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Widespread extrahepatic expression of acute-phase proteins in healthy chicken (*Gallus gallus*) tissues

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Abstract

Acute phase proteins (APP) are plasma proteins that can modify their expression in response to inflammation caused by tissue injury, infections, immunological disorders or stress. Although APP are produced mainly in liver, extrahepatic production has also been described. As a prerequisite to get insight the expression of APP in chicken during diseases, this study investigated the presence of five APP, including alpha1-acid glycoprotein (AGP), Serum Amyloid A (SAA), PIT54, C-Reactive protein (CRP) and Ovotransferrin (OVT) in twenty tissues collected from healthy chicken (*Gallus gallus*) by quantitative Real Time PCR and immunohistochemistry. As expected, APP gene abundance was higher in liver compared with other tissues. The mRNA coding for CRP, OVT and SAA was detected in all analyzed tissues with a higher expression in gastrointestinal tract, respiratory and lymphatic samples. SAA expression was particularly high in cecal tonsil, lung, spleen and Meckel's diverticulum, whereas OVT in lung, bursa of Fabricius and pancreas. AGP and PIT54 mRNA expression were detected in all tissues but at negligible levels. Immunohistochemical expression of AGP and OVT was variably detected in different organs, being identified in endothelium of every tissue. Positive cells were present in the epithelium of the mucosal layer of gastrointestinal tract and kidney. Lung and central nervous system stained for both proteins. No positive staining was detected in lymphoid tissues and muscle. These results suggest that most tissues can express different amount of APP even in healthy conditions and are therefore capable to mount a local acute phase reaction.

Keywords: acute phase proteins; animal welfare; chicken; extrahepatic expression; *Gallus gallus*

Introduction

Acute phase proteins (APP) are plasma proteins whose concentration is increased in the frame of the systemic response to inflammation caused by physical trauma, microbial infections, immunological disorders, neoplasia, or stress (Gabay and Kushner, 1999). From a diagnostic perspective, the increased concentrations of APP are meaningful as nonspecific biomarkers in the assessment of animal welfare (Murata et al., 2004).

APP may provide a similar use for poultry disease diagnostics. In chicken, at least five proteins, namely Alpha1-acid Glycoprotein (AGP), Serum Amyloid A (SAA), PIT54, Ovotrasferrin (OVT) and C-Reactive Protein (CRP) were found to be differentially expressed during diseases or stress (O'Reilly and Eckersall, 2014).

Elevated serum AGP levels have been observed in chickens infected with bacterial or viral pathogens (Inoue et al., 1997; Takahashi et al., 1998). SAA was overexpressed in broilers infected with *S. aureus* (Chamanza et al., 1999) and in chickens infected with Infectious Bronchitis Virus (IBV) (Asasi et al., 2013). PIT54 has been associated with *S. gallinarium* and *S. aureus* infections (Garcia et al., 2009), *E. coli* and *E. tenella* infections (Georgieva et al., 2010) and IBV infections (Asasi et al., 2013). CRP has been identified in birds after bacterial and viral infection (Patterson and Mora, 1964; Rauber et al., 2014; Seifi et al., 2014). Chickens infected with *Staphylococcus* spp., *E. coli* or parasites as *E. maxima* and *E. tenella* showed a significant increase in the levels of serum OVT (Chamanza et al., 1999; Rath et al., 2009). OVT concentrations were also higher when chickens were challenged with LPS (Horrocks et al., 2011; Koppenol et al., 2015).

Although liver is considered as the major source of APP, they can also be synthesized in other tissues and organs (Schrodl et al., 2016), even in healthy conditions. Extrahepatic expression of some APP has been previously described in farm animals such as SAA in cattle and horses (Berg et al., 2011; Lecchi et al., 2012), Hp in cattle (Dilda et al., 2012; Lecchi et al., 2012), AGP in cattle (Lecchi et al., 2009; Rahman et al., 2015). No information about the extrahepatic expression in poultry is presently available. The aim of the present study was to bridge this gap by investigating the distribution pattern of the five major APP in twenty tissues collected from six healthy chicken. The mRNA abundance was measured by quantitative PCR and the location on the protein was confirmed by immunohistochemistry where antibodies were available.

Material and methods

Tissue collection and preservation

The samples were collected during routinely slaughtering procedures from six healthy hybrid ROSS 708 female broilers, 55 days old (Table 1). The clinical status of the animals was assessed by ante- and post-mortem inspection.

Portions of each tissue were removed immediately after slaughtering, preserved in RNAlater (Sigma–Aldrich) or frozen into liquid nitrogen and afterwards stored at −80ºC before RNA extraction. Adipose tissue was stored without previous immersion in RNAlater. Samples for immunohistochemistry analysis were fixed in 10% buffered formalin.

Qualitative and quantitative mRNA expression

Total RNA was extracted using QIAzol lysis reagent according to the manufacturer's protocol (Qiagen) and treated with DNase I (Thermo Fisher Scientific, Fermentas). Total RNA was quantified using a NanoDrop ND-1000 UV–vis spectrophotometer. Reverse transcription was carried out with 1 µg RNA using the iScript cDNA Synthesis Kit (BioRad). The cDNA was used as template for PCRs.

Qualitative PCR was performed in 10 µL final volume containing 1x buffer, 1.5 mM $MgCl_2$, 0.2 mM each deoxynucleotide triphosphate (dNTP), 1 µM each primer and 0.025 U Taq polymerase (LeGene Biosciences). PCR conditions were 35 cycles of 94ºC for 30 s, 60ºC for 30 s and 72ºC for 45 s (Eppendorf Mastercycler). PCR products were visualized on 1.6% agarose gel stained with ethidium bromide. The same primers were used in qualitative and quantitative PCR (Table 2).

