



Short communication

The localization and differential expression of Serum Amyloid A in bovine liver and adipose tissue depots



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ABSTRACT

In this article the localization of the acute phase protein Serum Amyloid A (SAA) in different depots of bovine adipose tissue (AT) and liver is reported. Quantitative (Real Time) PCR was paired to immunohistochemistry after the production of a specific polyclonal antibody. SAA's mRNA was found in all analyzed AT depots included in the present study, the AT located in the withers being the major source of SAA mRNA. A polyclonal antibody was raised against bovine SAA and was used to validate gene expression analyses. Western Blotting confirmed that SAA is present in all the seven adipose tissue depots include in the present experiment. Anti-SAA polyclonal antibody also stained diffusely adipocytes. In liver, intracytoplasmic immunolabeling was observed in hepatocytes. Staining was generally mild and not diffuse: negative hepatocytes were intermixed with positive ones. A positive intracytoplasmic immunostaining was occasionally observed in endothelial cells lining small blood vessels within AT septa and liver parenchyma. Our data confirm that bovine AT may provide an important source of SAA in healthy subjects. It remains to be determined which is the contribution of AT in the serum concentration of SAA.

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1. Introduction

Serum Amyloid A (SAA) belongs to a family of structurally related proteins that are constitutively expressed (SAA4) or upregulated due to inflammatory stimuli (SAA1 to SAA3) (Uhlir and Whitehead, 1999). Serum amyloid A (SAA) is regarded as the major APP in most species including cow, pig, cat, dog, horse (Eckersall and Bell, 2010), Sheep (Miglio et al., 2013), goat and wild ruminants (González et al., 2008; Rahman et al., 2010a,b), and poultry (O'Reilly and Eckersall, 2014). Plasma concentration of SAA may rise up to 100–1000-folds in response to inflammation or infection (Eckersall and Bell, 2010). SAA1 and SAA2 are produced by liver and represent the main circulating isoforms (Molenaar et al., 2009) whereas the inflammatory isoform, namely SAA3, is produced by extrahepatic tissues (Jacobsen et al., 2005, 2006; Kjelgaard-Hansen et al., 2007).

The main biological functions of SAA remain still elusive. Being an apolipoprotein, SAA increases the export of cholesterol of phagocytosed cell membranes from cholesterol-laden macrophages via scavenger receptor B1. SAA fulfills also important roles in

inflammatory reaction, by acting on one side as an opsonin, and on the other side as chemoattractant molecule for monocytes and neutrophils (Ceciliani et al., 2012).

In cattle, SAA3 has been found to be expressed, beside liver, also in mammary gland (Weber et al., 2006), where it is upregulated after inflammatory challenge and plays a role in the mammary gland defense (Molenaar et al., 2009). It must also be said that SAA3 expression is not necessarily legated to an inflammatory status, since its presence has also been detected in tissues from healthy animals, such as forestomachs (Dilda et al., 2012) and many others (Berg et al., 2011; Lecchi et al., 2012). The serum concentration of SAA increases around peripartum, peaking in the week following parturition (Humblet et al., 2006), which is probably related to the upregulation of SAA due to delivery-related inflammatory status, caused by stress or lesions at the genital apparatus (Murata, 2007; Humblet et al., 2006). A recent investigation identified bovine SAA as an adipokine (Mukesh et al., 2010), and bovine AT was found to be also a major source of SAA, its gene expression being upregulated around peripartum (Saremi et al., 2013). The present study aims to precisely locate the SAA in bovine AT depots and liver by pairing quantitative gene expression studies and immunohistochemistry (IHC), as preliminary step to understand the precise physiological and inflammatory role of this protein in these tissues. In order to gather this information, and not being commercially available any

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antibody that can label SAA with IHC, A polyclonal antibody capable to immunolabel SAA in tissues was also raised.

2. Material and methods

2.1. Sample collection

Samples from bovine AT were collected during the routine slaughtering procedures from six multiparous non-pregnant Holstein cows in their late lactation period. The clinical status of the animals was assessed by ante mortem inspection. The animals enrolled in the study were clinically healthy and no gross lesions were recorded during common slaughterhouse inspection procedure. Samples from seven different fat depots, belonging to subcutaneous adipose tissue (from withers, tail head, and sternum) and visceral adipose tissue (omental, mesenteric, pericardial and perirenal), were collected, washed with sterile Phosphate Buffered Saline (PBS) and (a) snap frozen in liquid nitrogen and then stored at -80°C for Western Blotting, immunohistochemistry (IHC) cryosections and gene expression analyses or (b) fixed in formalin and then embedded in paraffin for immunohistochemistry (IHC). Liver samples were collected as well, washed with sterile PBS, stored in mRNA later (Sigma-Aldrich) at $+4^{\circ}\text{C}$ for 12 h and then frozen at -80°C for gene expression analyses, or fixed in formalin and then embedded in paraffin for IHC.

