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Identification of the pregnancy-associated glycoprotein family (PAGs) and some aspects of placenta development in the European moose (*Alces alces* L.)

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ABSTRACT

This study describes the identification and a broad-based characterization of the pregnancy-associated glycoprotein (*PAG*) genes expressed in the synepitheliochorial placenta of the *Alces alces* (Aa; N = 51). We used: (1) both size measurements (cm) of various Aa embryos/fetuses (crown-rump length) and placentomes (PLCs); (2) PCR, Southern and sequencing; (3) Western-blot for total placental glycoproteins; (4) deglycosylation of total cotyledonary proteins; and (5) double heterologous IHC for cellular immune-localization of the PAGs as pregnancy advanced (50–200 days *post coitum*). The crown-rump length and PLC size measurements permitted a novel pattern estimation of various pregnancy stages in wild Aa. The PLC number varied (5–21) and was the greatest at the mid and late stages of gestation in females bearing singletons or twins. The genomic existence of the identified PAG-like family was named *AaPAG-L*. Amplicon profiles of the *AaPAG-L* varied in the number and length (118–2000 bp). Southern with porcine cDNA probes confirmed specificity and revealed dominant *AaPAG-L* amplicons in males and females. Nucleotide sequences of the *AaPAG-L* amplicons shared 86.27% homology with the bovine *PAG1* (*bPAG1*) gene. Amino acid AaPAG sequences revealed *in silico* 88.23% to 100% homology with the bPAG1 precursor. Western-blots revealed a dominant mature 55 kDa AaPAG fraction, and the major ~48 kDa glycosylated form that was deglycosylated to ~44 kDa. The AaPAG-Ls was immuno-localized to mono- and bi-nucleated trophectodermal cells (TRD–chorionic epithelium), where signal intensity resembled intense TRD proliferation within developing PLCs as pregnancy advanced. This is the first study identifying the AaPAG-L family in the largest representative among the Cervidae.

1. Introduction

The genera of the moose and the Eurasian elk (*Alces alces*–Aa) are taxonomically the largest extant mammalian two sister subspecies among the deer family (Cervidae). Both subspecies have been classified in the international Red List of Threatened Species (www.iucnredlist.org),

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within the "least concern" category because they are still widespread and relatively abundant, despite fairly intense hunting in Russia, Scandinavia, and some other Asiatic areas. With the paucity of *post mortem* tissues for detailed study, and also many difficulties of serial sampling from wild animals, little scientific attention has been directed toward the placental development and identification of various secretory proteins involved in the maintenance/regulation of single or multiple gestations (until term 235–250 days *post coitum*; dpc).

The pregnancy-associated glycoproteins (PAGs) family is known as a large group of conserved genes encoding multiple secretory chorionic products, classified into two subfamilies of placental aspartic proteinases (EC 3.4.23), catalytically active and potentially inactive [1,2]. Among the identified complementary DNAs (cDNAs) of the *PAGs* (deposited in the GenBank/NCBI), at least 75 diversified cDNAs have been cloned in domesticated taxa only, including cattle, sheep, pig, horse, goat, cat, mouse, and water buffalo [1,3,4]. Only in a few wild taxa, a lower number of catalytically diversified *PAG* cDNAs (32) have been cloned: in the zebra [5], white-tailed deer (wtd) [6], American bison [7], wapiti [8], and giraffe [9]. Thus, in the wild species, this lower number of various *PAG* cDNAs is caused by many difficulties associated with the proper conditions for placenta collection required for high quality of total RNA isolation, allowing for effective cloning of full-length cDNA, including coding (ORF) and noncoding regions (5′UTR and 3′UTR).

Genomic analyses indicated the exonic-intronic structure (nine exons and eight introns, A–H) and specific promoter with a unique duplicated sequence within the bovine *PAG* (*bPAG1*) and porcine (*pPAG2*) cloned genes only [10,11]. Thus, most of the previous studies of the *PAG* gDNA templates were focused on domestic but not wild species.

Within each placenta type in various eutherians, different embryo-originated cells constitute the outer chorionic layer that forms a very precise interface with the uterus [12,13], where placental expression profiles of multiple *PAGs* are cell and pregnancy stage dependent [1,2]. Various distinct *PAG* transcripts have been identified in the pre-placental trophoblast (TR) during the peri-implantation period, whereas some other *PAGs* have been identified in the trophectoderm (TRD–chorionic epithelium) as the outer embryonic layer during the post-placental period until pregnancy term. So far, temporary specific placental *PAG* mRNA expression has been identified in: the cattle and sheep [14–16], the pig [17–19], the horse [5], the goat [20,21], the European bison [22], the white-tail deer [6], and some camelids, including the alpaca, dromedary, and Bactrian camel [23].

In domestic ruminants, the transcribed PAGs are comprised of two evolutionarily distinct groups. The first group, the 'modern' PAGs, is exclusively transcribed in specialized and moderately invasive TR/TRD, known as binucleate cells (BNCs). The second grouping, known as the 'ancient' PAGs, is transcribed in both cell types, mononucleate cells (MNCs) and BNC [14,15,24]. Also, immunoreactive PAGs were localized within various TRD cells (MNC/BNC) throughout placenta development in some taxa: cattle [25], pig, European bison, alpaca, and both camels [26–29]. Both cell types (MNC and BNC), producing multiple and diversified secretory PAGs, release their secretory granules into the maternal blood [1].

Protein studies allowed the identification of several distinct NH_2-terminal micro-sequences of native PAG isoforms (35–76 kDa) purified from the placenta of some domesticated species, including cows, goats, sheep [14,30–32], zebu [33], water buffalo [34], and wild American and European bison [35,36]. Also, *in vitro* studies revealed multiple secretory PAG isoforms produced by chorionic explants of domestic and wild species [22,37]. Other types of *in vitro* studies indicated the potential physiological importance of the PAGs, as embryo-originated signaling ligands interacting with different maternal gonadotropin receptors (gonadal and extra-gonadal) in cyclic pigs and cows [38] or pregnant pigs [39], and thus involved in the regulation of pregnancy maintenance.

The native and recombinant PAGs [40,41] have been applied for prenatal tests (radioimmunoassay and ELISA) to diagnose early pregnancy and to monitor fetus mortality, based on varying PAG concentrations in peripheral blood or milk of various domestic [1,42–47] or wild ruminants [1,48,49]. Moreover, PAG tests are useful for identification of fetal sex, single, twin, or multiple gestations, as well as to forecast miscarriages after embryo transfer and to detect pathological pregnancies [50,51].

Therefore, the objectives of our study were to identify the expected existence of the PAGs in Aa: (1) in the genome; (2) expression within the placental proteome as pregnancy advanced: including (2a) determination of glycosylation profiles, and (2b) cellular localization during subepitheliochorial/cotyledonary placenta development.

2. Materials and methods

2.1. Animals and tissue harvesting

Only wild Aa animals (N = 51); 40 males and 11 females during single: 50, 81, 103, 115, and 190 dpc or twin gestation: 72, 115, 175, and 200 (n = 2) dpc were used. All animals were bagged (September–March, 2008–2014) at the experimental hunting area of the Russian Research Institute of Game Management and Fur Farming, Kirov Region, in the east European part of the Russian Federation (58:3 N; 50:4 E). The annual quota of Aa to be shot is from 60 to 85 individuals on the territory of a scientific hunting area (64,000 hectares). All samples were culled with the appropriate required principles of ethics, traditions, and rules of hunting. Every Aa was bagged according to official local agreements/permissions for animal hunting for scientific purposes given by the Department of Conservation and Use of Wildlife of the Kirov Region, according to Federal Hunting Law (signed by the President of Russia on 24.07.2009), Federal Hunting Rules (signed by the Minister of Natural Resources and Ecology of Russia; 16.11.2010), and Hunting Realization Parameters (confirmed by the Governor of the Kirov Region; 14.12.2012).

Various tissue samples (skin, vas deferens, testes, and placentomes [PLCs]) were harvested *post mortem* from Aa of different ages (0.5–7.5 years). The start point of the dpc counting was generally used as September 10th, when the greatest activity of Aa rutting and pairing in the Kirov

region occurs, and the pregnancy stage (50–200 dpc) was recorded in this way previously [52]. However, it is obvious that not all females can be mated during the time of their first estrus during the rutting season. Also, enlarged body shape is very difficult (or almost impossible) to control, before wild early-pregnant (dpc) ruminant females are seasonally eliminated. Moreover, various placental developments resemble singleton or multiple gestations, and also locally restricted-nutrition in some areas determines a variety of the Aa population conditions. Therefore, the expected dpc in Aa (during various placenta sampling) were generally estimated on the basis of the potentially similar length (cm) of a single embryo/fetus, according to the previously generally standardized measurements of fetal growth rates/fetus crown-rump length (CRL) in domestic cattle [53], and also due to the available bovine fetal age calculator based on CRL (www.ansci.wisc.edu/jjp1/ansci_repro/lab/female_anatomy/crown_rump_calculators.htm).

Among the synepitheliochorial placenta type of Aa, only PLCs (created by fetal-originated cotyledons [CTs], with maternal-originated endometrial caruncles, [CARs]) were collected, due to the higher effectiveness of the PAG purifications from the PLCs, compared with lower effectiveness from the inter-PLC chorionic tissues in other wild ruminants [35,36]. For each Aa female, the sizes (cm) of the CRL and PLC were determined.

