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Differential gene expression during pre-implantation pregnancy in bos taurus

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List of abbreviations

18s rRNA	18S ribosomal RNA
28S rRNA	28S ribosomal RNA
ACTB	actin beta
AI	artificial insemination
bp	base pair
BST2	bone marrow stromal cell antigen 2
C1Q	complement component 1, q subcomponent
C1R	complement component 1, r subcomponent
C1S	complement component 1, s subcomponent
C2	complement component 2
C3	complement component 3
C3AR	complement component 3a receptor
C3bBb	C3 convertase
C3bBb3b	C5 convertase
C4	complement component 4
C5	complement component 5
C5AR	complement component 5a receptor
C6	complement component 6
C7	complement component 7
C8	complement component 8
C9	complement component 9
CD46	membran co factor protein
CD55	decay accelerating factor for complement
CEL	cell intensity files
CFB	complement factor B
CFD	complement factor D
CFH	complement factor H
CFI	complement factor I
CK8	cytokeratin 8
CL	corpus luteum
Cq	quantitative cycle
CR1	complement component receptor 1
CSF1	colony stimulating factor 1
D	day of the cycle
DF	degrees of freedom
EDTA	ethylenediaminetetraacetic acid
F	F-statistic test
FDR	false discovery rate
GAPDH	glyceraldehyde 3 phosphate dehydrogenase
GE	glandular epithelial cells
HIS3	histone H3
iC3b	inactive C3b
IDO	indoleamine 2,3-dioxygenase
IFI16	interferon-induced protein 16
IFI44	interferon-induced protein 44
IFIT2	interferon-induced protein with tetratricopeptide repeats 2
IFNT	interferon-tau
IgG	immunoglobulin G
IgM	immunoglobulin M
IL18	interleukin 18
IL1B	interleukin 1 beta
ISG	interferon stimulated genes
ISG15	ubiquitin-like modifier

LH	luteinizing hormone
LIF	leukemia inhibitory factor
LL	leukoLOCK RNA extraction method
LY	lysis RNA extraction method
MAC	membrane attack complex
miRNA	microRNA
mRNA	messenger ribonucleic acid
MS	mean square
MX1	myxovirus resistance 1
MX2	myxovirus resistance 2
NP	non-pregnant animals
nt	nucleotide
OAS1	2',5'-oligoadenylate synthetase 1
OOSP1	oocyte-secreted protein 1
P	probability value
P4	progesterone
PAX	PAXgene RNA extraction method
PBL	peripheral blood leukocytes
PCA	principal component analysis
$PGF_{2\alpha}$	prostaglandin $F_{2\alpha}$
PI	blood fractionation RNA extraction method
PLPE	paired local pooled error
R2	coefficient of determination
RBCs	red blood cells
RG	reference gene
RIN	RNA Integrity Number
RISC	RNA induced silencing complex
RMA	robust multi-array analysis
RNA	ribonucleic acid
Rpm	revolutions per minute
RT-qPCR	quantitative reverse transcription- polymerase chain reaction
SAM	significance analysis of microarrays
SC	stromal cells
SCC	somatic cell count
SEM	standard error of mean
SERPING1	serpin peptidase inhibitor, clade G (C1 inhibitor), member 1
SS	sum of squares
STAT1	signal transducer and activator of transcriptions 1
TG	target genes
TNFa	tumor necrosis factor alpha
UBQ	polyubiquitin 3
UCRP	ubiquitin cross-reactive protein
WB	whole blood RNA extraction method
WBC	white blood cells
XP	pregnant animals
YWHAZ	tyrosine 3-monooxygenase tryptophan 5-monooxygenase activation protein, zeta polypeptide

