Chapter 1: INTRODUCTION

HISTORICAL OVERVIEW ON PREGNANCY-ASSOCIATED GLYCOPROTEINS (PAGs) AND RELATED PROTEINS ISOLATED BY BIOCHEMICAL PROCEDURES

The mammalian placenta secretes a wide range of proteins and hormones. Many of them were isolated and characterized in the last 2 decades. Some cases, these products are identical to compounds synthesized by the nonpregnant adult (steroids and prostaglandins). Some of the placental proteins were found to be specific or at least associated to the pregnancy.

Butler *et al.* isolated two pregnancy specific proteins from the bovine placenta in 1982 using the following methodology: antisera produced against homogenates of whole bovine placenta were adsorbed with somatic tissues in order to remove antibodies against proteins not specific to the placenta. The antibodies selected by this method were used to follow the immunoreactive fractions throughout the isolation procedure. One of the proteins, the pregnancy specific protein- A (PSPA) exhibited a molecular mass of 65-70 kDa, a pI of 4.6-4.8 and showed an identity reaction with bovine α_1-fetoprotein in the immunodiffusion test. The second protein named pregnancy specific protein- B (PSPB) had a molecular weight of 47-53 kDa and a pI of 4.0-4.4. PSP-B represented a novel antigen.

In 1988, Beckers *et al.* reported the partial purification of a bovine chorionic gonadotrophin (bCG), which was later identified as a member of the aspartic proteinase family: the boPAG-2 (Xie *et al.*, 1994). This protein had an estimated molecular weight of 30 kDa in gel filtration. The same author reported the purification of a bovine pregnancy specific protein, which was more pure than that of the previous ones and did not have a luteotrophic activity (Beckers *et al.*, 1988b).

In 1990, Zoli *et al.* isolated partially an ovine pregnancy specific protein from the ovine fetal cotyledons. The purification procedure was followed by a RIA specific for the bovine pregnancy protein described by Beckers *et al.* (1988b). The molecular weight and the pI of the isolated protein were similar to those of bPSPB (60 kDa and 5.5, respectively) (Zoli *et al.* 1995).

In 1991, Zoli *et al.* performed a purification which was monitored by an antisera developed by Beckers *et al.* (1988b), produced against specific antigens of fetal cotyledons. The purification consisted of: extraction, $(NH_4)_2SO_4$ precipitation, Diethyl Amino Ethyl (DEAE)-cellulose chromatography, gel filtration and high performance liquid chromatographies (MonoQ and MonoP columns). This purification resulted in protein preparation having a molecular weight of 67 kDa, and four pI-s of 4.4, 4.6, 5.2 and 5.4 and

named pregnancy associated glycoprotein (PAG). PAG had the following NH_2-terminal sequence: Arg-Gly-Ser-x-Leu-Thr-Thr-His-Pro-Leu. These 4 isoforms differed in their sialic acid content. The pI, the sialic acid content and the immunoreactivity were closely related: the most basic form being the most immunoreactive.

A similar pregnancy-specific protein has also been reported by Camous *et al.* (1988). This protein had a molecular weight of 60 kDa and it was named pregnancy serum protein 60 (PSP60). Its 39-NH_2-terminal amino acid sequence is identical to that inferred from the cDNA of bPAG (Xie *et al.*, 1991b). Two residues of the NH_2-terminus of PSP60 which corresponds to asparagine in the cDNA of bPAG could not be identified because they were linked to oligosaccharides. The difference between the molecular masses of PSP60 and bPAG is probably the result of the different degree of glycosylation. It seems likely that bPAG and PSP60 proteins may correspond to one of the 5 forms of PSPB.

Four PAG related molecules were purified from the medium after culture of explants from Day 100 sheep placentas (Xie *et al.*, 1997). The purification included: $(NH_4)_2SO_4$ precipitation, sepharose blue, DEAE-Sepharose and high performance liquid (MonoS) chromatographies. These PAGs were cross-reacting with three different anti PAG-1 antisera. Several isoforms of proteins with molecular mass of 55, 60, 61 and 65 kDa were identified. These proteins did not present any proteolytic activity towards [^{14}C]methyl-hemoglobin (Xie *et al.*, 1997).

In 1997-1998, three different PAGs molecules were isolated and partially characterized by Garbayo *et al.* from the caprine placenta. The isolation procedure, monitored by a heterologous RIA, included extraction of soluble proteins at neutral pH, acidic and $(NH_4)_2SO_4$ precipitations, gel filtration and ion exchange chromatographies. Three PAGs differing in amino acid sequence and molecular masses (55, 59 and 62 kDa) were detected and each showed several isoforms with different pIs: PAG_{55} (pI: 5.3, 5.1, 4.9; NH_2-terminal sequence: Ile-Ser-Ser-Pro-Val-Ser-x-Leu-Thr-Ile), PAG_{59} (pI: 6.2, 5.9, 5.6; NH_2-terminal sequence: Arg-Gly-Ser-x-Leu-Thr-Thr-Leu-Pro-Leu) and PAG_{62} (pI: 5.1, 4.8; NH_2-terminal sequence: Arg-Asp-Ser-x-Val-Thr-Ile-Val-Pro-Leu) (Garbayo *et al.*, 1998).

Pregnancy-specific proteins were isolated, purified, and partially characterized from elk and moose placenta (Huang *et al.*, 1999). Bovine PSPB RIA was used to monitor the purification process (extraction, acidic and $(NH_4)_2SO_4$ precipitations, gel filtration, ion exchange and affinity chromatographies). The molecular weights of the isolated proteins from the

moose placenta were 58 and 31 kDa, while from the elk placenta 31, 45 and 57 kDa.

Evidence, that PAG-1 is also present in the zebu (*Bos indicus*) placenta was reported by Sousa *et al.* (2000). The immunological behavior of 6 month placental extracts isolated from *Bos taurus taurus* and *Bos taurus indicus* was investigated by using double radial immunodiffusion against anti boPAG-1 and anti boPAG-2 antisera (El Amiri *et al.*, 2000). Similar precipitation reaction was observed at both extracts against anti boPAG-1 (2 precipitation lines), while against anti boPAG-2 *Bos taurus taurus* extract gave 2, *Bos taurus indicus* gave 1 precipitation lines. After a purification procedure similar to that of Zoli *et. al.* (1991) a 67 kDa molecular weight protein was isolated, the N-terminus amino acid sequence of this protein was corresponding to the boPAG-1.

Three ovine PAGs were isolated from fetal cotyledons and characterized by El Amiri *et al.* (2002a,b).The immunoreactive fractions were followed through the isolation procedure by a heterologous RIA system. Three PAGs were reported by this author with several isoforms, they were termed according to their molecular weight: ovPAG-55 (pI: 4.0, 4.3, 4.7, 5.0, 5.9), ovPAG-57 (pI: 4.1, 4.5, 5.5), ovPAG-59 (pI: 4.5, 4.8, 5.0).

OVERVIEW ON THE FUNCTION OF PAGS

Molecular modeling study (Guruprasad *et al.*, 1996) showed that PAGs have a well-defined peptide-binding cleft with a preference to bind basic, relatively hydrophobic polypeptides. Further investigations also suggested that PAGs are unlikely to have proteolytictic activity.

According to the results of Xie *et al.* (1991b), Roberts *et al.* (1996) suggested that PAGs can have an endocrine function: with their binding clefts, they are able to bind specific cell surface receptors on maternal target cells. It is also likely that PAGs are able to sequester or transport peptide ligands such as Pepstatin A, in this way they can act as carrier molecules. The peptide-binding specificity of different PAG molecules is different, this variability may be a key to their function. Roberts *et al.* (1996) hypothesized that PAGs are able to compete with the MHC for peptides processed for antigen presentation and in this way, these molecules could interfere with activation of T-cells.

It is likely, that the PAGs act locally at the placental interface because of the following reasons:

a.) Some of them are expressed predominantly in mononucleated trophoblast cells and this cell population does not have an invasive character.

b.) PAGs are also expressed in the porcine and equine placenta, where there is no erosion of the uterine epithelium and access to the maternal circulation is limited.

The endocrine role of PSPB is suggested by Austin *et al.* (1999), who reported concentration-dependent increase in the release of an alpha chemokine which was identical with granulocyte chemotactic protein-2 (GCP-2). Similar GCP-2 induction effect was observed by IFN-tau in early gestation. Alpha chemokines may regulate adhesion, inflammation, angiogenesis associated with early implantation, they also can be involved in uterine remodeling after parturition.

Del Vecchio (1990, 1995a,b) examined the effect of PAG-1 on the progesterone, PGF_2alpha, PGE_2 and oxytocin production in both cultured luteal cells. He reported variable action of PSPB on Progesterone (P4) production: In some experiments PSPB did not have a direct stimulating effect on P4 production, it stimulated the PGE_2 production, which is a potent stimulator of luteal P4 production (luteotrophic compound) (Del Vecchio *et al.*, 1990). While in other studies he found that PSPB treatment increased the P4 production of luteal cells (Del Vecchio *et al.*, 1996). Similar results were reported by Weems *et al.* (1998) concerning to the effect of PSPB on P4 production by the bovine CL at mid pregnancy. It was concluded that PSPB could have an indirect role in maintaining the corpus luteum during pregnancy.

Pregnancy-associated glycoprotein inhibited the growth of bovine myeloid bone marrow cells (CFU-G) at 2400 and 3000 ng/ml concentrations (Hoeben *et al.*,1999). It was shown by Dosogne *et al.* (1999) and Hoeben *et al.* (2000), that the highest plasma PAG concentrations immediately preceded the alterations of blood polymorphonuclear neutrophil (PMN) leukocytes functions (phagocytosis, oxidative burst activity). Decrease in PMN's burst activity caused by PAG in vitro was investigated by Moreira da Silva *et al.* (1996a,b,c). Dosogne *et al.* (1999) suggested that the PAG is involved in the local immunosuppression which may be necessary in early pregnancy to reduce maternal immune response in order to protect against any host rejection, however peak concentrations of PAG are observed during late pregnancy.

HISTORICAL OVERVIEW ON MOLECULAR CLONING WORKS CONCERNING TO THE PAGS

In 1991, Xie *et al.* (1991a,b) described the molecular cloning of a bovine and an ovine PAG. Bovine and ovine conceptus and cotyledonary cDNA expression libraries were screened with an antiserum raised against PAG-1 (Zoli *et al.*, 1991). Two clones, from mid pregnant bovine and ovine cotyledonary libraries contained full open reading frames encoding polypeptides of 380 and 382 amino acids, respectively. These polypeptides were related in sequence to pepsinogen, cathepsin D and E, and they were clearly members of the aspartic proteinase gene family. Purified fractions of boPAG-1 and partially purified ovPAG were unable to hydrolyze denatured hemoglobin in a standard assay for aspartic proteinases, which suggested that both proteins are enzymatically inactive. Critical amino acid substitutions at the active site regions explained this observation, however PAG bound tightly to immobilized pepstatin and probably had an intact substrate-binding cleft. Bovine and ovine PAGs are also possessing a signal sequence and a pro-piece homologous to the one in pepsinogen. These two PAG molecules were reported to be expressed as mRNA of 1.7 kb in binucleate cells of conceptuses and in placental tissue until term.

In 1992, Lynch *et al.* found a high cDNA sequence identity between bPSPB and bPAG-1 and classified the bPSPB in the same aspartic proteinase family.

The cDNA of bovine PAG-2 was found to be structurally related to bovine PAG-1, ovine PAG-1 and pepsin (58%, 58% and 51% amino acid sequence identity, respectively) (Xie *et al.*, 1994). The cDNA of bovine PAG-2 was initially identified by screening a bovine placental cDNA with a PAG-1 cDNA. An identical PAG-2 cDNA was identified in the same library by antibody screening with an antiserum produced against a protein was able to bind to bovine LH receptors on the CL. The full length cDNA of 1258 bp was coding for a polypeptide of 376 amino acids. The mRNA of boPAG-2 is expressed in both mononucleate and binucleate cells of the trophectoderm and not in other fetal organs. It became evident, that boPAG-2 is synthesized by placental explants as a 70 kDa glycoprotein, which is processed to several smaller molecules having a molecular weight from 31 to 70 kDa.

The boPAG-1 gene isolated by Xie *et al.* (1995), contains 9 exons (size range 99-281 bp) and 8 introns (87-1800 bp) organized in a manner very similar to those of proteolytically active mammalian aspartic proteinases. It

seems likely, that exons 2 to 5 provide the N-terminal and exons 6 to 9 the C-terminal lobes of the mature protein. Southern blot analyses using exon-specific probe indicated the presence of 2 boPAG-1 genes. Probably the cDNA of this second boPAG-1 gene, highly related in sequence to boPAG-1 was isolated from bovine placental cDNA library. Several DNA fragments hybridized weakly to the probe during the Southern blot analysis, which indicated that several genes related in sequence to PAG-1 are present in the bovine genome.

Nagel *et al.* (1993) identified the ovPAG-2. Its expression was the strongest in the mononucleated cells.

Xie *et al.* (1997) reported the cloning 25 cDNA sequences closely related to ovPAG-1 and ovPAG-2 from Day 100 placenta. After sequencing they were classified into 7 groups and named ovPAG-3 to ovPAG-9. Two of the seven cDNA transcripts (ovPAG-3 and ovPAG-7) contained sequence corresponding to the amino terminus of the 65 kDa molecular weight ovPAG. *In situ* hybridization showed that ovPAG-3 and ovPAG-7 transcripts were restricted to the binucleate cells of the trophoblast. This localization was clearly observed near the tips of the chorionic *villi*.

Green *et al.* (2000) identified 21 bovine PAG and 9 ovine PAG cDNA. Phylogenetic analysis of the sequences indicated that the PAG are divided into two main groups (Table 1). This classification reflects their tissue localization as it was determined by *in situ* hybridization.

Two distinct types of PAG transcripts (poPAG-1, poPAG-2) were identified by screening porcine conceptus cDNA library with ^{32}P-labelled ovine and bovine cDNA (Szafranska *et al.*, 1995). This work was the first report confirming the presence of PAGs in a species not having synepitheliochorial, but epitheliochorial placentation as classified by Wooding *et al.* (1992). The two fully characterized cDNAs shared 48-57% amino acid sequence identity with ruminant PAGs. The open reading frames of the poPAG-1 and poPAG-2 cDNA encoded polypeptides of 389 and 387 amino acids, which were corresponding to molecular masses of 42.79 kDa and 42.57 kDa. The signal sequences were 15 amino acid long and highly conserved (93%) between the two pPAGs. *In situ* hybridization performed on frozen sections of Day 23 placenta indicated that poPAG-1 and poPAG–2 were expressed throughout the chorion, with no observable differences in the distribution of these PAGs. By Western blotting the proteins present in the medium after Day 20 and 25 tissue explants had been cultured, a major immunopositive band with the molecular weight of 70 kDa and several lower molecular weight bands were detected. The basic protein detected in the early porcine embryo during

expansion (Baumbach *et al.,* 1988) has been identified as a proteolytic fragment of poPAG-2 (Doré *et al.*, 1996).

Cloning and characterization of a PAG-like protein expressed in the placenta of the horse and zebra was performed by Green *et al.* (1999). The eqPAG was identified from a day 25 placental library, and had a predicted molecular weight of 42.90 kDa. It was secreted from cultured placental tissue as a mature and zymogen form. Surprisingly, this PAG had greatest sequence similarity not to other PAGs (52-57% amino acid identity), but to an aspartic proteinase called rabbit pepsinogen F (69% amino acid identity), which is expressed for short time within the newborn rabbit (Kageyama *et al.*, 1990). The Northern blot analysis revealed that the eqPAG transcript was highly expressed in pre-implantation conceptus tissue and its expression was restricted to the extraembryonic membranes. Transcripts similar to eqPAG (73% amino acid identity) has been cloned from the placenta of the domestic cat (Gan *et al.*, 1997).

Recently, Garbayo *et al.* (2000) isolated 11 cDNA from the goat placenta between Days 45 and 115 of pregnancy by using RT-PCR to generate PAG cDNA from pooled placental RNA. These 11 distinct cDNA differring by at least 5% in nucleotide sequence identity were fully characterized (caPAG-1 to caPAG-11). CaPAG-1, -3-7, -9-11 were expressed only after Day 45 of pregnancy and were localized to binucleate cells. CaPAG-2 was detectable only at Days 18-19 of pregnancy and was expressed in the trophectoderm. CaPAG-8 was present in the placentomes at all stages of pregnancy examined (Days 18 to 115).

EXPRESSION OF PAGS DURING PREGNANCY

Immunocytochemical studies showed that PAG-1 is localized to the granules of the binucleate cells of the trophectoderm in cattle and sheep (Zoli *et al.*, 1992a; Xie *et al.*, 1991a,b). The presence of antigens immunologically related to boPAG-1 in testicular and ovarian extracts was demonstrated by Zoli *et al.* (1990b,c).

The binucleate cells of the placenta start to appear just before the time of uterine attachment (around Day 17 in cattle). These cells are migrating towards the uterine epithelium, and forming trinucleate cells by fusing with uterine epithelial cells. After fusion their dense secretory granules are released from the basolateral face of the uterine epithelium.

The cloning study carried out by Green *et al.* (2000) revealed that the expression of PAGs are spatial and temporal during pregnancy. Some of the PAGs are expressed throughout the trophectoderm, while the expression of other PAG molecules are predominantly localized to the binucleate cells (Table 1).

Table 1. Expression pattern of the bovine and ovine PAG within the trophectoderm (Green *et al.*, 2000)

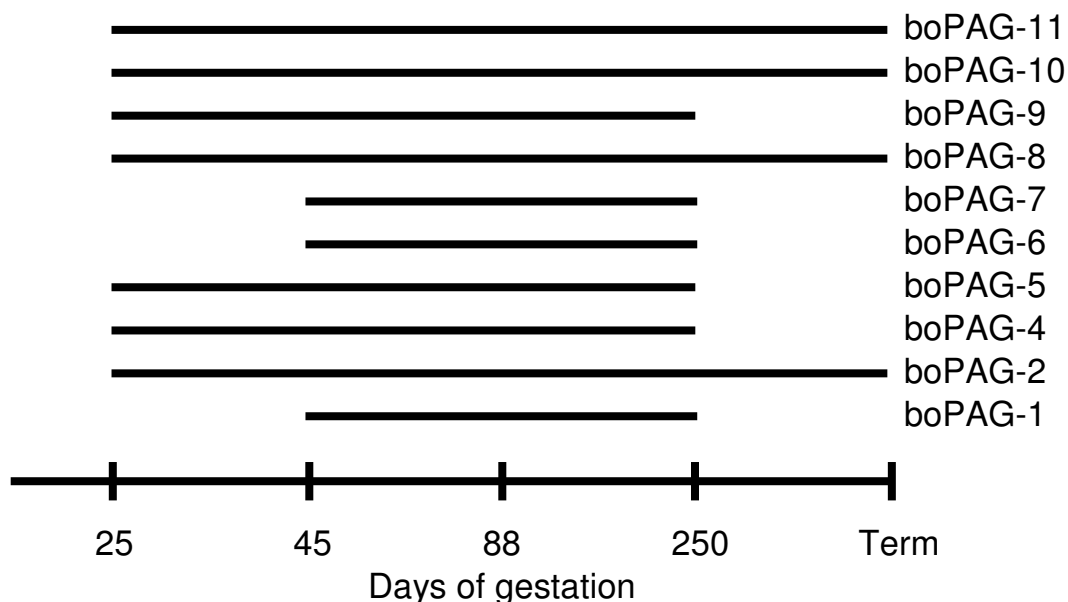| Binucleate cell expression			Expression throughout trophectoderm	
ovPAG-1	boPAG-1	boPAG-14	ovPAG-2	boPAG-2
ovPAG-3	boPAG-3	boPAG-15		boPAG-8
ovPAG-4	boPAG-4	boPAG-16		boPAG-10
ovPAG-5	boPAG-5	boPAG-17		boPAG-11
ovPAG-6	boPAG-6	boPAG-18		boPAG-12
ovPAG-7	boPAG-7	boPAG-19		boPAG-13
ovPAG-8	boPAG-9	boPAG-20		
ovPAG-9		boPAG-21		

Figure 1. Diagrammatic representation of bovine PAG expression throughout pregnancy (Green *et al.*, 2000). The expression of PAGs are shown as solid lines, the stages of pregnancy are displayed along the bottom

Molecular biology investigations also concluded, that during certain stages of pregnancy some PAGs were expressed, while others were absent (Garbayo *et al.* 1999, 2000; Green *et al.* 2000) (Figure 1). The PAG molecules which were predominantly expressed in the binucleate cells (like boPAG-1, -6, and -7) were expressed weakly or not in the Day 25 placenta, but they were present at the middle and the end of pregnancy. Others like boPAG-4, -5, and -9 were expressed at Day 25 and at earlier stages.

In species with epitheliochorial placenta, PAGs (eqPAG-1, poPAG-1, poPAG-2) were reported to be expressed throughout the chorion (Green *et al.*, 1999; Szafranska *et al.,* 1995)

POST-TRANSLATIONAL PROCESSING OF PAG-1

Generally, aspartic proteinases are synthesized as pre-pro forms and then undergo certain postranslational processing to achieve the catalytically active mature form (Davies *et al.*,1990). As predicted from the cDNA, the pre-pro forms of boPAG-1 and ovPAG-1 are consisting of 380 and 382 amino acids with molecular weights of 42.85 and 42.98 kDa (Xie *et al.,* 1991b). These structures contain a 15-residue signal sequence and a 38-residue pro-peptide. After the pro-piece removal, the polypeptide part of boPAG-1 and ovPAG-1 had a theoretical molecular weight of 36.73 and 36.80 kDa, while for the purified boPAG-1 analyzed by SDS-polyacrylamide gel electrophoresis 67 kDa was determined. Ovine PAG-1 is synthesized first in explant cultures as a ~ 70 kDa product, however later, the accumulation of smaller sized molecules was observed. Pulse-chase experiments using labelled amino acids, clearly showed, that the 70 kDa form precedes the formation of the 47 and 53 kDa forms.

Part of the molecular mass difference can be explained by the carbohydrate content of the protein, which is approximately 10%, the boPAG-1 contains 4 potential sites for N-linked glycosilation (Zoli *et al.*, 1991). The treatment of ovPAG-1 preparation with N-glycosidase F in order to remove oligosaccharides linked to the asparagine residues reduced the molecular weight to about 60 kDa (Xie *et al.*, 1996). Similar effect was observed, when explants from placenta were cultured in the presence of tunicamycin, which is an inhibitor of N-glycosylation. Treatment of ovPAG-1 with neuraminidase and O-glycanase reduced the molecular mass from 70 kDa to 63 and 59 kDa.

These results are suggesting that ovPAG-1 contains O-linked and N-linked oligosaccharides as well (Xie *et al.*, 1996).

Ovine PAG-1 incorporated ^{32}P into phosphoserine and phosphothreonine, but there was no incorporation of ^{35}S. The phosphate is associated with serine and threonine residues and not with the carbohydrate chain. This phosphor content is lost as the 70 kDa form is converted to smaller products (Xie *et al.,* 1996).

It was suggested by Roberts *et al.* (1995), that the binucleate cells of the placenta process the PAG molecules in some unusual manner.

PREGNANCY-ASSOCIATED GLYCOPROTEINS AS MEMBERS OF THE ASPARTIC PROTEINASE FAMILY

Aspartic proteinases are representing a major group of proteolytic enzymes having bi-lobed structure, same catalytic mechanism and aspartic acid residues around the highly conserved active site. They can be inhibited by pepstatin (Marciniszyn *et al.*, 1976a; Umezawa *et al.*, 1979) and certain active-site-directed reagents like diazoacetylnorleucine methyl ester and 1,2-epoxy-3-(p-nitro-phenoxy) propane (Rajagopalan *et al.,* 1966; Tang *et al.,* 1971). Their proteolytic specificity ranges from extremely broad like in the case of pepsin, to very narrow in the case of renin. Most of them are single chain enzymes with an approximate molecular weight of 35 kDa. Aspartic proteinases were isolated from various organisms: animals, plants, fungi and retroviruses. In the animals, these enzymes are well known by their role in the digestion process, tissue remodeling, regulation of blood pressure and several intracellular processes.

The catalytic activity of aspartic proteinases is dependent upon two conserved aspartic acid residues that are in close contiguity in the center of the substrate binding cleft. These residues are preceded by a hydrophobic amino acid (usually phenylalanine) and followed by invariant threonine and glycine (Davies *et al.,* 1990). The cleft is about 4 nm long and can accommodate a segment of substrate seven or eight amino acids long. A twofold symmetry, with a second conserved region centering around each aspartic acid is also required to polarize the bound water molecule which hydrolyses the scissile bond of the substrate (James and Sielecki 1986).

Common feature of eukaryotic aspartic proteinases that they are synthesized as inactive zymogens. In this form a 40-50 amino acids long

amino terminal propeptide folded into the active site cleft, inhibiting the access of substrate. It can be removed either autocatalytically at low pH, or by an other proteolytic enzyme (Marciniszym *et al.*, 1976b; James and Sielecki 1986; Koelsch *et al.,* 1994).

In the last decade, PAG molecules isolated from different species have been identified as members of the aspartic proteinase family. Sequence comparison of 8 PAG molecules from equine, porcine, ovine, bovine species showed that all PAG probably evolved from a pepsin-like progenitor molecule (Guruprasad *et al.,* 1996). On the basis of amino acid sequence differences between PAGs a phylogenetic tree was constructed (Tables 2-3).

The close similarity of boPAG-1 and ovPAG-1 to pepsin in sequence suggests that these three molecules would process similar three dimensional fold. The comparison of their three dimensional models to that of porcine pepsin and bovine chymosin proved that these models were closer to porcine pepsin.

Table 2. Phylogenetic tree for PAGs and related aspartic proteinases. The distances derived from the pairwise percentage sequence identities using the multiple sequence alignment program MALIGN were used for the tree construction (Guruprasad *et al.*, 1996)

	Pepsin	Chymosin	boPAG-1	boPAG-1v	boPAG-2	ovPAG-1	ovPAG-2	poPAG-1	poPAG-2	eqPAG
Pepsin	100									
Chymosin	59.5	100								
boPAG-1	49.5	42.5	100							
boPAG-1v	50.8	42.9	86.1	100						
boPAG-2	50.8	45.6	57.8	58.8	100					
ovPAG-1	49.4	42.3	70.6	71.6	58.5	100				
ovPAG-2	50.5	45.9	60.4	60.2	63.4	60.4	100			
poPAG-1	48.6	43.5	48.8	50.5	48.5	47.4	52.5	100		
poPAG-2	52.9	44.3	56.2	55.1	56.7	54.2	57.4	61.8	100	
eqPAG	58.6	52.3	54.9	55.2	55.4	55.5	54.6	55.3	58.5	100

Most of the PAG molecules was found to be catalitically inactive because of key mutations close to the active site (Xie *et al.*, 1999a,b; Szafranska *et al.*, 1995). For example: bovine PAG-1 has an alanine in the position of the normally invariant glycine at position 34 (pepsin numbering), while ovPAG-1

lacks one of the two aspartic acid residues at position 215 (pepsin numbering) that participates in catalysis and is substituted by glycine (Xie *et al.*, 1991b). Similar changes in the analogous aspartic acid in the amino terminal lobe of pepsin eliminates its proteolytic activity (Lin *et al.*, 1989). The above described glycine – alanine mutation in the boPAG molecule displaces the water molecule that normally resides between the two aspartic acids and is directly involved in the catalytic mechanism. Additionally, the access of peptide substrate to the catalytic center of the boPAG-1 and ovPAG-1 molecules is limited (Guruprasad *et al.*,1996). In contrast, the sequence analysis for eqPAG-1 and poPAG-2 suggested that these proteins are likely to have proteolytic activity (Guruprasad *et al.,*1996). This was confirmed by proteinase assays when purified preparation of recombinant eqPAG was capable to hydrolyze ^{14}C-hemoglobin and to catalyze the removal of its own propeptide (Green *et al*, 1999). Addition of pepstatin A to the reaction mixture completely inhibited the enzymatic process.

Table 3. Comparison of amino acid sequence identities between PAGs and aspartic proteinases (Green *et al.*, 1998)

	pPEP A	rhPEP	boPAG-1	ovPAG-1	boPAG-2	ovPAG-2	pPAG-1	pPAG-2	eqPAG	RPEP F	hCath D	hCath E
pPEP A	100											
rhPEP	86	100										
boPAG-1	48	49	100									
ovPAG-1	48	50	73	100								
boPAG-2	50	51	58	59	100							
ovPAG-2	49	49	59	60	65	100						
pPAG-1	49	49	49	48	50	53	100					
pPAG-2	51	52	54	53	57	57	64	100				
eqPAG	56	56	52	53	54	52	54	57	100			
rPEP F	56	57	51	50	53	58	56	58	69	100		
hCath D	49	46	40	40	40	45	36	40	42	44	100	
hCath E	54	54	42	42	44	44	43	46	46	46	52	100

pPEP A: pig pepsinogen; rhPEP: rhesus monkey pepsinogen A; rPEP F: rabbit pepsinogen F; hCath D: human cathepsin D; hCath E: human cathepsin E.

Several PAG molecules were reported to be capable to bind pepstatin (Xie *et al.,* 1991; Green *et al.,* 1999). This feature of PAGs was used to selectively remove them from placental conditioned media by affinity chromatography on pepstatin-agarose columns. The peptide binding specificity of PAG molecules differ significantly from each other and from pepsin. In the cases of boPAG-1 and ovPAG-1 there are binding preference for lysine- and arginine-rich peptides (Guruprasad *et al.,* 1996).

These observations are suggesting that PAGs represent a family where the peptide binding ability is retained, however the catalytic activity may be lost in most of the molecules.

BOVINE PLACENTA, BINUCLEATE CELLS

The placenta is a specialized extra-embryonic tissue which plays a special role in establishment, maintenance of pregnancy; a multi-functional organ with respiratory, nutritional, epurative, endocrine and immunological roles.

The bovine placenta, is synepitheliochorial containing 6 cellular layers between the maternal and the fetal blood circulation: endothelium of the maternal capillaries, uterine connective tissue, uterine epithelium, chorial epithelium, chorial connective tissue, endothelium of the chorial capillaries. The trophoblastic *villi* on the surface of the chorion are localized in restricted number of round or oval territories called cotyledons (localized cotyledonary type). The cotyledons with the outgrowths of the uterine mucosa (carunculas) are forming the placentomas. The number of cotyledons in the bovine placenta is 70-150. The placentomas are situated in 5 rows both in the gravid and in the nongravid horn (3 in the middle of the horn and 2 on its anterior part). The bovine cotyledons are convex and pedunculated. The intercaruncular area of the endometrium (paraplacenta) remains glandular during the gestation period while the caruncular area is nonglandular.

Unique feature of the ruminant placenta is the population of binucleate cells (BNC). These cells derive from the mononucleate cells of the trophectoderm by nuclear mitosis without cytoplasmic mitosis. The youngest BNCs are located deep in the trophectoderm, during maturation they migrate towards the maternal surface (Wooding *et al.*, 1983).

Binucleate cells first appear just before implantation (Wango *et al.*, 1990a,b), and can be recognized from days 16-17 in the bovine trophectoderm. These cells constitute about 15-20% of the trophectodermal cells, from implantation till one or two days before parturition when their number decreases rapidly (Wooding 1983; Wooding *et al.*, 1986). Morphometric quantitation studies showed, that 20% of the BNCs undergo migration along the villi through the chorionic tight junction to fuse with uterine epithelial cells and form trinucleate cells (maternal giant cells) (Wooding 1984; Wooding and Beckers 1987) (Figure 2). These trinucleate cells are short-lived

structures, after releasing their secretory granules by exocytosys they are resorbed by the trophectoderm (Wooding and Wathes, 1980).
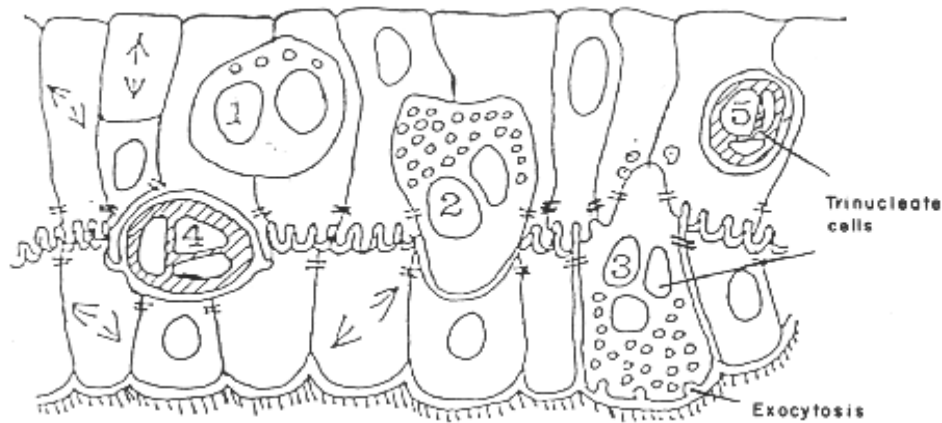

Figure 2. Binucleate cell development and migration (Wooding *et al.*, 1992). BNC migration (1-3) produces trinucleate cells which after exocytosys are resorbed by the trophectoderm (4-5)

Young BNCs are containing a small volume of dense ribosome-filled cytoplasm, which after cytoplasmic re-organization to a spherical or oval cell, has no contact with the basement membrane or the atypical trophectodermal tight junction. With their maturation an extensive array of rough endoplasmic reticulum and a large Golgy body will develop.

The BNCs are involved in the formation of fetomaternal syncytium which is essential for successful implantation and the subsequent placentomal growth. This cell population plays role in the production and delivery of different proteins and steroid hormones.

Binucleate cells are the sole source of placental lactogens (Verstegen *et al.*, 1985; Wooding *et al.*, 1992). Investigations using immunocytochemistry proved that protein hormones of BNCs are synthesized via a rough endoplasmic reticulum to Golgi body sequence and stored in the granules during growth and maturation (Wooding 1981; Wooding and Beckers, 1987). It was found that BNCs are responsible for the synthesis of PAGs, PSPB (Reimers *et al.,* 1985a; Gogolin-Ewens *et al.,* 1986; Morgan *et al.,* 1989; Zoli *et al.,* 1992a; Atkinson *et al.,* 1993).

Binucleate cells are involved in the production of progesterone (Wango *et al.*, 1991, 1992; Wooding *et al.*, 1996). Isolated BNCs are able to produce prostaglandins (Reimers *et al.,*1985b) which can play role in the paracrine

control of the progesterone synthesis. Wooding (1992) suggested that the placental BNC population probably contains subpopulations with different proteins in the granules. These secretory products are stored in the granules of the BNCs and their content is delivered into the maternal system after the BNC migration (Wooding 1984).

PAG, PSPB AND PSP60 RADIOIMMUNOASSAYS

Bovine PAG, PSPB and PSP60 preparations were used to raise antisera in rabbits, which allowed the development of specific radioimmunoassays in order to detect these products in biological fluids (Sasser *et al.*, 1986; Humblot *et al.*, 1988a,b; Mialon *et al*, 1993; Zoli *et al* 1992b).

The specific double-antibody RIA developed by Zoli *et al.* (1992b) allowed the measurement of bPAG in placental extracts, fetal serum, fetal fluids, and serum or plasma of pregnant cows. The minimal detection limit of the assay was 0.2 ng/ml. This author reported that about 20% of the unbreed heifers and nonpregnant cows presented detectable PAG concentrations.

Higher PAG concentrations were observed in maternal than in fetal serum confirming that this protein is delivered preferentially in the maternal system (Zoli *et al.*, 1992b). This particularly differs from placental lactogen, that was found in higher concentration in the fetal than in the maternal circulation.