All collected tissues were preserved (−20 °C), then transported to the UWM laboratories (Olsztyn, PL), and stored (−75 °C), until further analyses.

2.2. Examination of PAG-L amplicons with genomic templates of wild Aa

Genomic DNA (gDNA) samples of Aa were isolated from skin or testes (not contaminated by CT-expression of expected *PAG* transcripts) with the use of various commercial kits (FavorPrep Tissue DNA Extraction Mini Kit, Favorgen Biotech Corp., Taiwan; and Genomic Mini AX Tissue, A&A Biotechnology, PL). Only high-quality templates of obtained gDNA were used for PCR amplifications (300–500 ng/20 μL) of the *AaPAG-L* gene fragments.

To amplify expected homological regions encompassing nine exons and eight introns within the predictable structure of the *AaPAG-L* gene family, nine par primers were applied (Table 1; designated on various porcine/bovine *PAG* cDNAs and gDNAs deposited in the GenBank/NCBI database) that have previously been useful for many cross-species productions of gDNA amplicons [1,11,23,54]. For effective PCR-amplicon synthesis, AT MaxTaq Hot Start polymerase (Vivantis, Malaysia) was used during initial activation (95 °C/5 minutes), then 40 following cycles were: 95 °C (30 seconds) for the denaturation of gDNA templates, 60 °C decreased to 46 °C (by 2°C/4–6 cycles) for primer annealing (2 minutes), and 72 °C (2 minutes) for final amplicon synthesis. Obtained amplicons: *AaPAG-L* gDNA, porcine PAG10 (*pPAG10*) cDNA–used as a positive control, and negative control (without templates) were separated in agarose gels, parallel to a marker (100–3000 bp), UV-visualized using fluorescent intercalations: ethidium bromide (0.5 μg/mL), or Midori Green DNA (0.005%) stains; and archived (G:Box, Syngene, UK).

2.3. Specificity examination of various PAG-L amplicons by double Southern hybridizations

Because homologous AaPAG probes are not yet available (based on cDNA or gDNA templates), the expected specificity of multiple PCR-produced *AaPAG-L* amplicons was examined and confirmed by cross-species (heterologous, [ht]) double Southern hybridizations, as previously [17,23], with some modifications. Briefly, the obtained *AaPAG-L* amplicons were denatured in gel (to obtain

Table 1
Primer specificity applied for a partial cross-species amplification (directed toward nine different exonic and intronic regions) of the PAGs with genomic templates of the Aa.

Regions	Primers	Sequence (5′→3′)	Amplified gene fragments: exons (with introns)	Expected amplicon length by *pPAG2* [bp]	
				cDNA	gDNA
1	SeATG	TGGACCCAGGAAAGAAGCATG	Exons 1–2 (A)	170	1263
	2As	TTCTGGATCATGTTGTAAGG			
2	SeATG	TGGACCCAGGAAAGAAGCATG	Exons 1–5 (A, B, C, D)	582	4213
	5As	GGGATAGGCCAGGCCCAGGA			
3	2Se	TACAACATGATCCAGAATCT	Exons 2–3 (B)	115	1439
	314rar	TTCCAATGGTGATGTTACCCA			
4	2Se	TACAACATGATCCAGAATCT	Exons 2–5 (B, C, D)	315	2967
	5As	GGGATAGGCCAGGCCCAGGA			
5	4PR	CCATCTACTGCAAAAGCAAG	Exons 3–5 (C, D)	234	1467
	5As	GGGATAGGCCAGGCCCAGGA			
6	5Se	TCCTGGGCCTGGCCTATCCC	Exons 5–6 (E)	194	1121
	6As	CAGGGGCACCCACTTGAGGT			
7	5Se	TCCTGGGCCTGGCCTATCCC	Exons 5–9 (E, F, G, H)	606	3364
	PagC	CAGGCCAATCCTGTTCTGTCCT			
8	6Se	ACCTCAAGTGGGTGCCCCTG	Exons 6–9 (F, G, H)	432	2261
	PagC	CAGGCCAATCCTGTTCTGTCCT			
9	7Se	CCGGGACGTCGATGCTGCA	Exons 7–9 (G, H)	317	694
	PagC	CAGGCCAATCCTGTTCTGTCCT			
1–9 (pPAG-3 or -*10* cDNA probes)	SeATG	TGGACCCAGGAAAGAAGCATG	Exons 1–9	1173	—
	PagC	CAGGCCAATCCTGTTCTGTCCT		1173	—

single-strained DNA), neutralized, and then vacuum-transferred onto positively charged nylon membranes (0.2 μm Nytran; Schleicher & Schuell, Germany). Transferred *AaPAG-L* amplicons were immobilized to nylon membranes (5 min/312 nm UV and 1 h/80 °C), and then as single-strained DNA subjected to specific Southern hybridizations.

2.4. Production of various PAG-L probes required for autoradiographic Southern hybridizations

Generally, the PAG probes are not commercially available because each probe requires specific cDNA or gDNA templates [1]. During this study, two (α-^{32}P) dATP-labeled double stranded probes were produced with the use of the previously cloned *pPAG3* and *pPAG10* cDNAs (GenBank: AF315377 and AY775784, respectively) as available plasmid templates [19]. The pPAG3 probe is specific for the *pPAG1-L* gene subfamily (potentially catalytically inactive). The second produced pPAG10 probe allows specific identification of the *pPAG2-L* gene subfamily (catalytically active).

Both pPAG-L probes (pPAG1-L and pPAG2-L) were similarly produced by useful double round PCRs, as previously described for various cross-species hybridizations [17,22], with some required modifications. During this study, the first round (cold-PCR), based on plasmid *pPAG3* or *pPAG10* cDNA templates (in pBluescript SK +/− vector), with conserve redundant primers for several pPAG-variants (see Table 1; SeATG and pagC), allowed the production of two specific cDNA amplicon templates, representing the entire ORF of the *pPAG3* and *pPAG10* (1173 bp). The subsequent second-round PCR was completed with (α-^{32}P) dATP of 20 MBq (185 TBq/mmol) specific activity (Hartman Analytic GmbH, Germany). The [α-^{32}P]-pPAG3 and [α-^{32}P]-pPAG10 cDNA probes were purified by column chromatography (Sephadex G 50, Pharmacia, Sweden), and specific activity (1470 Wizard, Wallac, USA) was approximately 1.95×10^8 and 5.05×10^7 cpm/probe, respectively.

Both denatured and appropriately diluted pPAG3 and pPAG10 probes (556,800 and 145,500 cpm/mL, respectively) were used for double nylon membrane hybridizations of the *AaPAG-L* amplicons. A non-specific binding of nylons was blocked (2–4 h/42 °C) in the prehybridization buffer (50% formamide, 5 × SSC, 5 × Denhardt, 0.5% SDS, 0.1 mg/mL fragmented salmon sperm DNA). The blocked nylon membranes were initially hybridized (24 h/42 °C) with a denatured (5 min/95 °C) and chilled pPAG10 probe. After hybridization, the nylon membranes were washed (3 × 5 minutes) in posthybridization buffer (5 × SSC/0.5% SDS) and used for autoradiography. All nylon membranes were exposed (−70°C/70–120 h) to radiographic films (RTG XMB, Kodak, USA) in cassettes supplied with intensifying screens (Perlux Extrarapid 200, Germany). After exposure, the radiographic films were developed (Developer X-OMAT M43 Processor; Kodak, USA), photographed, and digitally archived (G:Box, Syngene, UK). After autoradiographic visualization of the pPAG10 probe, all nylon membranes were stripped (2 min/95 °C; 5 × SSC/0.5% SDS) to remove the previously hybridized probe, then subjected to the next ht-Southern hybridization with the pPAG3 probe.

2.5. Sequence identification within exon 1 and intron A of the AaPAGs

To identify the initial and partial nucleotide sequence of the *AaPAGs*, the gDNA amplicons were produced with the most useful primers SeATG and Ex2as (Table 1), which should amplify the conserved coding region (of exons 1 and 2), including nonconserved noncoding region (intron A) of the *PAG-L*, according to only two known organizations of previously cloned entire structures of *bPAG1* and *pPAG2* genes [10,11]. The presently obtained *AaPAG-L* amplicons were electrophoresed, gel out purified, and used as templates for automated sequencing (3130 Genetic Analyzer, Applied Biosystems, USA) in both directions (sense and antisense). The gDNA amplicon-labeling was performed with the BigDye Terminator v3.1 Cycle Sequencing Kit (Applied Biosystems, USA), but with some modifications to the original procedure. Briefly, the labeling conditions were: initial denaturation (at 96 °C for 1 minute), and then 25 cycles (96°C/10 seconds, 50°C/5 seconds, 60°C/4 minutes). Each labeling amplification (10 μL) contained: 1 μL (5–10 ng) of appropriate amplicon template, 2 μL Ready Reaction Mix, 3 μL BigDye Terminator v1.1/3.1 Sequencing buffer (5x), and 4 μL H_2O. Labeled amplicons were purified with the BigDye X Terminator Purification Kit (Applied Biosystems, USA) and separated in capillaries filled with POP-7 polymer. Obtained *AaPAG-L* sequence data were analyzed by DNAsis v.3.0 software (Hitachi Miraibo, Japan).

