Molekulargenetische und proteinanalytische Untersuchungen trächtigkeitsassoziierter Glykoproteine beim Rind

Dissertation zur Erlangung des Doktorgrades (Dr. rer. nat.)

der Fakultät für Agrarwissenschaften

der Georg-August-Universität Göttingen

vorgelegt von

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geboren in Querfurt

Göttingen, im September 2020

D 7

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Tag der mündlichen Prüfung: 26.11.2020

Es gibt keine Landstraße für die Wissenschaft, und nur diejenigen haben Aussicht, ihre lichten Höhen zu erreichen, die die Mühe nicht scheuen, ihre steilen Pfade zu erklimmen.

Karl Marx

Diese Arbeit wurde durch die Landwirtschaftliche Rentenbank und H. Wilhelm Schaumann Stiftung gefördert.

Veröffentlichungen

Diese Arbeit basiert auf folgenden Veröffentlichungen:

Beiträge in referierten Fachzeitschriften

Wiedemann, I., Krebs, T., Momberg, N., Knorr, C., Tetens, J. (2018).
mRNA expression profiling in cotyledons reveals significant up-regulation of the two bovine pregnancy-associated glycoprotein genes boPAG-8 and boPAG-11 in early gestation. Veterinary Medicine and Science, 4(4): 341-350

Krebs, T., Kilic, I., Neuenroth, L., Wasselin, T., Ninov, M., Tetens, J., Lenz, C. (2022).
A Multiplexed Parallel Reaction Monitoring Assay to monitor bovine Pregnancy-Associated Glycoproteins throughout pregnancy and after gestation. PLoS ONE 17(9): e0271057

Krebs, T., Kilic, I., Mütze, K., Kleinhans, S., Lücking, D., Hennies, M., Tetens, J. (2021).
Establishment of a Sandwich-ELISA for simultaneous quantification of bovine pregnancy-associated glycoprotein in serum and milk. PLoS ONE 16(5): e0251414

Kongressbeiträge

Krebs, T., Blaschka, C., Sondermann, J., Wiedemann, I., Lenz, C., Hennies, M., Kleinhans, S., Mütze, K., Knipper, K., Knorr, C. (2018).
Entwicklung eines Trächtigkeitsschnelltests beim Rind (Vortrag Sektion II). 45. AET-d Jahrestagung, 07.-08.06.2018 in Mariensee

Wiedemann, I., Momberg, N., Krebs, T., Tetens, J., Knorr, C. (2018).
Expressionsanalyse ausgewählter boviner PAG-Gene (Vortrag D18). Vortragstagung der DGfZ/GfT, 12.-13.09.2018 in Bonn

Krebs, T., Blaschka, C., Wiedemann, I., Lenz, C., Hennies, M., Kleinhans, S., Mütze, K., Knipper, K., Tetens, J., Knorr, C. (2018).
Entwicklung eines milchbasierten Lateral-Flow-Tests zur Trächtigkeitsdiagnose beim Rind (Vortrag D20). Vortragstagung der DGfZ/GfT, 12.-13.09.2018 in Bonn

Krebs, T., Wiedemann, I., Blaschka, C., Lenz, C., Hennies, M., Kleinhans, S., Mütze, K., Knipper, K., Knorr, C., Tetens, J. (2019).
Entwicklung eines milchbasierten Lateral-Flow-Tests zur Trächtigkeitsdiagnose beim Rind (Vortrag D20). 52. Jahrestagung Physiologie & Pathologie der Fortpflanzung und gleichzeitig 44. Veterinär-Humanmedizinische Gemeinschaftstagung, 20.-22.02.2019 in Göttingen

Krebs, T., Wiedemann, I., Hennies, M., Lenz, C., Tetens, J. (2019).
Quantitative Bestimmung boviner PAG-Profile während der Gravidität (Vortrag A19). Vortragstagung der DGfZ/GfT, 11.-12.09.2019 in Gießen

Krebs, T., Wiedemann, I., Hennies, M., Lenz, C., Tetens, J. (2020).
Quantitation of bovine PAG profiles during pregnancy (Poster 49). 53. Jahrestagung Physiologie & Pathologie der Fortpflanzung und gleichzeitig 45. Veterinär-Humanmedizinische Gemeinschaftstagung, 26.-28.02.2020 in Rostock-Warnemünde

Inhaltsverzeichnis

Abbildungsverzeichnis

Tabellenverzeichnis

Abkürzungsverzeichnis

ANOVA	analysis of variance
AUC	area under the curve
bCG	bovine chorionic gonadotrophin
BLAST	basic local alignment search tool
BNC	binucleate cell
boPAG	bovine pregnancy-associated glycoprotein
BSA	bovine serum albumin
CDH1	Cadherin-1
cDNA	complementary DNA
CID	collision-induced dissociation
CV	coefficient of variation
dNTP	desoxyribonucleoside triphosphate
EDTA	ethylenediaminetetraacetic acid
ELISA	Enzyme-linked Immunosorbent Assay
FPLC	fast protein liquid chromatography
FWHM	full width at half maximum
Gal	Galactose
GalNAc	N-Acetylgalactosamin
GlcNAc	N-Acetylglucosamin
HRP	horseradish peroxidase
IGF-1	Insulinähnlicher Wachstumsfaktor 1
IgG	Immunglobulin G
INFT	Interferon tau
kDa	Kilodalton
MALDI	Matrix-unterstützte Laser-Desorption/Ionisation
mRNA	messenger RNA
MS	Massenspektrometrie
m/z	Masse-zu-Ladung-Verhältnis
NCBI	National Center for Biotechnology Information
NeuAc	N-Acetylneuraminic acid

NP-40	nonyl phenoxypolyethoxylethanol
NPV	negative predictive value
PAG	pregnancy-associated glycoprotein
PAR	Protease-aktivierter Rezeptor
PBS	phosphate buffered saline
p. c.	*post conceptionem*
PCR	polymerase chain reaction
PGE_2	Prostaglandin E2
p. i.	post insemination
PMF	peptide mass fingerprinting
PNGase F	peptide:N-glycosidase F
p. p.	post-partum period
PPV	positive predictive value
PRM	parallel reaction monitoring
PSP	pregnancy specific protein
PSP60	pregnancy serum protein 60
PTM	posttranslationale Modifikation
qPCR	real-time quantitative PCR
RIA	Radioimmunassay
ROC	receiver operating characteristic
RQN	RNA quality number
RT	room temperature
SDS	sodium dodecyl sulfate
SEM	standard error of the mean
SRM	single reaction monitoring
TBST	tris-buffered saline containing Tween 20
TierErzHaVerbG	Tiererzeugnisse-Handels-Verbotsgesetz
TMB	3,3’,5,5’-tetramethylbenzidine
TOF	Time of Flight
YWHAG	tyrosine 3-monooxygenase/tryptophan 5-monooxygenase activation protein gamma

1 Allgemeine Einleitung

Die schnell voranschreitenden Entwicklungen in Tierzucht und Management haben in den letzten Dekaden zu starken Veränderungen in der Rinderhaltung im Allgemeinen und in der Milchviehhaltung im Besonderen geführt. Um den weltweit wachsenden Bedarf an Milchprodukten zu decken, werden Kühe mit steigender Milchleistung in immer größeren Herden gehalten (Lucy, 2001; Lucy *et al.*, 2011). Gestation und Milchproduktion der Tiere sind dabei unabdingbar miteinander verknüpft und Milchkühe werden noch während der laufenden Laktation besamt. Daher sind eine Trächtigkeit und deren möglichst frühe Diagnose inhärent für eine erfolgreiche Milchviehhaltung (Lucy, 2001). Der zuverlässige Nachweis einer Trächtigkeit kann sowohl über direkte (z. B. rektale Palpation) als auch indirekte Verfahren (Nachweis von zirkulierenden Molekülen im maternalen System) ermöglicht werden (Pohler *et al.*, 2017).

Trächtigkeitsassoziierte Glykoproteine (Pregnancy-associated glycoproteins; PAGs) haben sich dabei in den letzten Jahrzehnten als besonders geeignete Trächtigkeitsmarker in der Milchviehhaltung herausgestellt. Ihr Nachweis im Blut oder der Milch des trächtigen Muttertiers stellt eine alternative Methode der Trächtigkeitsdiagnose (z. B. zur transrektalen Sonographie) ab der 4. Woche *p. i.* dar. Zusätzlich können PAGs auch als Indikator für die Lebensfähigkeit des Embryos und einer physiologischen Plazentafunktion dienen (Wallace *et al.*, 2015). Trotz ihres großen Nutzens für die Milchviehhaltung, wurden die genauen physiologischen Funktionen dieser Proteine während der Trächtigkeit bis heute nicht aufgeklärt. In den letzten Jahren konnten neue Methoden in der Transkriptomik und Proteomik entwickelt werden, welche eine genaue Analyse komplexer physiologischer Vorgänge erlauben. Dabei agieren Transkriptomik und Proteomik als komplementäre Ansätze und können so zusammen neue Einblicke in die Regulation der Proteinexpression und -funktion liefern (Maier *et al.*, 2009; Vogel & Marcotte, 2012; Berg *et al.*, 2018).

Ziel der hier vorliegenden Arbeit ist daher, die im Verlauf der Trächtigkeit exprimierten bovinen PAGs molekularbiologisch und proteinanalytisch während der Trächtigkeit zu untersuchen, um so ein besseres Verständnis über die potenziellen Funktionen dieser Proteine zu erlangen. Dafür wurden im ersten Teil der Arbeit detaillierte mRNA-

Expressionsprofile mittels real-time quantitativer PCR (qPCR) für ausgewählte PAG-Gene im Verlauf der Trächtigkeit erstellt. Im zweiten Teil wurde ein „Parallel Reaction Monitoring“ (PRM) Assay entwickelt und validiert, der es ermöglicht die relativen Proteinlevel verschiedener PAGs während der Trächtigkeit und nach der Geburt zu beschreiben. Zusätzlich wurde hierbei der Einfluss der Glykosylierung untersucht. Diese Erkenntnisse trugen dazu bei, einen neuen Sandwich-Enzyme-linked Immunosorbent Assay (Sandwich-ELISA) für die simultane Quantifizierung von PAG im Serum und der Milch zu etablieren. Dieser ELISA soll es dem Landwirt ermöglichen, zeitnah, kostengünstig und mit wenig Aufwand, Trächtigkeitsdiagnosen zu erhalten. Zudem bietet er den durchführenden Laboratorien eine Kostenersparnis, da sie für Blut- und Milchproben den gleichen Test verwenden können.

2 Literaturübersicht

2.1 Wiederkäuerplazenta

Die Reproduktionseffizienz in der Milchviehhaltung ist weltweit rückläufig (Lucy, 2001). Eine erfolgreiche Trächtigkeit ist jedoch unabdingbar, da nur diese eine Laktation initiiert (Lucy, 2001). Ein besonders kritischer Punkt ist hierbei die Plazentation. Bis heute ist dieser Vorgang - und die mit ihm verbundenen biochemischen Prozesse - Gegenstand intensiver Forschung (Roberts *et al.*, 2016). Viele Funktionen der Säugetierplazenta sind noch unbekannt und es gibt nur wenige Kenntnisse darüber, wie plazentare Produkte (Proteine und Hormone) an der fetomaternalen Schnittstelle funktionieren (Zoli *et al.*, 1991; Burton & Jauniaux, 2015; Roberts *et al.*, 2016).

Hinzu kommt, dass die plazentaren Strukturen unter den Säugetieren sehr variabel sind (Haig, 1993; Xie *et al.*, 1997; Roberts *et al.*, 2016). Die Wiederkäuerplazenta nimmt unter ihnen eine Sonderform ein. Sie wird als "synepitheliochorial" bezeichnet (Wooding, 1992). Ein Charakteristikum dieser Plazentaform sind die binukleären Zellen (binuclear cells, BNCs), welche durch azytokinetische Mitose aus mononukleären Trophoblastzellen entstehen (Wathes & Wooding, 1980; Wooding, 1982, 1983; Klisch *et al.*, 1999a, 1999b). BNCs haben dabei die Eigenschaft, durch Gewebe zu migrieren oder in dieses einzudringen (siehe Abbildung 1). Das Ausmaß dieser Eigenschaften ist von Spezies zu Spezies unterschiedlich (Hoffman & Wooding, 1993).

Nach dem Auflösen der *Zona pellucida* (am 10. Tag *p. i.*) kommt es zu einem starken Größenwachstum des Trophoblasten (Chang, 1952; Greenstein *et al.*, 1958; Leiser, 1975). Während dieses Prozesses konnte ab dem 17. Tag *p. i.* (kurz vor der Implantation) auch die erstmalige Bildung von BNCs beim Rind nachgewiesen werden (Wimsatt, 1951; Greenstein *et al.*, 1958; Leiser, 1975). Anschließend machen sie, von der Implantation bis kurz vor der Geburt des Kalbes, zwischen 15 % und 20 % des gesamten Trophektoderms aus. Ein bis zwei Tage vor der Geburt verringert sich ihre Anzahl rapide (Wooding *et al.*, 1986; Wooding, 1992).

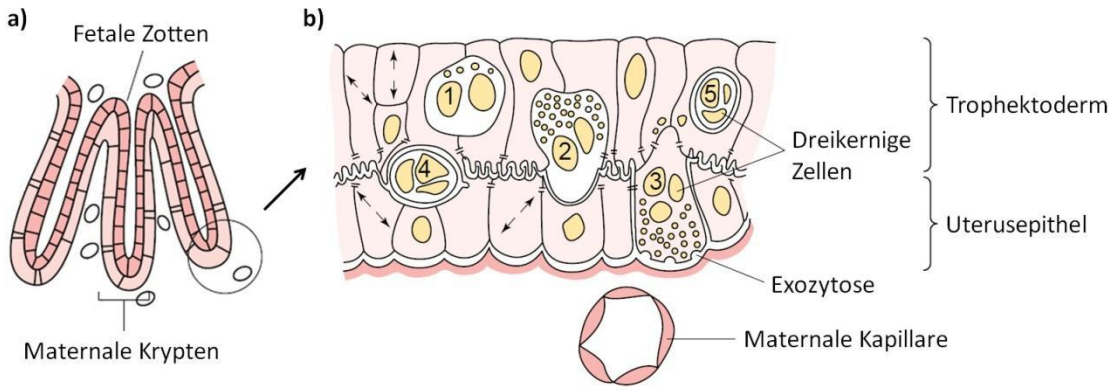

Abbildung 1: Zelluläre Veränderungen während der Implantation beim Rind (modifiziert nach Green *et al.*, 1998).
(a) Ausschnitt eines Plazentoms; Es zeigt das Penetrieren der fetalen Kotyledonen in die maternalen Krypten. Die Plazentome sind stark vaskularisiert.
(b) Die Bildung des fetomaternalen Synzytiums ist das Resultat der BNC-Migration (1-2). Ab Tag 40 der Trächtigkeit bilden die BNCs kurzlebige trinukleäre Zellen (3), welche nach erfolgter Exozytose wieder vom Trophektoderm resorbiert werden (4-5).

Nach der Implantation haben die binukleären Zellen vor allem zwei wichtige Funktionen. Zunächst verbinden sie sich mit uterinen Epithelzellen und bilden so ein fetomaternales Synzytium, welches beim Rind aus trinukleären Hybridzellen besteht (Wooding, 1992; Green *et al.*, 1998; Klisch & Leiser, 2003). Die Bildung dieser fetomaternalen Einheit ist ein wichtiger Schritt für eine erfolgreiche Implantation (Wooding, 1992) und erlaubt es dem Fetus außerdem einen direkteren Kontakt zum Muttertier aufzunehmen (Green *et al.*, 1998). Weitere Aufgaben der BNCs bestehen in der Bildung und Speicherung von Proteinen und Steroidhormonen, sowie deren Transport in das maternale System. BNCs wachsen im Verlauf der Zeit und bilden eine hohe Anzahl endoplasmatischer Retikula und einen großen Golgi-Apparat, welcher für die Bildung von Granula verantwortlich ist. Diese machen bei reifen BNCs mehr als 50 % des Gesamtvolumens aus (Wooding, 1992; Xie *et al.*, 1997; Green *et al.*, 1998; Klisch & Leiser, 2003; Klisch *et al.*, 2010). Binukleäre Zellen sind dann in der Lage, mittels Exozytose aus diesen Granula plazentares Laktogen (Duello *et al.*, 1986), Steroidhormone (Reimers *et al.*, 1985), Wachstumshormone und -faktoren (Munson *et al.*, 1996) sowie trächtigkeitsassoziierte Glykoproteine in den maternalen Blutkreislauf abzugeben (Wooding, 1992; Guruprasad *et al.*, 1996; Green *et al.*, 1998, 2000; Schlafer *et al.*, 2000; Reese *et al.*, 2016). Letztere sollen die Basis der hier vorliegenden Arbeit bilden.

2.2 PAG-Struktur

Die Arbeitsgruppe um Butler konnte 1982 erstmals zwei plazentare Antigene (pregnancy specific protein A und B; PSP-A und PSP-B) nachweisen, indem sie Extrakte aus boviner Plazenta in Kaninchen injizierte. PSP-A erwies sich als α-Fetoprotein, PSP-B hingegen als ein noch unbekanntes plazentares Protein (Butler *et al.*, 1982; Xie *et al.*, 1991; Green *et al.*, 1998). PSP-B konnte im weiteren Verlauf für die Entwicklung eines Radioimmunoassays (RIA) zur zuverlässigen Trächtigkeitsdiagnostik bei Rindern genutzt werden (Sasser *et al.*, 1986; Green *et al.*, 1998). Im gleichen Zeitraum konnte die Arbeitsgruppe um Beckers bovines Choriongonadotropin (bCG) (Beckers *et al.*, 1988a) und PSP aus fetalen Kotyledonen isolieren (Beckers *et al.*, 1988b). Im Jahr 1991 konnten Zoli *et al.* ein trächtigkeitsspezifisches Antigen aufreinigen, welches erstmals den Namen PAG trug (Zoli *et al.*, 1991). Man geht davon aus, dass alle genannten Proteine identisch sind oder zumindest eine hohe Ähnlichkeit besitzen (Wooding *et al.*, 2005). Nach diesen Entdeckungen konnte festgestellt werden, dass PAGs eine unerwartet hohe Komplexität aufweisen. Xie *et al.* (1997) gingen davon aus, dass bei Wiederkäuern um die 100 PAG-Gene existieren, die im plazentaren Gewebe exprimiert werden können.

Xie *et al.* (1991) klassifizierten die PAGs als Mitglieder der Familie der Aspartatproteasen. Diese stellen eine weit verbreitete Enzymklasse dar, die in Wirbeltieren, Pilzen, Pflanzen und Retroviren gefunden werden kann (Davies, 1990). Sie sind durch ihr pH-Optimum im sauren Bereich, zwei Asparaginsäure-Seitenketten im katalytischen Zentrum und ihre Spezifität für bestimmte Peptide charakterisiert (Davies, 1990). Ihre Bindungsstelle kann Substrate mit einer Länge von sieben bis acht Aminosäuren binden (Green *et al.*, 1998). Die meisten Aspartatproteasen sind einkettige Enzyme mit einem Molekulargewicht von etwa 35 kDa und einer Länge von ungefähr 330 Aminosäuren. Zu ihnen zählen neben den PAGs Pepsin, Chymosin, Cathepsin D und Renin (Davies, 1990). Des Weiteren sind sie durch eine konservierte Sequenz im Bereich des katalytischen Zentrums gekennzeichnet: (hydrophob)- in der Regel (Phe)-Asp-Thr-Gly-Ser an der N-terminalen Domäne und korrespondierend dazu (hydrophob)-Asp-Thr-Gly-Ser/Thr an der C-terminalen Domäne (Davies, 1990). PAGs zeigen eine Homologie der Aminosäuresequenz von 50 % mit Pepsin A und 45 % mit Chymosin (Xie *et al.*, 1997).

Untereinander haben bovine PAGs (boPAGs) eine Homologie der Aminosäuresequenz von etwa 68 %.

Beim Rind sind derzeit mindestens 21 verschiedene boPAGs und verschiedene Isoformen bekannt (Green *et al.*, 2000; Hughes *et al.*, 2000; Telugu *et al.*, 2009; Wallace *et al.*, 2015). Sie lassen sich in zwei phylogenetisch voneinander getrennte Gruppen einteilen: in eine ältere Gruppe, die vor rund 87 Millionen Jahren entstanden ist und in eine modernere Gruppe, die ungefähr vor 52 Millionen Jahren entstanden ist (Hughes *et al.*, 2000). Diese Daten stimmen sehr gut mit zwei wichtigen Zeitpunkten in der Evolution der Säugetiere überein: vor rund 83 Millionen Jahren kam es zur Abspaltung der *Artiodactyla*. Im weiteren Verlauf trennten sich dann, vor rund 52 Millionen Jahren, die *Ruminantia* von den *Suidae* ab (Kumar & Hedges, 1998). Somit kann das Auftreten der modernen boPAGs mit dem in Erscheinung treten der synephitelchorialen Plazenta der Wiederkäuer in Zusammenhang gebracht werden (Hughes *et al.*, 2000).

In den letzten Jahren kamen zahlreiche Studien zu dem Schluss, dass die boPAGs der unterschiedlichen phylogenetischen Gruppen in verschiedenen Zellen des Trophoblasten exprimiert werden, allerdings nicht alle boPAGs zur gleichen Zeit über die Trächtigkeit hinweg (Green *et al.*, 2000).

Moderne boPAGs werden dabei vor allem in den BNCs gebildet, während die älteren boPAGs in allen Trophoblastzellen, mononukleären als auch binukleären, gebildet werden (Green *et al.*, 2000; Wooding *et al.*, 2005; Touzard *et al.*, 2013; Wallace *et al.*, 2015). Touzard *et al.* (2013) zeigten, dass die boPAGs von bestimmten Zellsubpopulationen exprimiert werden, von denen jede Zellpopulation jeweils ein anderes PAG bildet und speichert. Dabei wird in den Kotyledonen vor allem die moderne Gruppe der boPAGs exprimiert, wohingegen im interkotyledonären Gewebe hauptsächlich die alten boPAGs gebildet werden (Touzard *et al.*, 2013).

Die genaue Regulation der PAG-Transkription ist nicht bekannt. Innerhalb der PAGs sind regulatorische Sequenzen vor dem Transkriptionsstart hoch konserviert und beinhalten mögliche Bindungsstellen für Transkriptionsfaktoren. Diese Regionen sind aber noch nicht systematisch untersucht worden, weshalb keine Rückschlüsse auf die Regulation der PAG-Transkription möglich ist (Telugu *et al.*, 2009; Wallace *et al.*, 2015).

Während beim Rind die PAG-Forschung auf Ebene der Genomik bereits einige Erkenntnisse in Bezug auf die Vielfalt dieser Genfamilie hervorgebracht hat, sind bisher wenig korrespondierende Erkenntnisse auf Proteinebene und hinsichtlich ihrer Glykosylierungsmuster vorhanden (Klisch *et al.*, 2005).

Bei boPAGs handelt es sich bei der Art der posttranslationalen Modifikation (PTM) meistens um N-Glykosylierungen (Patel *et al.*, 2004a; Klisch *et al.*, 2005, 2008). Die Glykosylierung ist die häufigste Veränderung an Proteinen in allen lebenden Organismen (Kornfeld & Kornfeld, 1985; Sharon & Lis, 1997; Herget *et al.*, 2009). N-Glykane stellen ihrerseits wiederum die am häufigsten vorkommende PTM in Vertebraten dar (Kornfeld & Kornfeld, 1985; Pavelka, 1997; Helenius & Aebi, 2001; Herget *et al.*, 2009). Etwa 50 % aller Proteine, die im Endoplasmatischen Retikulum und im Golgi-Apparat prozessiert werden, könnten N-glykosyliert sein (Apweiler *et al.*, 1999). Proteinsequenzen mit folgendem Motiv Asn-Xaa-Thr/Ser (wobei Xaa jede Aminosäure, mit Ausnahme von Prolin, darstellen kann) werden regelmäßig, aber nicht immer, an einem Asparaginrest glykosyliert (Sharon & Lis, 1997; Klisch *et al.*, 2005; Herget *et al.*, 2009).

In der Literatur finden sich verschiedene Angaben über die Molekulargewichte boviner PAGs mit Werten zwischen 48 kDa und 90 kDa (Butler *et al.*, 1982; Sasser *et al.*, 1989; Xie *et al.*, 1991, 1994; Zoli *et al.*, 1991; Green *et al.*, 2005; Klisch *et al.*, 2005; Szafranska *et al.*, 2006; Touzard *et al.*, 2013). Diese hohe Variabilität ergibt sich aus den verschiedenen Glykosylierungsmustern der einzelnen boPAGs (Klisch & Leiser, 2003; Klisch *et al.*, 2005, 2006; Touzard *et al.*, 2013). Zudem besitzen die verschiedenen boPAGs unterschiedlich viele Glykosylierungsstellen (siehe Tabelle 1) (Xie *et al.*, 1997; Green *et al.*, 2005; Klisch *et al.*, 2005; Touzard *et al.*, 2013). Eine Abspaltung der N-Glykane mittels Peptid:N-Glykosidase F (PNGase F) führt bei diesen Proteinen zu einer Massenreduktion (Klisch & Leiser, 2003; Klisch *et al.*, 2005, 2006; Touzard *et al.*, 2013), so dass nach Abspaltung der Signalsequenz und des Propeptids der Proteinkern der meisten boPAGs die erwartete Molekülmasse von 37 kDa aufweist (Klisch *et al.*, 2005).

Tabelle 1: Anzahl möglicher N-Glykosylierungsstellen ausgewählter boPAGs.

PAG	N-Glykosylierungsstellen	Quelle
boPAG1	4*	Xie *et al.*, 1997; Klisch *et al.*, 2005; Touzard *et al.*, 2013
boPAG2	1*	Touzard *et al.*, 2013
boPAG3	4	Xie *et al.*, 1997
boPAG5	2	Xie *et al.*, 1997
boPAG6	5	Xie *et al.*, 1997; Klisch *et al.*, 2005
boPAG7	6	Xie *et al.*, 1997; Klisch *et al.*, 2005
boPAG8	2	Xie *et al.*, 1997
boPAG9	3	Xie *et al.*, 1997
boPAG10	5	Xie *et al.*, 1997
boPAG11	1*	Touzard *et al.*, 2013
boPAG12	4	Xie *et al.*, 1997
boPAG17	3	Xie *et al.*, 1997; Klisch *et al.*, 2005

(*: nachgewiesene Glykosylierungsstellen)

Über histologische und massenspektrometrische Analysen konnten außerdem Veränderungen der Glykosylierung im Verlauf der Trächtigkeit festgestellt werden. Diese Veränderungen sind zum Beginn und zum Ende der Trächtigkeit besonders stark ausgeprägt (Lehmann *et al.*, 1992; Klisch *et al.*, 2006, 2008, 2010). Ferner konnten Klisch *et al.* (2008) eine tetra-antennäre kern-fukosylierte Struktur mit einem halbierenden N-Acetylglucosamin (GlcNAc) als dominantes N-Glykan der boPAGs bestimmen. Alle vier Antennen besitzen das Sd^a-Antigen (NeuAcα2-3[GalNAcβ1-4]Galβ1-4GlcNAc (Sd^a)-).

Solche komplexen Glykanstrukturen deuten auf eine stark regulierte und konservierte Glykosylierungsmaschinerie in den bovinen BNCs hin, denn da sich die beteiligten GlcNAc-Transferasen zum Teil gegenseitig inhibieren, ist eine strikte Separation im Golgi-Apparat erforderlich (Sasai *et al.*, 2003; Klisch *et al.*, 2008).

Interessant ist auch, dass das N-Acetylgalactosamin (GalNAc), welches Teil des Sd^a-Antigens ist, eher als typischer Zucker in O-Glykanen gefunden wird (Klisch & Leiser, 2003). Die funktionelle Relevanz des gekoppelten Sd^a-Antigens bleibt zum großen Teil ungeklärt. Es konnte gezeigt werden, dass es bis zum 30. Tag der Trächtigkeit und kurz vor

der Geburt des Kalbes (ungefähr ab dem fünften Tag *ante partum*) nicht vorhanden ist und sich dadurch die Serumhalbwertszeit der boPAGs in diesen Stadien erhöht. Es wird eine endokrine Kontrolle bei der Synthese dieses Glykantypen vermutet, welche hauptsächlich durch Östrogene gesteuert wird (Klisch *et al.*, 2008).

Eine vergleichende Darstellung der verschiedenen boPAGs auf Proteinebene über die gesamte Trächtigkeit hinweg, wie sie beispielsweise bei Touzard *et al*. (2013) und Telugu *et al*. (2009) auf Basis der relativen mRNA-Expression erfolgte, ist in der Literatur aktuell nicht zu finden. Es existiert lediglich eine Studie von Touzard *et al*. (2013), die Proteinlevel von drei verschiedenen boPAGs (boPAG-1; boPAG-2; boPAG-12) im Verlauf der Trächtigkeit beschreibt. Andere Studien (Green *et al.*, 2005; Klisch *et al.*, 2005) identifizierten verschiedene boPAGs zu bestimmten Zeitpunkten in der Mitte der Trächtigkeit.

2.3 PAG-Funktion

Obwohl die bovinen PAGs schon viele Jahre erforscht werden, ist ihre genaue Funktion noch unklar. Basierend auf ihrer Einteilung in ältere und moderne boPAGs, werden diesen Gruppen auch unterschiedliche physiologische Aufgaben zugeschrieben. Die älteren boPAGs haben typische Eigenschaften der Aspartatproteasen, daher wird bei ihnen eine enzymatische Aktivität diskutiert (Wooding *et al.*, 2005; Telugu *et al.*, 2009, 2010; Wallace *et al.*, 2015). Es wird vermutet, dass diese Gruppe der boPAGs eine entscheidende Rolle bei der Aktivierung von Wachstumsfaktoren spielt. Zusätzlich scheint eine wichtige Funktion bei der Anheftung zwischen Fetus und Muttertier (zu Beginn der Trächtigkeit) und dem Lösen dieser Verbindung (bei der Geburt) zu bestehen (Wooding *et al.*, 2005; Wallace *et al.*, 2015). Alternativ könnten die alten boPAGs mit ihrer proteolytischen Aktivität auch eine Funktion beim Schutz der Trophoblastzellen einnehmen und schädliche Moleküle oder Zellen zerstören (Wooding *et al.*, 2005).

Während die ältere boPAG-Gruppe lediglich aus einer kleinen Anzahl von Proteinen besteht (sechs der 21 bisher bekannten boPAGs), ist die moderne Gruppe deutlich größer (Green *et al.*, 2000; Wooding *et al.*, 2005). Ihr wird auch eine Funktion bei der Kontaktaufnahme von Trophoblastzellen und Endometrium zugeschrieben. Dabei spielen die Glykanstrukturen eine wichtige Rolle. Sie fungieren hier als eine Art Verbindung, mit

Glykanen auf der Seite der Trophoblasten und kohlenhydratbindenden Strukturen auf Seite des Endometriums (Lehmann *et al.*, 1992; Wooding *et al.*, 2005; Wallace *et al.*, 2015). Eine weitere wichtige Funktion könnte in der Beeinflussung des Immunsystems bestehen. Gerade die immunsuppressive bzw. -modulatorische Wirkung wird hier von einigen Autoren diskutiert (Mathialagan & Hansen, 1996; Hoeben *et al.*, 1999; Wooding *et al.*, 2005; Klisch *et al.*, 2006). Auch hier ist ein Einfluss der Glykosylierung sehr wahrscheinlich (Yoshimura *et al.*, 1996; Klisch *et al.*, 2008; Wallace *et al.*, 2015). Neben der immunomodulatorischen Komponente konnten auch luteotrope Effekte nachgewiesen werden. Diese Vermutungen basieren auf der Fähigkeit von boPAG-1 die Prostaglandin E_2 (PGE_2)-Ausschüttung zu erhöhen und somit auch die Progesteronsekretion des *Corpus luteum* (Weems *et al.*, 1998a, 1999, 2003; Wooding *et al.*, 2005; Wallace *et al.*, 2015). Dabei soll an dieser Stelle noch angemerkt werden, dass PGE_2 auch eine immunsuppressive Wirkung haben kann (Low & Hansen, 1988).

2.4 PAGs als Trächtigkeitsmarker

Obwohl die genauen physiologischen Funktionen der boPAGs bis jetzt noch nicht aufgeklärt werden konnten, haben diese sich als nützliche Trächtigkeitsmarker im Blut und in der Milch herausgestellt. Die meisten veröffentlichten Artikel zum Thema fokussieren sich auf die Entwicklung zuverlässiger Testsysteme für die Trächtigkeitsdiagnose beim Rind (Wallace *et al.*, 2015). Mit ihnen steht ein plazentares Produkt zur Verfügung, welches direkt ab der dritten Woche nach der Besamung nachgewiesen werden kann (Szafranska *et al.*, 2006). In Abbildung 2 sind typische PAG-Verlaufskurven im Blut und in der Milch einer Holstein-Friesian Kuh dargestellt. Bovine PAGs bieten als trächtigkeitsspezifische Marker entscheidende Vorteile gegenüber anderen Systemen.

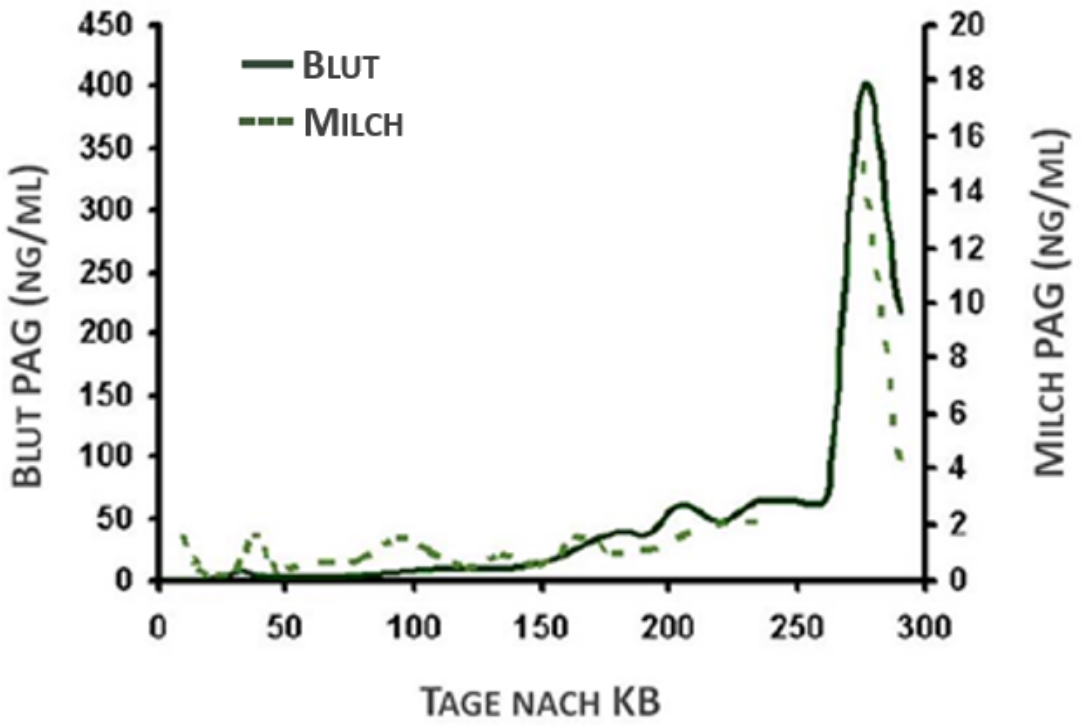

Abbildung 2: PAG-Verlauf im Blut und in der Milch einer Holstein-Friesian Kuh. Angegeben sind die PAG-Konzentrationen in Bezug auf die Zeit nach erfolgreicher Besamung (modifiziert nach Friedrich, 2006).

Im Vergleich zum häufig genutzten Progesteron oder dem „early pregnancy factor", sind sie deutlich zuverlässiger bei der Erkennung von nicht-tragenden Tieren (Reese *et al.*, 2016; Pohler *et al.*, 2017). Beim Rind wird auch Tau-Interferon (INFT) als möglicher Marker für die frühe Trächtigkeitsdiagnose (ab Tag 17 *p. i.*) diskutiert (Green *et al.*, 2010; Reese *et al.*, 2016; Pohler *et al.*, 2017). Es hat aber den entscheidenden Nachteil, dass es nicht direkt gemessen werden kann, sondern nur die erhöhte Expression bestimmter Gene unter Einfluss von INFT. Hinzu kommt, dass nicht alle diese Gene spezifisch für eine Trächtigkeit sind (Reese *et al.*, 2016; Pohler *et al.*, 2017). Die rektale Palpation und die Sonographie benötigen in aller Regel einen erfahrenen Untersucher und im Falle der Sonographie die nötige Technik (Reese *et al.*, 2016; Pohler *et al.*, 2017). Testsysteme zum Nachweis von boPAG in Milch und im Blut haben eine Genauigkeit zwischen 93 % und 96 % für die Detektion früher Trächtigkeiten (3 Wochen *p. i.*) und können somit als Alternative zu den oben genannten Methoden angesehen werden (Wallace *et al.*, 2015; Reese *et al.*, 2016; Pohler *et al.*, 2017). Zusätzlich konnten erste Studien nachweisen, dass die gemessene boPAG-Konzentration das Potential besitzt, neben der

Trächtigkeitsdiagnose, auch eine Aussage treffen zu können, ob eine Trächtigkeit erfolgreich ausgetragen wird oder ob es zu einem Abort kommen wird (Pohler *et al.*, 2013; Wallace *et al.*, 2015).

Die Detektion von PAGs in der Milch bietet, aufgrund seiner Vorteile gegenüber dem Blut, ein großes Potential für das Reproduktionsmanagement auf landwirtschaftlichen Betrieben. Die Milchproben können ohne großen Aufwand vor Ort vom Landwirt selbst genommen werden und der Stress einer Venenpunktion für die sonst stattfindende Blutabnahme wird vermieden (González *et al.*, 2001). Wie in Abbildung 2 bereits deutlich wird, ist die boPAG-Konzentration in der Milch deutlich geringer als im Serum. Zu Beginn der Trächtigkeit finden sich etwa 4,5 % - 16,7 % der gemessenen Blut-PAG-Konzentration in der Milch, im weiteren Verlauf (ab Tag 60 *p. i.*) pendelt sich dieser Wert auf ungefähr 3,2 % ein (Gajewski *et al.*, 2008; Friedrich & Holtz, 2010). Diese geringen Werte, gerade zu Beginn der Trächtigkeit, stellen eine große Herausforderung für das Testsystem dar, da sehr spezifische Antikörper vorhanden sein müssen, um die Detektion der geringen PAG-Mengen in der komplexen Matrix Milch zu gewährleisten. Daher gibt es zur Zeit nur ein kommerzielles ELISA-Testkit zur Bestimmung von PAG in Vollmilch, welches ab dem 28. Tag nach der Besamung eigesetzt werden kann (IDEXX Laboratories, 2019). Ein entscheidender Nachteil ist jedoch, dass dieser Test als qualitativer Test konzipiert ist und somit keine Aussagen über die PAG-Konzentration zulässt (Leblanc, 2013; Lawson *et al.*, 2014; Ricci *et al.*, 2015).