Pregnancy-associated glycoproteins can be detected in maternal circulation at around the time when the trophoblast forms definitive attachment to the uterine walls (Zoli *et al.*, 1992b; Sasser *et al.*, 1986). On Day 22 after fertilization 0.38±0.13 ng/ml serum PAG concentration was detected. In early and mid gestation PAG levels rose continuously. Sinclair *et al.* (1995) reported an exponential increase in the bPAG concentrations between Day 23 and 85 after AI. Dramatic increase was observed during the last 10 days of the gestation, when PAG levels reached peak concentrations 2462.4±1017.9 ng/ml (Figure 3). Using a PSP60 RIA Mialon *et al.* (1993) reported an 80 to 200 fold increase in the PSP60 concentration for the last 2 weeks of gestation. The PSPB profile showed similar increase for the prepartum period (Sasser *et al.*, 1986). After calving, PAG concentrations are decreasing, the detection limit is reached only by Day 100±20 postpartum (Zoli *et al.*, 1992b) (Figure 4). The half-life of this glycoprotein was estimated to be 7.4 to 9 days (Kiracofe *et al.*, 1993; Mialon *et al.*, 1993; Ali *et al.*, 1997).
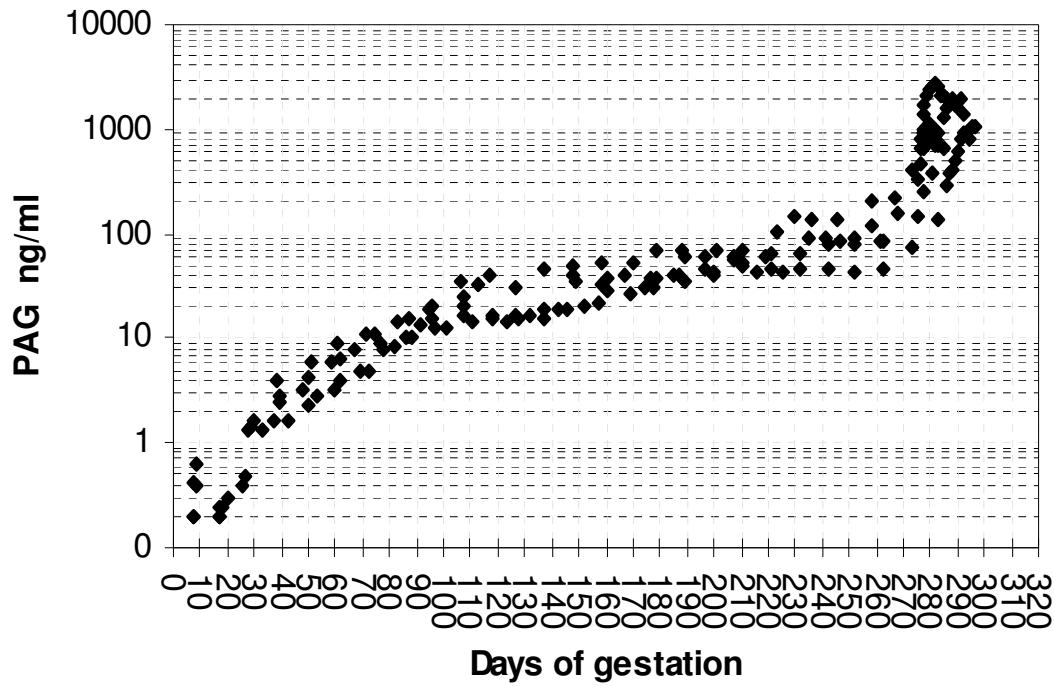
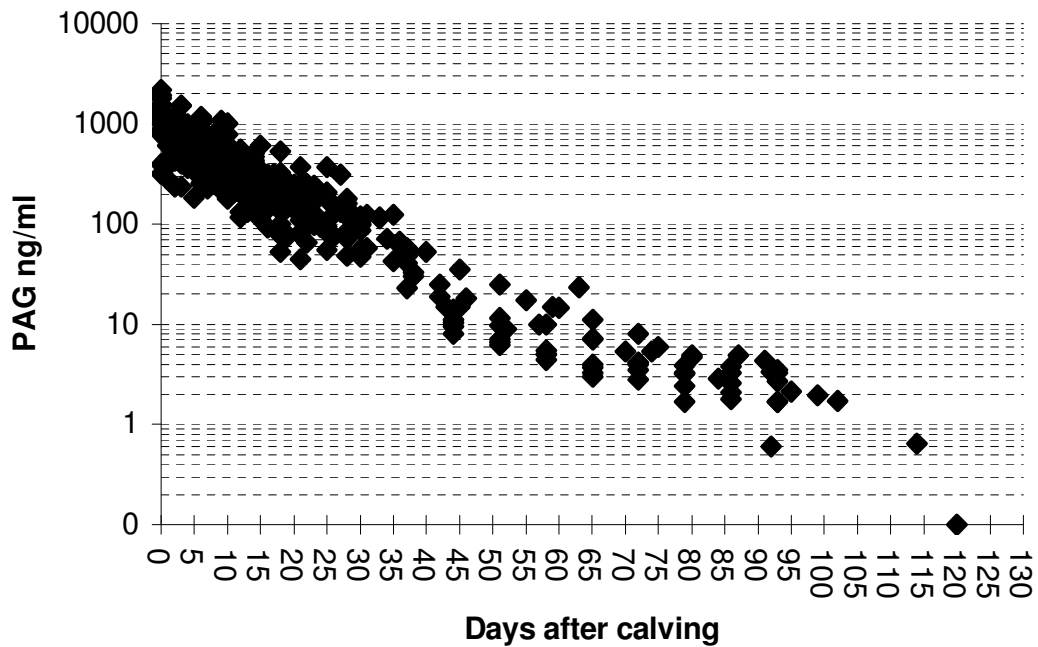Figure 3. PAG levels determined in cows during gestation (Patel *et al.*, 1997)

Figure 4. PAG levels determined in cows during the postpartum period (Sulon *et al.*, unpublished)

It was suggested, that the biexponential equation describes the best the clearance of bPAG from the peripheral circulation (Ali *et al.*, 1996, unpublished). Minor differences in clearance rate were detected between different breeds but not dietary level. This author suggested the two-compartmental model system for the distribution and metabolism of bPAG between the liver and the plasma pool.

Investigations performed in peripartum clearly demonstrated the positive effect of maternal environment and fetal genotype on peripheral blood concentration of bPAG-1. The experiments of interspecies fertilizations confirmed this hypothesis when the expression of antigens by trophoblast cells that are recognized as foreign bodies by the maternal immune system. The trophoblast of crossbreed fetuses expresses more similar antigens to the mother than fetuses unrelated to the breed of the recipient. Bovine PAG concentrations will be found to be more elevated in intra-species crossbreeds. (Beckers *et al.*, 1998, 1999). Mean peripartum PAG concentrations were higher in Hereford cows than in Holstein heifers or cows (Zoli *et al.*, 1992b). Mialon *et al* (1993) reported higher mean plasma PSP60 concentrations in Charolais cows than in Holstein and Normande breeds during the last two weeks of gestation. The time related changes in plasma bPAG concentrations were significantly ($p < 0.01$) affected by the stage of gestation and fetal number. Animals with twin pregnancies presented higher PAG, PSPB concentrations during gestation with the exception of the last 10 days (Patel *et al.*, 1995, 1997, 1998; Dobson *et al.*, 1993). Significantly higher amount of PSPB were produced at 40 days of gestation in twin pregnancies (Vasques *et al.*, 1995). Bovine PAG concentrations determined in twin-bearing cows are probably the result of dual attachment points and enhanced synthetic activity of twin placentas (Patel *et al.*, 1997). The birthweight of the calf was correlated to peripheral PSPB concentration in cows with single pregnancies, however this relationship decreased with the subsequent increase in fetal number (Patel *et al.*, 1995).

PREGNANCY DETECTION

Several reports are available on the day of earliest application of the PAG, PSPB and PSP60 RIA tests: Zoli *et al.*, (1992b) suggested that the PAG test can be used from 28 days after fertilization, according to Mialon *et al.*

(1993) detectable PSP60 concentrations can be measured in 100% of the pregnant females after Day 27, whereas Humblot *et al.*, (1988) and Delahaut *et al.*, (1996) reported that the PSPB test could be useful from Day 30.

Sasser *et al.* (1986) reported that from a commercial herd of 102 beef cows the PSPB RIA test could detect pregnancy earlier and more accurately than rectal palpation. This RIA was able to detect 177 of 187 nonpregnant cows. PSPB test allowed the detection of 90% of non pregnant females between Day 26 and Day 30-35 after AI (Humblot *et al.*, 1988c). Because of the appearance of PSPB later than 26 days in plasma of several pregnant cows, the accuracy of negative diagnosis did not approach 100% before Day 30-35 after AI. The accuracy of a positive PSPB test was always higher than 85%.

The accuracy of positive diagnoses by PSP60 RIA test was 90% in heifers and 74% in cows on Day 28. When the interval between calving and blood sampling was longer than 115 days, the accuracy of this method was 84% in cows on Day 28 (Mialon *et al.*,1994). The accuracy of negative diagnoses was higher than 90%.

In a field experiment, Zoli *et al.* (1992b) detected bPAG in the serum of 287 Holstein-Friesian animals (which had received transferred embryos) out of 430 at Day 35 postestrus. Heifers (n=267) presenting detectable levels of bPAG were confirmed to be pregnant by rectal palpation performed at Day 45. False positive results occurred in 6.9% (20/287) and false negative results were found in 2.1% (3/143) of the cases. The total accuracy of the test was 94.6% (407/430).

Plasma bPAG concentrations were successfully used to predict fetal age in maiden heifers. The regression coefficient of days of pregnancy on plasma $log_{10}bPAG$ concentration was significantly higher than one (Sinclair *et al.*, 1995).

Bovine PAG RIA compared with alternative methods of pregnancy diagnosis gave a sensitivity of 100%, specificity 93%, positive predictive value 97%, negative predictive value 100% and overall efficiency of 98% in a field experiment, based on 233 animals (Skinner *et al.*, 1996). Cows with false negative ultrasonographic diagnosis between 27 and 31 days after AI were reported to have bPAG concentrations above the threshold level (0.5 ng/ml) (Szenci *et al.*, 1998a).

When two pregnancy detection methods: bPAG RIA, bPSPB RIA were compared, the sensitivity of bPSPB and bPAG RIA test was similar (92.0% and 95.2-100%, respectively), the specificity of the bPAG test was significantly lower than that of the bPSPB test (56.7 and 79.0% respectively) (Szenci *et al.*,

1998b). When compared with calving results there were no significant differences among ultrasonography, bPAG RIA and bPSPB RIA tests.

The major source of false positive diagnoses may have been due to the samples being taken within the 100 day postpartum period. At this time these proteins are still present in the peripheral circulation because of their relatively long half-life. Therefore the use of these protein tests is limited under field conditions during the early stages of the postpartum period. Embryonic, fetal mortality resulting in residual bPAG in the maternal circulation may also have been the cause of false positive diagnoses.

PREGNANCY FOLLOW UP

Pregnancy-associated glycoprotein has been found in the serum of pregnant cattle and used as a pregnancy marker. As pregnancy failure occurs, PAG concentrations dropped and disappeared from maternal blood. Sequential assays of these proteins between Day 24 and term are useful for studying the course of pregnancy, although it does not allow discrimination between early embryonic mortality and non-fertilization (Mialon *et al.*, 1993). As PAG molecules are the products of the trophoblastic cells, it was suggested that their determination in maternal blood can be useful for prediction of fetal well-being and help to detect early placental abnormalities, embryonic mortality or abortion (Ectors *et al.*, 1996ab). Investigations performed on N'dama cows showed that PAG assay can be an efficient indicator of infertility in *Bovidae* (Dramé *et al.*, 2000).

Heifers expressing pregnancy failure between Days 16 and 35 after AI showed wide variation in PSP60 concentrations at Days 28 and 35. This phenomenon was explained by the variation in the time of embryo death (Mialon *et al.*, 1993). This author reported some cases where at Day 50 ultrasonic scanning and signs of estrus indicated that the embryo was already dead, however till this day the PSP60 concentrations were increasing. Decreasing PSP60 concentrations were observed in some animals before Day 50, where according to ultrasonic examination, the conceptus was still alive.

PSPB test in conjunction with milk progesterone test was used to determine the time of the embryonic death (Humblot *et al.*, 1988b). It was confirmed by analyzing the progesterone concentrations on Day 24 and by observing early return to estrus, that 60% of pregnancy failures result from non-fertilization or early embryonic death (Ayalon *et al.*, 1978; Diskin Sreenan

et al., 1980; Humblot *et al.,* 1986) before the antiluteolytic signal was present. In most of the early failures an elevation in the PSPB concentrations was not detectable, or only after Day 24. No coefficient of correlation between PSPB and progesterone concentration was significant at any days of gestation studied. Significant difference was found in the rate of mean PSPB levels between pregnant cows and cows showing late embryonic mortality between Days 24 and 30-35 after AI. In their study it was not possible to predict the occurrence of embryonic mortality.

Humblot (1988b) suggested that the measurement of progesterone parallel with PSPB in peripheral blood plasma or serum can be very useful to study the way in which various factors may chronologically affect embryonic mortality.

After in vitro production of embryos (IVF) or cloning, the PAG follow up in plasma samples collected weekly was able to monitor embryonic or fetal deaths (Ectors *et al.* 1996a,b, 1997). It was shown by Ectors *et al.* (1996a,b) comparing PAG levels in heifers received nuclear transfer (NT) and IVF embryos, that the recipients from the NT group presented higher PAG concentrations during late pregnancy. Probably this was the consequence of morphological abnormalities like major placental hypertrophy, associated with hydramnios and hydatiform molar, increase in number and in diameter of cotyledons which occurred more often in the NT group. It was suggested that these placental abnormalities are correlated with an incomplete maturation of oocytes at the time of fertilization, smaller follicles giving non competent or partially competent oocytes (Ectors *et al.* 1997). Heyman *et al.* (2002) found very high PSP60 concentrations (up to 400 ng/ml) in recipients that developed hydroallantois. In that study it was concluded that significantly increased PSP60 levels could indicate abnormal placental development. Ectors *et al.* (1997) reported the pregnancy follow-up of two heifers with early (week 7 and 8) and late (after Day 158) abortion. Cows that gave birth to a stillborn calf or to a schistosomus reflexus calf exhibited an aberrant PAG profile: in the first case the peripartum peak of bPAG was missing, while in the later case the animal expressed higher concentrations of PAG during the second trimester of the pregnancy (Patel *et al.*, 1997).

In veterinary practice, PAG and PSPB RIAs in biological fluids are helpful to confirm clinical diagnoses established by ultrasonography (Szenci *et al.*, 1998a). Cows presenting an initial positive and then negative ultrasonographic diagnosis expressed decreasing PAG concentrations (Szenci *et al.*, 1998a). The high progesterone and PAG concentrations confirmed the presence of a live conceptus and a functional corpus luteum at the time of the first

ultrasonographic examination. After the time of embryonic mortality occurred, the PAG levels had already decreased, while progesterone concentrations showed that the corpus luteum was maintained in three of the four cases. Following embryonic mortality using ultrasonography, PAG and PSPB concentrations decreased steadily, in one animal these protein concentrations did not reach the threshold level (Szenci *et al.*, 1998b). These authors were able to diagnose late embryonic and fetal mortality cases retrospectively in seven cows using PSPB RIA test and in four cows using PAG RIA test (Szenci *et al.*, 2000).

Because of the large individual variations in PAG concentrations in maternal blood, only the marked decrease or disappearance in serum/ plasma concentrations of these proteins can be a no controversial, predictive sign of embryonic or fetal death.

AIMS AND OBJECTIVES OF THE STUDY

The current study aimed to develop and characterize new, sensitive and specific radioimmunoassays for the measurement of PAG molecules in the serum and plasma of cows.

There were three specific objectives of this study:

Firstly, to validate two additional RIA systems using antisera produced against PAG molecules purified from caprine placenta. To describe the sensitivity, the accuracy, the precision of the systems. To determine the specificity of the 3 RIA systems focusing especially on the enzymatically active members of the aspartic proteinase family in order to investigate the possible effect of these products on the 3 RIA systems. To select the RIA system with the best ability to recognize PAG molecules in the maternal blood.

Secondly, to characterize the PAG profiles in cows with normal pregnancy after AI using the two additional RIA systems with frequent blood sampling. To determine the ratios between the concentrations determined by the newly developed RIA systems and the classical RIA system of Zoli *et al.* (1992b).

Thirdly, in a collaborative study to describe PAG and progesterone profiles in cows with pregnancy failure after AI or transfer of embryos produced by three different methods. To diagnose and to investigate the nature of pregnancy failures by the retrospective analysis of these profiles.

EXPERIMENTAL WORK

Chapter 2: **Investigation I: Validation of 2 RIA systems for measuring pregnancy-associated glycoproteins in bovine serum/plasma**

DEVELOPMENT OF THE RIA

Radioiodination

During the radioiodination, iodine is substituted on to the aromatic side-chain of tyrosine residues of the antigen resulting in a stable compound which forms a highly efficient tracer. Iodine can also substitute on to other amino acids, like histidine and phenylalanine, however the rate of the later reaction is 30-80 times less than that for tyrosine. At low levels of specific activity (1 atom of iodine per molecule or less) most of the substitutions are single (mono-iodotyrosine), while at higher levels of specific activity is diiodotyrosine formed.

In this study, the chloramine T method of Greenwood *et al.* (1963) was used to radiolabel PAG with I^{125}. The chloramine T is an oxidizing agent capable to convert iodide to a more reactive form. The antigen was dissolved in phosphate buffer (0.2 M, pH 7.5) to obtain 1 µg/µl concentration. The radioiodination mixture was prepared by adding 10 µl of chloramine T solution (5 mg/ml dissolved in water) and 10 µl of NaI^{125} (1 mCi, approximately 3.7×10^7 disintegrations per second) to 10 µl of antigen solution. After one minute of stirring 10 µl of metabisulphit solution (30 mg/ml dissolved in water) was added in order to terminate the reaction. This mixture was loaded onto a G-75 column, which was previously calibrated with Tris BSA buffer (0.025 M, pH 7.5) for the separation of free I^{125} and labeled antigen. Eluted fractions of 1 ml were collected. The aliquots were submitted to a test to determine their non-specific binding and ability to bind to the selected antisera. The specific radioactivity was determined according to the self-displacement method (Morris *et al.* 1976).

Antisera

Three different radioimmunoassays (RIA 1, RIA 2 and RIA 3) differing in the antiserum, were used in this experiment to measure pregnancy-associated glycoprotein concentrations in plasma samples. In RIA 1 anti PAG I$_{67}$ (Zoli *et al.* 1991), in RIA 2 anti PAG$_{55+62}$ and in RIA 3 anti PAG$_{55+59}$ polyclonal antisera (Beckers *et al.* 1998; 1999) were used. These antisera were raised in rabbit after immunizing them intradermally with the appropriate antigen.

Standard Curve

A PAG I_{67} preparation purified recently according to the protocol of Zoli *et al.* (1991) was used as standard and tracer for all assays.

The PAG measurements were performed according to the method of Zoli *et al.* (1992) with some modifications. Briefly, the previously weighed standards and the serum / plasma samples (0.1 ml) were diluted in 0.2 ml of Tris buffer at pH 7.5 (0.025 M Tris, 0.01 M $MgCl_2$, 0.01 % (wt/vol) Sodium-azide, 0.5 % (vol/vol) Tween 20). The standard curve ranged from 0.1 ng/ml to 25 ng/ml and in order to minimize nonspecific interference 0.1 ml virgin heifer serum / plasma was added to each tube of the standard curve. After the addition of appropriate dilutions of antisera the serum / plasma samples and the standards were incubated overnight at room temperature. Antisera titers were determined to obtain a tracer binding-ratio in the zero standards of approximately 20-30% (RIA 1: 1/500000, RIA 2: 1/250000, RIA 3: 1/1000000). The following day, tracer (0.1 ml or 25000 cpm) was added to all the tubes, and the tubes were incubated for 4 hours at room temperature. The total assay volume was 0.5 ml. The separation of the free and bound fractions was done by centrifugation (20 min at 1500 g, 10°C) after the addition of 1 ml second antibody polyethylene glycol (PEG) solution (0.17 % (vol/vol) normal rabbit serum, 0.83 % (vol/vol) sheep anti-rabbit IgG, 0.3 % (wt/vol) BSA, 4 % (wt/vol) polyethylene glycol 6000 in Tris buffer). After the tubes had been incubated for 1 hour with the second antibody PEG solution, 2 ml of Tris buffer was added and the tubes were centrifuged (20 min at 1500 g). The supernatant was aspirated and 4 ml of Tris buffer was added to each tube. The tubes were centrifuged (20 min at 1500 g) and the supernatant aspirated. The ^{125}I in the pellet was quantified using a gamma counter (LKB Wallac 1261 Multigamma counter, Turku, Finland) with a counting efficiency of 75 %.

Reproducibility

The reproducibility of the three RIA systems was tested at three different bPAG concentrations (0.6, 1.5 and 3.5 ng/ml). The precision is presented as intra- and inter-assay coefficient of variation. To determine the intra-assay coefficient of variation the same sample was assayed in duplicate, 20 times in

the three RIA systems. The inter-assay coefficient of variation was assessed by analyzing the same sample in duplicate, in 10 consecutive assays.

Accuracy

To determine the accuracy of PAG measurement in serum, known amounts of bPAG were added to a pool of virgin heifer sera. The percentage of recovery is presented as observed concentration (ng/ml) / expected concentration (ng/ml) X 100. Serial dilutions of serum samples were assayed in the three RIA.

Sensitivity

Sensitivity is defined as the minimal detection limit (MDL) of an assay or with other words 'the least concentration of unlabelled ligand which can be distinguished from a sample containing no unlabelled ligand'. MDL was determined as the mean concentration minus twice the standard deviation of 20 replicates of the zero standard (Skelley *et al.*, 1973).

Serum samples

Serum samples were collected from the jugular vein of 15 nonpregnant Holstein Friesian heifers into vacutainer tubes. Samples were allowed to clot, then centrifuged (15 min at 1500 g) and the serum was stored at -20 °C until assay.

RESULTS

Specific radioactivity

The specific radioactivity of the tracer was 3242.6 mCi/µmol as determined according to the self-displacement method (Morris *et al.* 1976).

Dilution Curves

Dilution curves of the 3 antisera (anti PAG I_{67}, anti PAG_{55+62}, anti PAG_{55+59}) used are pesented in Figure 1.
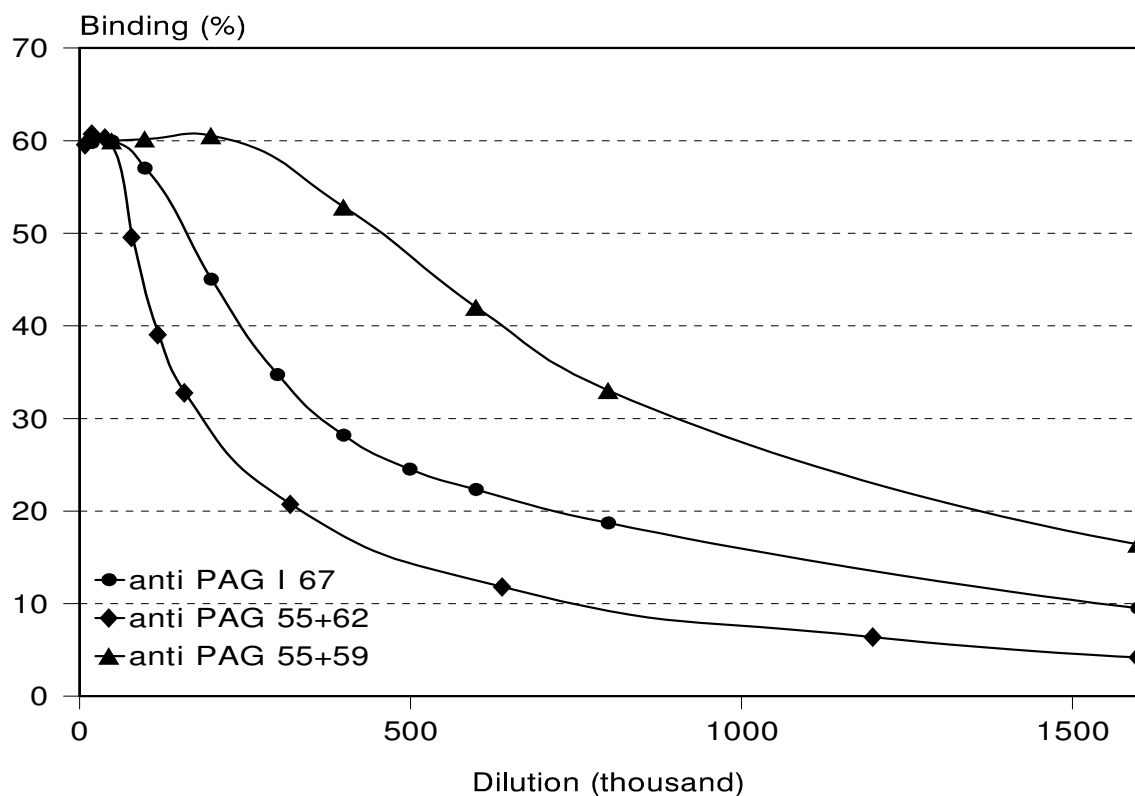
Figure 1. Dilution curves of anti PAG I_{67}, anti PAG_{55+62}, anti PAG_{55+59}

Standard curves

The characteristics of the standard curves of RIA 1, RIA 2 and RIA 3 are presented in Table 1.

Table 1. Characteristics of the standard curves of RIA 1, RIA 2 and RIA 3

RIA System	Slope[b]	Minimal Detection Limit (ng/ml)	Estimated dose at		
			20% B/B_0[c] (ng/ml)	50% B/B_0[c] (ng/ml)	80% B/B_0[c] (ng/ml)
RIA 1[a]	-0.59	0.05	2.68	0.85	0.268
RIA 2[a]	-0.65	0.06	4.96	1.71	0.586
RIA 3[a]	-0.63	0.11	6.29	2.20	0.768

[a] The tracer binding-ratio in the zero standards was 22%, the non specific binding was 0.52% of the total count

[b] The slope is an absolute value of the derivate of the curve at estimated dose 50%

[c] B/B_0= Tracer bound / tracer bound in the zero standard

Reproducibility

The intra- and inter-assay coefficient of variation for RIA 1,2 and 3 are presented in Tables 2 and 3.

Table 2. Intra-assay coefficients of variation of RIA 1, RIA 2 and RIA 3 systems (Intra-assay coefficients of variation were calculated for three concentrations of PAG: 0.6 ng/ml, 1.5 ng/ml, 3.5 ng/ml)

RIA System	Intra-assay CV at 0.6 ng/ml (%)	Intra-assay CV at 1.5 ng/ml (%)	Intra-assay CV at 3.5 ng/ml (%)
RIA 1	6.58	4.44	3.96
RIA 2	8.81	5.34	2.57
RIA 3	10.10	6.09	4.47

Table 3. Inter-assay coefficients of variation of RIA 1, RIA 2 and RIA 3 systems (Inter-assay coefficients of variation were calculated for three concentrations of PAG: 0.6 ng/ml, 1.5 ng/ml, 3.5 ng/ml)

RIA System	Inter-assay CV at 0.6 ng/ml (%)	Inter-assay CV at 1.5 ng/ml (%)	Inter-assay CV at 3.5 ng/ml (%)
RIA 1	6.88	5.31	4.16
RIA 2	11.32	5.89	3.04
RIA 3	15.55	10.17	5.30

Accuracy

Accuracy and mass recovery of RIA 1, 2 and 3 were assessed in the range of 1-200 ng/ml and are presented in Tables 4-6. The serial dilution samples showed dose-response curves (Figures 2-4) parallel to the standard curve.

Table 4. Recovery by RIA 1 of PAG added to bovine serum samples

Serum sample PAG concentration (ng/ml)	Amount of PAG added (ng/ml)	Theoretical PAG concentration (ng/ml)	Observed PAG concentration (ng/ml)	Recovery (%)
1.71	1	2.71	2.89±0.1	106.56±3.58
5.11	1	6.11	6.17±0.15	101.08±2.41
1.71	20	21.71	21.32±1.03	98.17±4.74
5.11	20	25.11	24.73±0.47	98.51±1.86
1.71	200	201.71	192.50±24.14	95.43±11.97
5.11	200	205.11	205.41±10.78	100.15±5.26

Table 5. Recovery by RIA 2 of PAG added to bovine serum samples

Serum sample PAG concentration (ng/ml)	Amount of PAG added (ng/ml)	Theoretical PAG concentration (ng/ml)	Observed PAG concentration (ng/ml)	Recovery (%)
3.97	1	4.97	5.08±0.18	102.25±3.60
13.66	1	14.66	14.59±0.05	99.50±0.36
3.97	20	23.97	23.90±0.60	99.70±2.50
13.66	20	33.66	34.91±0.45	103.70±1.34
3.97	200	203.97	200.37±11.83	98.24±5.80
13.66	200	213.66	210.44±7.91	98.49±3.70

Table 6. Recovery by RIA 3 of PAG added to bovine serum samples

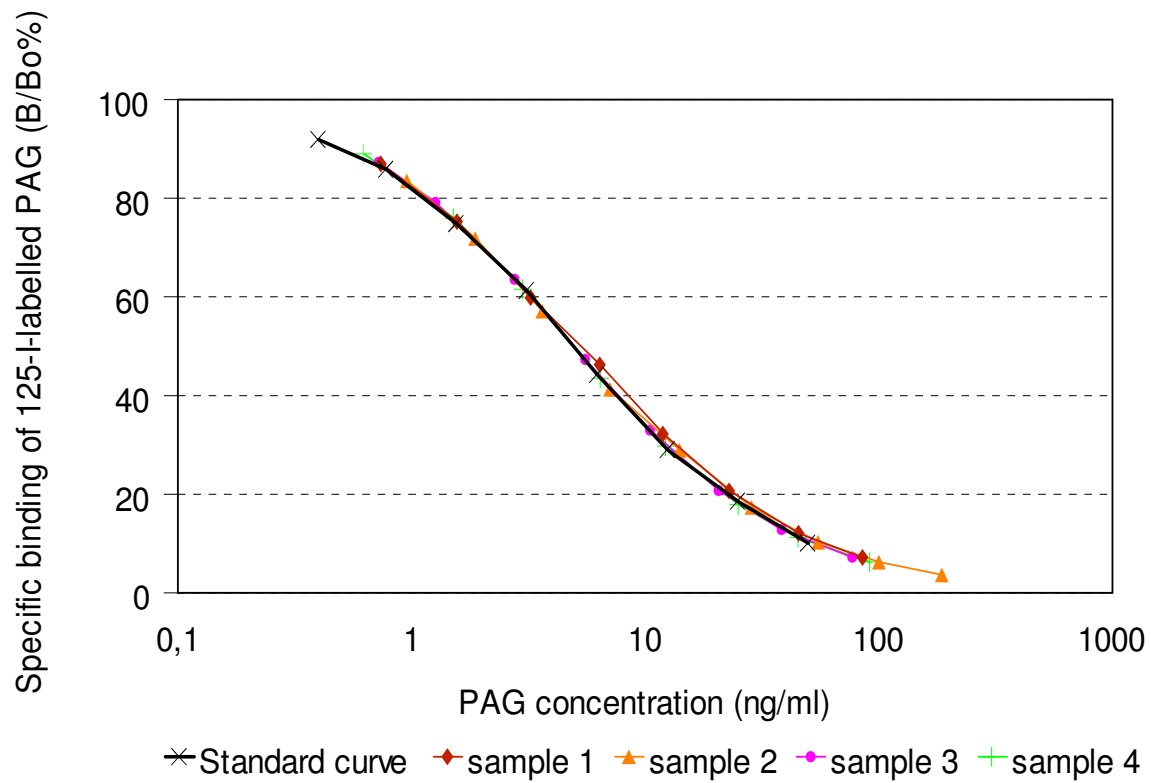| Serum sample PAG concentration (ng/ml)	Amount of PAG added (ng/ml)	Theoretical PAG concentration (ng/ml)	Observed PAG concentration (ng/ml)	Recovery (%)
3.61	1	4.61	4.46±0.05	96.75±1.17
11.86	1	12.86	12.88±0.21	100.20±1.66
3.61	20	23.61	24.34±0.25	103.08±1.06
11.86	20	31.86	33.43±1.14	104.94±3.58
3.61	200	203.61	196.23±17.47	96.38±8.58
11.86	200	211.86	207.57±16.20	97.97±7.65

Figure 2. Standard curve, serial dilution of samples in RIA 1
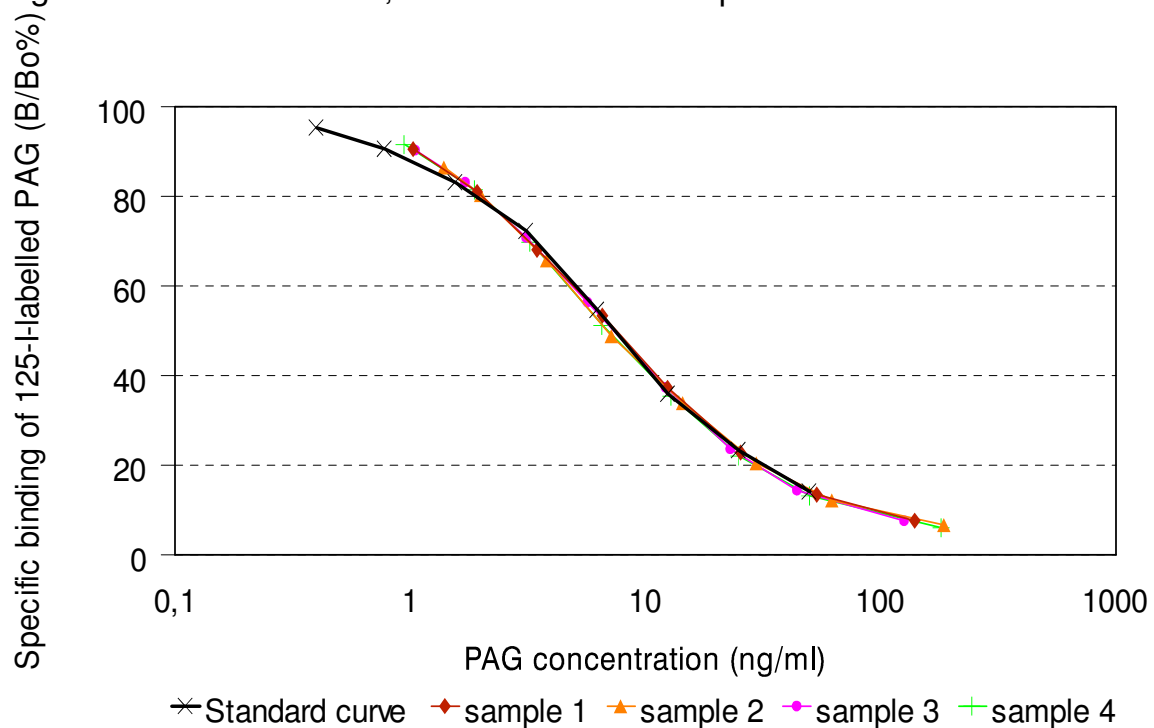

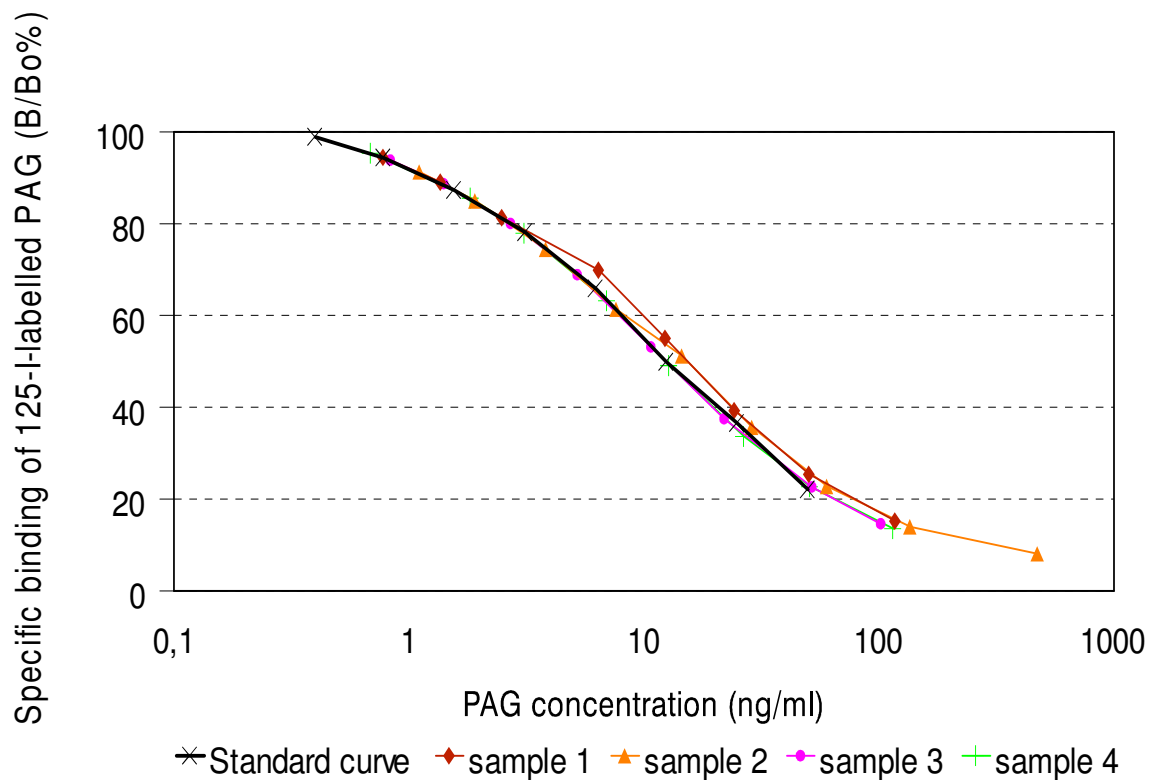Figure 3. Standard curve, serial dilution of samples in RIA 2

Figure 4. Standard curve, serial dilution of samples in RIA 3

Serum Samples

Pregnancy-associated glycoprotein concentrations were not detectable by RIAs 1, 2 and 3 in 73.33%, 80.00% and 50% of the serum samples respectively, originating from 15 nonpregnant heifers. In the rest of the samples 0.20±0.08, 0.38±0.22 and 0.34±0.29 ng/ml PAG concentration was determined by RIAs 1, 2 and 3, respectively.

