung lesions were rated using the following scale: 0: no

lesions; 1: 1 or 2 lesions < 15 mm in diameter; 2: 1 or 2 lesions (each < 50 mm in diameter) or a few scattered petechiae; 3: more than two lesions (each < 50 mm in diameter) or numerous scattered petechiae; 4: more severe than 3 [3]. The inoculation strain, *A. pleuropneumoniae* serotype 5B, was re-isolated from all 12 inoculated animals and from none of the 5 control animals. Ten of the 12 inoculated pigs developed characteristic, well-demarcated, lung lesions of variable severity (5 pigs with a lung lesion score (LLS) of 4 and 5 pigs with a LLS of 3). Two inoculated pigs (Nos. 29 and 35) showed no pathological changes deriving from lung infection (LLS = 0) and were therefore excluded from the gene expression analyses. No pathological changes were seen in lungs from the 5 control animals.

Tissue samples of approximately 500 mg were taken from liver, tracheobronchial lymph nodes, spleen, and tonsils. All samples were snap frozen in liquid nitrogen and stored at −80 °C until RNA extraction. Blood samples for RNA extraction were collected in tubes containing heparin (HEP tubes, Terumo, Herlev, Denmark). Leukocytes were immediately isolated by centrifugation (400 *g* for 10 min at 4 °C) after depletion of red blood cells using QIAamp hypotonic EL buffer (Qiagen, Albertslund, Denmark), and stored at −80 °C until RNA extraction [42]. Blood samples for serum ELISA were collected as unstabilized samples and allowed to clot for 1–2 h at room temperature or at 4 °C overnight before harvesting serum. Serum was kept at −20 °C until analysis. To re-isolate the inoculation strain, samples from lung, liver, tonsils and spleen were cultivated on PPLO agar (BD Diagnostic Systems, Brøndby, Denmark) and serotyped using latex agglutination [13].

Total RNA from liver, tracheobronchial lymph nodes, spleen, tonsils, and leukocytes were extracted as previously described using RNeasy midi kit, Qiagen (liver), RNeasy lipid midi tissue kit, Qiagen (lymph nodes, tonsils, and spleen), and QIAamp RNA blood, Qiagen (leukocytes) [42]. All RNA samples were treated with RNase-free DNase Set, Qiagen, according to the manufacturer's instructions. Quantity and quality of extracted total RNA was measured using a Nanodrop ND-1000 spectrophotometer (Saveen and Werner AB, Limhamn, Sweden) and the Agilent 2100 Bioanalyser (Agilent Technologies, Nærum, Denmark) respectively. The Agilent Bioanalyser assigns a RNA Integrity Number (RIN) from 1–10 to each total RNA sample, with 10 being non-degraded RNA. All samples in the present study had a RIN number above 7. Moderately degraded samples (RIN between 4 and 6) were previously shown to be applicable to expression analysis provided that careful normalization is performed [11].

2.2. Quantitative RT-PCR (qRT-PCR)

Total RNA was converted into first-strand cDNA by reverse transcription of RNA using QuantiTect Reverse Transcription (Qiagen) according to the manufacturer's instruction. The cDNA was stored at −20 °C until further use. Quantitative RT-PCR was performed on a RotorGene 3000 Detection System (Corbett Research, Sydney, Australia) using ampliQ Universal Real Time Master mix (Bie & Berntsen, Rødovre, Denmark), template cDNA, and gene specific primers (Tab. I) as described previously [42]. Melting curves were generated after each run to confirm a single PCR product. All reactions were performed in triplicate and outliers were excluded using the T_n test [31]. Standard curves generated from dilution series constructed from high responding samples were used to assign relative concentrations to all samples. The freely available software Primer3[1] was used for primer design, while gene sequences were obtained from public databases and from the Sino-Danish Pig Genome Project [30]. If possible, primers were designed to span intron/exon boundaries to prevent amplification of contaminating genomic DNA, if present. BLAST searches were performed to ensure the absence of intraspecies polymorphisms at the primer site.

Validity of qRT-PCR data relies on accurate normalization of gene expression data, as RNA recovery and cDNA synthesis efficiency might vary from sample to sample. In the present study, all data were carefully normalized to the geometric mean of the three most stably expressed reference genes as described by Vandesompele et al. [45]. Tissue specific reference genes were selected using the dedicated validation program geNorm, ranking six potential reference genes (hypoxanthine phosphoribosyl-transferase 1 (HPRT1), ribosomal protein L13a (RPL13A), phosphatidic acid phosphatase type 2 domain containing 1B (HTPAP), beta-2-microglobulin (B2M), glyceraldehyde-3-phosphate dehydrogenase (GAPDH), and β-actin) that were analysed in all tissues, from best to worst based on the stability of their expression. A normalization factor based on GAPDH, HPRT1, and HTPAP was used to standardize liver expression data between animals, as these genes were found to be most constantly expressed in this tissue using the geNorm software. Likewise, expression data were normalized with HPRT1, RPL13A, and HTPAP in leukocytes, HPRT1, β-actin, and HTPAP in tracheobronchial lymph nodes and RPL13A, B2M, and HPRT1 in tonsils, as well as HTPAP, β-actin, and GAPDH in

[1] http://frodo.wi.mit.edu/cgi-bin/primer3/primer3www.cgi.

Table I. Primers and conditions used for qRT-PCR.