2.6. Placentomal protein isolation, PAGE, and heterologous Western blotting of the AaPAGs

During this study of synepiteliochorial placenta type (PLC and inter-PLC regions), total Aa proteins were isolated only from various selected chorionic homogenates of frozen PLC tissues different in size (100 mg/small, middle, and large), due to a better method previously described for chorionic proteins isolation from CT-type than inter-CT tissues [35,36], or non-CT placenta type [55], with some modifications. Briefly, various AaPLC proteins (10–40 μg/sample/50, 120, 200 dpc), containing the AaPAG-L (expressed within the CT only), were separated by SDS-PAGE (12.5% gels), parallel to porcine placental proteins (used as a positive control of chorionic immunodetection), porcine endometrial proteins (negative control), and molecular markers (14.4–120 kDa; Fermentas, Thermo Fisher Scientific, USA). The mixture of separated total AaPLC proteins was stained with Coomassie Brilliant Blue dye (CBB). Duplicates of the SDS-PAGE–separated AaPLC proteins were semi-dry transferred onto nitrocellulose membranes (0.45 μm, Optitran BA-S58; Whatman, GE Healthcare Life Sciences, USA) and subjected to ht-Western analysis of the PLC protein detection with the primary rabbit polyvalent anti-porcine PAG polyclonals (anti-pPAG-Pv, 1:300; noncommercially available). The specificity of anti-pPAG-Pv polyclonals was previously defined by multiple homologous Western blotting of secretory native PAGs in pigs [55,56].

The immuno-complexes (AaPLC/anti-pPAG-Pv) on nitrocellulose membranes were visualized with secondary mouse anti-rabbit IgG monoclonals conjugated with alkaline phosphatase (1:150,000), and required NBT/BCIP

substrates, as done previously for various species [1,18,22,37,55]. Photographed gels and nitrocellulose-blots were archived by the FOTO/Analyst Archiver (Fotodyne, USA) or G:Box (Syngene, UK).

2.7. Deglycosylation of placentomal/cotyledonary proteins by glycopeptidase F treatment

To identify the removal of N-linked carbohydrate side chains linked to asparagines (N–x–T/S sequence) of the total PLC containing AaPAG-L proteins, *in vitro* deglycosylation was performed with glycopeptidase F treatments (TakaraBio, Japan), by a previously useful method for total 'secretory' porcine and bison chorionic proteins [22,56], presently modified for the total 'cellular' PLC proteins in the *Alces* genus (Aaa). Briefly, the cellular AaPLC proteins, harvested during various selected pregnancy stages (50 and 120 dpc), were deglycosylated (12 h/37 °C) with glycopeptidase F (2 mU/50 μg of protein). Parallel to the PLC Aa proteins, control digestion of the bovine fetuin was performed to confirm glycopeptidase F activity. Then, among all PLC, the native AaPAG-L proteins glycosylated (G) and *in vitro* deglycosylated (D) proteins were separated in denatured conditions (SDS-PAGE), semi-dry transferred onto the nitrocellulose membranes and then identified by ht-Westerns. Various blottings were done with many noncommercial anti-PAG polyclonals raised against various: native–N or recombinant–R porcine antigens (anti-pPAG: #Nd17–77 dpc and #RpPAG2) [18,55], and highly purified bovine antigen bPAG 67 kDa from 4 to 6 months of pregnancy (anti-bPAG67, #R726) [57], all polyclonals used in a similar titer (1:300).

2.8. Morphological staining with hematoxylin/eosin and heterologous double fluorescent immunohistochemistry (htdF-IHC)

Frozen PLC tissues of the Aa females (from different dpc) were sectioned at –20 °C (Leica CM3050, Germany), and the obtained cryo-sections (8–12 μm) were prepared as previously described for various different Artiodactylian taxa, including the pig and domestic ruminant species–as positive controls [18,26]. Similar heterologous IHC was required as previously for other species [27–29] because anti-PAG sera against placental Aa antigens are not yet commercially available. Briefly, the AaPLC sections were mounted on slides, fixed, dehydrated, and initially used for standard morphological staining with hematoxylin/eosin for a quality estimation of the PLC, affected by various transport conditions, from the area of sample collection (Kirov, Russia) to the area of all laboratory examinations (UWM, PL).

For htdF-IHC, the selected AaPLC sections were blocked (5 h/37 °C) with 2.5% ovalbumin solution, rinsed in PBS (3 × 10 min/RT), and subjected to specific immunodetection with the primary polyvalent polyclonals (24 h/4 °C), the same as for ht-Western (anti-pPAG-Pv; N + R; 1:300). The immuno-complexes (AaPAG-L/anti-pPAG-Pv) within various AaPLC sections were visualized with the secondary goat anti-rabbit polyclonals (1:1000) – conjugated with Alexa 488 fluorophore. After rinsing in PBS, the AaPLC sections were counterstained with propidium iodide to visualize the nuclei of the PLC cells. Negative controls were performed with diluted normal rabbit serum (1:300) and the complete omission of the primary anti-pPAG-Pv (as a mixture of 10 polyclonals raised against various N- or R-pPAG antigens).

Confocal and htdF-IHC examination verified immunopositive AaPAG-L signals, visualized by A488 (green), among all PLC cells with nuclei stained by propidium iodide (red). Archived morphological (hematoxylin/eosin) and images were analyzed using AnalySIS Software v3.2 (Olympus, Japan) and Leica Application Suite X Software (Leica, Germany).

2.9. Statistical analyses

The limited number of pregnant Aa females allowed duplicate calculation (mean ± standard error of the mean) of the number and the size of the PLCs (width × length). All analyses were performed using the Descriptive Statistics tool in Microsoft Excel software (Microsoft, USA).

3. Results

3.1. Evaluation of pregnancy stages by various validations of fetal age/growth rate (measurements) in the Aa, compared to domestic cattle

The measurements of Aa CRL allowed for a novel comparison of various pregnancy stages (dpc), between wild Aa and the most closely related and in size-similar domestic bovine species (Fig. 1). All estimated AaCRLs (left scale in Fig. 1C) were equivalent to the bovine CRLs [53] during singleton pregnancies. However, the AaCRLs were not comparable to the proposed CRLs by the fetal age calculator (www/.ansci.wisc.edu).

The AaPLCs varied in size (width × length) and increased as pregnancy advanced (Fig. 1A, B), with growing rates (right scale in Fig. 1C), from 2.5 × 3 cm (50 dpc; single fetus) up to 10 × 15 cm (200 dpc; twins). The AaPLC measurements in twin pregnancies could not be compared to bovine PLCs due to the lack of similar experimental data in domestic cattle. The PLC number varied from 5 to 21 (average 9.94 ± 1.0) and seemed to increase until late pregnancy (200 dpc) or until mid-gestation (115 dpc) in Aa females bearing singletons or twins, respectively. Furthermore, in the first half of pregnancy, we found the similar number of the PLCs (n = 5–14/fetus) during early single or twin pregnancies, while in contrast, the number of PLCs was higher (n = 14–21/fetus) in singleton gestation and fewer in twins (n = 8–12/fetus) during the second half/advanced gestation (Fig. 1D). Interestingly, in the singleton pregnancies the number of PLCs was 12 ± 2.73, whereas in twin pregnancies it was 9.08 ± 0.84. The different number and size of the PLCs, observed in various gestations, may be associated with a local and seasonally restricted nutrition conditions (start of March), especially during 175 to 180 dpc stages (Fig. 1C, D).

3.2. Identification of PAGs in the genome of the Aa (named AaPAG-L)

All gDNA templates isolated from various Aa tissues were effective for PCR-amplifications with various primers