Quantitative real time PCR (qPCR) was performed using 12 µL Eva Green Supermix (BioRad), 250 nM AGP and PIT54, 300 nM SAA, CRP and OVT and 400 nM GAPDH, YWHAZ and RPL4, using Eco Real-Time PCR System (Illumina). GAPDH, RPL4 and YWHAZ were selected as reference genes based on previous studies and literature (Yang et al., 2013; Yue et al., 2010). In order to evaluate the PCR efficiency using a relative standard curve, series of dilution were prepared by performing fourfold serial dilution starting from the pooled sample composed by a liver cDNA mix from six animals. Each sample was tested in duplicate. Non-reverse transcribed controls were performed by omitting reverse transcription and no template controls were conducted by adding nuclease free water. The thermal profile used (95°C for 10 min, 40 cycles of 95°C for 10 s and 60°C for 30 s; for melting curve construction, 55°C for 15 s and 80 cycles starting to 55°C and increasing 0.5°C each 10 s) was the same for each target gene. The MIQE guidelines were

followed (Bustin et al., 2009). For all genes studied, the standard curves derived from serial dilution of pooled sample gave correlation coefficients (R^2) greater than 0.990 and efficiencies greater than 94%. Results were compared using the comparative $^{\Delta\text{-}\Delta}$ Cq method.

Western blotting validation of cross-reactivity of polyclonal anti-boAGP antibody

Chicken and bovine serum were separated on a 12% sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS–PAGE) and blotted onto nitrocellulose membrane. Before gel separation, 1 µl ß-mercaptoethanol (Sigma Aldrich) was added to each sample. Immunolabeling was performed using a rabbit polyclonal anti-bovine AGP (anti-boAGP) (Ceciliani et al., 2007) as primary antibody (1:4000 dilution for 1 h min at RT), while an anti-rabbit IgG labelled with peroxidase (Vector Laboratories) was used as secondary antibody (1:4000 dilution for 45 min at RT). Both antibodies were diluted using Roti®-Block (Carl Roth). Immunoreactive bands were visualized by enhanced chemiluminescence (ECL) using Immobilon Western Chemiluminescent HRP Substrate (Millipore). In order to further assess the specificity of the antibody for chicken protein, Western blotting experiments were repeated after overnight incubation of the anti-boAGP with purified bovine AGP using a 1:1 molar ratio, in order to block every anti-AGP specific reactive sites and to detect any possible non-specific reactions. The solution was then utilized as primary antibody for immunohistochemistry. Bovine serum was used as positive control.

Immunohistochemistry

The immunolocalization was carried out as previously described (Rahman et al., 2015). Formalin-fixed tissue specimens were routinely processed for histology and paraffin embedded. Sections of 5 µm thick were obtained from paraffin blocks and mounted on poly-lysine-coated slides. The sections were then deparaffinized in xylene and rehydrated through a descending series of ethanol concentrations.

The endogenous peroxidase activity was blocked with 0.3% H_2O_2 in methanol for 30 min. Antigen retrieval was performed by heating the slides in citrate buffer solution (pH=6.5) in a water bath at 95°C for 30 min. The sections were cooled for 40 min at RT and then rinsed in Tris buffered saline (TBS). The slides were therefore incubated for 20 min at RT with normal goat serum (1:70) to block any nonspecific protein binding. Sections were incubated at 4°C overnight in a humidified chamber with the two primary antibodies anti-boAGP, 1:200 (Ceciliani et al., 2007) and rabbit polyclonal anti-chicken OVT, 1:800 (My BioSource, San Diego, CA, USA, Catalog nr. MBS71 5799).

The sections were then rinsed in TBS for 3 times of 3 min each and then incubated with PolyView mouse/rabbit nanopolymer detection reagents (Enzo Life Sciences, Inc.) for 20 min at RT. After three washes in TBS, the chromogen 3-amino-9-ethylcarbazole (AEC) (Vector Laboratories) was applied for 20 min and, after rinsing in tap water, slides were counterstained with Mayer's haematoxylin (Diapath srl) for 2 min. Slides were therefore rinsed in tap water for 5 min, dried in a stove at 45°C for 12 h and therefore mounted in aqueous mounting agent (Aquatex, Merck). Immunohistochemical staining was evaluated and semi-quantitatively scored as mild, moderate or intense.

Histological sections of liver served as positive controls. Additionally, paraffin embedded samples of oviduct from normal hens were retrieved from the departmental archives and served as positive controls for OVT. Negative controls were prepared by replacing the respective primary antibody with normal rabbit IgG (Santa Cruz Biotechnology, Inc.).

Statistical analysis

Statistical analysis was performed using XLSTAT for Windows (Addinsoft), mean and standard error of the mean (SEM) values were calculated.

Results

Detection and measurement of acute phase protein mRNA abundance in chicken tissues

In a preliminary set of experiments, the primers quality was assessed by qualitative PCR. Gene specific amplification was confirmed by a single band with the expected size in agarose gel electrophoresis as showed in Fig. 1 of supplemental material, and confirmed by a single peak in melt-curve analysis by qPCR.

qPCR was used to quantify extrahepatic expression of AGP, SAA, PIT54, CRP and OVT in the tissues listed in Table 1. Liver was used as reference tissue (mRNA expression=1) (Table 3).

mRNA coding for SAA, CRP and OVT was detected in all tissues analyzed, although its concentration appeared to vary considerably between different tissues (Fig. 1). Its amounts ranged from 0.002 to 0.72, 0.001 to 0.06 and 0.003 to 0.26, respectively, as compared to liver, with cecal tonsil and lung having the highest concentration. SAA mRNA expression was higher in cecal tonsil, lung, spleen, Meckel's diverticulum, pericardial adipose tissue and mucosa of gizzard (0.72, 0.69, 0.26, 0.21, 0.13 and 0.12, respectively). CRP mRNA expression was high just in brain and visceral adipose tissue (0.06 and 0.03, respectively). OVT was more expressed in lung, followed by pericardial adipose tissue and bursa of Fabricius (0.26, 0.19 and 0.13, respectively), whereas brain, cerebellum, pancreas, visceral adipose tissue and subcutaneous adipose tissue showed around 0.10 mRNA expression.