Gene	Sequence	Ta°C[a]	Total $MgCl_2$[b] (in mM)
AGP	F: AGTCCTGAGCCTCCTTCCTC R: GCCGAGCCGATATAATACCA	60	1.5
ALB	F: CAGAAGTTTTGGGGAAAATACCT R: TAAAGGAGTTCTGGGGCGTA	60	3.0
APOA1	F: GTTCTGGGACAACCTGGAAA R: GCTGCACCTTCTTCTTCACC	60	3.0
B2M	F: TGAAGCACGTGACTCTCGAT R: CTCTGTGATGCCGGTTAGTG	62	1.5
β-actin	F: CTACGTCGCCCTGGACTTC R: GCAGCTCGTAGCTCTTCTCC	62	1.5
CRP	F: CTTTTGCCCAGACAGACATGAT R: GAGTGGTTTGGTGAGCCTTG	60	1.5
FIB	F: GAATTTTGGCTGGGAAATGA R: CAGTCCTCCAGCTGCACTCT	59	3.0
GAPDH	F: ACCCAGAAGACTGTGGATGG R: AAGCAGGGATGATGTTCTGG	62	1.5
Hp	F: ACAGATGCCACAGATGACAGC R: CGTGCGCAGTTTGTAGTAGG	60	1.5
HPRT1	F: ACACTGGCAAAACAATGCAA R: TGCAACCTTGACCATCTTTG	62	1.5
HTPAP	F: GCTCTTGGCAGCTGTGATTG R: ATCCGTCAGGGGAGGGTAGT	59	3.0
PigMAP	F: ATGACAGCAAGCGAACAGTG R: GGGGATCCCTCTTGGTAATC	60	1.5
RPL13A	F: ATTGTGGCCAAGCAGGTACT R: AATTGCCAGAAATGTTGATGC	62	3.0
SAA2	F: TAAAGTGATCAGCAATGCCAAA R: TCAACCCTTGAGTCCTCCAC	60	1.5
TF	F: CTCAACCTCAAAACTCCTGGAA R: CCGTCTCCATCAGGTGGTA	60	3.0
TTR	F: TTGCCTTGGGGAAAACCA R: TGGTGTCCAATTCCACTTTG	60	1.5
IL-6	F: TGGGTTCAATCAGGAGACCT R: CAGCCTCGACATTTCCCTTA	60	3.0

[a] Ta°C: Primer annealing temperature. [b] $MgCl_2$: Total concentration of $MgCl_2$ in reaction mix.

spleen. GAPDH, β-actin, and RPL13A were used for data normalization in the analysis of gene expression differences between different tissues. To visualize differential expression of genes between the 10 out of 12 infected pigs revealing characteristic lung lesions, and the 5 non-infected control animals, the mean, normalized value for the infected group was displayed relative to the mean normalized value for the control group, set to 1. Results are presented as the mean ± standard error (SEM) and Wilcoxon rank sum test was performed to test for significance of gene expression. To demonstrate that the Wilcoxon rank sum test was robust with regards to the relatively low number of animals, the statistical power was estimated for eight APP expressed in the liver, using a permutation test with 10 000 resamplings.

2.3. Quantitative serum ELISA

Serum concentrations of the acute phase proteins CRP, SAA, Hp, and TTR were determined in two control and in all 12 infected animals at −1 and day 1 after the infection. CRP was analysed by a sandwich type ELISA using dendrimer-coupled cytidine diphosphocholine (a CRP-binding ligand) in the coating layer as described in [18] employing polyclonal rabbit anti-human antibodies with cross-reactivity towards porcine CRP followed by peroxidase-conjugated goat anti rabbit antibody for detection (both antibodies from DAKO, Glostrup, Denmark). The cross-reactivity of the anti human CRP antibody with pig CRP was demonstrated previously [17] and combining it with catching of CRP by diphosphocholine ensures

specificity for CRP. Pooled pig serum calibrated against a human CRP calibrator (DAKO A0073) was used as standard. The detection limit was 0.067 mg/L (human equivalents). A commercially available sandwich ELISA assay (Phase SAA assay, Tridelta Development Ltd., Kildare, Ireland) was used for determination of SAA. This assay is based on anti-human monoclonal antibodies in a sandwich set-up as originally described by McDonald et al. [33]. Samples were tested according to the manufacturer's instructions except that the lowest dilution was 1:20 to increase signal intensity. The detection limit of the assay was 2.5 mg/L (porcine SAA equivalents). Hp was determined by a sandwich ELISA using an in-house mouse anti-porcine Hp monoclonal antibody in the coating layer and biotinylated commercial rabbit anti human haptoglobin (DAKO A0030) as the detection antibody as described previously [43] with a detection limit of 3.3 mg/L (porcine Hp equivalents). All of the ELISA described above were developed using orthophenylenediamine (OPD)/peroxide and reading optical densities of wells at 490 nm subtracting unspecific coloration at 650 nm using an automatic plate reader (Thermo Multiskan Ex spectrophotometer, Thermo Scientific, Waltham, MA, USA). All samples including standards were determined in duplicate. Sample values were calculated from the curve fitted to the readings of the standard (using Ascent software v. 2.6, Thermo Scientific). TTR was measured using an in-house ELISA, as described previously by Campbell et al. [6]. Diluted serum samples or purified human prealbumin (TTR) (used as standard) (Sigma-Aldrich, Poole, UK) were used to coat microtiter plates (96 well, Corning Costar, Cambridge, UK) and TTR was detected using sheep anti-human prealbumin (IgG fraction) (ICN Biomedicals, Basingstoke, UK) followed by peroxidase-conjugated anti-sheep IgG (Sigma). Plates were developed with tetramethylbenzidine (TMB) substrate solution (KPL, Guildford, UK), the reaction was stopped with 1M HCl and absorbance read at 450 nm. TTR concentrations in the serum samples were compared to a standard curve of human TTR over a range of 0.03–2.00 μg/mL. Samples were run as single determinations at 1/400 dilution and the detection limit for the assay was 12.8 mg/L (human TTR equivalents). IL-6 serum concentrations were determined by a porcine IL-6 ELISA from R&D Systems (Duoset DY686). This is a sandwich ELISA using goat anti pig IL-6 for catching and biotinylated goat anti pig IL-6 for detection also including a porcine IL-6 standard. Development of plates was done with TMB peroxide color substrate from Kem-En-Tec (Taastrup, Denmark), following the manufacturer's instructions. Samples were run in duplicates in a dilution of 1:2 with a detection limit of 0.25 μg/L.