Chapter 3: Investigation II: Determination of the specificity of three RIA systems for the measurement of pregnancy-associated glycoproteins

The ruminant placenta expresses glycoproteins related to pregnancy. These proteins are belonging to the aspartic proteinase family (Xie *et al.* 1991a,b; Xie *et al.* 1994; Xie *et al.* 1995) and some of them are released in the maternal circulation allowing the development of different pregnancy tests. On the basis of these tests pregnancy diagnosis can be established (Zoli *et al.* 1992b), embryonic mortality and abortion can be better investigated (Zarrouk *et al.* 1999a,b).

Aspartic proteinases are a group of proteolytic enzymes sharing the same catalytic apparatus, acting through a distinct catalytic mechanism. Enzymes belonging to this class were the first known and first described enzymes (chymosin, pepsin).

Pepsin is the major component of the gastric juice, produced in the fundus of the adult mammals. During activation its zymogen form, the pepsinogen releases activation segments of 44-47 amino acids from the NH_2-terminal region in acidic conditions, as a result of an enzymatic process (Harboe *et al.* 1974; Stepanov *et al.* 1973).

Chymosin (rennin) is a neonatal gastric proteinase with high milk clotting activity released from the abomasal mucosa. Calf chymosin consists of three components chymosin A, B and C with different milk-clotting activity and absorbance at 278 nm, with a single peptide chain of 323 amino acid residues (Foltmann *et al.* 1979). The inactive precursor irreversibly converted into the active chymosin through an enzymatic process with a loss of 42 amino acid N-terminal residue (Pedersen *et al.* 1975). Alignment of calf chymosin with porcine pepsinogen showed that 204 amino acids (77%) residues were common in the two molecules. The term rennet is used for the extract originating from the abomasum of the young milkfed calf (Ernstrom *et al.* 1965). The main component of this product is the chymosin, however the presence of pepsin A in the calf rennet was confirmed also (Rothe *et al.* 1976).

Cathepsin D is a lysosomal endopeptidase present in most of the cells of different species (Barrett *et al.* 1977; Blum *et al.* 1992). Cathepsin D exists in two forms: a single polypeptide chain form of 46 kDa, and a two chain form with a 34 kDa heavy chain and a 12 kDa light chain (Takahashi *et al.* 1981). Five molecular forms of Cathepsin D were detected in the bovine milk: 45 and 46 kDa procathepsin D, pseudocathepsin D, single chained and two chained Cathepsin D (Larsen *et al.* 1995).

Renin is the product of the kidney, submaxillary gland (Inagami *et al.* 1985), and various other organs. It plays an important role in the regulation of blood pressure through its ability to cleave angiotensinogen. In bovine atretic

ovarian follicles significantly higher prorenin levels were reported by Mukhopadhyay *et al.* (1991). It was also suggested that the renin-angiotensin system participates in growth regulation and tissue function in the bovine uterus (Schauser *et al.* 1999).

Glycoproteins belonging to the aspartic proteinase family were found to be expressed in the trophectoderm of the ungulate placenta (ruminants, horse, pig) (Xie *et al.* 1997; Green *et al.* 1998; Green *et al.* 2000) and released in the maternal blood circulation. Molecular biology investigations performed by Xie *et al.* (1997) showed that the ruminant genome contains several pregnancy-associated glycoprotein (PAG) genes, most of them being expressed according to a spatial and temporal pattern (Green *et al.* 2000; Garbayo *et al.* 1999). It also became clear, that PAGs are closely related to each other and to other aspartic proteinases having high amino acid sequence identity (Xie *et al.* 1991; Xie *et al.* 1994). Sequence comparison studies showed that PAGs were most closely related to pepsins (Guruprasad *et al.* 1996). Bovine PAG-1 showed 60% amino acid sequence identity to pepsinogens, 49.5% to porcine pepsin, 57% to chymosins, 42.5% to bovine chymosin, and 58% to cathepsin D (Xie *et al.* 1991; Guruprasad *et al.* 1996). Distance phylograms showed that the PAG gene family probably diverged from the enzymatically functional aspartic proteinases (Xie *et al.* 1997). Guruprasad *et al.* (1996) suggested that the bilobed three-dimensional structure of bovPAG-1 was similar to that of pepsin and chymosin, moreover bovPAG-1 was able to bind pepstatin to its binding site like the other enzymatically active aspartic proteinases. These observations concerning the relationship between PAGs and other aspartic proteinases raise an important question: whether the RIA systems developed for the detection of PAG molecules are specific for PAGs or these assays are responding also to other substances belonging to the aspartic proteinase family and being present in biological fluids of the ruminant species.

As circulating pepsinogen was suggested to increase in various pathological changes of the digestive tract (Vörös *et al.* 1984), cathepsin D was found in milk (Larsen *et al.* 1993), renin was also shown in blood, in this study we aimed to test precisely the specificity of three different RIA systems against these enzymatically active members of the aspartic proteinase family till concentrations higher than expected in biological fluids.

MATERIALS AND METHODS

Three radioimmunoassay systems (RIA 1, RIA 2 and RIA 3) differing in the antiserum were used in this study. In RIA 1 anti PAG I$_{67}$, in RIA 2 anti PAG$_{55+62}$, and in RIA 3 anti PAG$_{55+59}$ were used (the number in the subscript refers to the molecular weight in kDa of the antigen used for the immunization) (Beckers *et al.* 1999). These antisera were produced in rabbits previously immunized with the appropriate antigen according to the method of Vaitukaitis *et al.* (1971). Pure PAG I 67 preparation purified according to the method of Zoli *et al.* (1991) was used as standard and for the tracer in all the three systems. The radiolabelling was carried out using iodine-125 according to the method of Greenwood *et al.* (1963).

The radioimmunoassays were performed according to the method of Zoli *et al.* (1992) with modifications in the buffer composition, and in the separation method. Tris buffer (0.025 M Tris, 0.007mM Pepstatin A, 0.5% Tween 20) with the pH of 7.5 was used for all the three systems. The standard curve was ranging from 0.1 ng/ml to 25 ng/ml. For the separation of bound and free fractions 1 ml of second antibody polyethylene glycol (PEG) solution (0.17 % (vol/vol) normal rabbit serum, 0.83 % (vol/vol) sheep anti-rabbit IgG, 0.3 % (wt/vol) BSA, 4 % (wt/vol) polyethylene glycol 6000 in Tris buffer) was used with an incubation time of 1 hour. Afterwards the tubes were washed with Tris buffer and centrifuged (1500 x g for 20 min). After the repetition of the previous washing step, the ^{125}I in the pellet was determined using a gamma counter (LKB Wallac 1261 Multigamma counter, Turku, Finland) with a counting efficiency of 75 %.

To test the specificity of the three RIA systems the following reagents from commercial origin were used: pepsinogen (from porcine stomach, Sigma, P-4656), pepsin (from porcine stomach mucosa, Sigma, P-6887), rennin (from calf stomach, Sigma, R-4879), Rennet (type I from calf stomach, Sigma, R-3376), renin (from porcine kidney, Sigma, R-2761), cathepsin D (from bovine kidney, Calbiochem, 219398). Bovine serum albumin (Fraction V, ICN Biomedicals, 160069) was used in this experiment as control to examine its effect on the binding of the tracer to the antisera. These products were dissolved in distilled water, then diluted in Tris buffer to obtain the following concentrations: 1 mg/ml, 0.5 mg/ml, 0.1 mg/ml, 50 µg/ml, 10 µg/ml, 5 µg/ml, 1 µg/ml, 0.5 µg/ml, 0.1 µg/ml, 50 ng/ml, 10 ng/ml. In the case of cathepsin D and renin the dilutions were ranging from 10 ng /ml to 0.1 mg/ml. Each step of the

dilution series of these products was assayed three times in the three RIA systems in quadruplicate as unknown samples. The amount of radiolabelled PAG bound to antisera in tubes containing no PAG (zero standard, B_0) was designated as 100% ^{125}I bound. The binding of I^{125}-labelled PAG to the antiserum was compared to the tracer binding to the zero standard (B/B_0).

The minimal detection limit (MDL) calculated for PAG I_{67} in RIA 1, RIA 2 and RIA 3 systems was 54 ± 18 pg/ml, 60 ± 18 pg/ml and 112 ± 57 pg/ml respectively. Compared to B_0 (MDL/B_0) these values were corresponding to 96.23%, 96.80% and 96.01% for RIA 1, RIA 2 and RIA 3, respectively. MDL was determined as the mean concentration minus twice the standard deviation of 20 replicates of the zero standard.

The statistic analysis was carried out using the Wilcoxon-Mann-Whitney test. The null hypothesis during the test was that the observed B/B_0 values for each dilution of the examined products were not significantly different from the determined MDL/B_0-values.

RESULTS

The specific binding of radiolabelled PAG to the anti PAG I_{67} (RIA 1), anti PAG$_{55+62}$ (RIA 2) and to the anti PAG$_{55+59}$ (RIA 3) in the presence of pepsinogen, pepsin, chymosin (rennin), rennet, renin and Cathepsin D was determined at each dilution of the examined products (Tables 2, 3 and 4). A certain dilution of a product was considered as crossreacting if the determined B/Bo-values were significantly lower than the MDL/B_0-values, or there was no significant difference between them. The concentration of products causing detectable crossreaction in RIA 1, RIA 2 and RIA 3 are given in Table 1.

In the examined concentration range (5 µg/l to 1 mg/l) albumin did not crossreact in the three RIA systems. Pepsinogen was reacting in all the three RIA systems over the concentration of 1 mg/ml, 50 µg/ml and 500 µg/ml for RIA 1, RIA 2 and RIA 3, respectively. Reaction was observed in the presence of pepsin in RIA 1, RIA 2 and RIA 3 over 1 mg/ml, 500 µg/ml and 1 mg/ml concentration respectively. Chymosin caused crossreaction in RIA 2 and RIA 3 systems at 1 mg/ml concentration. In the case of rennet, inhibition of binding was observed in RIA 2 at 1 mg/ml concentration. B/B_0-values determined for renin and cathepsin D were always significantly higher than MDL/B_0-values throughout the examined concentration range.

Table 1. Concentration of products causing detectable reaction in RIA 1, RIA 2 and RIA 3.

Product	Concentration of products causing detectable reaction		
	RIA 1	RIA 2	RIA 3
PAG	54 pg/ml	60 pg/ml	112 pg/ml
Albumin	-*	-*	-*
Pepsinogen	1mg/ml	50 μg/ml	500 μg/ml
Pepsin	1mg/ml	500 μg/ml	1 mg/ml
Rennin	-*	1mg/ml	1mg/ml
Rennet	-*	1mg/ml	-*
Cathepsin D	-*	-*	-*
Renin	-*	-*	-*

-* No crossreaction observed in the examined concentration range

Table 2. Specific binding of radiolabelled PAG to the antibody in RIA 1 in the presence of albumin and aspartic proteinases in different concentrations

Product	B/B_0 (%) determined in the following concentrations (ng/ml) of the examined products										
	10^1	5×10^1	10^2	5×10^2	10^3	5×10^3	10^4	5×10^4	10^5	5×10^5	10^6
Albumin	-	-	-	-	-	99.95 ±0.97***	100.93 ±1.53***	100.23 ±1.94**	99.8 ±1.57**	100.50 ±1.52***	99.58 ±1.23***
Pepsinogen	100.89 ±1.41***	100.87 ±1.86***	100.11 ±2.58***	100.26 ±2.86***	99.86 ±1.38***	100.81 ±1.65***	100.91 ±1.30***	99.71 ±1.41***	99.51 ±1.23***	99.16 ±1.29***	97.43 ±1.54
Pepsin	100.70 ±2.25**	99.68 ±0.66**	101.07 ±1.34**	99.79 ±1.22**	100.90 ±0.85**	99.67 ±0.51***	100.70 ±1.38***	99.72 ±1.68**	99.43 ±1.34***	98.62 ±1.08***	94.89 ±0.81*
Chymosin	102.89 ±1.68**	102.44 ±1.35**	101.01 ±2.84**	99.66 ±0.84**	99.95 ±0.62**	100.00 ±1.46**	98.74 ±0.70**	99.43 ±1.37**	100.83 ±1.30**	100.51 ±1.27**	100.29 ±0.75**
Rennet	99.06 ±1.91**	100.10 ±1.55**	100.47 ±0.77**	99.81 ±2.21*	100.04 ±2.46*	100.60 ±1.56***	99.32 ±1.66***	99.26 ±1.87***	99.16 ±2.24**	99.11 ±0.97***	97.96 ±1.45**
Cathepsin D	101.47 ±1.00**	100.82 ±2.49**	100.57 ±2.05**	100.80 ±0.99**	101.95 ±1.73**	101.16 ±0.66**	99.92 ±1.54**	98.72 ±0.88*	98.06 ±1.54*	-	-
Renin	100.76 ±1.94**	100.58 ±2.98*	102.36 ±0.99**	102.62 ±1.37**	101.62 ±0.58**	101.70 ±1.30**	101.47 ±0.75**	100.95 ±1.09**	100.21 ±2.50*	-	-

*p<0.05 versus minimal detection limit/B_0

**p<0.01 versus minimal detection limit/B_0

***p<0.001 versus minimal detection limit/B_0

Table 3. Specific binding of radiolabelled PAG to the antibody in RIA 2 in the presence of albumin and aspartic proteinases in different concentrations

Product	B/B_0 (%) determined in the following concentrations (ng/ml) of the examined products										
	10^1	5×10^1	10^2	5×10^2	10^3	5×10^3	10^4	5×10^4	10^5	5×10^5	10^6
Albumin	-	-	-	-	-	100.64 ± 1.40***	100.68 ± 1.37***	100.07 ± 2.00**	98.69 ± 0.46**	99.89 ± 2.02**	99.97 ± 1.96**
Pepsinogen	101.41 ± 1.05**	100.89 ± 1.18**	100.55 ± 1.89**	100.78 ± 1.47**	99.61 ± 1.48**	100.75 ± 1.80**	100.15 ± 2.38**	95.23 ± 2.43	94.92 ± 0.71**	94.25 ± 2.80*	93.00 ± 2.01**
Pepsin	99.92 ± 1.51**	99.27 ± 1.04**	100.68 ± 0.58**	100.89 ± 0.50**	102.00 ± 1.30**	100.96 ± 1.51***	100.50 ± 1.82***	101.01 ± 1.65***	99.87 ± 1.89***	95.81 ± 2.23	92.30 ± 2.04
Chymosin	102.32 ± 1.57**	102.18 ± 2.22**	99.80 ± 2.32*	100.56 ± 0.62**	99.56 ± 1.35**	99.41 ± 1.76*	98.96 ± 1.03**	98.76 ± 1.07*	99.76 ± 0.41**	99.53 ± 1.26**	97.75 ± 0.22
Rennet	100.85 ± 1.59**	100.72 ± 2.22**	100.41 ± 3.62*	99.32 ± 0.57**	99.71 ± 1.98*	101.25 ± 2.24***	100.26 ± 2.32***	100.98 ± 2.24***	99.29 ± 2.43**	98.76 ± 1.68*	98.26 ± 1.70
Cathepsin D	102.52 ± 1.19**	101.37 ± 1.04**	101.66 ± 0.92**	101.68 ± 2.49**	102.80 ± 1.27**	103.52 ± 1.98**	101.83 ± 1.81**	101.14 ± 1.39**	97.58 ± 1.99**	-	-
Renin	102.17 ± 1.32**	101.54 ± 2.31**	99.56 ± 1.01**	100.42 ± 0.57**	100.09 ± 0.79**	101.75 ± 1.53**	100.62 ± 1.33**	100.99 ± 1.63**	101.56 ± 0.45**	-	-

*$p < 0.05$ versus minimal detection limit/B_0

**$p < 0.01$ versus minimal detection limit/B_0

***$p < 0.001$ versus minimal detection limit/B_0

Table 4. Specific binding of radiolabelled PAG to the antibody in RIA 3 in the presence of albumin and aspartic proteinases in different concentrations

Product	B/B_0 (%) determined in the following concentrations (ng/ml) of the examined products										
	10^1	5×10^1	10^2	5×10^2	10^3	5×10^3	10^4	5×10^4	10^5	5×10^5	10^6
Albumin	-	-	-	-	-	100.63	101.14	99.34	101.25	99.55	100.02
						±0.81**	±1.53***	±2.30**	±2.37**	±1.26***	±1.36**
Pepsinogen	100.53	100.49	99.19	100.70	99.41	100.88	98.44	99.14	99.09	94.89	94.38
	±0.89**	±0.45**	±1.44**	±2.10**	±2.51**	±1.44***	±1.77**	±1.73***	±1.27***	±2.64	±2.56
Pepsin	99.51	101.15	98.89	99.96	100.04	100.60	100.80	100.83	98.17	97.81	94.67
	±0.99**	±2.37**	±0.28**	±1.64**	±0.79**	±1.13***	±1.22***	±1.68***	±0.96**	±1.33*	±1.37
Chymosin	101.63	102.24	101.56	101.14	100.17	100.80	99.92	99.92	99.84	100.11	97.78
	±1.35**	±1.57**	±1.41**	±2.13**	±1.10**	±1.93**	±0.97**	±0.38**	±0.62**	±0.59**	±1.35
Rennet	99.50	99.13	99.60	99.56	99.61	99.33	99.37	99.84	98.16	98.50	98.59
	±1.39**	±0.89**	±1.02**	±0.36**	±2.57**	±1.00***	±1.85***	±1.62***	±2.29*	±1.89**	±1.93**
Cathepsin D	100.74	101.99	102.93	101.64	102.40	102.23	101.02	102.25	99.60	-	-
	±0.68**	±1.45**	±0.69**	±0.65**	±1.07**	±0.51**	±1.55**	±0.95**	±0.65**		
Renin	101.45	100.74	100.72	100.16	100.82	101.65	99.98	100.49	99.52	-	-
	±0.66**	±1.42**	±2.23**	±0.72**	±2.49**	±1.01**	±2.23**	±1.38**	±0.88**		

*$p < 0.05$ versus minimal detection limit/B_0

**$p < 0.01$ versus minimal detection limit/B_0

***$p < 0.001$ versus minimal detection limit/B

Chapter 4: **Investigation III: Comparison of the ability of three Radioimmunoassay to detect pregnancy-associated glycoproteins in bovine plasma**

Part of this study was published in Reproduction in Domestic Animals
(Perényi *et al.*, 2002a)

This experiment was carried out to compare the ability of the three previously described RIA systems (RIA 1, RIA 2 and RIA 3) to detect PAG molecules in the maternal blood. The aim of this study was to determine the plasmatic PAG concentrations in pregnant cows during the first trimester of their gestation. The mean concentrations of PAG determined in the different RIAs were compared and the correlations between the results obtained by the three systems were analyzed.

MATERIALS AND METHODS

Experiment I

Blood samples were collected from the jugular vein of 103 Holstein Friesian cows into heparinized vacutainer tubes at 30-120 days after fertilization for pregnancy diagnosis (Laboratory Genes Diffusion, Douai, France). Samples were centrifuged (15 min at 1500 g) and the plasma was stored at -20 °C until assay.

Experiment II.

Holstein Friesian cows (n=103) were used in the experiment. Blood samples were collected from the jugular vein into heparinized vacutainer tubes at 21, 32 and 42 days after AI. The plasma obtained by centrifugation (15 min at 1500 g) was stored at -20°C until the PAG radioimmunoassay measurements. Transrectal ultrasonography as described previously (Szenci *et al.* 1998a) was performed on days 32 and 42 after AI, using a real-time, B-mode portable diagnostic ultrasound scanner (ANIVET 100, Budapest, Hungary) equipped with a 5 MHz linear-array rectal transducer. The recognition of the embryo proper with a beating heart was used as the criterion for a positive pregnancy diagnosis. Only the cows, which were classified as pregnant (n=49) at both ultrasonographic examinations (32 and 42 days after AI), were selected for this study. The PAG concentrations measured in the pregnant animals were used in the statistical analysis.

Data Analysis

The regression and the variance analysis were carried out by using the PSI – Plot v.4.0 and the GLM procedure of SAS, respectively.

RESULTS

The mean PAG concentrations determined by RIA 1, RIA 2 and RIA 3 in the samples from experiment II are presented in Tables 1 and 2. At Day 21 in RIA 1, RIA 2 and RIA 3 systems respectively, 19 (38.77%), 37 (75.51%) and 36 (73.47%) pregnant animals expressed detectable PAG concentrations. At Days 32 and 42 in the three systems all pregnant animals showed detectable PAG. At Day 21, the mean PAG concentrations measured by RIA 1 and RIA 2 systems were similar (0.43±0.24 ng/ml and 0.48±0.24 ng/ml, respectively), while the RIA 3 system provided significantly ($p<0.02$) higher values (0.64±0.37 ng/ml). At Days 32 and 42 similar PAG concentrations were found in RIA 2 (4.30±1.32 ng/ml and 5.56±1.95 ng/ml, respectively) and in RIA 3 (4.17±1.15 ng/ml and 5.60±1.89 ng/ml, respectively) while the RIA 1 system showed significantly ($p<0.0001$) lower PAG concentrations (2.43±0.81 ng/ml, 4.01±1.48 ng/ml, respectively).

Table 1. Concentrations (mean±SD) of PAG (ng/ml) obtained by RIA 1, RIA 2 and RIA 3 in females presenting detectable values 21 days after AI

	n^a=19	n^a=37	n^a=36
	RIA 1	RIA 2	RIA 3
PAG [ng/ml]	0.43±0.24[b]	0.48±0.24[b]	0.64±0.37[c]

[a] Females presenting detectable PAG concentrations
[b,c] Values with different superscripts differ between columns ($p<0.02$)

Table 2. Concentrations (mean±SD) of PAG (ng/ml) obtained by RIA 1, RIA 2 and RIA 3 32 and 42 days after AI

	Day 32			Day 42		
	n^a=49	n^a=49	n^a=49	n^a=49	n^a=49	n^a=49
	RIA 1	RIA 2	RIA 3	RIA 1	RIA 2	RIA 3
PAG [ng/ml]	2.43±0.81^b	4.30±1.32^c	4.17±1.15^c	4.01±1.48^d	5.56±1.95^e	5.60±1.89^e

^{a} Females presenting detectable PAG concentrations

^{b,c,d,e} Values with different superscripts differ between columns ($p<0.0001$)

The correlation coefficients (r) determined between the three RIA systems in the samples from experiment I and II are presented in Table 3. At Day 21, significant correlation was found between RIA 1 and RIA 2 ($p<0.0001$, r=0.870) and between RIA 1 and RIA 3 ($p<0.05$, r=0.598), while the correlation coefficient between RIA 2 and RIA 3 was low (r=0.276) and the correlation was not significant. Significant ($p<0.0001$) and similar correlations were determined in the first experiment for the whole 30-120 day period and for the second experiment 32 and 42 days after AI. The results of the regression analysis of the PAG concentrations determined by the three RIA systems for the 30-120 day period are shown in three regression systems (Figures 1-3.)

Table 3. Correlation coefficients between RIA 1, RIA 2 and RIA 3 at 21, 32, 42 and 30-120 days after AI

Days after AI	Correlation coefficient between		
	RIA 1 and RIA 2	RIA 1 and RIA 3	RIA 2 and RIA 3
Day 21	0.870**	0.598*	0.276
Day 32	0.939**	0.936**	0.973**
Day 42	0.964**	0.937**	0.960**
Days 30-120	0.940**	0.929**	0.974**

* $p<0.05$

** $p<0.0001$

y=1.639x+0.352
r=0.940
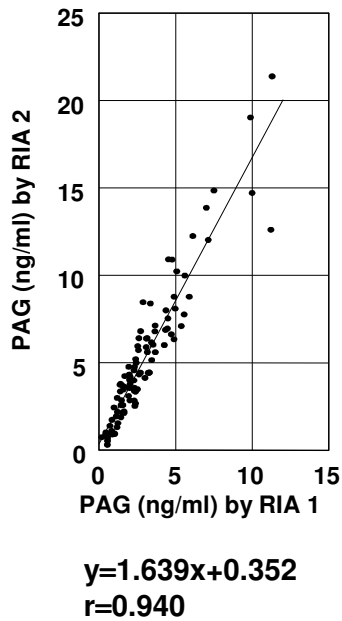
Figure 1. PAG concentrations
(ng/ml) obtained by RIA
2 versus RIA 1 systems
for 30-120 day period of
pregnancy

y=1.486x+0.017
r=0.929
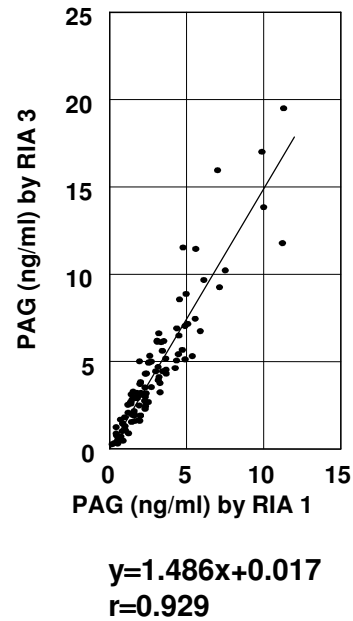
Figure 2. PAG concentrations
(ng/ml) obtained by RIA
3 versus RIA 1 systems
for 30-120 day period of
pregnancy

y=0.905x-0.232
r=0.974
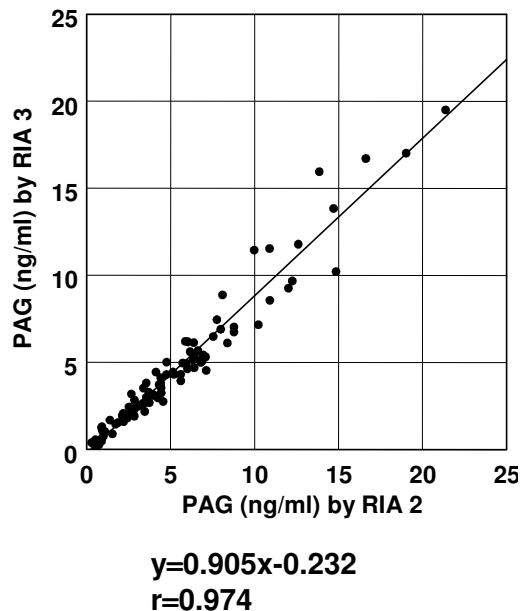
Figure 3. PAG concentrations (ng/ml) obtained by
RIA 3 versus RIA 2 systems for 30-120
day period of pregnancy

Chapter 4: Investigation III: Comparison of the ability of three Radioimmunoassay to detect
pregnancy-associated glycoproteins in bovine plasma

Chapter 5: Investigation IV: Determination of PAG profiles in heifers **and cows**

Part of this study was published in Reproduction in Domestic Animals
(Perényi *et al.*, 2002a)

In order to provide better reflection of the different PAG molecules probably released in the maternal blood during pregnancy, it was aimed to determine PAG concentrations in samples removed frequently from pregnant heifers/ cows.

MATERIALS AND METHODS

Blood samples were withdrawn from 7 Holstein Friesian heifers (209, 210, 579, 1654, 2792, 6178, 8368), from a Blonde d'Aquitaine heifer (9447) and cow (7177) frequently during their gestation (protocol number: 70) (Table 1). The plasma was removed after centrifugation (15 min at 1500 g) and stored at -20°C until assay. These animals were observed twice a day for the signs of estrus.

Table 1. Type of the females involved in this experiment and type of the males whose semen were used for AI

Animal n°	Breed of the female	Breed of the male
209	Holstein Friesian	Maine-Anjou
210	Holstein Friesian	Holstein Friesian
579	Holstein Friesian	Jersey
1654	Holstein Friesian	Maine-Anjou
2792	Holstein Friesian	Jersey
6178	Holstein Friesian	Holstein Friesian
7177	Blonde d'Aquitaine	Blonde d'Aquitaine
8368	Holstein Friesian	Holstein Friesian
9447	Blonde d'Aquitaine	Blonde d'Aquitaine

Six of the experimental animals delivered one healthy newborn calf, whereas cow N° 7177 delivered two calves after a normal length of pregnancy. Heifer n° 2792 showed late embryonic mortality. Heifer n° 8368 showed signs of abortion. The PAG profiles of the animals with pregnancy failure are presented separately from the profiles of animals with normal pregnancy.

Serological test (Immuno fluorescent antibody test) for the diagnosis of Neospora infection was performed on the samples removed from heifer n° 8368 on Days 9, 37, 66, 100, 131, 142.

As part of an other study, on heifer n° 1654 a laparotomy was performed in order to inject anti bPL into the muscular tissue of the fetus on Day 155 (protocol number: 125).

Some of the PAG profiles are presented partially because of the insufficient number of samples. The PAG concentrations obtained by the three RIA systems were used to calculate RIA 2/RIA 1 and RIA 3/RIA 1 ratios.

RESULTS

Animals with normal pregnancy

The PAG profiles of the experimental animals are analyzed in three different gestational stages:

I.   Days 0-60 of gestation
II.   Days 61-250 of gestation
III.   Days 251 of gestation till the end of the sampling

I. Days 0-60 of gestation

The complete individual PAG profiles of 5 pregnant animals during the first 60 days after AI measured by RIA 1, 2 and 3 are presented in Figures 1-5. The characteristics of 7 profiles are presented in Table 2. PAG molecules first appeared in the maternal blood in detectable concentrations at 19-28 days after AI. PAG concentrations measured by the three RIA systems were increased intensively till Days 33-37. After Days 35-40 the PAG profile of RIA 1 showed a plateau with concentration of PAG remaining constant, while in RIA 2 and RIA 3 profiles a decrease could be observed. In the cow n° 7177 and in heifer n° 9447 after Day 34 and Day 44, a decrease was detected in all the three RIA systems.

The heifer n° 9447 showed an unusual PAG profile during the first 60 day period of pregnancy. After an intensive increase in the PAG concentrations (Days 23-34), a plateau could be observed in the profiles (Days 34-44), then PAG concentrations started to fall (Days 44-60) reaching at about 1.2 ng/ml. This PAG concentration is close to the threshold value which is used in our laboratory during the routine PAG tests to distinguish between the pregnant and nonpregnant animals.

Except some points at the detection limit of the tests, the PAG concentrations measured by RIA 2 and 3 were higher than those of RIA 1. The mean RIA 2/RIA 1 and RIA 3/RIA 1 ratios determined in the first 60 days of pregnancy are shown in Table 3. From Days 21-25 till Days 51-55 these ratios were higher than 1.5. After Days 31-35 a decrease can be observed in these ratios in the examined period.

Table 2. Characteristics of the PAG profiles of 7 animals during the first 60 days after AI

Animal n°	Detectable PAG concentrations (Days after AI)			Intensive increase	plateau in RIA 2 & 3	decrease
	RIA 1	RIA 2	RIA 3		Days after AI	
209	19	22	22	19-33	33-41	41-50*
210	28	24	28	ND**	ND**	ND**
579	21	21	21	21-35	35-42	42-50*
1654	22	22	22	22-37	***	37-45*
6178	22	22	21	ND**	ND**	ND**
7177	22	22	22	22-34	***	34-48
9447	23	23	22	23-34	34-44	44-60