2.2. mRNA expression studies

Total RNA was extracted using TriZol (Invitrogen, Monza, Italia), treated with DNase I (Invitrogen, Monza, Italia) and quantified using a NanoDrop ND-1000 UV-vis spectrophotometer. The purity of RNA (A_{260}/A_{280}) was ~ 2 . Reverse transcription (RT) was carried out with $1\ \mu\text{g}$ RNA using the iSCRIPT cDNA Synthesis Kit (BioRad, Segrate, Italy). The cDNA was used as the template for qualitative PCR, which was performed in $10\ \mu\text{L}$ final volume containing $1\ \mu\text{L}$ cDNA, $1\times$ buffer (Vivantis, Oceanside, CA, USA.), $1.5\ \text{mM}$ MgCl_2 , $0.2\ \text{mM}$ each deoxynucleotide triphosphate (dNTP), $1\ \mu\text{M}$ each primer and $0.025\ \text{U}$ Taq polymerase (Vivantis, Oceanside, CA, USA.). Qualitative PCR was performed at the following conditions: 35 cycles of 94°C for 30 s, 60°C for 30 s and 72°C for 45 s (Eppendorf Mastercycler, Eppendorf, Germany). PCR products were visualized on 1.6% agarose gels stained with ethidium bromide. Primers are listed in Table 1 of Supplementary material and were used in both qualitative and quantitative PCR. Primers for SAA mRNA PCR were designed on SAA3 nucleotide sequence (NM_181016 | Bos taurus serum amyloid A 3 (SAA3)).

Quantitative reactions were performed in $12\ \mu\text{L}$ of Eva Green mix (BioRad, Segrate, Italy) and $400\ \text{nM}$ of GAPDH and LRP10, $500\ \text{nM}$ of HPCAL1 and $350\ \text{nM}$ of SAA primers (Table 1 Supplementary material) on Eco Real Time PCR detection System (Illumina, San Diego, Ca, USA). Each sample was tested in duplicate. To evaluate PCR efficiency, fourfold serial dilutions were prepared from reference samples (liver). The thermal profile for each target gene was 95°C for 90 s, 50 cycles of 95°C for 5 s and 60°C for 10 s; conditions for melting curve construction were 55°C for 60 s followed by 80 cycles starting at 55°C and increasing 0.5°C each 10 s. No-RT controls and no template controls were performed. The relative quantification of genes of interest was carried out after normalization of the sample using the geometric mean of the reference genes. The mRNA abundance data were analyzed by ANOVA using the General Linear Model of SAS (SAS/STAT, Version V8, 1999, SAS Inst). Significance was declared for $p \leq 0.05$.

2.3. Production of polyclonal anti-bovineSAA antibody

Aiming to localize SAA protein in bovine AT tissue by IHC and to detect the presence of specific protein species by Western Blotting, a polyclonal anti-bovine SAA antibody was raised in rabbits following standard immunization procedures, using $2\ \text{mg}$ of immunoreactive peptide synthesized as multiple antigenic peptide (MAP; Tam, 1988). In this MAP system, multiple copies of antigenic peptides are simultaneously bound to the α - and ϵ -amino groups of a non-immunogenic Lys-based dendritic scaffold. The polyclonal antibodies were raised against the peptide $_{86}\text{TDPLFKGTTSGQGQ}_{99}$, which is not present in the SAA sequence of rabbit. A simulation of hydrophathy of SAA (Kyte and Doolittle, 1982) identified the peptide within a hydrophilic domain.