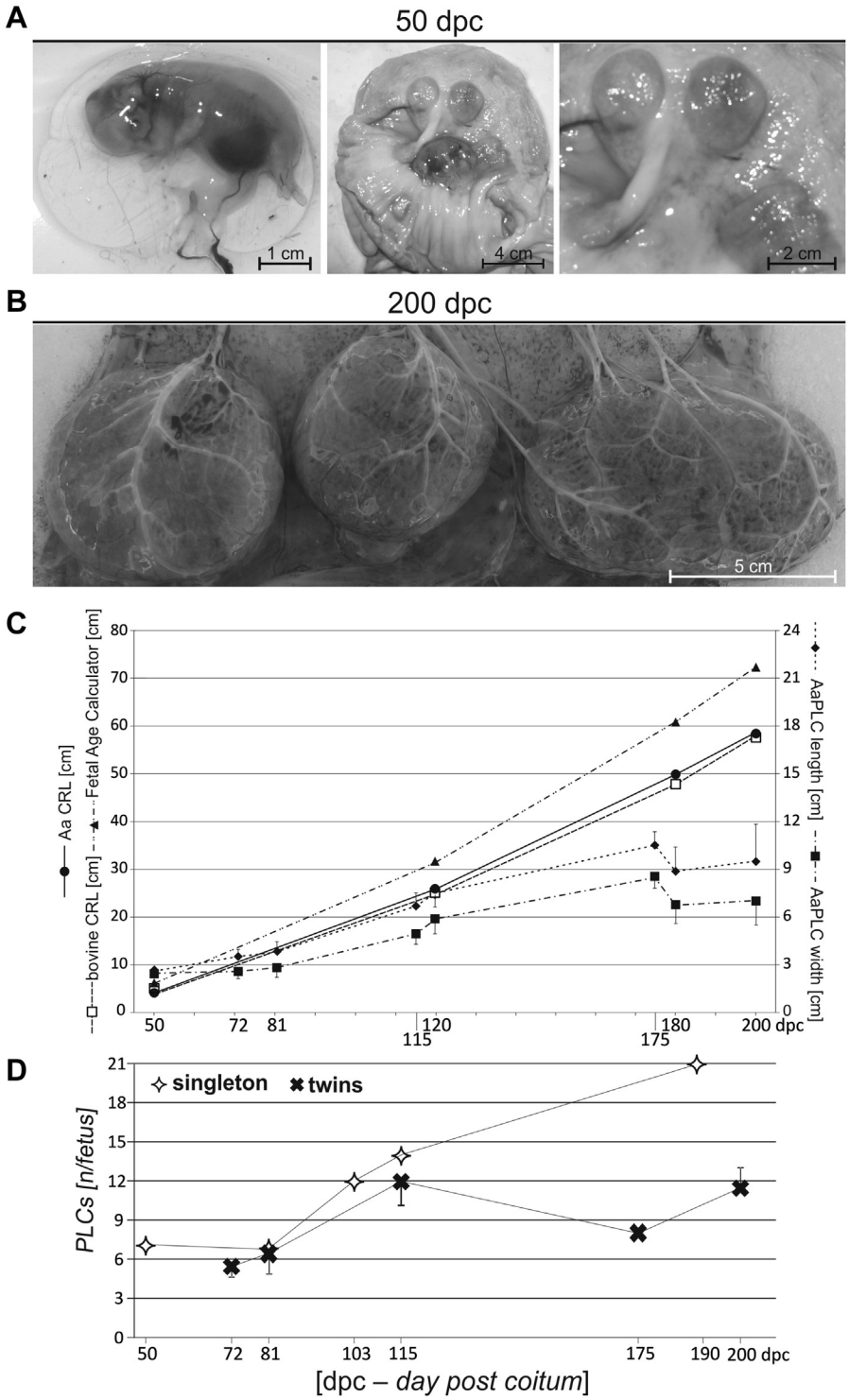

Fig. 1. Measurements (A, B) of the Aa embryo/fetus and various placentomes (PLCs) from different pregnancy stages (dpc). (C) Size estimation of the PLCs (width × length) and embryo/fetus (CRL–crown rump length) during pregnancy development of wild Aa taxon, compared to domestic cattle (age calculator). (D) Development of PLCs (n/fetus) in females bearing singletons or twins.

specific for different exonic-intronic regions of the *PAG-L* gene family (Table 1). Multiple PCR-amplifications performed in optimized conditions (46°C–60 °C) enabled the annealing of different heterologous (cross-species) pairs of the *PAG* primers. Electrophoretic separations of various *PAG-L* amplicons allowed preliminary UV-identification of

amplicon profiles of the *PAG-L* gene family, varying in number and length (118–2000 bp), presently named *AaPAG-L* in this genus (Table 2).

Southern hybridization (Fig. 2; Table 3) with [α-^{32}P] dATP-labeled cDNA probes (pPAG3 and pPAG10) confirmed the specificity of only some of the PCR-obtained *AaPAG-L* amplicons. Use of the positive control (Cp; *pPAG10* cDNA) during each PCR amplification allowed for evaluation of the effectiveness of the Southern hybridization. Lack of amplicons and/or hybridization signals in the negative control excluded contamination of amplification reagents.

Sequenced gDNA amplicons (fragment of promoter, entire exon 1, and fragment of intron A) revealed nucleotide sequences chromatograms, varied in length (110–300 bp). The 233-bp *AaPAG-L* amplicons (n = 10) shared 86.27% nucleotide sequence identity to the *bPAG1* gene (Fig. 3). Within exon 1 of the *AaPAG-L* sequences, seven single nucleotide polymorphisms (SNPs) were identified, whereas within a fragment of intron A only two SNPs and two InDel mutations were identified (Y = C/T, S = G/C, B = T/G, R = G/A, M = A/C, and W = A/T).

In silico translation of the *AaPAG-L* amplicons into the amino acid (17 aa) sequences coding the signal peptide of the AaPAG1-10 polypeptide precursors indicated 88.23 to 100% aa identity to the bPAG1 precursor (Fig. 4). Within standard 15, the aa-long signal peptide of the AaPAG-Ls, similar in all aspartic proteinases, a few aa substitutions ($L^7 \rightarrow V$ or G, $V^{10} \rightarrow G$ and $C^{15} \rightarrow G$) were identified in the AaPAG3, 5 to 8 and 10. In one precursor (AaPAG9), the seven aa missing sequence fragment of exon 1 was identified.

Table 2
Diversified profiles of the *AaPAG-L* amplicons identified by UV visualization within different nine exonic (E1–9) and eight intronic (InA–H) regions.

Primers	Amplified region (E + In)	Amplicon length [bp]	Animals [n]
SeATG/5As	E1–5, InA–D	582	1
2Se/314rar	E2–3, InB	118	2
2Se/5As	E2–5, InB–D	155, 429, 485, 560, 650, 765, 840, 890, 1100, 1200, 1430, 2000	2
4PR/5As	E4–5, InD	620, 700	1
		380	2
		254	1
5Se/6As	E5–6, InE	194	2
5Se/pagC	E5–9, InE–H	700	1
		670	2
		605	4
6Se/pagC	E6–9, InF–H	1785	1
		1500	2
		1000	4
		950	3
		150, 600, 900	1
		670	4
		655	3
		535	2
		415, 510	2
		431	17
		400	1
		200	19
7Se/pagC	E9–9, InG–H	1200	2
		1000	2
		900	3
		800	3
		600, 620, 770	1
		700	4
		565	3
		500	3
		360	8
		317	31

3.3. Heterologous Western blotting of the PAGs in the Aa placental proteome

Electrophoretic separation (SDS-PAGE) and CBB staining allowed for the identification of the protein profile of Aa placenta in different stages of pregnancy development (50–200 dpc). The placental proteome of all analyzed females was characterized with dominant 47 and 20 kDa proteins. During 120 and 200 dpc, CBB staining also revealed 26 and 34 kDa proteins, whereas 40 kDa form was detected only during 50 dpc (Fig. 5A). Western immunoblotting with the anti-porcine PAG-Pv polyclonals (Fig. 5B) revealed the dominant mature 55 kDa AaPAG fraction in all pregnancy stages (50–200 dpc), despite the size of the PLCs.

N-glycodiversity was detected that revealed various molecular ranges (26–61 kDa) of post-transcriptionally modified native mature N-glycosylated (G) or deglycosylated (D) AaPAGs isolated from various PLCs (50 and 120 dpc), parallel to the bovine fetuin–as a digestion control of glycopeptidase F activity (Fig. 6A). Duplicates of the G- and D-forms of SDS-PAGE AaPAGs were specifically immunodetected with the anti-bovine PAG67 polyclonals (#R726) during various dpc (Fig. 6B). The native mature G-forms of the AaPAGs, including the dominant ~48 and minor 37 kDa (50 dpc) and ~48, 37, 35, and 26 kDa (120 dpc) were deglycosylated (+glycopeptidase F) and converted to the faster migrating D-forms: ~44, 28, 26, and ~43, 33, 28, 23 kDa, respectively (Fig. 6B).

3.4. Morphological changes in Aa placenta development as pregnancy advances

Structural changes in placental development during pregnancy (50–200 dpc) have been identified by standard hematoxylin/eosin staining (Fig. 7). Within the synepitheliochorial placenta of Aa, two main cell groups were recognized: embryonic and maternal origin that form PLCs. Within the maternal part of the PLCs, the endometrial epithelium cells were grouped in CAR, whereas, the embryo-originated TRD cells formed CTs. The maternal CARs were found to be composed of dense connective tissue that become loose during early gestation as a result of cell elongation (Fig. 7A, B) but more clenched as pregnancy advanced (Fig. 7C–L). The specific CAR structure increased the surface of contact with strongly proliferating TRD cells. Among TRD cells that form CTs mainly cuboidal MNCs were present. Strong TRD proliferation resulted in the growth and branching of the CTs, where the BNCs have been identified (Fig. 7G, H, K).