*Decrease were observed in the PAG concentrations obtained by RIA 2 and RIA 3

**Not determined

***There was no plateau found in the RIA 2 and 3 profiles
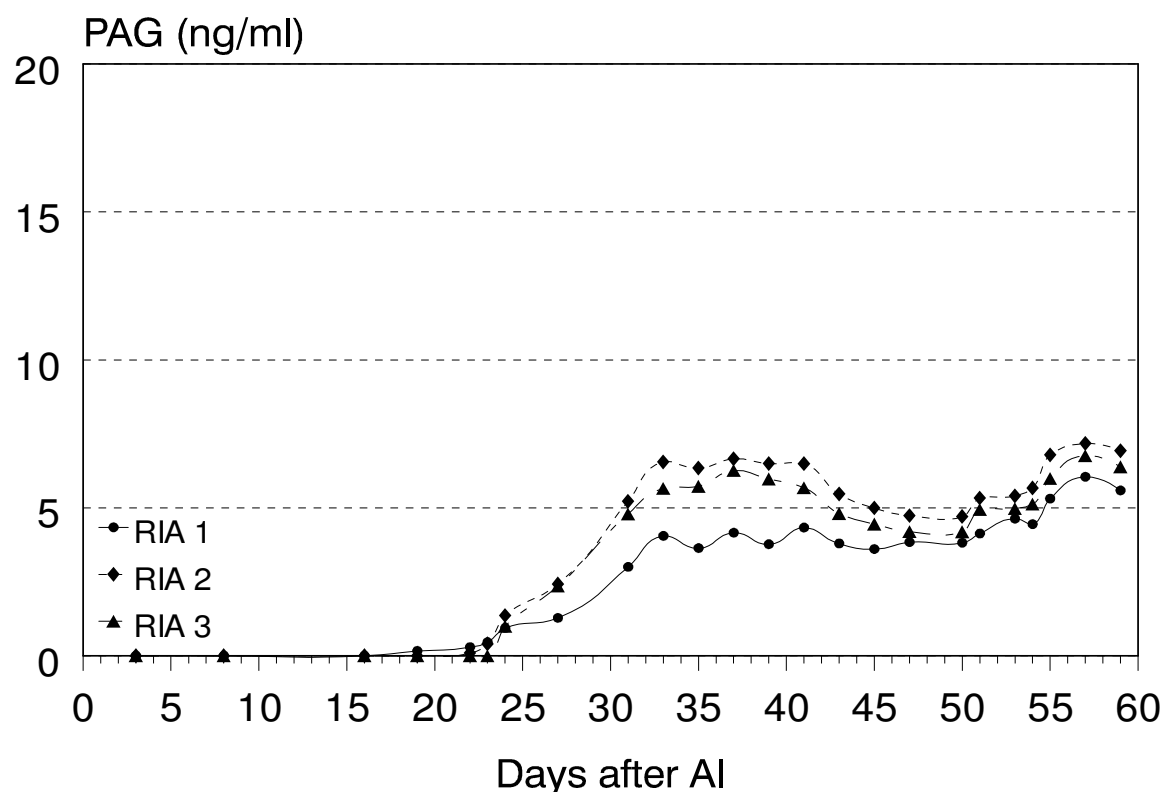
Figure 1. PAG profile of heifer n° 209 during the first 60 days after AI using RIA 1, 2 and 3
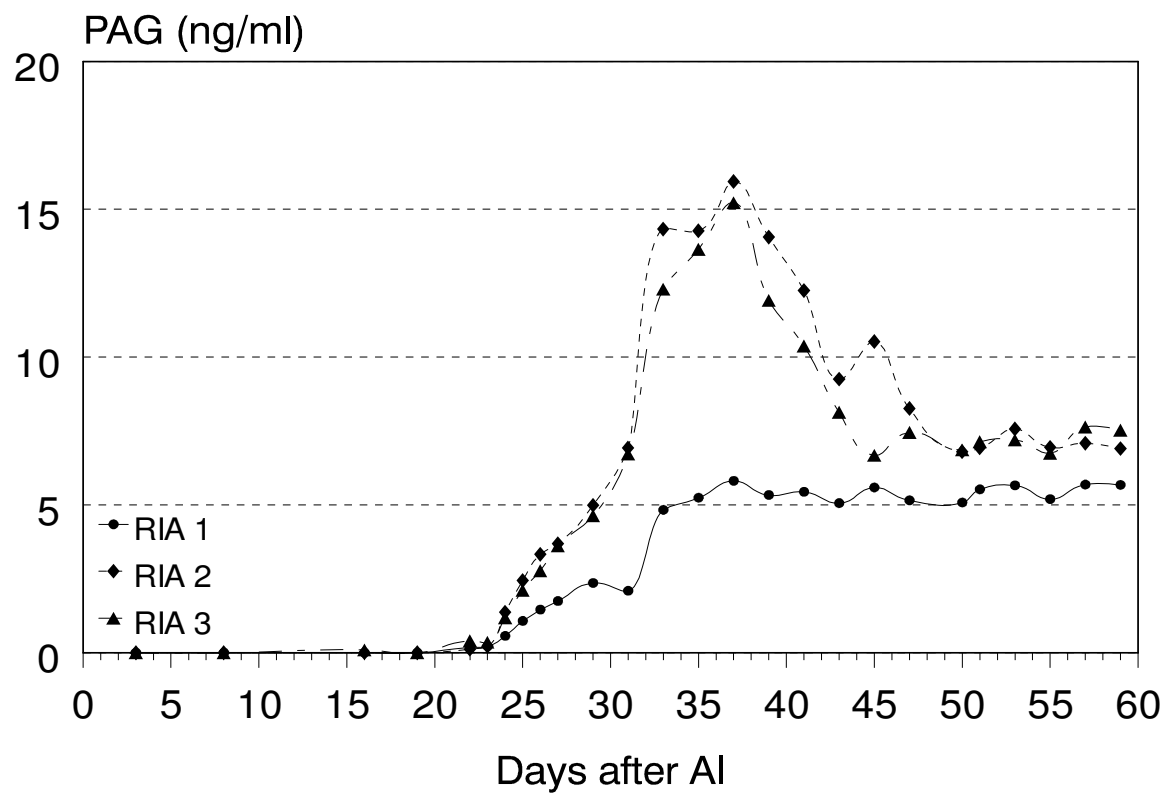
Figure 2. PAG profile of heifer nº 1654 during the first 60 days after AI using RIA 1, 2 and 3
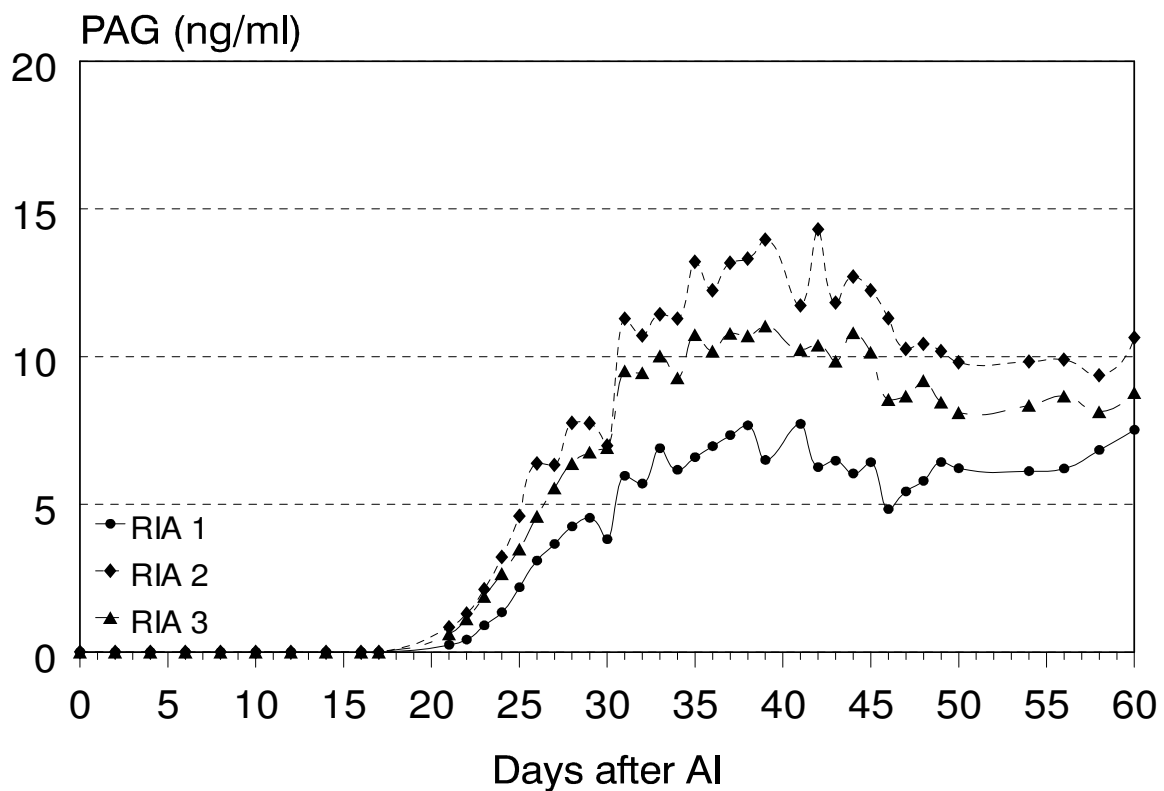
Figure 3. PAG profile of heifer nº 579 during the first 60 days after AI using RIA 1, 2 and 3
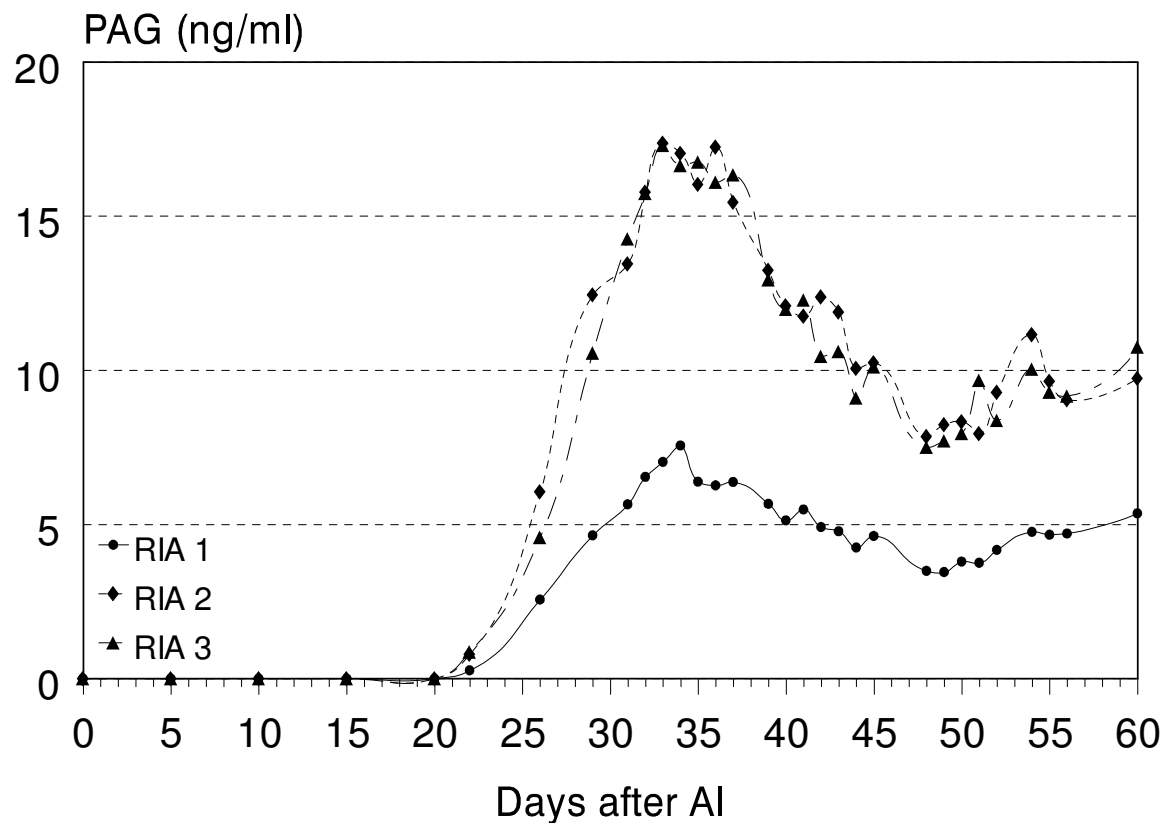
Figure 4. PAG profile of cow nº 7177 during the first 60 days after AI using RIA
1, 2 and 3
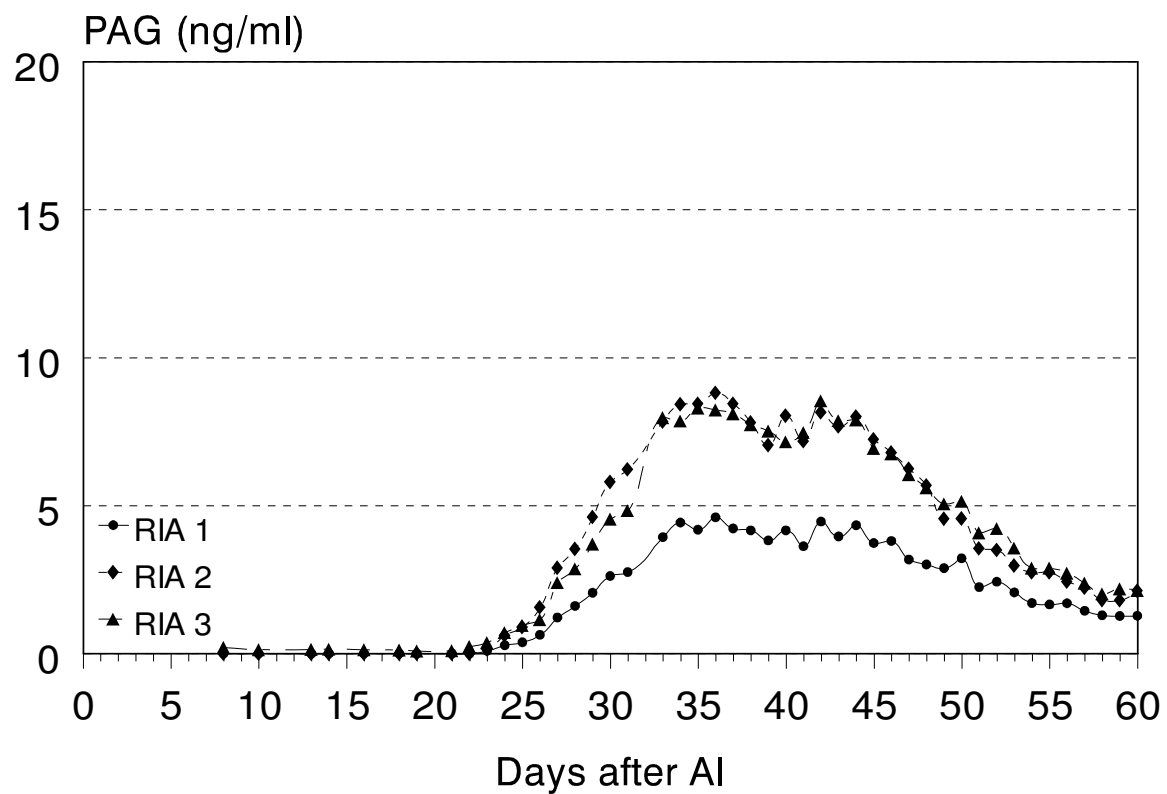
Figure 5. PAG profile of heifer nº 9447 during the first 60 days after AI using
RIA 1, 2 and 3

Table 3. RIA 2/ RIA 1 and RIA 3/ RIA 1 ratios (mean±SD) determined for the first 60 days of pregnancy in the seven experimental animals

Days after AI[*]	RIA 2/ RIA 1 Ratio	RIA 3/ RIA 1 Ratio
21-25	2.13±0.90	2.34±0.64
26-30	2.27±0.39	1.96±0.40
31-35	2.18±0.46	2.04±0.52
36-40	2.10±0.37	1.95±0.41
41-45	1.97±0.32	1.75±0.34
46-50	1.81±0.34	1.62±0.32
51-55	1.68±0.36	1.58±0.38
56-60	1.53±0.25	1.48±0.25

[*] RIA 2/ RIA 1 and RIA 3/ RIA 1 ratios were determined for 5 day periods where detectable PAG concentrations were measured

II. Days 61-250 of gestation

The individual PAG profiles of 3 pregnant animals during 61-250 days of pregnancy measured by RIA 1, 2 and 3 are presented in Figure 6-8. It can be observed that during the first part of this period the increase in the PAG concentrations was relatively slow, the PAG levels remained under 50 ng/ml till Days 154-169. After Days 150-170 a more intensive increase could be found in the PAG concentrations. At the end of this observation period heifer n° 579 and 1654 expressed 151.79-176.16 ng/ml and 161.85-180.26 ng/ml PAG concentrations, respectively, whereas in heifer n° 209 306.21-331.15 ng/ml PAG concentrations were determined. The mean RIA 2/RIA 1 and RIA 3/RIA 1 ratios determined for the 61-250- day period of pregnancy are shown in Table 4. At the beginning of this observation period until Days 91-100 a decrease can be observed in the RIA 2/ RIA 1 ratios, after RIA 2/ RIA 1 and RIA 3/ RIA 1 ratios showed variation between 1.05 and 1.17.
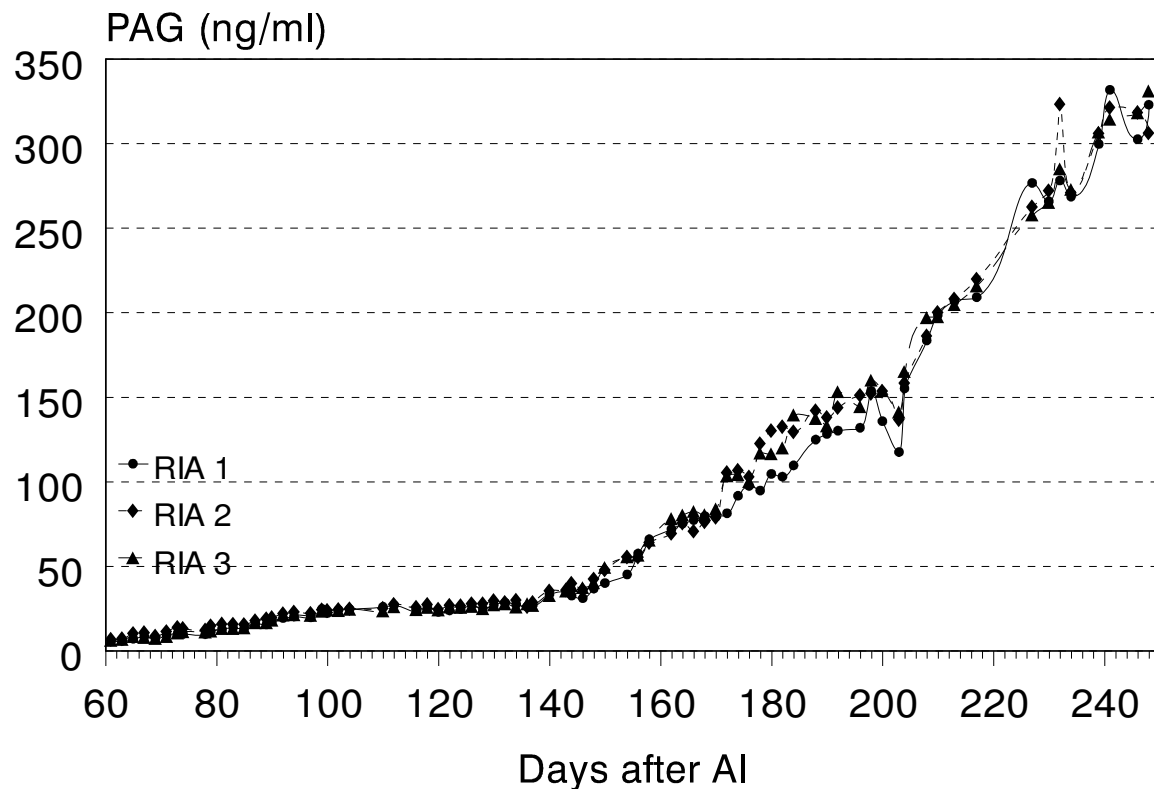
Figure 6. PAG profile of heifer nº 209 during 60-250 day period of pregnancy using RIA 1, 2 and 3
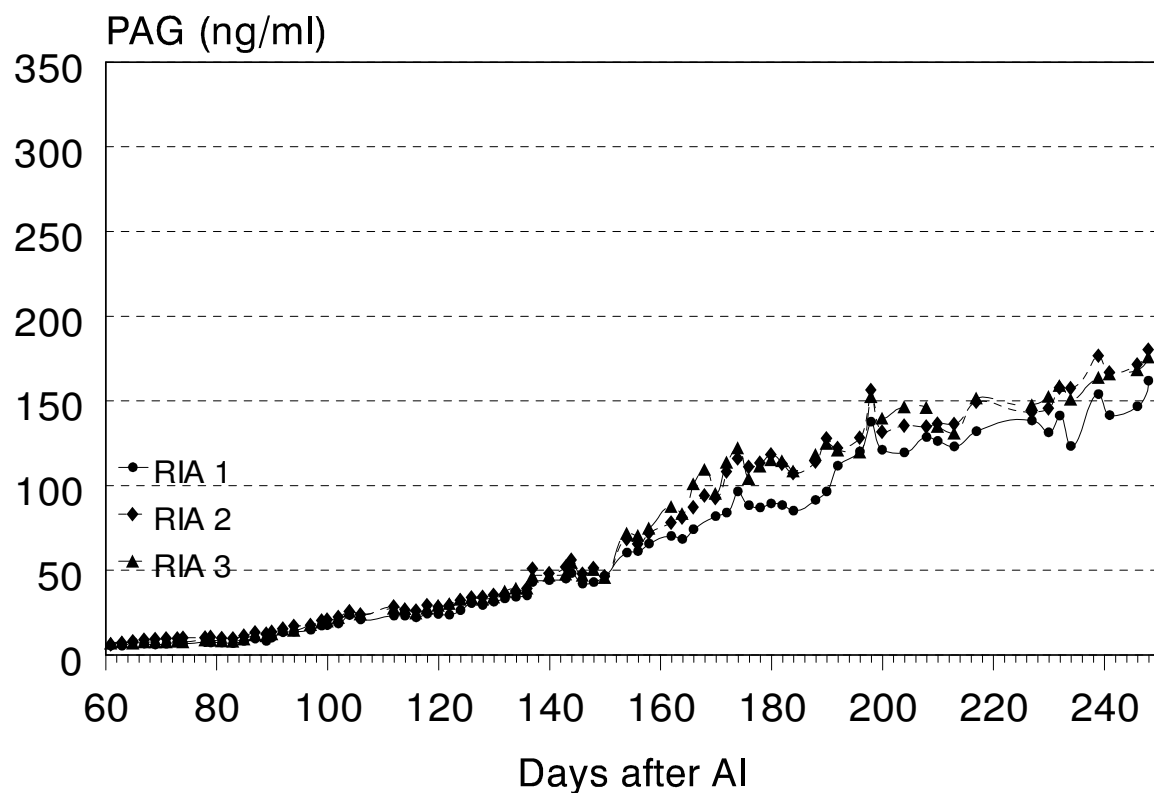
Figure 7. PAG profile of heifer nº 1654 during 60-250 day period of pregnancy using RIA 1, 2 and 3
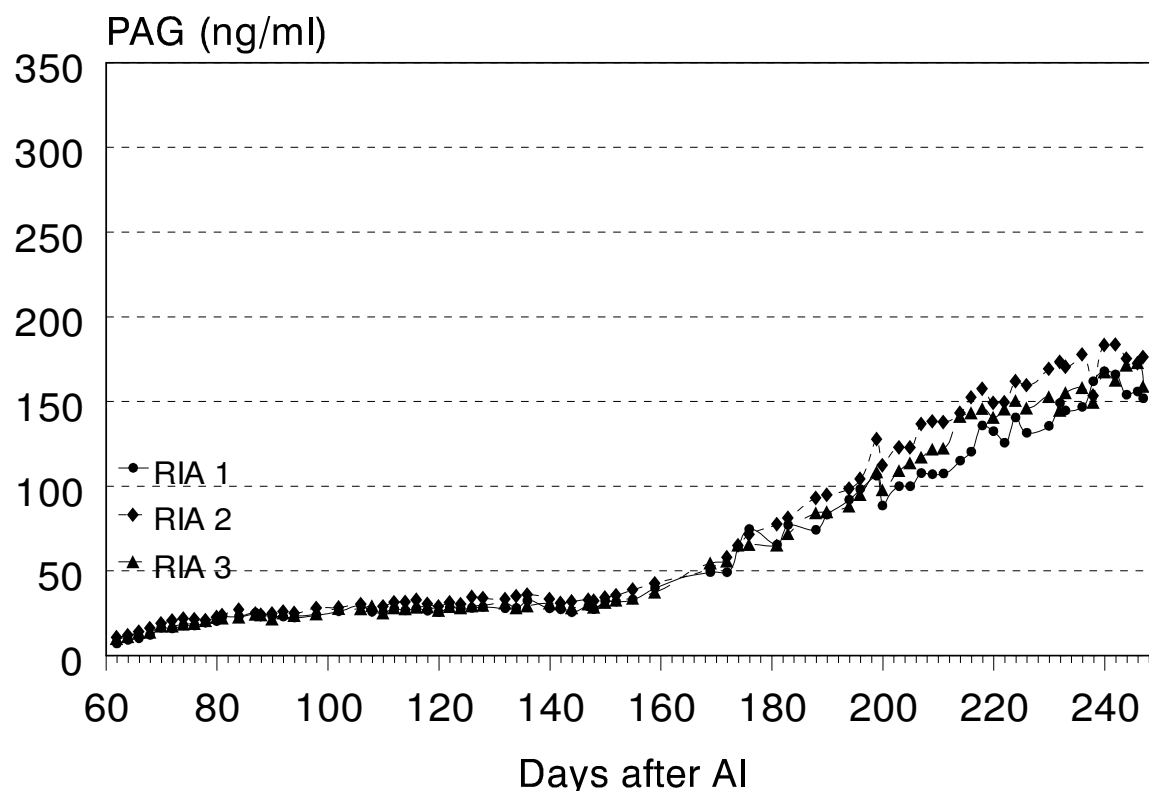
Figure 8. PAG profile of heifer nº 579 during 60-250 day period of pregnancy using RIA 1, 2 and 3

Table 4. RIA 2/ RIA 1 and RIA 3/ RIA 1 ratios (mean±SD) determined for 61-250 days of pregnancy in the seven experimental animals

Days after AI[*]	RIA 2/ RIA 1 Ratio	RIA 3/ RIA 1 Ratio
61-70	1.31±0.15	1.19±0.17
71-80	1.35±0.09	1.11±0.09
81-90	1.29±0.13	1.13±0.12
91-100	1.14±0.06	1.09±0.06
101-110	1.08±0.07	1.05±0.10
111-120	1.15±0.06	1.08±0.08
121-130	1.14±0.06	1.08±0.09
131-140	1.11±0.07	1.05±0.08
141-150	1.15±0.07	1.10±0.08
151-160	1.09±0.09	1.08±0.10
161-170	1.05±0.10	1.14±0.11
171-180	1.20±0.12	1.17±0.14
181-190	1.20±0.09	1.15±0.13
191-200	1.11±0.07	1.07±0.07
201-210	1.14±0.11	1.11±0.07
211-220	1.15±0.10	1.10±0.08
221-230	1.12±0.10	1.08±0.08
231-240	1.12±0.10	1.05±0.08
241-250	1.10±0.08	1.07±0.07

[*] RIA 2/ RIA 1 and RIA 3/ RIA 1 ratios were determined for 10 day periods where detectable PAG concentrations were measured

III. Days 251 of gestation till the end of the sampling

The PAG profiles of three heifers is presented in Figures 9-11 during the late pregnancy period. The characteristics of the PAG profiles concerning to this period are summarized in Table 5. Heifers n° 209 and 579 showed an intensive increase in the PAG concentrations 9-10 days before calving, PAG levels reached 1.8-2.8 µg/ml. Animal n° 1654 presented an increase in the PAG concentrations 4 days after the anti bPL injection and 4 days before the calving. The highest PAG concentrations determined in this animal during the prepartum PAG peak were 2.9-3.1 µg/ml. After this peak PAG concentrations started to fall. The RIA 2/ RIA 1 and RIA 3/ RIA 1 ratios (Table 6) were under 1.13.

Table 5. Characteristics of the PAG profiles concerning to the late pregnancy period

Animal n°	beginning of prepartum PAG peak Days after AI	Maximum PAG concentration Days after AI			Calving Days after AI
		RIA 1	RIA 2	RIA 3	
209	267	276	273	273	277
579	271	275	279	279	280
1654	259	262	262	263	263
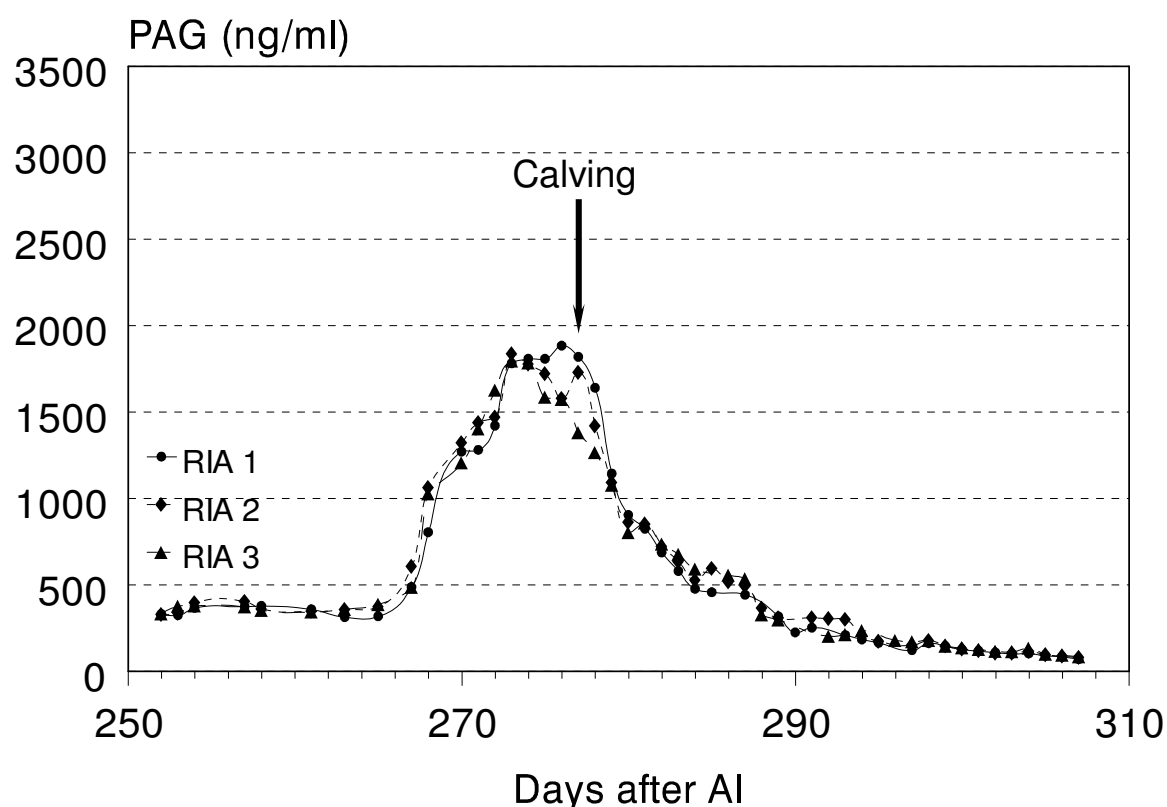
Figure 9. PAG profile of heifer nº 209 after Day 250 using RIA 1, 2 and 3
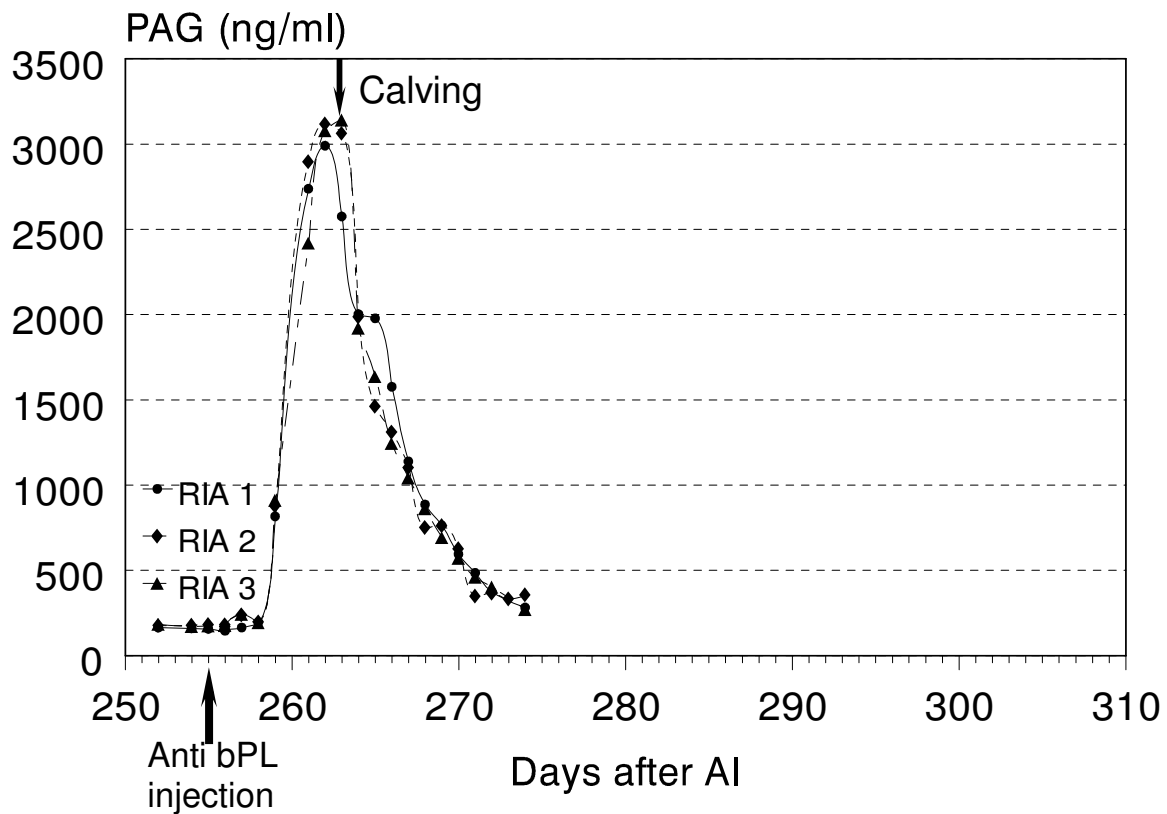
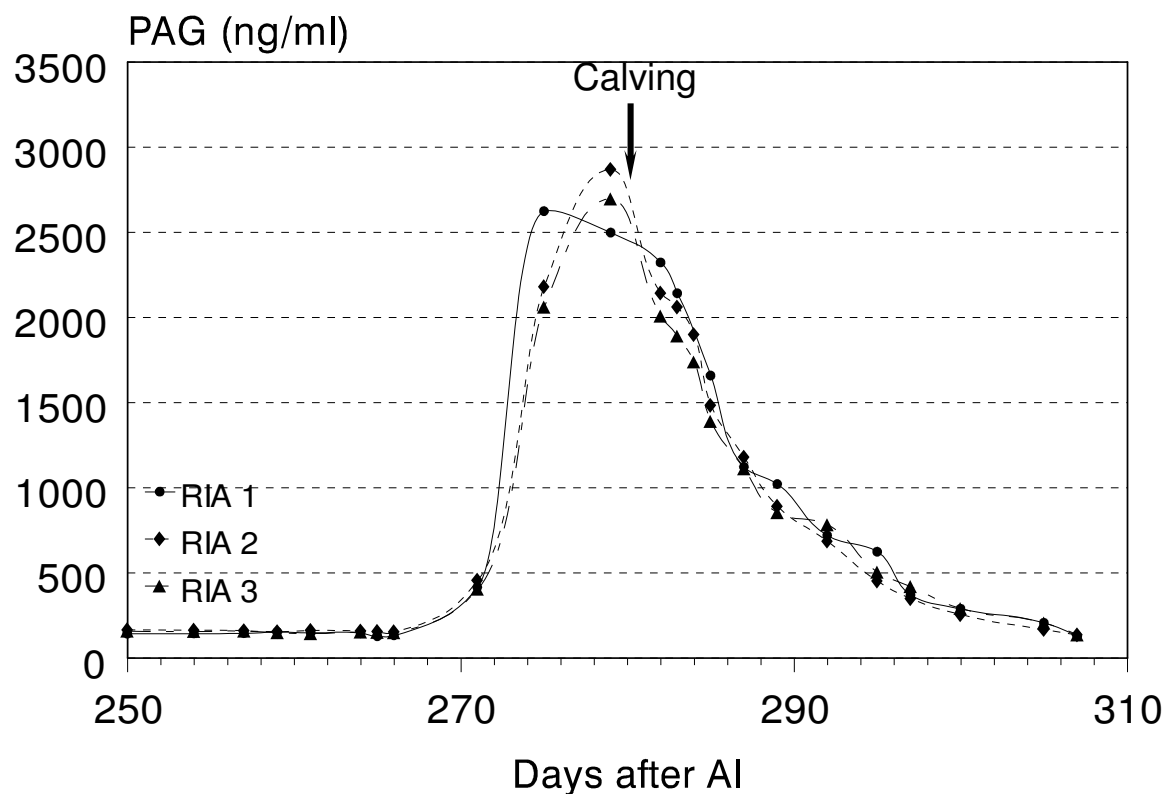Figure 10. PAG profile of heifer nº 1654 after Day 250 using RIA 1, 2 and 3

Figure 11. PAG profile of heifer nº 579 after Day 250 using RIA 1, 2 and 3

Table 6. RIA 2/ RIA 1 and RIA 3/ RIA 1 ratios (mean±SD) determined for the
late pregnancy period

Days after AI[*]	RIA 2/ RIA 1 Ratio	RIA 3/ RIA 1 Ratio
251-260	1.1±0.11	1.08±0.14
261-270	1.04±0.16	1.02±0.13
271-280	0.98±0.12	0.93±0.12
281-290	1.01±0.13	0.91±0.32
291-300	1.12±0.33	1.06±0.42
301-310	0.94±0.18	1.03±0.04
311-320	1.13±0.13	1.10±0.12
321-330	1.07±0.11	0.95±0.09

[*]RIA 2/ RIA 1 and RIA 3/ RIA 1 ratios were determined for 10 day periods
where detectable PAG concentrations were measured

Animals with spontaneous pregnancy failure

Heifer n° 2792 showed an increase in the PAG concentrations from Days 21, 22, 25 (RIA 3, RIA 2, RIA 1 respectively) (Figure 12). Between Days 40 and 49 a slight increase was recorded in the PAG levels, at Day 51 peak concentrations were detected. After, during the elimination of PAG molecules from the maternal blood PAG concentrations started to decrease. The progesterone profile of this animal showed a clear drop between Days 62 and 66. Right uterine horn pregnancy was detected by rectal palpation on Day 49. The clinical observations, the PAG and P4 profiles suggesting that this animal showed early fetal mortality / early abortion. The fetal death probably occurred between Days 51 and 58 after AI.
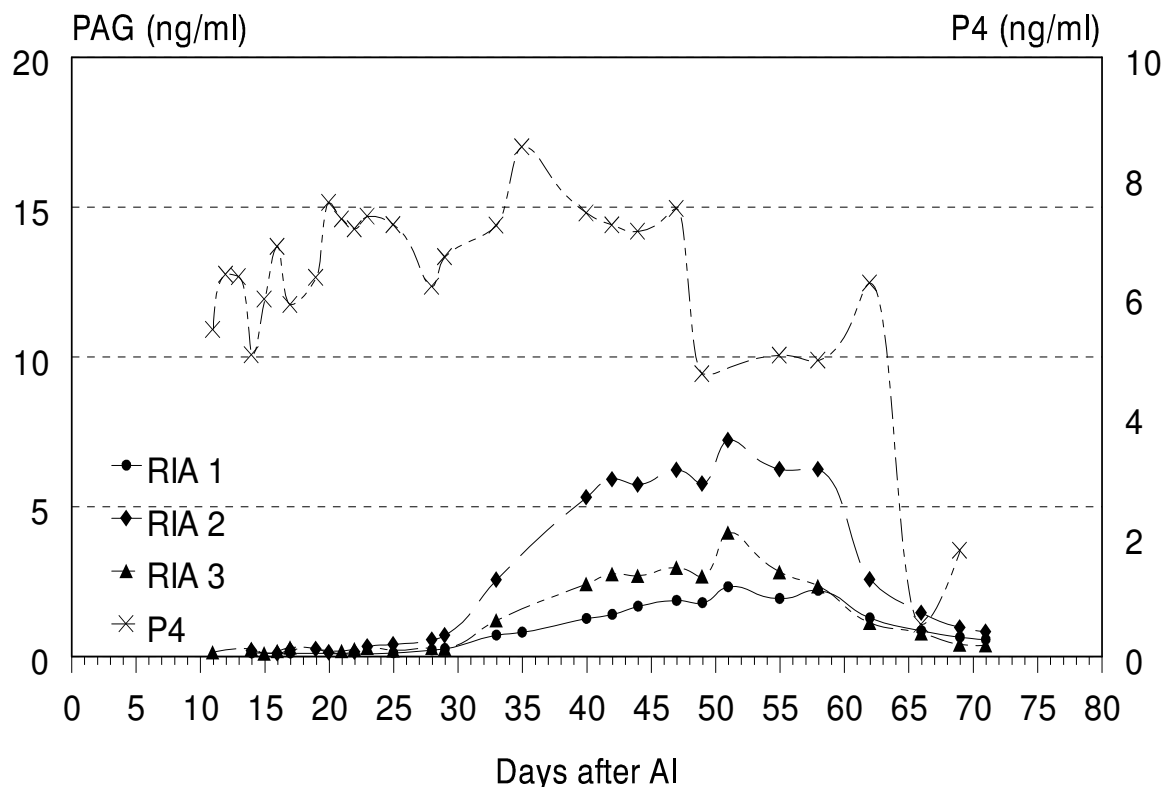
Figure 12. PAG and P4 profile of heifer nº 2792 using RIA 1, 2 and 3

Heifer n° 8368 presented detectable PAG concentrations from Days 21-22 after AI (Figure 13). Thereafter PAG levels increased rapidly till about Day 32. From Day 32 till about Day 66 the rate of increase was lower. From Day 21 till Day 59 the RIA 2/ RIA 1 and RIA 3/ RIA 1 ratios were higher than 1.5. A dramatic increase can be observed in the release of the PAG molecules from Day 66 till Days 86-95. After Day 95 PAG concentrations fell to about 40-45 ng/ml. Just before the time of abortion (Day 144) a smaller PAG peak was detected by the three RIA systems (Days 136-140 after AI) and after PAG concentrations started to decline steadily as this glycoprotein was eliminated from the maternal circulation.