2.4. Western Blotting analysis

The immunoreactivity of the antibody and the cross-reactivity with other bovine proteins was tested by Western Blotting against bovine serum: $100\ \text{ng}$ of total protein were separated in 12% acrylamide SDS-PAGE gels and blotted onto nitrocellulose membranes (BioRad, Segrate, Italy). Nitrocellulose blots were blocked for 20 min with 1% (v/v) Roti-Block® (Carl Roth GmbH, Karlsruhe, Germany) in PBS, 0.1% Tween at room temperature. The membranes were then incubated with different concentrations of polyclonal anti-SAA primary antibody (1:1000, 1:2000, 1:5000 and 1:10,000) diluted in 1% (v/v) Roti-Block® in PBS for 1 h at room temperature. After three washings with 1% (v/v) Roti-Block® in PBS (10 min each), the nitrocellulose membranes were further incubated with an anti-rabbit IgG polyclonal antibody (dilution 1:2000), conjugated to horseradish peroxidase (Sigma, Milano, Italy). Following three washes with PBS, 0.1% Tween, the immunoreactive bands were finally visualized by using enhanced chemiluminescence assay (Millipore, Vimodrone, Italy) and were exposed to X-ray film. To rule out unspecific cross-reactivity of the primary antibody. The specificity of antibody was further tested on nitrocellulose membrane after pre-incubation of the primary antibody with the immunoreactive peptide (Rahman et al., 2010a,b).

In a second part of the experiment, the presence of SAA in adipose tissue from different fat depots was validated by means of Western blotting analysis. Aliquots of $100\ \text{mg}$ of adipose tissue were prepared as previously described (Rahman et al., 2014) and brought to a final concentration of 1% protease inhibitor cocktail (Sigma-Aldrich, Milano, Italy). Aliquots of $50\ \mu\text{g}$ (total protein) were loaded onto each lane of a 12% sodium dodecyl sulphate-polyacrylamide (SDS-PAGE) gel and Western Blotting was carried out as previously described onto nitrocellulose membranes and immunolabeled with a primary anti-SAA dilution of 1:4000, which was previously shown to be effective for Western blotting experiments. Positive control was the MAP peptide which was used as immunogen for the preparation of the polyclonal antibody anti-SAA. The immunoreactive bands were visualized as previously described. To confirm that an equal amount of protein was loaded in each lane, the membrane was immunolabeled with mouse anti β -actin antibody (Calbiochem, Darmstadt, Germany) at a dilution of 1:10,000 as previously described (Lecchi et al., 2008).

2.5. Immunohistochemistry

Immunohistochemistry was performed on both frozen and formalin-fixed paraffin embedded (FFPE) tissue sections to identify the presence of the SAA proteins in the different cell types present in AT and in liver. Serial sections of $5\ \mu\text{m}$ were obtained from frozen samples, mounted on polylysine-coated slides and fixed in cold acetone (-20°C) for 2 min. The slides were then immersed in $100\ \text{mM}$ Tris buffer saline pH 7.5 (TBS) for 5 min and endogenous

peroxidase activity was therefore blocked with 0.3% H₂O₂ in TRIS for 30 min. Nonspecific protein binding was blocked by incubation for 20 min at room temperature (RT) with 10% normal goat serum. Polyclonal anti-SAA antibody was used at dilutions of 1:80, and incubated overnight at 4 °C in a humidified chamber. After 3 rinses in TBS for 5 min each, the sections were incubated with PolyView mouse/rabbit nanopolymer detection reagents (Enzo Life Sciences Inc., Lausen Switzerland) for 20 min at RT. After washing 3 times in TBS, the chromogen, 3-amino-9-ethylcarbazole (Vector Laboratories, Burlingame, CA, USA) was applied for 10 min. The slides were then rinsed in tap water, counterstained with Mayer hematoxylin (Diapath SpA, Martinengo, Italy) for 1 min and mounted with glycerin jelly (Kaiser's glycerol gelatin, Merck KGaA, Darmstadt, Germany.).

For the FFPE samples, 5 μm sections were obtained and mounted on poly-lysine-coated slides. The sections were deparaffinized in xylene and rehydrated through a descending series of ethanol concentrations. The endogenous peroxidase activity was blocked with 0.3% H₂O₂ in methanol for 30 min. Antigen retrieval was then performed by heating the slides in a pressure cooker for 10 min in a citrate buffer solution (pH 6.5). The sections were then rinsed in TBS. The IHC staining procedure was then performed as described above for the frozen specimens. Sections of liver were used as positive controls. Negative controls were prepared by replacing the respective primary antibody with normal rabbit serum (non-immune rabbit serum, Dako Denmark A/S, Glostrup, Denmark). In addition, to rule out unspecific cross-reactivity of the primary antibody, the anti-SAA specific sites were blocked by pre-incubation with the SAA peptide which was used to raise the antibody, as previously described (Rahman et al., 2010a,b).