3.5. Heterologous immunolocalization of the PAGs in the Aa placenta

The htdF-IHC and confocal immunodetections have allowed the identification of the cellular localization of

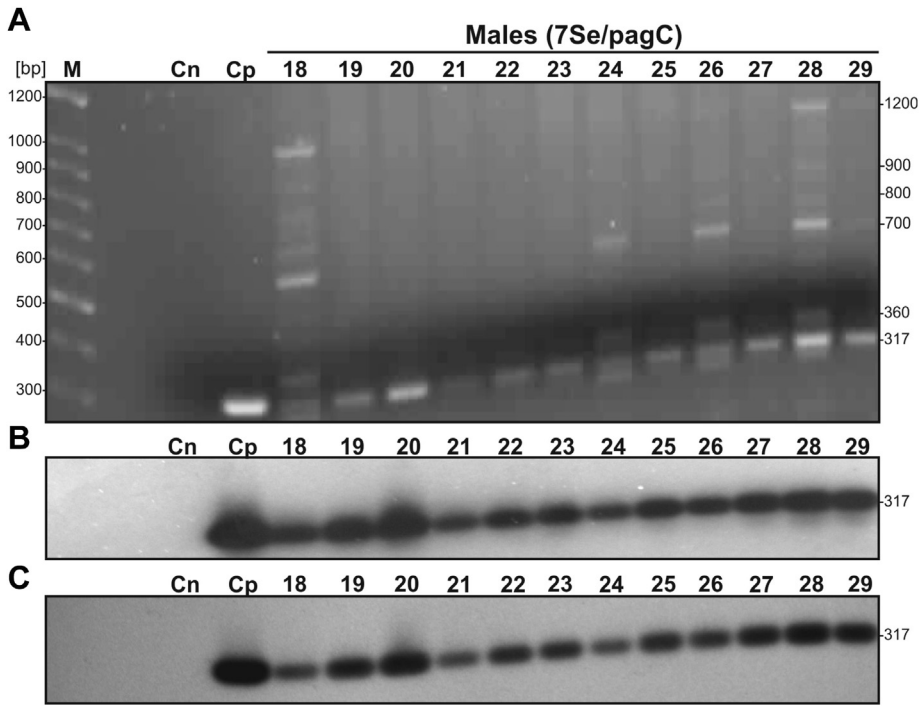

Fig. 2. Representative electropherogram (A) and autoradiograms of the *AaPAG-L* amplicons double hybridized with pPAG3 (B) or pPAG10 (C) probes. The *AaPAG-L* amplicons were produced with the use of the gDNA templates (300–500 ng) of 12 males (#18–29) and 7Se/pagC primers, then analyzed parallel to molecular mass marker (M), positive (Cp; *pPAG10* cDNA) and negative (Cn) controls.

AaPAG-L family expression during pregnancy (Figs. 8 and 9). The green signal of fluorescent intensity of the AaPAG-L immunocomplexes is associated with development of PLCs, mainly caused by intensive proliferation of TRD cells within CTs during pregnancy (50–200 dpc). Stronger AaPAG-L expression was identified in the TRD cells (CT) especially located in very close neighborhood of the uterine compartments (CAR). Among these CT cells, many specific positive AaPAG-L/TRD cells containing secretory granules were identified (Figs. 8B, J and 9B).

Table 3
Diversity of *AaPAG* amplicons for which specificity was confirmed by double Southern hybridizations with pPAG3 or pPAG10 probes.

Primers	Amplicon length [bp]	Animals [n]
pPAG3 probe		
SeATG/2As	170	1
2Se/314rar	115, 155	2
2Se/5As	429	2
5Se/6As	194	2
5Se/pagC	605	7
6Se/pagC	431	40
7se/pagC	317	29
pPAG10 probe		
2Se/314rar	115	2
2Se/5As	429	2
4PR/5As	254, 429	1
5Se/6As	194	2
5Se/pagC	670	7
	605	10
6Se/pagC	950	1
	850	7
	510	3
	431	40
	200	7
7se/pagC	317	39

4. Discussion

4.1. A novel pattern for precise validation of various pregnancy stages in wild Aa

The measurements of the CRL and PLC sizes permitted a novel pattern estimation of various pregnancy stages in wild Aa and allowed for comparison to domestic cattle, as the most closely related and in size-similar ruminant taxa

```
                                  promoter◄──────►EXON 1
bPAG1 1290    ttggatcca ggaaataaac ATGAAGTGGC TTGTGCTCCT CGGGCTGGTG
AaPAG    1    .....g... .....g..g. .......... .......... Y...SBR.B.
                                  EXON 1◄──────►intron A
bPAG1 1339  GCCTTCTCAG AGTGCATAGT CAAgtaa-ta tggggactat aagccacatc
AaPAG   50  .....Y.... ..B....... ...|....g.. .....r..g. ...atg....

bPAG1 1388  atat-ccttt ctcagatttt tcttttcatc tgattattct tccacccatc
AaPAG  100  ..c.t..... y..g..... .......... .......... .......m.t

bPAG1 1437  aggtttagaa tgtaatggaa tatttcccta agagtcttaa agctcaactc
AaPAG  150  .........t g.g..w.... .........g .c........ ..t.....c.

bPAG1 1487  ttacttattg ataagtaact tgctatagga actg 1520
AaPAG  200  .......... .......c.. ....t..... .... 233
```

Fig. 3. Nucleotide sequence comparison of the 233 bp *AaPAG* amplicons (n = 10) with the *bPAG1* gene region encompassing the promoter fragment (small letters), the entire exon 1 (capital letters), and of the intron A fragment (small letters). The standard ambiguity codes for single nucleotide polymorphism (SNP) identified within the *AaPAG* amplicons (Y = C/T, S = G/C, B = T/G, R = G/A, M = A/C, W = A/T). Start codon ATG (underlined), doted identical nucleotides (.) and inserted gaps (–) indicate InDel mutations.

```
bPAG1      1 MKWLVLLGLV AFSECIV
ampl 1       .......... .......
ampl 2       .......... .......
ampl 3       ......V..G .......
ampl 4       .......... .......
ampl 5       ......V... .......
ampl 6       ......V..G .......
ampl 7       .......... ....G..
ampl 8       ......G... ....G..
ampl 9       .......... -------
ampl 10      .......... .......
```

Fig. 4. *In silico* translation of the *AaPAG* amplicons (n = 10) into the amino acid (17 aa) sequences coding signal peptide of AaPAG precursor compared to the bPAG1 polypeptide precursor. Dots (.) indicate identical aa, capital letters (V, G) indicate aa substitutions in the AaPAG precursors, and gaps (–) indicate missing sequence fragment.

(Fig. 1). Our results revealed that Aa CRL were equivalent to bovine CRL [53] but not comparable to CRL proposed by the fetal age calculator (Fig. 1C). It seems that such a discrepancy in the measurements of CRL, between wild and domestic taxa, may occur through the occurrence of seasonal and restricted access to food during the winter season in pregnant wild females.

It is generally known that in domestic pregnant sheep restricted access to food, or harsh conditions results in reduced fetal mass, CRL, and the number of PLCs [58]. Our results indicate doubly increased PLC numbers in the singleton Aa pregnancies, and this resembled data

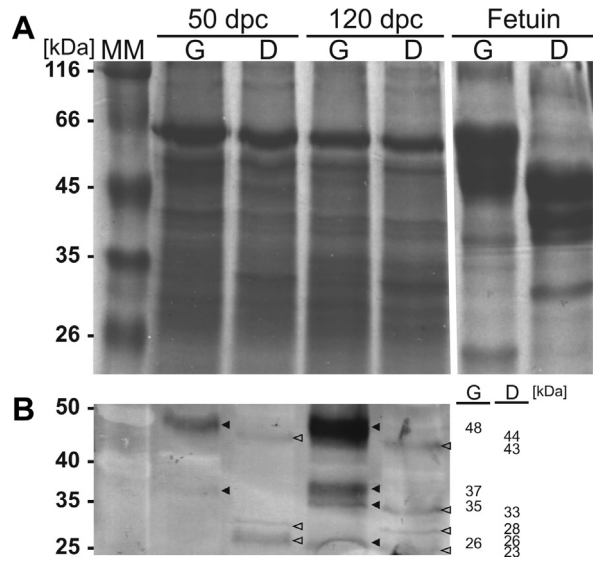

Fig. 6. Enzymatic deglycosylation of total cellular proteins (50 μg/lane) isolated from the Aa placentomes (50 or 120 dpc) and treated with the glycopeptidase F, parallel to bovine fetuin (as a digestion control of glycopeptidase F activity). (A) Separated placentomal proteins by SDS-PAGE and stained with standard CBB dye. (B) Western blotting of separated AaPAG protein forms (23–48 kDa): glycosylated (G–black arrowheads) and deglycosylated (D–empty arrowheads), detected by anti-bovine PAG67 polyclonals (#R726). Both SDS-PAGEs were with MM–mass markers (standard or prestained).

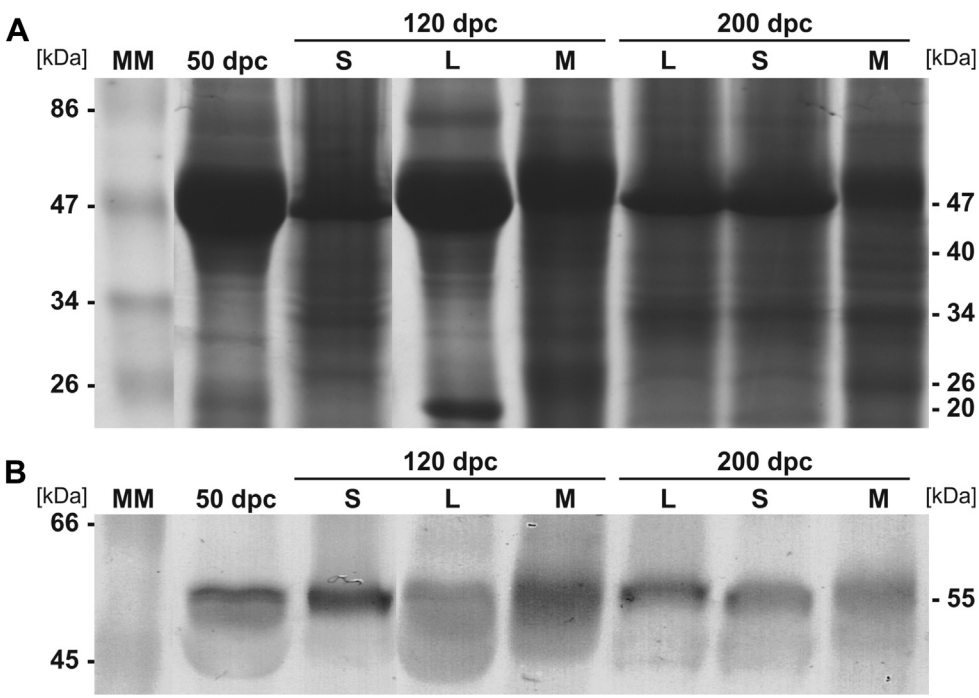

Fig. 5. Heterogeneous profile of the total Aa placentomal proteins in different stages of pregnancy development (50–200 dpc), separated by SDS–PAGE, then stained with CBB dye (A); or the heterologous Western blotting of the AaPAGs (55 kDa) performed with primary rabbit polyvalent anti-porcine PAG polyclonals (B). dpc, *day post coitum*; S, small, M, medium, and L, large placentomes; MM, mass markers (standard or prestained).