Acute phase protein concentrations at 1 day post infection (dpi) were compared for each animal individually to the mean of the concentration of the acute phase protein in question of all animals at −1 dpi, testing significance using two-tailed unpaired *t*-test. The Spearman rank correlation coefficient rho was used to assess possible correlations between LLS, IL-6 protein, IL-6 mRNA and number of tissues from which the inoculation strain was re-isolated. To test whether the observed value rho was statistically significant, we performed a one-sided *t*-test on $\rho/\sqrt{(1-\rho^2)/(n-2)}$, with n the number of data points in each group with critical values depending on the reaction pattern (positive/negative) of the protein.

3. RESULTS

3.1. Gene expression

Gene expression in liver tissue of CRP, FIB, Hp, IL-6, PigMAP, and SAA2 were significantly ($p < 0.05$) higher within the infected group ($n = 10$). Whereas gene expression of AGP, ALB, APOA1, TTR, and, TF were found to be significantly down-regulated ($p < 0.05$). Furthermore, qRT-PCR was used to quantitate the extrahepatic expression of several APP in the following tissues: tracheobronchial lymph nodes, spleen, tonsils, and leukocytes (Tab. II and Fig. 1). mRNA coding for ALB, APOA1, CRP, Hp, PigMAP, SAA2, TF, TTR and IL-6 was detected in all tissues investigated. Expression of AGP was found in all tissues but tonsil, while expression of FIB was only found in tracheobronchial lymph nodes, spleen, and liver. CRP, Hp, SAA2, and IL-6 were up-regulated ($p < 0.05$) in leukocytes and tracheobronchial lymph nodes of the infected pigs. In the spleen PigMAP, FIB, SAA2, Hp, and IL-6 were significantly up-regulated in the infected animals while TF and APOA1 were significantly down-regulated. In the tonsils IL-6, PigMAP, and SAA2 were up-regulated ($p < 0.05$) in the infected group (Tab. II). The biological variation in the infected group was not correlated to the exact time of death within the 4 h interval used for sacrifice.

Expression level differences of CRP, Hp, SAA2, and IL-6 between liver, leukocytes,

Table II. Extrahepatic and hepatic expression of APP and IL-6 ± standard error of the mean. The mean value of the 10 infected animals is shown relative to the 5 control animals as described in Materials and methods.

Gene	Tissue				
	Liver	Leukocytes	Spleen	Lymph	Tonsil
AGP	0.46 ± 0.04**	E (3/5 10/10)[a]	E (1/5 5/10)	E (2/5 2/10)	Not expressed
ALB	0.16 ± 0.02***	E (5/5 9/10)	E (5/5 1/10)	E (1/5 1/10)	0.75 ± 0.08
APOA1	0.53 ± 0.08*	0.53 ± 0.23	0.74 ± 0.09*	2.03 ± 1.26	1.16 ± 0.46
CRP	2.10 ± 0.41*	4.88 ± 1.72**	E (3/5 5/10)	3.70 ± 0.81***	E (2/5 5/10)
FIB	3.02 ± 0.21***	Not expressed	4.12 ± 1.11***	E (4/5 9/10)	Not expressed
Hp	1.92 ± 0.23**	23.23 ± 5.24***	21.55 ± 4.93***	3.42 ± 0.81**	0.94 ± 0.41
PigMAP	7.47 ± 0.48***	E (5/5 10/10)[b]	4.36 ± 0.62***	5.43 ± 1.72*	2.27 ± 0.55**
SAA2	68.19 ± 12.90***	95.66 ± 21.30***	24.52 ± 9.70***	668 ± 160**	2.11 ± 0.28*
TF	0.17 ± 0.02***	0.45 ± 0.12	0.37 ± 0.05**	1.30 ± 0.26	1.32 ± 0.27
TTR	0.04 ± 0.0***	E (1/5 1/10)	0.39 ± 0.06	0.83 ± 0.18	E (4/5 6/10)
IL-6	11.16 ± 8.30*	7.46 ± 3.31*	14.08 ± 7.49***	167 ± 130**	4.37 ± 1.81*

[a] Expressed in 3 out of 5 control animals and 10 out of 10 infected animals.
[b] Due to very low expression in a few control animals we were not able to quantify PigMAP in leukocytes.
* $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$ Wilcoxon rank sum test.

tracheobronchial lymph nodes, spleen, and tonsils were further compared in the 10 infected animals (Fig. 1). Expression levels for each gene product measured in each tissue was compared to leukocyte expression, set to 1, as shown in Figure 1 to visualize tissue specific gene expression. As seen (Fig. 1) all APP were highly expressed in the liver. Furthermore, SAA2 was expressed at a significantly higher level in tonsils and in regional lymph nodes compared to leukocytes ($p = 0.001$). Hp was expressed at a significantly higher level in the lymph nodes and liver compared to leukocytes, and at a significantly lower level in spleen and tonsil compared to leukocytes (Fig. 1). CRP was expressed at a significantly higher level in liver and lymph nodes compared to leukocytes (Fig. 1). CRP expression levels from the spleen and tonsils were not included in Figure 1 as only five of the ten infected animals expressed CRP (see Tab. II). Expression of PigMAP was found to be at a significantly higher level in spleen (× 10), in lymph nodes (× 62) and in liver (× 1000) compared to leukocytes. To demonstrate that the Wilcoxon rank sum test was robust with regards to the low number of animals, the statistical power was estimated for a subsample of APP expressed in the liver. Statistical power was found to be 1 for 6 out of 8 APP tested, as no overlap was present between data for healthy and infected tissue, and 0.75 and 0.80, respectively, for the last two, indicating that the number of biological replicates used in the present study was sufficient due to relative low variation within the two groups.