Ziel der vorliegenden Arbeit war es, bovine PAGs molekulargenetisch und proteinanalytisch zu untersuchen. Erkenntnisse aus diesen Studien haben dazu beigetragen, einen neunen Sandwich-ELISA für den Nachweis von PAG im Blut und in der Milch zu etablieren. Insgesamt wurden drei Studien durchgeführt. Die erste Untersuchung beschäftigt sich mit der Erstellung detaillierter mRNA-Expressionsprofile ausgewählter boviner PAG-Gene im Verlauf der Trächtigkeit mittels real-time quantitativer PCR (qPCR) (Kapitel 3). In der zweiten Studie wurde ein PRM-Assay entwickelt und validiert, der es ermöglicht, die relativen Proteinlevel 18 verschiedener PAGs während der Trächtigkeit und nach der Geburt zu beschreiben. Dabei wurden die Proben nativ und deglykosyliert untersucht, um einen möglichen Effekt der Glykosylierung zu detektieren (Kapitel 4). Die Ergebnisse aus den ersten beiden Studien liefern wichtige und neue Startpunkte für zukünftige

Untersuchungen boviner PAGs, welche dazu beitragen können die physiologischen Funktionen und den Einfluss posttranslationaler Modifikationen dieser Proteine während der Trächtigkeit besser zu verstehen. Die dritte Studie beschäftigt sich mit der Etablierung eines Sandwich-ELISA für den Trächtigkeitsnachweis bei Rindern auf Basis der PAGs (Kapitel 5). Er erlaubt eine die simultane Quantifizierung von PAG im Serum und der Milch mit derselben Testprozedur und bietet somit eine Zeitersparnis für die Landwirte und ist kosteneffizienter für die durchführenden Laboratorien.

3 mRNA expression profiling in cotyledons reveals significant up-regulation of the two pregnancy-associated glycoprotein genes boPAG-8 and boPAG-11 in early gestation

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veröffentlicht in

Veterinary Medicine and Science (2018), 4(4): 341-350

URL: https://onlinelibrary.wiley.com/doi/10.1002/vms3.123

DOI: 10.1002/vms3.123

Abstract

The multigene family of pregnancy-associated glycoproteins (PAGs) belongs to a group of aspartic proteases that are exclusively expressed by trophoblast cells in the placenta of even-toed ungulates. In Bovidae, 22 different PAG genes (boPAGs) with a wide range of temporal and spatial expression- and glycosylation patterns have been reported to date. In this study we describe the mRNA expression patterns using real-time quantitative PCR (qPCR) for selected modern (boPAG-1, -9, -21) and ancient bovine PAGs (boPAG-2, -8, -10, -11, -12) in cotyledonary tissue. The highest mean expression was detected in boPAG-8 and lowest in boPAG-10 ($p < 0.05$). Furthermore, boPAG-8 and -11 were significantly greater expressed in early gestation compared with later pregnancy stages. The characterization of boPAG mRNA-expression levels gives important insights for further protein analyses which will be valuable information for the development of new pregnancy detection systems.

Keywords: bovine, pregnancy, pregnancy-associated glycoproteins, mRNA-expression, placenta

Introduction

Changes in gene expression are associated with the development of the blastocyst from the morula to the embryo. Some genes that are transcribed only in the trophectoderm are activated during pregnancy for the first time (Green *et al.,* 2000).

Subsequent research in this field has revealed that multigene families are expressed in the reproductive tract of mammalian species during this period of life (Green, 2004; Telugu *et al.* 2009). Pregnancy associated glycoproteins (PAGs) are an example of this type of family. PAGs and PAG-like proteins can be found in numerous different species (e.g.de Sousa *et al.* 2006; Szafranska *et al.* 2006), but they are mainly expressed by the trophoblast cells of members of the *Cetartiodactyla* order (Wallace *et al.* 2015) where they represent one of the major secretory products (Szafranska *et al.* 1995; Xie *et al.* 1997a; Xie *et al.* 1997b; Garbayo *et al.* 1998; Green *et al.* 2000; Brandt *et al.* 2007).

PAGs belong to the vertebrate aspartic proteinase family. Therefore, they are directly related with a number of enzymes such as pepsin, chymosin, cathepsin D or renin (Hughes *et al.* 2003). Based on this relationship, a proteolytic activity in some PAGs has been discussed by different authors (Green *et al.* 1998; Telugu *et al.* 2009, 2010). They are suspected to play a role in the biochemical processing of latent growth factors at the placenta-uterine interface (Wooding *et al.* 2005). On the other hand, there are a lot of bovine PAGs (boPAGs) incapable of being enzymatically active due to amino acid substitutions around the catalytic site (Guruprasad *et al.* 1996; Xie *et al.* 1997b; Wooding *et al.* 2005 Szafranska *et al.* 2006). These PAGs are hypothesized to bind other proteins or consist of an atypical binding site as described in several plasmepsins (Berry *et al.* 1999; Banerjee *et al.* 2002; Wooding *et al.* 2005). Despite the above-mentioned functions, PAGs have been proposed to have immunomodulatory and luteotrophic actions (Wallace *et al.* 2015).

In the Bovidae 22 different PAG genes with a wide range of temporal and spatial expression- and glycosylation patterns have been identified (Telugu *et al.* 2009; Wallace *et al.* 2015). Bovine PAG are the subject of intense research but the exact number and function of boPAG and their closely related variants remain unclear (Telugu *et al*. 2009). There are different scientific perspectives on grouping the 22 boPAGs. Phylogenetic

analyses have indicated that PAGs can be clustered into at least two groups that were termed 'ancient' and 'modern' based on the time when each group arose (Hughes *et al.* 2000). The ancient group is thought to have arisen around 87 million years ago, whereas the modern one has arisen around 52 million years ago. Beside the difference in the date of origin both groups are transcribed in different cell types. The modern group (e.g. boPAG-1, -9, -21) is expressed only in a subset of trophoblast cells, so called binucleated cells (BNC). They are formed in the early stages of pregnancy and have been suspected to play an important role in implantation and placentation (Ishiwata *et al.* 2003; de Sousa *et al.* 2006). In addition, they are ideally positioned to adapt or regulate the maternal immune system to pregnancy (Wooding *et al.* 2005; Szafranska *et al.* 2006). Furthermore, boPAG-1 is the most commonly used PAG for pregnancy diagnosis in cattle (Wallace *et al.* 2015). The ancient group (e.g. boPAG-2, -8, -10, -11, -12) can be found in all trophoblast cells (Green *et al.* 2000; Hughes *et al.* 2000; Wooding *et al.* 2005; Telugu *et al.* 2009; Wallace *et al.* 2015;). A group of PAG genes (e.g. boPAG-2) that are expressed mainly at the fetal maternal interface may have an impact on the fetal-to-maternal surface attachment or the establishment of an immune barrier between both surfaces (Wallace *et al.* 2015).

To date there are only a few studies about detailed boPAG expression patterns throughout pregnancy. Comparison between these studies is difficult as the results are either inconsistent or show a low coverage of pregnancy days (e.g. only specific test days but no expression profiles). Although modern PAGs are well characterized in the maternal circulation (Sasser *et al.* 1986; Friedrich & Holtz 2010) detailed information about their mRNA expression up to day 60 *post conceptionem* (p. c.) are missing. The aim of this study was to illustrate detailed mRNA expression profiles for selected modern (boPAG-1, -9, -21) and ancient (boPAG-2, -8, -10, -11, -12) bovine PAGs with a high density of measurements, especially in early gestation and throughout pregnancy using real-time quantitative PCR (qPCR). The characterization of boPAG mRNA-expression levels provides important insights for further protein analyses which are valuable information for developing new pregnancy detection systems.

Materials and Methods

Sample Collection

Bovine cotyledonary tissue was collected at a local abattoir. Two samples from three different cotyledons per animal were taken as biological replicates. Cotyledons of similar size and location were collected by manual separation of placentomes within 30 min of slaughter. The pregnancy stage was estimated via fetal crown-rump-length (Rexroad *et al.* 1974) and divided into three groups: early pregnancy (day 20 - 90, N = 12), mid pregnancy (day 91 - 180, N = 11) and late pregnancy (day 181 - parturition, N = 1). Fifty mg of tissue per cotyledon were washed with 1xPBS (137mM NaCl, 2.7mM KCl, 4.3mM $Na_2HPO_4 \bullet 2H_2O$, 1.4mM KH_2PO_4) and transferred in 1.5 ml RNAlater (Sigma Aldrich, Germany), stored at 4°C overnight and at -20°C until RNA extraction.

RNA extraction

Total RNA was extracted using TriZol Reagent (Life Technologies, USA). The protocol was followed according to the manufacturer's recommendation with minor modifications: Samples were removed from RNAlater and briefly washed with 1x PBS, made free from fetal and maternal membranes, dissected in Petri dishes and subsequently homogenized in 1 ml TriZol Reagent using a FastPrep® FP120 Cell Disrupter (Qbiogene, USA) two times for 15 sec at 4 m/s. RNA was precipitated in ice-cold isopropyl alcohol and then washed in 1 ml of 80 % ethanol. RNA pellets were eluted in 50-150 µl RNase-free water (Ambion, USA) depending on the pellet size. The RNA quantity and quality were checked immediately after RNA extraction, samples were then reverse transcribed and stored at -80°C afterwards.

Assessment of RNA quantity and quality

The RNA concentration was measured at a wavelength of 260 nm, and the purity of RNA was assessed by the absorbance at 230 nm and 280 nm with a NanoPhotometer® P-360 spectrophotometer (Implen, Germany). The RNA quality number (RQN) was evaluated

using a microchip electrophoresis on a Fragment Analyzer (Advanced Analytical Technologies Inc., USA) in a core laboratory (Transcriptome and Genome Analysis Laboratory Goettingen, Germany).

Genomic DNA contamination was tested by endpoint PCR using intron spanning primers specific to bovine *CDH1* and PAG-genes (Table 1).

cDNA synthesis

Reverse transcription was performed using the High Capacity cDNA Reverse Transcription Kit (Applied Biosystems, USA). A fixed amount of 500 ng total RNA was reverse transcribed at 37 °C in a final volume of 20 µl containing 2x RT-Buffer, 2x RT Random Primers, 50 U MultiScribe™ Reverse Transcriptase, 4 mM dNTPs and 20 U RNase Inhibitor. CDNA samples were stored at -20°C.

qPCR analysis

Relative expression levels of selected PAG-genes in cotyledons were determined using real-time quantitative PCR (qPCR) with EvaGreen detection. Cadherin-1 (*CDH1*) and Tyrosine 3-Monooxygenase/Tryptophan 5-Monooxygenase Activation Protein Gamma (*YWHAG*) were selected as reference genes for normalization. Primer pairs for CDH1, boPAG-1, -2, -8, -9, -11, -12 and -21 were designed using primer3 software (http://bioinfo.ut.ee/primer3-0.4.0/) and their specificity checked with NCBI Primer-BLAST (https://www.ncbi.nlm.nih.gov/tools/primer-blast/). Primers for *YWHAG* and boPAG-10 were obtained from Telugu *et al.* (2009). Each PCR-fragment was sequenced and aligned to the corresponding reference sequence. The specificity was evaluated by Nucleotide-BLAST (https://blast.ncbi.nlm.nih.gov/Blast.cgi?PROGRAM=blastn&PAGE_TYPE=BlastSearch&LINK_LOC=blasthome) prior to qPCR-analyses to assess the amplification of the correct gene. All primers used in this study including their applications are given in Table 1.

Table 1: Oligonucleotides used for qPCR to measure relative expression levels of bovine PAG-genes during pregnancy

Gene	Accession Number	Primer	Sequence (5'-3')	Product size (bp)	
				cDNA	gDNA
boPAG-1	NM_174411.2	for	TAGGCTCATCGGTGCCATAC	116	203
		rev	GACCTGGCACTGGGTAGTTG		
boPAG-2	NM_176614.1	for	TCCTGGAGGAACAAGCTTACA	149	1111
		rev	TGAGCCTGTGTCAAAGACGA		
boPAG-8	NM_176619.3	for	GACACCGGCTCATCTGACTT	139	229
		rev	CAGAGCCGTAGACGAGTTCA		
boPAG-9	NM_176620.2	for	TGAAGTGGATTGTGCTCCTC	149	1228
		rev	CTGGGACAGTCTGTAAGGATGC		
boPAG-10	NM_176621.3	for	TTGAGCAGTCAGAAAGAGAACG	137	-
		rev	TTCATGGAGATGCTGTCTATGTTT		
boPAG-11	NM_176623.2	for	GAAGATGACAGGAGGCAGGATAC	138	221
		rev	GTGGATACCGGGACATCACT		
boPAG-12	NM_176622.1	for	TCCTGGAAGAACGAGCTTACA	149	1113
		rev	TGAGCCTGTGTCAAAGACGA		
boPAG-21	NM_176630.2	for	TACAGGCTCATCTGACTTTTGG	141	240
		rev	CTCCCAGATCCATAGGTGATGC		
CDH1	NM_001002763.1	for	TGCCCAGAAAATGAGAAAGG	99	225
		rev	TTGGCCAGTGATGCTGTAGA		
YHWAG	NM_174793.2	for	AGCACATGCAGCCCACTC	121	-
		rev	TCGTCGAAGGCGGTCTTG		

PCR-products were amplified using 2 µl of cDNA, 5 mM dNTPs (Roche, Switzerland), 10 µM of each primer (Sigma Aldrich, Germany), 0.5 µl EvaGreen (JenaBioscience, Germany) and 1.5 U FastStart Taq-Polymerase in 1x PCR buffer containing $MgCl_2$ and 1x GC-Solution (Roche, Switzerland) in a final volume of 25 µl. For amplification of boPAG-11 1x Sulfolane (Sigma Aldrich, Germany) was added to the reaction mix.

PCRs were performed in a Stratagene Mx3005P real-time cycler (Agilent, USA) with the following thermal profile: 38 cycles of 30 s at 95 °C, 30 s at 56 °C and 30 s at 72 °C with an additional 10-min denaturation in the first cycle. The amplification was followed by a melting curve measurement with 1 min at 95 °C, 30 s at 70 °C then heating the samples to 95 °C, where the change in fluorescence was measured at each 0.5 °C rise.

Amplification efficiencies were calculated using standard curves generated by serial dilutions of known concentrations of the target amplicons with six orders of magnitude. Samples were quantified by the $2^{-\Delta\Delta CT}$ method (Livak & Schmittgen 2001) or Pfaffl

method (Pfaffl 2001) respectively. For each transcript, PCR was performed in triplicate as technical replicates.

Statistical analysis

The experimental results were analyzed with R 3.2.2 (R Development Core Team, Austria). Expression levels were compared within different PAG-genes (boPAG-1, boPAG-2, boPAG-8, boPAG-10, bo-PAG-11, boPAG-12, boPAG-21) and different pregnancy stages (early, middle and late) with PAG-gene-pregnancy stage interactions using a two-way ANOVA. Post hoc evaluation was performed with Tukey's Honest Significant Difference (HSD) Test.

Results

Sample collection and RNA-extraction

Cotyledonary tissue from 24 animals was collected and used for RNA extraction. RNA-samples were observed to have mean spectrometry values of 1.99 ± 0.09 for absorbance ratios A260/A280, on the other hand mean OD A260/A230 ratios were 1.4 ± 0.4. RQN values derived from a Fragment Analyzer (Advanced Analytical Technologies Inc., USA) were between 5.8 and 9.9. After quality assessment a total of 21 samples with two or three biological replicates remained in the study (early pregnancy: N = 11; mid pregnancy: N = 9; late pregnancy: N = 1).

qPCR

Standard curves of all genes had efficiencies in the range of 95.1 % to 103 % and slopes in the range of -3.24 to -3.45. Gene expression ratios for boPAG-8, -9, -11, -12 and -21 were determined by the $2^{-\Delta\Delta CT}$ method (Livak & Schmittgen 2001) and according to Pfaffl (2001) for boPAG-1, -2 and -10 due to higher differences in primer efficiencies between reference genes and boPAG-genes of interest (Figure 1).

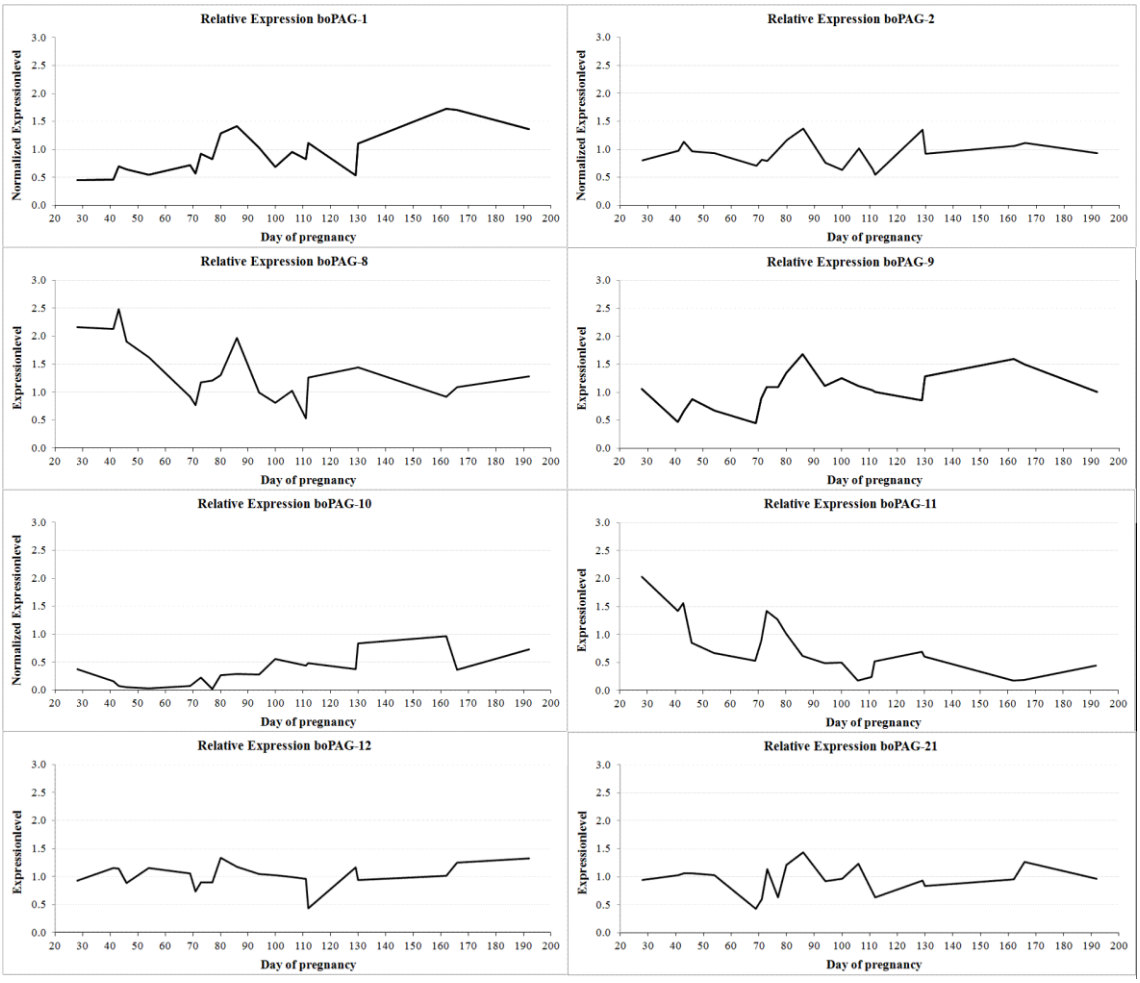

Figure 1: relative expression patterns of boPAG-1, -2, -8, -9, -10, -11, -12, -21. Expression levels for boPAG-1, -2 and -10 derived from efficiency-normalization by Pfaffl-method. Expression levels for boPAG-8, -9, -11, -12 and -21 were determined by $2^{-\Delta\Delta CT}$ method.

All boPAG-genes of interest were detectable and could be quantified throughout the available pregnancy stages. A 2-way ANOVA indicated significant differences in the expression levels between PAGs ($p < 0.001$) and an interaction between boPAG-gene and

pregnancy stage ($p < 0.001$) on the relative expression level. A significant overall pregnancy stage effect was not detected. In the next step a pairwise comparison using Tukey's HSD test was performed.

BoPAG-1 shows highest expression levels around the end of the first and second trimester. A similar pattern is given in boPAG-9 and, for the early pregnancy, in boPAG-21, which are members of the modern boPAG-group.

BoPAG-2 and -12 are the most closely related PAG-members in this investigation. Nevertheless, the expression patterns are only slightly similar with an increase towards the end of the early pregnancy and a decline between day 110 and 120. BoPAG-8 has the highest mean expression level and boPAG-10 the lowest mean expression level, a significant difference in the mean expression level was observed in comparison with the other investigated PAGs ($p < 0.05$).

Besides that, boPAG-8, -10 and -11 transcripts show a mirror-inverted expression in cotyledonary tissue where boPAG-8 and -11 expressions are higher in the early gestation while boPAG-10 expression increases throughout pregnancy. BoPAG-1, -2, -9, -11, -12 and -21 are expressed on similar level throughout the different pregnancy stages (Figure 2).

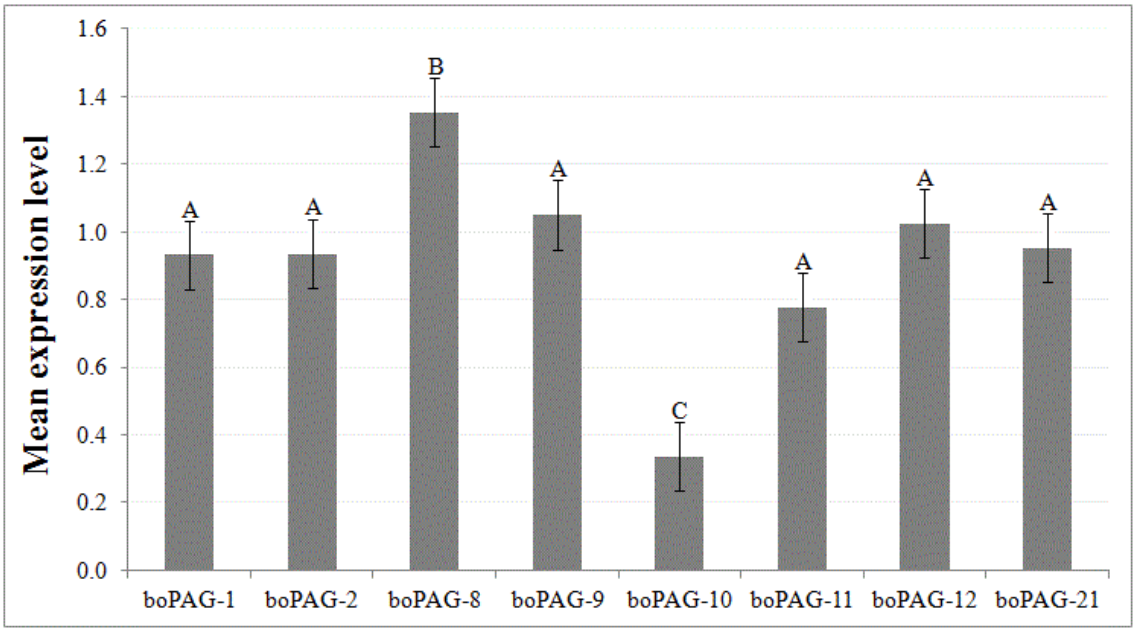

Figure 2: Differences in gene expression levels between observed boPAG-genes.

Pairwise comparison revealed that boPAG-8, which had the highest mean expression level in early pregnancy stage, decreases significantly from early pregnancy to mid-pregnancy ($p < 0.05$) and remained at steady-state levels between mid-pregnancy and late pregnancy. A similar effect to boPAG-8 was observed in boPAG-11 with a significant decrease in mean expression between early pregnancy and mid pregnancy ($p < 0.001$) (Figure 3). In all other examined boPAGs no significant differences between the relative expression levels within the pregnancy stages were observed.

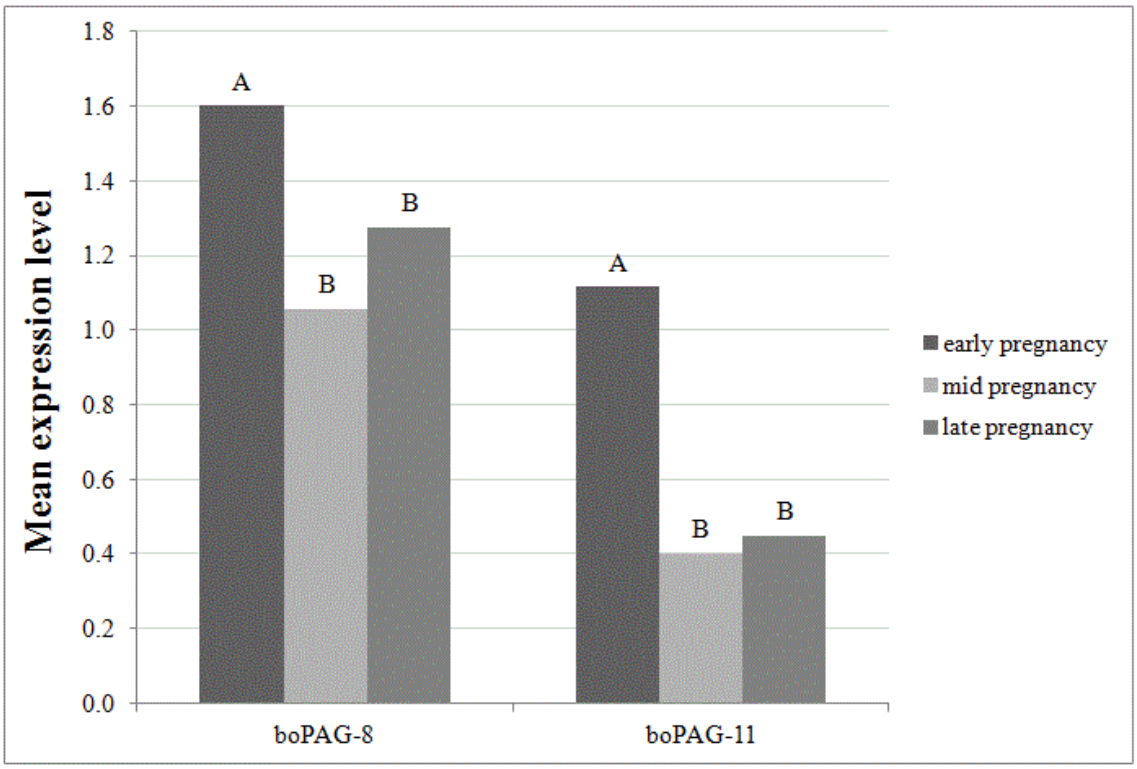

Figure 3: Differences in gene expression levels between different pregnancy stages. Letters indicate significant differences between pregnancy stages within boPAG-8 ($p < 0.05$) and boPAG-11 ($p < 0.001$).

Discussion

To our knowledge, this is the first study describing mRNA expression profiles of both ancient and modern boPAG genes in cotyledons before day 60 p. c.. In contrast to other studies that examined expression levels from different boPAGs, we did not examine specific pregnancy days but collected samples continuously. Therefore, we have been able

to illustrate detailed patterns of boPAG expression, especially in early gestation from day 28 p. c. onwards.

Some of the first studies on the relative quantification of bovine PAG transcripts in placental tissue were reported by Patel *et al.* (2004) and Telugu *et al.* (2009). Patel *et al.* (2004) observed a significantly higher expression of boPAG-9 on day 30 of gestation compared to boPAG-1. Our results correspond to these findings, regarding a generally higher relative expression of boPAG-9 in cotyledonary tissue although we could not determine any significant differences. Furthermore, our results do not indicate a continuous increase of boPAG-9 expression from day 30 to day 60 as reported by Patel *et al.* (2004). Instead there is a decrease in-between.

Touzard *et al.* (2013) described lowest boPAG-1 levels at day 60 of gestation and a significant increase until day 80. This expression pattern is in line with the present data, but boPAG-1 abundance increases from the beginning of gestation until day 60.

In former studies performed by Telugu *et al.* (2009) boPAG-2 was found as the most abundant transcript. Although this cannot be confirmed by our results regarding relative expression levels, the absolute expression of boPAG-2 is higher than of other transcripts. Its most closely related transcript - boPAG-12 - did not show significant differences in relative expression patterns but its absolute abundance is lower (results not shown). So far, boPAG-21 expression was analyzed in only one study with alternating up- and down-regulation (Touzard *et al.* 2013). Our results verify previous findings and additionally show that boPAG-21 is more highly expressed in the very early pregnancy and down-regulated until day 60 p. c.

The regulation of PAG transcription has not yet been elucidated (Wallace *et al.* 2015). The pre-transcriptional regulatory sequences are highly conserved within the gene family and include potential binding sites for transcription factors that might be involved in transcriptional regulation (Telugu *et al.* 2009).

Our results show that the relative expression of boPAG-8 is significantly higher throughout the whole pregnancy compared to the other PAG-genes. This confirms the study of Touzard *et al.* (2013) who also described the highest expression in boPAG-8.

Furthermore, we confirm earlier studies on expression patterns of ancient boPAG-genes (Telugu *et al.* 2009): the expression of boPAG-8 and boPAG-11 is significantly higher in the early gestation and declines with progression of pregnancy with a short increase around day 80. In contrast to that boPAG-10 expression peaks in the end of second trimester, beginning of the last trimester respectively. Nevertheless, it is the least expressed transcript in our study. In general, the present study confirms further findings e.g. by Patel *et al.* (2004), Telugu *et al.* (2009) and Touzard *et al.* (2013) and expands the knowledge by more detailed information about boPAG-expression in early gestation. We collected samples from an abattoir and did not slaughter animals at fixed timepoints but determined the age of the fetuses by measuring the crown-rump-length which is an approximate standard for the normality of Holstein-Friesian fetal growth (Rexroad *et al.* 1974). Nevertheless, we obtained similar expression patterns as previously described. This shows that the method by Rexroad *et al.* (1974) is still a reliable tool for estimating the age of bovine fetuses, although breeding progress took place in the Holstein-Friesian cattle bread e.g. increased body size (Schönmuth & Löber 2006).

The low amount of samples for late pregnancies in this study is due to several reasons: on the one hand we generally observed better RNA-quality in samples from early gravidities compared to samples from mid or late pregnancies. This might be a result of higher time exposure during sample collection as the conjunction of placentomes is stronger and the detachment of cotyledonary tissue is more complicated. Previous studies already described that there is an effect of time exposure during tissue handling and RNA-integrity (Copois *et al.* 2007; Fajardy *et al.* 2009). The variability of gene expression profiles is dependent on the homogeneity of the starting material. The placenta consists of a large pattern of different cell types, fetal and maternal areas including blood of both individuals (Mondon *et al.* 2005; Fajardy *et al.* 2009;). This leads to different expression patterns even in samples from one organism according to the (cell) composition of each sample. As a consequence, samples or biological replicates, especially from pregnancies > 100 days, were excluded from the analyses to ensure reliable results. Nevertheless, a minimum of two biological replicates per sample remained in the study. Another reason is a law that prohibits slaughtering healthy mammals (except goats and sheep) in the last third of gestation for commercial reasons, which came into force in September 2017

(TierErzHaVerbG, § 4). On the other hand, we had a high coverage of measurement points especially for < 100 days of pregnancy.

According to the different expression patterns of the analyzed boPAG genes, we suppose that boPAGs with a higher expression in early pregnancy (boPAG-8 and boPAG-11) seem to be important factors during placentation. Touzard *et al.* (2013) pointed out the restriction of boPAG-11 to binucleate cells located in the chorionic plate of the cotyledon. Therefore, they are ideally situated to accumulate at the placenta-uterine interface by acting as bridging molecules during cell-cell adhesion (Wallace *et al.* 2015).

The expression levels of boPAG-1, boPAG-9 and boPAG-10 are higher in the mid/late gravidity, which leads us to the suggestion that these PAGs might play a crucial role in maintenance of pregnancy. For boPAG-1, luteotrophic capabilities have already been described (Weems *et al.* 1998). Similar functions can be assumed for boPAG-2, boPAG-12 and boPAG-21.

Conclusion

In summary, it was possible to derive distinct longitudinal mRNA expression patterns and varying overall expression levels of eight different bovine PAGs in cotyledonary tissue across subsequent pregnancy stages. Although late pregnancy stages were underrepresented in our data, clear differences between early (pregnancy days 20-90) and later stages were found, especially for boPAG-8 and -11 indicating a possible role of these PAGs in placentation and the maintenance of early pregnancy.

Acknowledgement

The authors are deeply indebted to Julia Sondermann for the excellent technical assistance with the sample collection.

Source of Funding

The study was financially supported by initial funding provided by the University of Goettingen to CK.

Contributions

CK, IW and JT designed the study, TK collected samples, IW an NM performed molecular genetic analyses, TK analyzed the data, IW, TK and JT drafted the paper

Conflict of interest

The authors declare that they do not have any financial and personal relationships with other people or organizations that could inappropriately bias or influence their work.

Ethical Statement

The study is in accordance with the German legal and ethical requirements of appropriate animal procedures. Animals were not purposely euthanized for this study. Samples were taken during the conventional slaughter process.

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4 A Multiplexed Parallel Reaction Monitoring Assay to monitor bovine Pregnancy-Associated Glycoproteins throughout pregnancy and after gestation

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veröffentlicht in

PLoS ONE (2022), 17(9): e0271057

URL: https://doi.org/10.1371/journal.pone.0271057

DOI: 10.1371/journal.pone.0271057

Abstract

Bovine pregnancy-associated glycoproteins (**boPAGs**) are extensively glycosylated secretory proteins of trophoblast cells. Roughly 20 different boPAG members are known but their distribution patterns and degree of glycosylation during pregnancy are not well characterized. The objective of the present study was the development of a parallel reaction monitoring-based assay for the profiling of different boPAGs during pregnancy and after gestation. Furthermore, we investigated the effects of N-glycosylation on our analytical results. BoPAGs were purified from cotyledons of four different pregnancy stages. The assay detects 25 proteotypic peptides from 18 boPAGs in a single run. The highest abundances were found for boPAG 1 in both, glycosylated and deglycosylated samples. Strongest effects of glycosylation were detected during mid and late pregnancy as well as in afterbirth samples. Furthermore, we identified different boPAG-clusters based on the observed relative protein abundances between glycosylated and deglycosylated samples. A linkage between the impact of glycosylation and potential N-glycosylation sites or phylogenetic relation was not detected. In conclusion, the newly developed parallel reaction monitoring-based assay enables for the first time a comprehensive semi-quantitative profiling of 18 different boPAGs during pregnancy and post-partum on protein level, thereby investigating the influence of glycosylation. The results of this study provide new and important starting points to address further research on boPAGs to better understand their physiological role during pregnancy and for the development of new pregnancy detection tests.

Introduction

Pregnancy-Associated Glycoproteins (**PAGs**) are expressed in trophoblast cells of the placenta of species within the Cetartiodactyla order and secrete in maternal blood and milk [1]. They belong to the aspartic proteinase family and are therefore related to pepsin [2,3], cathepsin D, cathepsin E [4], chymosins and renin [4–6]. PAGs can be phylogenetically divided into an ancient group, which is predicted to have originated 87 million years ago, and a modern group, which is predicted to have arisen 52 million years ago [1,7]. Most of the PAGs belong to the modern group, which can only be found in the Ruminantia with their synepitheliochorial placenta, and they are particular numerous in the *Bovidae* [1,8–10].

To date, in cattle roughly 20 different PAGs and related paralogs are known, with largely varying temporal and spatial expression patterns during gestation [1,9,10]. Phylogenetic analysis discovered that six of those bovine PAGs (**boPAGs**) belong to the ancient group [7,9,11]. Initial studies suggest that ancient boPAGs are predominantly expressed in both, mono- and binucleate cells, whereas modern boPAGs are expressed only in binucleate cells [2,9,11]. A more recent study by Touzard et al. (2013) demonstrated that modern boPAGs are expressed in cotyledons and ancient boPAGs are expressed in the intercotyledonary chorion [12].

The ancient boPAGs are thought to be active aspartic proteinases, whereas modern boPAGs have lost their catalytic activity due to amino acid substitutions within the binding sites [3,5,11,13]. The enzymatic activity for some members of the ancient group was experimentally confirmed [14]. In the modern boPAG group the binding function may be retained and enables this group to bind or interact with peptides or proteins without hydrolyzing them [3,5,11,13].

An unusual feature of boPAGs is their high degree of glycosylation. The expected molecular mass of the protein core without posttranslational modifications and after the removal of the signal sequence is around 37 kDa [15,16]. The mean molecular weight of boPAGs with attached N-glycans is around 67 kDa [12,16–18]. Therefore, the degree of N-glycosylation seems to be the major factor in boPAG molecular mass [16]. The different boPAGs have up to six potential N-glycosylation sites [4,12,16]. A tetraantennary

core-fucosylated structure with a bisecting N-acetylglucosamine (GlcNAc) with all antennae carrying a terminal Sda-antigen (NeuAcα2-3[GalNAcβ1- 4]Galβ1-4GlcNAc-) could be identified as the most abundant N-glycan in boPAGs expressed in binucleate cells [19]. This N-glycan structure shows a relatively high uniformity, which is indicative for a highly regulated glycosylation process in the binucleate cells and therefore suggests that the glycans might have specific functions during pregnancy [15,19,20]. Furthermore, the attached N-glycans and their change during the course of pregnancy seem to be an important factor for the boPAG-clearance from the maternal blood. The absence of the Sda-antigen in the beginning (before day 30 of gestation) and to the end of pregnancy causes a higher serum half-life of boPAGs at these timepoints [19,20]. This led to the assumption that the glycosylation process in cattle is under endocrine control. The change in estradiol concentration might be the main regulatory element in this process, but the exact mechanisms remain unclear [19]. Despite many years of research, the overall physiological role of boPAGs and the mechanism of their possible function are unknown. It has been hypothesized in the past that PAGs may act in protecting fetal or placental antigens from the maternal immune system, process growth factors, influence the secretion of progesterone, or facilitate adhesion (at implantation) and detachment (at birth) processes at the fetal maternal interface [1]. Therefore, their existence seems to be essential for a successful pregnancy outcome. To date, there are only a few studies that give insights into the protein level of different boPAGs [12,16–19,21]. However, these studies are often limited to few PAGs and a specific gestational period (e.g. mid pregnancy) and thus do not cover the entire pregnancy.