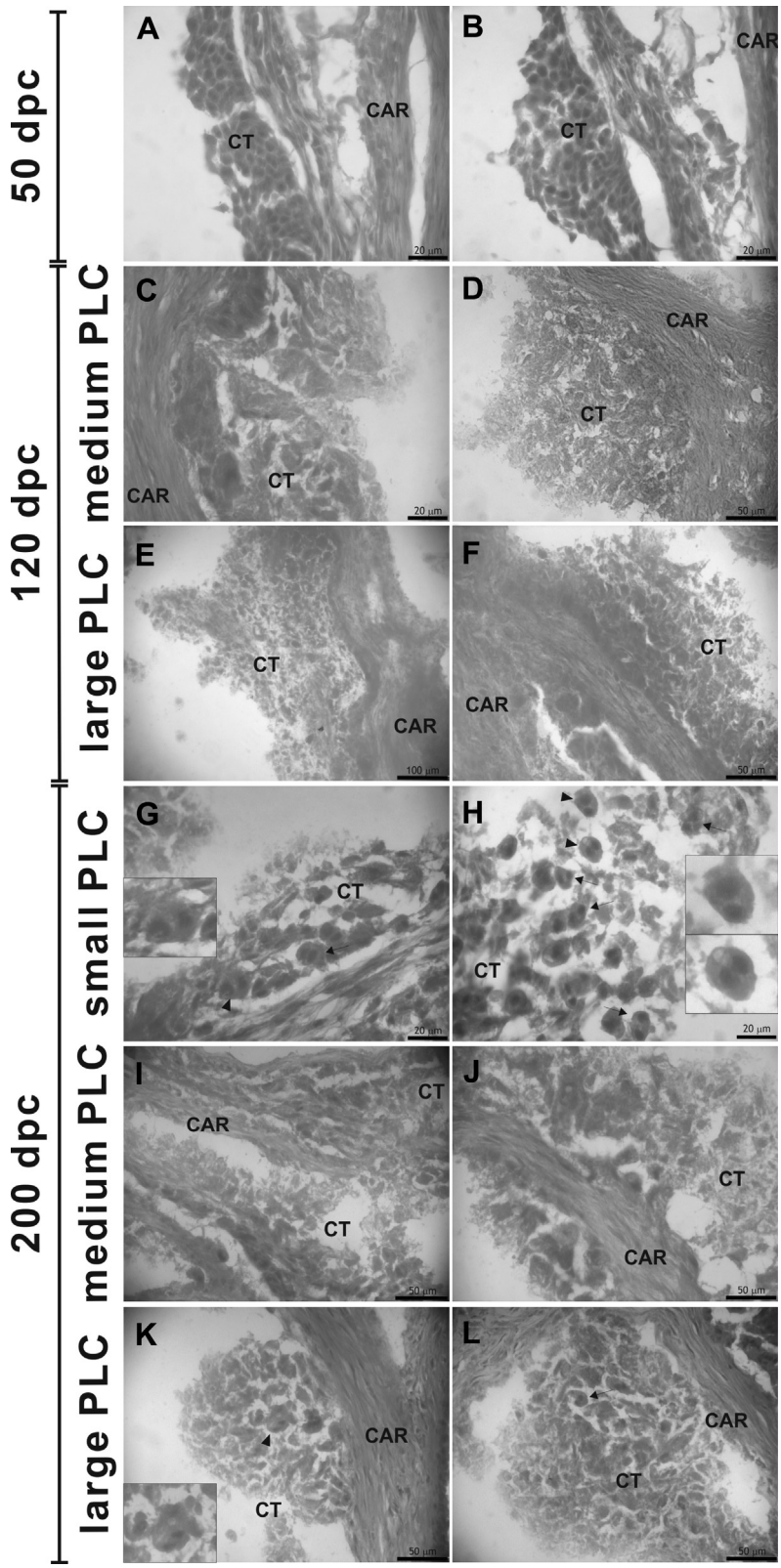

Fig. 7. Morphological staining (hematoxylin/eosin) of various size of the Aa placentomes (PLCs) on 50 (A–B), 120 (C–F), and 200 (G–L) days of pregnancy (dpc). Arrows indicate binuclear cells (BNCs), whereas arrowheads (in G, H, K), inserts of BNCs digitally magnified (2×). The size bars are indicated (20–100 μm). CT, embryonic cotyledons; CAR, caruncles.

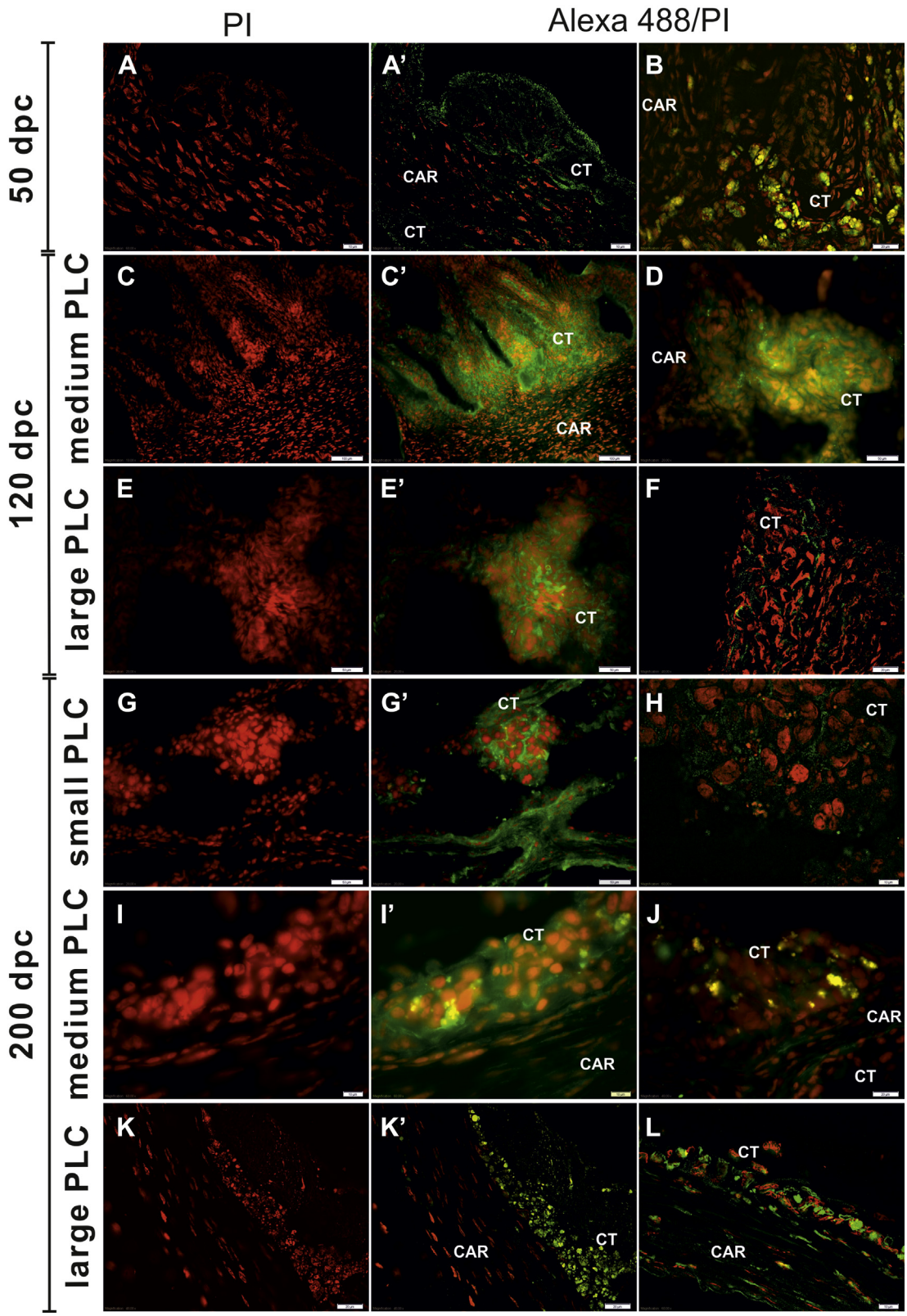

Fig. 8. Placentomal (PLC) localization of the AaPAGs in the Aa on 50 (A–B), 120 (C–F), and 200 (G–L) days of pregnancy (dpc) identified in the trophectodermal cell layer by heterologous immunodetection with the use of polyvalent rabbit anti-porcine PAG-Pv polyclonals (raised against various porcine secretory native antigens produced *in vitro*) visualized by goat anti-rabbit IgG-conjugated with fluorescent Alexa 488 dye (green) among all placental cells, in which fluorescent