3.2. Protein concentrations in serum

Serum protein analyses were performed on samples from all 12 inoculated pigs and two pigs from the control group (Fig. 2). Prior to inoculation all pigs had CRP-values below 6 μg/mL (mean ($n = 14$): 1.13 μg/mL, SD: 1.57) while 14–18 h after infection CRP concentrations were significantly increased in all animals of the inoculated group (14.8 μg/mL, SD: 5.38 ($n = 11$)), with the exception of animal No. 29, the CRP concentration of which (2.79 μg/mL) was not statistically different from the pre-inoculation mean concentration. The same general pattern was seen with SAA, except that one pre-inoculation control showed a slightly raised value (12.10 μg/mL, pig No. 32); apart from that, all the pre-inoculation values were below the detection limit of the assay (2.5 μg/mL). The mean post-inoculation level was 224.48 μg/mL (SD: 48.75, $n = 11$).

Also, for SAA, post-inoculation values of both pigs 29 and 35 were lower than for the rest of the infected animals, however pig No. 29 was clearly the lowest (below the detection limit) and the only

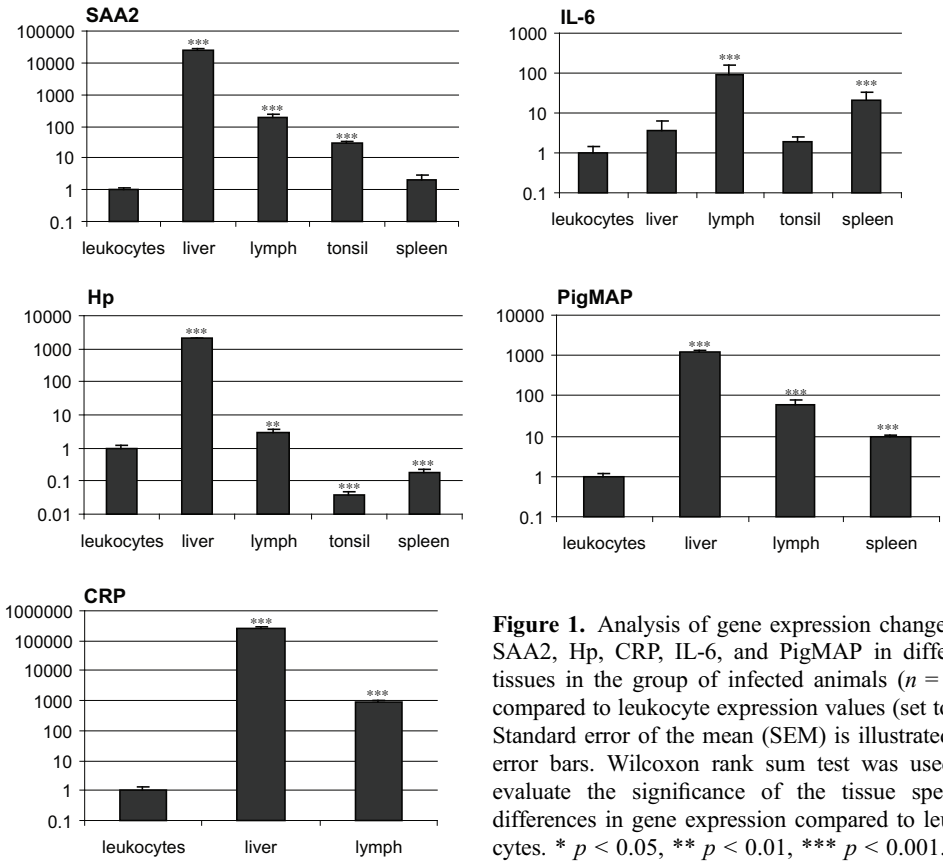

Figure 1. Analysis of gene expression changes of SAA2, Hp, CRP, IL-6, and PigMAP in different tissues in the group of infected animals (n = 10), compared to leukocyte expression values (set to 1). Standard error of the mean (SEM) is illustrated by error bars. Wilcoxon rank sum test was used to evaluate the significance of the tissue specific differences in gene expression compared to leukocytes. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$.

one not significantly different from the pre-inoculation mean. The two other APP measured, Hp (positive) and TTR (negative) did not respond within 14–18 h after inoculation, pre-inoculation concentrations not being different from post-inoculation concentrations. Although the mean Hp concentration tended to increase after inoculation this was not significant (SD were more than 230 µg/mL). Samples were not hemolysed to a degree affecting the Hp analysis. IL-6 was determined in the same samples (Tab. III) and while all pre-inoculation samples had IL-6 concentrations below the detection limit of the assay (250 pg/mL) except for pig No. 27, all post-inoculation samples, except pigs Nos. 25, 29 and 35, were above the detection limit with a wide variation of concentrations (from ca. 400 pg/mL to > 6 000 pg/mL). Correlations were found between LLS and IL-6 protein (rho = 0.68), LLS and IL-6 mRNA (rho = 0.78) as well as LLS and the number of tissues from which the inoculation strain was re-isolated (rho = 0.76) using the Spearman rank correlation test. Moderate correlation was found between serum concentration of IL-6 and CRP (rho = 0.43) as well as between IL-6 and SAA (rho = 0.63). All correlations with LLS were found highly significant ($p < 0.001$). The correlation between IL-6 and SAA was significant ($p < 0.01$), while the correlation between IL-6 and CRP was close to being statistically significant ($p = 0.06$).