The two expelled fetuses were positive for *Neospora caninum* by PCR analysis performed on their brain samples. The samples removed on Days 9, 37, 66 and 100 were negative in the immuno fluorescent antibody test. The plasma sample withdrawn on Day 131 was doubtful, the sample from Day 142 was slightly positive in the same serological test.
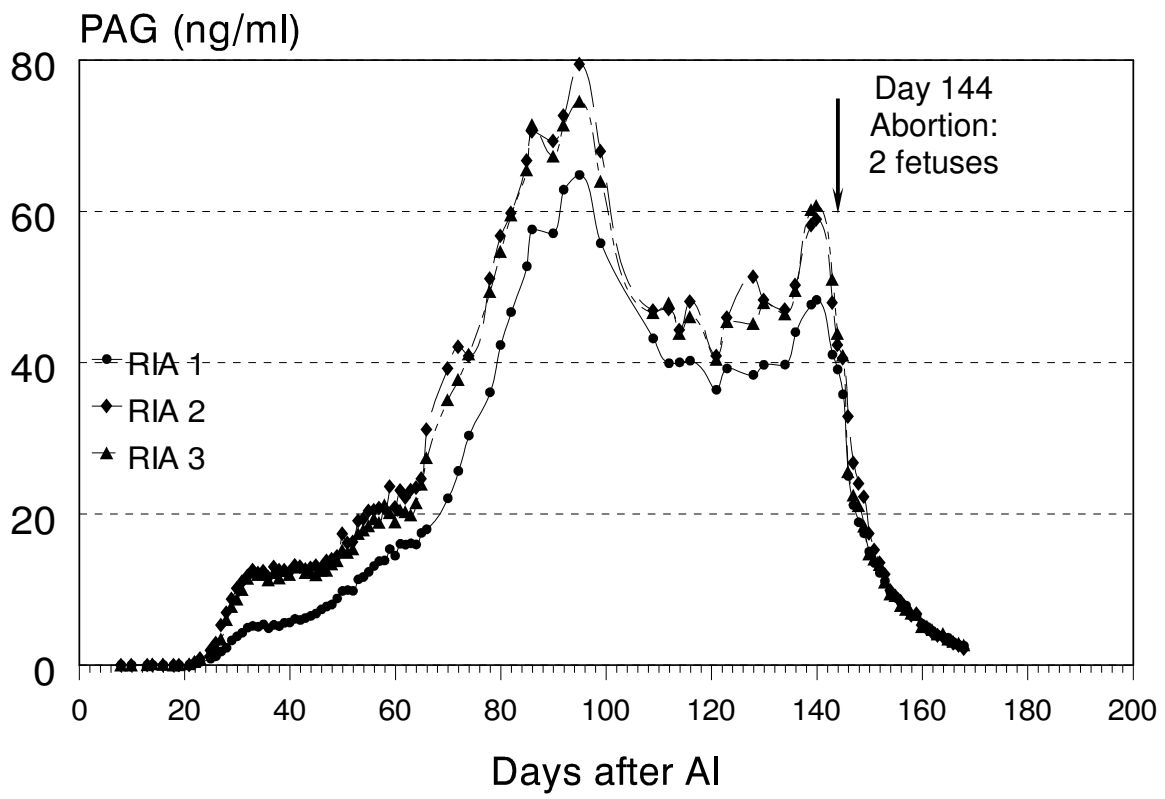
Figure 13. PAG profile of heifer nº 8368 using RIA 1, 2 and 3

Chapter 6: Investigation V: Determination of PAG profiles in cows after Embryo Transfer

In a collaborative study 268 embryo recipients were followed by clinical observation and blood sampling at short time interval. In this study PAG and P4 profiles are presented which were determined from maternal blood in order to better understand the origin and the pathogeny of the pregnancy failure.

MATERIALS AND METHODS

In this experiment, 268 embryos produced by three different methods were used for embryo transfer. The three procedures were the following: standard MOET (n=118) (multiple ovulation and embryo transfer) procedure, IVP embryos cultured in a system which included buffalo rat liver co-culture cells and 10% fetal calf serum (Cocult) (n=44) and IVP embryos cultured in a synthetic oviduct fluid (SOF) (n=106) culture system (van Wagtendonk *et al.*, 2000).

For the in vitro production of embryos, cumulus oocyte complexes were collected by ultrasound-guided ovum pick up procedure. Oocyte maturation and subsequent fertilization (Day 0) with frozen-thawed, Percoll separated semen from different breeding bulls were carried out according to procedures published before (van Wagtendonk *et al.*, 2000). Eighteen hours after the fertilization (Day 1), presumptive zygotes were transferred into the culture medium. On Day 6 to 8, embryos were evaluated for their developmental stage and grade of quality. Grade 1 and 2 morulae and blastocysts were loaded individually into 0.25 ml straws (ZA481, IMV, l'Aigle, France) (van Wagtendonk *et al.*, 1998). These embryos were transferred within three to nine hours after completion of the production process into the uterus of Holstein Friesian recipients which were housed indoors at the experimental farm of Holland Genetics. The transfer was performed on Day 7 or 8 of the natural estrous cycle. If there were more embryos than recipients, embryos were frozen, using conventional slow freezing in a 10% glycerol solution (van Wagtendonk *et al.*, 1995). Only expanded blastocysts were frozen.

Blood samples were collected into heparinized tubes from the jugular vein every three days from Day 7 until Day 35, then once a week until Day 119. The animals were controlled for the signs of estrus each day, if return to estrus was observed, then on the same day a last blood sample was withdrawn. Samples were centrifuged (15 min at 1500 g) and the plasma was stored at -25°C until assay.

PAG and progesterone concentrations were determined in the plasma samples. When signs of pregnancy failure were not recorded during the observation period RIA 1 system was used to measure PAG concentration. In the cases of pregnancy failure RIA 1, RIA 2 and RIA 3 systems were used to characterize the PAG profiles.

Concentrations of progesterone in the peripheral blood were estimated by validated solid-phase ^{125}I RIA method (Coat-A-Count TKPG; Diagnostic Products Corporation, Los Angeles, CA, USA) as described by Dieleman and Bevers (1987).

The embryonic mortality was confirmed on the basis of detection of return to estrus, by ultrasonography and rectal palpation, and retrospectively by determining and analyzing the P4 and PAG concentrations.

For the whole data set of the transfer of 268 embryos a complete analysis is to be published. The P4 and PAG concentrations determined in the animals with successful pregnancy (control group) were used in order to determine the threshold P4 and PAG concentrations, which are corresponding to the one tailed confidence interval of 95% each days after reference estrus. The recipients with pregnancy loss were classified according to the characteristic of their PAG and P4 profiles. The P4 and PAG concentrations below the one tailed confidence interval of 95% determined in the control group were considered as subnormal. The days after reference estrus where subnormal P4 and PAG concentrations had been found were indicated for each animal. The beginning of a drop in P4 and PAG concentrations was defined as a day where the sample removed gave lower concentrations than those determined in the previous sample.

RESULTS

The summarized results of the experiment is presented in Table 1.

Table 1. Total number of transfer of *in vitro vs. in vivo* derived embryos, pregnancy failures according to the time of return to estrus

Group	Total number of		Return to estrus after reference estrus[1]			Total number of recipients		
	transfers	pregnancy failures[2]	before Day 24	Days 24-119	after Day 119	with pregnancy failure, estrus was not recorded[1]	removed	calved[2]
MOET	118	62	34	22	4	2	3	53
		52.5%	54.8%	35.5%	6.5%	3.2%		44.9%
Cocult	44	23	13	7	2	1	0	21
		52.3%	56.5%	30.4%	8.7%	4.4%		47.7%
SOF	106	67	39	25	0	3	0	39
		63.2%	58.2%	37.3%		4.5%		36.8%
Totals[3]	268	152	86	54	6	6	3	113
		56.7%	32.1%	20.2%	2.2%	2.2%	1.1%	42.2%

[1]proportions relative to number of pregnancy failures of one group
[2]proportions relative to number of transfers of one group
[3]proportions relative to total number of transfers

From the whole experiment after the transfer of 268 embryos, part of the PAG and P4 profiles of recipients that calved have been analyzed by Vos *et al.* (2001) showing no significant differences in the P4 and PAG concentrations between recipients of the different embryo groups. The P4 and PAG concentrations determined in the animals with successful pregnancy (control group) are presented in Figure 1.

The result of the analysis of individual profiles from recipients suffered pregnancy failure between Days 24 and 119 are presented in Tables 2-4. At each animal the interval was calculated between the earliest abnormality (drop or subnormal concentration) in the PAG (based on the results of RIA 1 system) and P4 profiles. If the abnormality occurred earlier in the P4 profile this number was considered as positive, otherwise negative. The recipients were arranged in Tables 2-4 according to decreasing order of the interval calculated above. The pregnancy failures where abnormality first occurred in the P4 profile were considered as type A pregnancy failure. The data of these recipients are presented in Table 2. The pregnancy loss was considered as type B

pregnancy failure if the PAG profile showed earlier abnormality than the P4 profile (Table 4). In several cases the abnormalities of P4 and PAG profiles occurred at the same time (type C pregnancy failures, Table 3).
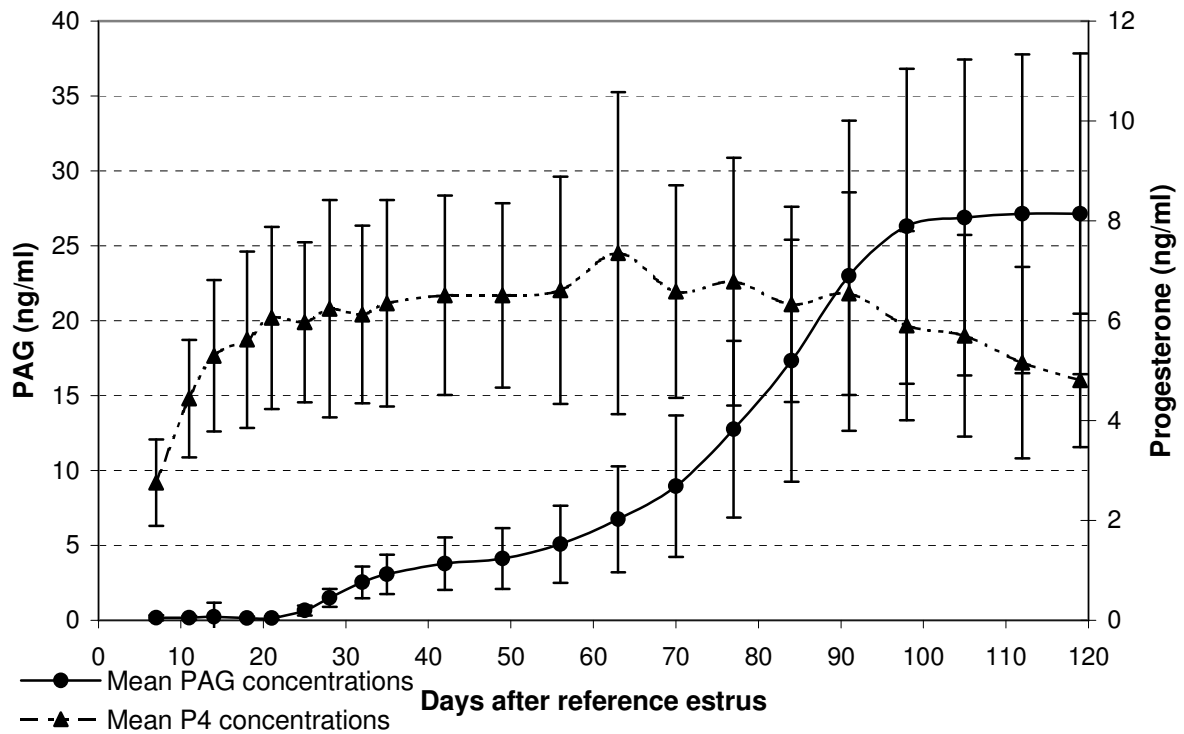

Figure 1. PAG and progesterone concentrations (Mean±SD) during the first 119 days of pregnancy after embryo transfer in the control group

Table 2. Characteristics of the PAG and P4 profiles of the animals suffered type A pregnancy failure between Days 24 and 119 after reference estrus

Recipient ID	Embryo Origin	Days after reference estrus					Interval (days)
		Drop in PAG concentration	Drop in P4 concentration	Return to estrus	Subnormal PAG	Subnormal P4	
B 1654	Cocult	-a	25	25	-b	14, 21→	NDc
R 394	MOET	-a	18-25	25	-b	21→	NDc
R 586	Cocult	-a	21-25	25	-b	21→	NDc
V 223	SOF	77-98	56-70	-d	77→	14, 70	63
R 634	MOET	35	32, (56)	-d	32→	11-25, 32→	21
B 1558	MOET	77-98	91-98	98	28→	7-14, 98	21
R 483	SOF	35	35	36	32→	11, 25,35	21
B 1668	SOF	35-42	35-42	42	25→	7, 35→	18
R 593	SOF	32-56	-e	60	25→	7-56	18
V 225	SOF	-a	25-28	29	25→	7, 28	18
R 650	MOET	-f	25-32	31	25→	7, 28→	18
R 638	MOET	42	32-42	42	25-28	7, 25, 35→	18
B 1622	MOET	42-64	25-42, (60-64)	42	49→	35-49	17
R 599	SOF	21-56	-e	52	28→	7-56	14
B 1653	MOET	49-67	35-49	49	56→	42-56	14
B 1690	SOF	32-49	49	49	25→	14, 49	11
V 210	MOET	42-49	32-49	49	42→	35→	10
R 459	Cocult	35-42	32-42	42	28→	18, 35→	10
B 1666	MOET	21-25	21-25	25	25	11, 21→	10
B 1600	Cocult	35	28-35	35	35	32→	7
R 622	MOET	32-42	18-21, (42)	42	25→	21-32, 42	7
R 588	MOET	35-42	18-21, (42)	42	25→	21-28, 42	7
R 625	MOET	42	35-42	42	-b	35→	7
B 1594	Cocult	42-49	35-49	48	-b	42→	7
R 578	MOET	-f	18-25	24	25	21→	7
R 621	SOF	-f	18-25, (42)	-d	25→	21-32, 42	7
B 1613	MOET	-f	18-25	25	25	21→	7
B 1659	MOET	-a	18-25	25	25	18→	7
B 1613	MOET	-f	18-25, (49)	49	25→	21-35, 49	7
R 644	SOF	-f	21-25	24	25	21→	4
R 643	SOF	-f	21-25	24	25	25	4
R 643	SOF	-f	21-25	24	25	21→	4
B 1655	SOF	-f	21-28	25	25	21→	4
B 1685	MOET	-f	21-25	25	25	21→	4
R 653	MOET	-f	21-25	-d	25	25	4
R 578	SOF	-f	21-25	25	25	25	4
R 667	SOF	-f	21-25	25	25	21→	4
V 225	MOET	-f	21-25	25	25	25	4
B 1682	SOF	-f	21-28	27	25→	25→	4
B 1658	SOF	35	25-35	35	28→	32→	3

a No decrease in the PAG concentrations
b PAG concentrations were in the normal range compared to the control group
c There was no abnormality found in the PAG profile, so the interval was not determined
d Estrus was not recorded
e P4 concentrations were not detectable
f PAG concentrations were not detectable

Table 3. Characteristics of the PAG and P4 profiles of the animals suffered type C pregnancy failure between Days 24 and 119 after reference estrus

Recipient ID	Embryo Origin	Days after reference estrus					Interval (days)
		Drop in PAG concentration	Drop in P4 concentration	Return to estrus	Subnormal PAG	Subnormal P4	
B 1678	SOF	21-28	21-28	28	25→	25→	0
R 602	SOF	42	42	41	-[b]	42	0
B 1631	Cocult	77-84	77-84	84	77→	77→	0
B 1689	SOF	-[f]	25-28	27	25→	25→	0

[b] PAG concentrations were in the normal range compared to the control group
[f] PAG concentrations were not detectable

Table 4. Characteristics of the PAG and P4 profiles of the animals suffered type B pregnancy failure between Days 24 and 119 after reference estrus

Recipient ID	Embryo Origin	Days after reference estrus					Interval (days)
		Drop in PAG concentration	Drop in P4 concentration	Return to estrus	Subnormal PAG	Subnormal P4	
R 628	MOET	42-49	35-49	46	32→	35→	-3
R 611	MOET	28-42	32-42	41	28→	32→	-4
B 1693	SOF	49-70	56-70	69	49→	63→	-7
B 1653	SOF	35-49	35-42	-[d]	28→	42→	-7
R 467	SOF	11-25	18-25	25	-[b]	21→	-7
R 521	SOF	11-42	18-21, (42)	42	28→	18-28, 42	-7
B 1678	MOET	-[a]	35-42	41	25→	35→	-10
R 526	MOET	11-25	21-25	25	-[b]	21→	-10
B 1533	SOF	35-42	35-42	42	25→	42→	-10
B 1611	Cocult	42	42	42	25-28	35→	-10
B 1538	SOF	49	42-49	50	28→	49	-14
R 464	SOF	35-49	49	49	42→	49	-14
B 1604	MOET	42-57	42-57	56	28, 42→	56→	-14
B 1639	SOF	42-49	49	48	25→	49	-24
R 481	SOF	42-49	49	49	25→	-[g]	-24
B 1629	Cocult	63-70	-[h]	-[d]	32-35, 49→	-[g]	ND[i]

[a] No decrease in the PAG concentrations
[b] PAG concentrations were in the normal range compared to the control group
[d] Estrus was not recorded
[g] P4 concentrations were in the normal range compared to the control group
[h] No decrease in the P4 concentrations
[i] There was no abnormality found in the P4 profile, so the interval was not determined
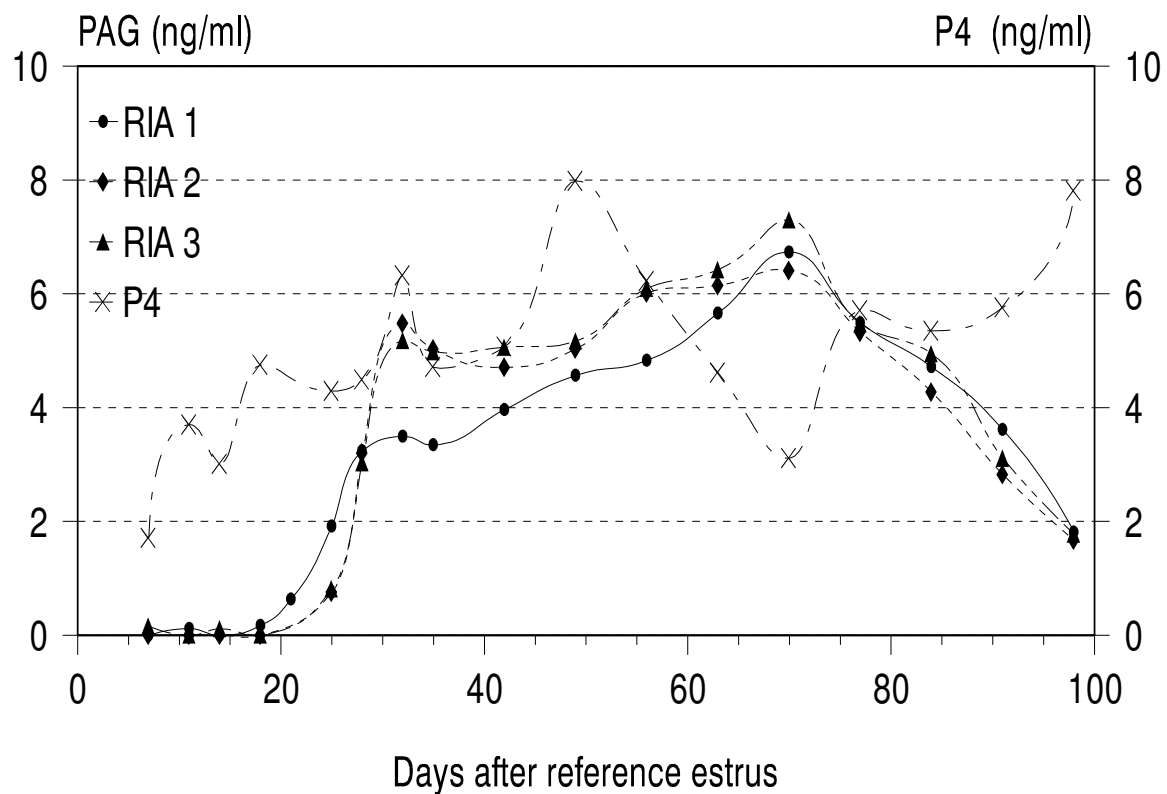
Pregnancy failure type A

V 223

Figure 2. PAG and P4 profiles of recipient n° V 223

The recipient n° V 223 presented P4 concentrations in the physiological range except Days 14 and 70. After Day 49 decrease could be observed in P4 concentrations till Day 70. Thereafter P4 concentrations were recovered.

Pregnancy-associated glycoproteins first appeared in the maternal blood at Day 18. Their concentrations increased till Day 70, and after fell in the rest of the observation period.

The fall in the P4 concentrations between Days 49 and 70 was probably responsible for the embryonic loss occurred at around Day 70, P4 and PAG profile clearly indicate the embryonic death with a surviving CL.
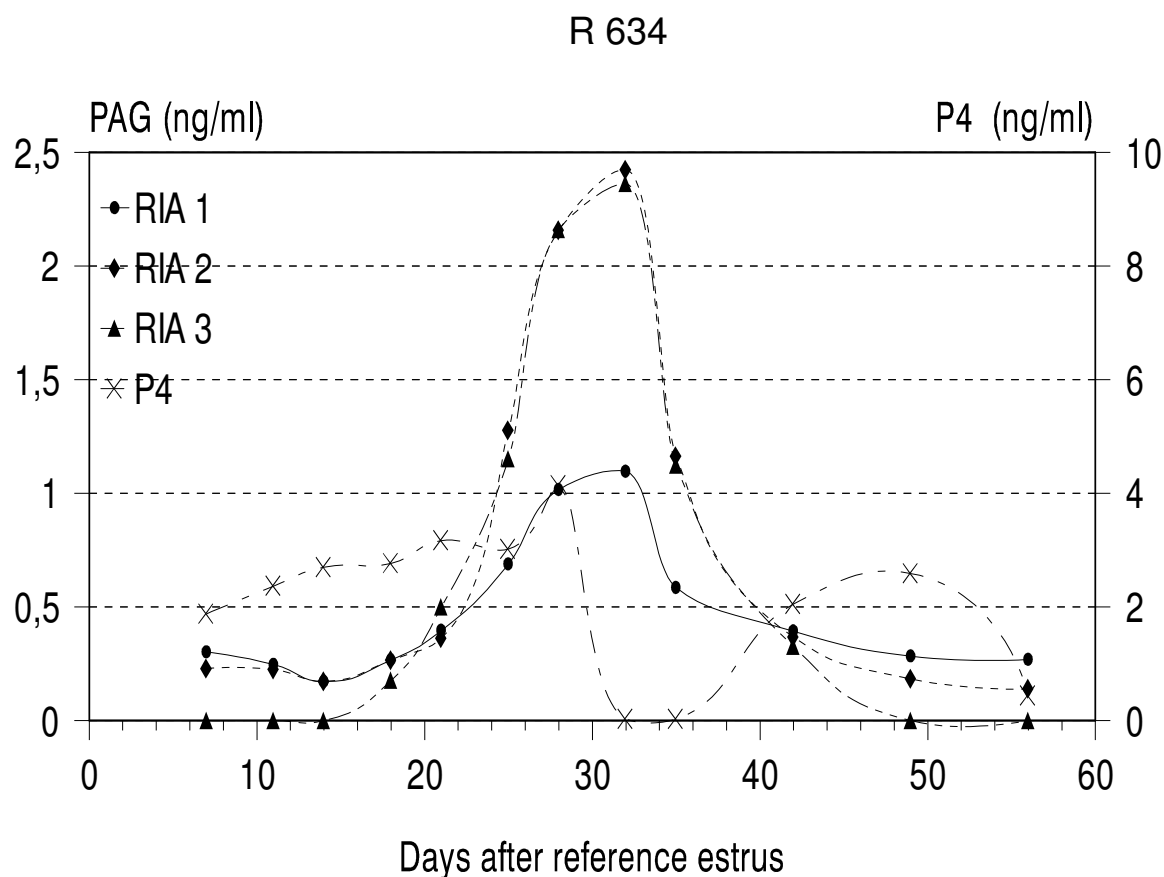
R 634

Figure 3. PAG and P4 profiles of recipient n° R 634

Progesterone concentrations were increasing slowly in the first part of the profile and were remaining under 3.5 ng/ml except at Day 28. Between Days 28 and 32 P4 concentrations were decreasing sharply reaching undetectable levels at Days 32 and 35, however during this time no signs of estrus were detected. After Day 35, P4 levels were increasing again, and finally fall after a period corresponding to the normal lifespan of the CL. P4 concentrations were subnormal between Days 11-25 and after Day 32.

The PAG concentrations began to increase after Day 18, reaching peak values at around Day 32. After Day 32 sharp drop could be observed in the PAG concentrations.

In conclusion, this recipient was not suitable for embryo transfer because of the low P4 concentrations (insufficient luteal function).

B 1558
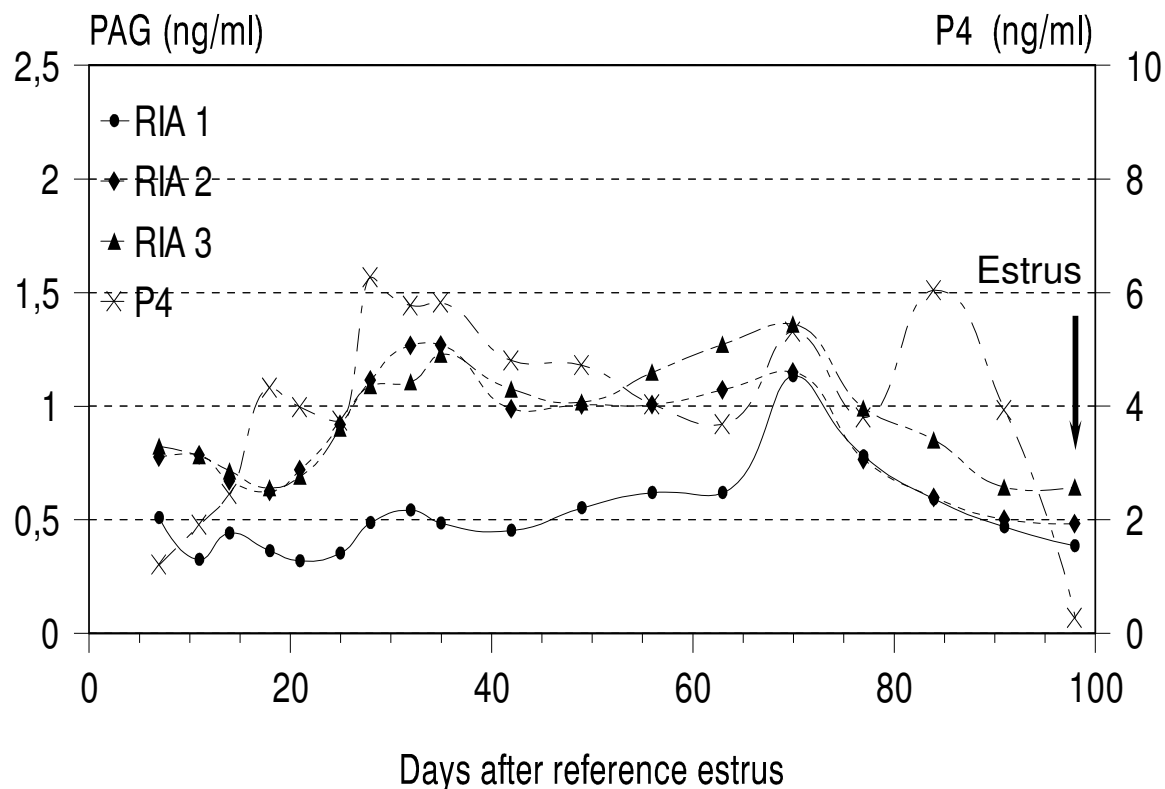
Figure 4. PAG and P4 profiles of recipient n° B 1558

The animal n° B 1558 showed slowly decreasing P4 concentrations between Days 28 and 63. From Day 84 sharp decrease was observed. Estrus was recorded on Day 98. Slowly decreasing PAG levels were found till Days 18-21. These results are probably corresponding to the elimination of the PAG from the maternal blood originating from the previous calving. After Day 18-21 slow PAG increase could be observed with very low PAG concentrations. RIA 2 and 3 showed remarkably higher concentrations than RIA 1. From Day 70 PAG concentrations showed a definite decrease.

According to the routine PAG test (RIA 1), this animal would be considered as nonpregnant except Day 70, whereas RIA 2 and 3 indicates the presence of a conceptus between Days 18 and 70.

This animal clearly indicates the possibility of a new diagnosis: pregnant but endangered pregnancy.

R 483
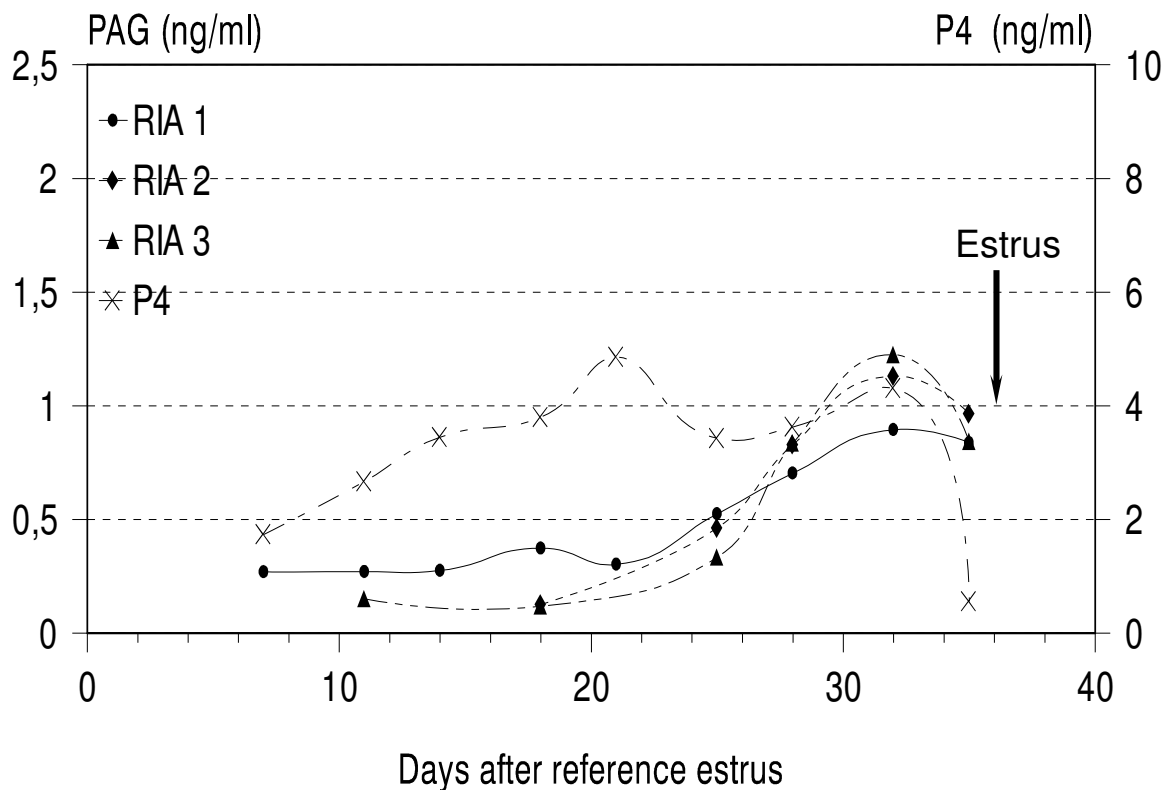
Figure 5. PAG and P4 profiles of recipient n° R 483

Progesterone levels were increasing slowly, remained around 4 ng/ml till Day 32 and decreased under 1 ng/ml between Days 32 and 35. Subnormal P4 concentrations were detected at Days 11, 25 and 35. Estrus was detected on Day 36.

PAG concentrations began to increase after Day 21. The PAG levels were subnormal from Day 32.

According to the earliest abnormality in the profiles (subnormal P4 concentrations at Days 11 and 25), this animal was considered to have type A pregnancy failure.

B 1622
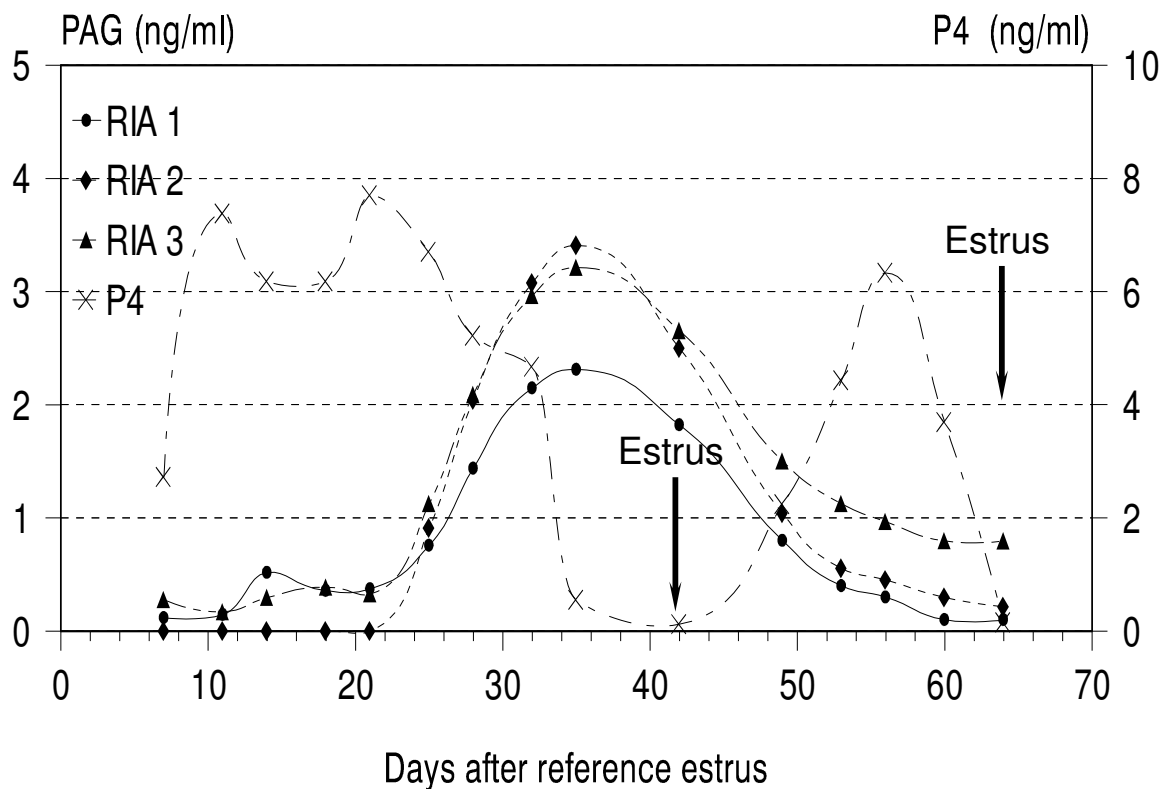
Figure 6. PAG and P4 profiles of recipient n° B 1622

The P4 profile of animal n° B 1622 showed a clear decrease after Day 32. Estrus was observed at Day 42, after this P4 level increased and changed according to a normal luteal phase. The second estrus was recorded on Day 64.

The increase in the PAG concentrations could be observed after Day 21. Peak PAG concentration was detected at Day 35, after PAG level started to decrease. From Day 49 PAG concentrations were subnormal.

R 599
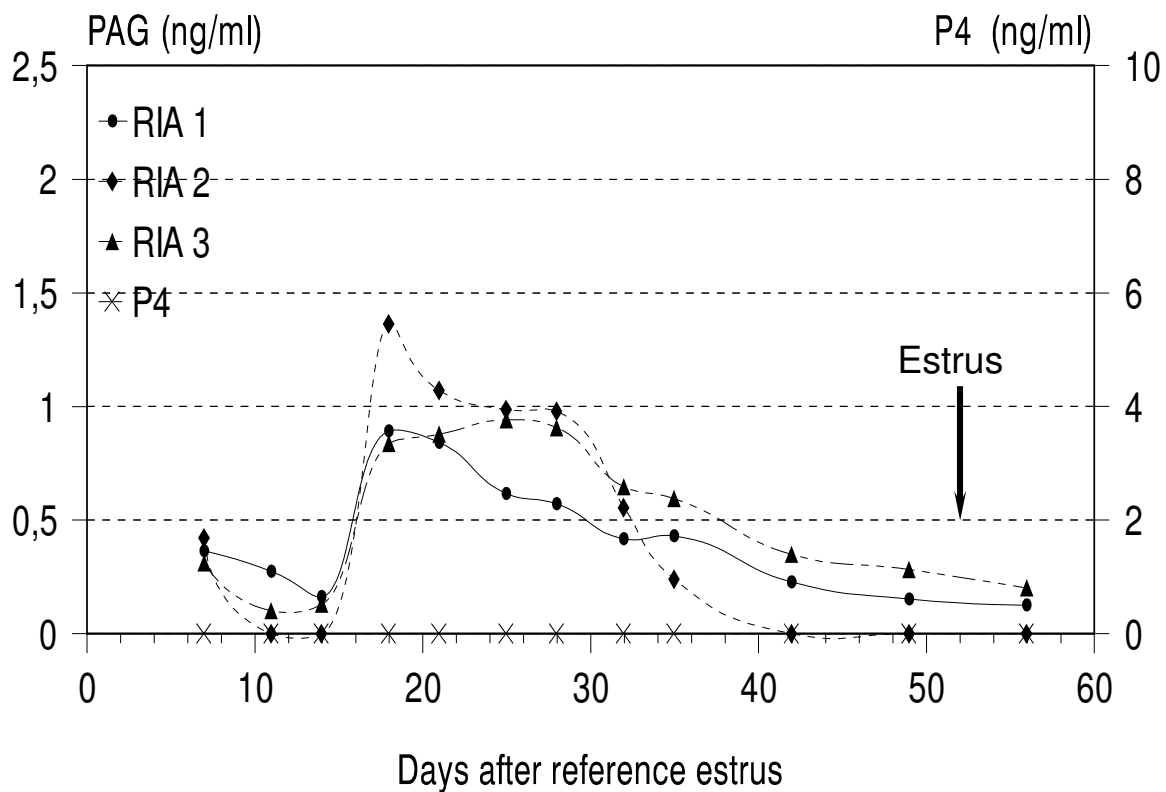
Figure 7. PAG and P4 profiles of recipient n° R 599

During the observation period the cow n° R 599 did not express detectable levels of P4, however the embryo transfer was performed on Day 7 after the sign of the last estrus. At Day 52 signs of estrus were recorded.