3. Results and discussion

The presence of SAA mRNA in the seven adipose tissue depots was first examined by qualitative PCR confirming the presence of SAA mRNA in all the AT tissue depots included in the present study (Data not reported). For more accurate and reliable normalization of gene-expression data, the authors calculated a normalization factor based on multiple control genes starting from data previously reported about adipose tissue (Hosseini et al., 2010; Mukesh et al., 2010), as suggested by the software geNormTM (Vandesompele et al., 2002). The mRNA abundance of SAA was then quantified by qPCR and was 20–40% less as compared to liver. Results are presented in Fig. 1A. The withers SAA mRNA abundance, on the contrary, was very close to that of liver, while the tail head expressed much lower mRNA SAA as compared to that of other adipose tissue depots. The mRNA abundance of SAA from withers and tail head was statistically significantly different as compared to that of the other AT depots. The present results mostly agreed with those from Saremi et al. (2013), except that the major source of SAA in AT in the present finding was withers, whereas omental AT was previously found to be the main source of SAA (Saremi et al., 2013). The SAA gene expression in adipose tissue is related to the lactation period and reaches the peak around peripartum, to steadily decrease thereafter (Saremi et al., 2013). The animals selected to be included in this study were in their late lactation period. Therefore, the lower expression level of SAA mRNA in AT as compared to that of liver is consistent with the previous observation that SAA's mRNA abundance decreases in parallel with the lactation period.

In order to localize the SAA protein in adipose tissue and liver, a polyclonal antibody specific for a peptide uniquely present in some ruminant species, was raised. To the best of the knowledge of the authors, commercially available antibody that can label bovine SAA with immunohistochemistry are lacking. Some antibodies reacting, or cross-reacting, with bovine SAA, (McDonald

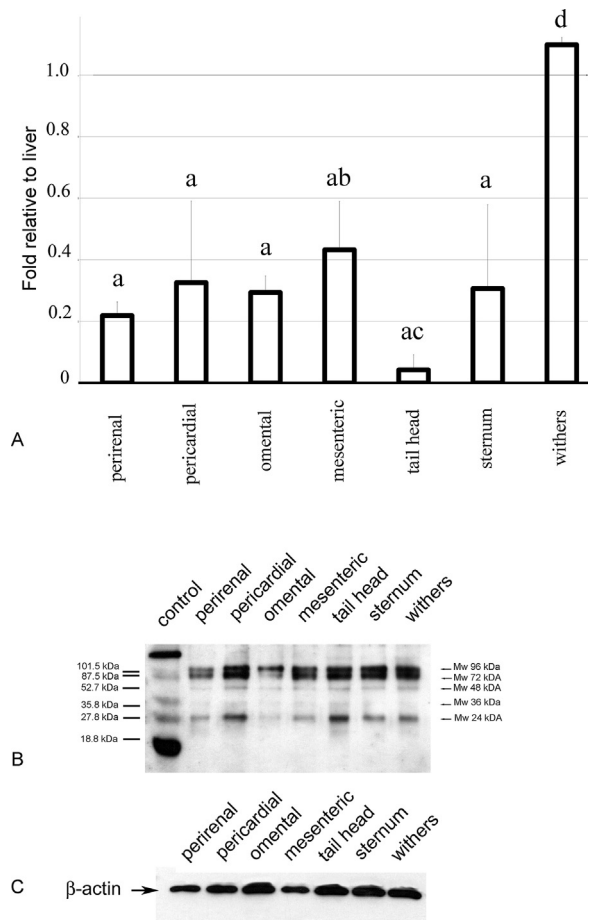


Fig. 1. Serum Amyloid A expression in adipose tissue.

(A) Relative abundance of SAA in different bovine adipose tissue depots collected from clinically healthy dairy cows. The values are expressed as the fold difference relative to the liver and are normalized using GAPDH, LRP10 and HPCAL1 as reference genes. The data are the means \pm standard errors of the means. The adipose tissues indicated with (a) are not different from the others. The adipose depots, indicated with (b) and (c) are statistically different each from the other, but not different from those marked. The adipose depots marked with (d) is statistically different from all the others.

(B) Western Blotting results for SAA in different bovine adipose tissue depots collected from clinically healthy dairy cows ($n=6$). Positive control was the MAP peptide which was used as immunogen for the preparation of the polyclonal antibody anti-SAA, as specified in Materials and Methods.