4. DISCUSSION

We report here the expression of porcine APP, induced by an acute experimental infection, in several peripheral lymphoid tissues compared to the liver, which is the main source of APP. The

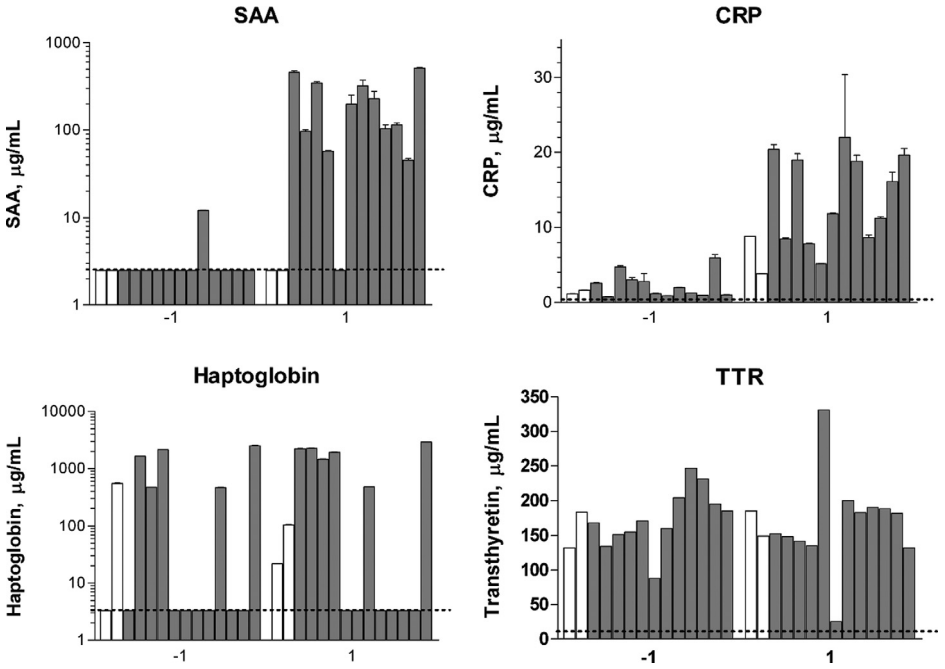

Figure 2. Serum concentrations in individual pigs of SAA, CRP, Hp and TTR before (−1 dpi) and after (1 dpi) inoculation with *A. pleuropneumoniae*. White bars represent uninfected controls (Nos. 23 and 24), and grey bars represent samples from inoculated animals (Nos. 25–36, depicted in numerical order). Detection limits are indicated by horizontal, dotted lines. Protein concentrations were determined by ELISA as described in the text. Mean and SD of two determinations are shown except for TTR (single determinations).

acute phase response was induced by infection of pigs with the lung pathogen *A. pleuropneumoniae* and was studied within the first 14–18 h after initiating the infection. As expected, the hepatic expression of a number of genes known to be involved in the acute phase protein response was dramatically affected by the infection. Up-regulated hepatic expression was seen for genes encoding CRP, FIB, Hp, PigMAP, SAA2, and IL-6, whereas genes for AGP, ALB, APOA1, TTR, and TF were down-regulated. This is in agreement with previous findings using cDNA microarrays [16] where hepatic expression of CRP, FIB, SAA, Hp, ALB, APOA1 and TTR was demonstrated after infection with *A. pleuropneumoniae*.

The hepatic response was accompanied by expression in tonsillar, splenic, and tracheobronchial lymphatic tissue as well as in leukocytes of ALB, APOA1, CRP, Hp, PigMAP, SAA2, TF, TTR, and IL-6. Extrahepatic production of ALB, APOA1, SAA, Hp, and PigMAP has previously been described in pigs [12, 32, 36, 40]. However, the current study provides to our knowledge the first example of extrahepatic expression of AGP, CRP, FIB, Hp, PigMAP, TF, and TTR in porcine peripheral lymphoid tissues. This firmly establishes that the APP response is not confined to the liver but is widely disseminated. Induction patterns were tissue specific with the spleen being most similar to the liver (Tab. II).

As seen in Table II, CRP, Hp, PigMAP, SAA2, and IL-6 were significantly induced after infection in most of the tissues with the tonsils being the least responsive tissue. TF, TTR, and APOA1, all negative APP, were decreased in a more restricted number of tissues. Notably, APOA1 and TF were significantly decreased in the spleen after infection. Extrahepatic expression and

Table III. Individual variation in lung lesion score, serum IL-6 concentration (dpi −1 and 1), IL-6 mRNA level in leukocytes relative to mean of control group (dpi 1 only) and re-isolation of inoculum strain of *A. pleuropneumoniae*.

Pig No.[a]	Lung lesion score[b]	IL-6 protein (pg/mL)		IL-6 mRNA (relative conc.) 1 dpi	Re-isolation[c]
		−1 dpi	1 dpi		
23	0	–	–	0.653	–
24	0	–	–	0.722	–
25	4	–	–	0.801	ton.
26	3	–	391	1.080	lu., ton.
27	3	392	810	2.522	lu., ton.
28	3	–	1429	3.316	lu., ton., li.
29	0	–	–	0.743	ton.
30	4	–	6114	25.906	lu., ton., li., spl.
31	4	–	2097	4.520	lu., ton., li., spl.
32	3	–	1628	1.180	lu., ton., li., spl.
33	3	–	461	1.694	lu., ton.
34	4	–	739	7.707	lu., ton., li., spl.
35	0	–	–	0.858	ton.
36	4	–	4614	25.841	lu., ton., li., spl.

[a] Control animals: 23 and 24, inoculated animals: 25–36. [b] Lung lesion scores: 0: no lesions, 1: 1–2 lesions, < 15 mm diameter, 2:1–2 lesions (each < 50 mm diam.) or a few scattered petechiae, 3: More than 2 lesions (each < 50 mm diam.) or numerous scattered petechiae, 4: More severe than 3. [c] Re-isolation: lu.: lung, li.: liver, spl.: spleen, ton.: tonsil.

regulation of a range of APP, especially SAA, has been documented in a number of species. For example, in a study of mice challenged systemically by intraperitoneal injection of bacterial endotoxin, acute phase response expression patterns of several APP, including SAA, ceruloplasmin, AGP, and Hp were reported for kidney, spleen, thymus, heart, brain, lung, and testis [23]. However, the present study demonstrates expression as well as significant extrahepatic up- or down-regulation of APOA1, CRP, Hp, FIB, PigMAP, SAA2, and TF in infected pigs compared to non-infected controls. Also, this is the first description of porcine TF being down-regulated in the liver after infection, indicating that it may be classified as a negative APP in the pig. This is in agreement with Kramer et al. [24], who found TF to be decreased in serum samples from pigs infected with *Salmonella*.