Around Day 18 a small peak was observed in the PAG concentrations indicated by RIA 1-2 systems, and at Day 25 by RIA 3 system. Thereafter the PAG levels started to decrease till the end of the observation period.

B 1653
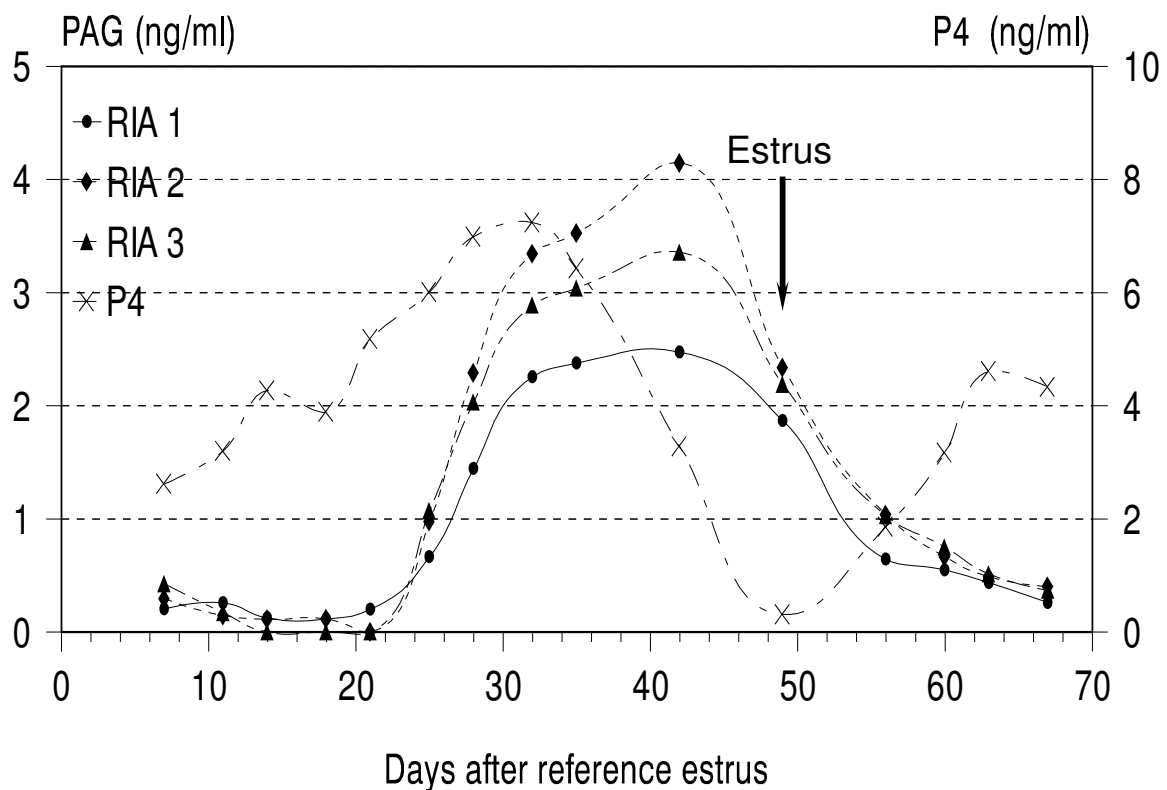
Figure 8. PAG and P4 profiles of recipient n° B 1653

The P4 profile of the cow n° B 1653 showed relatively low concentrations during the first 18 days. Sharp decrease was observed after Day 32. Estrus was recorded on Day 49.

PAG concentrations started to increase intensively after Day 21. After Day 32 increase with lower magnitude was observed. From Day 41 the PAG profile showed a decrease.

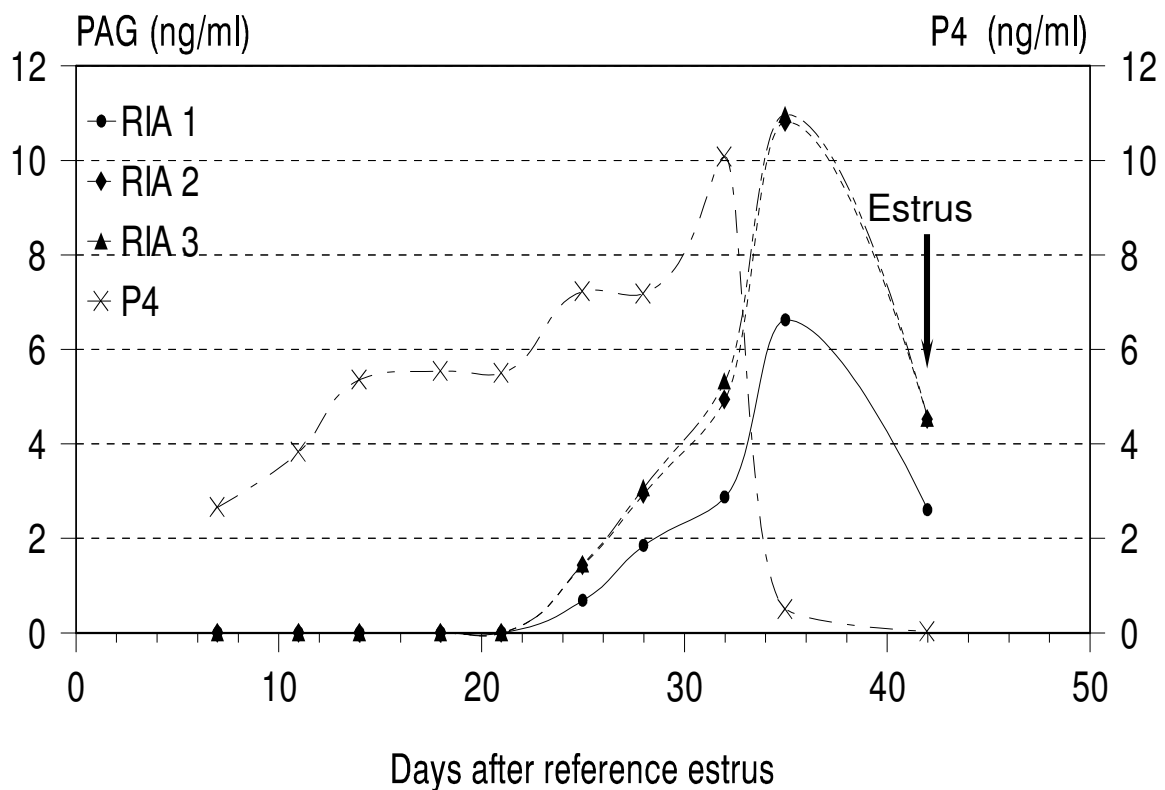The cause of the embryonic death was probably the decrease in the P4 concentrations.

R 625

Figure 9. PAG and P4 profiles of recipient n° R 625

Progesterone concentrations of cow n° R 625 were in the normal range till Day 32. P4 began to decrease rapidly after Day 32 reaching about 0 ng/ml. Estrus was recorded on Day 42.

PAG concentrations started to increase after Day 21, reached a peak value at around Day 35, then started to decrease.

The cause of the embryonic death was probably the decrease in the P4 concentrations.
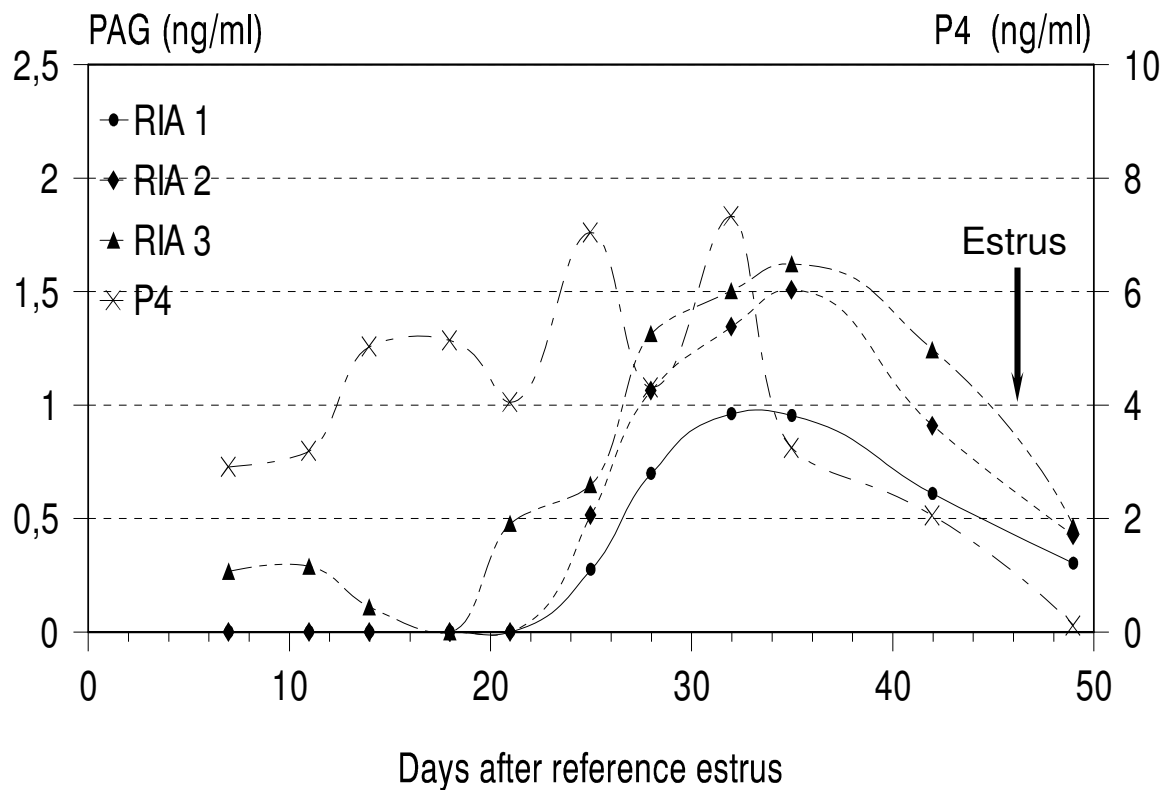
Pregnancy failure type B

R 628

Figure 10. PAG and P4 profiles of recipient n° R 628

The P4 concentrations seem to be in the physiological range (with remarkable fluctuations between Days 21 and 32). After Day 32 a decrease could be observed. Estrus was recorded on Day 46.

Radioimmunoassay 3 showed rising concentration from day 18 while the other 2 systems presented increasing PAG concentrations from Day 21. The 3 RIAs showed a peak around day 32, 35 however the concentrations obtained by RIA 1 were lower compared to the control group. After Day 35 PAG concentrations were decreasing slowly.
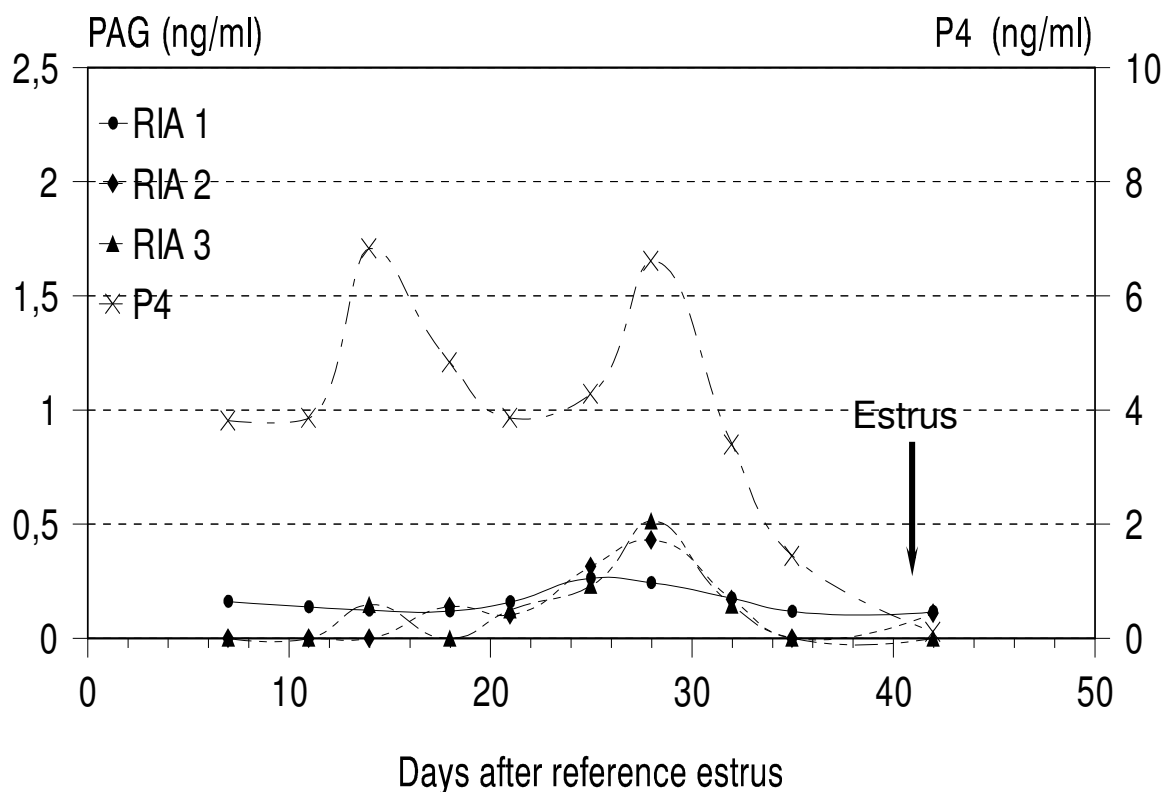
R 611

Figure 11. PAG and P4 profiles of recipient n° R 611

The P4 profile of animal n° R 611 showed 2 peaks at around Day 14 and Day 28. P4 concentrations were in the normal range, however at around Days 21 and 25, P4 concentrations were relatively low close to 4 ng/ml (valley). After Day 28, P4 concentrations fell to around 0 ng/ml (Day 42). Estrus was recorded on Day 41.

PAG levels were subnormal from Day 28, all the three RIA systems presented very low concentrations.

The relatively low and after Day 25 decreasing PAG concentrations are suggesting that in this case the quality of the embryo was responsible for the pregnancy loss.
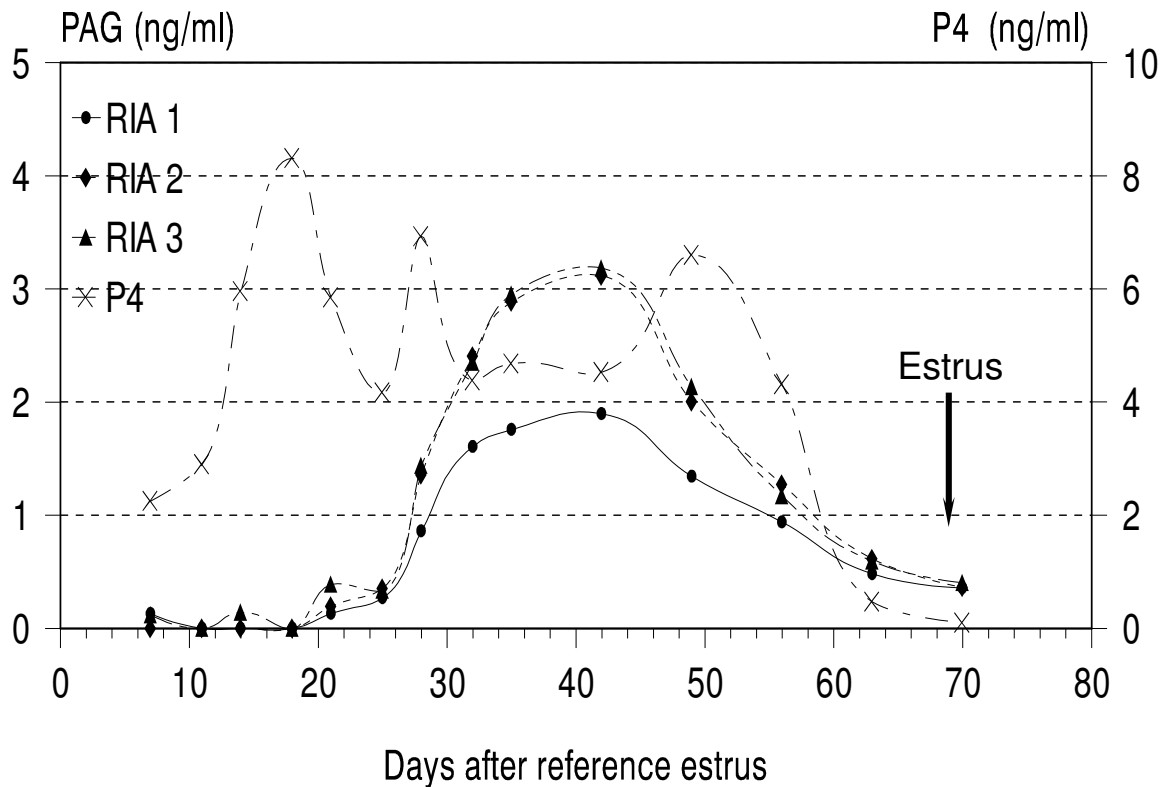
B 1693

Figure 12. PAG and P4 profiles of recipient n° B 1693

Progesterone concentrations were in the normal range till Day 56, however 2 increases and decreases could be observed within the first 35 days (1st decrease Days 18-28, 2nd decrease Days 28-32). After Day 49 P4 fell finally. Estrus was recorded on Day 69.

PAG levels showed intensive increase between Days 18 and 32. After, a plateau could be observed in the PAG profile, the decrease started at around Day 42.

As P4 fell after Day 18, we can suggest that the embryonic signal was poor. The drop of P4 (Days 28-32) followed by relatively low levels of P4 (Days 32-42) and plateau in the PAG profile (Days 32-42) can suggest the maternal origin of the pregnancy loss, however according to our classification criterias this recipient had a type B pregnancy failure.
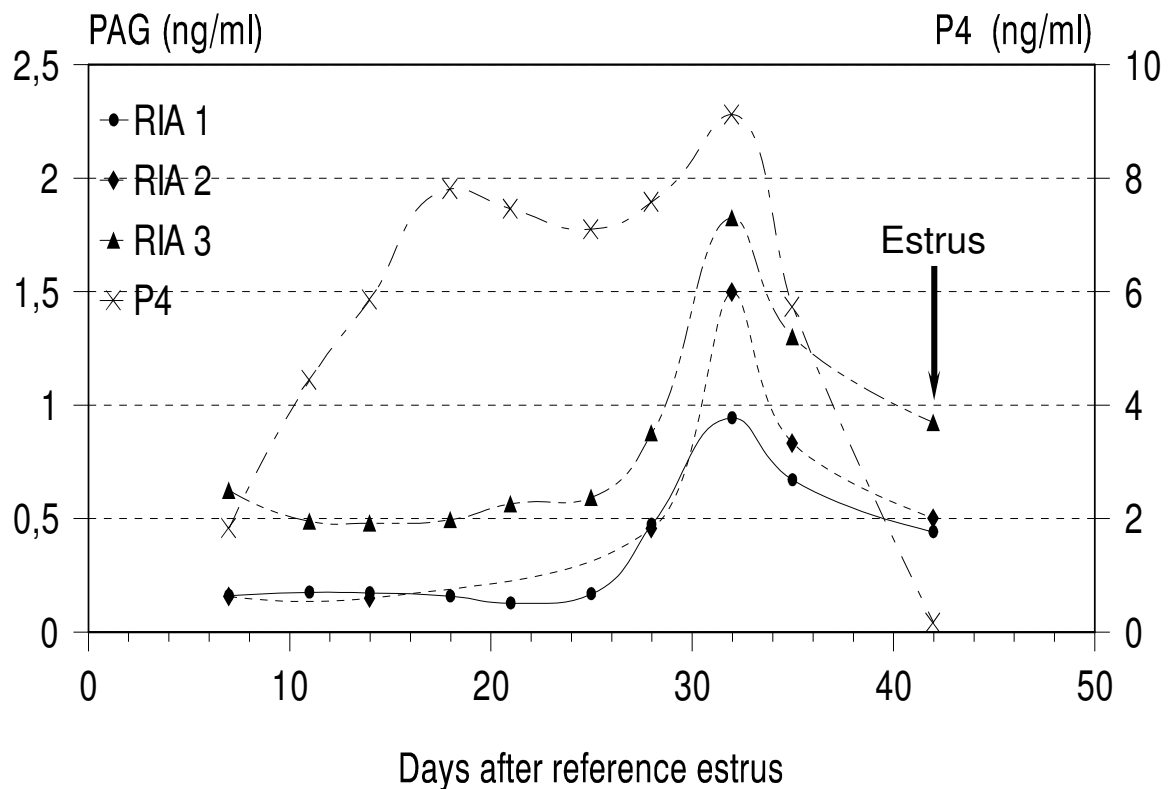
B 1533

Figure 13. PAG and P4 profiles of recipient n° B 1533

Progesterone profile showed a decrease after Day 32. At day 42, P4 concentration is around 0 ng/ml, at the same time, signs of estrus were observed.

PAG levels were low till Day 25, when they started to rise. RIA 3 presented relatively high concentrations in the first part of the profile. The PAG levels determined after Day 25 were subnormal indicating the embryonic origin of the pregnancy failure. Decrease could be observed in the PAG level after Day 32.
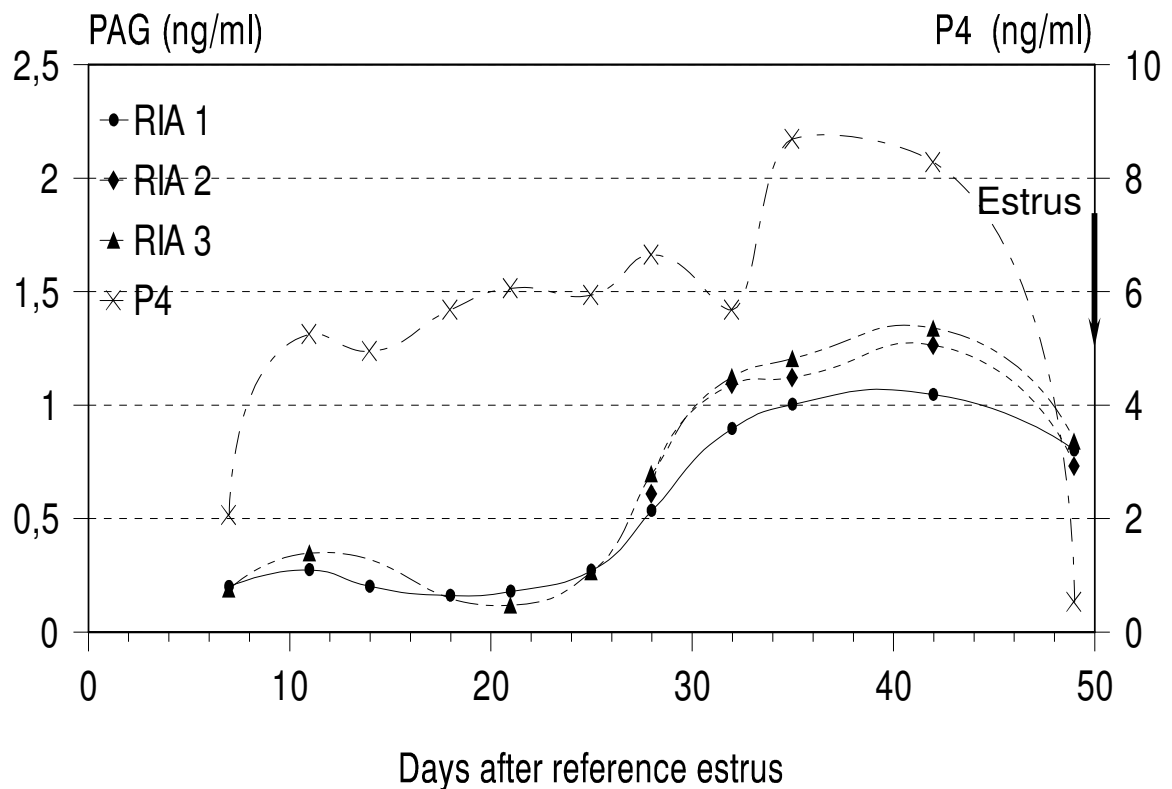
B 1538

Figure 14. PAG and P4 profiles of recipient n° B 1538

The P4 concentrations were in the normal range till Day 42, when P4 started to decrease. Estrus was recorded on Day 50.

PAG increase was detected after Day 21, PAG concentrations rose very slowly to reach a low plateau. After Day 28 PAG concentrations were in the subnormal range. After Day 42 decrease was observed by the three RIA.

The low PAG concentrations suggest the embryonic origin of the pregnancy failure.
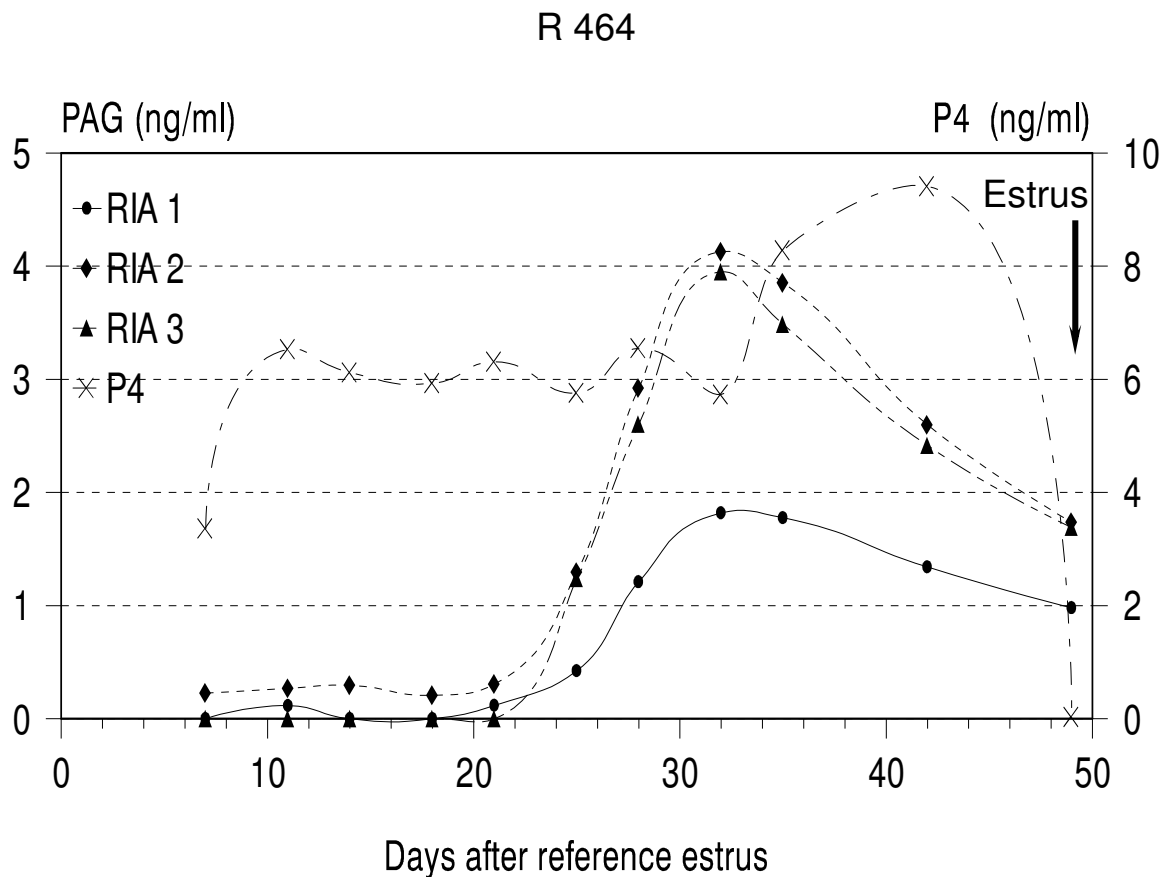
R 464

Figure 15. PAG and P4 profiles of recipient n° R 464

Progesterone concentrations were in the physiological range till Day 42, after P4 fell rapidly. Estrus was observed on Day 49.

Increasing PAG concentrations were detected after Day 21. The highest PAG concentrations were found at Day 32, followed by a gradual decrease.

The decrease in the PAG concentrations prior to the decrease in the P4 concentration indicates the embryonic origin of the pregnancy failure.
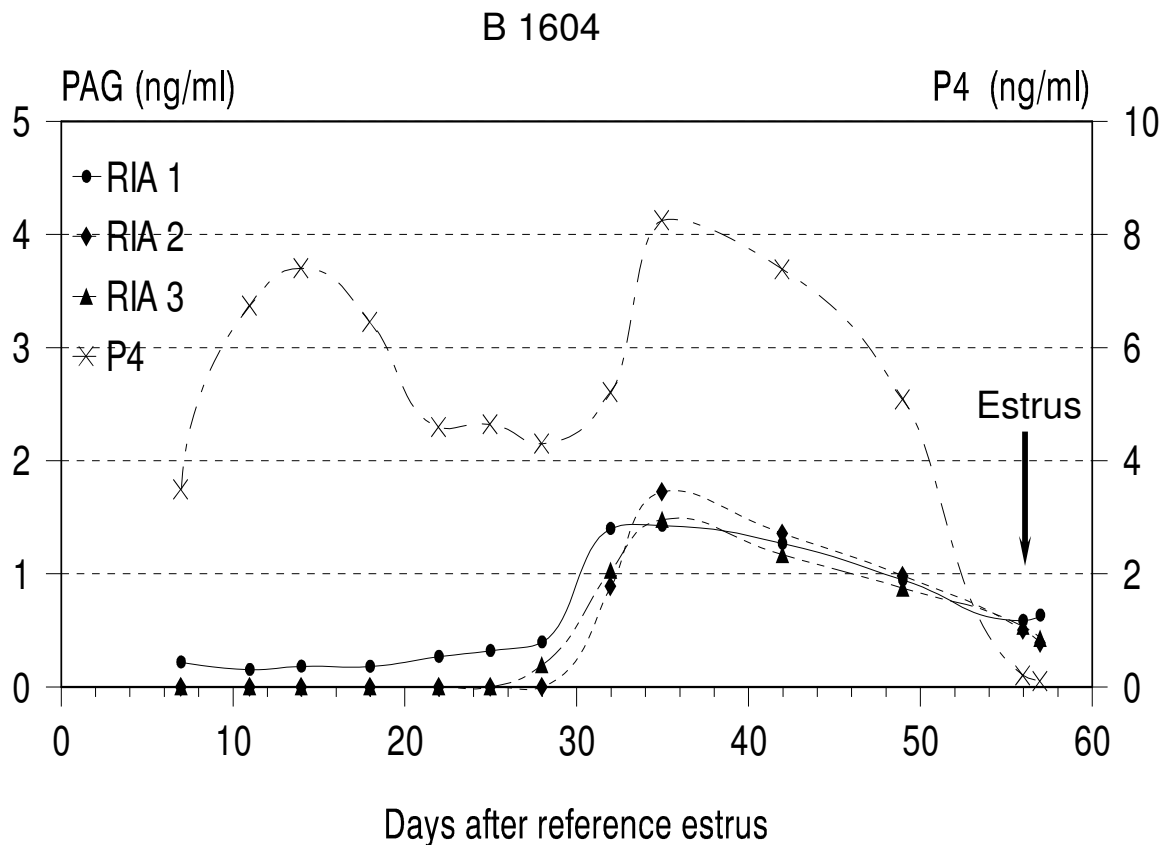
B 1604

Figure 16. PAG and P4 profiles of recipient n° B 1604

The P4 profile of animal n° B 1604 showed two peaks at around Day 14 and Day 35. After Day 35 P4 concentrations started to decrease reaching undetectable levels. Estrus was recorded on Day 56.

PAG profiles showed an increase after Day 28 till Days 32-35. From Day 35 till Day 57 slow decrease could be observed in the PAG concentrations. Subnormal PAG concentrations were observed at Day 28 and after Day 42.
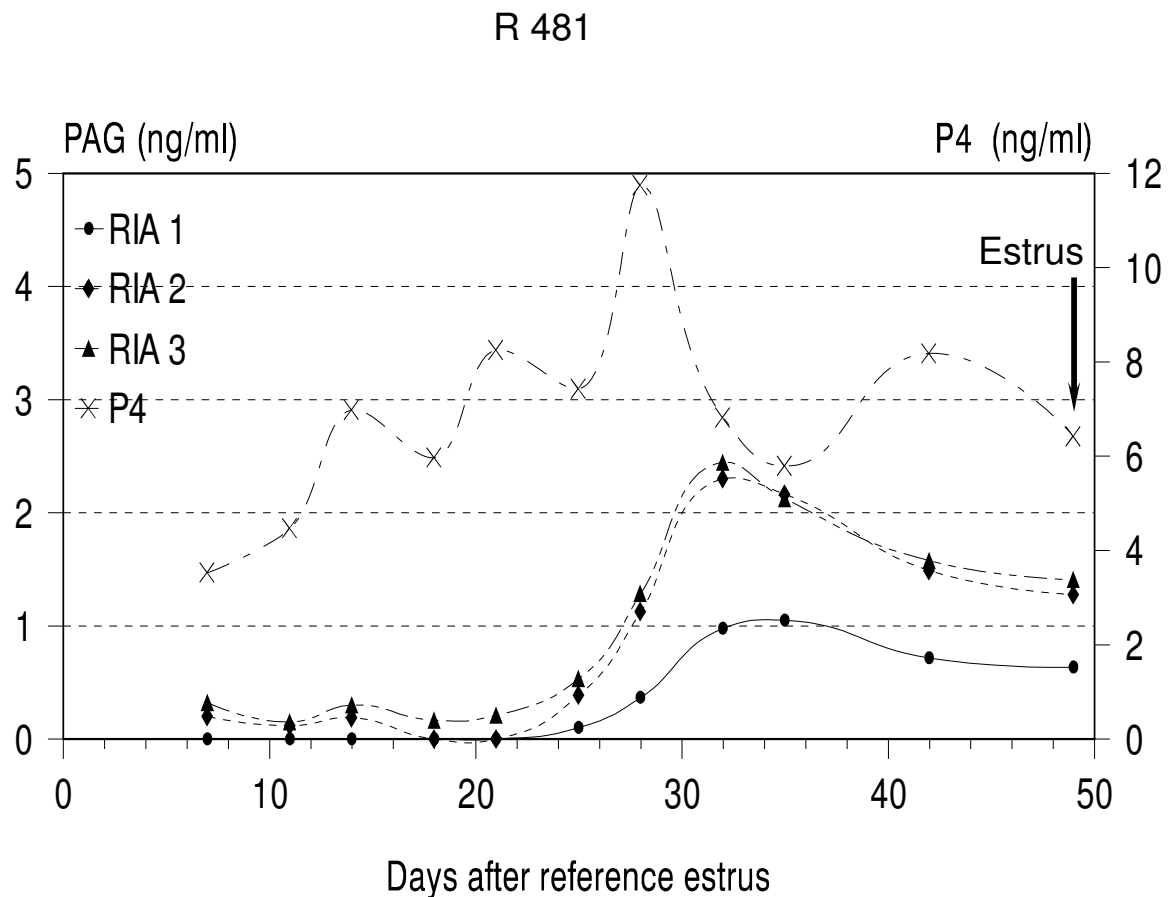
R 481

Figure 17. PAG and P4 profiles of recipient n° R 481

Progesterone levels of recipient n° R 481 were in the physiological range during the observation period. Signs of estrus were detected at Day 49, however at this time the P4 concentration was still high (6.42 ng/ml).

Slow PAG increase was detected after Day 21. The PAG concentrations remained relatively low in RIA 1, subnormal from Day 25 and started to decrease after Days 32-35.