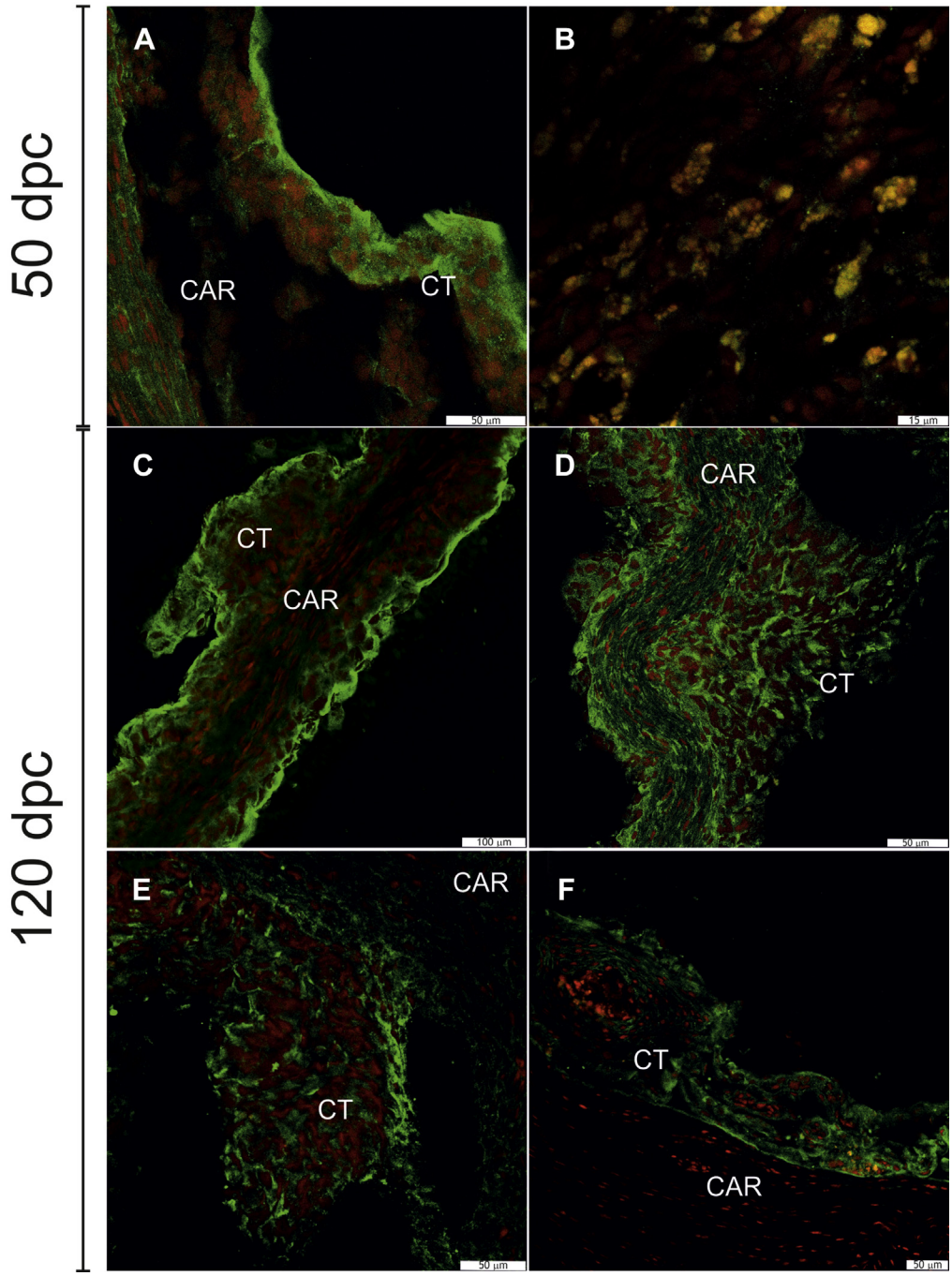

Fig. 9. Confocal localization of the chorionic AaPAGs on 50 (A–B) and 120 (C–F) days of pregnancy (dpc) identified in trophectodermal cells by heterologous immunodetection, with the use of primary polyvalent rabbit antiporcine PAG-Pv polyclonals (raised against various porcine secretory native antigens produced *in vitro*), visualized by goat anti-rabbit IgG-conjugated with fluorescent Alexa 488 dye (green) among all placental cells, in which fluorescent propidium iodine stained nuclei (red). The size bars are indicated (15–100 μm). CT, embryonic cotyledons; CAR, caruncles. (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

observed in the wtd [59], in which however, the PLC number is lower (n = 6). Moreover, significant relationships exist between body mass index (BMI) and pregnancy length in many wild mammalian marine and land species, from the Killer whale (*Orcinus orca*) to the Common shrew (*Sorex araneus*), both taxa belonging to the Cetartiodactyla

propidium iodine (PI) stained nuclei (red). The size bars are indicated (10–100 μm). CT, embryonic cotyledons; CAR, caruncles. (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

order [60]. Thus, we suggest that CRL and PLC measurements are robustly and individually required for each taxon.

4.2. Genomic identification of the AaPAG-L family

This study describes for the first time the identification of the *PAG* family in the Aa genome (Tables 2 and 3; Fig. 2 as an example), presently named *AaPAG*, according to Latin nomenclature (*Alces alces*). The *AaPAG-L* identification was difficult due to the lack of cDNA or gDNA sequences in the GenBank base in this species. Ultraviolet-visualization and heterologous Southern hybridizations revealed the *AaPAG-L* amplicon profiles vary in number and length (118–2000 bp). Heterologous Southern hybridizations with both probes; the pPAG10 (for the *pPAG2-L* subfamily encoding active members) and pPAG3 (for the *pPAG1-L* subfamily catalytically inactive members), report high-sequence homology and confirm the specificity of dominant *AaPAG-L* amplicons (Table 3) due to multiple gene numbers or their fragments within the Aa genome.

Nucleotide sequence comparisons of the *AaPAG-L* amplicons (fragment of promoter, entire exon 1 and fragment of intron A) indicated 86.27% identity with the bPAG1 gene (Fig. 3). Within the *AaPAG-L* consensus sequence, we identified seven SNPs (in exon 1), and then two SNPs and two InDel mutations (in intron A). *In silico* translation of the *AaPAG-L* amplicon sequences revealed entire signal peptide sequences of the AaPAG1–10 polypeptide precursors (as possible products of different genes or the same gene and differences between individuals) that share 88.23% to 100% aa identity with the bPAG1 precursor (Fig. 4). The AaPAG-9 precursor, with missing sequence fragment (7 aa) of exon 1 was also identified, similarly to previously missing sequence fragments of many shortened bPAG7 and the bPAG4 polypeptide precursors (without exon 6 or 8) were reported by *in silico* translations of cDNA [14,15]. Thus, we suggest that the localization of the SNPs identified (with gDNA templates) within the signal peptide of the AaPAG precursors may have a potential impact on protein maturation and their activity.

Previous studies pointed out the large number and diversity of cloned cDNA of the *PAG* genes in various eutherians [1]. Among the Cervidae family, the wtd is the only species in which 10 *wtdPAG* cDNAs (entire ORF, or fragments) were cloned using dominant cotyledonary mRNAs during 85 to 90 dpc [6]. The *wtdPAG9* is similar to *wtdPAG8*, but the sequence coding exon 7 is missing. Among the Bovidae family, numerous *PAG* cDNAs were cloned and deposited in the GenBank database: 22 in cattle [14,15] and 19 in water buffalo [3,4]. Also, some shortened or full length cDNAs have recently been identified in the placenta of other ruminants including American bison [7], wapiti [8], and giraffe [9].

Most of the presently applied primers were designed for the previously identified nucleotide of cloned cDNAs in the pig: *pPAG1* and *pPAG2* [17], and then *pPAG-3, -4, -5, -6, -8, -10* [19], as well as the exon-intron structure or the *pPAG2* gene promoter [11]. It can be assumed that the lower amplification efficiency for some *AaPAG-L* gene exonic-intronic regions was affected by heterologous primers to amplified gDNA templates and resulting from interspecies sequence differences of the *PAGs* in the genome of the pig (Suidae) and the Aa (Cervidae). Similar lower amplification efficiency was observed with various gDNA templates of camelids (Camelidae), i.e., alpaca, Bactrian camel, and dromedary [23]. So far, Southern hybridization of gDNA templates suggests at least 100 phylogenetically 'modern' or 'ancient' *PAGs* that are expected in various ruminant genomes [14]. The evolutionary multiplicity of the *PAG* family arrived from many duplications of the entire genes or their fragments that can appear in a tandem or in dispersed locations [61,62]. Such *PAG* family location, identified by FISH, should be confirmed by a laser microdissection of selected chromosomes, and then by Southern hybridizations of gDNA templates [63]. Thus, various gDNA (isolated from leukocytes, muscles, or hair roots) may provide very useful alternative templates for the identification of the *PAG* gene family in various wild species, in which access to high-quality mRNA (required for cDNA cloning) is difficult or even impossible, especially during various pregnancy stages [1].