(C) Western Blotting results for β -actin.

et al., 1991; Molenaar et al., 2009) were not validated for immunohistochemistry. In general, raising antibodies against SAA proteins is always problematic: the protein in native state show peculiar biochemical characteristics, can produce multimers and/or associate with other proteins and its sequence is pretty conserved. Indeed, many authors have reported difficulties in raising/using anti-SAA antibodies, given the background that this protein can lose its solubility during the purification procedure (Soler et al., 2013). The utilization of a peptide overcame the low solubility of SAA that drives the protein to precipitate (Yamada, 1999). By using a concentration of primary antibody at 1:5000, two major bands were detected at 26/28 kDa and 56/60 kDa, correspondent to SAA dimer and tetramer, respectively (Fig. 1 Supplemental). The positive signal completely disappeared when tested on nitrocellulose membrane stained with the pre-incubated antibodies, thus confirming the specificity of rabbit polyclonal anti-bovine SAA (data not reported). The presence of SAA protein was then determined in the seven AT depots and results are presented in Fig. 1B. To confirm that an equal amount of protein was loaded in each lane, membranes were also

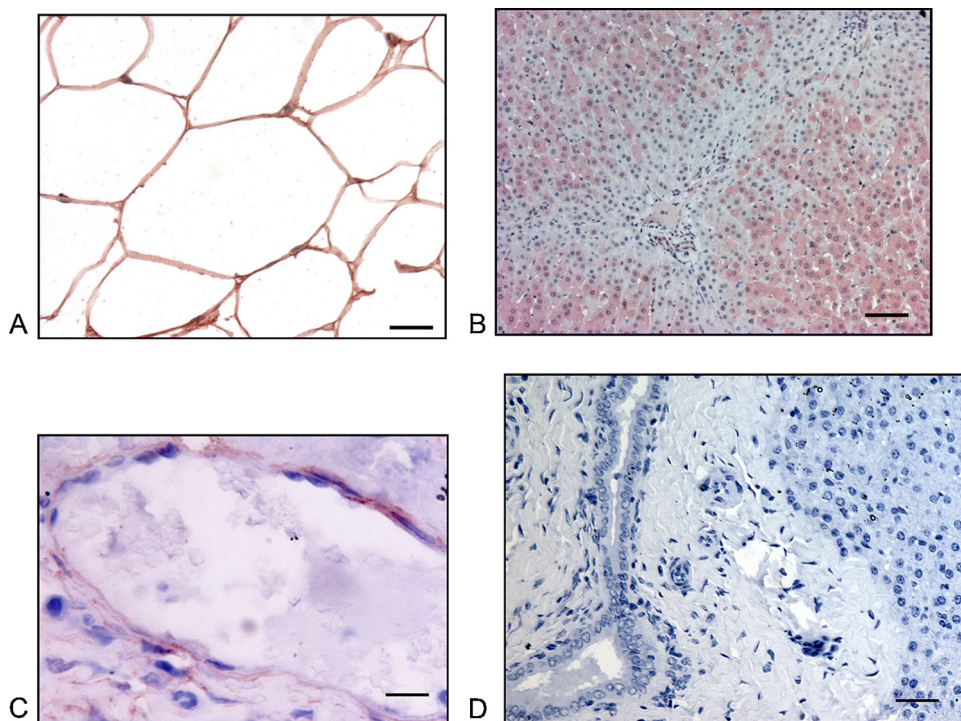


Fig. 2. Immunohistochemical localization of SAA in bovine tissues.

(A) Adipose tissue (omental), anti-SAA immunolabeling, AEC (red) chromogen. Intense positive immunostaining defines adipocyte cell borders. Bar 50 μm .

(B) Liver, anti-SAA immunolabeling, AEC (red) chromogen. Diffuse, moderate positive intracytoplasmic immunostaining is detectable in about 80% of hepatocytes in the section. Negative hepatocytes are mostly located around the portal triad (functional zone 1). Bar 100 μm .

(C) Detail of a blood vessel within liver parenchyma, anti-SAA immunolabeling, AEC (red) chromogen. Focal, positive intracytoplasmic immunostaining is visible in a single endothelial cell (arrowhead). Bar 20 μm .