During the last years, new mass spectrometers and data acquisition schemes for the characterization and quantification of proteins have been developed [22–24]. In targeted MS methods, only a specific subset of analytes is measured in predefined *m/z* ranges and known retention time windows, e.g. a set of predefined tryptic peptides as surrogates for the proteins of interest [25,26]. These methods have become the gold standard for large-scale quantification and verification of proteins, even when applied to complex biological samples [22,23,26,27]. Parallel Reaction Monitoring (**PRM**) is a targeted method where all product ions of mass-selected peptides are monitored in parallel with one ion injection and full scan mass analysis [22,24]. This method has several

advantages. All potential product ions of a target peptide are available for peptide identification and there is no need of preselection of target transitions before analysis [22]. Furthermore, PRM has a high tolerance for co-isolated background peptides and can be multiplexed where the product ions of several target peptides are comingled and detected in a single-scan [22,28].

The aim of the present study is the development and validation of a multiplexed PRM assay for boPAGs in order to allow for the first time a comprehensive semi-quantitative profiling of boPAGs during pregnancy and post-partum. Furthermore, we investigate glycosylated and deglycosylated samples to detect possible effects of protein modifications on the results obtained using our methods. The establishment of a new method for the characterization of boPAGs on protein level will enable future studies investigating the physiological role of boPAGs during pregnancy and developing new pregnancy detection tests.

Materials and methods

The study is in accordance with the German legal and ethical requirements of appropriate animal procedures. Animals were not purposely euthanized for this study. Tissue samples were taken during the conventional slaughter process.

Tissue collection and protein purification

The detailed workflow of tissue collection and subsequent protein purification is described in Krebs et al., 2021 [29]. In brief, cotyledon samples were collected from an abattoir and a local dairy farm. Based on the crown-rump length of the fetuses [30], they were divided into four pregnancy stages: 35-90 days of gestation (early pregnancy), 91-180 days of gestation (mid pregnancy), 181-240 days of gestation (late pregnancy) and afterbirth samples. Subsequently, protein extraction from cotyledonary tissue was performed according to the protocols of Zoli et al. (1991) [17] and Klisch et al. (2005) [16]

with some modifications. Further protein purification was performed using a multistep fast protein liquid chromatography (**FPLC**). A scheme of the different FPLC steps can be found in Fig S1 in the supplementary. The resulting boPAG-containing fractions were stored at -20 °C until further analysis [29].

Peptide-N-Glycosidase F (EC 3.5.1.52) treatment

Aliquots of protein samples from chromatography analysis were subjected to Peptide-N-Glycosidase F (**PNGaseF**) digestion to remove N-gylcans according to the manufacturer's instructions (New England Biolabs, Germany). Therefore, 10 µg of protein extract were denatured for 10 min at 100 °C in glycoprotein denaturing buffer 10x (New England Biolabs, Germany) and LiChrosolv H_2O (Merck, Germany) (if necessary) in a 10 µl reaction volume. After cooling for 5 min on ice, the samples were centrifuged for 10 sec at full speed (13,300 rpm). Following this, 100 IU PNGaseF, 2 µl GlycoBuffer 2 (10x) (New England Biolabs, Germany), 2 µl 10 % NP-40 (New England Biolabs, Germany) and 6 µl LiChrosolv H_2O were added to the samples to reach a total reaction volume of 20 µl. After incubation for 1 h at 37 °C, deglycosylated protein samples were used for in gel digestion followed by mass spectrometric analysis.

Western blot analysis

Purified protein samples from homogenized cotyledonary tissue of all pregnancy stages were either deglycosylated with PNGase F or left glycosylated. All samples were diluted to a final concentration of 1 µg protein/lane in either deglycosylation buffer (deglycosylated samples) or with dd H_2O (glycosylated samples). In addition, NuPAGE LDS sample buffer 4x (Thermo Fisher Scientific, USA) was added to all samples, followed by an incubation for 10 min at 70 °C prior loading.

Gel electrophoresis was performed using NuPAGE 4-12 % Bis-Tris 1.0 mm gradient gels (Thermo Fisher Scientific, USA). Gels were run at 80 V until the samples were migrated out of the wells. Afterwards the voltage was increased to 150 V to finish the run. Molecular weight standard (Protein Marker VI (10-245) prestained, Applichem, Germany) was

run simultaneously. Gels were either stained with Coomassie Brilliant Blue or proteins were transferred onto nitrocellulose membrane (GE Healthcare, USA) in transfer buffer (25 mM Tris, 192 mM glycine diluted in 20 % C_3H_8O) [31]. The transfer was performed by 80 V for 1.5 h at 4 °C.

After transfer, the nitrocellulose membrane was briefly rinsed with ddH_2O and stained with Ponceau S solution (0.1 % Ponceau diluted in 5 % CH_3COOH) to check the protein transfer quality. Afterwards Ponceau stain was rinsed off with washes of Tris-buffered saline containing Tween 20 (TBST) (20 mM Tris, 136.5 mM NaCl diluted in 0.01 % Tween 20; pH 7.4).

The membranes were blocked with 1 % BSA in TBST at room temperature (**RT**) for 30 min, washed three times with TBST, and probed with different boPAG antisera at a concentration of 13 µg antibody/membrane. Polyclonal boPAG antisera were produced using seven boPAG-fractions of different pregnancy stages from chromatography. A detailed description of the polyclonal antibody production is given in Krebs et al. (2021) [29]. Afterwards, membranes were incubated overnight at 4 °C while shaking. On the next day, the nitrocellulose membranes were washed three times with TBST and incubated with a fluorescence-labeled goat anti-rabbit IgG (1:50,000) (IRDye 680RD Goat anti-Rabbit IgG Secondary Antibody, LI-COR Biosciences, USA) for 1 h at room temperature in the dark. After three TBST washes, western blots were developed at 700 nm with Odyssey CLx Near-Infrared Fluorescence Imaging System (LI-COR Biosciences, USA).

Mass spectrometric protein analysis – sample preparation

Acetonitrile and H_2O used in these experiments were purchased from Merck, Germany in LiChrosolv hypergrade.

Both glycosylated samples from chromatography and deglycosylated samples from PNGaseF digestion were subjected to in-gel digestion with trypsin (EC 3.4.21.4). Therefore, all samples were diluted to a final concentration of 10 µg protein/lane in NuPAGE LDS sample buffer 4x, NuPAGE Sample Reducing Agent 10x (Thermo Fisher Scientific, USA) and H_2O, followed by incubation for 10 min at 70 °C prior to loading. Gel

electrophoresis was performed on a NuPAGE 4-12 % Bis-Tris 1.0 mm gradient gel in an 8x8 cm vertical unit (Bio-Rad, USA) and stained with Coomassie Brilliant Blue for visualization. For an initial shotgun analysis to determine sample purity and detect PRM peptide candidates, gels were run full distance and cut into 15 equidistant slices irrespective of staining. For the final PRM assays sample were run ~1 cm into the gel for purification purposes only and excised as a single band. Gel slices were washed 5 min at room temperature under shaking. Afterwards, the supernatant was discarded and 100 % acetonitrile (Merck, Germany) added to the gel pieces. After incubation for 15 min at room temperature on a shaker (750 rpm), the supernatant was again discarded and the gel pieces were dried in a vacuum centrifuge (SpeedVac Savant SPD111V, Thermo Fisher Scientific, USA).

Reduction was performed by incubation with dithiothreitol (10 mM $C_4H_{10}O_2S_2$ in 100 mM NH_4HCO_3, 50 min, 56 °C). Following centrifugation, the supernatant was removed and the gel pieces dried using 100 % acetonitrile again. Alkylation was then achieved with iodoacetamide (55 mM C_2H_4INO in 100mM NH_4HCO_3, 20 min, RT, darkness). After removal of the supernatant, gel pieces were washed again with 100 mM NH_4HCO_3. Samples were dried using 100 % acetonitrile again twice, and the gel pieces dried in a vacuum centrifuge. Endoprotease lysis was performed overnight at 37 °C using modified porcine trypsin (Trypsin Gold, Promega, USA) in digestion buffer (50 mM NH_4HCO_3, 5 mM $CaCl_2$) at an enzyme-to-protein ratio of 1:50.

Peptides were extracted from the gel by incubation with water (20 µl, 15 min, 37 °C). 80 µl neat acetonitrile were added and further incubated 15 min at 37 °C. The supernatants were transferred to fresh tubes, the gel pieces incubated again with 5 % aqueous formic acid (65 µl, 15 min, 37 °C), centrifuged, and incubated again after addition of 65 µl neat acetonitrile. Subsequently, the gel pieces were centrifuged and the supernatants pooled with supernatants from the first extraction. Resulting peptide solutions were dried in a vacuum centrifuge and stored at -20 °C until further use.

Prior to MS analysis, peptide mixtures were reconstituted in loading buffer (1 % acetonitrile, 0.1 % CH_2O_2 in H_2O) to a nominal concentration of 100 fmol/µl, and spiked with 100 fmol/µl Escherichia coli ß-galactosidase (EC 3.2.1.23) tryptic digest to avoid

adsorption effects [32] and 100 fmol/µl of pepstatin A (Sigma-Aldrich, USA) to inhibit proteolytic activity of boPAGs [13,14,33] during analysis.

Mass spectrometric protein analysis

For initial protein identification, samples were enriched on a self-packed reversed phase-C18 precolumn (0.15 mm ID x 20 mm, Reprosil-Pur120 C18-AQ 5 µm, Dr. A. Maisch HPLC GmbH, Germany) and separated on an analytical reversed phase-C18 column (0.075 mm ID x 200 mm, Reprosil-Pur 120 C18-AQ, 3 µm, Dr. A. Maisch HPLC GmbH, Germany) using a 30 min linear gradient of 5-35 % acetonitrile/0.1 % formic acid (v:v) at 300 nl/min). The eluent was analyzed on a Q Exactive hybrid quadrupole/orbitrap mass spectrometer (Thermo Fisher Scientific, Germany) equipped with a FlexIon nanoSpray source and operated under Excalibur 2.4 software using a data-dependent acquisition method. Each experimental cycle was of the following form: one full MS scan across the 350-1600 *m/z* range was acquired at a resolution setting of 70,000 full width at half maximum (**FWHM**), and AGC target of $1*10e^6$ and a maximum fill time of 60 ms. Up to the 12 most abundant peptide precursors of charge states 2 to 5 above a $2*10e^4$ intensity threshold were then sequentially isolated at 2.0 FWHM isolation width, fragmented with nitrogen at a normalized collision energy setting of 25 %, and the resulting product ion spectra recorded at a resolution setting of 17,500 FWHM, and AGC target of $2*10e^5$ and a maximum fill time of 60 ms. Selected precursor *m/z* values were then excluded for the following 15 s. Two technical replicates per sample were acquired.

For PRM assays, samples were analyzed on a nanoflow chromatography system (Eksigent nanoLC425) hyphenated to a hybrid triple quadrupole-TOF mass spectrometer (TripleTOF 5600+) equipped with a Nanospray III ion source (Ionspray Voltage 2400 V, Interface Heater Temperature 150°C, Sheath Gas Setting 12) and controlled by Analyst TF 1.7.1 software build 1163 (all AB Sciex, Germany). Peptides dissolved in loading buffer were enriched on a micro pillar array trapping column (µPac 1 cm, 5 µm, PharmaFluidics, Belgium) and separated on an analytical micro pillar array column (µPac 50 cm, 2.5 µm, PharmaFluidics, Belgium) using a 30 min linear gradient of 5-35 % acetonitrile/0.1 % formic acid (v:v) at 300 nl/min.

Targeted LC/MS/MS analysis was performed using a Top12 parallel reaction monitoring acquisition consisting of a MS survey scan of *m/z* 350–1250 accumulated for 150 ms at a resolution of 30,000 FWHM, and up to 12 MS/MS scans of *m/z* 180–1600 accumulated for 150 ms at a resolution of 17,500 FWHM and a precursor isolation width of 0.7 FWHM, resulting in a total cycle time of 2.1 s. Precursors were chosen from a retention-time encoded list of peptide *m/z* values of interest, which were selected for MS/MS in a time window of RT±4 min above a threshold MS intensity of 125 cps. MS/MS activation was achieved by CID using nitrogen as a collision gas and the manufacturer's default rolling collision energy settings. Two technical replicates per sample were acquired.

PRM Peptide selection and data analysis

Protein and peptide identification was achieved using MASCOT Software version 2.4 (Matrix Science Ltd, USA) [34] or MaxQuant Software version 1.5.7.4 (Max Planck Institute for Biochemistry, Germany) [35]. Proteins were identified against the UniProtKB bovine reference proteome (v2020.01) along with a set of common lab contaminants. Searches were performed with trypsin (excluding proline-proximal cleavage sites) as enzyme and iodoacetamide as cysteine blocking agent. Up to two missed tryptic cleavages were allowed for, and methionine oxidation and protein N-terminal acetylation variable modifications. Instrument type ‘Orbitrap’ was selected to adjust for MS acquisition specifics. MASCOT searches were performed using precursor mass tolerances of 15 ppm and a fragment mass tolerance of 0.1 Da. MaxQuant searches used an internal pre-calibration for a final search with tolerances of 4.5 ppm (MS) and 20 ppm (MS/MS), respectively. Protein and peptide results lists were thresholded at False Discovery Rates (**FDR**) of 0.01, respectively, using a forward-and-reverse decoy database approach. Afterwards, protein identification results were imported into Scaffold version 5.0.0 (Proteome Software, USA) [36] for analysis of sequence coverage of different boPAGs.

PRM data were analyzed using Skyline version 20.1.0.76 (University of Washington, USA) [37]. In Skyline, all peaks were automatically integrated and the 6 most intense transitions (y and b ions) for each precursor were selected. Furthermore, all peaks were manually inspected and the peak boundaries were adjusted to avoid interferences and to

confirm correct detection. Afterwards the Total Area Fragment data from each peptide was exported from Skyline and further analyzed using R 3.6.1 (R Development Core Team, Austria).

PRM for qualitative measurements of boPAGs in the protein digests were performed targeting at least one prototypic peptide for each boPAG (Table 1). Selected peptides were verified to be unique to the protein of interest by an online BLAST analysis (Program: NCBI BLASTP, database: NCBI Protein Reference Sequences database, Organism: Bos Taurus (taxid:9913), 2020/06/22). Peptides containing potential missed cleavage sites, methionine or cysteine were excluded; doubly charged precursor ions were favored. Furthermore, the selected peptides must have a length between 8 and 25 amino acids and *m/z* values between 450 and 800.

Statistical analysis

All experimental results were statistically analyzed with R 3.6.1 (R Development Core Team, Austria). Peptides with a high coefficient of variation (**CV**) of their measured peak area between two technical replicates (CV > 30 %) were not considered for further analysis. The relative protein abundances (expressed in percentage) of each boPAG (glycosylated and deglycosylated) in each pregnancy stage were calculated by the ratio of its peptide peak area to the total peptide peak area from all boPAGs of each pregnancy stage. This is a common method, which is also described in the literature [38,39]. Relative protein levels and the influence of glycosylation during pregnancy were examined using a one-way ANOVA. In order to evaluate the differences within the classes of boPAGs, a post hoc test with Bonferroni correction was performed. The Bonferroni correction was applied to correct for the number of comparisons resulting from multiple testing. The results were considered significant at $P < 0.05$.

The shotgun MS proteomics data have been deposited to the ProteomeXchange Consortium via the PRIDE [40] partner repository with the dataset identifier PXD027383. The PRM raw data have also been deposited to the ProteomeXchange Consortium via the PRIDE [40] partner repository with the dataset identifier PXD034108.

Results

In this study we developed a multiplexed PRM assay for boPAGs with the aim to provide an overview of the relative abundances of different boPAGs on protein level in the course of pregnancy and after parturition.

BoPAGs were purified from cotyledons from different gestation stages by FPLC. A detailed description of the purification process can be found in Krebs et al. (2021) [29]. A scheme of the different FPLC steps is shown in Fig S1 in the supplementary. Overall, we analyzed six different purified poolsamples from four different gestation stages out of 16 different pregnancies (early pregnancy poolsample was purified from cotyledonary tissues of five male and five female fetuses, mid pregnancy, late pregnancy, and afterbirth poolsamples were purified from cotyledonary tissues of one male and one female fetus each) by mass spectrometry. The early pregnancy poolsample consisted of cotyledonary tissues of ten pregnant cows in order to obtain sufficient protein amounts. At this point, we would also like to mention that our workflow for developing the presented PRM assay makes use of multi-stage chromatographic separation upfront to the actual proteomic analysis (see Materials and Methods). The required effort technically precludes the use of high n sampling numbers.

Upon gel electrophoresis, boPAG-fractions isolated from the cotyledonary tissue gave one major band at around 67 kDa in the native form and at approximately 37 kDa after deglycosylation (Fig 1). These apparent molecular weights are consistent with earlier data [1,3,16–18] including the mass shift after deglycosylation [12,16,20].

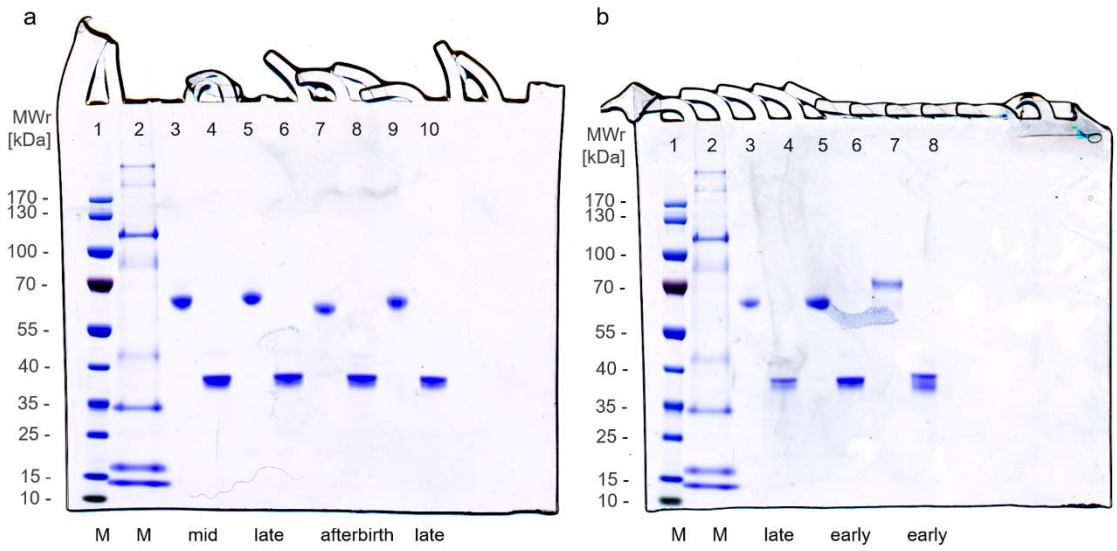

Fig 1. Gel images of seven different purified bovine pregnancy-associated glycoproteins (boPAG) -samples from four different pregnancy stages. The protein samples (1 µg/lane) were either enzymatically deglycosylated with Peptide-N-Glycosidase F (PNGase F) (lane 4, 6, 8, 10) or left untreated (lane 3, 5, 7, 9). Molecular weights of the marker (M)-bands (lane 1, 2) are indicated on the left (kDa). Early pregnancy samples of lane 7 (b) and lane 8 (b) were not analyzed by mass spectrometry.

For an initial overview of the purity and composition of the different protein fractions, the glycosylated and deglycosylated samples were subjected to in-gel trypsin digestion followed by shotgun-MS analysis. From the data, a total of 9 different boPAGs (boPAG 1, boPAG 3, boPAG 4, boPAG 6, boPAG 7, boPAG 10, boPAG 16, boPAG 20 and boPAG 21) could be identified in the samples of the respective gestation stages (Table S1-S12). We used the untargeted peptide identification results to construct a first list of candidate peptides for a targeted PRM assay. Due to the known limitations of shotgun mass spectrometry analyses to detect especially low abundant proteins [22,41–43], we then extended the list to all 21 known boPAGs using predicted tryptic peptides. Protein sequences from UniprotKB and NCBI databases were theoretically digested with trypsin, and the resulting predicted peptide sequences were selected for suitability based on empirical criteria described in the Material and Methods section of this article. Due to the high degree of sequence homology among boPAGs, the resulting list of potentially ‘proteotypic’ unique peptide proxies was limited (Table S13).

We tested our PRM method based on this inclusion list of measured and predicted peptides on pooled reference samples that contained equal volumes of each of the boPAG samples from different gestational stages. After careful manual evaluation of peptide detectability, a final inclusion list of peptides (Table 1) was generated. Note that for boPAGs 12, 13 and 19, no suitable peptides could be found that fulfilled the criteria. As consequence, they were not considered in the further analysis. Based on the selected peptides and optimized parameters, we analyzed all samples of the respective pregnancy stages according the protocol described in the Material and Methods section. In summary, the final PRM analysis monitors 25 peptides (with their 6 most intense transitions) from 18 boPAGs using a scheduled inclusion list. A figure with the mapping of the chosen peptides onto the 18 sequences can be found in the supplementary materials (BoPAG sequence data S1)

Table 1. List of proteins and proteotypic peptides.

PAG	Peptide Sequence	Precursor Charge	Average Measured RT (sec)	Precursor *m/z*	Mean CV (%)
PAG 1 (NP_776836.1)	R.VSSSTETWYLGDVFLR.L	3	42.48	620.644	8.72
PAG 2 (NP_788787.1)	K.TFNPQNSSSFR.E	2	42.13	642.80	11.75
	R.NYLDTAYVGNITIGTPPQEFR.V	3	38.34	790.39	16.07
PAG 3 (NP_001291497.1)	K.VSSSTETWILGDVFLR.V	3	40.71	603.98	16.58
PAG 4 (NP_788788.1)	K.ALVDTGSSDIVGPSTLVNNIWK.L	3	40.32	762.73	20.32
PAG 5 (NP_788789.1)	R.HLESSTSGLTQK.T	2	40.96	644.33	13.11
	K.ENTVSTSTETWILGDVFLR.L	3	42.29	723.36	10.86
PAG 6 (NP_788790.1)	K.GIPFDGILGLSYPNK.T	3	41.11	530.95	10.33
PAG 7 (NP_001103448.1)	R.HLQSSTFRPTNK.T	3	33.16	472.58	19.30
	K.WVPLIQAVDWSVHVDR.I	3	43.35	640.67	8.86
PAG 8 (NP_788792.2)	K.NLGTSETWILGDVFLR.L	3	41.72	607.65	11.46
PAG 9 (NP_788793.1)	K.GELNWIPLIEAGEWR.V	3	32.91	594.97	18.38
PAG 10 (NP_788794.2)	R.IGNLVSVAQPFGLSLK.E	3	42.04	548.32	12.34
	R.TITGANPIFDNLWK.Q	2	42.50	795.41	13.69
PAG 11 (NP_788796.1)	K.QQGAISEPIFAFYLSTR.K	3	42.13	643.33	11.24
	R.VVFDTGSSDLWVPSIK.C	3	40.46	583.97	11.52
PAG 14 (XP_002699292.1)	R.NISFSGAIPIFYK.L	2	30.58	728.89	27.29
PAG 15 (NP_788797.1)	R.LSQISFHGSNLTIHPLR.N	3	43.36	640.68	4.47
PAG16 (NP_788798.1)	R.HFQSSTFRPTTK.T	2	41.72	718.86	4.54
	K.NQGAISDPIFAFYLSK.D	3	42.43	590.97	7.96
PAG 17 (NP_788800.1)	K.EHTYSLSQISSR.G	3	29.57	469.90	10.12
PAG 18 (NP_788799.1)	K.LSFSGAIPIFDNLR.N	3	23.35	517.28	9.14
PAG 20 (NP_788802.1)	R.FDGVLGLNYPNISFSK.A	3	42.43	590.97	9.62
	R.STEFWILGEAFLR.L	3	40.16	523.60	12.99
PAG 21 (NP_788803.1)	K.NEGAISEPIFAFYLSK.K	3	41.22	595.97	11.77

PAG profiles in glycosylated samples during gestation and post-partum

For the evaluation of the relative abundances from the 18 different boPAGs during gestation and post-partum, we analyzed the six different purified samples from the different pregnancy stages using the developed PRM assay. The values of the late pregnancy samples were averaged. These data were used to calculate the relative abundance of each boPAG in the respective pregnancy stage. The mean relative abundances of boPAGs in the course of pregnancy and post-partum are shown in Fig 2.

Analysis of the data with ANOVA indicated a significant effect of boPAG-type on relative abundances ($P < 0.001$). BoPAG 1 was the most abundant boPAG in all pregnancy stages and showed a significant difference ($P < 0.001$) in mean relative abundance compared to the other investigated boPAGs. Furthermore, boPAG 18 showed significantly higher relative abundances ($P < 0.001$) in comparison to boPAG 2, boPAG 3, boPAG 4, boPAG 5, boPAG 6, boPAG 7, boPAG 8, boPAG 9, boPAG 10, boPAG 11, boPAG 14, boPAG 15, boPAG 17, boPAG 20, boPAG 21 and boPAG 16 ($P = 0.005$). BoPAG 16 had a higher level of mean relative abundance ($P < 0.001$) compared to boPAG 2, boPAG 3, boPAG 4, boPAG 5, boPAG 6, boPAG 7, boPAG 8, boPAG 9, boPAG 10, boPAG 11, boPAG 14, boPAG 17, boPAG 20, boPAG 21 and boPAG 15 ($P = 0.005$) throughout the period of gestation studied. The additionally observed differences between boPAGs did not reach statistical significance. Nevertheless, we detected distribution patterns in regard to boPAG concentrations at different stages in the course of pregnancy. The first group (boPAG 1; boPAG 5; boPAG 15) showed nearly equal relative mean abundances in early, mid and late pregnancy and decreased levels of protein post-partum. A second group (boPAG 2; boPAG 3; boPAG 4; boPAG 6; boPAG 7; boPAG 8; boPAG 9; boPAG 14; boPAG 21) displayed highest levels of relative mean abundances at early and late pregnancy stage in comparison to the levels observed at mid pregnancy and after gestation. BoPAG 17 and boPAG 18 exhibited a profile with highest levels during mid and late pregnancy. The last set comprised boPAG 10, boPAG 16 and boPAG 20. Within this group, we detected highest levels of relative abundance in the afterbirth sample.

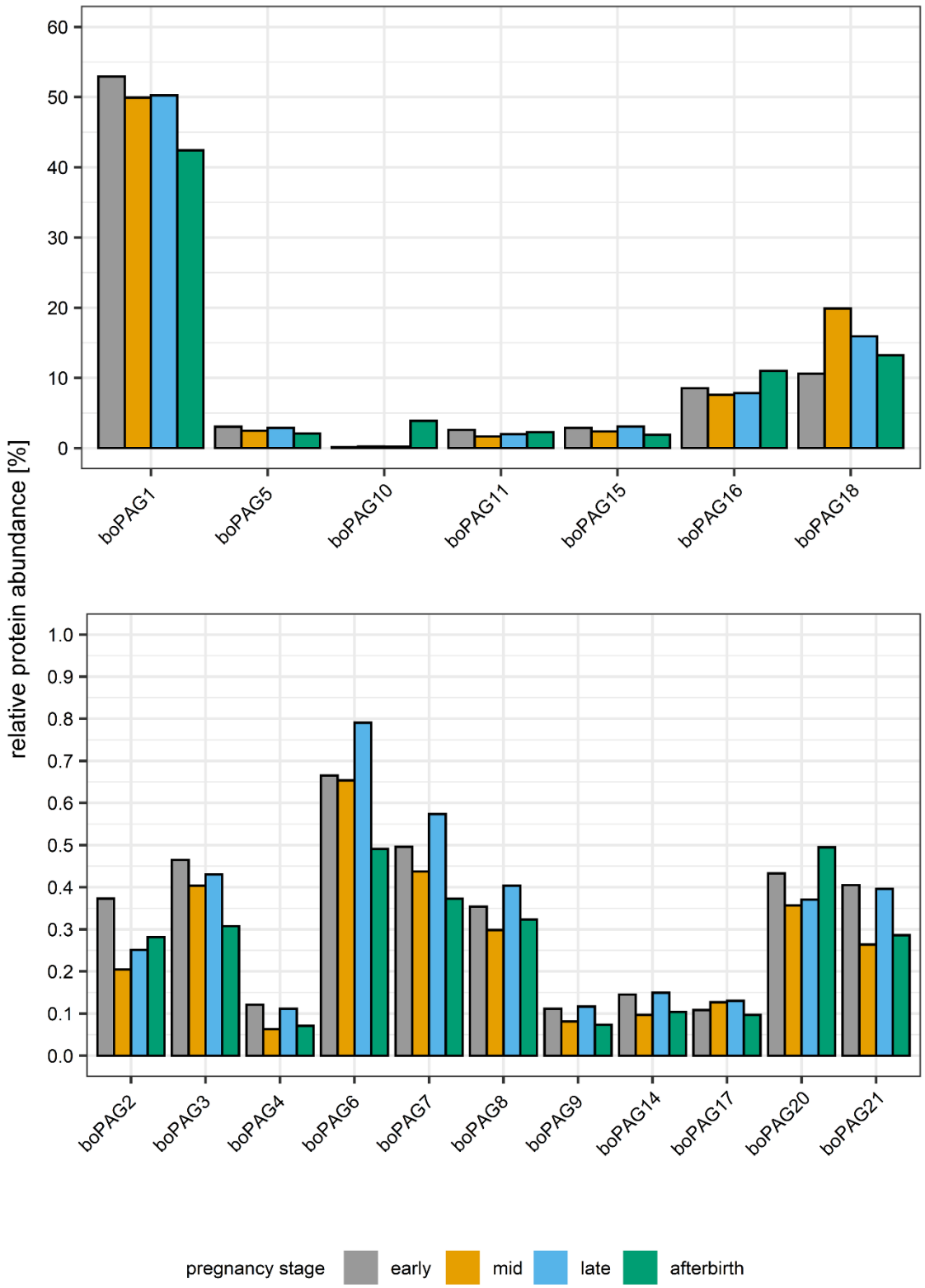

Fig 2. Visualization of relative protein abundances measured by Parallel Reaction Monitoring (PRM) mass spectrometry in glycosylated samples during pregnancy and postpartum.

PAG profiles in deglycosylated samples during gestation and post-partum

The possible effects of glycosylation on mass spectrometry analyses are well known. Their complexity and associated physical properties can lead to an overall poorer detection of glycosylated proteins or peptides [42,44–47]. Since boPAGs are highly glycosylated [15,16,19,20], we decided to examine the effect of N-glycosylation on the outcome of our analysis.

We subjected six samples purified from cotyledonary tissues at different pregnancy stages to enzymatic deglycosylation. The success of the Peptide-N-Glycosidase F (PNGase F) treatment was verified by gel electrophoresis (Fig 1). The observed differences in apparent molecular weight between glycosylated and deglycosylated samples already indicate the major effect of N-glycosylation on this this group of proteins. Changes in the mean relative abundances of deglycosylated boPAGs in the course of pregnancy and post-partum as detected by PRM mass spectrometry are shown in Fig 3.

ANOVA pointed out significant differences in the relative abundance of boPAGs ($P < 0.001$). Again, boPAG 1 was the most abundant boPAG at all pregnancy stages and showed significant differences ($P < 0.001$) in mean relative abundance compared to the other investigated boPAGs. Furthermore, boPAG 16 showed significantly higher relative abundances in comparison to boPAG 2 ($P < 0.001$), boPAG 3 ($P = 0.009$), boPAG 4 ($P = 0.006$), boPAG 5 ($P = 0.005$), boPAG 6 ($P = 0.01$), boPAG 7 ($P < 0.001$), boPAG 8 ($P = 0.008$), boPAG 9 ($P = 0.006$), boPAG 10 ($P = 0.003$), boPAG 11 ($P = 0.04$), boPAG 14 ($P = 0.007$), boPAG 17 ($P = 0.007$), boPAG 20, ($P < 0.001$) and boPAG 21 ($P = 0.008$). The observed differences between other boPAGs did not reach statistical significance.

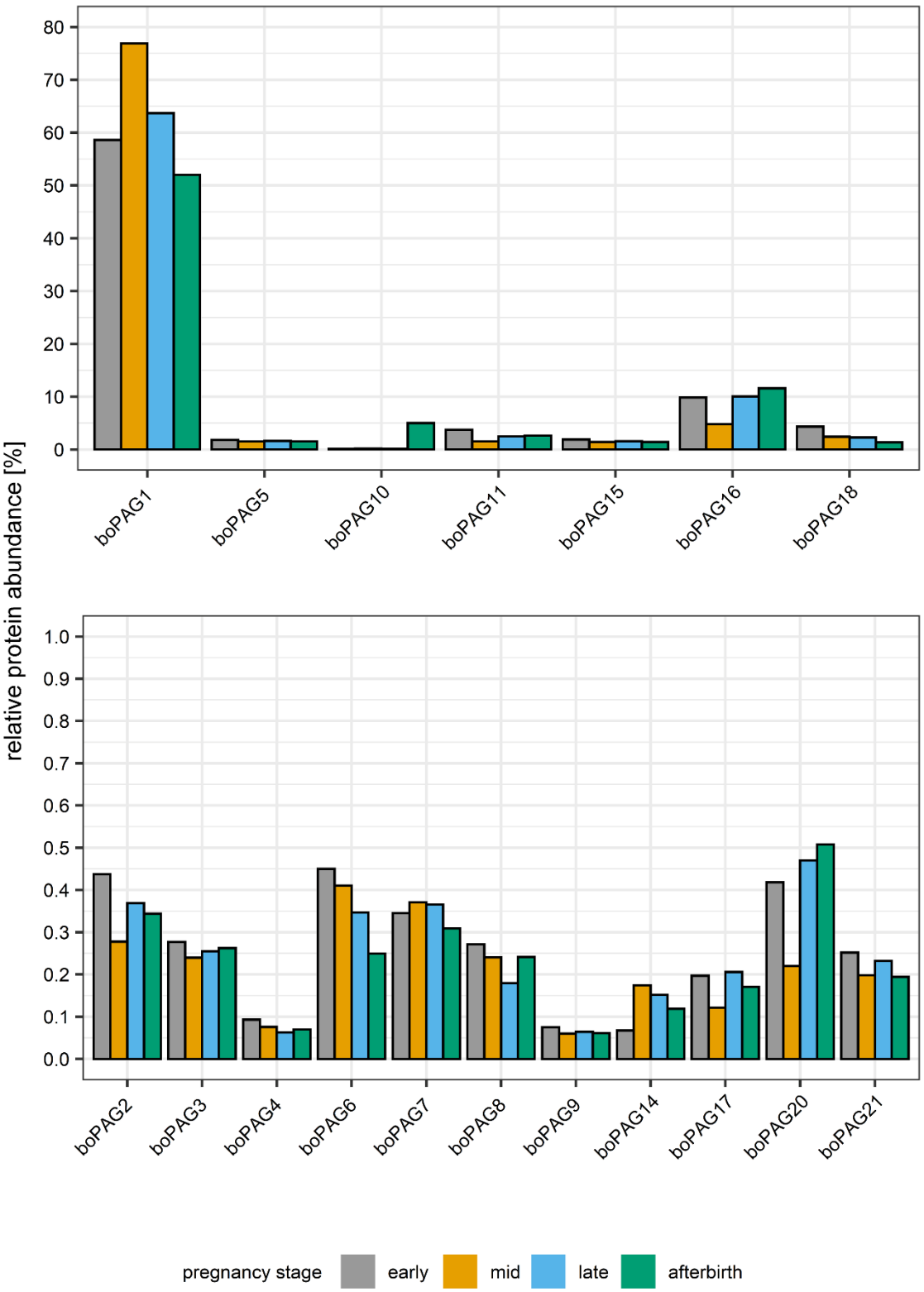

Fig 3. Visualization of relative protein abundances measured by Parallel Reaction Monitoring (PRM) mass spectrometry in deglycosylated samples during pregnancy and post-partum.

Nevertheless, mean relative boPAG levels could be assigned to different groups of nearly identical distribution patterns. BoPAG 1, boPAG 7 and boPAG 14 showed highest relative levels in mid pregnancy compared to the other pregnancy stages. Another group (boPAG 5; boPAG 9; boPAG 15) showed nearly equal relative mean abundances during gestation and after parturition. BoPAG 2, boPAG 3, boPAG 4, boPAG 17 and boPAG 21 had highest levels of mean relative abundances during early and late pregnancy compared to their levels during mid pregnancy and post-partum. Some boPAGs, such as boPAG 4, boPAG 6, boPAG 8 and boPAG 18 showed decline in relative abundances in the course of pregnancy with highest levels reached at early pregnancy stage. Two boPAGs (boPAG 4 and boPAG 8) had increased levels in the post-partum sample. The last set comprised boPAG 10, boPAG 16 and boPAG 20 which exhibited highest levels of relative mean abundances in the afterbirth sample.

Comparison between glycosylated and deglycosylated samples

Overall, the mean Total Area Fragment between glycosylated and deglycosylated samples increased by 58.5 %. Distributions of the relative proportion of boPAG abundances at the different pregnancy stages for glycosylated samples and deglycosylated samples are shown in Fig 4. In glycosylated samples, we found an equal distribution of the relative proportions among the different pregnancy stages. Upon deglycosylation, the distribution is slightly shifted. The relative proportions of mean Total Area Fragment during mid and late pregnancy increased whereas the proportions during early pregnancy and post-partum decreased.

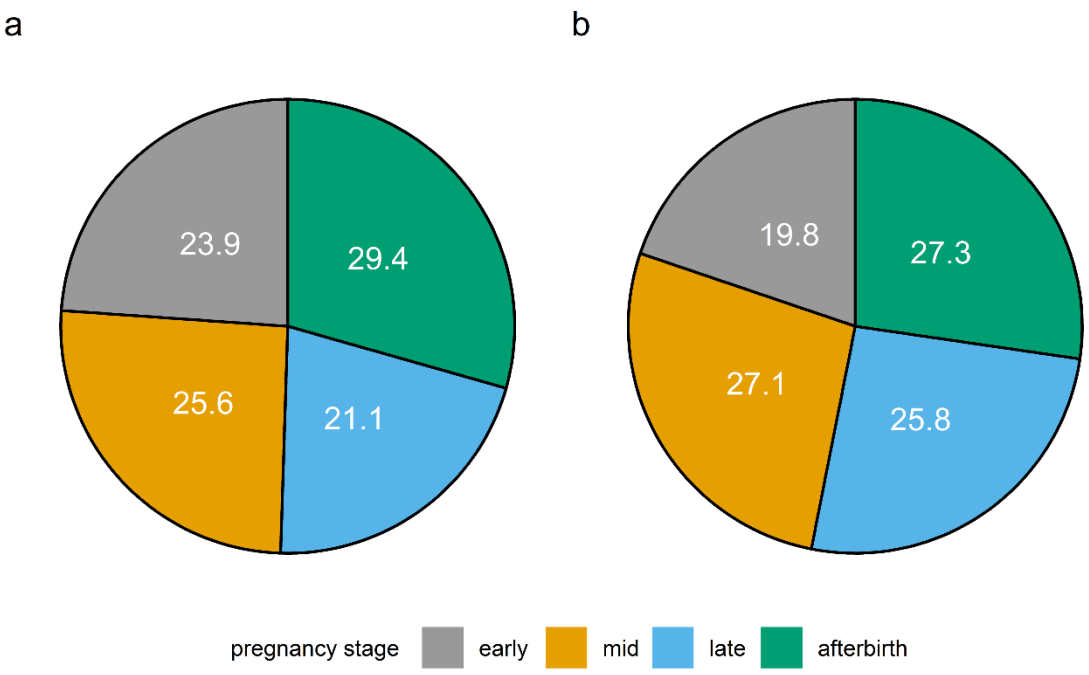

Fig 4. Relative proportions among the different pregnancy states for (a) glycosylated samples and (b) deglycosylated samples. Note the different basic population of Total Area Fragment (a = 4,774,159; b = 7,567,626).