Expression of AGP and PIT54 was negligible as compared to liver (from 0.0001 to 0.001). Nonetheless, both APP were detected in all the tissues.

Localization of acute phase proteins in chicken tissues by immunohistochemistry

To the best of our knowledge, no antibodies specific for chicken CRP, PIT54 and SAA has been reported so far in literature. Therefore, histological immunolocalization was carried out only for OVT, by using a commercially available antibody, and AGP, by using the anti-boAGP that was shown as cross-reacting with a most prominent band with a molecular weight (MW) of 55–65 kDa in serum (Fig. 2, Supplemental). In order to assess whether the reactive bands were related to AGP, blocking of the anti-boAGP specific sites was carried out by incubating an aliquot of antibody with purified bovine AGP. The mixture was then utilized as primary antibody on Western blotting membrane. Chicken serum did not show any reactive band after reaction with anti-boAGP

polyclonal antibody incubated with purified AGP (Fig. 2, Supplemental), thus confirming that the bands were derived from AGP. The results indicated that anti-boAGP monoclonal antibody (a) specifically reacts with bovine and chicken plasma and (b) does not react with other proteins in bovine and chicken plasma.

Immunohistochemical expression of AGP and OVT was variably detected in tested tissues, with similar pattern of expression for both markers (Fig. 2 and 3). Detailed results are given in Table 4. The presence of AGP and OVT proteins was also clearly identified in endothelium of every tissue, confirming they are circulating proteins that can easily reach any tissue by bloodstream. At immunohistochemical examination, liver and oviduct were used as positive controls and stained for AGP and both AGP and OVT, respectively.

Briefly, multifocal positivity was detected in adipose and connective tissue for both AGP and OVT. Scattered positive cells, for both markers, were variably present in the epithelium of the mucosal layer of organs of the gastrointestinal tract (crop, mucosa of proventriculus, duodenum, caecum and Meckel's diverticulum). In the liver, hepatocytes were mildly and diffusely positive, whereas ductal epithelium exhibited intense multifocal granular intracytoplasmic positivity, as also seen in kidney tubular epithelium. Pancreatic islets were intensively positive but acini always negative. Lung was diffusely positive in its different anatomic regions. Central nervous tissue was multifocally positive according to different areas. No positive staining was detected in lymphoid tissues (thymus, intestinal Peyer's patches, bursa of Fabricius, spleen) and muscle.

Discussion

The present study reports for the first time the extrahepatic expression of five APP, namely AGP, SAA, PIT54, CRP and OVT, in 20 tissues collected from healthy chicken. Where antibodies were commercially available, protein localization was also determined by immunohistochemical studies. Acute phase proteins mRNA was detected in all tissues included in this experiment, although liver was confirmed to be the main source of APP. Extrahepatic production of APP mRNA was found to be particularly abundant in the gastrointestinal tract, lymphatic system and lung.

Among the five APP, SAA mRNA was the most abundantly expressed. SAA is a major APP in vertebrates and can increase its concentration up to 1000-fold as response to a stimuli (Gabay and Kushner, 1999). SAA belongs to a family of apolipoproteins that are incorporated into high-density lipoprotein to be afterward released into the circulation (Benditt and Eriksen, 1977). In mammals, multiple SAA genes have been described (Uhlar and Whitehead, 1999; Upragarin et al., 2005). In chickens, on the other hand, only one SAA gene has been identified so far (Ovelgönne et al., 2001). SAA exhibits significant immunological activity being chemotactic for neutrophils and mast cells, and takes part to the so called cytokine-serum amyloid A-chemokine network (De Buck et al., 2016). In humans, SAA can act as pathogenic pattern recognition protein by opsonizing Gram-negative bacteria, increasing meanwhile macrophages and neutrophil phagocytosis (Shah et al., 2006). Furthermore, SAA can activate the inflammasome cascade (Eklund et al., 2012). Given the background of its immune-modulatory activity, it is not surprising that the extrahepatic tissues where SAA was found to be mostly expressed were those related to immune defenses, such as for example cecal tonsils, in which SAA mRNA abundance almost equals the amount produced by liver (72%). This finding supports the hypothesis that cecal tonsil, which is the major lymphoid district within the gut-associated lymphoid tissue (GALT) (Yun et al., 2000), is the second main source of SAA in chicken. Spleen was the other tissue where SAA was found to be abundantly expressed (26% as compared with liver), in agreement with previous findings reporting splenic expression of SAA in chickens orally infected with *S. enteritidis* (Matulova et al., 2012) and *A. galli* (Dalgaard et al., 2015). The mRNA abundance of SAA in the cecal tonsil and spleen tissues confirms in chicken that, as has been demonstrated in humans and laboratory rodents, SAA is also involved in the development of adaptive immunity, as shown by its capability to overexpress IL-17A and IL-17F from CD4+ T cells (Ather et al., 2011; 2013). This hypothesis is also supported by the finding of SAA mRNA in Meckel's diverticulum, which is regarded as a fully mature lymphoid tissue in chicken (Casteleyn et al., 2010). SAA mRNA expression was also detected in pancreas, in

a similar way already reported by Lecchi et al. (2012) and, for the first time, in gizzard's mucosa, which is a part of the digestive tract of birds. The potential presence of SAA in this district is interesting, since provides the evidence that also locally produced innate-immunity related proteins can protect the mucosa of the mechanical stomach of poultry. The last tissue where SAA was highly expressed was lung (60% as compared with liver). Respiratory system provides the second main entrance for many pathogens in mammals as well as in avian species in birds (Smiałek et al., 2011). The finding of SAA expression in lung is consistent with what has been recently reported about lung and spleen SAA overexpression in chickens experimentally challenged with after H5N1 influenza virus (Burggraaf et al., 2014).