(D) Immunolabeling of bovine liver section with the pre-incubated SAA antibody. AEC (red) chromogen, Mayer's hematoxylin counterstain. No positive staining is present: hepatocytes, stroma and biliary ducts are stained only with Mayer's (blue) counterstain. Bar 100 μm . (For interpretation of the references to color in this figure legend, the reader is referred to the web version of the article.)

immunolabeled with mouse anti β -actin antibody (Fig. 1C). Positive signals for SAA were detected ranging from 26 to 28 kDa to 96 kDa bands, corresponding to the multimers of this protein. The presence of different bands of bovine SAA was recently reported (Saremi et al., 2013), in agreement with what was already reported in other species like the pig (Soler et al., 2013). There are a number of references in the bibliography that described the ability of locally expressed SAAs to form multimers in physiological conditions (Molenaar et al., 2009; Eckhardt et al., 2010). The functional implication of the presence of such multimers in bovine fat depots remains, however, to be determined in the future.

In the final part of the study, the specific polyclonal anti-SAA antibody was used to locate the presence of SAA in adipose tissue. Results are presented in Fig. 2. An anti-SAA positive immunostaining was detected in frozen as well as in FFPE sections prepared from visceral AT, subcutaneous AT and liver. Both visceral and subcutaneous AT showed the same intense positive signals in all the analyzed depots (Fig. 2A). Immunostaining was localized at the periphery of adipocyte cytoplasm, lining cell borders. A positive intracytoplasmic immunostaining was also observed in endothelial cells lining small blood vessels within AT septa. Immunohistochemistry on liver parenchyma showed anti-SAA immunolabeling in hepatocytes and in scattered endothelial cells of blood vessels. Immunostaining in hepatocytes was intracytoplasmic, generally mild to weak and often patchy or with negative hepatocytes intermingled with positive hepatocytes. In some animals, positive immunolabeling was more intense in hepatocytes of functional zones 2 (midzonal) and 3 (centrilobular), whereas hepatocytes in the functional zone 1 (periportal) were mostly not immunolabeled (Fig. 2B). A positive intracytoplasmic immunostaining was

occasionally observed in endothelial cells lining small blood vessels within AT septa and liver parenchyma. On the background of the histological location of SAA, we speculate that SAA can be involved in the specific functions of zone 2 and 3 of the liver, which include glycolysis, lipogenesis and cytochrome P-450-based drug detoxification, instead of those carried out in zone 1, which include gluconeogenesis and β -oxidation (Hijmans et al., 2014). These results are preliminary and have to be confirmed on a large number of animals. As already presented in Western Blotting experiments, the positive immunolabeling of control and fat tissues completely disappeared when tissue sections were stained with the pre-incubated antibody, thus confirming the specificity of anti-SAA antibody for its antigen on tissues as well (Fig. 2D).

The biological role of SAA remains mostly undisclosed. There is a growing interest for SAA derived from adipocytes due to its function as a mediator between hypertrophied adipocytes and macrophages by regulating the adipocyte cholesterol efflux (Poitou et al., 2009). The linking between SAA presence and a possible role in adipose tissue is provided by the finding that SAA activates PPAR γ through ERK 1/2-dependent COX-2 expression (Liu et al., 2011). PPAR γ is highly expressed in bovine AT, where it fulfills an essential role of fine tuning of adipogenesis (Bionaz et al., 2013) by inducing adipogenic differentiation of vascular stromal cells (Ohyama et al., 1998). Furthermore, AT PPAR γ plays also a role in Long Chain fatty Acid oxidation by regulating the expression of carnitine palmitoyltransferase 2 and carnitine *o*-acetyltransferase (Sharma et al., 2012). One of the possible role of SAA may be to regulate adipogenesis and reorganization of adipose tissue and potentiate fatty acid oxidation by interacting with PPAR γ receptor.

The present study provides a detailed characterization of the expression pattern of SAA in different AT depots and liver of lactating cows. Our findings parallel what has been recently reported (Saremi et al., 2013), extending the investigation to other AT depots (pericardial adipose tissue) and localizing the precise site of expression by IHC. New insights in the expression pattern of SAA in liver were also provided. SAA is an acute phase protein, and therefore any further knowledge about its expression in adipose tissue would contribute to better understanding the modifications of its concentration in pathophysiological conditions. The production of a specific anti-SAA antibody will provide a useful mean to measure the concentration of SAA in bovine biological fluids.

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at <http://dx.doi.org/10.1016/j.vetimm.2015.08.004>.

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