The low PAG levels are suggesting that in this case the origin of the pregnancy failure was the poorly developing embryo.
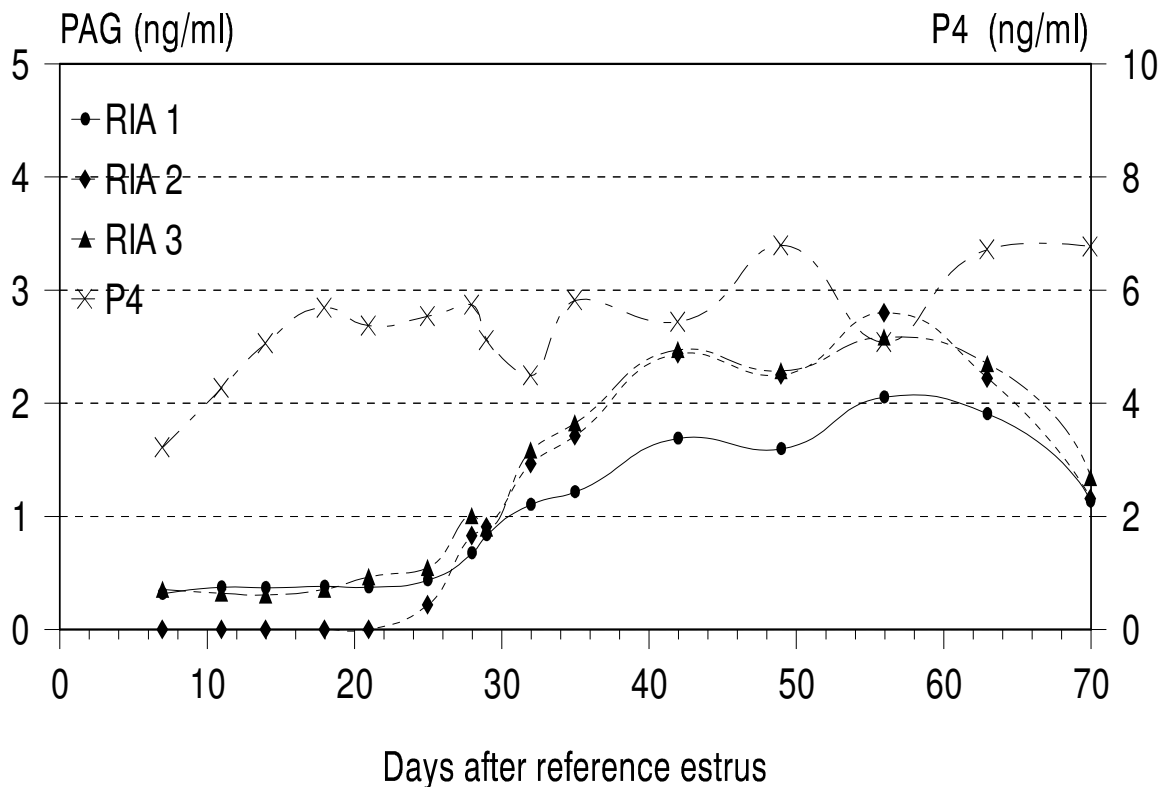
B 1629

Figure 18. PAG and P4 profiles of recipient n° B 1629

The P4 profile of recipient n° B 1629 was physiological in the observation period.

PAG concentrations showed remarkably slow increase after Day 21. After Day 56 decrease could be observed and at Day 70 the PAG concentration was close to 1 ng/ml. The peak PAG concentrations around Day 25-50 detected by RIA 2 and 3 (often observed in normal pregnancy) were missing.

By rectal palpation performed at Day 53 the cow was considered as pregnant, at Day 60 the ultrasonographic examination detected only fluid in the uterus.

Pregnancy failure type C

B 1678
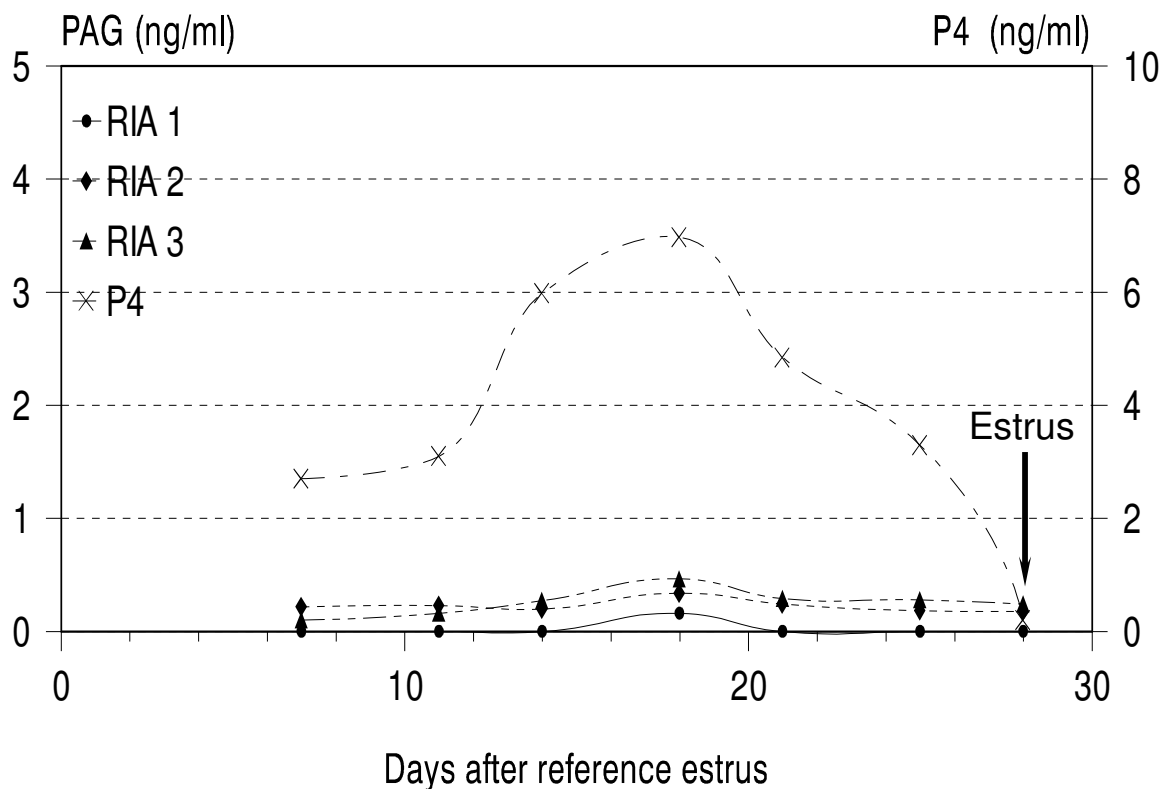
Figure 19. PAG and P4 profiles of recipient n° B 1678

Animal n° B 1678 showed decrease in P4 concentration from Day 18 till Day 28. Estrus was recorded on Day 28. Low PAG concentrations could be detected by all the three RIA systems at Day 18, after PAG levels decreased to basal values.

The small peak in the PAG profiles indicates that the embryo transferred was developed till around Day 18. The prolongation of the length of the estrous cycle also suggest that living conceptus was present in the uterus providing embryonic signal.

R 602

Figure 20. PAG and P4 profiles of recipient n° R 602

Progesterone concentrations were in the normal range. A sharp decrease can be observed in the P4 levels between Days 35 and 42. Estrus was observed on Day 41.
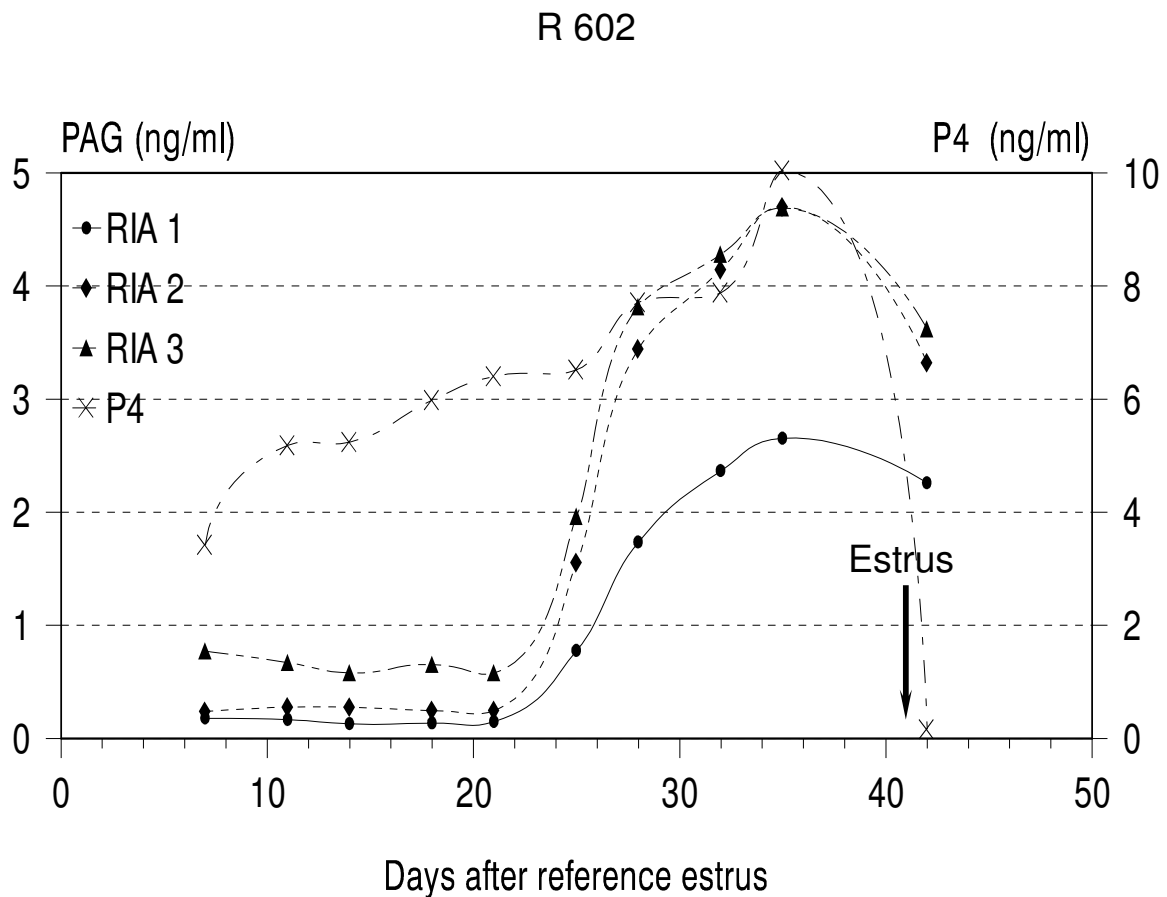
PAG concentrations started to increase after Day 21, reached peak values around Day 35, then they slowly decreased.

Chapter 7: DISCUSSION, CONCLUSIONS & PERSPECTIVES

In the present work, two additional RIA systems (RIA 2 and 3) were developed to measure PAG molecules in bovine maternal serum/ plasma. Their sensitivity (in terms of minimal detection limit), accuracy and precision were determined and compared to those of the RIA system (RIA 1) used as reference.

The specificity of the 3 RIA systems against the enzymatically active members of the aspartic proteinase family (pepsinogen, pepsin, rennin, rennet, renin, cathepsin D) was precisely investigated.

PAG concentrations were determined using the 3 RIA systems (including RIA 1) in samples obtained from pregnant cows at different intervals after AI. The ability of the 3 RIA systems to recognize PAG molecules in the maternal blood was compared.

PAG profiles determined by the 3 RIA tests in females with normal pregnancy were presented. By determining the RIA 2/RIA 1 and RIA 3/ RIA 1 ratios, these profiles were compared.

In our last experiment developed in cooperation with the Society of Holland Genetics and with the University of Utrecht, PAG and progesterone profiles determined in recipients after embryo transfer were presented.

Radioimmunoassays

The circulation of trophoblast derived proteins (PAG, PSPB, PSP60) over a certain concentration in the maternal blood is a good indicator of the presence of a living conceptus. In several diagnostic laboratories, immunoassays (RIA, ELISA) are used for routine pregnancy diagnosis. The limit of this approach lies in the relatively low concentration of PAG I_{67}, PSPB, PSP60 in blood during early stages of pregnancy. This is probably the result of the expression of these glycoproteins later in pregnancy (Green *et al.*, 2000).

This study was the first to describe the application of RIA systems in the bovine species that employs antisera produced against PAG molecules of smaller size isolated from the caprine placenta. These heterologous RIA systems were compared to a homologous RIA and were shown as more efficient to detect the circulating PAG molecules.

The characteristics of RIA systems developed for the detection of trophoblast derived proteins are shown in Table 1.

The sensitivity defined as the lowest standard amount distinguishable from zero determined in RIA 1 was 0.05 ng/ml. This concentration was lower than those of determined for the similar RIA systems by Zoli *et al.* (1992b) (0.20 ng/ml) and Ali *et al.* (unpublished) (0.65 ng/ml). The minimal detection limit (MDL) of RIA 3 (0.11 ng/ml) was the highest compared to the other two systems, RIA 2 (0.06 ng/ml) presented slightly higher MDL than that of RIA 1 (0.05 ng/ml). Compared to the sensitivity of RIA 2 and 3 reported by González *et al.* (1999) (0.4 and 0.1 ng/ml, respectively) our RIA 2 system was more sensitive (0.06 ng/ml) and RIA 3 presented similar sensitivity (0.11 ng/ml).

Table 1. Characteristics of RIA systems developed for the detection of trophoblast derived proteins

Reference	detected protein	antiserum dilution	sensitivity (ng/ml)	intra assay CV (%)	inter assay CV (%)
Sasser *et al.*, 1986	PSPB	1:300000	0.13	6.8±0.9	20.47±7.0
Zoli *et al.*, 1992b	PAG	1:250000	0.20	6.87±2.48	11.59±0.63
Mialon *et al.*, 1993	PSP60	1:1800000	0.20	6.0	12.0
Perényi *et al.*, 2002	PAG (by RIA 1)	1:450000	0.05	3.96-6.58	4.16-6.88
Perényi *et al.*, 2002	PAG (by RIA 2)	1:250000	0.06	2.57-8.81	3.04-11.32
Perényi *et al.*, 2002	PAG (by RIA 3)	1:1000000	0.11	4.47-10.10	5.30-15.55

The results we obtained from the newly validated RIA systems are suggesting that these tests provide valuable tools for further studies related to the bovine (ruminant) pregnancy.

Specificity of three RIA systems against the enzymatically active members of the aspartic proteinase family

In our second investigation it was found that the presence of pepsinogen, pepsin, chymosin and rennet over a certain concentration could decrease the binding of the radiolabelled PAG tracer to the antibodies. As the pepsin, pepsinogen, chymosin, PAG are belonging to the aspartic proteinase family,

the binding inhibition caused by these products can be explained by sequence identity between them, and probably by the ability of the antisera to recognize epitops having the same amino acid sequence. As pepsin is an endopeptidase, enzymatic degradation of the tracer and the antiserum could be also possible during the incubation phase of the assay but in this case the crossreaction should be observed in all the three RIA systems with the same intensity. In our study, the quantity of pepsin and pepsinogen causing observable crossreaction was different in each RIA system. This can probably be explained by the different recognition of these substances by the appropriate antisera, because of the epitop modification caused by the release of the N-terminal polypeptide segment during enzyme activation. However from these results it is not clear if the reacting epitops were located in the activation segments or in the mature protease.

As a normal serum pepsinogen concentration in bovine blood 44±12 ng/ml and 20±8 ng/ml were reported for heifers and cows, respectively (Harvey-White et al., 1983). The integrity of the abomasal mucosa can be altered by intestinal worms like Ostertagia species, Trichostrongylus axei, Haemonchus spp., abomasitis, abomasal displacement which can result in increased permeability of the abomasal wall towards macromolecules like pepsinogen. Plasma pepsinogen levels higher than 3000 mU tyrosine (> 97.71 ng/ml pepsinogen) were reported to have a diagnostic value for ostertagiasis (Selman et al., 1977). Calves with naturally acquired Ostertagia infection presented serum pepsinogen concentrations of 3500 to 9300 mU of tyrosine (114 to 288 ng/ml) (Harvey-White et al., 1983). Increased pepsinogen levels were reported by Vörös et al. (1984) as a result of diseases like abomasal ulceration, abomasitis (125-443 ng/ml), left (77±5.2 ng/ml) and right (59±3.7 ng/ml) abomasal displacement. The serum pepsinogen concentrations reported by these authors never reached the concentration range (50-1000 µg/ml) where pepsinogen was able to crossreact in the three RIA systems examined. So our PAG test cannot be suspected to be influenced by increased pepsinogen concentrations even in pathological conditions like helminthosis and abomasal diseases.

According to our results renin concentrations reported in the bovine plasma (0.026 GU/l) (Deinum et al., 1990; Nielsen et al, 1991) and in the uterus (0.043-0.67 GU/kg) (Schauser et al., 1999) can not cause detectable crossreaction with the three RIA systems used in this study.

Cathepsin D is an indigenous endopeptidase of the bovine milk, its concentration together with procathepsin D was estimated to be 0.4 µg/ml in bovine skim milk (Larsen et al., 1996).

As PAG molecules have been found in detectable levels by RIA in milk of pregnant goats (González *et al.*, 2001), their determination in the milk of different ruminant species became object of interest. During the incubation phase of RIA tests performed in milk samples, milk proteins mainly casein are often precipitated causing non desired interferences like increased non specific binding. Our study clearly showed that rennet does not crossreact in the examined RIA systems (under the concentration of 1 mg/ml), so it can be applied before the assay in order to coagulate casein, eliminating with this a main source of inaccurate results. The precipitated milk proteins can be separated later by centrifugation.

In conclusion, crossreaction between the RIA systems examined and the aspartic proteinases could not result in the detection of higher PAG concentrations in plasma samples, than really being present in the blood. In this study using very high concentrations of various aspartic proteinase members, we showed that crossreaction exists between the three RIA systems and pepsin, pepsinogen, chymosin and rennet however these interferences are out of the concentration range as these enzymes occur in blood. According to our results, RIA 1, 2 and 3 systems can be considered as specific for the detection of PAGs against pepsinogen, pepsin, chymosin, renin, cathepsin D in the range of concentration in plasma or serum as they were reported in the literature.

PAG concentrations determined in nonpregnant cows and pregnant animals after AI

Determination of PAGs in maternal blood can be an important tool to diagnose early pregnancy in the cow and to follow up ongoing pregnancies.

In our first experiment, some of the nonpregnant heifers presented low, detectable PAG concentrations by the three RIA systems. Similar observations were reported by Zoli *et al.* (1992b) in unbred heifers and bulls. In humans Rosen *et al.* (1982) observed the production of trophoblast-specific proteins (SP$_1$) by nontrophoblastic cells. Zoli *et al.* suggested the extraplacental production of pregnancy specific proteins in the gonads (testes, ovaries) of the ruminants (1990bc). These results mean that the detection of very low levels of PAG may not necessarily indicate pregnancy. In contrast, Sasser *et al.*

(1986) found that the pool of virgin heifer sera did not cause any inhibition in the binding of ^{125}I-PSPB.

In our third experiment PAGs already at Day 21 were detected in the maternal blood in 38.77%, 75.51% and 73.47% of the pregnant animals by RIA 1, RIA 2 and RIA 3 respectively. The PAG concentrations measured by RIA 1 at Day 21 after AI (0.41±0.23 ng/mL) were similar to those reported by Zoli *et al.* (1992b) in cows Day 22 after AI (0.38±0.13 ng/mL) and higher than those showed by Szenci *et al* (1998a) (0.26±0.04 ng/mL). Compared to our results Patel *et al.* (1997) reported slightly higher PAG concentrations (0.5±0.1 ng/mL) at Day 20 after AI in cows with a single conceptus, while in the twin-bearing animals, a 1.0±0.6 ng/mL PAG concentration was found. Using a sensitive PSP-60 RIA test, Patel *et al.* (1998) reported 0.9±0.2 ng/ml PSP-60 concentration in cows Day 20 postoestrus.

The serum profiles of PAG determined by the 3 RIA systems were similar to the PAG profiles reported by Zoli *et al.* (1991). Similar changes were reported in the plasmatic concentration of PSPB (Sasser *et al.*, 1986) and PSP60 (Mialon *et al.*, 1993) after fertilization, which probably confirm that PSPB and PSP60 correspond to a member of the PAG family.

The appearance of PAG molecules in detectable concentrations was observed from Days 19-28, with high individual variation. Sasser *et al.* (1986) reported a variation from Day 15 to Day 28 postbreeding concerning to the earliest day of detection of PSPB in the blood of 21 cows using a double antibody RIA for bPSPB.

After the appearance of PAG molecules in blood intensive increase was observed by the 3 RIA systems. A short plateau phase and or a decrease was found in our study after Days 33-34. For this period of pregnancy a decrease in the PSP60 secretion was found (between Days 35 and 50) by Mialon *et al.* (1993), whereas Sasser *et al.* (1986) observed relative stable levels (3±0.6 ng/ml) of PSPB (between Days 42 and 70). It is remarkable that, for the first 50-60 day period of pregnancy the PAG profiles determined by our RIA 2 and RIA 3 systems showed discrepancy (characterized by higher RIA 2/ RIA 1 and RIA 3/ RIA 1 ratios) compared to the RIA 1 reference system. Heifer n° 9447 showed such an intensive decrease between Days 44-60 that at Day 60 using a routine PAG RIA test it could be mistakenly considered as nonpregnant. The decrease in the PAG secretion can be the result of a change in the regulation of the development of the foeto-placental unit.

Major increase was detected by our RIA systems at Day 268-271, at the end of the pregnancy. The PAG levels determined in our study were higher than those reported by Sasser *et al.* (1986) for PSPB and Mialon *et al.* (1993).

Heifer n° 1654 showed a relative early dramatic PAG rise, it began four days after the anti bPL injection, its evolution was shorter (3-4 days) than it was observed in the other two heifers (4-8 days). This animal calved 4 days after the beginning of the PAG increase while this interval was 9 days at the other two animals. The explanation of this sudden increase in the PAG levels is unknown. Some authors suggested that these proteins are probably involved in the induction of parturition through an unknown pathway or it is possible that this phenomenon is only the result of certain physical, hormonal changes in the placenta (Sasser *et al.*, 1986; Mialon *et al.*, 1993; Zoli *et al.*, 1992b). It seems likely that in heifer n° 1654 the intrafetal injection of anti bPL induced the early termination of pregnancy. We can hypothesize that the puncture of the fetus induced a stress and a consequential activation of the fetal suprarenal glycocorticoid synthesis which could participate in the initiation of the hormonal process of parturition.

RIA 2 and RIA 3 systems showed detectable concentrations of PAG at around the same time than RIA 1. The early increase detected by RIA 2 and 3 was more intensive than that of RIA 1.

After the first 60 days of pregnancy there was no remarkable difference detected in the PAG profiles determined by the 3 RIA.

Since the work of Xie *et al.* (1997) it is known that there are probably more than 100 PAG genes in the ruminant genome and most of them being expressed in the superficial layers of the placenta. Till now only one PAG had been purified from bovine placental cotyledons (Zoli *et al.*, 1991). Using RNase protection assays, Green *et al.* (2000) showed, that in bovine placentomes, the PAG I_{67} transcripts were not present at Day 25 after AI, these transcripts became first detectable 45 days after AI.

The fact that, in agreement with our results various authors reported detectable, increasing PAG, PSPB concentrations before Day 45: 22 day pc. Zoli *et al.* (1992b), 27 days after fertilization Mialon *et al.* (1993), 24 days after AI Sasser *et al.* (1986) suggest that these RIA systems probably were able to detect also PAG molecules different and earlier expressed than PAG I_{67}. However it should be considered that there is a quite high individual variation among cows in date of the first increase in the PAG concentrations (Zoli *et al.*, 1992b; Sasser *et al.*, 1986).

RIA 2 and RIA 3 gave higher plasma PAG concentrations in the observation periods of our experiments, showing that anti PAG_{55+59}, and anti PAG_{55+62} antisera in conjunction with the PAG I_{67} tracer are probably able to better recognize the earlier forms of circulating PAGs in the plasma of pregnant cows, than the RIA 1 system. The higher percentage of animals

showing detectable concentrations of PAG in RIA 2 and 3 at 21 days after AI in the third experiment also confirms this hypothesis. In the individual profiles between Days 33-44 peak PAG concentrations were determined by RIA 2 and 3 with higher RIA 2/ RIA 1 and the RIA 3/ RIA 1 ratios than in the other parts of the curves. These observations suggest that RIA 2 and RIA 3 also detected additional PAG molecules, non identical with PAG I_{67} which are secreted into the maternal blood in this period. As the only component, which was different in RIA 1, RIA 2 and RIA 3 was the antiserum, these results might be explained by the different ability of the antisera to recognize certain PAG molecule-specific epitops. The antisera against PAG_{55+59} and PAG_{55+62} used in this experiment were specific for the PAG family and were able to recognize the individual PAG family members with different affinities. The higher plasma concentrations found by using RIA 2 and RIA 3 throughout the observation periods suggest a possibility to detect PAGs expressed earlier in the trophoblast. At Day 21 after AI the 0.598 correlation coefficient is the result of the different efficiency of RIA 2 and 3 to detect PAG molecules; however it can also be the result of the low PAG concentrations close to the detection limits of the three assay systems. Together with these observations, the significantly higher PAG plasmatic concentration obtained by RIA 3 could prove that this assay system is able to detect better a PAG secreted earlier into the maternal blood.

In conclusion, our results clearly showed that the ability of a PAG RIA system to recognize early PAGs can be improved by using antisera raised against different forms of PAGs ($PAG_{55,59,62}$). The 2 additional RIA systems (besides RIA 1) has proven useful for studying the course of pregnancy.

Possible detection of additional PAG family members ?

Molecular biology studies showed that there is a great sequence identity between different genes encoding PAG molecules (Xie *et al.*, 1997). PAG transcripts isolated from different species are showing high sequence identity to each other (Xie *et al.*, 1997). In some cases the sequence identity was higher between PAG transcripts originating from different species, than between PAG transcripts isolated from the same species. The NH_2-terminal sequence of 3 PAG molecules used for the antibody production in our

experiment were compared to the inferred amino acid sequence of various boPAG transcripts (Table 2.).

The data of Table 2 suggest the following hypotheses:

- Because of similarity of the structure of PAG I_{67} (boPAG-1) and PAG_{55}, PAG_{59}, PAG_{62}, the antisera produced against the three forms of caprine PAGs are able to recognize epitops located on PAG I_{67}.

- It can be seen in Table 2, considering the 27 NH_2-terminal amino acids, that PAG_{55} shares more sequence identity with boPAG -4-7, -9, -11, -16-18 than with PAG I_{67}, PAG_{59} share more identity with boPAG -16-17 than with PAG I_{67}, PAG_{62} shared more identity with boPAG -2, -4-7, -9, -15-18 than with PAG I_{67}.

We can suspect that the proteins sharing more sequence identity with each other have more identical epitops also. This would mean that the antisera produced against $PAG_{55,59,62}$ could probably better recognize the epitops located on certain more identical boPAG molecules (*e.g.* boPAG-2, -4-7, -9, -11, -15-18).

Table 2. Sequence comparison between PAG_{55}, PAG_{59}, PAG_{62} proteins and boPAGs (the base of the comparison was the amino terminal sequence (27 amino acids) of PAG_{55}, PAG_{59}, PAG_{62} proteins, and the sequences inferred from the cDNA of the boPAGs obtained from SWISSPROT)

	Sequence identity (%)						
	PAG I_{67} boPAG-1	boPAG-2	boPAG-4	boPAG-5	boPAG-6	boPAG-7	boPAG-8
PAG_{55}	68.18	59.26	77.27	77.78	72.73	77.27	38.46
PAG_{59}	74.07	56.00	66.67	70.37	66.66	66.67	33.33
PAG_{62}	62.96	69.23	70.37	70.37	78.95	74.07	44.44
PAG I_{67}	100.00	57.89	78.16	74.87	80.00	77.42	52.85

	Sequence identity (%)						
	boPAG-9	boPAG-10	boPAG-11	boPAG-12	boPAG-13	boPAG-14	boPAG-15
PAG_{55}	77.27	34.61	70.00	59.26	59.26	68.18	66.67
PAG_{59}	66.67	38.46	65.21	51.85	56.00	70.37	69.23
PAG_{62}	66.67	42.31	57.69	62.96	62.23	55.56	68.00
PAG I_{67}	78.16	56.40	59.74	60.32	59.09	72.99	82.37

	Sequence identity (%)					
	boPAG-16	boPAG-17	boPAG-18	boPAG-19	boPAG-20	boPAG-21
PAG_{55}	77.27	85.18	74.07	63.64	63.64	68.18
PAG_{59}	81.48	77.78	69.23	62.96	74.07	74.07
PAG_{62}	66.67	66.67	64.00	59.26	62.96	62.96
PAG I_{67}	82.89	79.21	81.63	81.32	81.32	80.79

Green *et al.* (2000) investigated the expression of boPAG –1, -2 and –4-11 throughout different periods of pregnancy. In that study it was found that between Days 25-45 after fertilization boPAG-2, -4, -5, -8-11 were expressed in the bovine trophectoderm, whereas PAG I_{67}, boPAG-6, -7 were not expressed.

As during this period the PAG I_{67} was not expressed, and in our study we could measure detectable concentrations of PAG using the 3 RIA systems, we can suggest considering the sequence comparison results presented in Table 2, that with RIA 2 and RIA 3 we probably detected better boPAG-2, -4, -5, -9, -11 and boPAG-4, -5, -9, -11 respectively. During the 25-45 day period of pregnancy, RIA 1 system could probably detect better boPAG-4, -5, -9 (as they are related closer to PAG I_{67}) than the also expressed boPAG-2, –8, -11 molecules.

In the individual profiles presented in investigation 4, the discrepancy between RIA 2, RIA 3 and RIA 1 suggested that during the first 50-60 days of gestation additional PAG molecules different from PAG I_{67} secreted in the maternal blood.

Our results suggest that by the use of RIA 2 and RIA 3 we are able to detect PAG I_{67} and probably some additional (identified by molecular biology but not isolated from tissue) PAG molecules in the maternal blood circulation of the bovine species. Till now there is no such a bovine PAG molecule known what is expressed only during the first trimester of pregnancy according to the pattern that our results suggest. In the goat, caPAG-2 was found to be expressed only in early pregnancy (Days 18 and 19) (Garbayo *et al* 2000). The existence of such a molecule in the bovine species, similarly to the goat, is likely. As there is no information available concerning to the expression of boPAG-12-21, further investigations should be performed in that field.

Despite several attempts in our laboratory aiming to purify PAG molecules from bovine cotyledonary extracts using classical purification procedures (Zoli *et al.*, 1991) and purification methods that employ pepstatin agarose affinity chromatography, we could only detect and sequence the PAG I_{67}. It seems likely that these bovine PAG molecules are sensitive for the degradation which probably occurred just after the placenta collection and during the purification. It is also possible that their quantity in the placenta is very low and their detection during the different steps of the purification requires highly sophisticated equipments and procedures.

Our results suggest that there is a sense to use RIA systems employing antisera produced against PAG molecules isolated from an other species to determine PAG concentrations in the maternal blood during pregnancy in the bovine species. The purification of bovine PAGs different from PAG I_{67} is difficult. The purification of PAG molecules from other species can help to better investigate the PAG molecules expressed in the bovine species, as the purification of these non bovine PAG molecules can be easier, and some of them likely to share high sequence identity with the bovine PAGs.

Pregnancy follow up I.

From the experimental animals presented in the 4th investigation two heifers showed the signs of pregnancy failure. In heifer n° 2792, the decrease in the PAG profile (after Day 51) occurred earlier than the drop in the P4 profile (after Day 62) which suggest that the trophoblastic function was decreased prior to the luteal function of the mother. The lower intensity in the increase of PAG concentrations observed between Days 33-51 could be the result of the alteration of the trophoblastic function.

Heifer n° 8368 aborted on Day 144. The PCR analysis performed on brain samples of the 2 aborted fetus suggested that the cause of the abortion was *Neospora Caninum* infection. Abortion, occurring during mid gestation, is the primary clinical sign of Neospora infection in cattle. (Anderson *et al.*, 2000). The PAG profile of this animal showed that the synthetic activity of the trophoblast was already modified between Days 95 and 110, when the first remarkable decrease was observed in the PAG concentrations. In contrast, according to the immuno fluorescent antibody test performed at Day 100, the mother was serologically negative. Positive serological result was obtained only 2 days before abortion. We cannot rule out the possibility that between Days 95 and 110 the parasite was already present in the mother and the tachyzoites caused destruction in the trophoblastic tissue. From these results it is likely that there is a relationship between the Neospora infection and abortion, but the role of this parasite in the decrease of PAG concentrations observed between Days 95 and 110 is unclear.

Embryo transfer, Pregnancy follow up II.

In our fifth investigation, we observed pregnancy failures in 56.7% of the total number of transfers. 32.1% of the recipients returned to estrus before Day 24, which was higher than the pregnancy loss reported by Heyman *et al.* (1985) for the first 24 days of pregnancy. These authors reported that 24.3% of the recipients suffered pregnancy failure between Days 24 and 90, which is similar to our observation (22.4%) for the period between Days 24-119. The incidence of pregnancy failures was similar in the MOET (52.5%) and in the Cocultured group (52.3%), higher incidence of pregnancy failure was found in the SOF group (63.2%). Higher incidence of embryonic loss was also reported after non-surgical transfer of IVF embryos (Greve *et al.*, 1993; Horta *et al.*, 1993). Horta *et al.* (1993) showed that, heat-stressed cows receiving IVF embryos suffered higher embryo/ fetal losses than heat-stressed cows after AI.

Farin *et al.* (2001) reported that bovine placentas from embryos produced *in vitro* had increased volume densities of BNCs, BNCs invading the caruncular endometrium and cotyledonary pyknotic cells compared to placentas from embryos produced *in vivo*. Theoretically this can result in higher maternal blood concentrations of products synthesized in the BNCs. However, in samples from animals previously received embryos produced with the three different methods and calved in term, Vos *et al.* (2000) did not observe significant difference in the PAG concentrations.

The maintenance of the early pregnancy in cow requires extended lifespan of the functional corpus luteum (Lauderdale *et al.*, 1972; Millar *et al.*, 1974; Kosugiyama *et al.*, 1978; Lindell *et al.*, 1980). Decrease in the luteal function caused by luteolytic treatment resulted in rapid loss of the conceptus (Kastelic *et al.* 1989). The relationship between inadequate luteal function and infertility in cattle was shown by Odde *et al.* (1980). Luteal insufficiency can be characterized by corpora lutea which have a short lifespan or by corpora lutea having normal lifespan but decreased progesterone secretion (Gaverick and Smith, 1986). In the background of luteal insufficiency, incorrect preovulatory follicular development, depressed luteotrophic stimulation, events affecting the ovulation can be found (Hunter *et al.*, 1991; Gaverick *et al.*, 1992).

In our experiment, in the case of pregnancy failures classified as type A, deficiency could be observed in the luteal function (characterized by the falling P4 levels) prior to the death of the embryo indicated by decreased synthesis of PAG molecules by the trophoblast. In these animals the consequences of low progesterone levels were the insufficient endometrial gland secretion and

increasing myometral contractivity, which lead to metabolic disorder and the loss of the conceptus.

In the case of recipient R 599, the embryo was transferred into a heifer where there was no detectable P4 secretion. This animal demonstrated that the embryo could not develop (successful pregnancy cannot be established) without the presence of functional corpus luteum. The lack of P4 prevented to prepare the endometrium to satisfy the increasing demands for histotrophic support by the growing conceptus. It seems that the embryo transferred developed and produced PAG earlier and for a limited period of time, the successful attachment and implantation of the embryo was failed, however this environment probably stimulated the earlier PAG secretion of the surviving trophectoderm. Perhaps only trophectoderm cells were able to survive in the uterus for a few weeks before being cleared by the immune system or dying for other reasons. We can hypothesize also, that the CL of this animal synthesized a form of progesterone (5 alpha or 5 beta progesterone) that probably was not detected by our RIA system. Because acyclicity is also relatively frequent in heifers, we can hypothesize that this animal was in anoestrus.

In the case of embryonic mortalities classified as type B, the luteal function of the mother could be considered as physiological according to the P4 profile till the day of the embryonic death. The reason of the embryonic loss was probably in the embryo. Genetic factors, chromosomal abnormalities can cause early embryonic mortality in cattle. Homozygous recessive embryos for deficiency of uridine-5`-monophosphate (UMP) synthase are believed to die before Day 40 of gestation. Dilution procedures (Massip *et al.*, 1993), culture conditions (used mediums, gases, protein supplement, presence of bovine oviduct epithelial cells)(Fukui *et al.*, 1991) have strong influence on the viability, development rate of the embryos. Placental abnormalities, increased fetal losses were often observed after the use of *in vitro* procedures or cloning techniques (Hill *et al.*, 2000). Fetuses with subnormal placental development, slowly starve because of the insufficient supply of nutrients. These changes can negatively affect the protein synthesis in the trophoblastic cells which result in decreasing PAG concentrations in the blood of the recipient. Higher risk of pregnancy failure was reported for twin pregnancies through the gestational period (Day *et al.*, 1997; Lopez-Gatius *et al.*, 2002). In these cases the insufficient uterine space for two or more embryos is the major cause of the pregnancy loss. The protein described by Beckers *et al.* (1988a) and identified as PAG-2 (Xie *et al.*, 1994) was reported to have a luteotrophic activity. We can hypothesize that the decreased trophoblastic synthesis of this

protein can be a factor that plays role in the decreased P4 synthesis of the corpus luteum, which was observed after the decrease in the PAG concentrations.