Porcine SAA2 was expressed in tracheobronchial lymph, tonsils, leukocytes, and spleen at levels ranging from 0.01% to 1% of that found in the liver (Fig. 1). This was comparable to the findings of Meek and Benditt [35] where various SAA isotypes were expressed in 15 different extrahepatic tissues at 0.1% to 10% of the levels found in the liver of mice. Also in non-hepatic tissues, SAA2 was subject to a dramatic acute phase regulation (Tab. II). Hp has previously been demonstrated in intestinal lymph nodes of pigs [44], in lung tissue from pigs suffering from bronchopneumonia using immunohistochemistry [19] and different levels of mRNA coding for Hp have been found in lung and other tissues of mice and baboon [23, 48]. However, the highly regulated expression of non-hepatic Hp after infection has not been shown in peripheral lymphoid tissue of pigs before. The same is true for TTR [4, 22], FIB [15, 38], AGP [23, 29], PigMAP [9] and CRP [26], the expression of which has been demonstrated in various non-hepatic tissues in different species but has never been described in pigs during an acute phase response.

APOA1 and ALB have recently been described in skeletal muscle tissue of newborn pigs suffering from intrauterine growth restriction (approximately 30% decrease in APOA1 protein, [47]) as well as in mandibular lymph nodes in wild boars (*Sus scrofa*) naturally infected with *Mycobacterium bovis* (APOA1 approximately 40% decreased at the protein level, [36]). In con-

trast to the decreased APOA1 protein levels, Naranjo et al. [36] found mRNA levels of APOA1 to be up-regulated in the same infected pigs, suggesting a large extent of post-transcriptional regulation. We show here that mRNA levels of APOA1 were significantly down-regulated in the spleen and liver of infected animals (Tab. II).

Extrahepatic APP are most probably produced by local resident tissue cells, as several APP were expressed at a significantly different level in the various tissues compared to leukocytes (Fig. 1), see e.g. FIB which was not expressed in leukocytes but induced four fold in the spleen after infection (Tab. II), and by PigMAP which was expressed in spleen, lymph nodes, and in liver 10 times, 62 times, and 1 000 times more, respectively, compared to the level of expression in leukocytes. This means that the observed tissue patterns are reflecting the specific expression of the tissue in question, not biased by influx of other cell types as e.g. blood leukocytes.

Serum protein concentrations of three positive and one negative APP as well as IL-6 were also determined in two controls and all 12 inoculated pigs. Two proteins previously shown to be rapidly reacting APP (CRP and SAA) and two more slowly reacting APP (Hp and TTR) [3, 6, 17, 21] were compared before and after the infection. In general, infections with *A. pleuropneumoniae* induce an immediate IL-6 response of short duration followed by SAA, CRP and then Hp responses [21, 28]. We found prominent increases in serum protein concentrations of IL-6, SAA, and CRP after infection, while Hp and TTR concentrations were not affected within the time span of the present study (14–18 h).

Comparing individual animals, correlation between serum IL-6 concentrations, IL-6 leukocyte mRNA levels (qRT-PCR), and SAA and CRP serum concentrations could be seen with some additional correlation to the LLS except for pig 25 (Tab. III and Fig. 2). Notably, the zero-pathology animals (Nos. 29 and 35) in which the inoculation strain was only re-isolated from the tonsils also showed zero or low IL-6 (protein and mRNA) and SAA (protein) responses. This was confirmed by the Spearman rank test showing correlation between both SAA, CRP and IL-6 and lung lesion score.

In conclusion, the data presented here show for the first time that extrahepatic expression of APP is occurring widely in peripheral lymphoid tissues and cells including leukocytes, tonsils, spleen and tracheobronchial lymph nodes and, importantly, that their synthesis is dramatically altered as a part of the systemic acute phase response within 14–18 h after experimental lung infection with *A. pleuropneumoniae* concurrently with the hepatic response. As we clearly find non-hepatic responses (e.g. up regulation of SAA2, CRP, FIB, and PigMAP) in animals where the pathogen was not found outside tonsils and lung (animals Nos. 26, 27 and 33, data not shown) acute phase induced host factors might be involved in triggering this disseminated response. Although the liver do harbor cells capable of producing pro-inflammatory cytokines including IL-6 [37, 41] as also shown in the present study the currently preferred model for the hepatic APP response precisely incorporates the induction of hepatic APP by blood-borne circulating host factors [5]. It is quite plausible that such circulating APP-inducing factors could also affect non-hepatic tissues within the time frame of the hepatic acute phase response. This, however, does not explain the role of local IL-6 production as found in all tissues in the present study.

The present results support the concept that extrahepatically synthesized APP might play important, yet so far unrecognized roles in the innate defence response to infection, and that many different cell types in the organism have the potential to be induced to produce APP, being a more generalized disseminated systemic phenomenon that previously thought. The production of pro-inflammatory mediators and APP in several different peripheral lymphoid tissues of the organism as an early/immediate response would suggest that these reactions are beneficial to the host even in non-systemic infections. Work is in progress to analyse transcriptional responses at different locations of the infected lung. It will be very interesting to elucidate the nature and the magnitude of cytokine and APP responses in such directly affected organs compared to the APP response of peripheral types of tissue described in the present paper.

Acknowledgements. This work was supported in part by a grant from The Danish Research Council (274-07-0389). Thanks are due to Dr Tim K. Jensen for evaluation of pathology, to Dr Øystein Angen for assistance with experimental infection of animals and to Dr Anders Stockmarr for assistance with the statistics. We also thank Nanna Skall Sørensen, Patrik Wernhoff and Heidi Mikkelsen for helpful discussions.

REFERENCES

[1] Abernethy T.J., Avery O.T., The occurrence during acute infections of a protein not normally present in blood, J. Exp. Med. (1941) 73:173–182.