Zusammenfassung

Ein besseres Verständnis der biologischen Prozesse bei der Fortpflanzung von Rindern kann zu einer früheren und zuverlässigen Diagnose einer Trächtigkeit führen und somit zu einer Verbesserung der Zuchtleistung beitragen. Während der Präimplantationsphase sind auch immunologische Regulationsmechanismen von großer Bedeutung, da das mütterliche Immunsystem mit den väterlicherseits vererbten fremden Antigenen der Blastozyste konfrontiert wird. Das Komplementsystem als ein Teil des angeborenen Immunsystems spielt eine primäre Schlüsselrolle bei der Abwehr von Pathogenen und ist auch maßgeblich bei der Abstoßungsreaktion von Allotransplantaten beteiligt. Interferon-tau (INFT), das von den Trophoblastenzellen synthetisiert wird, steuert die Genexpression verschiedener Faktoren des Immunsystems wie Zytokinen und anderer Interferon stimulierter Gene (ISG) im Uterus. Aktuelle Studien bei dieser Spezies zeigen, dass während der frühen Trächtigkeit zahlreiche Faktoren des Immunsystems nicht nur lokal in der Gebärmutter, sondern auch in den Leukozyten des peripheren Blutes (PBL) vorhanden sind und aktiviert werden.
Ziel dieser Studie war es, den regulatorischen Einfluss des Embryos auf das mütterliche Immunsystem zu untersuchen. Unter diesem Gesichtspunkt sollten zum einen die Schlüsselfaktoren des Komplementsystems im Endometrium untersucht und zum anderen das Genexpressionmuster der PBL und der somatischen Milchzellen von trächtigen und zyklischen Tieren analysiert werden. Da die Leukozyten aus dem Blut durch die sogenannte Diapedesis in die Milch gelangen, wurde in dieser Studie untersucht, ob sich in Blut- und Milchzellen ein vergleichbares trächtigkeitsabhängiges Genexpressionsmuster feststellen lässt. Dieses könnte diagnostisch als früher Hinweis auf eine Trächtigkeit genutzt werden, da sich bei Milchkühen Milchproben in einer nicht invasiven Weise gewinnen lassen.
Um die lokale Wirkung des Embryos auf Endometriumszellen zu betrachten, wurde in zwei verschiedenen *in vivo* Modellen am Tag 18 nach der künstlichen Besamung die Genexpression ausgewählter Kandidatengene des Komplementsystems mittels quantitativer real-time PCR (RT-qPCR) gemessen. Die Rolle von INFT bei der Aktivierung des Komplementsystems wurde zusätzlich in Zellkulturmodellen geprüft. Auch hier wurden nach einer Behandlung der endometrialen Zellen mit INFT die Genexpression dieser Kandidatengene mit RT-qPCR getestet.
Expressionsveränderungen in PBL wurden mit Hilfe der Microarray Technologie und RT-qPCR bestimmt. Hierzu wurde zunächst die Expression von Kandidatengene aus den PBL von Färsen während des Zyklus und während der frühen Trächtigkeit untersucht. Im Falle einer Regulation wurden diese auch in Milchproben laktierender Kühe mit RT-qPCR bestimmt. Um das beste Ergebnis hinsichtlich der Reproduzierbarkeit der

Genexpressionsdaten zu gewährleisten wurden mehrere Protokolle zur Extrahierung von Ribonucleinsäuren (RNA) aus Leukozyten des Blutes und aus somatischen Zellen der Milch verglichen. Die Datenauswertung erfolgte mit biostatischen Methoden wie der Principal Component Analyse (PCA).

Die Ergebnisse aus der Analyse der Endometriumproben weisen auf eine trächtigkeitsabhängige Aktivierung des klassischen Pfades des Komplementsystems hin. Obwohl INFT im Zellkulturmodell die Genexpression verschiedener Faktoren des klassischen Weges erhöht, wird eine mütterliche Immunantwort auf den semi-allogenen Embryo verhindert. Die genauen Mechanismen dieser embryo-maternalen Kommunikation sind nicht bekannt und bedürfen noch weiterer Untersuchungen.

Anhand der Untersuchungen der Genregulation in PBL und somatischen Milchzellen von Färsen beziehungsweise laktierenden Kühen lassen sich in beiden Fällen von einer frühen Trächtigkeit abhängige Genexpressionmuster feststellen. Auch weisen Blut und Milch hierbei ein ähnliches Expressionsprofil auf. Es wurde dargelegt, dass die Auswertung der Daten der Genexpressionsergebnisse mit PCA erfolgreich dazu beitragen konnte, nicht-trächtige (NP) von trächtigen Tieren (XP) zu unterscheiden. Eine bestimmte Gruppe von Genen zeigte nicht nur an Tagen 16, 18 und 20 der Trächtigkeit eine differenzielle Genexpression zwischen trächtigen und nicht-trächtigen Tieren, sondern auch an Tag 8 in PBL und an Tag 12 in Milch somatischen Zellen.