Furthermore, we observed changes in abundances and distribution patterns depending on the glycosylation status of the samples. The levels of boPAG 1 were significantly increased and the levels of boPAG 18 were significantly decreased in the deglycosylated samples during pregnancy and postpartum in comparison to the glycosylated samples ($P < 0.001$). The additionally observed differences in the abundance of other glycosylated and deglycosylated boPAGs did not reach statistical significance. Nevertheless, we discovered changes in the distribution pattern of the different boPAGs. The above mentioned boPAG 1 had increased relative abundances in deglycosylated samples during mid and late pregnancy compared to the glycosylated ones. BoPAG 2 exhibited a nearly equal distribution among glycosylated and deglycosylated samples with a slight increase in relative abundances in deglycosylated samples. BoPAG 3, boPAG 9 and boPAG 21 displayed similar distribution pattern between glycosylated and deglycosylated samples with a small decrease in relative abundances in deglycosylated samples. BoPAG 4, boPAG 6 and boPAG 8 showed a major change in their distribution pattern which was dependent on the glycosylation state of the samples. For those proteins, in the

glycosylated samples highest abundances were observed in early and late pregnancy. Upon deglycosylation they exhibited decreasing levels throughout gestation. BoPAG 7 and boPAG 14 displayed distribution pattern in glycosylated samples which resembled the profile of boPAG 4, boPAG 6 and boPAG 8. However, in the deglycosylated samples they showed an increase in relative abundances during mid pregnancy. An opposite effect was monitored in regard to boPAG 17. Of note, boPAG 16 and boPAG 20 showed a decrease in relative abundance during mid pregnancy and a slight increase during late pregnancy when comparing glycosylated with deglycosylated samples. Overall, boPAG 18 had lower abundances in deglycosylated samples than in glycosylated ones. Another set of boPAGs comprised boPAG 5, boPAG 10, boPAG 11 and boPAG 15. These proteins displayed no glycosylation-dependent difference in their distribution.

We observed major differences in the percentage change of Total Area Fragment values from peptides between deglycosylated and glycosylated samples during mid and late pregnancy as well as post-partum (Fig 5). One-way ANOVA indicated an effect of the pregnancy stage on the percentage change (P = 0.07). During early pregnancy the majority of the peptides showed changes in their Total Area Fragment in the range of 30 %.

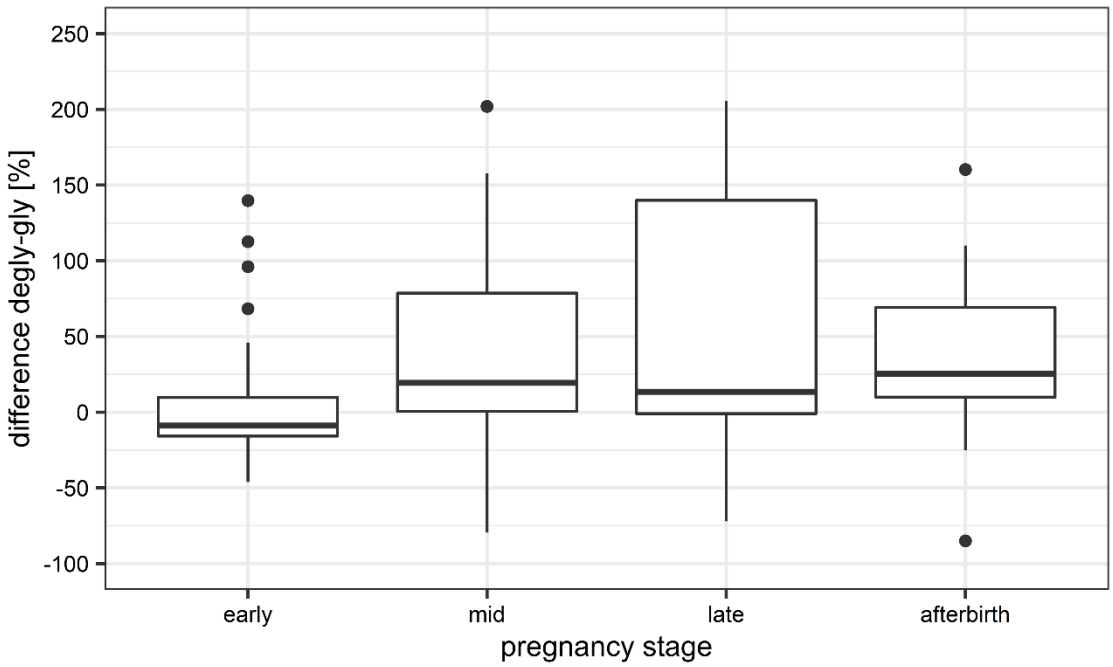

Fig 5. Box-and-whisker plot visualization of the percentage change of Total Area Fragment values from peptides between deglycosylated and glycosylated samples in the course of pregnancy and post-partum.

These findings are further supported by the results of our Western blot analysis (Fig S2). Fig 6 summarizes the signal intensities of the six different boPAG-antisera within the different pregnancy stages and between the two sample types. The largest alterations in signal strength between deglycosylated and glycosylated samples could be observed during mid and late pregnancy as well as post-partum. Analysis with ANOVA showed a significant effect of glycosylation on signal intensities (P = 0.04). Further Bonferroni-corrected t-test revealed that binding of the six boPAG-antisera to deglycosylated samples is significantly (P = 0.03) enhanced over all investigated pregnancy stages. This suggests that binding from the polyclonal sera may be sterically hindered by the attached N-glycans, especially during mid pregnancy, late pregnancy and post-partum.

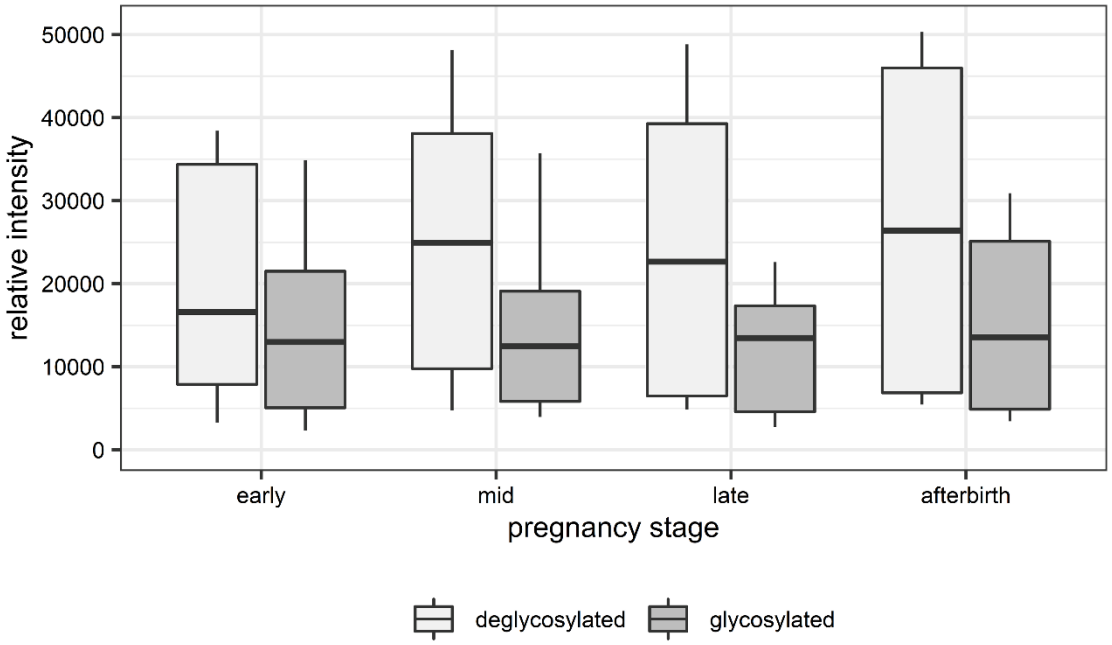

Fig 6. Overview of the results from Western blot analyses. Box-and-whisker plot visualization represents the intensities of the fluorescence signal.

Based on the detected variations in the PRM assay, boPAGs were assigned to two different sets as a function of their percentage change within the different pregnancy stages (Fig 7). The first set included boPAG 3, boPAG 5, boPAG 6, boPAG 7, boPAG 8, boPAG 9, boPAG 15 and boPAG 21 which showed only minor differences with either an increase or a decrease in the two sample types in a range of 30 % within a pregnancy stage. The

second set comprised boPAG 1, boPAG 2, boPAG 4, boPAG 10, boPAG 11, boPAG 14, boPAG 16, boPAG 17 and boPAG 20. These boPAGs seemed to be strongly glycosylated during pregnancy with an increase in the Total Area Fragment between glycosylated and deglycosylated samples within a range of 35.1 % - 205.6 %. The only exception of this grouping is the peptide assigned to boPAG 18. Here we observed highly negative percentage changes within pregnancy and post-partum (early pregnancy -46.1 %; mid pregnancy -79.6 %; late pregnancy -72.1 %; post-partum -85.0 %).

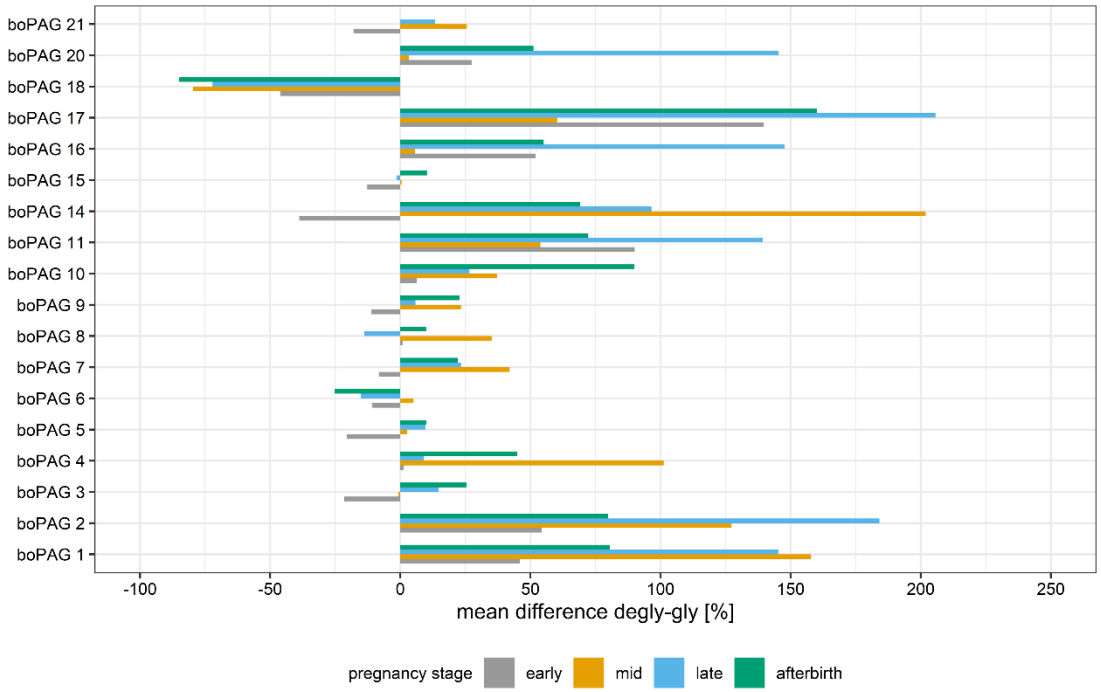

Fig 7. Mean percentage change of Total Area Fragment values from boPAGs between deglycosylated (degly) and glycosylated (gly) samples in the course of pregnancy and post-partum.

Discussion

The development of new mass spectrometers and data acquisition schemes for the characterization and quantification of proteins with high sensitivity have driven tremendous advances in proteomics over the past years [22–24,43]. As a new targeted acquisition workflow, PRM has gained particular interest because of its precision and ease of

method development [22,24,43,48]. In this study, a PRM assay for the determination of the relative protein abundances of 18 different boPAGs during pregnancy and after calving was developed. Furthermore, we investigated glycosylated and deglycosylated samples to assess possible effects of the glycosylation status on the outcome of the applied analytical method and on changes in the levels of relative abundances.

There are only a few studies that give insights into the protein level of different boPAGs [12,16–19,21]. Zoli et al. (1991) [17] and Sousa et al. (2002) [21] identified boPAG 1 by N-terminal sequencing in a mix of cotyledon tissue derived from 2- to 6-month-gestations [17] and in cotyledon tissue isolated from zebus at 3 different gestational ages (10-11 weeks; 20-21 weeks; 30-31 weeks) [21]. In 2005 the first mass spectrometric analysis of boPAGs were conducted by two independent research groups. Green et al. (2005) identified boPAG 4, boPAG 6, boPAG 7, boPAG 16, boPAG 17, boPAG 20 and boPAG 21 by MALDI-TOF mass spectrometry with the peptide mass fingerprinting (PMF)-method from two placental extracts (obtained from cotyledons of 18 cm and 40 cm crown-rump fetuses) [18]. Klisch et al. (2005) purified different boPAGs (boPAG 1, boPAG 6, boPAG 7, boPAG 17) from cotyledonary tissue of different mid pregnancy placentas (day 100; day 155; day 180) and analyzed them by MALDI-TOF/TOF mass spectrometry with same method as Green et al. (2005) [16,18]. No differences were detected between the different examined gestational stages. The most intensive band (66 kDa) on a Coomassie blue stained gel was identified as boPAG 1 [16]. Taken together, these results indicate that boPAG 1 is probably the most abundant boPAG in cotyledonary tissue, especially during mid-pregnancy. This is in accordance with our results as we also identified boPAG 1 as the most abundant boPAG during pregnancy. Additionally, our PRM assay also detected the other boPAGs found in the studies of Klisch et al. (2005) [16] and Green et al. (2005) [18] in the respective gestational stages.

The study of Touzard et al. (2013) investigated three different boPAG profiles (boPAG 1; boPAG 2; boPAG 12) during gestation using Western blot analysis [12]. Therefore, they generated specific antibodies against the respective boPAGs which were then used to examine the expression levels of boPAGs in cotyledonary and intercotyledonary regions in 60 to 220-day-old bovine placentas [12]. Our protein profiles of the respective boPAGs in glycosylated samples showed high conformity in terms of the distribution pattern,

when looking at results of the cotyledonary samples from day 80 onwards. Touzard et al. (2013) found highest abundance levels of boPAG 1 in early pregnancy (day 80) and a slight decrease of protein levels from early to mid (day 100) and late pregnancy (day 220). BoPAG 2 exhibited clearly a difference between protein levels from early pregnancy (day 80) in comparisons to protein levels in mid (day 100) and late pregnancy (day 220) and protein abundance of boPAG 11 remained at nearly same abundance levels during all gestation periods which were considered in this study [12]. Furthermore, Touzard et al. (2013) could not detect significant differences on protein level for the respective boPAGs in samples collected at day 80, day 100 and day 220 [12]. The only discrepancy between our study and the study conducted by Touzard et al. (2013) [12] are the lower protein levels of boPAG 1 and boPAG 2 observed in samples collected at day 60 compared to the ones obtained at the early pregnancy stage in our study. The reasons for the differences are probably the range of our early pregnancy stage compromising samples from 35-90 days of gestation in comparison to a specific sample timepoint and the use of a different antibody-based method in the study of Touzard et al. (2013) [12].

In summary, the comparison of our findings with the few existing proteomic studies on boPAGs shows that the developed PRM assay provides reliable and comparable results. Furthermore, we were able to expand the existing knowledge of previous protein analysis studies on boPAGs due to the advantages of PRM.

The available body of literature shows that boPAGs can have up to six potential N-glycosylation sites and that the degree of N-glycosylation seems to be the major factor in boPAG molecular mass [4,12,16]. N-glycans are usually attached to the amido group of an N side chain in a particular consensus sequence (NxS or NxT where x ≠ P) but such sequences may be glycosylated only partially or not at all [45,49]. It is known that N-glycans are involved in important cellular processes including cell-cell and receptor ligand interactions, immune response or apoptosis [46,50]. All those functions are also discussed for boPAGs during placentation and ongoing pregnancy highlighting the important functional role of the attached N-glycans in these proteins [1,11,19]. However, despite the obvious biological importance, our knowledge of in vivo N-glycosylation sites and the regulation of boPAG-glycosylation during pregnancy is still very limited [19]. For the stated reasons and the possibility to detect a signal shift between glycosylated

samples and deglycosylated samples with modern LC-MS technologies [46,47], we decided to examine the effect of glycosylation in our study.

Enzymatic digestion of all samples with PNGase F resulted in changes of relative boPAG abundances and of their distribution pattern. The overall mean of the Total Area Fragment between glycosylated and deglycosylated samples increased by 58.5 %. This increase in Total Area Fragment was not equally distributed over the different boPAGs and pregnancy stages. In our study, the largest influence of glycosylation was detectable in mid and late pregnancy samples as well as post-partum. These results were further verified by immunoblotting. As already described, the detected band signals in all investigated samples shifted their apparent molecular weights after PNGase F treatment [12,16,19] and we found a much more intensive binding of the six different boPAG antisera to the deglycosylated samples compared to the glycosylated samples. However, this method is limited to the analysis of proteins with available antisera and can only identify protein-wide glycosylation occupancy [47]. Western blotting is not able to distinguish the extent of glycosylation of different boPAGs and at different pregnancy stages. PRM can overcome both of these limitations and provides an analysis tool that can be used for site-specific analysis of protein glycosylation [47,48]. PRM-based protein assays do not require an antibody and have the advantage of multiplexed detection of analytes [48].

As a result, we were able to divide the boPAGs into groups. One group consisting of eight boPAGs (boPAG 3; boPAG 5; boPAG 6; boPAG 7; boPAG 8; boPAG 9; boPAG 15; boPAG 21) showed only minor differences with either an increase or a decrease between the two sample types within a pregnancy stage in a range of 30 %. It seems likely that these differences arose from the normal variability of the measurement, especially for the boPAGs with a low abundance. The change in the distribution pattern of the relative protein abundances between glycosylated and deglycosylated samples in this set of boPAGs originate in the greater increase in the Total Area Fragment of the other boPAG group within the different pregnancy stages. It seems quite likely that the boPAGs of this group are not heavily glycosylated in any pregnancy stage.

The other group of boPAGs (boPAG 1; boPAG 2; boPAG 4; boPAG 10; boPAG 11; boPAG 14; boPAG 16; boPAG 17; boPAG 20) showed an increase in the Total Area Fragment between glycosylated and deglycosylated samples by 35.1 %-205.6 %. It seems that the second set of boPAGs have a higher or more complex degree of glycosylation during gestation compared to the first set and the deglycosylation process leads to a better detection of boPAGs in this group [45].

Given these data we asked if there is any correlation between group belonging and the number of potential glycosylation sites or the distance between potential glycosylation sites and the monitored proteotypic peptides. After sequence analysis (BoPAG sequence data S1), we could not find such type of correlation. Furthermore, both groups consist of boPAGs which are monitored by proteotypic peptides with potential glycosylation sites. The lack of this relationship can have different reasons. It is known that the canonical glycosylation sequon is not an adequate predictor of glycosylation [47]. Only 70 % of sequons carry a N-glycan and there is experimental evidence for N-glycosylation on consensus sequences different from the canonical one [46,47,50]. Additionally, the biochemical properties of the amino acid immediately proximal to the glycosylated N, the presence of either a NxS sequon or NxT sequon and the position of an asparagine within its protein sequence contributes to the extent or probability of glycosylation [47,50–52]. Therefore, proof that a potential N-glycosylation site is occupied by a glycan requires experimental evidence [50]. A good example of the discrepancy between the existence of potential glycosylation sites and experimental proof is boPAG 2. The protein sequence of boPAG 2 consist of 6 sequons. Touzard et al. (2013) used a progressive enzymatic N-deglycosylation protocol with PNGase F to determine the number of occupied glycosylation sites of boPAG 1, boPAG 2 and boPAG 11 from late pregnancy placenta (day 220) by Western blot [12]. The digestion of the immunoreactive boPAG 2 protein indicated, that boPAG 2 has only one occupied N-glycosylation site in the respective pregnancy stage. Touzard et al. (2013) hypothesized that this result could have been related to folding of the protein [12]. The results from our PRM assay indicate that boPAG 2 seems to be glycosylated at least at one glycosylation site during early pregnancy and at two glycosylation sites during mid and late pregnancy and after gestation. In this study boPAG 2 was monitored by two proteotypic peptides. Both of them have a potential N-

glycosylation site in their sequence. The peptide NYLDTAYVGNITIGTPPQEFR showed a decrease of the Total Area Fragment in early pregnancy sample (-27.2 %) and an increase in mid (137.8 %), late (139.3 %) and afterbirth (110.0 %) samples. The other peptide TFNPQNSSSFR showed an increase of the Total Area Fragment over all pregnancy stages (early pregnancy: 96.1 %; mid pregnancy: 123.6 %; late pregnancy: 199.2 %; afterbirth: 76.1 %). Altogether this supports the findings of different studies which show that boPAG glycosylation undergoes major changes during pregnancy [12,19,20]. Nevertheless, the role of these observed changes and the mechanism of glycosylation in boPAGs remain unclear [19,20] and more research is needed.

The PRM assay developed and described in this study provides a practical and efficient method that promises to be a powerful tool for further research on boPAGs. With this assay it is possible to compare protein abundances between cotyledons and intercotyledonary chorion during gestation and at term. In combination with a stepwise deglycosylation of the samples this might improve our knowledge of the mechanisms behind the observed changes of boPAG glycosylation. Furthermore, the PRM assay can be adapted to blood and milk samples. Accurate quantification of boPAGs in these body fluids is achievable by any of the commonly used stable isotope labelling techniques [26,43,48,53]. This information provides insights into which boPAGs are released in the maternal circulation. This knowledge can be utilized for the development of new pregnancy detection systems. Güzel et al. (2018) showed that PRM can be used as an attractive alternative for immunoassay [53]. Therefore, the PRM assay itself can be applied as sensitive and reliable tool for pregnancy detection based on quantitation of 18 different boPAGs in blood or milk.

Conclusions

In conclusion, we developed a PRM assay for the determination of the relative protein abundances of 18 different boPAGs during pregnancy and after calving. To our knowledge, this is the first study which addresses the detection of the different boPAGs

in parallel in the time of pregnancy and afterbirth samples on protein level, thereby investigating the influence of glycosylation.

The detected boPAG distribution pattern in glycosylated samples confirmed the results of other proteomic studies. Highest degrees of glycosylation appeared in mid and late pregnancy samples as well as in afterbirth samples. Additionally, we identified a group of boPAGs that seems not heavily glycosylated in any pregnancy stage. A linkage between the impact of glycosylation and potential N-glycosylation sites or phylogenetic relation was not detected. The PRM assay itself and the results of this study give new starting points to address further research on boPAGs to better understand the physiological role during pregnancy and achieve a real knowledge of these proteins and their posttranslational modifications. For these reasons, the designed assay shall be improved and applied for the detection of individual boPAGs in maternal blood and milk in the near future. This improvement will also allow us to efficiently screen higher numbers of animals. Reliable identification and quantification will be ensured by the use of labelled synthetic peptides.

Supporting information

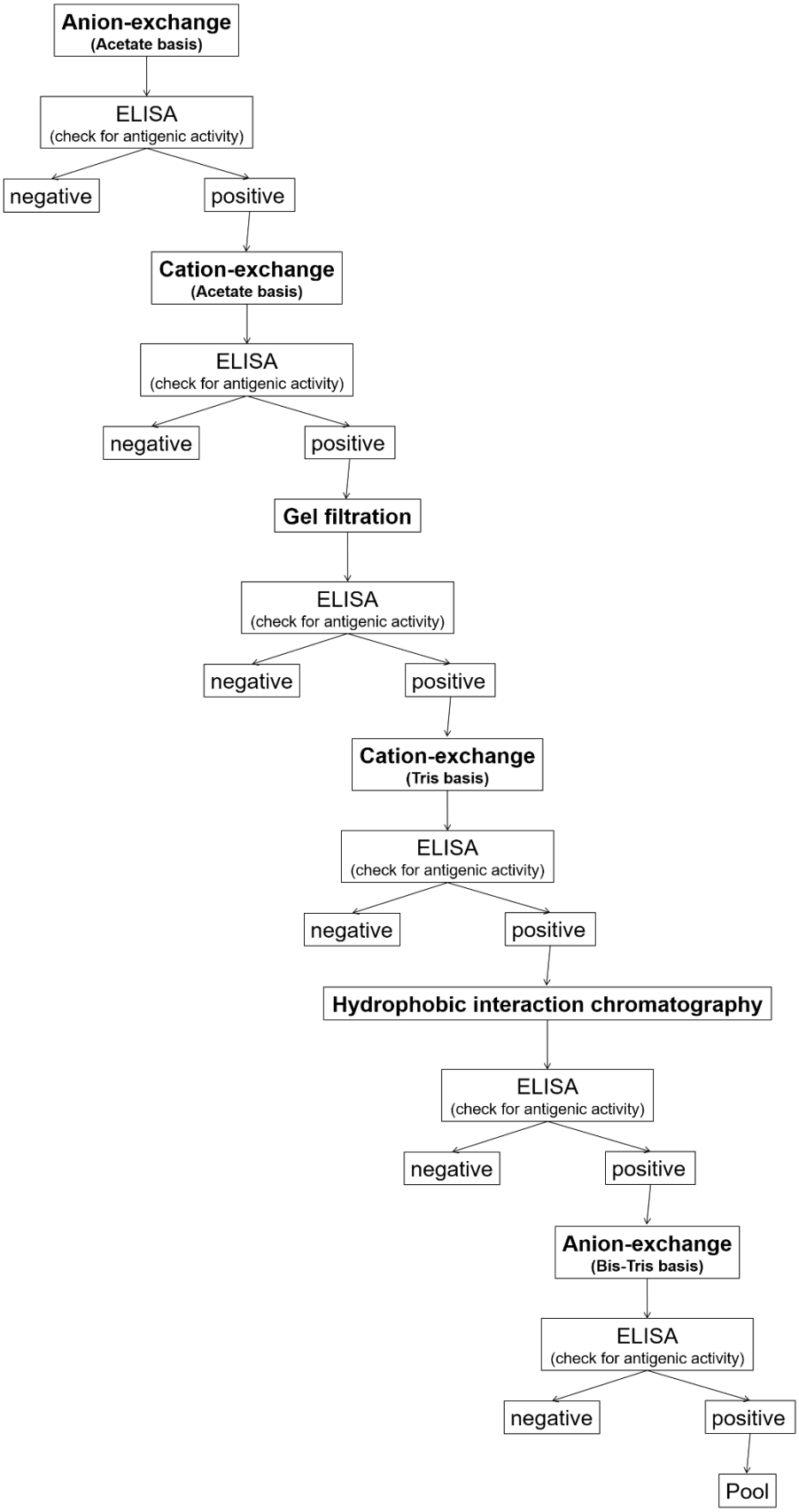

Fig S1. Overview of the FPLC-Workflow.

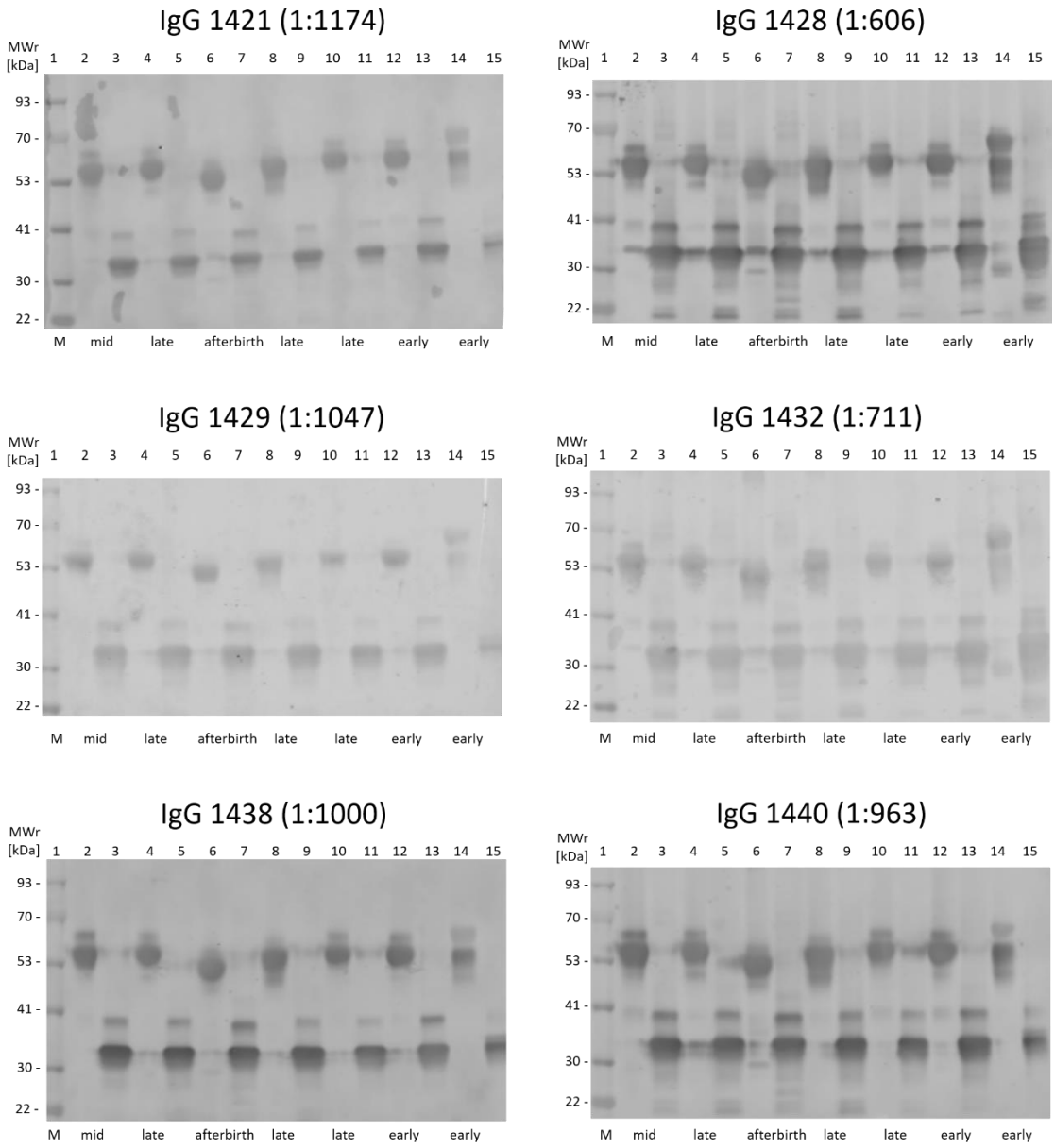

Fig S2. Overview of the six Western blots. Blots were probed with six different bovine Pregnancy-Associated Glycoprotein (boPAG) antisera (IgG 1421-IgG 1440). Dilutions are indicated inside the parentheses.

Lane 1: Marker
Lanes 2, 4, 6, 8, 10, 12, 14: glycosylated samples
Lanes 3, 5, 7, 9, 11, 13, 15: deglycosylated samples

The three late pregnancies and the two early pregnancies are represented by different samples as described in the results part. Samples of Lane 14 and Lane 15 were not analyzed by Mass Spectrometry. Relative intensity-values were analyzed for the 53 kDa band and above for glycosylated samples and between 30 kDa and 41 kDa for deglycosylated ones.

BoPAG sequence data S1. Sequence Analysis of the 18 different boPAG.

```
Ruler 1          1         10        20        30        40        50
Consensus        ----------------MKWLVLLGLVAFSECIVKIPLRRVKTMRKTLSGKNmLNNFLKEH
boPAG1           ----------------MKWLVLLGLVAFSECIVKIPLRRLKTMRNVVSGKNMLNNFLKEH
boPAG2           ----------------MKWLVLLGLVALSECIVILPLKKMKTLRETLREKNLLNNFLEEQ
boPAG3           ----------------MKWLVLLGLVAFSECIVKIPLRRVKTMRNTVSGKNILNNILKEH
boPAG4           ----------------MKWLVLLGLVAFSECIVKIPLRRVKTMTKTLSGKNMLNNFVKEH
boPAG5           ----------------MKWLVLLGLLTSSECIVILPLTKVKTMRKTLSEKNMLNNFLKEQ
boPAG6           ----------------MKWLVLLGLVAFSECIVKIPLRRVKTMRNAISGKNTLNNILKEH
boPAG7           ----------------MKWLVVLGLVAFSECIVKIPLRRVKTMRKTLSGKNMLNNFLKED
boPAG8           ----------------MKWLVLLGLVALSECIVKIPLTKMKTMQEAIREKQLLEDFLDEQ
boPAG9           ----------------MKWIVLLGLVAFSECIVKIPLRQVKTMRKTLSGKNMLKNFLKEH
boPAG10          ----------------MKWLGLLGLVALSECMVIIPLRQMKTMRETLRERHLLTNFSEEH
boPAG11          ----------------MKWLVFLGLVAFSECIVIMLLTKTKTMREIWREKKLLNSFLEEQ
boPAG14          MLKTQTSLSTWSQERSMNWLVLLGLVAFSECIVKIPLRRVKTMRNTVSGKNMLDNFLKEH
boPAG15          ----------------MKWLVLLGLVAFSECVVKIPLRRVKTMTKTLSGKNMLNNFLKEH
boPAG16          ----------------MKWLVLLGLVAFSECIVKIPLRRVKTMRKTLSGKNTLNNFLKEH
boPAG17          ----------------MKWLVLLWLVAFSECIVKIPLRQVKTMRKTLSGKNTLNNFLKEH
boPAG18          ----------------MKWLVLLGLVAFSECIVKIPLRRVKTMRKTLSGKNILNNFLKEH
boPAG20          ----------------MKWLVLLGLVAFSECIFKIPLRRVKTMRKTLSGKNMLNNFLKEH
boPAG21          ----------------MKWVVLLGLVAFSECIVKIPLRRVKTMRKTLSGKNMLNNFLKEH
Sequence Logo
Ruler 2          1         10        20        30        40        50
```

```
Ruler 1                   70        80        90       100       110
Consensus        aYRLSQISFRGSNL-TiHPLRNIrDIfYVGNITIGTPPQEFQVVFDTGSSDLWVPSiFCt
boPAG1           AYSLSQISFRGSNL-TTHPLRNIKDLVYMGNITIGTPPQEFQVVFDTASSDLWVPSDFCT
boPAG2           AYRLSK---NDSKI-TIHPLRNYLDTAYVGNITIGTPPQEFRVVFDTGSANLWVPCITCT
boPAG3           VYRLSQISFRGSNL-TTHPLRNIKDLIYVGNITIGTPPQEFQVVFDTGSSDFWVPSDFCT
boPAG4           AYRLSQISFRGSNL-TIHPLRNIRDFFYVGNITIGTPPQEFQVIFDTGSSELWVPSIFCN
boPAG5           AYRLSQISSRGSNI-TIHPLRNIMDMVYVGKITIGTPPQEFQVVFDTGSSELWVPSVFCP
boPAG6           AYRLPQISFRGSNL-T-HPLRNIRDLFYVGNITIGTPPQEFQVIFDTGSSDLWVASIFCN
boPAG7           PYRLSQISFRGSNL-TIHPLRNIRDIFYVGNITIGTPPQEFQVIFDTGSSDLWVPSIDCN
boPAG8           PHSLSQHSDPDKKF-SSHQLKNFQNAVYFGTITIGTPPQEFQVNFDTGSSDLWVPSVDCQ
boPAG9           PYRLSQISFRGSNL-TIHPLRNIMNLVYVGNITIGTPPQEFQVVFDTGSSDLWVPS-FCT
boPAG10          PYNLSQKAANDQNIIYHHPLRSYKDFSYIGNINIGTPPQEFQVLFDTGSSSLWVPSIYCQ
boPAG11          ANRMSDDSASDPKL-STHPLRNALDMAYVGNITIGTPPKEFRVVFDTGSSDLWVPSIKCI
boPAG14          AYRLSHISFRGSNL-TSHSLRNIRDLFYVGNITIGTPPQKFQVIFDTGSFFLWVPSIFCN
boPAG15          AYRLSQISFHGSNL-TIHPLRNIRDLFYMGNITIGTPPQEFLVVFDTGSSDLWVPSDFCT
boPAG16          PYRLSHISFRGSNL-TTLPLRNIRDMLYVGNITIGTPPQEFQVVFDTGSSDLWVPSDFCT
boPAG17          TYSLSQISSRGSNL-TIHPLRNIMDMLYVGNITIGTPPQEFQVVFDTGSSDLWVPSVFCQ
boPAG18          AYRLSQISSCGSNL-TFHPLRNIKDRLYVGNITIGTPPQEFQVIFDTGSSDLWVTSVFCT
boPAG20          PYKLSQISFRGSNL-TTLPLRNIWDIFYIGTITIGTPPQEFQVVFDTASSDLWVPSIICN
boPAG21          GNRLSKISFRGSNL-TTLPLRNIEDLMYVGNITIGTPPQEFQVVFDTGSSDFWVPSDFCT
Sequence Logo
Ruler 2                   70        80        90       100       110
```