Beside SAA, also OVT was heavily expressed in lungs (26% as compared to liver). The product of expression, namely the OVT protein, was also detected at the protein level by immunohistochemistry, identifying lungs as another important source of APP in chicken. Ovotransferrin provides an useful biomarker of inflammatory diseases in chicken (Rath et al., 2009), but no information about its extrahepatic expression was available before the present study. The high level of expression of OVT in lungs may also explain the early appearance of this protein in serum after experimental challenging with avian influenza virus (Sylte and Suarez, 2012). Ovotransferrin is a multi-functional protein with a major role in avian natural immunity (Giansanti et al., 2012). It was shown that OVT could permeate *E. coli* outer membrane, accessing to their inner membrane and causing ion leakage inside bacteria, eventually driving to the uncoupling the respiratory-dependent energy production (Aguilera et al., 2003). Consistently with its immune-related function, OVT mRNA was found expressed also in bursa of Fabricius (13% as compared with liver), which is a central lymphoid organ for B cells production and lymphocyte maturation (Ratcliffe, 2002). On the background of its distribution along the mucosa and the immune districts associated to gastro enteric system, we may therefore speculate that OVT may contribute to the first line of defense against invading pathogens. High expression of OVT was also detected on pancreas (11%), pericardial adipose tissue (19%) and brain (11%). Its identification in brain suggests that OVT may fulfill its activity of iron-binding uptake (Taylor and Morgan, 1991) also in chicken central nervous system. We may also not rule out the possibility that one of OVT function in brain includes the scavenging of iron radical in order to protect neural cells from reactive oxygen species damages. The physiological role of OVT in pancreas remains elusive.

CRP mRNA was found to be moderately expressed in brain and visceral adipose tissue (6% and 3% as compared to liver, respectively). CRP has the ability to bind directly to necrotic cells, cellular debris and polysaccharides on bacteria, fungi, and parasites, thus acting as an opsonin. In as

such, CRP activates the complement system when bound to one of its ligands and binds to phagocytic cells, modulating both humoral and cellular components of inflammation (Petersen et al., 2004). In humans, CRP overespression in brain and adipose tissue is associated to brain diseases, or metabolic disorders related to inflamed adipose tissue, respectively (Brooks et al., 2010; Islam, 2016). The biological significance of CRP expression in these two tissues in chickens has yet to be elucidated.

Although detected in all tissues included in this studies, the extrahepatic mRNA expression of AGP and PIT54 was found to be negligible as compared to liver (<0.01). For what concerns AGP, these findings look somehow contradictory if compared with immunohistochemistry result, since the presence of the protein was demonstrated in many tissues. This result can be partially explained by the long half-life of AGP after its synthesis (Ceciliani and Pocacqua, 2007). It cannot also be ruled out the possibility that the protein found in the tissues also derive, at least partially, from the liver and carried out to tissues via blood circulation.

In conclusion, the data presented here demonstrated that the five acute phase proteins AGP, SAA, PIT54, CRP and OVT, are constitutively expressed in all chicken tissues even in absence of a systemic acute phase response. The APP mRNA abundance of SAA and OVT was found to be higher in three systems, namely respiratory, gastrointestinal and lymphoid system. The results presented in this study suggest the hypothesis that extrahepatic APP, in particular those produced at mucosal levels, might play important roles in the innate and adaptive immunity by providing the first line of defense against pathogens or locally modulating the inflammation.

Further research is needed to investigate the relationship between local and systemic reactions and to determine the significance of its local production in avian species. Understanding where APP are produced and secreted is the first step for their proper utilization as biomarkers during diseases.

Conflict of interest statement

We wish to confirm that there are no known conflicts of interest associated with this publication and there has been no significant financial support for this work that could have influenced its outcome. Moreover, none of the authors has any financial or personal relationships that could inappropriately influence or bias the content of the paper.

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426 **Table 1.** Samples list.

Adipose tissue	Subcutaneous adipose tissue Visceral adipose tissue Pericardial adipose tissue	
Muscular system	Pectoral muscle	
Lymphatic system	Thymus Spleen Cecal tonsil Bursa of Fabricius	
Central nervous system	Brain Cerebellum	
Urogenital system	Kidney	
Respiratory system	Lung	
Gastrointestinal tract	Crop	
	Stomach	Mucosa of proventriculus Mucosa of gizzard
	Pancreas	
	Intestine	Duodenum Caecum
	Meckel's diverticulum	
	Liver	

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428

429 **Table 2.** Sequences of oligonucleotide primers for acute phase proteins and reference genes.