Diese Studie belegt, dass eine veränderte Genexpression durch eine frühe Trächtigkeit nicht nur lokal im Uterus, sondern auch in PBL und Milchzellen nachgewiesen werden kann. Damit besteht die Option einer sehr frühen Trächtigkeitserkennung, welche in weiteren Untersuchungen sowohl hinsichtlich des Fortpflanzungsgeschehens als auch in der milchwirtschaftlichen Praxis entwickelt werden könnte.

Abstract

A better understanding of the biological processes during the fertilization period of cattle may improve the efficiency of their reproduction by enabling an early and reliable pregnancy diagnosis. During the preimplantation phase regulatory mechanisms are of special importance, since the maternal immune system is confronted with the inherited paternal antigens of the bovine blastocyst. The complement system as a part of the innate immune system plays a key role in the primary defense against pathogens and contributes significantly in the rejection of allografts. Interferon-tau (IFNT) which is secreted by trophoblast cells regulates the gene expression of several proteins in the uterus, such as cytokines and interferon stimulated genes (ISG). Recent studies indicated that during early pregnancy several components of the immune system are present and activated not only locally in the uterus, but also in bovine peripheral blood leukocytes (PBL).

The aim of this study was to analyze the ability of the conceptus to influence the maternal immune system. Therefore, it was necessary to examine particular key factors of the complement system in the endometrium and to analyze the gene expression pattern on PBL and milk somatic cells from pregnant and cyclic animals. Since leukocytes present in blood can get into the milk through a process called diapedesis, it was investigated whether a similar gene expression pattern can be determined in PBL and milk somatic cells. This could be used diagnostically as an early indication of pregnancy, since in dairy cows milk sampling is non invasive.

In order to investigate the local effect of the conceptus on the endometrium, selected candidate genes of the complement system were analyzed in two different *in vivo* models at day 18 after insemination by quantitative real-time PCR (RT-qPCR). The role of INFT in the activation of the complement components was also tested in cell culture models. After treatment of endometrial cells with IFNT the gene expression of candidate genes was analyzed by RT-qPCR.

To determine the expression changes in PBL, microarray technology and RT-qPCR were applied. Therefore, the expression of candidate genes in PBL of heifers during the cycle and during early pregnancy was investigated. In the case of a regulation, these were also determined by RT-qPCR in milk samples of lactating cows. In order to guarantee the best outcome concerning the reproducibility of gene expression data, several protocols to extract ribonucleic acid (RNA) out of leukocytes from blood and somatic cells from milk were investigated. Biostatistical tools as principal component analysis (PCA) were then applied to analyze the results.

Results of the endometrium tissue analysis imply a pregnancy dependent activation of complement components of the classical pathway. Although IFNT in the cell culture model increases the expression of different factors of the classical pathway, a maternal immune response towards the semi-allogenic embryo is prevented. The exact mechanisms of this embryo maternal communication are yet unknown and require further investigation.

In the study of gene regulation in PBL and in milk somatic cells of heifers or lactating cows respectively an early pregnancy dependent gene expression pattern can be detected in both cases. Furthermore, blood and milk have a similar gene expression profile. It was demonstrated that the data evaluation of gene expression results using PCA could successfully be used to separate non-pregnant (NP) from pregnant animals (XP). A specific set of genes showed differential gene expression between pregnant and non-pregnant animals not only at days 16, 18 and 20 of pregnancy, but also at day 8 in PBL and at day 12 in milk somatic cells.

This study confirms that changes in gene expression during early pregnancy can be demonstrated locally in the uterus, in PBL and in milk somatic cells. This provides the option to detect pregnancy in an earlier stage, which could be used in further investigations regarding reproductive events and in milk production practice.

1 Introduction

1.1 Bovine estrous cycle

Cattle are non-seasonally polyestrous, meaning that animals can go into heat several times in a year if pregnancy does not occur. The length of the estrous cycle is 21 days on average, and this interval can range from 16 to 24 days (figure 1). The cycle is divided into four periods: estrus, metestrus, diestrus, and proestrus (Liebich 2003, Hafez & Hafez 2000), in this study estrus is considered at day 21, metestrus at days 1 - 4, diestrus at days 5 - 18 and proestrus at days 19 - 20.