Red Markings: N-glycosylation sequons

Blue Markings: proteotypic peptides (listed in Table 1)

```
Ruler 1                130       140       150       160       170
Consensus   SpaCSTHVRFRHIQSSTFRITNKTFsItYGSGRMKGVVvHDTVRIGDLVSTDQPFGLSxe
boPAG1      SPACSTHVRFRHLQSSTFRLTNKTFRITYGSGRMKGVVVHDTVRIGNLVSTDQPFGLSIE
boPAG2      SPACYTHKTFNPQNSSSFREVGSPITIFYGSGIIQGFLGSDTVRIGNLVSPEQSFGLSLE
boPAG3      SRACSTHVRFRHLQSSTFRLTNKTFRITYGSGRMKGVVAHDTVRIGDLVSTDQPFGLSVE
boPAG4      SSTCSKHDRFRHLESSTFRLSRRTFSITYGSGRIEALVVHDTVRIGDLVSTDQQFGLCLE
boPAG5      SSACSTHIRFRHLESSTSGLTQKTFSITYGSGSTKGFLAYDTVRIGDLLSTDQEFGLSME
boPAG6      SSSCAAHVRFRHHQSSTFRPTNKTFRITYGSGRMKGVVVHDTVRIGDLVSTDQPFGLCLK
boPAG7      STSCATHVRFRHLQSSTFRPTNKTFRIIYGSGRMNGVIAYDTVRIGDLVSTDQPFGLSVE
boPAG8      SPSCSKHKRFDPQKSTTFQPLNQKIELVYGSGSMKGVLGSDTIQIGNLVIVNQIFGLSQN
boPAG9      MPACSAPVWFRQLQSSTFQPTNKTFTITYGSGSMKGFLAYDTVRIGDLVSTDQPFGLSVV
boPAG10     SSSCYKHNSFVPCNSSTFKATNKIFNTNYTATSIKGYLVYDTVRIGNLVSVAQPFGLSLK
boPAG11     SPACHTHITFDHHKSSTFRLTRRPFHILYGSGMMNGVLAYDTVRIGKLVSTDQPFGLSLQ
boPAG14     SSTCSTHIRFRHLQSSTFRLINKTFSIIYGSGRIKGVVVHDTVQIGDLVSTDQPLGLCME
boPAG15     SPACSKHFRFRHLQSSTFRLTNKTFSIEYGSGTMEGIVAHDTVRIGDLVSTDQPFGLSMT
boPAG16     SPACSTHVRFRHFQSSTFRPTTKTFRIIYGSGRMKGVVAHDTVRIGNLVSTDQPFGLSMA
boPAG17     SLACATKVMFIHLHSSTFRHTQKVFNIKYNTGRMKGLLVYDTVRIGDLVSTDQPFCISLA
boPAG18     SPTCSTHVMFRHFDSSTFRPTKKTFSINYGSGRMKGVVVHDTVRIGDLVSTDQPFGLSVV
boPAG20     SSTCSTHVRFRHRQSSTFRLTNKTFGITYGSGRMKGVVVHDTVRIGDLVSTDQPFGLSVA
boPAG21     SPDCITHVRFRQHQSSTFRPTNKTFSITYGSGRMRGVVVHDTVRIGDLVSTDQPFGLSVS
Sequence Logo
Ruler 2                130       140       150       160       170
```

```
Ruler 1                190       200       210       220       230
Consensus   Ey-G-FEGrp-FDGVLGLNYPNiSFSGAIPIFDKLKNqGAISEPVFAFYLSKDKqEGSVV
boPAG1      EY-G-FEGRI-YDGVLGLNYPNISFSGAIPIFDKLKNQRAISEPVFAFYLSKDEREGSVV
boPAG2      EY-G-FDSLP-FDGILGLAFPAMGIEDTIPIFDNLWSHGAFSEPVFAFYLNTNKPEGSVV
boPAG3      EY-G-FEGRAYYDGVLGLNYPNISFSGAIPIFDNLKNQGAISEPVFAFYLSKDEQEGSVV
boPAG4      ES-G-FEGMR-FDGVLGLSYTNISPSGAIPIFYKLKNEGAISEPVFAFYLSKDEREGSVV
boPAG5      EH-G-FEDLP-FDGVLGLNYPDMSFITTIPIFDNLKNQGAFSEPVFAFYLGK--VKGSVV
boPAG6      DS-G-FKGIP-FDGILGLSYPNKTFSGAFPIFDKLKNEGAISEPVFAFYLSKDKQEGSVV
boPAG7      EY-G-FAHKR-FDGILGLNYWNLSWSKAMPIFDKLKNEGAISEPVFAFYLSKDKREGSVV
boPAG8      QSSGVLEQVP-YDGILGLAYPSLAIQGTTPVFDNLKNREVISEPVFAFYLSSRPENISTV
boPAG9      EY-G-LEGRN-YDGVLGLNYPNISFSGAIPIFDNLKNQGAISEPVFAFYLSKNKQEGSVV
boPAG10     EF-G-FDDVP-FDGILGLGYPRRTITGANPIFDNLWKQGVISEPVFAFYLSSQKENGSVV
boPAG11     QF-G-FDNAP-FDGVLGLSYPSLAVPGTIPIFDKLKQQGAISEPIFAFYLSTRKENGSVL
boPAG14     ES-G-FEGVS-FDGILGLSYRNISFSGAIPIFYKLKNEGAISEPVFAFYLSKDKQEGSVV
boPAG15     ES-G-FEGIP-FDGVLGLNYPNISFSGAIPIFDKLKNQGAISEPVFAFYLSKDEQEGSVV
boPAG16     EY-G-LESRR-FDGILGLNYPNLSCSGAIPIFDKLKNQGAISDPIFAFYLSKDKREGSVV
boPAG17     EV-G-FDGIP-FDGVLGLNYPNMSFSGAIPIFDNLKNEGAISEPVFAFYLSKDKREGSVV
boPAG18     EL-G-FDGIP-FDGVMGLNYPKLSFSGAIPIFDNLRNQGAISEPVFAFYLSKDEQEGSVV
boPAG20     EY-G-FEGRR-FDGVLGLNYPNISFSKAIPIFDKLKNEGAISEPVFAFYLSKDKQKGSVV
boPAG21     EY-G-FKDRA-YDGILGLNYPDESFSEAIPIFDKLKNEGAISEPIFAFYLSKKKREGSVV
Sequence Logo
Ruler 2                190       200       210       220       230
```

Red Markings: N-glycosylation sequons

Blue Markings: proteotypic peptides (listed in Table 1)

```
Ruler 1                 250       260       270       280       290
Consensus   MFGGVDHRYYKGELNWVPLIQAGDWsVHMDRISMKRKVIACSdGCeALVDTGTSIIvGPr
boPAG1      MFGGVDHRYYEGELNWVPLIQAGDWSVHMDRISIERKIIACSDGCKALVDTGTSDIVGPR
boPAG2      MFGGVDHRYYKGELNWIPVSQTSHWQISMNNNISMNGTVTACSCGCEALLDTGTSMIYGPT
boPAG3      MFGGVDHRYYEGELNWVPLIEAGDWIVHMDRISMKRKIIACSGSCEAIVDTGTSAIEGPR
boPAG4      MFGGADHRYYKGELNWIPLMKAGDWSVHMDRISMKRKVIACSGGCKALVDTGSSDIVGPS
boPAG5      MFGGVDHTYYKGELNWVPLIQAGEWSLHMDRISMKRKVIACSGGCEAFYDTGTSLILGPR
boPAG6      MFGGVDHRYYKGELNWVPLIQVGDWFVHMDRTTMKRKVIACSDGCKALVDTGTSDIVGPS
boPAG7      MFGGVDHRYYKGELKWVPLIQAVDWSVHVDRITMNREVIACSEGCAALVDTGSSNIQGPR
boPAG8      MFGGVDHTYHKGKLQWIPVTQARFWQVAMSSMTMNGNVVGCSQGCQAVVDTGTSLLVGPT
boPAG9      MFGGVDHQYYKGELNWIPLIEAGEWRVHMDRISMKRTVIACSDGCEALVHTGTSHIEGPG
boPAG10     MFGGVNRAYYKGELNWVPVSQVGSWHINIDSISMNGTVVACKRGCQALLDTGTAFLRGPR
boPAG11     MLGGVDHSYHKGKLNWIPVSQTKSWLITVDRISMNGRVIGCEHGCEALVDTGTSLIHGPA
boPAG14     MFGGVDHRYYKGELKWVPLIRAGDWSVHMDRISMKRKIIACSDCCKALVDTGTSDIAGPR
boPAG15     MFGGVDHRYYKGELKWVPLIEAGDWIVHMDCISMRRKVIACSGGCEAVVDTGVSMIKGPK
boPAG16     MFGGVDHRYYKGELNWVPLIRAGDWIVHVDRITMKREVIACSDGCAALVDTGTSLIQGPG
boPAG17     MFGGVDHRYYKGELNWVPLIQAGGWTVHVDRISMKRKIIACSGGCEALVDTGTALIKGPR
boPAG18     MFGGVDHRYYKGELNWIPLIQAGDWSVHMDSISMKRKVIACSGGCKAVVDTGTSLIEGPR
boPAG20     MFGGVDHRYYKGELNWVPLIRAGDWSVHVDRITMKGEVIGCSDGCTAMVDTGSSNIQGPG
boPAG21     MFGGVDHRYYKGELNWVPLIEEGDWSVRMDGISMKTKVVACSDGCEAVVDTGTSLIKGPR
Sequence Logo
Ruler 2                 250       260       270       280       290
```

```
Ruler 1                 310       320       330       340       350
Consensus   RLVNNIqKLIGAtPR-GSeHYVSCSAVNTLPSIIFTINGINYPVPARAYILKDSRGrCYT
boPAG1      RLVNNIHRLIGAIPR-GSEHYVPCSEVNTLPSIVFTINGINYPVPGRAYILKDDRGRCYT
boPAG2      KLVTNIHKLMNARLE-NSEYVVSCDAVKTLPPVIFNINGIDYPLRPQAYIIKIQ-NSCRS
boPAG3      KLVNKIHKLIGARPR-HSKYYISCSAVNTLPSIIFTINGINYPVPGRAYVLKDSRGRCYS
boPAG4      TLVNNIWKLIGATPQ-GSEHYVSCSAVNSLPSIIFTIKSNNYRVPGQAYILKDSRGRCFT
boPAG5      RLVNNIQKLIGATPQ-GSEHYISCFAVISLPSIIFTINGINIPVPARAYIHKDSRGHCYP
boPAG6      TLVNNIWKLIRARPL-GPQYFVSCSAVNTLPSIIFTINGINYRLPARAYIHKDSRGRCYT
boPAG7      RLIDNIQRIIGATPR-GSKYYVSCSAVNILPSIIFTINGVNYPVPPRAYILKDSRGHCYT
boPAG8      HLVTDILKLINPNPILNDEQMLSCDAINSLPTLLLTINGIVYPVPPDYYIQRFSERICFI
boPAG9      RLVNNIHRLIRTRPF-DSKHYVSCFATKYLPSITFIINGIKYPMTARAYIFKDSRGRCYS
boPAG10     GSVSKIQKLIHARPI-DREHVVSCQAIGTLPPAVFTINGIDYPVPAQAYIQSLS-GYCFS
boPAG11     RPVTNIQKFIHAMPY-GSEYMVLCPVISILPPVIFTINGIDYSVPREAYIQKISNSLCLS
boPAG14     RLVNNIQKLIGSTPR-GSEYYISCSAVNTLPSIIFTINGINYPVPARAYILKDSRGRCYT
boPAG15     TLVDNIQKLIGATLR-GFKHYVSCSAVDTLPSITFTINGINYRVPARAYILKDSRGCCYS
boPAG16     RVIDNIHKLIGATPR-GSKHYVSCSVVNTLPSIIFTINGINYPVPAPAYILKDSRGYCYT
boPAG17     RLVNNIQKLIGTTPR-GSKHYVSCSVVNTLPSIIFTINGINYPVPARAYILKDSESNCYT
boPAG18     RLVNNIQKLIRAMPR-GSEYYVSCSAVNTLPPIIFTIKGINYPVPAQAYILKDSRGHCYT
boPAG20     RVIDNIHKLIGATPR-GSKHYVSCSAVSALPSVVFTINGINYPVPARAYVLKDFTGNCYT
boPAG21     KLVNKIQKLIGATPR-GSKHYVYCSAVNALPSIIFTINGINYPVPARAYILKDSRGRCYT
Sequence Logo
Ruler 2                 310       320       330       340       350
```

Red Markings: N-glycosylation sequons

Blue Markings: proteotypic peptides (listed in Table 1)

```
Ruler 1                370       380       390       400
Consensus   t F K E n r v s - - - s - S T E T W I L G D V F L R L Y F S V F D R G N D R I G L A R A V
boPAG1      T F Q E N R V S - - - S - S T E T W Y L G D V F L R L Y F S V F D R G N D R I G L A R A V
boPAG2      V F Q G G T E N - - - S - S L N T W I L G D I F L R Q Y F S V F D R K N R R I G L A P A V
boPAG3      M F Q E N K V S - - - S - S T E T W I L G D V F L R V Y F S V F D R G N D R I G L A R A V
boPAG4      A F K G H Q Q S - - - S - S T E M W I L G D V F L R L Y F S V F D R R K D R I G L A T K V
boPAG5      T F K E N T V S - - - T - S T E T W I L G D V F L R L Y F S V F D R G N D R I G L A Q V -
boPAG6      A F K E H R F S - - - S - P I E T W L L G D V F L R R Y F S V F D R G N D R I G L A R A V
boPAG7      T F K E K R V R - - - R - S T E S W V L G E V F L R L Y F S V F D R G N D R I G L A R A V
boPAG8      S F Q G G T E I L K N L G T S E T W I L G D V F L R L Y F S V Y D R G N N R I G L A P A A
boPAG9      A F K E N T V R - - - T - S R E T W I L G D A F L R R Y F S V F D R G N D R I G L A R A V
boPAG10     N F L V R P Q R - - - V N E S E T W I L G E V F L R L Y F S V F D R G N N R I G L A P A V
boPAG11     T F H G D - - - - - - - - D T D Q W I L G D V F L R L Y F S V Y D R G N N R I G L A P A V
boPAG14     T F R E H R F S - - - S - S T E T W I L G A V F L R L Y F S V F D Q G N D R I G L A P A V
boPAG15     S F Q E T T V S - - - P - S T E T W I L G D V F L R L Y F S V F D R G N D R I G L A R A V
boPAG16     A F K E Q R V R - - - R - S T E S W L L G D V F L R L Y F S V F D R G N D R I G L A R A V
boPAG17     T F K E N T V R - - - T - S R E T W I L G D V F P R L Y F S V F D R G N D R I G L A R A V
boPAG18     T F K E D R L S - - - P P S T E T W I L G D V F L R R Y F S V F D R G N D R I G L A R A V
boPAG20     T F K E K R V R - - - R - S T E F W I L G E A F L R L Y F S V F D R G N D R I G L A R A V
boPAG21     A F K K Q R F S - - - S - S T E T W L L G D A F L R V Y F S V F D R G N G R I G L A Q A V
Sequence Logo
Ruler 2                370       380       390       400
```

Red Markings: N-glycosylation sequons

Blue Markings: proteotypic peptides (listed in Table 1)

List of identified boPAGs in the sample from mid gestation (for an SDS-PAGE gel image of the samples please see Figure 1a: Lane 3 and 4).

Table S1. List of identified boPAGs in the sample from mid gestation (glycosylated).

PAG	Protein Identification Probability [%]	Protein Percentage of Total Spectra [%]	Exclusive Unique Peptide Count	Exclusive Unique Spectra Count	Total Spectrum Count	Percentage of Amino Acids Identified [%]
PAG 1 (NP_776836.1)	100	0.80	19	38	2850	61.32
PAG 3 (NP_001291497.1)	100	0.11	1	3	401	48.03
PAG 4 (NP_788788.1)	100	0.11	7	8	384	28.42
PAG 6 (NP_788790.1)	100	0.11	7	9	407	41.42
PAG 7 (NP_001103448.1)	100	0.22	1	1	795	35.79
PAG 10 (NP_788794.2)	100	0.10	2	3	357	48.03
PAG16 (NP_788798.1)	100	0.22	5	5	781	42.42
PAG 20 (NP_788802.1)	100	0.21	1	1	731	49.47
PAG 21 (NP_788803.1)	100	0.16	1	2	579	10.26

Table S2. List of identified boPAGs in the sample from mid gestation (deglycosylated).

PAG	Protein Identification Probability [%]	Protein Percentage of Total Spectra [%]	Exclusive Unique Peptide Count	Exclusive Unique Spectra Count	Total Spectrum Count	Percentage of Amino Acids Identified [%]
PAG 1 (NP_776836.1)	100	0.87	21	37	3115	78.68
PAG 3 (NP_001291497.1)	100	0.15	1	2	528	41.99
PAG 4 (NP_788788.1)	99	0.13	0	0	462	27.89
PAG 6 (NP_788790.1)	100	0.15	5	6	543	30.87
PAG 7 (NP_001103448.1)	100	0.26	1	1	944	35.26
PAG 10 (NP_788794.2)	100	0.11	1	2	398	33.60
PAG16 (NP_788798.1)	100	0.25	3	3	890	31.31
PAG 20 (NP_788802.1)	100	0.27	1	1	969	47.63
PAG 21 (NP_788803.1)	100	0.17	2	3	595	14.47

List of identified boPAGs in the sample from late gestation (for an SDS-PAGE gel image of the samples please see Figure 1a: Lane 5 and 6).

Table S3. List of identified boPAGs in the sample from late gestation (glycosylated).

PAG	Protein Identification Probability [%]	Protein Percentage of Total Spectra [%]	Exclusive Unique Peptide Count	Exclusive Unique Spectra Count	Total Spectrum Count	Percentage of Amino Acids Identified [%]
PAG 1 (NP_776836.1)	100	0.65	18	35	2288	61.32
PAG 3 (NP_001291497.1)	100	0.09	1	2	309	28.08
PAG 4 (NP_788788.1)	100	0.10	4	5	333	12.63
PAG 6 (NP_788790.1)	100	0.08	1	1	294	15.30
PAG 7 (NP_001103448.1)	100	0.22	1	1	759	47.37
PAG 10 (NP_788794.2)	100	0.09	1	2	307	28.87
PAG16 (NP_788798.1)	100	0.20	4	4	713	28.28
PAG 20 (NP_788802.1)	100	0.15	1	1	523	28.42
PAG 21 (NP_788803.1)	100	0.15	1	2	523	8.16

Table S4. List of identified boPAGs in the sample from late gestation (deglycosylated).

PAG	Protein Identification Probability [%]	Protein Percentage of Total Spectra [%]	Exclusive Unique Peptide Count	Exclusive Unique Spectra Count	Total Spectrum Count	Percentage of Amino Acids Identified [%]
PAG 1 (NP_776836.1)	100	0.84	17	31	3016	65.00
PAG 3 (NP_001291497.1)	100	0.13	1	3	472	25.72
PAG 4 (NP_788788.1)	93	0.13	0	0	454	12.63
PAG 6 (NP_788790.1)	100	0.13	3	3	469	26.12
PAG 7 (NP_001103448.1)	100	0.25	1	1	915	35.79
PAG 10 (NP_788794.2)	100	0.09	1	1	322	24.93
PAG16 (NP_788798.1)	100	0.24	3	4	849	22.98
PAG 20 (NP_788802.1)	100	0.21	1	1	741	28.42
PAG 21 (NP_788803.1)	100	0.15	1	2	546	8.42

List of identified boPAGs in the sample from afterbirth (for an SDS-PAGE gel image of the samples please see Figure 1a: Lane 7 and 8).

Table S5. List of identified boPAGs in the sample from afterbirth (glycosylated).

PAG	Protein Identification Probability [%]	Protein Percentage of Total Spectra [%]	Exclusive Unique Peptide Count	Exclusive Unique Spectra Count	Total Spectrum Count	Percentage of Amino Acids Identified [%]
PAG 1 (NP_776836.1)	100	0.61	16	27	2207	58.68
PAG 3 (NP_001291497.1)	100	0.10	1	1	351	28.61
PAG 4 (NP_788788.1)	100	0.12	13	18	439	54.74
PAG 6 (NP_788790.1)	100	0.10	2	2	353	21.90
PAG 7 (NP_001103448.1)	100	0.24	1	1	860	34.47
PAG 10 (NP_788794.2)	100	0.19	2	3	668	55.91
PAG16 (NP_788798.1)	100	0.24	4	5	871	36.11
PAG 20 (NP_788802.1)	100	0.22	2	2	776	56.84
PAG 21 (NP_788803.1)	100	0.17	2	5	620	15.00

Table S6. List of identified boPAGs in the sample from afterbirth (deglycosylated).

PAG	Protein Identification Probability [%]	Protein Percentage of Total Spectra [%]	Exclusive Unique Peptide Count	Exclusive Unique Spectra Count	Total Spectrum Count	Percentage of Amino Acids Identified [%]
PAG 1 (NP_776836.1)	100	0.79	16	24	2889	66.32
PAG 3 (NP_001291497.1)	100	0.16	1	2	583	28.61
PAG 4 (NP_788788.1)	100	0.17	1	1	623	54.74
PAG 6 (NP_788790.1)	100	0.14	3	3	507	22.16
PAG 7 (NP_001103448.1)	100	0.29	1	1	1046	32.11
PAG 10 (NP_788794.2)	100	0.25	2	3	918	59.06
PAG16 (NP_788798.1)	100	0.29	4	6	1050	36.11
PAG 20 (NP_788802.1)	100	0.31	2	2	1117	57.89
PAG 21 (NP_788803.1)	100	0.17	1	2	604	10.79

List of identified boPAGs in the sample from late gestation (for an SDS-PAGE gel image of the samples please see Figure 1a: Lane 9 and 10).

Table S7. List of identified boPAGs in the sample from late gestation (glycosylated).

PAG	Protein Identification Probability [%]	Protein Percentage of Total Spectra [%]	Exclusive Unique Peptide Count	Exclusive Unique Spectra Count	Total Spectrum Count	Percentage of Amino Acids Identified [%]
PAG 1 (NP_776836.1)	100	0.62	18	35	2210	61.32
PAG 3 (NP_001291497.1)	100	0.09	1	3	304	28.61
PAG 4 (NP_788788.1)	100	0.11	15	19	405	55.00
PAG 6 (NP_788790.1)	100	0.10	1	1	340	19.53
PAG 7 (NP_001103448.1)	100	0.20	1	1	702	50.53
PAG 10 (NP_788794.2)	100	0.09	1	2	303	30.97
PAG16 (NP_788798.1)	100	0.19	6	8	666	45.20
PAG 20 (NP_788802.1)	100	0.26	5	9	919	61.84
PAG 21 (NP_788803.1)	100	0.13	1	4	444	13.16

Table S8. List of identified boPAGs in the sample from late gestation (deglycosylated).

PAG	Protein Identification Probability [%]	Protein Percentage of Total Spectra [%]	Exclusive Unique Peptide Count	Exclusive Unique Spectra Count	Total Spectrum Count	Percentage of Amino Acids Identified [%]
PAG 1 (NP_776836.1)	100	0.86	21	36	2896	82.11
PAG 3 (NP_001291497.1)	100	0.13	1	3	426	31.23
PAG 4 (NP_788788.1)	100	0.14	1	1	470	53.95
PAG 6 (NP_788790.1)	100	0.15	4	5	497	27.44
PAG 7 (NP_001103448.1)	100	0.28	1	1	935	38.68
PAG 10 (NP_788794.2)	100	0.09	1	2	311	35.43
PAG16 (NP_788798.1)	100	0.28	6	7	934	44.70
PAG 20 (NP_788802.1)	100	0.39	3	5	1304	60.53
PAG 21 (NP_788803.1)	100	0.16	1	2	548	11.05

List of identified boPAGs in the sample from late gestation (for an SDS-PAGE gel image of the samples please see Figure 1b: Lane 3 and 4).

Table S9. List of identified boPAGs in the sample from late gestation (glycosylated).

PAG	Protein Identification Probability [%]	Protein Percentage of Total Spectra [%]	Exclusive Unique Peptide Count	Exclusive Unique Spectra Count	Total Spectrum Count	Percentage of Amino Acids Identified [%]
PAG 1 (NP_776836.1)	100	0.49	18	30	1731	60.53
PAG 3 (NP_001291497.1)	100	0.07	1	2	246	24.93
PAG 4 (NP_788788.1)	100	0.07	4	5	232	12.89
PAG 6 (NP_788790.1)	100	0.07	4	5	259	25.59
PAG 7 (NP_001103448.1)	100	0.15	1	1	527	45.26
PAG 10 (NP_788794.2)	100	0.05	0	0	171	12.34
PAG16 (NP_788798.1)	100	0.14	3	3	493	20.20
PAG 20 (NP_788802.1)	100	0.12	1	1	416	26.58
PAG 21 (NP_788803.1)	100	0.11	1	4	381	8.16

Table S10. List of identified boPAGs in the sample from late gestation (deglycosylated).

PAG	Protein Identification Probability [%]	Protein Percentage of Total Spectra [%]	Exclusive Unique Peptide Count	Exclusive Unique Spectra Count	Total Spectrum Count	Percentage of Amino Acids Identified [%]
PAG 1 (NP_776836.1)	100	0.69	18	29	2181	66.05
PAG 3 (NP_001291497.1)	98	0.11	1	2	358	20.73
PAG 4 (NP_788788.1)	87	0.08	0	0	261	12.63
PAG 6 (NP_788790.1)	100	0.11	3	3	359	26.39
PAG 7 (NP_001103448.1)	100	0.20	1	1	626	35.26
PAG 10 (NP_788794.2)	95	0.06	0	0	179	13.65
PAG16 (NP_788798.1)	100	0.19	2	2	612	16.92
PAG 20 (NP_788802.1)	100	0.16	1	1	499	28.42
PAG 21 (NP_788803.1)	100	0.14	1	2	437	8.16

List of identified boPAGs in the sample from early gestation (for an SDS-PAGE gel image of the samples please see Figure 1b: Lane 5 and 6).

Table S11. List of identified boPAGs in the sample from early gestation (glycosylated).

PAG	Protein Identification Probability [%]	Protein Percentage of Total Spectra [%]	Exclusive Unique Peptide Count	Exclusive Unique Spectra Count	Total Spectrum Count	Percentage of Amino Acids Identified [%]
PAG 1 (NP_776836.1)	100	0.51	19	32	1699	59.21
PAG 3 (NP_001291497.1)	100	0.07	1	3	246	27.03
PAG 4 (NP_788788.1)	100	0.08	3	4	266	12.63
PAG 6 (NP_788790.1)	100	0.11	10	14	373	52.24
PAG 7 (NP_001103448.1)	100	0.20	1	1	669	46.32
PAG 10 (NP_788794.2)	100	0.06	0	0	189	12.34
PAG16 (NP_788798.1)	100	0.16	2	2	522	18.69
PAG 20 (NP_788802.1)	100	0.14	1	1	467	46.58
PAG 21 (NP_788803.1)	100	0.11	1	4	381	10.26

Table S12. List of identified boPAGs in the sample from early gestation (deglycosylated).

PAG	Protein Identification Probability [%]	Protein Percentage of Total Spectra [%]	Exclusive Unique Peptide Count	Exclusive Unique Spectra Count	Total Spectrum Count	Percentage of Amino Acids Identified [%]
PAG 1 (NP_776836.1)	100	0.72	17	27	2602	64.21
PAG 3 (NP_001291497.1)	100	0.12	1	2	432	23.10
PAG 4 (NP_788788.1)	93	0.10	0	0	369	12.63
PAG 6 (NP_788790.1)	100	0.12	3	3	440	26.39
PAG 7 (NP_001103448.1)	100	0.22	1	1	780	36.32
PAG 10 (NP_788794.2)	89	0.07	0	0	247	9.97
PAG16 (NP_788798.1)	100	0.20	2	2	732	19.44
PAG 20 (NP_788802.1)	100	0.19	1	1	676	46.58
PAG 21 (NP_788803.1)	100	0.13	2	3	472	12.37

Table S13. Initial list of proteins and proteotypic peptides.

PAG	Peptide Sequence	Precursor Charge	Precursor *m/z*
PAG 1 (NP_776836.1)	K.ALVDTGTSDIVGPR.R	2	700.87
	R.AISEPVFAFYLSK.D	2	736.39
	R.VSSSTETWYLGDVFLR.L	3	620.64
PAG 2 (NP_788787.1)	R.QYFSVFDR.K	2	531.25
	K.TFNPQNSSSFR.E	2	642.80
	R.NYLDTAYVGNITIGTPPQEFR.V	3	790.39
PAG 3 (NP_001291497.1)	K.VSSSTETWILGDVFLR.V	3	603.98
PAG 4 (NP_788788.1)	K.ALVDTGSSDIVGPSTLVNNIWK.L	3	762.73
PAG 5 (NP_788789.1)	K.TFSITYGSGSTK.G	2	624.80
	R.HLESSTSGLTQK.T	2	644.33
	K.ENTVSTSTETWILGDVFLR.L	3	723.36
PAG 6 (NP_788790.1)	K.ALVDTGTSDIVGPSTLVNNIWK.L	3	767.40
	K.GIPFDGILGLSYPNK.T	3	530.95
	R.HHQSSTFRPTNK.T	2	720.36
PAG 7 (NP_001103448.1)	R.STESWVLGEVFLR.L	2	761.89
	R.HLQSSTFRPTNK.T	3	472.58
	K.WVPLIQAVDWSVHVDR.I	3	640.67
PAG 8 (NP_788792.2)	K.NLGTSETWILGDVFLR.L	3	607.65
PAG 9 (NP_788793.1)	R.QLQSSTFQPTNK.T	2	689.85
	K.GELNWIPLIEAGEWR.V	3	594.97
	K.YLPSITFIINGIK.Y	2	739.93
PAG 10 (NP_788794.2)	K.AANDQNIIYHHPLR.S	3	554.62
	R.IGNLVSVAQPFGLSLK.E	3	548.32
	R.TITGANPIFDNLWK.Q	2	795.41
PAG 11 (NP_788796.1)	K.LLNSFLEEQANR.M	2	717.37
	K.QQGAISEPIFAFYLSTR.K	3	643.33
	R.VVFDTGSSDLWVPSIK.C	3	583.97
PAG 12 (NP_788795.1)	K.GELNWIPVSQTR.Y	2	700.37
PAG 14 (XP_002699292.1)	R.NISFSGAIPIFYK.L	2	728.89
	R.DLFYVGNITIGTPPQK.F	3	588.31
PAG 15 (NP_788797.1)	R.LSQISFHGSNLTIHPLR.N	3	640.68
PAG16 (NP_788798.1)	R.STESWLLGDVFLR.L	2	761.89
	R.HFQSSTFRPTTK.T	2	718.86
	K.NQGAISDPIFAFYLSK.D	3	590.97
PAG 17 (NP_788800.1)	K.EHTYSLSQISSR.G	3	469.90
	K.GELNWVPLIQAGGWTVHVDR.I	3	749.72
PAG 18 (NP_788799.1)	K.AVVDTGTSLIEGPR.R	3	472.25
	R.LSPPSTETWILGDVFLR.R	3	644.34
	K.LSFSGAIPIFDNLR.N	3	517.28
PAG 20 (NP_788802.1)	R.FDGVLGLNYPNISFSK.A	3	590.97
	R.STEFWILGEAFLR.L	3	523.60
PAG 21 (NP_788803.1)	R.QHQSSTFRPTNK.T	2	715.86
	R.IGDLVSTDQPFGLSVSEYGFK.D	3	753.71
	K.NEGAISEPIFAFYLSK.K	3	595.97

Acknowledgements

The authors are deeply indebted to Julia Sondermann for the excellent technical assistance with the sample collection and preparation.

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Financial Disclosure

This work was supported by the H. Wilhelm Schaumann Stiftung, Hamburg, Germany (https://www.schaumann-stiftung.de/; received TK) and the Landwirtschaftliche

Rentenbank, Frankfurt am Main, Germany [grant number 785745] (https://www.rentenbank.de/; received TK, JT). Furthermore, the authors acknowledge support by the Open Access Publication Funds of the University of Goettingen. The H. Wilhelm Schaumann Stiftung provided support in form of salaries for the author TK. The Landwirtschaftliche Rentenbank provided support in the form of salaries for authors [TK] and research material [TK, JT]. The Open Access Publication Funds of the University of Goettingen only cover the publication costs. The funders of the study did not have any additional role in the study design, data collection and analysis, decision to publish, or preparation of the manuscript. The specific roles of these authors are articulated in the "author contributions'" section. There was no additional external funding received for this study.

Competing Interest

The authors have declared that no competing interests exist.

Data availability

The shotgun MS proteomics data have been deposited to the ProteomeXchange Consortium via the PRIDE [40] partner repository with the dataset identifier PXD027383. The PRM raw data have also been deposited to the ProteomeXchange Consortium via the PRIDE [40] partner repository with the dataset identifier PXD034108.

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5 Establishment of a Sandwich-ELISA for simultaneous quantification of bovine pregnancy-associated glycoprotein in serum and milk

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veröffentlicht in

PLoS ONE (2021), 16(5): e0251414

URL: https://doi.org/10.1371/journal.pone.0251414

DOI: 10.1371/journal.pone.0271057

Abstract

Bovine pregnancy-associated glycoproteins (**boPAG**) are expressed by trophoblast cells in the bovine placenta. The multigene family of boPAG belongs to the group of aspartic proteases. The accumulation and circulation in maternal blood and milk has made boPAG very useful and important for pregnancy diagnosis in cattle. The goal of the present study was to develop and validate a new Sandwich-ELISA which allows the detection of boPAG in maternal serum and whole milk. Therefore, 984 serum and 928 milk samples were collected monthly from 231 Holstein Friesian cows (*Bos Taurus*) from one week after insemination (**p.i.**) until six weeks postpartum. The ELISA is able to identify a cow as being pregnant at day 30 p.i. in serum and at day 40 p.i in milk with threshold values of 1.0 ng/ml in serum and 0.0165 ng/ml in milk. The postpartum half-life of boPAG was estimated to be 6.4 days in serum and 7.1 days in milk. The boPAG profile established during pregnancy in serum and milk showed a typical pattern. The amount of boPAG found in milk was 1.5 % of the amount of boPAG present in serum

In conclusion, a Sandwich-ELISA has been developed to quantify boPAG in serum and in whole milk simultaneously with the same test procedure. This is time saving for farmers and more efficient for laboratories.

Introduction

An accurate and timely pregnancy diagnosis is of considerable economic relevance in livestock management, especially in the cattle industry. Traditionally, pregnancy testing is done by manual or ultrasonographic examination per rectum or with the detection of progesterone as a non-pregnancy specific marker in serum or milk. In the last three decades, the identification and immunological detection of "pregnancy-associated glycoproteins" (**PAG**) has turned out to be an alternative method for pregnancy diagnosis [1–4].

These proteins are expressed by different cell types of the placenta. They are products of an unusual gene-family, which phylogenetically belongs to aspartic proteinases and is present in the Cetartiodactyla order [5]. PAG are particularly numerous in the Bovidae with their synepitheliochorial placenta [5, 6]. In cattle, roughly 20 different PAG members and related paralogs are known with largely varying temporal and spatial expression and glycosylation patterns during gestation [5, 7, 8]. Until now, the knowledge about the exact number and function of bovine PAG (**boPAG**) is incomplete [7], but the accumulation and circulation in maternal blood and milk have made boPAG a very useful and important tool for pregnancy diagnosis in cattle [5, 9–12]. Different studies demonstrated that boPAG can be used as reliable pregnancy markers in serum and milk, applicable as early as approximately day 25 post breeding [5, 13–15]. Thus, the detection of boPAG in serum or milk is an alternative method to transrectal ultrasonography or progesterone assays. Furthermore, boPAG-determination allows the direct identification of a placental product present in the maternal system that can be used as a marker of a viable pregnancy [16]. Some studies already figured out that boPAG are effective at identifying cows that will undergo early fetal mortality or late embryonic loss and are potential markers for a healthy placental function [15, 17, 18]. For these reasons, the measurement of boPAG in blood or milk is an important and powerful diagnostic tool for livestock management.

Today, there are different ELISAs available for detection of PAG in bovine milk and serum but most of the milk ELISA use skimmed milk instead of whole milk. In some studies,

PAG has been measured in unskimmed milk, but using a commercial test kit, which cannot quantify PAG concentrations in milk [11, 12, 19].

The present study addresses the establishment and validation of a new ELISA that quantifies boPAG concentrations in blood or whole milk samples in one ELISA system within a few hours. This is time saving for farmers and more efficient for laboratories, since only one test is necessary which enables a parallel measurement of blood and milk samples.

Materials and methods

The study is in accordance with the German legal and ethical requirements of appropriate animal procedures. Animals were not purposely euthanized for this study. Tissue samples were taken during the conventional slaughter process. The consultation of the institutional Animal Welfare Body is documented under no. E5-18.

Tissue collection for protein purification

For protein purification, cotyledon samples from different pregnancy stages were collected from an abattoir located in Germany, afterbirth cotyledons were obtained from a local dairy farm directly after calving. Uteri of pregnant cows (*n* = 16) were opened approximately 20–30 min after killing. Thereafter, the cotyledons were dissected from the caruncula and extensively washed with 0.9 % NaCl. Subsequently, the samples were immediately stored on ice and transported to the laboratory where they were stored at -20˚C until further processing. The gestation stage was estimated by measuring the crown-rump-length of the fetuses [20]. Overall, the collected cotyledons were divided into four different pregnancy stages: 35–90 days of gestation (early pregnancy), 91–180 days of gestation (mid pregnancy), 181–240 days of gestation (late pregnancy) and afterbirth samples.

Protein Extraction

The protein extraction was performed according to Zoli et al. [1] and Klisch et al. [21] with some modifications. Cotyledonary tissue was thawed, weighed and homogenized with an Ultra-Turrax (Ultra-Turrax T18 digital, IKA, Germany) in potassium phosphate buffer (0.01 M KH_2PO_4, 0.1 M KCl; pH 7.6) at 4 ˚C. The buffer tissue ratio was 5:1 (v/w). Protease inhibitors (0.2 mM phenylmethylsulfonyl fluoride, 0.2% sodium EDTA) were added during the homogenization process. The mixture was stirred for 20 min at 4 ˚C. Then the homogenate was centrifuged at 3,000 x *g* and 4 ˚C for 30 min. The pellet was discarded. The supernatant was transferred in a beaker and stirred. Ammonium sulfate was slowly added to achieve 40% saturation. Thereafter the supernatant solution was gently stirred at 4 ˚C for 1 h. Then it was centrifuged at 3,000 x *g* and 4 ˚C for 30 min. Again, the pellet was discarded and the supernatant was adjusted to 80 % ammonium sulfate saturation. After stirring the sample at 4 ˚C for 1 h, it was centrifuged at 27,000 x *g* and 4 ˚C for 1 h. The pellet was retained and dissolved in Tris/Cl buffer (0.01 M; pH 7.6). Following this step, the sample was stored at -20 ˚C until further analysis.