Gene	GenBank	Primer Forward (5'-3')	Primer Reverse (5'-3')	Length (bp)
AGP	NM_204541.2	GGTGTACATCATGGGTGCCT	CGCATGTTTCATTCAGCCTCA	143
SAA	XM_003206257.1	TGCTTCGTGTTGCTCTCCAT	CATGTCCCGGTATGCTCTCC	123
PIT54	XM_003202017	GCCAGTGCAATTTGTTCAGA	TCCCGTAAATCCCAGTTGTC	146
CRP	EU106581.1	ATCCCATGCTCAACTTCACC	CCGACGTAGAAGCGGTACTC	145
OVT	NM_205304.1	AGCCATTGCGAATAATGAGG	ATGGGCTTCAGCTTGTATGG	90
GAPDH	GQ184819.1	GATCCCTTCATCGACCTGAA	ACAGTGCCCTTGAAGTGTCC	77
RPL4	XM_003209573.1	TGTTTGCCCCAACCAAGACT	TCCTCAATGCGGTGACCTTT	136
YWHAZ	XM_003205203.1	TTCCCTTGCAAAAACGGCTT	TTCAGCTTCGTCTCCTTGGG	148

430 SAA, PIT54, CRP, GAPDH, RPL4 and YWHAZ were from Marques et al. (2016). AGP and OVT primers were
431 designed using Primer 3 (http://bioinfo.ut.ee/primer3-0.4.0).

432

433 **Table 3.** Extrahepatic expression of APP ± standard error of the mean (SEM) compared to liver
434 expression values (set to 1). Data shows the mean value of 6 animals.

Tissues	Genes				
	AGP	SAA	PIT54	CRP	OVT
Subcutaneous adipose tissue	5.30E-05 ± 0.0000	7.26E-02 ± 0.0495	9.49E-05 ± 0.0001	6.26E-03 ± 0.0030	6.67E-02 ± 0.0130
Visceral adipose tissue	3.05E-05 ± 0.0000	1.33E-02 ± 0.0033	2.10E-05 ± 0.0000	3.17E-02 ± 0.0197	9.93E-02 ± 0.0503
Pericardial adipose tissue	5.27E-04 ± 0.0005	1.38E-01 ± 0.0310	3.65E-04 ± 0.0003	9.38E-03 ± 0.0030	1.89E-01 ± 0.0330
Pectoral muscle	9.70E-06 ± 0.0000	2.38E-03 ± 0.0010	8.24E-06 ± 0.0000	2.65E-04 ± 0.0002	3.41E-03 ± 0.0009
Thymus	2.05E-05 ± 0.0000	7.09E-02 ± 0.0201	3.27E-04 ± 0.0001	1.91E-03 ± 0.0009	8.55E-03 ± 0.0019
Spleen	8.70E-04 ± 0.0003	2.57E-01 ± 0.0631	9.99E-04 ± 0.0003	1.08E-03 ± 0.0004	1.91E-02 ± 0.0028
Brain	1.23E-04 ± 0.0000	9.32E-03 ± 0.0037	1.45E-04 ± 0.0000	5.90E-02 ± 0.0576	1.08E-01 ± 0.0273
Cerebellum	7.58E-05 ± 0.0000	1.68E-02 ± 0.0070	1.02E-04 ± 0.0000	3.53E-04 ± 0.0001	1.07E-01 ± 0.0330
Kidney	9.67E-04 ± 0.0004	6.56E-02 ± 0.0274	5.59E-04 ± 0.0002	3.69E-03 ± 0.0013	3.44E-02 ± 0.0097
Lung	5.33E-04 ± 0.0003	6.94E-01 ± 0.3031	2.91E-04 ± 0.0002	8.14E-03 ± 0.0033	2.59E-01 ± 0.0676
Crop	3.48E-05 ± 0.0000	3.79E-02 ± 0.0276	4.76E-05 ± 0.0000	2.99E-04 ± 0.0002	1.40E-02 ± 0.0073
Mucosa of proventriculus	3.40E-04 ± 0.0001	1.36E-02 ± 0.0036	2.26E-04 ± 0.0001	7.35E-03 ± 0.0056	2.78E-02 ± 0.0044
Mucosa of gizzard	1.55E-04 ± 0.0001	1.16E-01 ± 0.0507	2.06E-04 ± 0.0002	5.22E-03 ± 0.0020	3.51E-02 ± 0.0103
Pancreas	1.45E-04 ± 0.0001	3.30E-02 ± 0.0117	2.67E-04 ± 0.0001	2.21E-03 ± 0.0010	1.12E-01 ± 0.0253
Duodenum	1.61E-05 ± 0.0000	1.83E-02 ± 0.0068	1.26E-04 ± 0.0001	6.41E-04 ± 0.0003	1.78E-02 ± 0.0135
Meckel's diverticulum	6.61E-06 ± 0.0000	2.06E-01 ± 0.1290	1.34E-05 ± 0.0000	5.44E-03 ± 0.0047	3.13E-02 ± 0.0227
Caecum	1.77E-05 ± 0.0000	2.41E-02 ± 0.0059	7.65E-06 ± 0.0000	5.71E-04 ± 0.0001	8.39E-03 ± 0.0012
Cecal tonsil	5.08E-05 ± 0.0000	7.22E-01 ± 0.2917	1.93E-05 ± 0.0000	8.26E-04 ± 0.0002	4.11E-02 ± 0.0113
Bursa of Fabricius	5.23E-05 ± 0.0000	3.00E-02 ± 0.0134	3.39E-05 ± 0.0000	2.68E-03 ± 0.0013	1.26E-01 ± 0.0280

435

436

437 **Table 4.** Results of the immunohistochemical examination.