Estrus is defined as the period of time when the female organism is receptive for mating, showing common signs as swollen vulva, clear vaginal discharge (mucus), roughened tail head or pink vaginal mucosa. This period lasts from 6 to 24 hours, with 15 hours being the average length. During estrus, estrogen is produced by the growing follicle and induces a peak release of pulsatile luteinizing hormone (LH) which causes ovulation at the end of estrus. The corpus luteum (CL) evolved from an ovarian follicle produces the steroid hormone progesterone (P4) throughout the diestrus, reaching its plateau at approximately day 12. If no pregnancy signal is received by the endometrium, release of prostaglandin $F_{2\alpha}$ ($PGF_{2\alpha}$) leads to luteolysis at the end of diestrus (Liebich 2003, Hafez & Hafez 2000).

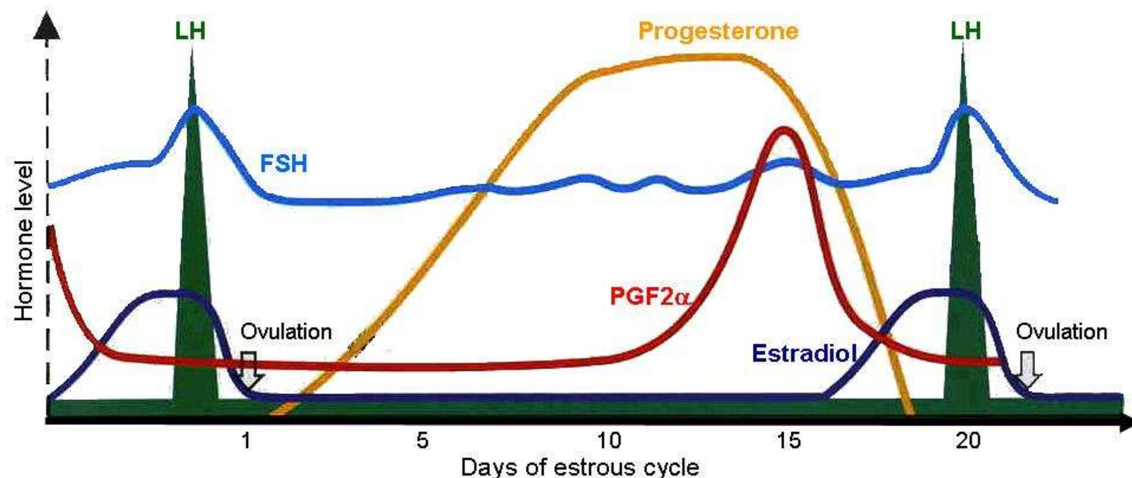


Figure 1: Schematic representation of hormone concentration changes in plasma during the bovine estrous cycle (following (Pineda *et al.* 2003)).

The prostaglandin secretion is driven by luteal oxytocin secretion and requires the interaction of circulating oxytocin with its receptors, which is located on endometrial epithelial cells

(Demmers *et al.* 2001). The prostaglandins bind to the receptor on the CL leading to luteal regression. The prostaglandins also cause further release of oxytocin from the CL, which induces more $PGF_{2\alpha}$ release. This generates a positive feedback mechanism to ensure luteal death and initiates a return to estrus (Demmers *et al.* 2001, Goff 2002, Spencer *et al.* 2004).

In case of pregnancy the luteolytic cascade is blocked and P4 concentration remains constant. Due to the increasing secretion of the ruminant pregnancy recognition signal IFNT by the preimplantation trophoblast, the conceptus ensures continued exposure of the endometrium to high circulating concentrations of P4. Thus, secretory activity of the endometrial glands to provide the nutrients and signaling factors such as growth factors and cytokines required for the development of the blastocyst is maintained (Bazer & Spencer 2005, Demmers *et al.* 2001, Goff 2002, Roberts *et al.* 1992, Spencer *et al.* 2004).

1.2 Activation of the complement system during pregnancy

During pregnancy, the survival of the semi-allogenic embryo depends on functional complexes to modulate the immune response of the mother. For example, in early pregnancy paternal antigens present in the semi-allogenic conceptus could be considered as a non-innate surface by the immune system of the mother and therefore activate components of the complement system (Emond *et al.* 2004, Richani *et al.* 2005b). To prevent embryo rejection, the activation of regulatory complement proteins may also take place adjusting the immune response of the mother.

The complement system is an essential part of the innate immune system consisting of multiple serum proteins which opsonize pathogens and subsequently induce a cascade of proteins and proteases to encourage inflammatory responses and trigger pathogen inactivation. The complement system can be activated via the classical pathway by the presence of antigen–antibody complexes and via the alternative pathway through spontaneous complement component 3 (C3) hydrolysis or further antigens (figure 2) (Monsinjon *et al.* 2001, Schraufstatter *et al.* 2002).