Chromatography

For protein purification, a fast protein liquid chromatography (**FPLC**) with the following steps was used: anion-exchange (Acetate basis), cation exchange (Acetate basis), gel filtration, cation exchange (Tris basis), hydrophobic interaction chromatography and anion exchange (Bis-Tris basis). Between the different FPLC steps the fractions were checked for boPAG content by using an available ELISA previously established by Friedrich and Holtz [9]. This ELISA was established on the basis of an existing RIA [22] and uses an anti-boPAG-1-IgG polyclonal rabbit antiserum for specific binding of PAG [9]. Prior to chromatography, samples were thawed and filtered through a syringe filter (0.22 µm; polyethersulfone, Carl Roth, Germany) to avoid clogging of the different columns. In the first step, the filtered samples were desalted in Tris/Cl buffer (0.01 M; pH 7.6) by passing it through a Sephadex G-25 column (HiTrap Desalting, 5 ml, GE Healthcare, USA). Following this, the samples were loaded to a Source 30Q (GE

Healthcare, USA) packed column (20 ml, HiScale 16, GE Healthcare, USA), which was equilibrated with Tris/Cl buffer (0.01 M; pH 7.6). After unbound protein had washed through, elution of the loaded samples (40 ml) was performed using six steps of increasing ionic-strength buffer by adding NaCl (0.02 M, 0.04 M, 0.08 M, 0.16 M, 0.32M and 1 M). The flow rate was 10 ml/min and the absorbance was measured at 280 nm. Fractions were collected automatically and were checked for boPAG content by ELISA [9]. The fractions with antigenic activity were pooled. The pools were submitted to buffer exchange using a HiTrap Desalting column equilibrated in ammonium acetate buffer (0.01 M; pH 5.2). The concentrated fractions were further purified on a Source 30S (GE Healthcare, USA) packed column (Tricorn 10/100, GE Healthcare, USA) equilibrated with the sample buffer. The loaded samples (90 ml) were eluted with the above-mentioned gradient of NaCl. The protein content was monitored by measuring the UV absorbance at 280 nm. Again, all fractions were analyzed by ELISA [9]. The boPAG-containing fractions were pooled and submitted to gel filtration on a Superdex 200 column (Superdex 200 Prep Grade in a XK 26/70 Column, GE Healthcare, USA), equilibrated in PBS (0.68 M NaCl, 0.0405 M Na_2HPO_4, 0.0075 M KH_2PO_4, 0.0135 M KCl; pH 7.3) plus 0.5 M NaCl buffer. A maximum of 10 ml per sample was loaded on the column. Fractions of 5 ml were collected. The flow rate was 2 ml/min and the absorbance was recorded at 280 nm. In the next step, all boPAG-containing and pooled fractions were subjected to a buffer exchange to cation buffer (15 mM Na_2HPO_4, 15 mM HCOONa, 35 mM $C_2H_3NaO_2$; pH 5.25) using the desalting column. Subsequently, a second cation exchange was carried out using the Source 30S packed column equilibrated with the same buffer. The bound proteins were eluted with the exponential gradient of NaCl. The protein content was monitored by measuring the UV absorbance at 280 nm. After cation exchange, ELISA-checked and pooled fractions were loaded onto a column (1 ml HiTrap Phenyl HP, GE Healthcare, USA) for hydrophobic interaction chromatography after the addition of the same volume of 4 M $(NH_4)_2SO_4$. Previously, the columns had been equilibrated with a buffer containing 50 mM Na_2HPO_4 and 2M $(NH_4)_2SO_4$ (pH 7). Proteins were eluted using a linear gradient to water. Fractions of 0.5 ml were collected and assayed. Those with high antigenic activity were pooled, and buffer was exchanged to an anion exchange buffer (35 mM BisTris, 25 mM Tris; pH 9.0) using a fresh desalting column. Following this, a second anion exchange was performed using a Source 30Q packed column

(Tricorn 10/100 equilibrated with anion exchange buffer). After elution of the unbound proteins, the exponential NaCl gradient was applied at a flow rate of 3 ml/min. 1 ml fractions were automatically collected and analyzed by ELISA [9]. The boPAG-containing fractions were pooled and equilibrated in PBS buffer using a desalting column. Afterwards, they were stored at -20 ˚C until further processing.

Polyclonal Antibody Production

Anti-boPAG antibodies were produced using seven boPAG-fractions of different pregnancy stages from chromatography. Immunizations of rabbits against PAG from early, mid, late pregnancy and afterbirth were performed by ImmunoGlobe Antikörpertechnik GmbH (Himmelstadt, Germany). In total seven rabbits were immunized with boPAG in PBS (early pregnancy (2 rabbits), mid pregnancy (1 rabbit), late pregnancy (3 rabbits) and afterbirth (1 rabbit)). Each rabbit received 5–7 times multiple intra dermal injections with approximately 250 µg purified boPAG-fraction with Montanide ISA 206 (Seppic, France) as adjuvant. Blood collection were carried out two and three weeks after each antigen injection starting after the third boost and final collection after the last boost. The antisera within rabbits were pooled.

Afterwards, each antibody was tested with itself and the other antibodies for coating and as biotinylated antibodies. In contrary to expectations, two different antibodies against late pregnancy PAG preparations (IgG 1438 and IgG 1440) were found to be most suitable for the development of the Sandwich-ELISA. This pair of antibodies showed the best differentiation between pregnant and non-pregnant animals in combination with high specific PAG binding and low background in the assay system.

Sandwich-ELISA for Detection of PAG in Serum or Milk

The PAG-Sandwich-ELISA utilizes 96 well microtiter plates (Costar 2592, Corning, USA). These plates were coated with 100 µl of anti-PAG polyclonal rabbit antibody (IgG 1438; raised against PAGs from late pregnancy) at a concentration of 1 µg/ml in coating buffer (0.05M $NaHCO_3$; pH 9.6). Antibodies were purified by using rmp Protein A Sepharose

Fast Flow (GE Healthcare, USA). After overnight incubation at 4 ˚C, the wells were blocked with washing buffer (10 % PBS, 0.05% Tween 20) and then washed five times with 350 µl washing buffer. Plates were stabilized by using 300 µl of 20 % sucrose solution. After decantation, the plates were dried at room temperature and stored with silica gel at 4 ˚C until use. A standard stock solution of 10 ng/ml was prepared from a cotyledonary extract (crude extract) from mid pregnancy in standard buffer (PBS-T (PBS with 0.05 % Tween 20), 0.1 M NaH_2HPO_4, 10 % PAG free bovine serum (from non-pregnant animals)) and stored at -20 ˚C until use. The boPAG content of the extract was determined with the same ELISA mentioned above [9]. For the preparation of a standard curve, the standard stock was diluted 1:20 in dilution buffer (PBS-T, 0.1 M NaH_2HPO_4, 10 % PAG free-bovine serum). Afterwards, two-fold serial dilutions were prepared freshly before every use. This procedure resulted in seven standards with the following concentrations: 500 pg/ml, 250 pg/ml, 125 pg/ml, 62.5 pg/ml, 31.3 pg/ml, 15.6 pg/ml and 7.8 pg/ml. Dilution buffer was used as 0-Standard (0 pg/ml) and negative control. Positive control samples were prepared from two serum or milk samples with high and midrange boPAG concentrations. For this purpose, the serum sample with a high concentration (33.5 ng/ml) was diluted 1:100 in dilution buffer and the serum sample with a medium concentration (3.5 ng/ml) was diluted 1:20. The milk sample with a high (442.6 pg/ml) and medium concentration (48.4 pg/ml) was used undiluted. The same control samples were used on each plate throughout the experiment.

Bovine serum samples were diluted in dilution buffer 1:10 in early pregnancy (<30 d post insemination (**p.i.**)), 1:100 in mid pregnancy (>30 d p.i.) and 1:1,000 in later pregnancy (> 150 d p.i.). Milk samples were used undiluted in the beginning of gestation (< 160 d p.i.) and diluted 1:10 until the end of gestation (> 160 d p.i.). The dilutions at the aforementioned gestation stages were necessary to allow accurate concentration measurement of the samples in the range of the standard curve. All standards, samples and controls were assayed in duplicate.

For the first step, 50 µl of matrix solution (0.05 % PBS-T, 0.1 M NaH_2HPO_4, 10 % PAG-free bovine milk (from non-pregnant animals), 10 % PAG free-bovine serum) were added into each well. Afterwards, 50 µl of prepared standard, control or pre-diluted sample were added, and the mix was incubated for 2 h in the dark and at room temperature

(20–25 ˚C) on a shaker (500 rpm). After incubation, the plate was washed three times with 350 µl of diluted washing buffer before adding biotin-conjugated anti-boPAG polyclonal rabbit antiserum (35 ng/ml) to the wells. The antibody (IgG 1440; raised against PAGs from late pregnancy, Protein A purified) was conjugated to Biotinamidohexanoic acid N-hydroxysuccinimide ester (**biotin**). The biotin antibody conjugate was diluted in biotinylated antibody buffer (0.136 M NaCl, 0.02 M Na_2HPO_4, 0.01 M EDTA, 0.005 % chlorhexidine digluconate, 0.1 % gelatine, 0.05 % Tween 20, 1 % BSA, 10 % normal rabbit serum, 50 % PAG free- bovine milk). To each well, 100 µl of diluted biotin antibody conjugate was added, followed by 30 min incubation in the dark at room temperature on a shaker (500 rpm). After three washing steps with 350 µl of diluted washing buffer, 100 µl of streptavidin conjugated to horseradish peroxidase (EC 1.11.1.7) (**HRP**) was added into each well. The streptavidin-HRP conjugate was diluted 1:500 in HRP buffer (0.136 M NaCl, 0.02 M Na_2HPO_4, 0.01 M EDTA, 0.005 % chlorhexidine digluconate, 0.1 % gelatine, 0.05 % Tween 20, 1 % BSA, 10 % normal rabbit serum). The plate was incubated for 30 min in the dark at room temperature on a shaker (500 rpm), followed by 5 additional washes with 350 µl of diluted washing buffer. Then 100 µl of 3,30,5,50-Tetramethylbenzidin (**TMB**) substrate (TMBS, SurModics, MN, USA) were added, followed by an incubation for 20 min in the dark at room temperature on a shaker (500 rpm). The enzyme reaction was stopped by the addition of 100 µl of 1 M HCl. The color changed from blue to yellow and the color intensity was measured spectrophotometrically at 450 nm with a 650 nm reference filter using an EMax Plus Microplate reader (Molecular Devices, USA) with software SoftMax Pro 6.5.1 (Molecular Devices, USA). Automatic data reduction was done using a 4-parameter logistic (4-PL) curve fit.

For development and validation, the new boPAG Sandwich-ELISA was standardized with boPAG-1 which was used in an well-established double-antibody ELISA in our laboratory [9]. During assay development, 37 serum samples were measured in both assays. The results are shown in the S1 Fig of the supplementary material. For further validation, all collected blood samples in this study were analyzed on both ELISA systems. The measurement of milk samples with the double-antibody ELISA was not possible, as this ELISA is not sensitive enough to analyze boPAG-content in milk.

In order to determine the intraassay variability of the new Sandwich-ELISA, four serum (515 pg/ml; 155 pg/ml; 65 pg/ml; 46 pg/ml) and four milk samples (87 pg/ml; 51 pg/ml; 44 pg/ ml; 17 pg/ml), were analyzed 10 times in duplicate within one plate in three independent assays. On the other hand, the interassay variability was determined measuring two milk and serum samples in duplicate on 20 plates. These samples are the same as those used as positive controls. The detection limit was determined measuring 20 0-Standard samples in duplicate plus three standard deviations. A total of 52 assays were performed for the analysis of all samples in the validation study (26 serum assays and 26 milk assays).

Serum and Milk Collection

For the validation of the assay and the establishment of PAG profiles, 984 blood and 928 milk samples were collected monthly from 231 Holstein Friesian cows (*Bos Taurus*) from one week after insemination (**p.i.**) until six weeks postpartum (**p.p.**). The last calving of the sampled cows was a minimum of 87 days ago. The animals were housed on different farms in Lower Saxony and Hesse (Germany). The blood sample and the corresponding milk sample for each animal were collected on the same day. Confirmation of pregnancy in these animals was carried out by regular analysis of all serum samples using the well-established ELISA by Friedrich and Holtz [9]. Furthermore, the pregnancy was verified by the birth of a healthy calf. This information was provided directly from the farmers. Cattle were considered non-pregnant if the date of the last insemination was at least 60 days in the past and the pregnancy tests performed until then had shown a negative result. These animals continued to be sampled at monthly intervals until a new insemination was performed by the farmer. Samples from animals with an abort, animals that had no more than one sample or animals which have left the farms for different reasons (e.g. udder diseases, unsuccessful rebreeding etc.) were not included in the further analysis. In total 5 animals had an abortion (between days 96 and 163 after insemination). Three of them had lower PAG concentrations in serum and milk than animals that carried their calves to term. One animal showed higher PAG concentrations in serum and normal concentrations in milk compared to the animals that calved at term and the last

animal showed no differences in serum and milk concentrations in comparison to animals with a normal pregnancy. Similar results are described in literature [17, 18, 23]. We excluded these animals from the analysis due to the small sample size. Milk samples were not examined in the dry period.

All blood samples in this study were collected from tail blood vessels. Approximately 12 ml of whole blood was captured in sample tubes for serum collection with separating agent (KABE LABORTECHNIK GmbH, Germany). They were immediately cooled and shipped to our laboratory. Then the blood was centrifuged at 2,800 x *g* for 10 min at 4 ˚C. The serum was stored at -20˚C until further processing. Milk samples (approximately 8 ml from each cow) were stripped from a healthy quarter before milking and stored in milk preservation tubes with ProClin as preservative (KABE LABORTECHNIK GmbH, Germany) at -20 ˚C until assayed. All serum and milk samples were vortexed following thawing and before dilution or analysis steps.

Statistical Analysis

All experimental results were analyzed with R 3.6.1 (R Development Core Team, Austria). A nonlinear regression (for all samples) and a linear regression (for samples from gestation day 30 onwards) were used to estimate the correlation between boPAG-concentration and days after insemination. To determine the earliest gestation day at which the test can significantly differentiate between pregnant and nonpregnant animals, a one-sided ANOVA was used. Post hoc evaluation was performed with a two-sided Dunnett T-Test [24]. The results were considered significant at $P < 0.05$.

The clinical sensitivity, clinical specificity, the positive predictive value (**PPV**) and the negative predictive value (**NPV**) were calculated for various threshold values. Therefore, the Rpackage “pROC” [25] was used. All blood and milk samples collected throughout gestation were included in this analysis. The clinical or diagnostic sensitivity indicates the probability of how well the ELISA correctly identifies a pregnant cow as pregnant, whereas the clinical or diagnostic specificity indicates the probability of how well the ELISA correctly identifies a cow as open. These terms should not be mistaken with the

analytical sensitivity or specificity of a test system. Since the diagnostic sensitivity and specificity do not give any information about the probability of the test giving the correct diagnosis in the population tested, we calculated the positive predictive value and the negative predictive value. The positive predictive value of the test system is the likelihood that a cow with a positive test result actually is pregnant. The negative predictive value is the opposite of the positive predictive value. It is the likelihood that a cow with a negative test result is not pregnant.

A receiver operating characteristic **(ROC**) analysis was done [26] to determine the optimal cutoff value for serum and milk pregnancy testing. Samples were taken as experimental units in this analysis. The Youden's index was used to find the best cutoff value that optimizes sensitivity and specificity. It is the threshold, that maximizes the distance to the identity line [27]. Furthermore, the area under curve (**AUC**) was calculated to measure the ability of the test to correctly classify pregnant and nonpregnant cows. A perfect test has an AUC of 1.0.

Results

The standard curve of the Sandwich-boPAG-ELISA showed a linear pattern. The characteristics of the ELISA are shown in Table 1. As expected, the highest variation was observed in the control sample with the lowest concentration. The correlation of the serum concentrations between the Sandwich-boPAG-ELISA and the established ELISA in our lab was $r = 0.96$ ($P < 0.001$), which is a clear indication that boPAG (and therefore pregnancy) recognition of both assay types is comparable. Recovery of serial dilution of serum and milk samples was 109.5 % and 112.1 %, respectively.

Table 1. Properties of the Sandwich-ELISA for measuring pregnancy-associated glycoprotein in serum and milk.

	Serum	Milk
Sample volume (µl)	50	50
Measuring range (pg/ml)	78 - 5,000	7.8 - 500
Detection limit (pg/ml)	7.43	0.74
Dilution linearity (%)	109.5	112.1
Mean recovery rates (%)	99.5	100.5
Intraassay CV (%)	1.9	2.5
Interassay CV (%)		
3.5 ng/ml	4.6	
33.5 ng/ml	6.1	
48.4 pg/ml		11.4
442.6 pg/ml		7.0

Serum Analysis

155 serum samples were collected from 62 nonpregnant animals and 666 serum samples were collected from 154 pregnant animals monthly throughout pregnancy until six weeks postpartum. These were the samples that met requirements for further analysis. Unless otherwise stated, results in the following section are presented as 10-day-means ± SEM. Individual boPAG-concentrations in the course of pregnancy are shown in Fig 1. BoPAG was detectable in serum before day 30 p.i., but only in very low concentrations. The serum boPAG concentration began to rise in maternal blood around day 30 p.i. reaching an average concentration of 2 ng/ml ± 0.2 ng/ml by day 40 p.i. and then were stable until day 70 p.i.. Afterwards, the circulating boPAG rose steadily through the rest of pregnancy. Approximately at day 250 p.i., the average serum concentration of boPAG declined to 86.7 ng/ml ± 15.4 ng/ml. Thereafter, the serum boPAG concentrations rose until parturition.

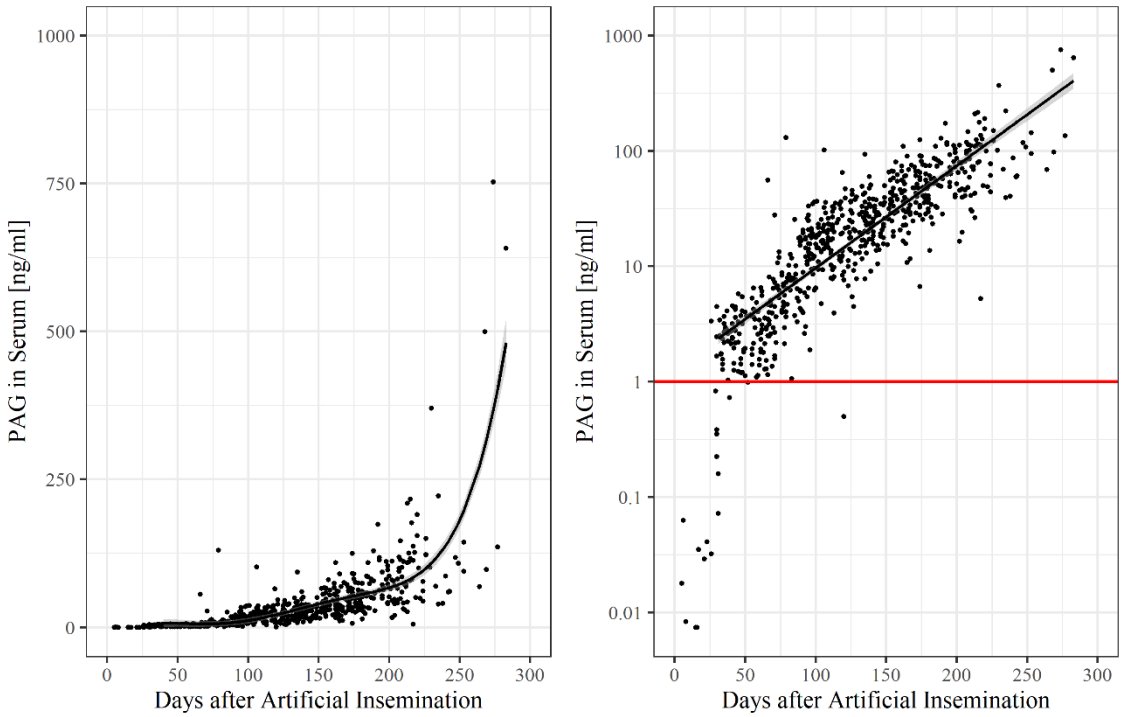


Fig 1. Pregnancy-associated glycoprotein concentrations during pregnancy in serum. The correlation between pregnancy-associated glycoprotein concentration and days after insemination was estimated with a non-linear regression for all samples ($R^2 = 0.61$, $P < 0.001$) (left) and with a linear regression for samples from gestation day 30 onwards ($R^2 = 0.74$, $P < 0.001$) (right). The red line indicates the threshold value. Note the log-scale of the y-axis in the right figure.

The overall mean boPAG concentration of non-pregnant cows was 0.12 ng/ml ± 0.03 ng/ml and the overall mean boPAG concentration of pregnant cows was 34.1 ng/ml ± 2.1 ng/ml. 10-day means of boPAG serum concentration throughout pregnancy in comparison to the nonpregnant control group are shown in Fig 2. Average boPAG-values for pregnant cows were significantly different from those of the nonpregnant control group from day 100 onwards ($P = 0.002$, Dunnett-Test). This is the result of a considerably high variation in the concentrations of boPAG in serum, as indicated by the standard errors from the average values.

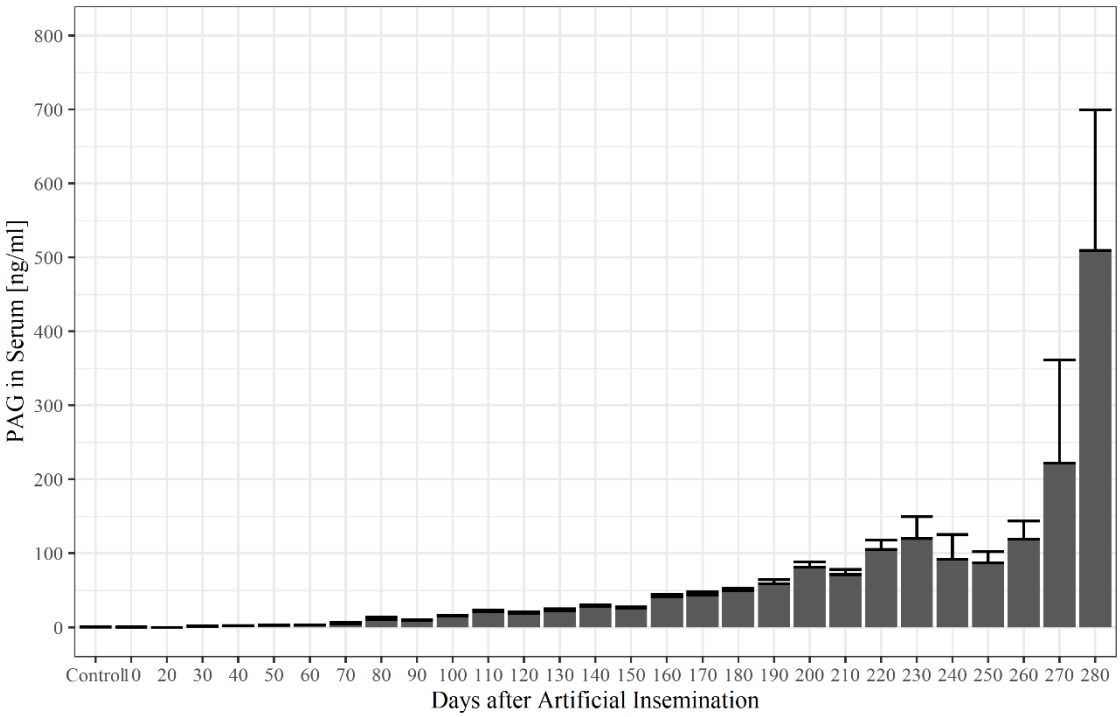


Fig 2. Serum pregnancy-associated glycoprotein concentrations (10-day-means ± SEM) in pregnant and non-pregnant control cows (control).

The results of the ROC analysis at various threshold values are shown in Table 2. The corresponding ROC curve (S2 Fig) and confusion matrices (S1–S5 Tables) can be found in the supplemental material. Across all serum samples, this approach resulted in an optimal threshold value of 1.0 ng/ml with an AUC of 0.988, a sensitivity of 97.1 %, and a specificity of 95.5 %. The PPV is 98.9 % and the NPV is 88.6%. A serum sample < 1.0 ng/ml would identify a cow as open, whereas a sample with a serum boPAG concentration > 1.0 ng/ml would identify a cow as being pregnant. On average, this value was reached at day 30 p.i. by a pregnant cow.

Table 2. Accuracy of a pregnancy diagnosis based on the serum and milk pregnancy-associated glycoprotein level assessed with the newly established ELISA at various threshold values.

	Serum					Milk				
Threshold (ng/ml)	0.4	1.0	1.5	2.0	2.5	0.01	0.0165	0.02	0.025	0.16
Sensitivity (%) (no./no.)	97.7 (651/666)	97.1 (647/666)	94.0 (626/666)	91.1 (607/666)	88.4 (589/666)	97.3 (616/633)	95.3 (603/633)	93.4 (591/633)	90.8 (575/633)	56.2 (356/633)
Specificity (%) (no./no.)	89.0 (138/155)	95.5 (148/155)	98.7 (153/155)	99.4 (154/155)	99.4 (154/155)	85.2 (121/142)	91.5 (130/142)	93.0 (132/142)	95.1 (135/142)	98.6 (140/142)
PPV (%) (no./no.)	97.5 (651/668)	98.9 (647/654)	99.7 (626/628)	99.8 (607/608)	99.8 (589/590)	96.7 (616/637)	98.0 (603/615)	98.3 (591/601)	98.8 (575/582)	99.4 (356/358)
NPV (%) (no./no.)	90.2 (138/153)	88.6 (148/167)	79.3 (153/193)	72.3 (154/213)	66.7 (154/231)	87.7 (121/138)	81.3 (130/160)	75.9 (132/174)	69.9 (135/193)	33.6 (140/417)
Accuracy (%) (no./no.)	96.1 (789/821)	96.8 (795/821)	94.9 (779/821)	92.7 (761/821)	90.5 (743/821)	95.1 (737/775)	94.6 (733/775)	93.3 (723/775)	91.6 (710/775)	64.0 (496/775)

The numbers within the parentheses indicate the number of samples.

Post-partum samples were obtained from eight animals (total number of samples *n* = 9) in the period from three weeks after calving to six weeks after calving. Except for one cow, all animals were sampled only once. In total, there were four samples from the third week p.p. (days 17; 20; 20; 21), one sample from the fourth week p.p. (day 25), one sample from the fifth week p.p. (day 35) and three samples from the sixth week p.p. (days 37; 39; 40). The mean boPAG concentration in the post-partum period is 135.7 ± 38.4 ng/ml. The average concentration of PAG in serum decreases from 246.1 ± 36.9 ng/ml in week three after parturition to 31.6 ± 7.2 ng/ml by post-partum week six. A simple linear regression model was fitted to the data after ln transformation (Fig 3) to estimate boPAG half-life in the post-partum period and to estimate the time point when boPAG become undetectable for the ELISA. The estimated post-partum half-life of serum boPAG was 6.4 days and boPAG was estimated to be undetectable for the ELISA three months postpartum given a first-order process of boPAG elimination.

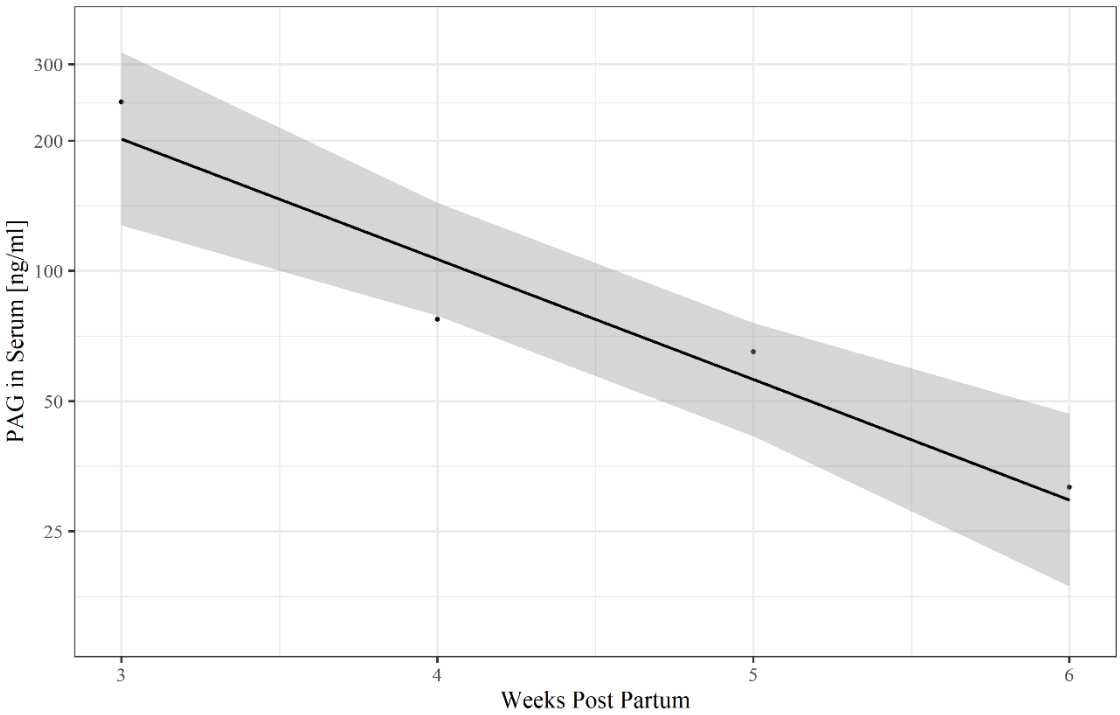


Fig 3. A ln-PAG-time graph to estimate PAG half-life in the postpartum period and to estimate the time at which PAGs became undetectable (R^2 = 0.90, *P* = 0.03).

Milk Analysis

633 milk samples were collected from 154 pregnant animals and 142 milk samples were collected from 62 non-pregnant animals that were also chosen for the serum sampling. Milk samples were not examined in the dry period from day 250 p.i. until calving. Unless otherwise stated, results in the following section are presented as 10-day-means ± SEM.

Individual concentrations of boPAG in milk during pregnancy are shown in Fig 4. BoPAG were detectable in milk before day 30 p.i., but only in very low concentrations. The boPAG concentration in milk began to rise at day 20 p.i. and reached a stable concentration of 0.025 ng/ml ± 0.006 ng/ml around day 30 p.i.. From day 60 p.i. until day 190 p.i., we found a slight increase in milk boPAG concentration up to 0.88 ng/ml ± 0.11 ng/ml. After 200 d p.i., the circulating boPAG rose steadily thought the rest of pregnancy. Nonpregnant animals had a mean boPAG concentration of 0.014 ± 0.006 ng/ml, whereas pregnant animals had a mean concentration of 0.47 ± 0.04 ng/ml. 10-day means of boPAG milk concentration in comparison to the non-pregnant control group is shown in Fig 5. Average boPAG values for pregnant cows were different from those of the nonpregnant control group from day 140 p.i. onwards (P = 0.008, Dunnett-Test).

The results of the ROC analysis for milk samples at various threshold values are shown in Table 2. The corresponding ROC curve (S3 Fig) and confusion matrices (S6–S10 Tables) can be found in the supplemental material. On the scale of all milk samples, this approach resulted in an optimal cut-off value of 0.0165 ng/ml with an AUC of 0.969, a sensitivity of 95.3%, and a specificity of 91.5%. The PPV is 98.0% and the NPV is 81.3%. A milk sample < 0.0165 ng/ml would identify a cow as open, whereas a sample with a milk boPAG concentration > 0.0165 ng/ml would identify a cow as being pregnant. On average, this value is reached at day 40 p.i. by a pregnant cow.

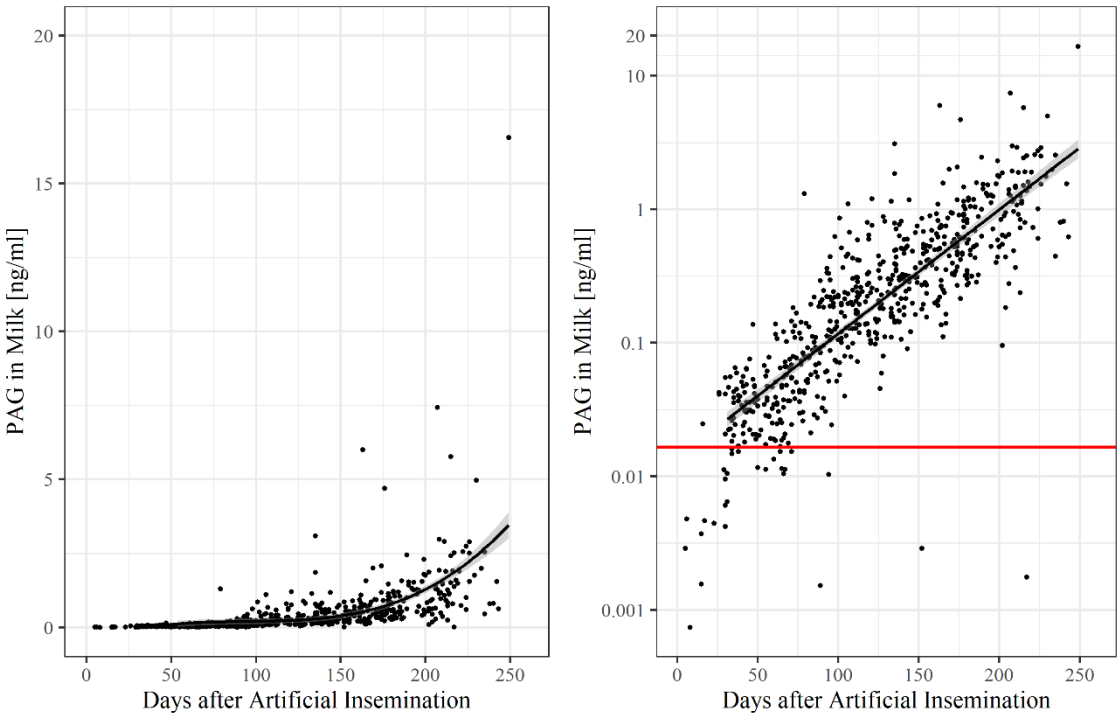


Fig 4. Pregnancy-associated glycoprotein concentrations during pregnancy in milk. The correlation between pregnancy-associated glycoprotein concentration and days after insemination was estimated with a non-linear regression for all samples (R^2 = 0.34, $P < 0.001$) (left) and with a linear regression for samples from gestation day 30 onwards (R^2 = 0.67, $P < 0.001$) (right). The red line indicates the threshold value. Note the log-scale of the y-axis in the right figure.

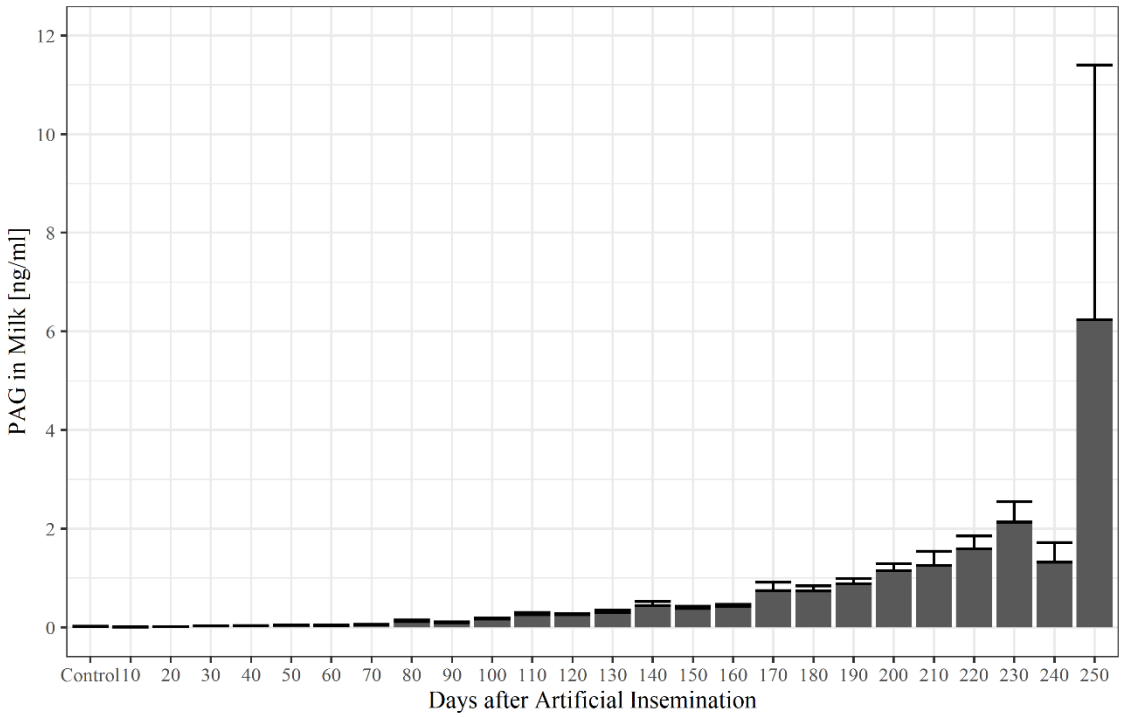


Fig 5. Milk pregnancy-associated glycoprotein concentrations (10-day-means ± SEM) in pregnant and nonpregnant control cows (control).

In the post-partum period (three weeks p.p. to six weeks p.p.), the mean milk boPAG-concentration was 1.39 ± 0.41 ng/ml. The samples were collected from the same animals as described in the serum part. The mean milk boPAG concentration declined from 2.27 ± 0.5 ng/ml three weeks p.p. to 0.3 ± 0.06 ng/ml six weeks p.p. A simple linear regression model with ln-transformed boPAG as dependent variable was used (Fig 6) to estimate boPAG halflife in the post-partum period and to estimate the time at which boPAG becomes undetectable for the ELISA. Based on the estimated coefficient, the half-life of milk boPAG is 7.1 days. The time where milk boPAG become undetectable for the ELISA was estimated by linear regression to be two months postpartum given a first order elimination pattern.

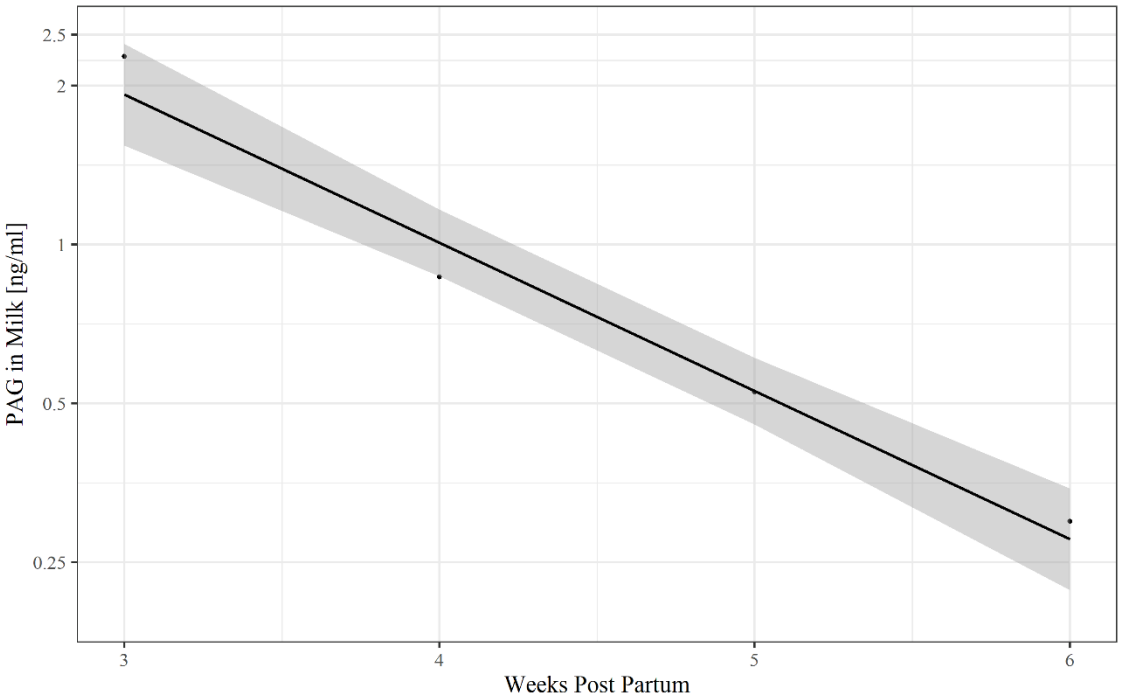


Fig 6. A ln-PAG-time graph to estimate PAG half-life in the postpartum period and to estimate the time at which PAGs became undetectable (R^2 = 0.97, *P* = 0.008).