[2] Baarsch M.J., Scamurra R.W., Burger K., Foss D.L., Maheswaran S.K., Murtaugh M.P., Inflammatory cytokine expression in swine experimentally infected with *Actinobacillus pleuropneumoniae*, Infect. Immun. (1995) 63:3587–3594.

[3] Baarsch M.J., Foss D.L., Murtaugh M.P., Pathophysiologic correlates of acute porcine pleuropneumonia, Am. J. Vet. Res. (2000) 61:684–690.

[4] Baehr C., Reichel V., Fricker G., Choroid plexus epithelial monolayers – a cell culture model from porcine brain, Cerebrospinal Fluid Res. (2006) 3:13.

[5] Baumann H., Gauldie J., The acute phase response, Immunol. Today (1994) 15:74–80.

[6] Campbell F.M., Waterston M.V., Andresen L.O., Sorensen N.S., Heegaard P.M.H., Eckersall P.D., The negative acute phase response of serum transthyretin following *Streptococcus suis* infections in the pig, Vet. Res. (2005) 36:657–664.

[7] Carpintero R., Piñeiro M., Andrés M., Iturralde M., Alava M.A., Heegaard P.M.H., et al., The concentration of apolipoprotein A-I decreases during experimentally induced acute-phase processes in pigs, Infect. Immun. (2005) 73:3184–3187.

[8] Ceciliani F., Giordano A., Spagnolo V., The systemic reaction during inflammation: the acute-phase proteins, Protein Pept. Lett. (2002) 9:211–223.

[9] Daveau M., Jean L., Soury E., Olivier E., Masson S., Lyoumi S., et al., Hepatic and extra-hepatic transcription of inter-alpha-inhibitor family genes under normal or acute inflammatory conditions in rat, Arch. Biochem. Biophys. (1998) 350:315–323.

[10] Eckersall P.D., Young F.J., Nolan A.M., Knight C.H., McComb C., Waterston M.M., et al., Acute phase proteins in bovine milk in an experimental model of Staphylococcus aureus subclinical mastitis, J. Dairy Sci. (2006) 89:1488–1501.

[11] Fleige S., Pfaffl M.W., RNA integrity and the effect on the real-time qRT-PCR performance, Mol. Aspects Med. (2006) 27:126–139.

[12] Geisert R.D., Ashworth M.D., Malayer J.R., Expression of inter-alpha-trypsin inhibitor heavy chains in endometrium of cyclic and pregnant gilts, Reproduction (2003) 126:621–627.

[13] Giese S., Stenbæk E., Nielsen R., Identification of *Actinobacillus pleuropneumoniae* serotype 2 by monoclonal or polyclonal antibodies in latex agglutination tests, Acta Vet. Scand. (1993) 34:223–225.

[14] Gruys E., Toussaint M.J., Niewold T.A., Koopmans S.J., Acute phase reaction and acute phase proteins, J. Zhejiang Univ. Sci. B (2005) 6:1045–1056.

[15] Haidaris P.J., Induction of fibrinogen biosynthesis and secretion from cultured pulmonary epithelial cells, Blood (1997) 89:873–882.

[16] Hedegaard J., Skovgaard K., Mortensen S., Sørensen P., Jensen T.K., Hornshøj H., et al., Molecular characterisation of the early response in pigs to experimental infection with *Actinobacillus pleuropneumoniae* using cDNA microarrays, Acta Vet. Scand. (2007) 49:11.

[17] Heegaard P.M.H., Klausen J., Nielsen J.P., González-Ramón N., Piñeiro M., Lampreave F., Alava M.A., The porcine acute phase response to infection with *Actinobacillus pleuropneumoniae*. Haptoglobin, C-reactive protein, major acute phase protein and serum amyloid A protein are sensitive indicators of infection, Comp. Biochem. Physiol. B Biochem. Mol. Biol. (1998) 119:365–373.

[18] Heegaard P.M.H., Pedersen H.G., Jensen A.L., Boas U., A robust quantitative solid phase immunoassay for the acute phase protein C-reactive protein (CRP) based on cytidine 5′-diphosphocholine coupled dendrimers, J. Immunol. Methods (2009) in press.

[19] Hiss S., Willbrenning G.S., Suntz M., Reinacher M., Sauerwein H., Immunohistochemical localization of haptoglobin in porcine lungs, Anat. Histol. Embryol. (2007) 37:196–199.

[20] Huang H., Potter A.A., Campos M., Leighton F.A., Willson P.J., Haines D.M., Yates W.D., Pathogenesis of porcine *Actinobacillus pleuropneumonia*, part II: roles of proinflammatory cytokines, Can. J. Vet. Res. (1999) 63:69–78.

[21] Hultén C., Johansson E., Fossum C., Wallgren P., Interleukin 6, serum amyloid A and haptoglobin as markers of treatment efficacy in pigs experimentally infected with *Actinobacillus pleuropneumoniae*, Vet. Microbiol. (2003) 95:75–89.

[22] Jacobsson B., Carlström A., Platz A., Collins V.P., Transthyretin messenger ribonucleic acid expression in the pancreas and in endocrine tumors of the pancreas and gut, J. Clin. Endocrinol. Metab. (1990) 71:875–880.

[23] Kalmovarin N., Friedrichs W.E., O′Brien H.V., Linehan L.A., Bowman B.H., Yang F., Extrahepatic expression of plasma protein genes during inflammation, Inflammation (1991) 15:369–379.

[24] Kramer T.T., Saucke L., Griffith R.W., Kunesh J.P., Desferoxamine and iron dextran in acute

Salmonelle cholerae-suis infection in pigs, Am. J. Vet. Res. (1986) 47:1452–1457.

[25] Kristensen C.S., Angen Ø., Andreasen M., Takai H., Nielsen J.P., Jorsal S.E., Demonstration of airborne transmission of *Actinobacillus pleuropneumoniae* serotype 2 between simulated pig units located at close range, Vet. Microbiol. (2004) 98:243–249.