The classical pathway is triggered by the C1 complex which is composed of complement component 1, r subcomponent (C1R), complement component 1, q subcomponent (C1Q) and complement component 1, s subcomponent (C1S) (Janeway 2005). Activation of C1 complex occurs when C1Q binds to immunoglobulin M (IgM) or immunoglobulin G (IgG) complexed with antigens, or when C1Q binds directly to the surface of the pathogen (Janeway 2005). The inhibition of C1 complex is controlled by the inhibiting factor serpin peptidase inhibitor (SERPING1) (Davis, III 2004). The alternative pathway is triggered by spontaneous C3 hydrolysis forming C3a and C3b. The activation of the complement system

via either one of these pathways results in activation of an active C3 convertase (Janeway 2005).

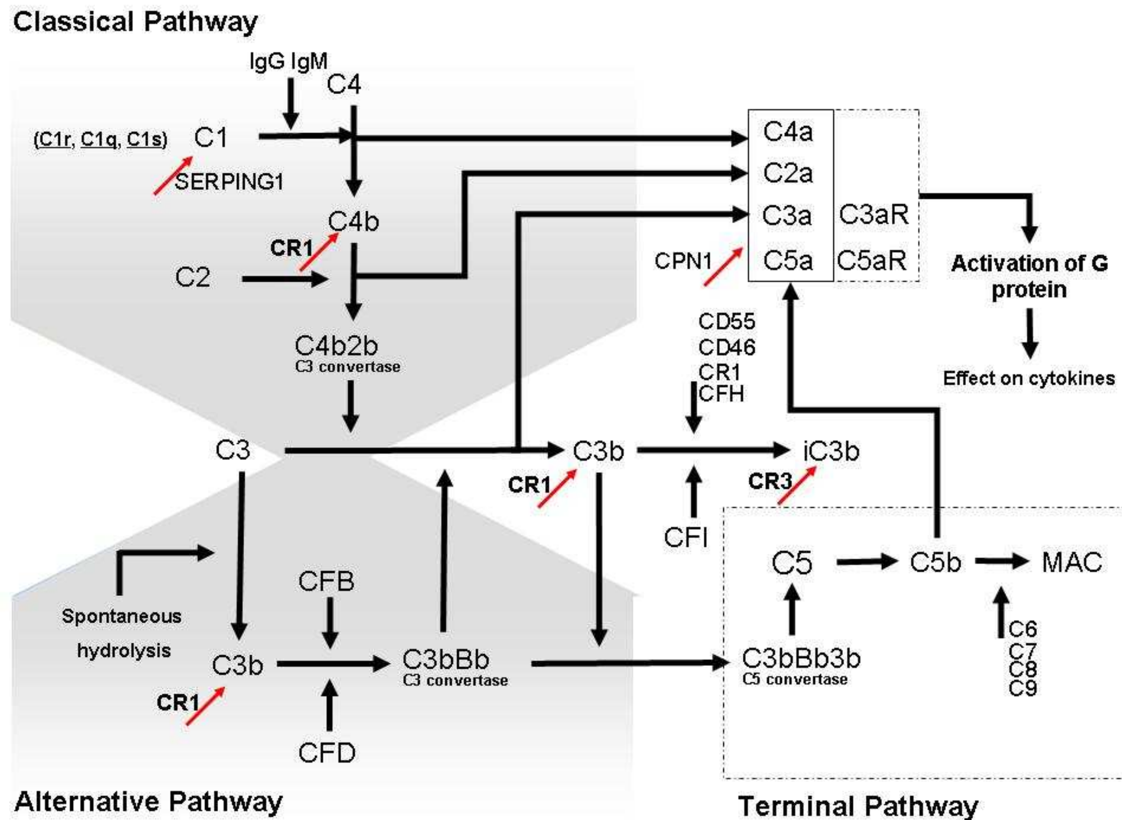


Figure 2: Schematic representation of the complement system activation. Red arrows indicate inhibitory and regulatory complement components which play an important role by controlling the activation of the casacade through inactivation of its target component.