Correlation of serum and milk concentrations

BoPAG-concentrations in milk amounted to 1.5 % in pregnant animals and 11.7 % in nonpregnant animals of that measured in serum. The correlation between milk and serum boPAG values was r = 0.58 (*P* < 0.001) in pregnant and r = 0.11 (*P* = 0.17) in nonpregnant cows (Fig 7).

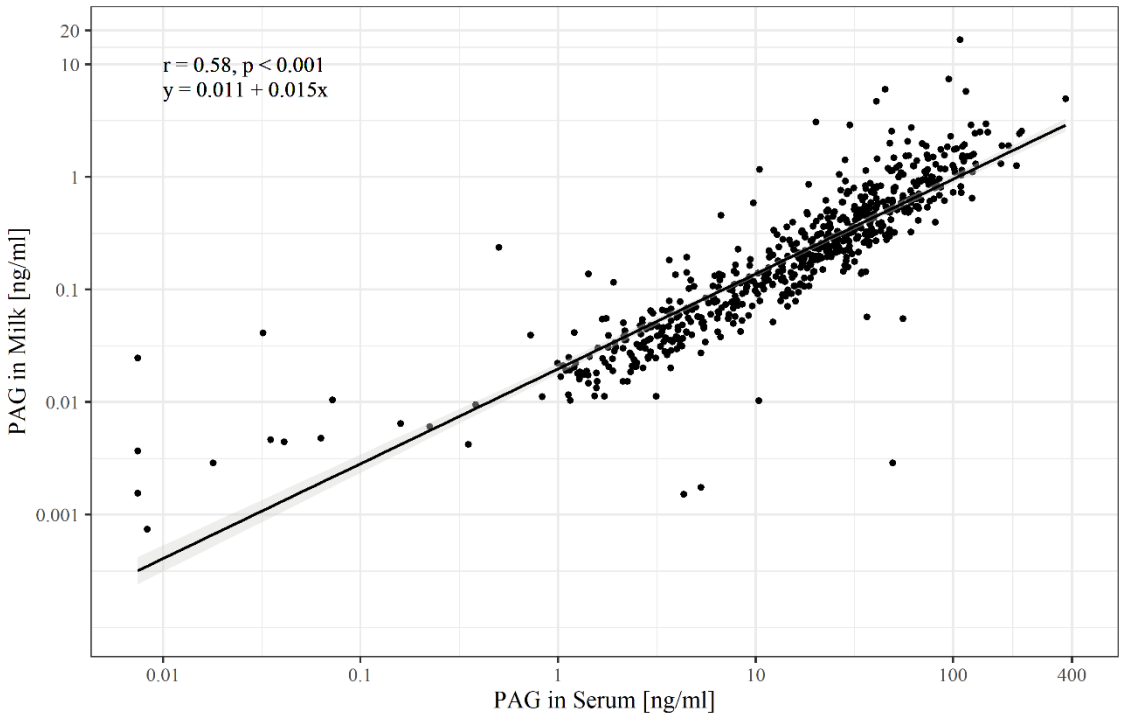


Fig 7. Comparison of concentrations of pregnancy-associated glycoproteins between plasma and milk of pregnant animals. Note the log-scale of both axes.

The rise of boPAG concentrations through the time of pregnancy is much sharper in serum compared to that in milk. Furthermore, the slope of the regression line reflects the greater concentrations of boPAG in serum compared with whole milk.

Discussion

In cattle, an early and reliable pregnancy diagnosis is of considerable economic interest. ELISA based on the measurement of pregnancy-associated glycoproteins in serum or in milk offer an early possibility (28 days after insemination) to realize this intent. The detection of pregnancy specific proteins in serum or in milk in bovine species is a well-established diagnostic method [2, 9–11, 22, 28]. Pregnancy-associated glycoproteins are detectable in the maternal serum and in the milk throughout gestation and, therefore, are advantageous over progesterone testing. Progesterone testing has a prevalence for

false positive results and the date of mating or artificial insemination is essential for correct pregnancy diagnosis [14, 22]. Ultrasonography is another valuable tool for detection of pregnancies as early as 26 days of gestation [14, 29]. However, the accuracy is limited under field conditions before day 30 of gestation and the pregnancy status is only guaranteed at the time of screening [14, 30]. Furthermore, expensive equipment and technical skills or a trained person are required when performing the ultrasound procedure [13, 29]. Regarding these facts, PAG assays are an alternative, reliable method for diagnosing pregnancies in cattle. Additional benefits of a PAG assay using milk are the avoidance of stressful effects during sampling (e.g. of venepuncture) and the omission of special equipment or experience.

Therefore, the goal of the present work was the development of an ELISA that is able to quantify boPAG concentrations in serum and milk simultaneously. To the best of our knowledge, only a few assays are described in literature, which are able to quantify PAG in bovine milk [9, 31, 32]. There is only one commercially available ELISA for detection of PAG in bovine milk [11, 12, 33], which is designed as a qualitative test and does not determine actual PAG concentrations. Instead, the results are classified as "not pregnant", "recheck" or "pregnant" [11, 12], based on the subtrahend (S-N value) from the optical density of the sample (S) and a negative control (N).

For our newly developed Sandwich-ELISA we decided to use polyclonal instead of monoclonal antibodies, as they have several advantages for our particular application. In cattle, roughly 20 different PAG members and related paralogs are known with largely varying temporal and spatial expression and glycosylation patterns during gestation [5, 7, 8]. Therefore, it is advantageous to use a broad spectrum of different antibodies to different epitopes, to ensure a reliable pregnancy diagnosis throughout pregnancy. Furthermore, the detection of multiple epitopes offers better sensitivity for detecting proteins that are present in low concentrations in a sample, which is the case for boPAG in milk and polyclonal antisera are less prone to posttranslational modifications of native proteins [34].

The validation of our newly developed boPAG-Sandwich-ELISA was performed on the basis of serum and milk samples from 216 cows throughout gestation. The earliest

possible detection of boPAG in serum with our Sandwich-ELISA turned out to be day 17 p.i.. At this time point the concentration of boPAG in serum is very low (35 pg/ml). Such low concentrations of boPAG in early pregnancy should be looked at with caution. Zoli et al. [22] supposed that boPAG is formed in extraplacental tissues and that threshold values for a pregnancy diagnosis should take background or nonspecific levels (< 0.5 ng/ml) into account. Therefore, results before day 28 p.i. should be interpreted with caution [22]. In the present study, the optimal threshold value for pregnancy testing in serum with the best sensitivity and specify after ROC analysis turned out to be 1.0 ng/ml. This cut-off value is higher than the value described by Zoli et al. (0.5 ng/ml) [22], almost the same as described by Green et al. (0.922 ng/ml) [10], and lower than the value described by Friedrich and Holtz (2ng/ml) [9] and is reached on average around day 30 p.i.. The differences in cut-off values do arise from the use of different antisera against boPAG. Due to the high variability of the PAG in bovids, the antisera thus probably detect different kinds of epitopes. The overall sensitivity and specificity of the test is quite similar to those determined by others using boPAG serum assays [9, 22, 35, 36]. The PAG profile obtained in the presented Sandwich-ELISA shows a nearly equal pattern as observed by others [2, 10, 32, 37].

In our study, we found a linear decline in ln-serum boPAG concentration in the postpartum period. Following a first-order elimination pattern, the postpartum serum half-life of boPAG was estimated to be 6.4 days. These findings are in line with those reported by other working groups with described half-lives ranging from 4.3 days to 8.86 days [5, 10, 22, 38]. Pohler et al. [18] and Wallace et al. [5] put the differences of serum half-life down to the detection of different members of the boPAG family or the detection of differentially glycosylated variants of the same boPAG family instead of a change in half-life during the course of pregnancy. With our assay, we estimated that PAG in serum were detectable until 90 days postpartum. Therefore, it is no problem for farmers to breed cows again after 50–70 days in milk. The assay can be used from the fourth week after insemination onwards. Thus, PAG from an earlier pregnancy are not an issue for the test at this point of time.

Studies about PAG-concentration in milk of cattle throughout pregnancy are rare in literature. There are only a few assays described, which quantify boPAG in milk [9, 31, 32,

39]. All those assays used skimmed milk instead of whole milk. In some studies, PAG has been measured in unskimmed milk, but using a commercial test kit, which cannot quantify PAG concentrations in milk [11, 12, 19].

With aid of our newly developed Sandwich-ELISA boPAG-detection in whole milk is possible as early as day 26 p.i. At this time point, the concentration of boPAG in milk is 41.2 pg/ml. This is an earlier time point as described by Friedrich and Holtz [9], who detected boPAG in milk at day 60 p.i. and as described by Gajewski et al. [32], who could not detect boPAG in milk during the first 3 weeks of pregnancy. In the present study, the optimal threshold value for pregnancy testing in milk turned out to be 16.5 pg/ml. On average, this value was exceeded around day 40 p.i.. This value is much lower than that reported by Gajewski et al., [32] who proposed a value of 0.2 ng/ml. Metelo et al. [31] and Friedrich and Holtz [9] did not specify threshold values, because those concentrations cannot be used as reference for confirming pregnancies. González et al. [40] proposed a cut-off value of 1.6 ng/ml for pregnancy testing in goat milk. The test can be used as early as day 32 p.i.. However, PAG concentrations in goat milk are ten times higher than for cows [40] The overall sensitivity and specificity of the Sandwich- ELISA using milk was high with 95.3 % and 91.4 % respectively. As a result, the PPV in our experiment was high (96.8 %) compared with the NPV (87.6 %). The overall accuracy of the test was 94.3 %. These results agree with others who have conducted milk assays [11, 19, 32, 35, 39]. Leblanc [12] reported a very high sensitivity (99.2 %) and specificity (95.5 %) of pregnancy diagnosis with the commercial milk test, but he used the test for confirmation of pregnancy after day 60 p.i. in cows previously diagnosed as pregnant by rectal palpation. Therefore, the number of nonpregnant cows depends on the accuracy of the rectal palpation [12].

The boPAG profile obtained in the Sandwich-ELISA shows lower concentrations in milk compared with other assays used for boPAG quantification in milk [9, 31, 32, 39]. The obtained average boPAG levels rose from 23.6 pg/ml ± 6.1 pg/ml on day 30 p.i. to 381.6 pg/ ml ± 42.1 pg/ml on day 150 p.i. After day 150 p.i., a faster increase of boPAG levels was detectable until day 250 of pregnancy with an average concentration of 6.2 ng/ml. About day 200 p. i., the average concentration of PAG in milk exceeded 1.0 ng/ml. The average concentration of nonpregnant animals was

14.2 pg/ml ± 6.5 pg/ml. Friedrich [41] found a mean PAG-concentration of nonpregnant animals of 0.46 ± 0.10 ng/ml. In pregnant animals he described an average concentration of 0.67 ng/ml until day 100 p.i, a concentration of 0.83 ng/ml between day 100 p.i. and day 150 p.i. and a concentration of 1.28 ng/ml between day 150 p.i. and day 200 p.i. [41]. Before day 60 p.i., he could not determine concentrations above 1.0 ng/ml with the exception of two animals. In his study, he detected a rise in concentration at day 150 p.i., which is a similar date as found in our study [41]. Gajewski et al. [32] obtained a boPAG milk profile varying from 0.06 ng/ml in the 6th week of pregnancy, through 0.20 ng/ml on average on day 119 of pregnancy, 1.28 ng/ml on day 168 p.i., to 4.84 ng/ml on day 201 p.i.. Furthermore, in their study they found a similar surge of boPAG concentration in milk around day 150 of pregnancy. Overall, we can conclude from these results, that our test found nearly the same pattern of boPAG in milk but lower concentrations. This may be due to the fact, that we used whole milk instead of skimmed milk. Since PAG are water soluble and associated with the aqueous portion of the milk, fat in whole milk may act as a source of interference [9, 19, 40]. Nevertheless, our newly developed Sandwich-ELISA is sensitive enough to be a useful tool for pregnancy diagnosis in whole milk as early as day 40 p.i..

In our study, we found a linear decline in ln-milk PAG concentration in the postpartum period as already described for serum samples. Following a first-order elimination pattern, the postpartum half-life of PAG in milk was estimated to be 7.1 days, which is quite similar to the half-life found in serum and it seems that there is no difference in the elimination rate of boPAG in serum and milk. However, the only difference is the lower concentration of boPAG in milk compared to serum which makes them undetectable for other assays around day 30 postpartum [41, 42]. With our assay, we estimated that PAG were detectable until 60 days postpartum.

In a last step, we compared the serum and milk PAG concentration throughout pregnancy. We found a correlation of $r = 0.58$ ($P < 0.001$). The amount of PAG found in milk was 1.5 % of the amount of PAG present in serum, but the profiles were nearly parallel with exception of an aberration in late pregnancy. In literature information about the correlation between milk and blood PAG concentration is manifold ranging from 0.64 (whole milk and plasma of Holstein cows from 25 to 102 d in gestation [11]), 0.70

(skimmed milk and plasma of Holstein cows from 25 to 220 d in gestation [32]) and 0.79 (whole milk and plasma of Holstein cows from 23 d in gestation; [19]) to 0.81 (skimmed milk and serum of Holstein cows from day 51 to 250 in gestation [9]). Reasons for the relatively low correlation coefficient of our study in comparisons to the findings of the other studies are the individual variability of the boPAG concentration and the use of the more complex matrix whole milk instead of skimmed milk.

There are also different information in literature about the amount of PAG in milk compared to blood ranging from 0.6 % - 16.7 % in quantitative assays [9, 31, 32, 39] and 50 % in the commercial test kit [11, 19]. To some extent the amount fluctuates along the course of pregnancy [32, 41]. The reasons for those phenomena are multifactorial. BoPAG levels are negatively correlated with increased milk production [11]. Furthermore, the concentration of circulating PAG in blood or in milk is also influenced by other factors such as breed, body weight, parity status of the dam, foetal sex, foetal number and foetal birth weight [5]. The transport mechanisms of PAG from plasma across the mammary gland into milk are not fully understood until now. There are roughly 20 different boPAG members and related paralogs with a huge range in their temporal and spatial expression and glycosylation patterns during gestation [5, 7, 8]. Furthermore, it should be kept in mind, that every assay detects different members of the PAG family or differentially glycosylated variants of the same PAG with the result of often perplexing differences in studies [5]. Probably there is an influence of glycosylation on the transport mechanism and therefore not every PAG can be found in milk.

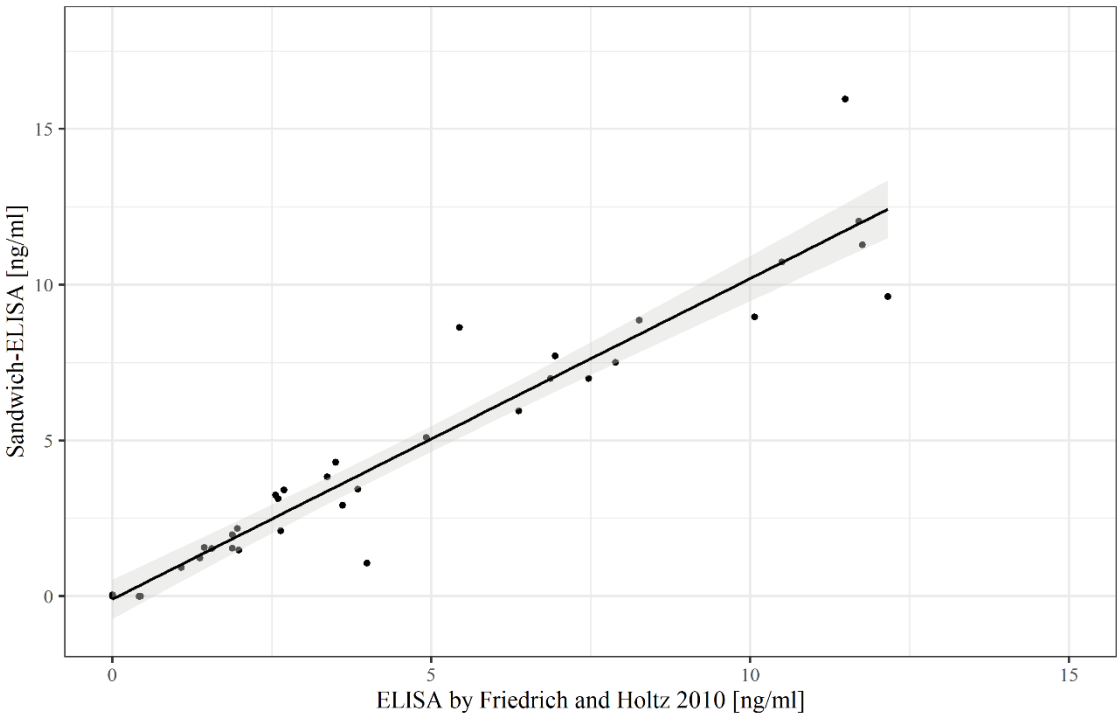
Conclusions

In conclusion, a new Sandwich-ELISA was developed for the detection of boPAG in serum and milk of pregnant cattle within one system. This is time saving for farmers and more efficient for laboratories. From fourth week after insemination onwards, the Sandwich-ELISA was able to identify a cow as being pregnant with high sensitivity (97 % in serum; 95 % in milk) and specificity (96 % in serum; 91 % in milk). The detected boPAG-profile

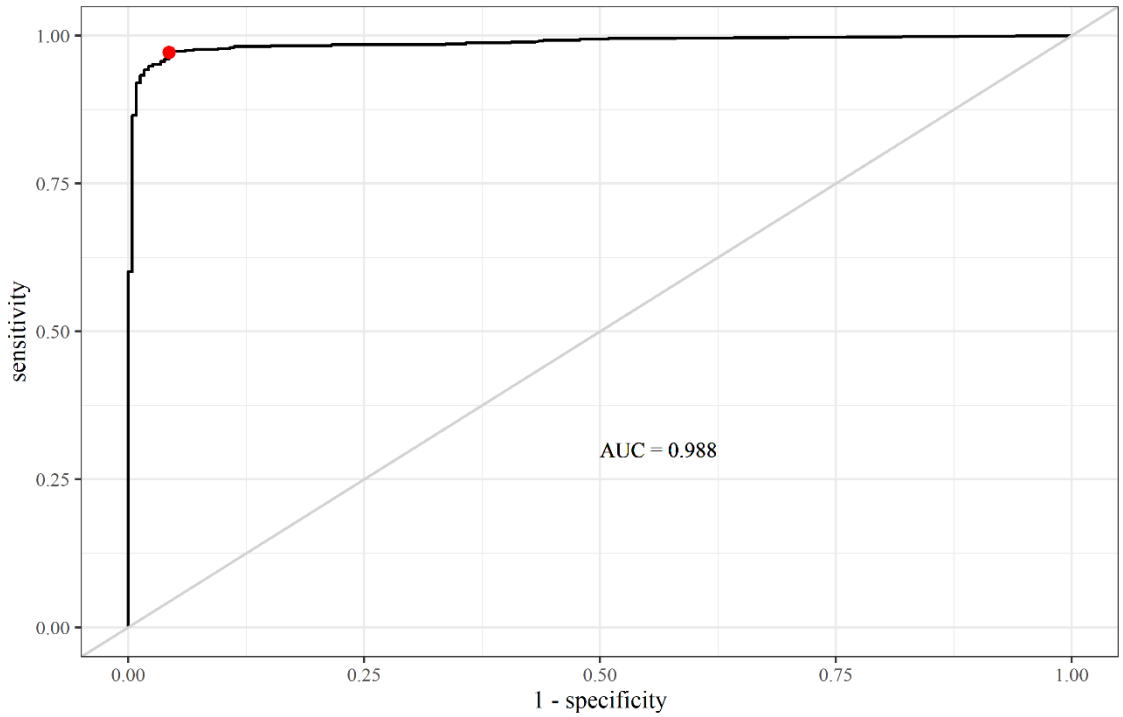
showed a typical pattern as described by other studies. With the possibility to measure boPAG concentration in whole milk, stressful effects during sampling (e.g. of venepuncture) are avoided and there is no need for special equipment or experience. Furthermore, to the best of our knowledge, only a few assays are described, which are able to quantify PAG in bovine milk and there is only one commercially available ELISA for detection of PAG in bovine milk which is designed as qualitative ELISA. The use of a quantitative assay has some advantages over a qualitative assay in research and clinical purposes. The quantification of an analyte gives more detailed information about the concentration range in different physiological states and over time (e.g. during pregnancy). Furthermore, it allows the comparison of concentrations between different individuals. This is not possible with qualitative or semi quantitative results.

For the reasons mentioned above, the described quantitative Sandwich-ELISA could be a very useful tool for pregnancy diagnosis in cattle.

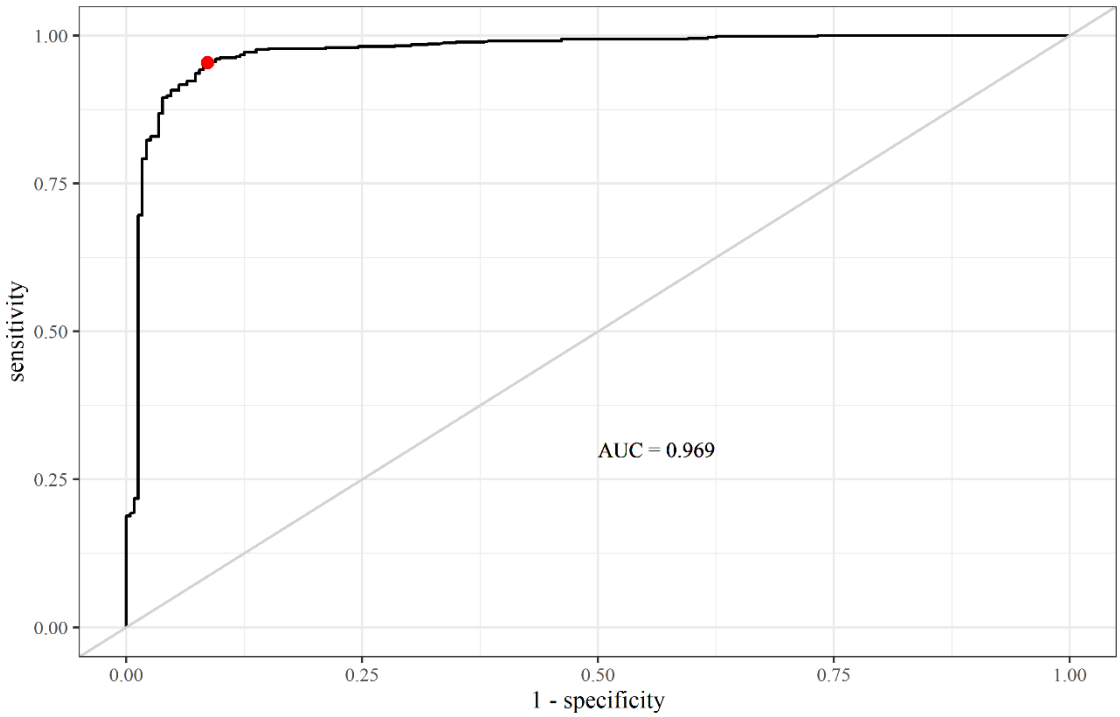
Supporting information



S1 Fig. Comparison of concentrations from 37 serum samples of pregnancy-associated glycoproteins between the new developed Sandwich-ELISA and the ELISA established by Friedrich and Holtz (2010) [9] after standardization. The correlation was estimated with a linear regression ($y = 1.017\ x$, $R^2 = 0.91$, $P < 0.001$).



S2 Fig. ROC curve to determine the optimal cutoff value for serum pregnancy testing. The Youden's index was used to find the best cutoff value that optimizes sensitivity and specificity (indicated by the red dot).



S3 Fig. ROC curve to determine the optimal cutoff value for milk pregnancy testing. The Youden's index was used to find the best cutoff value that optimizes sensitivity and specificity (indicated by the red dot).

S1 Table. Confusion matrix for evaluation of sensitivity, specificity, positive predictive value, negative predictive value, and accuracy in serum at a threshold value of 0.4 ng/ml.

Threshold 0.4 ng/ml			
PAG-ELISA	Pregnant	Non-Pregnant	Total ∑
Pregnant	651	17	688
Non-Pregnant	15	138	153
Total ∑	666	155	821

S2 Table. Confusion matrix for evaluation of sensitivity, specificity, positive predictive value, negative predictive value, and accuracy in serum at a threshold value of 1.0 ng/ml.

Threshold 1.0 ng/ml			
PAG-ELISA	Pregnant	Non-Pregnant	Total ∑
Pregnant	647	7	654
Non-Pregnant	19	148	167
Total ∑	666	155	821

S3 Table. Confusion matrix for evaluation of sensitivity, specificity, positive predictive value, negative predictive value, and accuracy in serum at a threshold value of 1.5 ng/ml.

Threshold 1.5 ng/ml			
PAG-ELISA	Pregnant	Non-Pregnant	Total ∑
Pregnant	626	2	628
Non-Pregnant	40	153	193
Total ∑	666	155	821

S4 Table. Confusion matrix for evaluation of sensitivity, specificity, positive predictive value, negative predictive value, and accuracy in serum at a threshold value of 2.0 ng/ml.

Threshold 2.0 ng/ml			
PAG-ELISA	Pregnant	Non-Pregnant	Total ∑
Pregnant	607	1	608
Non-Pregnant	59	154	213
Total ∑	666	155	821

S5 Table. Confusion matrix for evaluation of sensitivity, specificity, positive predictive value, negative predictive value, and accuracy in serum at a threshold value of 2.5 ng/ml.

Threshold 2.5 ng/ml			
PAG-ELISA	Pregnant	Non-Pregnant	Total ∑
Pregnant	589	1	590
Non-Pregnant	77	154	231
Total ∑	666	155	821

S6 Table. Confusion matrix for evaluation of sensitivity, specificity, positive predictive value, negative predictive value, and accuracy in milk at a threshold value of 0.01 ng/ml.

Threshold 0.01 ng/ml			
PAG-ELISA	Pregnant	Non-Pregnant	Total ∑
Pregnant	616	21	637
Non-Pregnant	17	121	138
Total ∑	633	142	775

S7 Table. Confusion matrix for evaluation of sensitivity, specificity, positive predictive value, negative predictive value, and accuracy in milk at a threshold value of 0.0165 ng/ml.

Threshold 0.0165 ng/ml			
PAG-ELISA	Pregnant	Non-Pregnant	Total ∑
Pregnant	603	12	615
Non-Pregnant	30	130	160
Total ∑	633	142	775

S8 Table. Confusion matrix for evaluation of sensitivity, specificity, positive predictive value, negative predictive value, and accuracy in milk at a threshold value of 0.02 ng/ml.

Threshold 0.02 ng/ml			
PAG-ELISA	Pregnant	Non-Pregnant	Total ∑
Pregnant	591	10	601
Non-Pregnant	42	132	174
Total ∑	633	142	775

S9 Table. Confusion matrix for evaluation of sensitivity, specificity, positive predictive value, negative predictive value, and accuracy in milk at a threshold value of 0.025 ng/ml.

Threshold 0.025 ng/ml			
PAG-ELISA	Pregnant	Non-Pregnant	Total ∑
Pregnant	575	7	582
Non-Pregnant	58	135	193
Total ∑	633	142	775

S10 Table. Confusion matrix for evaluation of sensitivity, specificity, positive predictive value, negative predictive value, and accuracy in milk at a threshold value of 0.16 ng/ml.

Threshold 0.16 ng/ml			
PAG-ELISA	Pregnant	Non-Pregnant	Total ∑
Pregnant	356	2	358
Non-Pregnant	277	140	417
Total ∑	633	142	775

Acknowledgements

The authors are deeply indebted to Julia Sondermann, Andrea Hartmann and Leonie Sterzl for the excellent technical assistance. We also thank Michael Baumann and farmers of the HVL e.V. for support in sample collection.

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Financial Disclosure

This work was supported by the H. Wilhelm Schaumann Stiftung, Hamburg, Germany (https://www.schaumann-stiftung.de/) in the form of funds awarded to TK, the Landwirtschaftliche Rentenbank, Frankfurt am Main, Germany (https:// www.rentenbank.de/) in the form of a grant and research materials awarded to TK, KM, SK, DL, MH, and JT (785745), TECOdevelopment GmbH (https://www.tecomedical.com/) in the form of salaries for DL and MH, and Hessischer Verband für Leistungs- und Qualitätsprüfungen in der Tierzucht e.V. (https://www.hvl-alsfeld.de/) in the form of salaries for SK and KM. The specific roles of these authors are articulated in the 'author contributions' section. The Open Access Publication Funds of the University of Goettingen were used to cover the publication costs. The funders of the study did not have any additional role in the study design, data collection and analysis, decision to publish, or preparation of the manuscript.

Competing Interest

The authors have read the journal's policy and have the following competing interests: Landwirtschaftliche Rentenbank provided a grant and research materials for the study.

MH and DL are employees of TECOdevelopment GmbH. KM and SH are employees of Hessischer Verband für Leistungs- und Qualitätsprüfungen in der Tierzucht e.V. This does not alter our adherence to PLOS ONE policies on sharing data and materials. There are no patents, products in development or marketed products associated with this research to declare.

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6 Diskussion

Die Biologie und Physiologie unserer Nutztiere sind zum großen Teil von der Domestikation und der selektiven Zucht beeinflusst. Die Reproduktionsphysiologie der Milchkühe hat sich in den letzten 50 Jahren geändert und einer der Hauptgründe ist die genetische Selektion auf eine steigende Milchproduktion (Lucy, 2001). Moderne Milchviehherden haben im Vergleich zur traditionellen Milchviehhaltung ein verlängertes Intervall bis zur ersten Ovulation, eine höhere Anzahl an unregelmäßigen Lutealphasen, niedrigere Serum-Progesteron- und IGF-1-Konzentrationen, häufigeres Auftreten von multiplen Ovulationen mit anschließenden Zwillingsträchtigkeiten und eine höhere Inzidenz an embryonalen Verlusten (Lucy, 2001). Ein Weg diese Probleme zu lösen, ist die bessere Erforschung der komplexen biologischen und physiologischen Mechanismen hinter den ökonomisch wichtigen Merkmalen. Ein verbessertes Wissen in diesem Bereich kann insgesamt dazu beitragen, eine nachhaltige Balance zwischen Produktivität, Produktqualität und Tierwohl zu schaffen (Bendixen *et al.*, 2011).

In den letzten Jahrzehnten hat sich vor allem die Transkriptomik als vielversprechender Ansatz in der Charakterisierung von biologischen Prozessen herausgestellt (Young, 2000; Tian *et al.*, 2004). Häufig werden mRNA-Konzentrationen als stellvertretende Größe für die korrespondieren Proteinkonzentrationen genutzt. Dabei ist der Grundgedanke, dass die Transkriptabundanz die Hauptdeterminante für die Proteinabundanz darstellt (Maier *et al.*, 2009; Ghazalpour *et al.*, 2011; Vogel & Marcotte, 2012). Die rasanten technischen Fortschritte im Bereich der Massenspektrometrie - und die damit einhergehende Entwicklung neuer Messmethoden - haben zu einer besseren Charakterisierung der proteomischen Komponenten in biologischen Systemen geführt (Cravatt *et al.*, 2007; Vogel & Marcotte, 2012). Dabei konnte auch festgestellt werden, dass zwar generell ein Zusammenhang zwischen Proteinkonzentration und mRNA-Abundanz besteht, dieser in der Regel aber mäßig bis schlecht ausgeprägt ist ($R^2 \approx 0.4$) (Tian *et al.*, 2004; de Sousa Abreu *et al.*, 2009; Vogel & Marcotte, 2012). Transkriptomik und Proteomik liefern komplementäre Informationen und sind somit sehr gut dafür geeignet, einen besseren Einblick in komplexe biologische und physiologische Vorgänge zu erhalten (Tian *et al.*, 2004; Maier *et al.*, 2009; Ghazalpour *et al.*, 2011; Almeida *et al.*, 2014).

Bis heute gibt es nur wenige detaillierte Studien über die bovine PAG-Expression und die Verteilung der Proteine über den gesamten Verlauf der Trächtigkeit. PAGs können neben ihrer Funktion als wichtiger Trächtigkeitsmarker in der modernen Milchviehhaltung auch als Indikator für die Lebensfähigkeit des Embryos und einer normalen Plazentafunktion dienen (Wallace *et al.*, 2015). Dadurch kann ein besseres Verständnis über die Verteilung dieser Proteingruppe während der Trächtigkeit zu wichtigen Erkenntnissen beitragen, welche die Reproduktionseffizienz in der Rinderhaltung verbessert.

Aus diesen Gründen, war das Ziel der vorliegenden Arbeit, trächtigkeitsassoziierte Glykoproteine molekulargenetisch und proteinanalytisch zu charakterisieren. Zusätzlich konnten diese Erkenntnisse dazu beitragen, einen neunen Sandwich-ELISA zu etablieren, der die simultane Quantifizierung von boPAG in Blut und Milch ermöglicht.

Für die Expressionsanalysen boviner PAG-Gene (Kapitel 3) wurden Kotyledonen auf einem kommerziellen Schlachthof gesammelt (Wiedemann *et al.*, 2018). Im Gegensatz zu bisherigen Studien (Patel *et al.*, 2004b; Telugu *et al.*, 2009; Touzard *et al.*, 2013) erfolgte dabei die Probennahme kontinuierlich über den gesamten Trächtigkeitszeitraum hinweg und war nicht auf bestimmte Trächtigkeitstage beschränkt. Insgesamt konnten Kotyledonen von 23 tragenden Kühen gesammelt werden, die einen Trächtigkeitszeitraum zwischen 28 und 192 Tagen nach erfolgter Besamung abdeckten (Wiedemann *et al.*, 2018). Dadurch war es uns möglich, einen detaillierten Einblick in die boPAG-Expression, insbesondere während der frühen und mittleren Trächtigkeitsphase, zu erlangen (Wiedemann *et al.*, 2018). Dieser Bereich ist mit Hinblick auf weiterführende Forschungsfragen besonders wichtig. Zum einen können boPAGs, die voranging während der frühen Trächtigkeit exprimiert werden, mögliche Kandidaten für einen verbesserten Trächtigkeitstest sein (Green *et al.*, 2005), zum anderen konnten die größten Änderungen in der Glykosylierung der boPAGs zu Beginn und zum Ende einer Trächtigkeit beobachtet werden (Klisch *et al.*, 2008).

Die späte Trächtigkeit konnte in dieser Studie vor allem aus zwei Gründen nicht besser abgedeckt werden: zum einen trat während der Probensammlung das Verbot zur Schlachtung tragender Tiere im letzten Trächtigkeitsdrittel in Kraft (TierErzHaVerbG). Zum anderen haben die Probensammlung und Präparation der Kotyledonen im letzten

Trächtigkeitsdrittel aufgrund des Größenwachstums und der stärkeren Verbindung mit dem maternalen Gewebe mehr Zeit in Anspruch genommen (Wiedemann, 2018; Wiedemann *et al.*, 2018). Das hatte zur Folge, dass Proben und biologische Replikate, besonders aus diesem Zeitraum, aufgrund unzureichender Qualität von der weiteren Analyse ausgeschlossen wurden (Wiedemann, 2018; Wiedemann *et al.*, 2018).

Trotz dieser Einschränkungen decken sich die Ergebnisse der Untersuchung mit bereits vorhanden Studien (Patel *et al.*, 2004b; Telugu *et al.*, 2009; Touzard *et al.*, 2013). Zusätzlich konnte durch das kontinuierliche Probenintervall die Datenlage während der frühen und mittleren Trächtigkeit ergänzt werden. Insbesondere während der frühen Phase (Zeitpunkt der Plazentation, Änderung in der Glykosylierungsstruktur der boPAGs) können die Expressionsprofile eine wichtige Grundlage für den Abgleich mit proteomischen Daten darstellen und somit dazu beitragen, neue Erkenntnisse über die Funktionen der boPAGs innerhalb dieses Zeitraumes zu gewinnen.

Im Gegensatz zu Transkriptomanalysen, in denen die mRNA-Expression diverser boPAGs untersucht wurden (Xie *et al.*, 1997; Green *et al.*, 2000; Patel *et al.*, 2004b; Telugu *et al.*, 2009; Touzard *et al.*, 2013; Wiedemann *et al.*, 2018), ist die Informationslage zu boPAGs auf Proteinebene deutlich schlechter (Klisch *et al.*, 2005). Zoli *et al.* (1991) und Sousa *et al.* (2002) konnten durch eine NH_2- terminale Mikrosequenz Analyse mittels Edman-Abbau boPAG-1 in fetalen Kotyledonen von Rind (Zoli *et al.*, 1991) und Zebu (Sousa *et al.*, 2002) nachweisen. Erste Studien, die verschiedene boPAGs mittels Massenspektrometrie identifizierten, wurden im Jahr 2005 publiziert (Green *et al.*, 2005; Klisch *et al.*, 2005). Beide Arbeitsgruppen nutzten dabei die Peptidmassenfingerprint-Methode (PMF) (Henzel *et al.*, 1993; James *et al.*, 1993; Mann *et al.*, 1993; Pappin *et al.*, 1993; Yates *et al.*, 1993). Green *et al.* (2005) identifizierten dabei boPAG-1, -4, -6, -7, -16, -17, -20, -21 in zwei Kotyledonenextrakten 95 und 150 Tage alter Feten. Im Gegensatz dazu konnten Klisch *et al.* (2005) aus Kotyledonen der mittleren Trächtigkeit (100, 155, und 180 Tage alte Feten) lediglich boPAG-1, -6, -7 und -17 nachweisen. Die PMF-Methode benötigt stark aufgereinigte Proteine. Verunreinigungen oder komplexe Proben (mehr als 2 Proteine) können dazu führen, dass die Proteine nur mit großer Unsicherheit oder gar nicht identifiziert werden (Jensen *et al.*, 1997; Aebersold & Mann, 2003; Wright *et al.*, 2012).

Diese Einschränkungen können mit neueren Verfahren der gezielten Proteomanalyse (Targeted Proteomics) umgangen werden (Aebersold *et al.*, 2016). Diese sind weit weniger durch eine hohe Probenkomplexität und Probenhintergrund beeinträchtigt (Domon & Aebersold, 2010; Gallien *et al.*, 2012; Peterson *et al.*, 2012). Das grundsätzliche Prinzip beruht dabei auf der selektiven Messung von proteotypischen Peptiden (stellvertretend für die Proteine) in einem definierten Retentionszeitfenster und *m/z*-Bereich (Makawita & Diamandis, 2010; Gallien *et al.*, 2012; Rauniyar, 2015).