Tissue		AGP	OVT
Subcutaneous adipose tissue	**Adipocytes**	+ (intense, with scattered less positive)	+ (multifocal to diffuse intense)
	Connective tissue	+ (multifocal)	+ (multifocal)
Visceral adipose tissue	**Adipocytes**	+ (intense multifocal to diffuse)	+ (multifocal)
	Connective tissue	+ (multifocal)	+ (multifocal)
Pericardial adipose tissue	**Adipocytes**	+ (diffuse)	+ (multifocal)
Pectoral muscle	**Muscular fibers**	Negative	Negative
Thymus	**Lymphocytes**	Negative	Negative
Spleen	**Lymphocytes**	Negative	Negative
Brain	**Neurons**	+ (mild diffuse)	+ (mild diffuse)
	Axons	Negative	Negative
	Choroid plexus	+ (diffuse)	+ (diffuse)
Cerebellum	**White matter**	Negative	Negative
	Granule cell layer, Purkinje cell layer	Negative	Negative
	Molecular layer	+ (mild)	+ (mild)
Kidney	**Tubular epithelium**	+ (multifocal cytoplasmic)	+ (multifocal granular cytoplasmic)
	Glomeruli	Negative	Negative
Lung	**Parabronchi, atria, air capillaries, bronchi**	+	+
Crop	**Epithelium**	stratum corneum: +, stratum lucidum and stratum granulosum: -, stratum spinosum and stratum basale: +	stratum corneum: +, stratum lucidum and stratum granulosum: -, stratum spinosum and stratum basale: +
	Muscle	Negative	Negative
Mucosa of proventriculus	**Mucosal columnar epithelium**	+ (in the basilar zone of the cytoplasm)	+ (in the basilar zone of the cytoplasm)
	Ductal epithelium	+ (faint diffuse)	+ (moderate multifocal)
	Oxynticopeptic cells	+ (faint patchy)	+ (faint patchy)
Mucosa of gizzard	**Epithelium, koilin, muscle**	Negative	Negative
Pancreas	**Islets**	+ (intense)	+ (intense)
	Acini	Negative	Negative
Intestine	**Duodenum, ceacum epithelium, Meckel's diverticulum epithelium**	+ (mild scattered single cells)	+ (mild scattered single cells)
	Lymphoid follicles (GALT, cecal tonsil)	Negative	Negative
Bursa of Fabricius	**Lymphocytes**	Negative	Negative
	Epithelium	+ (mild multifocal)	+ (mild multifocal)
Liver	**Hepatocytes**	+ (faint diffuse)	+ (faint multifocal)
	Ductal epithelium	+ (mild multifocal)	+ (intense multifocal)

438 +/- = mild, + = moderate, ++ = intense

439

Figure 1. Relative extrahepatic expression of SAA, OVT and CRP in chicken healthy tissues studied by qPCR. Liver was used as reference tissue and data are means ± SEM of six animals.