The P4 and the abnormal PAG profiles of recipient B 1629 together with the clinical observations (rectal palpation, ultrasonography) suggested clearly that in this case not the corpus luteum function was the cause of the embryonic loss. If the embryo had damaged inner cell mass already at the time of transfer, the degeneration of the cells forming the embryonic disc could result in the development of trophoblastic vesicle. The growing trophoblastic vesicle could accumulate fluid, which can be detected by ultrasonography as a liquid containing structure in the uterine lumen. It has been demonstrated that after transplantation of trophoblastic vesicles into recipient heifers and ewes, the corpus luteum can be maintained until Day 37 and Day 54, respectively (Heyman *et al.* 1984). It is also possible that after the transfer the embryo had an asynchronous, delayed trophectoderm development with normal inner cell mass development. This could be due to culture conditions and it has been shown, at least with cloned embryos, that poor placentation takes place with reduced vascularisation of the placentome (Hill *et al.*, 2000). This results in increased embryonic loss within the first 2 months of the pregnancy.

The embryonic losses classified as type C, expressed decrease in P4 and PAG profiles beginning at about the same day after reference estrus. In these cases the origin of the embryonic death can be in the recipient (decreased, abnormal luteal function) or/and in the embryo (decreased viability of the cells of the embryonic disc or trophoblast).

This work brought a new investigation approach to better understand the nature of late embryonic mortalities occurring after embryo transfer. The results of this study suggest that the determination of P4 and PAGs in the maternal blood combined with ultrasonographic detection of the embryonic heart beating could be very efficient tool in the pregnancy follow up.

Conclusions

The results we obtained from the newly validated RIA systems are suggesting that these tests provide valuable tools for further studies related to the bovine pregnancy.

The lack of interference with the enzymatically active members of the aspartic proteinase family (pepsinogen, pepsin, rennin, rennet, renin, cathepsin D) at physiological and pathological concentrations they occur in the blood means that these RIA systems can be considered as specific for the detection of PAG molecules.

Our results clearly show that the ability of a PAG RIA system to recognize early PAGs can be improved by using antisera raised against different forms of PAGs ($PAG_{55,59,62}$). The 2 additional RIA systems (RIA 2 and RIA 3) have proven useful for studying the course of pregnancy, especially during the first trimester.

Our fifth investigation brought a new approach to better understand the nature, the evolution and the pathogeny of late embryonic mortalities occurring after transfer of embryos from different origin. The results of this study show that the determination of P4 and PAGs in the maternal blood are very efficient tools in the pregnancy follow up.

Perspectives

It seems that PAG I_{67} is more abundant and stable than the other PAG molecules expressed in the bovine trophectoderm. This could be the reason that this PAG was isolated first by Zoli *et al.* (1991). From our results it can be suggested, that further investigations should be carried out in order to isolate and characterize the PAG molecules released in early pregnancy and detected with higher efficiency with RIA 2 and RIA 3 in the bovine species. RIA 2 and 3 systems would be efficient tools to follow the immunoreactive PAG containing fractions during a purification procedure. Affinity chromatography using antisera against $PAG_{55,59,62}$ bound to the agarose matrix could be used for such a purification. The individual PAG profiles suggesting that such a purification should be performed using cotyledons collected from cows during the first 60 days of their pregnancy.

Production of monoclonal antibodies directed against PAG specific epitops could help to improve the specificity of the RIA. During biochemical purification the immunoreactive (PAG containing) fractions between the different purification steps are controlled by RIA tests and Western blot. The application of PAG specific monoclonal antibodies in RIA or Western blot could improve these techniques by decreasing the binding of the antibodies to non PAG molecules, and it could allow the isolation of PAG molecules different from PAG I_{67}.

Determination of PAG concentrations at different times after AI in a large number of cows belonging to the same breed would permit to establish breed specific PAG profiles. Like this, the stage of pregnancy could be predictable on the basis of serum/ plasma concentration of PAG. This system would be especially useful, when the breeding dates are not known (in extensive animal keeping conditions).

The detection of the prepartum PAG peak using PAG test performed during late pregnancy could help to determine the approximate time of calving. This could be especially useful in the case of valuable breeding cattles or cows participating cloning experiment where veterinary intervention seems to be necessary at the calving.

The determination of P4 and different PAG molecules in plasma samples collected frequently combined with ultrasonographic detection of the embryonic heart beating and rectal palpation could be very efficient tool in the pregnancy follow up and in studies of embryonic mortality.

Since a large part of embryos obtained by *in vitro* techniques does not develop correctly, a better monitoring of recipients (PAG determination, ultrasonographic examination) could allow to detect the abnormal placental and fetal development earlier. The availability of an earlier diagnosis will permit to make a rapid decision concerning to the endangered pregnancy and to determine the best conditions for in vitro embryo production and manipulation

The analysis of P4 and PAGs profiles from maternal blood of animals receiving embryos produced in vitro using different culture media allows to investigate the influence of those culture media on the development of the embryo and the trophoblast.

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Summary

Glycoproteins expressed in the ruminant placenta have been isolated and characterized during the last two decades: PSPB (Butler *et al.*, 1982); PAG (bPAG1, PAG I_{67}, bovPAG 1) (the number in the subscript refers to molecular weight in kDa) (Zoli *et al.*, 1991); PAG_{55}, PAG_{59}, PAG_{62} (Garbayo *et al.*, 1998).

Pregnancy specific proteins were identified and characterized as early as 1982 by Butler *et al.* (1982) as PSPA and PSPB. The first, PSPA was considered as corresponding to bovine α-fetoprotein and thus was not strictly specific for pregnancy. The second one, the PSPB later named bPSPB was rapidly proposed as a good marker, detectable in peripheral blood, for pregnancy diagnosis and follow up (Sasser *et al.*, 1986). In 1991 Zoli *et al.* (1991) isolated and characterized placental glycoproteins having a molecular weight of 67 kDa and four isoelectric points (4.4, 4.6, 5.2 and 5.4) with the following NH_2-terminal sequence: Arg-Gly-Ser-x-Leu-Thr-Thr-His-Pro-Leu. Soon after, molecular cloning investigations on bovine and ovine placenta, showed that PAGs belonged to the aspartic proteinase family sharing sequence identity with pepsin (57%), pepsinogen (49.5%), cathepsin D (58%) and E, chymosin (42.5%) and renin (Xie *et al.*, 1991b; Xie *et al.*, 1994; Xie *et al.*, 1995). Distance phylograms showed that the PAG gene family probably diverged from the enzymatically functional aspartic proteinases (Xie *et al.*, 1997). Guruprasad *et al.* (1996) suggested that the bilobed three-dimensional structure of bovPAG-1 was similar to that of pepsin and chymosin, moreover bovPAG-1 was able to bind pepstatin to its binding site like the other enzymatically active aspartic proteinases. In 1992, Lynch *et al.* found a high cDNA sequence identity between PSPB and bovPAG I_{67} and classified the PSPB in the same aspartic proteinase family. Only the PAG I_{67} was characterized in term of carbohydrate and sialic acid content (Zoli *et al.*, 1991). In the following years other pregnancy-associated glycoprotein sequences were identified in the ruminant placenta by molecular biology studies (Xie *et al.*, 1997; Green *et al.*, 2000).

In 1998, some PAG molecules purified from caprine placental extracts were characterized as PAG_{55} (NH_2-terminal sequence: Ile-Ser-Ser-Pro-Val-Ser-x-Leu-Thr-Ile), PAG_{59} (NH_2-terminal sequence: Arg-Gly-Ser-x-Leu-Thr-Thr-Leu-Pro-Leu), PAG_{62} (NH_2-terminal sequence: Arg-Asp-Ser-x-Val-Thr-Ile-Val-Pro-Leu) (Garbayo *et al.*, 1998).

Molecular biology investigations showed that there are probably more than 100 PAG genes in the ruminant genome (Xie *et al.*, 1997) most of them being expressed in the superficial layers of the placenta. For example, in the bovine species, two different patterns of expression were found: some of the PAGs like bovPAG-2, -8, -10 and -11 were expressed in the mono- and binucleate cells of the trophectoderm, while other proteins like bovPAG-1, -4-7, -9 were localized in the binucleate cells (Green *et al.*, 2000). Molecular biology investigations also concluded, that during certain stages of pregnancy some PAGs were expressed, while others were absent (Garbayo *et al.*, 1999; Green *et al.*, 2000). In trophectoderm of conceptuses removed on Day 25, 45, 60, 88, 150, 250 after fertilization, Green *et al.* (2000) detected the mRNA encoding for PAG I_{67} from Day 45, while the mRNA for bovPAG-4 and bovPAG-9 were predominantly present at Day 25. An important question remained unanswered: were PAGs secreted into the maternal circulation earlier and is it possible to evidence their presence in the maternal blood. Surprisingly, compared to molecular biology studies, the biochemical approaches allowed the purification of a few different molecules.

Three different radioimmunoassay systems (RIA 1, RIA 2 and RIA 3) using antisera produced against PAG I_{67} (RIA 1), PAG_{55+62} (RIA 2) and PAG_{55+59} (RIA 3) were used in this work. Two of these additional RIA systems (RIA 2 and RIA 3) were developed and validated in this study for the detection of pregnancy-associated glycoproteins. Their sensitivity (in term of minimal detection limit), accuracy, precision were characterized and compared to those of RIA 1. The specificity of the three RIA systems against the commercially available, enzymatically active members of the aspartic proteinase family like pepsinogen, pepsin, chymosin, rennet, cathepsin D, renin were determined (Perényi *et al.*, 2002b). These products were tested in a wide concentration range (10 ng/ml to 1 mg/ml). Pepsinogen crossreacted in RIA 1, RIA 2 and RIA 3 over 1 mg/ml, 50 μg/ml and 500 μg/ml concentrations, respectively. In the presence of pepsin, crossreaction was observed in RIA 1, RIA 2 and RIA 3 over 1 mg/ml, 500 μg/ml and 1 mg/ml concentrations, respectively. Chymosin, rennet, could crossreact in RIA 2 and RIA 3, while renin and cathepsin D did not decrease the binding of the tracer to antisera more, than that of the minimal detection limit. As the plasma / serum concentrations of the examined aspartic proteinases reported in the literature were outside the concentration range where crossreaction was observed, it can be concluded that these RIA systems were specific for the detection of PAGs in biological fluids.

The three RIA systems were used in order to measure the PAG concentration in plasma samples withdrawn from pregnant cows and heifers

throughout different periods following artificial insemination (AI) (Perényi *et al*, 2002a). These systems were able to detect PAG molecules in the maternal blood as early as 21 days after AI in different concentrations (RIA 1: 0.43±0.24 ng/ml, mean±SD; RIA 2: 0.48±0.24 ng/ml; RIA 3: 0.64±0.37 ng/ml). On Day 32 and 42 RIA 2, (4.30±1.32 ng/ml, 5.56±1.95 ng/ml) and RIA 3 (4.17±1.15 ng/ml, 5.60±1.89 ng/ml) presented significantly (p<0.0001) higher PAG concentrations than those of RIA 1 (2.43±0.81 ng/ml, 4.01±1.48 ng/ml), respectively. After Day 21, significant correlations (p<0.0001; r≥0.929) were determined between the three systems. This study clearly indicated that the ability of a RIA test to recognize PAG molecules in the maternal blood can be improved by carefully selecting the antiserum. As the PAG I_{67} transcripts became first detectable 45 days after AI, our observations, suggest that all the three RIA systems are probably detecting PAGs different and earlier expressed than PAG I_{67}.

We also aimed to determine PAG concentrations in samples removed frequently from pregnant heifers. Using three PAG RIA systems, we presented two partial and five complete individual PAG profiles from our experimental animals. Five of the profiles were representing normal ongoing pregnancies, whereas two of them pregnancies with abortion and embryonic loss. The ratios between the concentrations determined by the newly developed RIA systems and RIA 1 system were calculated. Decrease could be observed during the first 60 days of pregnancy in these ratios. The analysis of individual PAG profiles presented showed that PAG molecules secreted in the maternal blood between 21 and 60 days after AI were better recognized by RIA 2 and 3 systems.

From our observations it can be suggested, that further investigations should be carried out in order to isolate and characterize the PAG molecules released in early pregnancy and detected with higher efficiency with RIA 2 and RIA 3. RIA 2 and 3 systems would be efficient tools to follow the immunoreactive PAG containing fractions during a purification procedure.

The individual PAG profiles suggesting that such a purification should be performed using cotyledons collected from cows during the first 60 days of their pregnancy.

In a collaborative study 268 embryo recipients were followed by clinical observation and blood sampling at short time interval (every three day from Day 7 until Day 35, then once a week until Day 119). In this study PAG and P4 profiles are presented which were determined from maternal blood. The analysis of the profiles allowed to classify the recipients into 3 groups: Group

A: abnormality first occurred in the P4 profile; Group B: abnormality first occurred in the PAG profile; Group C: abnormality occurred at the same time in the P4 and PAG profiles. Pregnancy failures classified as type A were probably originating from the mother, while type B pregnancy failures from the embryo. In the case of type C pregnancy failures the reason of the embryonic loss was in the mother or/and in the embryo. By the retrospective analysis of these profiles valuable informations were gathered concerning to the origin and the pathogeny of the pregnancy failure.

The determination of P4 and different PAG molecules in plasma samples collected frequently combined with ultrasonographic detection of the embryonic heart beating and rectal palpation could be very efficient tool in the pregnancy follow-up.

The analysis of P4 and PAGs profiles from maternal blood of animals receiving embryos produced in vitro using different culture media allows to investigate the influence of those culture media on the development of the embryo and the trophoblast.

In conclusion, new efficient tools for the pregnancy detection and follow up in cattle were developed in the first part of our study. The efficiency of these RIA systems was verified in normal and failed pregnancies followed by frequent blood sampling in our experimental farm. These assays were used to characterize a large series of pregnancies after transfer of embryos of different origin.

Résumé

Durant les vingt dernières années, des glycoprotéines exprimées dans le placenta des ruminants ont été isolées et caractérisées: la PSPB (Butler *et al.*, 1982), la PAG (bPAG 1, PAG I$_{67}$ ou encore bov PAG-1) (le nombre en indice fait référence ou poids moléculaire exprimé en kDa) (Zoli *et al.*, 1991) et les PAG$_{55}$, PAG$_{59}$, PAG$_{62}$ (Garbayo *et al.*, 1988).

Des protéines spécifiques de la gestation ont été identifiées et caractérisées dés 1982 par Butler *et al.* en tant que PSPA et PSPB. La première, PSPA était considérée comme correspondant à l'α fœtoprotéine bovine et de ce fait elle n'était pas strictement spécifique de la gestation. La seconde, la PSPB dénommée plus tard bPSPB, a rapidement été proposée comme un bon marqueur détectable dans le sang périphérique pour le diagnostic et le suivi de la gestation (Sasser *et al.*, 1986). En 1991, Zoli *et al.* isolait et caractérisait des glycoprotéines placentaires présentant une masse moléculaire de 67 kDa et 4 points isoélectriques 4.4, 4.6, 5.2 et 5.4 avec la séquence peptidique terminale: Arg-Gly-Ser-x-Leu-Thr-Thr-His-Pro-Leu. Peu après, des investigations par clonage moléculaire sur des placentas bovins et ovins, montraient que les PAGs appartiennent à la famille des protéases aspartiques partageant des identités de séquences avec la pepsine (57%), le pepsinogène (49.5%), la cathepsine D (58%) et E la chymosine (42.5%) et la rénine (Xie *et al.*, 1991b; Xie *et al.*, 1994; Xie *et al.*, 1995). Les arbres phylogéniques montrent que la famille des PAGs a probablement divergé de protéases aspartiques fonctionnelles sur le plan enzymatique (Xie *et al.*, 1997). Guruprasad *et al.*, (1996) ont suggéré que la structure tridimensionnelle bilobée de la bovPAG-1 était similaire à celle de la pepsine et de la chymosine, de plus, la bovPAG-1 était capable de lier la pepstatine à son site de fixation comme les autres protéases aspartiques actives enzymatiquement. En 1992, Lynch *et al.* trouvent une identité élevée dans les séquences cDNA entre la PSPB et la bovPAG I$_{67}$. De ce fait, ils classifièrent la PSPB dans la même famille des protéases aspartiques. A ce jour, seule, la PAG I$_{67}$ a été caractérisée en terme de contenu en carbohydrates et en acide sialique. Dans les années qui ont suivi, d'autres séquences correspondant à des glycoprotéines (sites de glycosylation) ont été identifiées dans le placenta des ruminants sur base d'études par biologie moléculaire (Xie *et al.*, 1997; Green *et al.,* 2000).

En 1998, plusieurs formes moléculaires de la PAG ont été purifiées à partir d'extraits de placenta caprins et caractérisées comme PAG$_{55}$ (séquence

N terminale: Ile-Ser-Ser-Pro-Val-Ser-x-Leu-Thr-Ile), PAG_{59} (séquence N terminale: Arg-Gly-Ser-x-Leu-Thr-Thr-Leu-Pro-Leu) et PAG_{62} (séquence N terminale: Arg-Asp-Ser-x-Val-Thr-Ile-Val-Pro-Leu) (Garbayo *et al.*, 1998). Des investigations par biologie moléculaire ont montré qu il existe probablement plus de 100 gènes de PAG dans le génome des ruminants (Xie *et al.*, 1997), la plupart d'entre eux étant exprimés dans les couches superficielles du placenta. Par exemple, dans l'espèce bovine deux images différentes d'expression se retrouvent: plusieurs PAGs comme les bovPAG-2, -8, -10 et -11 sont exprimées dans les cellules mono et binucléées du trophectoderme, tandis que d'autres protéines comme les bovPAG-1, -4-7, -9 sont localisées dans les cellules binucléées (Green *et al.*, 2000). Des investigations par biologie moléculaire ont aussi conclu que durant certaines périodes de la gestation, plusieurs PAGs sont exprimées tandis que d'autres sont absentes (Garbayo *et al.*, 1999; Green *et al.*, 2000). Dans le trophectoderme de *conceptus* recueillis au jour 25, 45, 60, 88, 150 et 250 après la fécondation, Green *et al.* (2000) ont détecté le mRNA codant pour la PAG I_{67} à partir du 45e jour alors que le mRNA codant pour la bovPAG-4 et celui codant pour la bovPAG-9 étaient surtout présents au jour 25. Une question importante est restée sans réponse: des molécules de PAG étaient-elles sécrétées plus précocement dans la circulation sanguine maternelle et était-il possible de mettre leur présence en évidence dans le sang maternel?

Trois systèmes différents de dosages radioimmunologiques (RIA 1, RIA 2 et RIA 3) basés sur l'utilisation d'antisérum générés contre la PAG I_{67} (RIA 1) la PAG_{55+62} (RIA 2) et la PAG_{55+59} (RIA 3) ont été caractérisés et utilisés dans notre étude (thèse) les systèmes 2 et 3 (RIA 2 et RIA 3) ont été développés et validés pour la première fois dans cette étude pour la détection des glycoprotéines associées à la gestation chez les bovins. Les différentes caractéristiques de sensibilité, précision exactitude ont été comparées à celles du système 1 (RIA 1).

La spécificité des 3 systèmes a été vérifiée vis à vis des membres actifs de la famille des protéases aspartiques: pepsinogène, pepsine, chymosine, renette, cathepsine D et rénine. Ces différents produits ont été testés sur une large échelle de concentrations (10 ng/ml à 1 mg/ml). Le pepsinogène interférait dans les RIA 1, 2 et 3 seulement à des concentrations supérieures à 1 mg/ml, 500 µg/ml et 1 mg/ml respectivement. La chymosine, et la renette pourraient interférer dans les RIA 2 et 3 tandis que la rénine et la cathepsine D ne modifiaient pas la liaison plus que la dose minimale détectable. Etant donné que les concentrations des protéases aspartiques examinées, rapportées dans la littérature se situent en dessous de celles qui pourraient donner lieu à une

interférence, il a été conclu que les différents systèmes de dosages radioimmunologiques (RIA 1, 2 et 3) étaient spécifiques pour la détection des PAGs dans les liquides biologiques (Perényi *et al.,* 2002b).

Les trois systèmes (RIA) ont été utilisés afin de mesurer la concentration de PAG dans les échantillons de plasma recueillis de vaches et de génisses gestantes a différentes périodes suivant l'insémination artificielle (IA). Ces systèmes ont été capables de détecter les molécules de PAG aussi tôt que 21 jours après IA et ce à différentes concentrations (RIA 1: 0.43±0.24 ng/ml, moyenne±DS; RIA 2: 0.48±0.24 ng/ml; RIA 3: 0.64±0.37 ng/ml). Au 32e et 42e jour, le RIA 2 (4.30±1.32 ng/ml, 5.56±1.95 ng/ml) et le RIA 3 (4.17±1.15 ng/ml, 5.60±1.89 ng/ml) présentaient des concentrations de PAG significativement différentes (p<0.0001) de celles du RIA 1 (2.43±0.81 ng/ml, 4.01±1.48 ng/ml) respectivement. Après le 21e jour, des corrélations significatives (p<0.0001; r≥0.929) ont été déterminées entre les trois systèmes. Cette étude a clairement indiqué que l'aptitude d'un RIA à reconnaître les molécules de PAG dans le sang maternel peut être améliorée par une sélection minutieuse de l'antisérum.

Ensuite notre étude a visé à déterminer les concentrations de PAG dans des échantillons de sang prélevés à faibles intervalles de temps chez des génisses et vaches gestantes. Dans ce document, nous avons présenté deux profils partiels et 3 complets des concentrations de PAG mesurées durant la gestation au moyen des 3 systèmes de dosage. Cinq femelles ont mené à bien leur gestation tandis que 2 ont présenté une mortalité embryonnaire ou un avortement.

Les rapports de concentrations mesurées dans les 2 nouveaux systèmes comparés au système de référence (RIA 1) ont été calculés et analysés. Après 60 jours de gestation les rapports décroissent suggérant que les formes moléculaires mieux reconnues par les antisérums des RIA 2 et 3 se retrouvent essentiellement durant la période allant du 25e au 60 jour de gestation.

Dans une étude réalisée en collaboration avec la Faculté d'Utrecht et la société <<*Holland Genetics*>>, 268 femelles receveuses d'embryons ont été suivies par observation clinique et prélèvement de sang à faible intervalle de temps: tous les 3 jours à partir du jour 7 et jusqu' au jour 35, ensuite une fois par semaine jusqu' au jour 119. Dans cette étude, la PAG et la progestérone ont été mesurées et leurs profils de concentration sont présentés. 113 femelles ont mené une gestation sans problème particulier et vêlèrent a terme. Les résultats des dosages ont été publiés par Vos *et al.* (2001). L'analyse des profils de concentrations chez les femelles qui ont souffert de pertes

embryonnaires ou fœtales a fait l'objet d'un soin particulier. Parmi ces profils seules 60 sont présentées dans ce document. Leur analyse permet de classifier les receveuses en 3 groupes : groupe A, l'anomalie affecte le profil de progestérone en premier lieu, groupe B, l'anomalie survient dans le profil de la PAG en premier lieu, groupe C, l'anomalie affecte les 2 profils en même temps. Les échecs de gestation classifiés type A sont probablement d'origine maternelle, tandis que ceux classifiés de type B sont probablement d'origine embryonnaire. Dans le cas du type C, l'origine peut être maternelle et fœtale ou encore un asynchronisme entre le développement embryonnaire et les modifications histo-physiologiques et endocrines de la receveuse. Ainsi, l'analyse rétrospective de ces différents profils a permis de clarifier l'origine et la pathogénie des échecs gestatifs.

Notre travail permet d'ouvrir de nouvelles perspectives dans le domaine. Nos observations relatives aux rapports de concentrations relevés dans les RIA 2 et 3 comparés au RIA 1 indiquent clairement la sécrétion dans le circulation maternelle de formes moléculaires mieux reconnues par les antisérum anti PAG_{55}, PAG_{59} et PAG_{62}. Dès lors il serait intéressant d'isoler les formes précoces pour développer un dosage radioimmunologique focalisé sur le début de la gestation. Les applications seraient d'intérêt pour le diagnostic précoce de la gestation et pour l'étude des mortalités embryonnaires.

Ensuite nos observations rassemblées grâce l'analyse des profils de concentration de PAG et P4 après transfert embryonnaire ouvrent de nouvelles perspectives d'étude pour mieux décrire les relations fœto-maternelles. Il serait intéressant de suivre jour par jour les processus dans les cas d'échec de façon à établir la pathogénie ou plutôt les pathogénies des mortalités embryonnaires et fœtales. Ce type de suivi permettra d'améliorer les conditions de culture des embryons afin de réduire les mortalités. Des investigations dans ce sens devraient comporter des examens par échographie de façon à suivre la croissance et les battements cardiaques du fœtus.

En conclusion notre étude a permis d 'améliorer les dosages radioimmunologiques pour suivre la gestation chez les bovins. L'application de ces dosages dans les cas d'échecs gestatifs a amélioré le diagnostic et la connaissance de la pathogénie des mortalités embryonnaires et fœtales.

Abbreviations

AI	Artificial insemination
B	Tracer bound
bCG	Bovine chorionic gonadotrophin
BNC	Binucleate cell
Bp	Base pairs
bPL	Bovine placental lactogen
BSA	Bovine serum albumin
CL	Corpus luteum
cpm	count per minute
CV	Coefficient of variation
DEAE-cellulose	Diethylaminoethyl-cellulose
ELISA	Enzyme-linked immunosorbent assay
GCP-2	Granulocyte chemotactic protein-2
GLM	General linear model
GU	Goldblatt unit
IFN-τ	Interferon-tau
IVF	*In vitro* fertilization
LH	Luteinizing hormone
MALIGN	Multiple sequence alignment program
MDL	Minimal detection limit
MHC	Major histocompatibility complex
MOET	Multiple ovulation and embryo transfer
p	Probability
P4	Progesterone
PAG	Pregnancy-associated glycoprotein (Zoli *et al.*, 1991)
pc	Post coitum
PEG	Polyethylene glycol
pI	Isoelectric point
PMN	Polymorphonuclear neutrophil
PSP60	Pregnancy serum protein 60 (Mialon *et al.*, 1993)
PSPA	Pregnancy specific protein-A (Butler *et al.*, 1982)
PSPB	Pregnancy specific protein-B (Butler *et al.*, 1982)
r	Correlation coefficient
RIA	Radioimmunoassay
RT-PCR	Reverse transcriptase polymerase chain reaction
SAS	Statistical Analysis System
SD	Standard deviation
SOF	Synthetic oviduct fluid
UMP	Uridine-5'-monophosphate

Publications derived from this thesis

Zs Perényi, JM Garbayo, J Sulon, L Duwez, O Szenci, JF Beckers. PAGs molecules as markers of pregnancy in the bovine: comparison of concentrations obtained by using three different antisera. 15th Annual Meeting AETE, 10-11 September 1999, Lyon France, p 162 (Abstract+Poster)

Zs Perényi, JM Garbayo, J Sulon, L Duwez, O Szenci, JF Beckers. PAGs molecules as markers of pregnancy in the bovine: comparison of concentrations obtained by using three different antisera. First Belgian workshop on Animal Endocrinology, 13 October 1999, Gembloux Belgium, Biotechnol Agron Soc Environ 2000; 4(1): 30-31. (Abstract)

Zs Perényi, J Sulon, O Szenci, JF Beckers. The inactive members of the aspartic proteinase family in the ruminant placenta: specificity of three different radioimmunoassay systems. Second Belgian Workshop on Animal Endocrinology, 15 November 2000, Leuven Belgium, Biotechnol Agron Soc Environ 2001; 5(1): 26-27. (Abstract)

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associated glycoproteins, Accepted for publication in Reproduction in Domestic Animals (01-52-RO-52) (2002b).

Publications related to this thesis

JF Beckers, PV Drion, JM. Garbayo, Zs Perényi, A Zarrouk, J Sulon, B Remy, O Szenci. Pregnancy-associated glycoproteins in ruminants: inactive members of the aspartic proteinase family. Middle European Buiatrics Congress, 21-23 May 1998, Siófok Hungary, p 125-130. (Communication)

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PAGs MOLECULES AS MARKERS OF PREGNANCY IN THE BOVINE: COMPARISON OF CONCENTRATIONS OBTAINED BY USING THREE DIFFERENT ANTISERA

ZS. PERÉNYI, J. M. GARBAYO, J. SULON, L. DUWEZ, O. SZENCI, J. F. BECKERS

Bd Colonster 20. B41, B 4000 Liège, Faculty of Veterinary Medicine, University of Liège, Belgium

Pregnancy- associated glycoproteins (PAGs) are synthesized in the ruminant trophoblast and secreted in the maternal blood circulation (Zoli *et al.*, Biol. Repr., 1991, 45, 1-10). The goal of this study was to compare the ability of three different antisera to measure the PAG concentrations in plasma samples collected from heifers or cows mated or inseminated 30-120 days before the veni puncture.

In the three systems pure 67 kDa PAG preparation was used as tracer (labelled by iodine- 125 isotope using the lactoperoxidase method) and as standard. In RIA 1, the antiserum was raised against a pure preparation of 67 kDa PAG purified from bovine placenta by Zoli *et al.* 1991. In RIA 2 and 3 the antisera were raised against two preparations of PAG purified from caprine cotyledons according to Garbayo *et al.* (Biol. Repr., 1998, 58, 109-115)

The results are shown in three regression systems (Fig. 1, Fig. 2, Fig. 3).

y=1.4862x+0.0168
r=0.9294

y=1.6394x+0.3523
r=0.9403

y=0.9052x-0.2317
r=0.9738
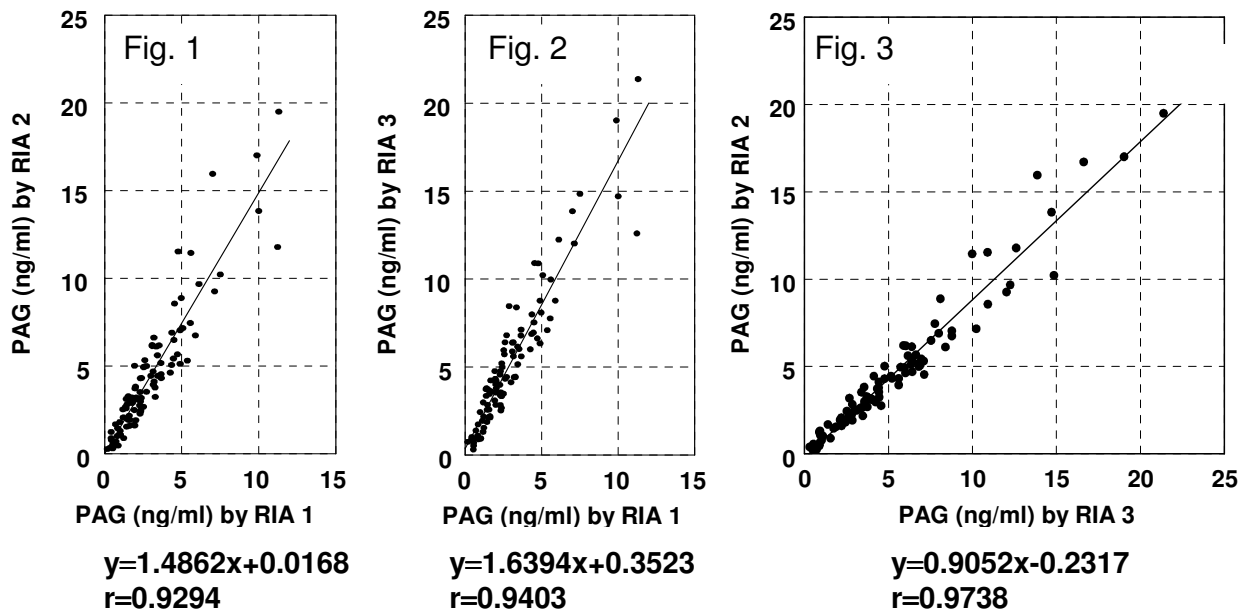
The parameters included in each regression system were calculated with PSI-Plot version 4.0. Y and x are the parameters of the regression line, r is the correlation coefficient.

It can be seen that the values obtained by RIA 2 and RIA 3 systems were everytime higher than the values measured by RIA 1. The correlation between the three systems was high however the correlation between RIA 3 and RIA 2 was the best (r=0.9738).

These data clearly show that antisera raised against PAG purified from goat placenta fully recognize the bovine PAGs in maternal blood. This study confirms the similarity of different PAGs in ruminants and the ability of various RIA systems to detect them in plasma.

ABILITY OF THREE DIFFERENT ANTISERA TO RECOGNIZE PREGNANCY-ASSOCIATED GLYCOPROTEINS IN HEIFERS DURING THE FIRST FIFTY DAYS OF GESTATION

PERÉNYI ZS., DESBULEUX H., SULON J., SZENCI O., BANGA-MBOKO H., SOUSA N. M., EL AMIRI B., BECKERS J. F.

Bd. Colonster 20. B41, B 4000 Liège, Faculty of Veterinary Medicine, University of Liège, Belgium

Pregnancy-associated glycoproteins (PAGs) are expressed in the superficial layer of the ruminant placenta according to a temporal and spatial expression pattern and probably the PAG 67 is not the first molecule expressed in the trophoblast (Green *et al.*, Biol. Repr., 2000, 62, 1624-1631). Some of them are secreted into the maternal blood circulation providing a useful tool to follow up an ongoing pregnancy. This study was undertaken to determine the ability of three different antisera to recognize PAGs in blood samples collected frequently from three heifers through the first 50 days of pregnancy.

In the three RIA systems, a pure 67 kDa PAG preparation was used as tracer (labelled by iodine-125 isotope using the chloramin T method) and as standard. In RIA 1, the antiserum was produced against a pure preparation of 67 kDa PAG (PAG I_{67}) purified according to Zoli *et al.* (Biol. Repr., 1991, 45, 1-10). In RIA 2 and 3, the antisera were obtained by immunizing rabbits against two preparations of PAG purified from caprine cotyledons according to Garbayo *et al.* (Biol. Repr., 1998, 58, 109-115). The PAG profiles for the 3 heifers are shown in Fig. 1, Fig. 2 and Fig. 3, respectively.
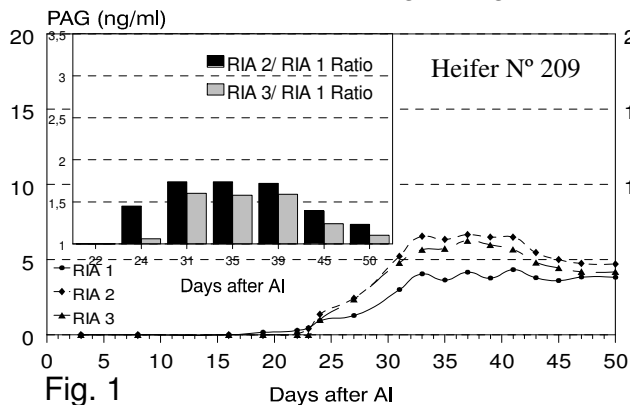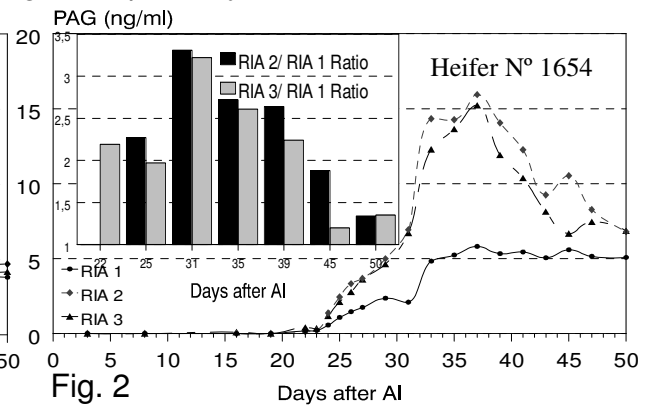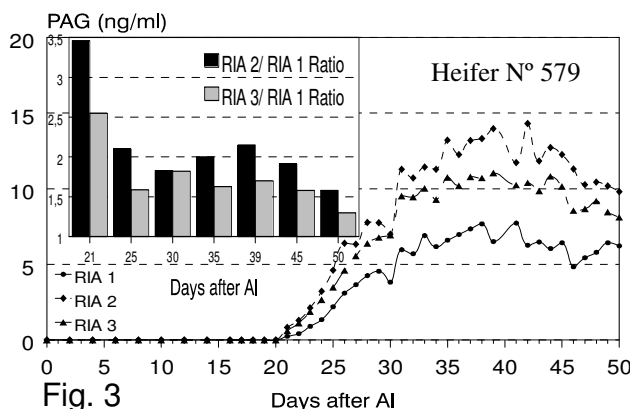
Fig. 1

Fig. 2

Fig. 3

In the first days after fertilization PAG levels were undetectable in all the three RIA. PAG levels started to increase intensively from day 20-23 after AI. Between day 30 and 40, RIA 2 and RIA 3 systems showed higher concentrations when compared to those of RIA 1, the RIA 2/RIA 1 and the RIA 3/RIA 1 ratios were higher than 1.5 (small diagrams in the left part of the figures).

Our results showed that between 30 and 40 days after AI, PAGs better recognized by RIA 2 and 3 are secreted into the maternal blood. These observations support the hypothesis on the early expression of PAG molecules different from PAG I_{67}.