Ein Verfahren der gezielten Proteomanalyse ist das 2012 erstmals beschriebene PRM (Peterson *et al.*, 2012). Beim PRM werden alle Produkt-Ionen des proteotypischen Peptides (beim "Single Reaction Monitoring" (SRM) lediglich 3-5 vorausgewählte Transitions (Peterson *et al.*, 2012; Rauniyar *et al.*, 2017)) mit einer hohen Auflösung und genauen Massenbestimmung simultan erfasst. Dies führt dazu, dass die Identität des gefundenen Peptides sicherer bestimmt werden kann und man keine vorherige Auswahl der Transitions treffen muss. Zusätzlich gibt es eine höhere Toleranz gegenüber co-isolierten Hintergrundpeptiden oder anderen Störeffekten. Durch die hohe Auflösung der Messung und das Erfassen aller Produkt-Ionen können diese „Störfaktoren“ besser identifiziert und herausgefiltert werden (Peterson *et al.*, 2012; Schilling *et al.*, 2015; Thomas *et al.*, 2015; Rauniyar *et al.*, 2017).

Daher war es das Ziel im zweiten Teil der hier vorliegenden Arbeit, einen PRM-Assay für boPAGs zu etablieren und zu validieren. Durch die Entwicklung und Vorteile der oben beschriebenen Methoden ist es nun möglich, die bis dato vorhandenen Erkenntnisse vorangegangener proteinanalytischer Studien (Sousa *et al.*, 2002; Green *et al.*, 2005; Klisch *et al.*, 2005) zu boPAGs zu erweiterten. Dadurch soll auch ein besserer Abgleich mit den vorhanden transkriptomischen Daten ermöglicht werden.

Mit dem in dieser Arbeit etablierten PRM-Assay (Kapitel 4) ist es möglich, 18 verschiedene boPAGs über den gesamten Verlauf der Trächtigkeit und nach der Geburt zu detektieren.

Als Ergebnis der Messungen konnten die relativen Proteinabundanzen von 18 verschiedenen boPAGs in den untersuchten Trächtigkeitsstadien und in der Nachgeburt bestimmt werden. Zusätzlich wurde ein Einfluss der Glykosylierung gefunden. Basierend

auf den gewonnenen Erkenntnissen wurden die boPAGs in zwei verschiedene Gruppen eingeteilt. Die erste Gruppe (boPAG-3, -5, -6, -7, -8, -9, -15, -21) zeigte dabei fast keine Änderungen der gemessenen Abundanzen zwischen glykosylierten und deglykosylierten Proben. Die zweite Gruppe (boPAG-1, -2, -4, -10, -11, -14, -16, -17, -20) hingegen zeigte eine Zunahme der gemessenen Abundanzen von bis zu 205,6 % in deglykosylierten Proben. Diese Ergebnisse lassen vermuten, dass die zweite Gruppe der boPAGs deutlich stärker während der Trächtigkeit glykosyliert ist als die erste.

Die relativen Proteinabundanzen der 18 boPAGs und der gefundene Einfluss der Glykosylierung lassen nun erstmals eine Zuordnung verschiedener boPAGs zu den vermuteten Funktionen dieser Proteingruppe zu. BoPAGs der zweiten Gruppe, mit einer hohen Abundanz während der frühen Trächtigkeitsphase, könnten eine wichtige Rolle während der Plazentation spielen. N-Glykane dieser boPAGs könnten von Lektinen gebunden werden (Wooding *et al.*, 2005; Wallace *et al.*, 2015). Lektine sind bi- oder polyvalent kohlenhydratbindende Proteine (Goldstein *et al.*, 1980; Barondes, 1981, 1984) welche membranständig (z. B. Selektin) oder frei gelöst (z. B. Galektin) (Kaltner & Stierstorfer, 1998) in vielen verschieden Organismen zu finden sind (Goldstein *et al.*, 1980; Barondes, 1981; Lis & Sharon, 1986). Aufgrund ihrer Eigenschaften könnten Lektine spezifische Glykanmotive der boPAGs erkennen und binden, wodurch sie einen wichtigen Beitrag zur Adhäsion zwischen maternalem Endometrium und fetalem Allantochorion leisten (Clark, 2015; da Anunciação *et al.*, 2017; Wawrzykowski *et al.*, 2019). Eine Veränderung der Glykanstruktur von boPAGs zum Zeitpunkt der Geburt (Klisch *et al.*, 2006, 2008) könnte dann wiederum eine entscheidende Rolle beim Lösen dieser Verbindung spielen (Clark, 2015; Wawrzykowski *et al.*, 2019). Veränderungen bei diesen physiologischen Abläufen (z. B. Abnormitäten in den Glykanstrukturen) werden als Gründe für Funktionsstörungen (z. B. *Retentio secundinarum*) vermutet (Clark, 2015; da Anunciação *et al.*, 2017; Hooshmandabbasi *et al.*, 2018; Wawrzykowski *et al.*, 2019). Weiterhin könnten die boPAGs, egal ob sie enzymatisch aktiv sind oder nicht, mithilfe ihres katalytischen Zentrums andere Proteine (z. B. Integrine) binden und so einen wichtigen Beitrag bei der Kontaktaufnahme zwischen Muttertier und Fetus leisten (Wooding *et al.*, 2005; Wallace *et al.*, 2015). Des Weiteren sind die boPAGs der zweiten Gruppe, mit einer hohen Abundanz während der mittleren Trächtigkeit, vermutlich wichtige Faktoren für die

Aufrechterhaltung einer Trächtigkeit. Diese Rolle können sie indirekt, über die Regulation der Prostaglandin- und Progesteronsynthese, ausüben (Del Vecchio *et al.*, 1996; Weems *et al.*, 1998a; 1998b; Wooding *et al.*, 2005; Wallace *et al.*, 2015).

Viele boPAGs der zweiten Gruppe haben hohe oder maximale Proteinabundanzen in der späten Trächtigkeitsphase und nach der Geburt. Innerhalb dieser Stadien wurden im Vergleich zur frühen und mittleren Trächtigkeit zudem die größten Unterschiede zwischen glykosylierten und deglykosylierten Proben festgestellt. In diesen Phasen der Gestation könnten die von Wooding *et al.* (2005), Klisch *et al.* (2008) und Wallace *et al.* (2015) postulierten immunmodulatorischen Funktionen der boPAGs und ihrer Glykanstrukturen eine entscheidende Rolle spielen. Hierbei wird von verschiedenen Autoren eine immunsuppressive Funktion diskutiert (Klisch *et al.*, 2006, 2008; Wallace *et al.*, 2015; Hooshmandabbasi *et al.*, 2018). Die verschiedenen boPAGs können dann im Zusammenspiel mit Progesteron wirken. Die Immunsuppression während der Trächtigkeit ist vermutlich ein wichtiger Mechanismus, um eine immunologische Rejektion des Fetus durch das Muttertier zu verhindern (Weinberg, 1987; Goff & Horst, 1997; Hansen, 2013). Somit sind boPAGs auch eine mögliche Erklärung für die nachgewiesene Immunsuppression kurz vor der Geburt, da die Progesteronkonzentration im peripartalen Zeitraum abnimmt (Goff & Horst, 1997; Hansen, 2013). Neben dieser immunsuppressiven Einflussnahme einiger boPAGs, könnte im Gegensatz dazu bei anderen eine inflammatorische Wirkung bestehen. Es wird davon ausgegangen, dass boPAGs diese Wirkung weniger über einen klassischen Rezeptor vermitteln, vielmehr scheint es, dass einige von ihnen eventuell über ihre noch vorhandene proteolytische Aktivität Liganden erzeugen, welche anschließend die Signalwirkung vermitteln. Auch die direkte Aktivierung von Rezeptoren über eine proteolytische Abspaltung ist möglich (Wallace *et al.*, 2015). Eine Gruppe G-Protein-gekoppelter Rezeptoren, die für einen solchen Wirkmechanismus in Frage kommen könnte, sind Protease-aktivierte Rezeptoren (PARs). Sie spielen sowohl bei anti- als auch proinflammatorischen Prozessen in verschiedenen Organsystemen eine Rolle (Russell & McDougall, 2009; Russell *et al.*, 2010; Fu *et al.*, 2015). Da boPAGs auch die Prostaglandin E_2-Synthese fördern (Weems *et al.*, 1998a, 2003; Thompson *et al.*, 2012), ist auch darüber eine immunsuppressive oder proinflammatorische Wirkung möglich.

BoPAG-10, welches vor allem in der Nachgeburtsprobe vorhanden war, zeigte einen der auffälligsten Verläufe während der Trächtigkeit. Da boPAG-10 zu der phylogenetisch älteren Gruppe gehört, kann es durch seine potenzielle proteolytische Aktivität eine besondere Bedeutung bei der Geburt haben. Ein Abfall des pH-Wertes an der fetomaternalen Schnittstelle zum Zeitpunkt der Geburt könnte die proteolytische Aktivität von boPAG-10 fördern, welches infolgedessen das Lösen der fetalen Kotyledonen vom maternalen Gewebe bewirkt (Wooding *et al.*, 2005; Wallace *et al.*, 2015).

Viele der genannten möglichen Aufgaben der boPAGs deuten auf wichtige Funktionen der Glykanstrukturen hin. In unserer Untersuchung konnten wir jedoch eine Gruppe von boAPGs identifizieren, welche scheinbar wenig bis gar nicht glykosyliert sind. Für diese erste Gruppe erscheint daher eine Sezernierung in das maternale System als eher unwahrscheinlich. Die Glykosylierung von Proteinen ist ein wichtiger Faktor, welcher die Proteinhalbwertszeit im Serum reguliert und die Proteine vor Proteolyse schützt (Klisch *et al.*, 2006, 2008; Mitra *et al.*, 2006; Russell *et al.*, 2009; Goettig, 2016). Daher ist ein parakriner oder autokriner Wirkmechanismus wahrscheinlich. Mit der Ausnahme von boPAG-8, gehören alle boPAGs in der ersten Gruppe (mit geringen Unterschieden zwischen glykosylierten und deglykosylierten Proben) zu den modernen PAGs welche vermutlich keine proteolytische Aktivität aufweisen (Telugu *et al.*, 2009). Mithilfe ihres katalytischen Zentrums könnten sie dennoch als Transporter fungieren oder an spezifische Rezeptoren binden (Green *et al.*, 1998). Während der frühen Trächtigkeitsphase können sie als Brückenmoleküle fungieren und somit eine wichtige Rolle während der Plazentation spielen (Green *et al.*, 1998; Wooding *et al.*, 2005; Wallace *et al.*, 2015).

Zusammenfassend liefern die Ergebnisse dieser Studie eine wichtige Basis für die zukünftige boPAG-Forschung. Weiterführende Untersuchungen mittels PRM-Assay in anderen Geweben (z. B. im interkotyledonären Raum, den Karunkeln etc.) und im peripheren maternalen System (Serum und Milch) können zu einem detaillierten Überblick über die Verteilung der verschiedenen boPAGs beitragen. Diese Untersuchungen sind wichtige Bausteine, um ein besseres Verständnis der Glykosylierungsmechanismen und der genauen Funktionen der einzelnen boPAGs zu erlangen.

Mithilfe der Ergebnisse aus den ersten beiden Studien dieser Arbeit (Kapitel 3 und Kapitel 4) ist es möglich die Korrelation zwischen mRNA-Expression und relativer Proteinabundanz zu berechnen. Dafür wurden zunächst die mRNA-Expressionen der einzelnen boPAGs über den Zeitraum der untersuchten Trächtigkeitsphasen in der PRM-Studie gemittelt. Da keine Nachgeburtsproben in der RNA-Studie untersucht wurden, sind diese von proteinanalytischer Seite auch nicht mit in die Korrelationsrechnung eingegangen. Der umgekehrte Fall gilt für boPAG-12, für das zwar mRNA-Expressionsprofile vorliegen, aber keine Proteinabundanzen.

Zwischen der mRNA-Expression und boPAG-Leveln findet man Pearsons-Korrelationskoeffizienten von $r = 0{,}17$ ($p > 0{,}05$; glykosylierte Proben) bzw. $r = 0{,}2$ ($p > 0{,}05$; deglykosylierte Proben). Beide Werte untereinander sind deutlich niedriger als der in der Literatur beschriebene mittlere Wert von $R^2 = 0{,}4$ (Tian *et al.*, 2004; de Sousa Abreu *et al.*, 2009; Vogel & Marcotte, 2012). Dieser Unterschied zu dem über viele Studien festgestellten Durchschnittswert, lässt sich durch technische und biologische Gründe erklären. Ein wichtiger Punkt ist hierbei bereits die Art der Probengewinnung. In beiden hier vorgestellten Studien wurden die Proben auf einem kommerziellen Schlachthof gesammelt. Vom Tod des einzelnen Tieres bis zur Probensammlung vergingen durchschnittlich 20 - 30 min. Obwohl direkt eine fachgerechte Konservierung und Lagerung erfolgte, kann dieser Zeitraum einen nur schwer determinierbaren Einfluss auf die Probenqualität haben. Fast alle bekannten Studien, die den Zusammenhang zwischen mRNA-Expression und Proteinabundanz untersucht haben, nutzten *in vitro* Systeme (Nie *et al.*, 2006; de Sousa Abreu *et al.*, 2009; Vogel *et al.*, 2010; Maier *et al.*, 2011; Schwanhäusser *et al.*, 2011) oder gängige Tiermodelle (Ghazalpour *et al.*, 2011), wodurch Umwelteinflüsse minimiert werden können. Ein weiterer wichtiger technischer Einflussfaktor, ist die hier vorliegende Analyse unterschiedlicher Proben. Zwar handelt es sich bei beiden Studien um Gewebeproben von Kotyledonen aus den gleichen Trächtigkeitsstadien, jedoch stammen diese Proben von unterschiedlichen Tieren. Zudem erfolgten die Analysen zu unterschiedlichen Zeitpunkten. Als letzter technischer Faktor bleibt die Messungenauigkeit der verwendeten Methoden. Sie kann über 30 % Variation in der RNA-Protein-Korrelation ausmachen (Nie *et al.*, 2006), wobei die Messung der Proteinlevel einen höheren Anteil hat, als die der mRNA-Level (Nie *et al.*, 2006; Vogel *et al.*, 2010; Ghazalpour

et al., 2011; Vogel & Marcotte, 2012). Mithilfe von verschieden Markierungstechniken zur absoluten Quantifizierung von Proteinleveln können diese Variationen dennoch auf einem sehr niedrigen Niveau gehalten werden und Vergleichsmessungen zwischen verschieden Replikaten zeigen hohe Übereinstimmungen (Vogel *et al.*, 2010; Ghazalpour *et al.*, 2011; Schwanhäusser *et al.*, 2011).

Die genannten technischen Faktoren, welche die Korrelation zwischen mRNA-Leveln und Proteinabundanz beeinflussen können, lassen sich in zukünftigen Studien berücksichtigen. Eine absolute Quantifizierung der verschiedenen boPAGs, beispielsweise mit Markierungstechniken (z. B. stabile Isotope) (Domon & Aebersold, 2010; Gallien *et al.*, 2012; Peterson *et al.*, 2012; Schilling *et al.*, 2015), ist in unseren PRM-Assay integrierbar. Um den Einfluss der Probensammlung zu minimieren und die Probenqualität zu verbessern, würde sich eine Trophoblastenzellkultur anbieten (Green *et al.*, 2005), welche, vor Ort etabliert, lange Transportwege vermeidet.

Weiterhin müssen auch biologische Prozesse in die Betrachtung miteinbezogen werden. Um die Genexpression der boPAGs und damit auch ihre Rolle im reproduktionsphysiologischen Kontext besser zu verstehen, sollten vier fundamentale zelluläre Prozesse Berücksichtigung finden: die Transkription, die mRNA-Degeneration, die Translation und die Proteindegeneration (Maier *et al.*, 2009; Vogel *et al.*, 2010; Schwanhäusser *et al.*, 2011). Dabei haben diese genannten Prozesse keinen einfachen linearen Zusammenhang. Sie sind eng miteinander verbunden und können sich gegenseitig über Feedbackmechanismen regulieren. Cis- und trans-wirkende Elemente bilden dabei ein großes Repertoire von Systemen, welche die Synthese von Proteinen verstärken oder hemmen können (Maier *et al.*, 2009; Vogel *et al.*, 2010; Schwanhäusser *et al.*, 2011; Vogel & Marcotte, 2012). Verschiedene Studien haben gezeigt, dass nicht allein die Transkription Hauptdeterminante der Proteinabundanz ist. Es hat sich herausgestellt, dass mindestens zu einem gleichen Teil posttranskriptionelle Faktoren, wie die Translation und die Proteindegradation, wichtige Rollen spielen (Vogel *et al.*, 2010; Schwanhäusser *et al.*, 2011; Vogel & Marcotte, 2012). Hierbei hat sich auch gezeigt, dass spezifische Kombinationen von mRNA- und Proteinstabilität mit verschieden biologischen Funktionen einhergehen (Nie *et al.*, 2006; Schwanhäusser *et al.*, 2011). Gene mit stabilen mRNAs und instabilen Proteinen kodieren häufig extrazelluläre Proteine. Das ist in gewisser Weise

zu erwarten, da Proteine die aus der Zelle sekretiert werden, in der Regel eine kurze zelluläre Halbwertszeit haben (Schwanhäusser *et al.*, 2011). Ein Teil der boPAGs könnte in diese Gruppe fallen. Die starke posttranslationale Modifikation einiger boPAGs könnte dann auch ein möglicher Versuch der Zellen sein, die extrazelluläre Halbwertszeit zu verlängern.

Insgesamt wäre eine Erhebung dieser vielfältigen biologischen Daten für die boPAGs interessant. Zum einen, weil es eine phylogenetische Aufteilung dieser Glykoproteinfamilie gibt und zum anderen, weil sie unterschiedlichste biologische Funktionen aufzuweisen scheint, die sowohl intrazellulär als auch extrazellulär ihre Wirkung entfalten. Welche Rolle die Glykosylierung dabei spielt, sollte ebenfalls mit einbezogen werden. Alle Daten könnten dann, zusammen mit einer multiplen Regressionsanalyse, unter Umständen einen Großteil der boPAG-Abundanz erklären und somit einen wichtigen Beitrag zu Erforschung dieser Proteinfamilie leisten. Für solch einen systembiologischen Ansatz liefern die ersten beiden Studien in dieser Arbeit (Kapitel 3 und Kapitel 4) einen wichtigen Grundbaustein. Ihre Ergebnisse geben jeweils für sich, als auch gemeinsam betrachtet, wichtige Hinweise zur temporalen Verteilung, zu biochemischen Eigenschaften und möglichen Funktionen der boPAGs.

Die letzte Studie der hier vorliegenden Arbeit (Kapitel 5) beschreibt die Etablierung und Validierung eines neuen Sandwich-ELISA zur parallelen Quantifizierung von boPAGs in Serum und Vollmilch von tragenden Tieren. Für die Immunisierung und Erzeugung der polyklonalen Antiseren wurden dieselben aufgereinigten Homogenisate verwendet, deren boPAG-Zusammensetzungen auch durch die PRM-Studie (Kapitel 4) bestimmt worden sind. Damit ist auch gewährleistet, dass zu einem großen Teil bekannt ist, gegen welche boPAGs die erzeugten Antiseren gerichtet sind. Eine zu 100 % sichere Aussage kann an dieser Stelle nicht getroffen werden. Das hat zwei Gründe: zum einen war es nicht möglich den PRM-Assay für alle rund 20 bekannten boPAGs zu etablieren, da aufgrund der Auswahlkriterien nicht für jedes boPAG repräsentative Peptide ermittelt werden konnten. Zum anderen ist nicht bekannt, ob weitere boPAGs existieren, die bislang nicht beschrieben sind. Verfahren der gezielten Proteomanalyse sind in der Regel hypothesen-basierte Verfahren. Sie können somit keine neuen Proteine in einer Probe entdecken (Domon & Aebersold, 2010).

Bei der Testetablierung stellten sich zwei Antiseren, welche aus Homogenisaten der späten Trächtigkeit generiert worden sind, als beste Kombination für den Sandwich-ELISA heraus. Diese Antikörperkombination zeigte die besten Ergebnisse in der Differenzierung zwischen tragenden und nicht-tragenden Tieren sowie eine hochspezifische boPAG-Bindung bei einem niedrigen Hintergrundsignal. Die möglichen Gründe dafür sind vielfältig. Die Anzahl der Planzentome und ihre Größe nehmen während der Trächtigkeit stark zu (Schlafer *et al.*, 2000; Estrella *et al.*, 2017; Wawrzykowski *et al.*, 2019). Hinzu kommt, dass gerade in der letzten Phase der Trächtigkeit die Granula der BNCs mit boPAGs angereichert sind (Schlafer *et al.*, 2000; Wooding *et al.*, 2005). Dadurch spricht vieles dafür, dass die Homogenisate aus diesen Trächtigkeitsstadien einen besonders hohen Anteil an boPAGs aufweisen und infolgedessen nach der Immunisierung der Kaninchen spezifischere Antiseren produzieren. Zusätzlich muss der individuelle Tiereffekt bei der polyklonalen Antiseren-Produktion berücksichtigt werden (Liddell, 2005). Die boPAG-Affinität der Antiseren ist für jedes immunisierte Kaninchen unterschiedlich. Das liegt an der individuellen Immunantwort und an bereits vor der Immunisierung vorhandenen Hintergrundantikörpern (Liddell, 2005). Da die PRM-Studie gezeigt hat, dass es innerhalb der einzelnen boPAGs keinen signifikanten Einfluss des Trächtigkeitsstadiums gibt, ist es daher auch nicht nötig ein spezifisches Antiserum zu verwenden, welches gegen spezielle boPAGs eines bestimmten Trächtigkeitsstadiums gerichtet ist. Der so etablierte Sandwich-ELISA liefert mit der Literatur vergleichbare Ergebnisse und bietet Vorteile für die Landwirte und Tiere (Entnahme von einer Vollmilchprobe vs. Entnahme einer Blutprobe) sowie für die Labore, die den Test durchführen. Durch die parallele Quantifizierung wird nur noch ein Testkit benötigt, um beide Probentypen zu untersuchen.

Zusammen mit dem hier beschriebenen PRM-Assay (Kapitel 4) kann dieser neue Sandwich-ELISA auch weitere Vorteile für die Landwirte und die Forschung bieten. Neben der frühen und zuverlässigen Trächtigkeitsdiagnose, welche eine Alternative gegenüber dem Progesteronnachweis oder der transrektalen Sonografie darstellt, könnte auch eine Aussage über den möglichen Fortgang einer Trächtigkeit getroffen werden. In verschieden Studien an Milch- und Fleischrindern konnte gezeigt werden, dass Tiere mit einem Abort in der späten embryonalen und frühen fetalen Phase einen signifikant niedrigeren

boPAG-Wert zwischen dem 25. und 32. Trächtigkeitstag aufwiesen, als Tiere, die eine Trächtigkeit bis zum Schluss ausgetragen haben (Breukelman *et al.*, 2012; Pohler *et al.*, 2013, 2016). Breukelman *et al.* (2012) konnten vier verschiedene boPAG-Profile in der frühen Trächtigkeit bei Tieren mit einem späteren Abort identifizieren. Anhand dieser unterschiedlichen Profile könnte es möglich sein, den Grund der Aborte (maternal oder embryonal) herauszufiltern (Breukelman *et al.*, 2012): eine physiologische boPAG-Konzentration zum Beginn einer Trächtigkeit mit einer nachfolgenden Abnahme könnte ein Indiz für einen abrupten Abbruch der Implantation darstellen. Diese Art des Aborts hat vermutlich maternale Gründe, wie beispielsweise eine unzureichende Vorbereitung des Endometriums für die Aufnahme eines Fetus. Eine pathologische Embryonalentwicklung aufgrund einer verzögerten Plazentation könnte mit einer verspäteten Detektion von boPAG-Konzentrationen einhergehen. Einen ähnlichen kausalen Zusammenhang kann man zwischen humanen Choriongonadotropin-Werten und nachgewiesenen Aborten bei Frauen finden (Lenton *et al.*, 1988; Macklon *et al.*, 2002; Breukelman *et al.*, 2012).

Anhand dieser Beispiele wird deutlich, dass bovine PAGs eine wichtige Funktion in der Diagnose und frühzeitigen Erkennung von pathophysiologischen Veränderungen an der Plazenta haben. Die frühzeitige Detektion eines möglichen Aborts hat auch einen erheblichen ökonomischen Wert, vor allem in der Milchviehhaltung (Lucy, 2001; Heyman *et al.*, 2002; Silke *et al.*, 2002; Pohler *et al.*, 2016). Alle genannten Ergebnisse, die den Zusammenhang zwischen boPAG-Konzentration und möglichem Trächtigkeitsausgang beschreiben, beruhen auf der Analyse von Blutproben mittels RIA oder ELISA. Ob sich der gleiche Effekt auch in Milch-PAG-Werten niederschlägt ist unbekannt. Der in dieser Arbeit vorgestellte Sandwich-ELISA ist in der Lage, boPAG sowohl in Blut als auch in Vollmilch-Proben zuverlässig und sensitiv zu quantifizieren, wodurch die Möglichkeit geschaffen worden ist, diese Fragestellung zu beantworten. Dies wäre ein wichtiger Schritt, das Reproduktionsmanagement in der Milchviehhaltung zu verbessern, da es den Landwirten dann möglich ist, selbst anhand einer Vollmilchprobe den Trächtigkeitsstatus sowie das Risiko eines möglichen Aborts ihrer Tiere zu überprüfen.

Ausblickend könnte der PRM-Assay bei der Aufklärung dieser Konzentrationsunterschiede einen wichtigen Beitrag leisten. Mithilfe des Sandwich-ELISA können zunächst Tiere ausfindig gemacht werden, die ein entsprechendes boPAG-Profil zeigen.

Anschließend werden diese Tiere dann engmaschig überwacht und beprobt (z. B. Serum, Milch, Plazentagewebe, abortierter Embryo oder Fetus etc.). Die genaue boPAG-Zusammensetzung der Proben könnte dann mittels PRM-Assay ermittelt werden. Ein anschließender Vergleich mit physiologischen Trächtigkeiten (z. B. mit den Ergebnissen der PRM-Studie) kann Aufschluss darüber geben, ob bestimmte boPAGs bei pathophysiologischen Veränderungen in bestimmten Geweben und im maternalen Kreislauf signifikant häufiger auftreten als andere. Da auch ein Einfluss der Glykosylierung bei diesen Prozessen als sehr wahrscheinlich gilt (Constant *et al.*, 2011), sollte hier auch eine Analyse der Glykane mittels Massenspektrometrie erfolgen. Hierbei bestünde die Möglichkeit einen PRM-Assay für die Seiten-spezifische Quantifizierung von posttranslationalen Veränderungen (z. B. N-Glykanen) bei Proteinen zu entwickeln (Thomas *et al.*, 2015).

Zusammenfassend liefern die Ergebnisse der vorliegenden Arbeit neue und wichtige Erkenntnisse für die weitere Erforschung der boPAGs. Sie zeigen auch, dass aufgrund ihrer biochemischen Eigenschaften und der damit einhergehenden Vielfalt ein komplementärer Ansatz für weiterführende Untersuchungen gewählt werden sollte. Trotz intensiver Bemühungen in den letzten Jahrzehnten, konnten die genauen Funktionsmechanismen der boPAGs nicht bestimmt werden. Ihre Existenz während der gesamten Trächtigkeit scheint aber ein essentialer Baustein für deren Erfolg zu sein. Zusätzlich haben sie als zuverlässige Trächtigkeitsmarker eine große Bedeutung für die praktische Landwirtschaft. Das Zusammenspiel von molekulargenetischen, proteinanalytischen und immunologischen Methoden könnte so zu einem großen Erkenntnisgewinn in Wissenschaft und Praxis beitragen.

7 Zusammenfassung

Die vorliegende Dissertation beschäftigt sich mit molekulargenetischen und proteinanalytischen Untersuchungen trächtigkeitsassoziierter Glykoproteine (PAGs) beim Rind und deren praktische Anwendung als Trächtigkeitsmarker.

Bovine PAGs (boPAGs) werden in den Trophoblastzellen der Plazenta gebildet und gehören zu einer Multigenfamilie, welche mit den Aspartatproteasen verwandt ist. Aufgrund ihrer Akkumulation und Zirkulation im maternalen System (Blut und Milch) haben sie sich in den letzten Jahrzehnten als nützlicher und zuverlässiger Trächtigkeitsmarker in der praktischen Landwirtschaft herausgestellt. Die genauen Steuerungsmechanismen ihrer Bildung und Sekretion sowie ihre physiologische Rolle während der Trächtigkeit sind in weiten Teilen noch ungeklärt.

In der ersten Studie der vorliegenden Arbeit (Kapitel 3) wurden mRNA-Expressionsprofile für ausgewählte moderne (boPAG-1, -9, -21) und anzestrale (boPAG-2, -8, -10, -11, -12) bovine PAGs in Kotyledonengewebe erstellt. Hierbei konnte die höchste Expression für boPAG-8 ermittelt werden, die niedrigste für boPAG-10. Desweitern waren boAG-8 und boPAG-11 während der frühen Trächtigkeit (20 - 90 Tage) signifikant höher exprimiert. Das könnte auf eine wichtige Rolle dieser beiden PAGs während der Plazentation und Aufrechterhaltung der frühen Trächtigkeit hindeuten. Die Charakterisierung von boPAG mRNA-Expressionsprofilen liefert insbesondere während der frühen Phase der Trächtigkeit (Zeitpunkt der Plazentation, Änderung in der Glykosylierungsstruktur der boPAGs) eine wichtige Grundlage für den Abgleich mit proteomischen Daten. Sie können somit dazu beitragen neue Erkenntnisse über die Funktionen der boPAGs innerhalb dieses Zeitraumes zu gewinnen.

Die zweite Studie der vorliegenden Dissertation (Kapitel 4) beschäftigt sich mit der Entwicklung und Validierung eines „Parallel Reaction Monitoring“ (PRM) Assays, der es ermöglicht, die relativen Proteinlevel 18 verschiedener boPAGs während der Trächtigkeit und nach der Geburt zu beschreiben. Zusätzlich wurde hierbei der Einfluss der Glykosylierung untersucht. Die signifikant höchsten Proteinabundanzen konnten sowohl in glykosylierten als auch in deglykosylierten Proben für boPAG-1 ermittelt werden. Der größte Einfluss der Glykosylierung konnte während der mittleren und späten

Trächtigkeit sowie in der Nachgeburt gefunden werden. Zusätzlich konnte dabei eine Gruppe von boPAGs (boPAG-3, -5, -6, -7, -8, -9, -15, -21) identifiziert werden, welche scheinbar während der Trächtigkeit nur sehr schwach glykosyliert ist. Ein Zusammenhang zwischen dem Einfluss der Glykosylierung und der Anzahl an potenziellen Glykosylierungsstellen oder der phylogenetischen Verwandtschaft der boPAGs konnte nicht gefunden werden. Der PRM-Assay und die Ergebnisse dieser Studie liefern neue Ausgangspunkte für die weitere Erforschung der boPAGs, welche dazu beitragen können ein besseres Verständnis über die genauen physiologischen Funktionen dieser Proteingruppe während der Trächtigkeit zu erlangen.

Der dritte Teil der Arbeit (Kapitel 5) beschreibt die Etablierung eines Sandwich-ELISA für den Trächtigkeitsnachweis bei Rindern auf Basis der PAGs. Dieser erlaubt die simultane Quantifizierung von PAG im Serum und der Milch mit derselben Testprozedur. Für die Validierung des ELISA wurden 984 Serumproben und 928 Milchproben monatlich im Zeitraum von einer Woche nach der Besamung bis sechs Wochen post-partum von 231 Holstein Friesian Kühen gesammelt und analysiert. Der ELISA ermöglicht eine sichere Trächtigkeitsdiagnose ab dem 30. Tag nach der Belegung im Serum und ab dem 40. Tag nach der Belegung in der Milch. Die ermittelte postpartum Halbwertszeit von boPAG im Serum beträgt 6,4 Tage, in der Milch 7,1 Tage. Die mittlere boPAG-Konzentration in der Milch entsprach 1,5 % der mittleren Serumkonzentration. Des Weiteren zeigten die ermittelten boPAG-Profile im Serum und der Milch tragender Tiere typische Verlaufsmuster. Dieser ELISA ermöglicht dem Landwirt zeitnah, kostengünstig und aufwandsarm Trächtigkeitsdiagnosen zu erhalten. Zudem bietet er den durchführenden Laboratorien eine Kostenersparnis, da sie für Blut- und Milchproben den gleichen Test verwenden können.

8 Summary

The dissertation addresses molecular genetic and protein analytical studies of bovine pregnancy-associated glycoproteins (boPAGs) and their practical application as pregnancy markers.

Bovine PAGs are expressed by trophoblast cells in the placenta and are part of a multigene family that belongs to the group of aspartic proteases. The accumulation and circulation in maternal blood and milk have made boPAGs very useful and important for pregnancy diagnosis in cattle during the last decades. However, the exact mechanisms of their formation and secretion as well as their physiological role during pregnancy remain largely unknown.

In the first study of this dissertation (chapter 3), mRNA expression profiles were determined for selected modern (boPAG-1, -9, -21) and ancient (boPAG-2, -8, -10, -11, -12) bovine PAGs in cotyledon tissue. The highest expression was found for boPAG-8, the lowest for boPAG-10. Furthermore, it was found that boPAG-8 and -11 are significantly higher expressed in early gestation (pregnancy days 20 - 90). In conclusion, they could have a possible role in placentation and the maintenance of early pregnancy. The characterization of boPAG mRNA expression profiles provides an important basis for the further comparison with proteomic data, especially during the early phase of pregnancy (time of placentation, change in the glycosylation pattern of boPAGs). Thus, they could contribute to gain new knowledge about the function of the boPAGs within this period.

The second study of this dissertation (chapter 4) addresses the development and validation of a multiplexed parallel reaction monitoring (PRM) assay for the determination of the relative protein abundances of 18 different boPAG during pregnancy and after calving. Furthermore, we investigated glycosylated and deglycosylated samples to detect possible effects of the glycosylation status on the used method and changes in the relative abundances. The significantly highest protein abundances were found for boPAG-1 in both, glycosylated and deglycosylated samples. Highest degrees of glycosylation appeared in mid and late pregnancy samples as well as in afterbirth samples. Additionally, we identified a group of boPAGs (boPAG-3, -5, -6, -7, -8, -9, -15, -21) that seems not heavily glycosylated in any pregnancy stage. A linkage between the impact of

glycosylation and potential N-glycosylation sites or phylogenetic relation was not detected. The PRM assay itself and the results of this study give new starting points to address further research on boPAG to better understand the physiological role during pregnancy and achieve a real knowledge of these proteins.

The third part of the thesis (chapter 5) describes the establishment of a new Sandwich-ELISA which allows the simultaneous detection of boPAG in maternal serum and whole milk. Therefore, 984 serum and 928 milk samples were collected monthly from 231 Holstein Friesian cows from one week after insemination until six weeks postpartum. The ELISA is able to identify a cow as being pregnant at day 30 *p. i.* in serum and at day 40 *p. i.* in milk. The postpartum half-life of boPAG was estimated to be 6.4 days in serum and 7.1 days in milk. The amount of boPAG found in milk was 1.5 % of the amount of boPAG present in serum. Furthermore, the boPAG profile established during pregnancy in serum and milk showed a typical pattern. The developed ELISA quantifies boPAG concentrations in blood or whole milk samples in one ELISA system within a few hours. This is time saving for farmers and more efficient for laboratories, since only one test is necessary.

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10 Erklärung über den geleisteten Eigenanteil an der Arbeit

Hiermit erkläre ich den geleisteten Anteil an den in die Dissertationsschrift aufgenommenen Beiträgen.

Im ersten Beitrag mit dem Titel „mRNA expression profiling in cotyledons reveals significant up-regulation of the two pregnancy-associated glycoprotein genes boPAG-8 and boPAG-11 in early gestation", sind folgende Bereiche von mir übernommen worden: Probensammlung in Zusammenarbeit mit Frau Julia Sondermann und statistische Auswertung des Versuchs. Mitarbeit beim Verfassen des Beitrags in Abstimmung mit den anderen Autoren. Dieser Beitrag ist auch Teil der Dissertation von Frau Dr. Isabel Kilic (geb. Wiedemann) aus dem Jahr 2018. Die jeweiligen Beiträge der einzelnen Autoren sind im Teil „Contributions" im Artikel aufgeführt.

Im zweiten Beitrag mit dem Titel „A Multiplexed Parallel Reaction Monitoring Assay to monitor bovine Pregnancy-Associated Glycoproteins throughout pregnancy and after gestation ", sind folgende Bereiche von mir übernommen worden: Probensammlung in Zusammenarbeit mit Frau Julia Sondermann, die Probenaufarbeitung und Aufreinigung in Zusammenarbeit mit Frau Julia Sondermann und Herrn Dr. Mark Hennies (TECOdevelopment GmbH). Die Planung und Durchführung der Western-Blots und deren Auswertung erfolgten in Zusammenarbeit mit Dr. Momchil Ninov. Die Planung und Durchführung der massenspektrometrischen Versuche und deren Auswertung erfolgten in Zusammenarbeit mit Frau Lisa Neuenroth und Herrn Dr. Christof Lenz. Die statistische Auswertung des Versuchs erfolgte in Zusammenarbeit mit Herrn Dr. Christof Lenz. Das Verfassen und Überarbeiten des Beitrags erfolgten in Abstimmung mit den aufgeführten Autoren des Artikels. Die jeweiligen Beiträge der einzelnen Autoren sind zusätzlich im Teil „Contributions" im Artikel aufgeführt.

Im dritten Beitrag mit dem Titel „Establishment of a Sandwich-ELISA for simultaneous quantification of bovine pregnancy-associated glycoprotein in serum and milk", sind folgende Bereiche von mir übernommen worden: Probensammlung in Zusammenarbeit mit Frau Julia Sondermann, Frau Katja Mütze (HVL e.V.) und Frau Dr. Sonja Kleinhans (HVL e.V.), die Probenaufarbeitung und Aufreinigung in Zusammenarbeit mit Frau Julia Sondermann und Herrn Dr. Mark Hennies (TECOdevelopment GmbH). Etablierung und

Validierung des ELISA in Zusammenarbeit mit Herrn Dr. Mark Hennies (TECOdevelopment GmbH) und Herrn Daniel Lücking (TECOdevelopment GmbH). Die statistische Datenauswertung wurde von mir durchgeführt. Das Verfassen und Überarbeiten des Beitrags erfolgten in Abstimmung mit den aufgeführten Autoren des Artikels. Die jeweiligen Beiträge der einzelnen Autoren sind zusätzlich im Teil „Contributions“ im Artikel aufgeführt.

11 Eidesstattliche Erklärung

1. Hiermit erkläre ich, dass diese Arbeit weder in gleicher noch in ähnlicher Form bereits anderen Prüfungsbehörden vorgelegen hat.
   Weiter erkläre ich, dass ich mich an keiner anderen Hochschule um einen Doktorgrad beworben habe.

   Göttingen, den .....

   .....
   (Unterschrift)

2. Hiermit erkläre ich eidesstattlich, dass diese Dissertation selbständig und ohne unerlaubte Hilfe angefertigt wurde.

   Göttingen, den .....

   .....
   (Unterschrift)