4.3. Protein diversity of the AaPAG-L family expressions in the placenta

Our Western immunoblotting (Fig. 5) allowed identification of protein profiles of the AaPAG family (55 kDa) in the placental proteome at various pregnancy stages (50–200 dpc). Diversified masses of multiple PAG fractions were identified in various pregnancy stages in some Cetartiodactyla [1], in which different placenta types are developed [64]. Among the Cervidae, 31 and 58 kDa, or 31, 45, and 57 kDa PAGs were identified, in the Alaskan moose (m) and elk-red deer (e), alternatively named mPSPB and ePSPB, respectively [65]. In other wild species with cotyledonary placentas (e.g., red deer and buffalo), 45 and 66 kDa are the major fractions during early or late pregnancy, respectively [66]. In wtd, the dominant wtdPAGs are diversified, including 35 and 55 kDa fractions isolated from CTs extracts; and 55 kDa fractions from inter-PLC extracts on 85 to 90 dpc [6], whereas in fallow deer (fd), the fdPAG are 36, 39, 56, and 62 kDa on 110 dpc [67]. Among the Camelidae, in alpaca (Lp), the LpPAGs are 39 to 64 kDa, but in the dromedary (Cd) the mass of the dominant CdPAGs are elevated to 69.9 and 76.4 kDa [66].

Among Bovidae, in water buffalo (wb), various wbPAGs (55–70 kDa) were identified during late (8 months) pregnancy [34]. In European bison, (Eb) multiple EbPAGs (45–85 kDa) were identified on 45 to 150 dpc [22,36], while in American bison (Amb), AmbPAGs on 90 to 120 dpc were 72 and 74 kDa in various CTs, and an additional 76 kDa fraction in the liquids obtained during CTs defrosting [35]. Such huge diversity was also determined in other species. In the pig, cellular PAG forms (39.7–66.1 kDa) isolated from placental tissues [66], as well as, secretory PAG forms (43–70 kDa) were *in vitro* produced by various chorionic explants, then isolated from media and used as numerous native antigens for effective production of various polyclonals [18,55,68]. Moreover, such differences in the mass of the PAGs come from not only homologous or heterologous immuno-detections, in which various epitopes are

recognized by different antisera but also from specific posttranslational modifications during various pregnancy stages [1].

N-glycosylation diversity of the AaPAG-L proteins was identified during PLC development, by comparison of the naturally G-glycosylated and enzymatically D-deglycosylated forms by heterologous Western-blotting (Fig. 6). Presently, we detected various profiles of the G- and D-forms of the AaPAG-L proteins, similarly as previously for the secretory EbPAGs [22] and pPAGs [56]. Also, cellular bPAG isolated from CT and inter-CT tissues (220 dpc) reported 67, 53, and 44 kDa G-form, effectively deglycosylated to six D-forms: 57, 50, 41, 45, 38, and 21 kDa [69]. Multiple N-glycosylation sites (2–7) of the PAGs were confirmed *in silico* by cDNA analyses in the pig [17], cattle and sheep [14], and goat [20]. It seems that the heterogeneity of the AaPAG-L family may be due to various activation processing (by degradation) of their distinct precursors, and due to post-translational modifications, involving various antennary oligosaccharide chains, similarly as in other species [1].

4.4. Identification of the cellular localization of AaPAG-L expression

We identified morphological changes in Aa placenta development during various stages (50–200 dpc) of pregnancy (Fig. 7). Within the synepitheliochorial placenta of Aa two main cell groups were recognized: embryonic (CT) and maternal (CAR) origin that form PLCs. The CARs were found to be composed of dense connective tissue that becomes unclenched as a result of cell elongation during early gestation but then becomes more clenched tissue as the pregnancy advances. Within the PLCs, noticeable strongly proliferating TRD cells and their intense expansion progress (CT), between flat stratified squamous epithelium (CAR) structures, increased the vascularized feto-maternal contact surface (interface). Within the cellulary diversified CT, rare and larger BNCs among smaller cuboidal MNCs were identified in the Aa placenta. Our hematoxylin/eosin staining revealed many morphological structure changes during the placental development of Aa that resembled the PLC enlargement in some other wild species. A similar PLC structure was observed in African elephant [70], European bison [27], and giraffe [71], where the layer of the elongated and flattened maternal epithelial cells is in close proximity to the expanding TRD cells. Despite a different placenta type, intense TRD cell proliferation also occurs in the diffuse placenta of the pig, alpaca, and both camels [26,28,29]. The BNCs were also observed in some other wild ruminants, that is, in European bison [27] and giraffe [71], while a migration of the BNCs toward the maternal parts of the placenta was observed in elk [72].

Our htdF-IHC and confocal microscopy allowed identification of the cellular localization of the AaPAG-L family expression during various pregnancy stages (Figs. 8 and 9). The signal intensity of the AaPAG-L immunocomplexes was related to the PLCs size, mainly robust proliferation of the TRD cells within the CTs, as pregnancy advanced (50–200 dpc). During fast expansion of the chorionic villi of the CTs and intensive branching of the TRD folds, the strongest AaPAG-L signals were identified (Fig. 8), especially within the enlarged TRD cells (with multiple secretory granules) that were located toward neighboring uterine CARs (Fig. 8I', J and K'). Immunolocalization of AaPAG-L(s) resembled expression of PAGs in the placenta of several other species including white-tailed deer, cattle, bison, alpaca, Bactrian camel, dromedary, and pig [6,25–29,73].

Multigranulated embryo-originated TRD/CT cells expressing (Figs. 8B, J and 9B) and then secreting AaPAGs (into the peripheral blood) have been observed in some other ruminants only [1]. Initially, in the Bovidae, some granules within the PLCs, containing the placental lactogens and also the SBU3 antigens, as the previous alternative named PAGs, were identified [74]. Recently, among the Cervidae [67], a complex isolation of various PLC proteins (using monitoring by various polyclonals) revealed many diversified NH_2-terminal sequences of a few novel mature isoforms (A–D) of the fdPAGs (39–62 kDa)—isolated from fetal parts of the PLCs (FCT), that is, from the embryonic CTs (deposited in the EMBL-EBI database Swiss-Prot, Acc. Nos.: C0HJC7–9 and C0HJD0). Also, various alfa-fetoprotein isoforms (56–63 kDa)—isolated from both the tissues: the embryo-originated CTs (FCT; 56 kDa), and/or from the maternal uterine CARs (MCT; 60 and 63 kDa). Both micro-sequences have been deposited as two alfa-fetoprotein isoforms (56 or 63 kDa from FCT or MCT, respectively) with the same shorter or longer sequences (Acc. No. C0HJD1). However, the C0HJD1 isoforms were recognized within the FCT and the MCT by the same polyclonals (#R438), raised against total bovine precipitated cellular proteins (at 40% to 80% ammonium sulfate saturation) and used for effective affinity chromatography (*Vicia villosa* agarose or Sepharose 4B Ig columns) during efficient fdPAG identifications [67].

In many eutherians [75], the placenta interfaces the fetal and maternal environments. Located at the placental villous surface, in direct contact with maternal blood, is the TRD layer, which mediates the crucial maternal-fetal exchange of gases, nutrients, and waste products, produces hormones that support the pregnancy and provides immunological defense [64]. The presence of multiple granules within TRD cells in the CTs of many eutherians is associated with the endotheliochorial relationship [76,77]. In the human, various placental cells with multiple granules (with high microRNA expression) were identified, which are packaged into extracellular vesicles of diverse sizes, including exosomes [78] and are capable of communicating distinctive signals to maternal and/or fetal tissues. Within the feto-maternal interface, trophoblastic debris, shed from the syncytiotrophoblast and phagocytosed by epithelial cells, trigger maternal endothelial cell activation [77]. The proximity of the TRD cells expressing the PAGs allows for growing their concentrations in the maternal blood, and for pregnancy diagnoses in white-tailed deer [79], in semi-domesticated reindeer [48,49], and some other wild and domestic ruminants [see: 1,2]. In bovine females with single and twin gestations, the different sensitivities of the commercial ELISAs and radioimmunoassays for pregnancy tests depend on many heterologous tracers and mainly polyclonals recognizing various PAG epitopes [45,80]. Therefore, the AaPAGs,

secreted into maternal blood vessels, will be a good prenatal marker for the future evaluation of pregnancy and embryonic mortality in wild ruminants maintained in conservation parks or zoos.

4.5. Conclusion

This study describes for the first time the PAG family in the genome and placental proteome of the moose from the European population (Kirov Region). Southern hybridization of multiple *PAG* genes can be helpful for generating genome maps of various wild, especially endangered, mammals. The PAG family is strongly expressed in various chorionic cells and can be involved in the regulation of placental development of Aa and various endangered eutherians that are at serious risk of extinction. Our methods can be helpful for monitoring animal population diversity within various natural conservation areas.

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A. Lipka performed experimental work and drafted the manuscript. G. Panasiewicz assisted in the study design, coordinated all experimental work, and had important input into the manuscript writing, statistical analyses, and figure preparation; M. Majewska conducted the ht-dflHC; M. B.-Kobuszewska participated in the Southern analyses; A. P. Saveljev and A. P. Pankratov collected Aa tissues and measured macromorphologicaly the embryos and uteri; and B. Szafranska conceived the study design and helped in writing the final version of the manuscript.

Competing Interests

The authors declare that they have no conflict of interest.

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