[26] Kuji T., Masaki T., Li L., Cheung A.H., Expression of C-reactive protein in myointimal hyperplasia in a porcine arteriovenous graft model, Nephrol. Dial. Transplant. (2007) 22:2469–2475.

[27] Kushner I., The Phenomenon of the Acute Phase Response, Ann. N.Y. Acad. Sci. (1982) 389:39–47.

[28] Lauritzen B., Lykkesfeldt J., Skaanild M.T., Angen Ø., Nielsen J.P., Friis C., Putative biomarkers for evaluating antibiotic treatment: an experimental model of porcine *Actinobacillus pleuropneumoniae* infection, Res. Vet. Sci. (2003) 74:261–270.

[29] Lecchi C., Avallone G., Giurovich M., Roccabianca P., Ceciliani F., Extra hepatic expression of the acute phase protein alpha 1-acid glycoprotein in normal bovine tissues, Vet. J. (2008) doi:10.1016/j.tvjl.2007.12.027.

[30] Li H., Gene sequencing. China, Denmark team up to tackle the pig, Science (2000) 290:913–914.

[31] Mandel T., Accuracy and precision: evaluation and interpretation of analytical results, in: Kolthoff I.M., Elving P.J. (Eds.), Treatise on analytical chemistry, 2nd ed., Wiley, New York, 1978, pp. 283–285.

[32] Matsumoto S., Okumura K., Ogata A., Hisatomi Y., Sato A., Hattori K., et al., Isolation of tissue progenitor cells from duct-ligated salivary glands of swine, Cloning Stem Cells (2007) 9:176–190.

[33] McDonald T.L., Weber A., Smith J.W., A monoclonal antibody sandwich immunoassay for serum amyloid A (SAA) protein, J. Immunol. Methods (1991) 144:149–155.

[34] McDonald T.L., Larson M.A., Mack D.R., Weber A., Elevated extrahepatic expression and secretion of mammary-associated serum amyloid A 3 (M-SAA3) into colostrum, Vet. Immunol. Immunopathol. (2001) 83:203–211.

[35] Meek R.L., Benditt E.P., Amyloid A gene family expression in different mouse tissues, J. Exp. Med. (1986) 164:2006–2017.

[36] Naranjo V., Villar M., Martín-Hernando M.P., Vidal D., Höfle U., Gortazar C., et al., Proteomic and transcriptomic analyses of differential stress/inflammatory responses in mandibular lymph nodes and oropharyngeal tonsils of European wild boars naturally infected with *Mycobacterium bovis*, Proteomics (2007) 7:220–231.

[37] Núñez A., Gómez-Villamandos J.C., Sánchez-Cordón P.J., Fernández de Marco M., Pedrera M., Salguero F.J., Carrasco L., Expression of proinflammatory cytokines by hepatic macrophages in acute classical swine fever, J. Comp. Pathol. (2005) 133:23–32.

[38] Parrott J.A., Whaley P.D., Skinner M.K., Extrahepatic expression of fibrinogen by granulosa cells: potential role in ovulation, Endocrinology (1993) 133:1645–1649.

[39] Petersen H.H., Nielsen J.P., Heegaard P.M.H., Application of acute phase protein measurements in veterinary clinical chemistry, Vet. Res. (2004) 35:163–187.

[40] Ramsay T.G., Caperna T.J., Ontogeny of adipokine expression in neonatal pig adipose tissue, Comp. Biochem. Physiol. B Biochem. Mol. Biol. (2009) 152:72–78.

[41] Seki S., Habu Y., Kawamura T., Takeda K., Dobashi H., Ohkawa T., Hiraide H., The liver as a crucial organ in the first line of host defence: the roles of Kupffer cells, natural killer (NK) cells and NK1.1 Ag+ T cells in T helper 1 immune responses, Immunol. Rev. (2000) 174:35–46.

[42] Skovgaard K., Mortensen S., Poulsen K.T., Angen Ø., Heegaard P.M.H., Validation of putative reference genes for qRT-PCR normalization in tissues and blood from pigs infected with *Actinobacillus pleuropneumoniae*, Vet. Immunol. Immunopathol. (2007) 118:140–146.

[43] Sorensen N.S., Tegtmeier C., Andresen L.O., Piñeiro M., Toussaint M.J., Campbell F.M., et al., The porcine acute phase protein response to acute clinical and subclinical experimental infection with *Streptococcus suis*, Vet. Immunol. Immunopathol. (2006) 113:157–168.

[44] Vaerman J.P., Langendries A., Pabst R., Rothkötter H.J., Contribution of serum IgA to intestinal lymph IgA, and vice versa, in minipigs, Vet. Immunol. Immunopathol. (1997) 58:301–308.

[45] Vandesompele J., De Preter K., Pattyn F., Poppe B., Van Roy N., De Paepe A., Speleman F., Accurate normalization of real-time quantitative RT-PCR data by geometric averaging of multiple internal control genes, Genome Biol. (2002) 3:1–12.

[46] Vernooy J.H., Reynaert N., Wolfs T.G., Cloots R.H., Haegens A., de Vries B., et al., Rapid pulmonary expression of acute-phase reactants after local lipopolysaccharide exposure in mice is followed by an interleukin-6 mediated systemic acute-phase response, Exp. Lung Res. (2005) 31:855–871.

[47] Wang J., Chen L., Li D., Yin Y., Wang X., Li P., et al., Intrauterine growth restriction affects the proteomes of the small intestine, liver, and skeletal muscle in newborn pigs, J. Nutr. (2008) 138:60–66.

[48] Yang F., Friedrichs W.E., Navarijo-Ashbaugh A.L., deGraffenried L.A., Bowman B.H., Coalson J.J., Cell type-specific and inflammatory-induced expression of haptoglobin gene in lung, Lab. Invest. (1995) 73:433–440.

[49] Xu L., Badolato R., Murphy W.J., Longo D.L., Anver M., Hale S., et al., A novel biologic function of serum amyloid A. Induction of T lymphocyte migration and adhesion, J. Immunol. (1995) 155:1184–1190.