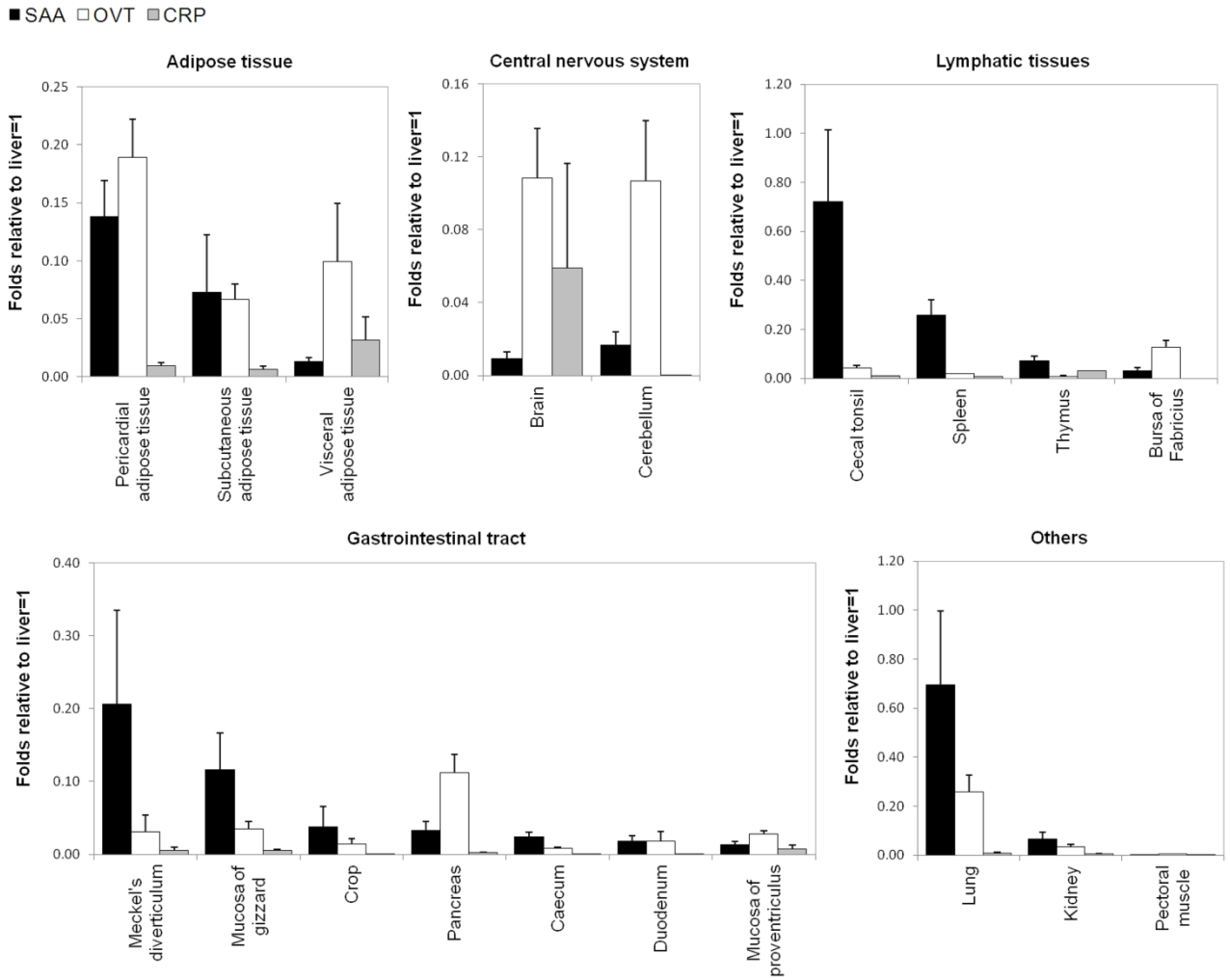

Figure 2. Immunohistochemical staining of chicken tissues for OVT (A, C, E, G) and AGP (B, D, F, H) (standard ABC method, AEC red chromogen). A-B. Brain: neurons are positively stained while white matter is negative. C-D. Kidney: mild, multifocal staining of tubular epithelium. E-F. Adipose tissue: diffuse positive staining of adipocytes. G-H. Lung: moderate, diffuse positive staining of capillary epithelium. Bar, 12.5, 25 and 50 micrometers.

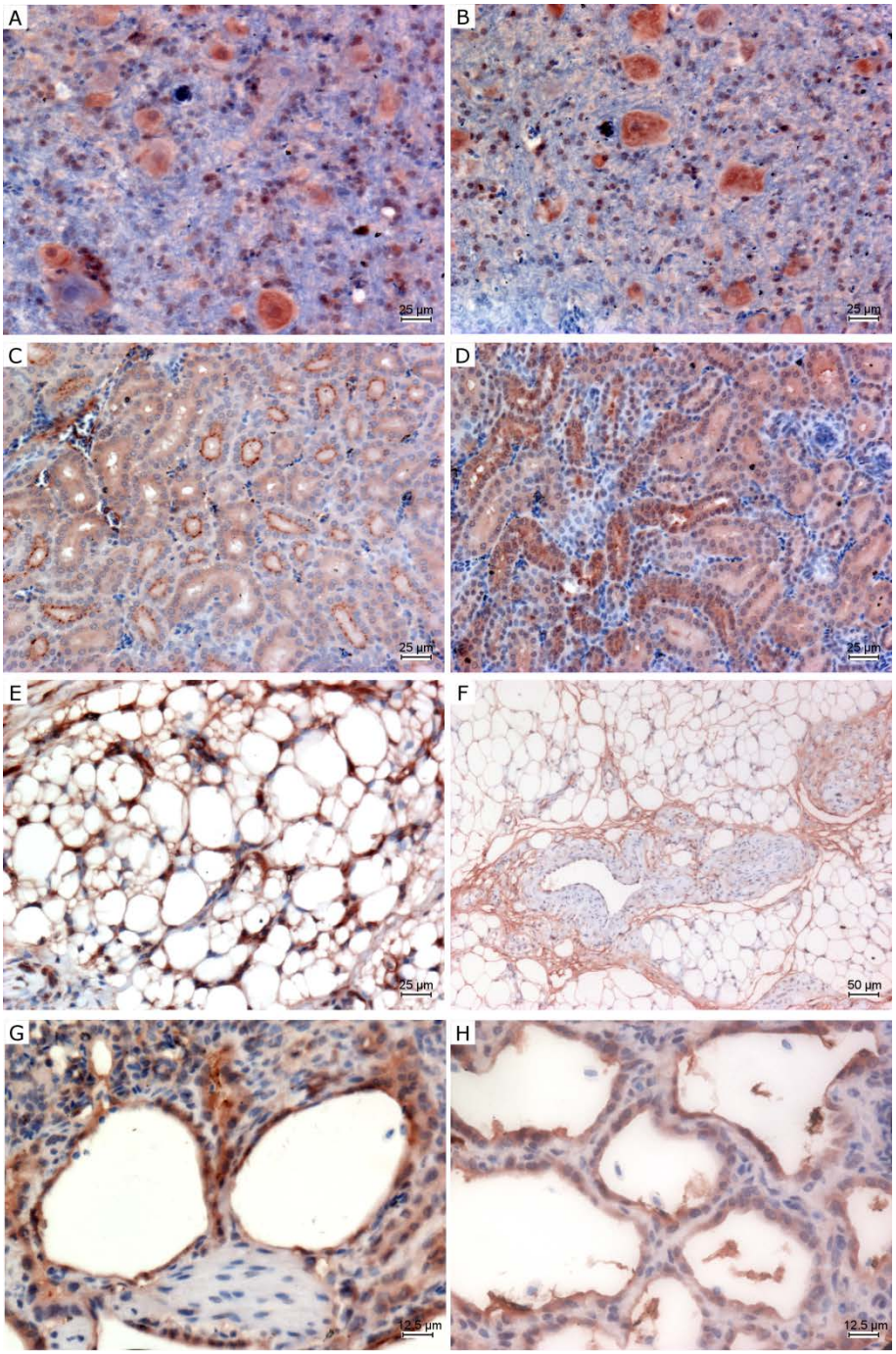

Figure 3. Immunohistochemical staining of chicken tissues for OVT (I, K, M, O) and AGP (J, L, N, P) (standard ABC method, AEC red chromogen). I-J. Pancreas: intense positive staining of endocrine islet. Exocrine pancreas is negative. K-L. Liver: mild diffuse staining of hepatocytes and moderate to intense staining of bile duct epithelium. M-N. Proventriculus: diffuse, moderate positive staining of the basilar portion of lining epithelium. O-P. Crop: diffuse, intense positive staining of squamous epithelium (stratum corneum and stratum basale). Bar, 25 and 50 micrometers.

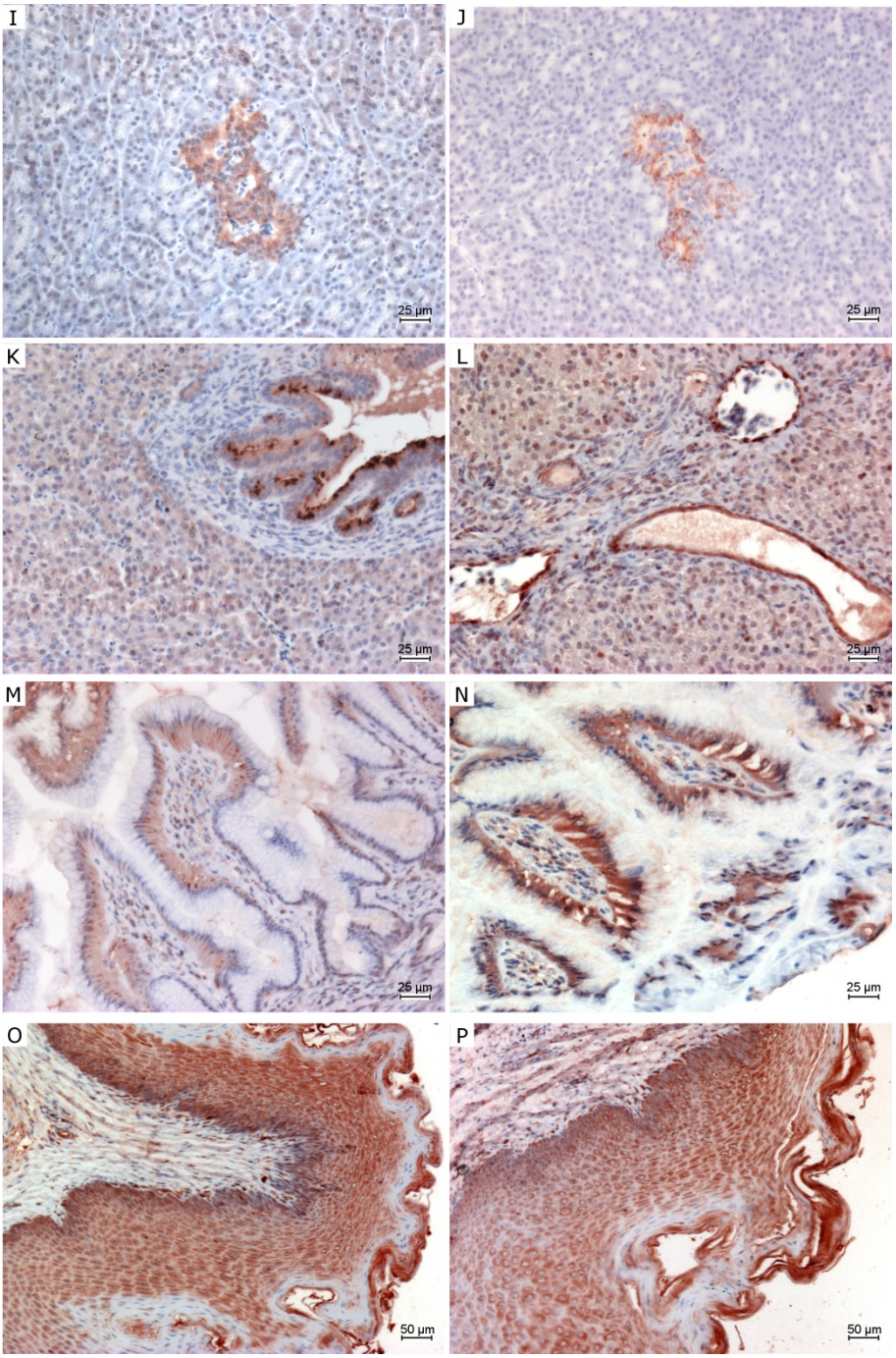

Figure 1. Supplemental material.

PCR amplification products of AGP, SAA, PIT54, CRP and OVT in liver chicken mRNA separated by agarose gel electrophoresis (1.6%) stained with ethidium bromide. Non-template reaction was used as negative control.

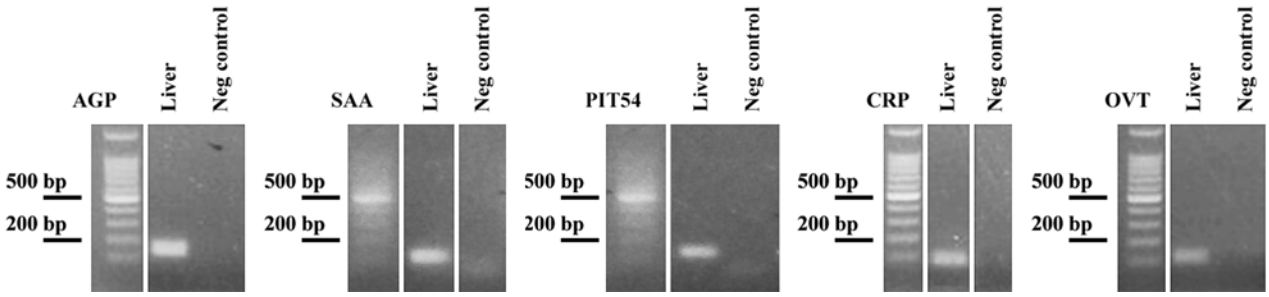

Figure 2. Supplemental material.

Detection of chicken AGP by Western blotting in serum. Left panel: anti-boAGP primary antibody. Right panel: anti-boAGP primary antibody after blocking specific sites with purified bovine AGP.