Spontaneous production of C3b allows the binding of complement factor B (CFB), which allows complement factor D (CFD) to cleave CFB to form the complex C3 convertase (C3bBb). This C3 convertase, although produced in small amounts, can cleave multiple C3 proteins into C3a and C3b, thus amplifying C3b production (Ehrnthaller *et al.* 2010). To prevent adverse immune reactions towards the host and to inhibit autologous complement activation, there are several important regulatory proteins. Regulatory factors as CFH, CFI, CR1, CD55 and CD46 play an important role in controlling the amplification loop. This process is tightly controlled by the binding of one of these factors to C3b to form the inactive C3b (iC3b) (Janeway 2005).

Additionally, through hydrolysis of complement factors, chemotactic active anaphylatoxins (C3a and C5a) are released providing early defense mechanisms against pathogen-associated molecular patterns (Carroll 2004, Monsinjon *et al.* 2001, Richani *et al.* 2005b, Schraufstatter *et al.* 2002). Anaphylatoxins are considered to be potent pro-inflammatory mediators and powerful chemo-attractants which recruit neutrophils, macrophages and mast cells at the inflammatory site (Carroll 2004, Monsinjon *et al.* 2003). The cells respond to anaphylatoxins via interaction with specific receptors, which are functionally coupled to G-proteins (Monsinjon *et al.* 2003). It was demonstrated that an effect on cytokine production can occur by binding of anaphylatoxin to its G-protein coupled receptor (Monsinjon *et al.* 2001). These products, may modulate innate and adaptive immunity by activating further biochemical cascades.

Binding of C3b to the C3 convertase, formed either by the activation of the classical or the alternative pathway, creates C5 convertase (C3bBb3b). C5 convertase cleaves complement component 5 (C5) into C5a and C5b, which is a key component for the association of C6, C7, C8 and C9 (Girardi *et al.* 2003, Rampersad *et al.* 2008). The result of this activation cascade is the formation of the membrane attack complex (MAC) causing cell lysis and death.

In humans, a significant increase of complement system activation is associated with different pregnancy disorders, as for example spontaneous abortions and intra-uterine growth retardation (Rampersad *et al.* 2008, Richani *et al.* 2005a). Studies performed in mice showed that blockade of the anaphylatoxin C5a receptor (C5aR) prevents fetal loss and growth restriction during mid pregnancy (Girardi *et al.* 2003).

A previous holistic approach by using microarray analysis has shown that pregnancy increases the expression of specific factors from the classical pathway (C1S, C1R, SERPING1 and C4) in the bovine endometrium at day 18 (Klein *et al.* 2006). These results were reinforced by *in situ* hybridization where C1S, C1R and SERPING1 were localized in endometrial glandular epithelial cells (GE) (Klein et al. 2006). Whether bovine pregnancy recognition signal IFNT activates components of the complement system eventually triggering immune responses towards the semi-allogenic preimplantation conceptus is up to date not clear.

1.3 Early pregnancy in cattle and signaling responses

The development of the embryo starts with the fertilization of an oocyte by a sperm in the oviduct approximately 12 - 24 hours after ovulation. During the migration from 3 to 4 days through the oviduct, the zygote develops into a blastocyst (Michel 1995, Ulbrich *et al.* 2006). In cattle, hatching of the blastocyst from the zona pellucida occurs between days 8 - 10 after fertilization (Maddox-Hyttel *et al.* 2003). By day 12, elongation of the trophoblast is initiated

and until implantation the conceptus increases in size more than 1000 -fold. The conceptus remains detached in the uterus until implantation, which in cattle starts around day 19 after fertilization (Bazer & Spencer 2005).

In the uterine lumen, the trophoblast of the bovine embryo rapidly elongates and secretes high amounts of IFNT inhibiting luteolysis. IFNT is a member of the IFN type I family, which includes IFN-alpha and IFN-beta (Samuel 1991). During the prolonged preimplantation phase, the embryo requires nutrient supply via the uterine milk (Chelmonskasoyta 2002, Demmers *et al.* 2001, Roberts *et al.* 1992). The production of IFNT increases with conceptus elongation, inducing local gene expression changes of ISG such as chemokines and cytokines in the endometrium (Emond *et al.* 2000, Emond *et al.* 2004, Forde *et al.* 2011). Failure of the conceptus to signal its presence can lead to pregnancy loss (Chelmonskasoyta 2002, Demmers *et al.* 2001)

In contrast to primates and rodents, ruminants show a late implantation and a less invasive type of placentation known as placenta synepitheliochorialis, where binucleated trophoblast cells invade the uterine epithelium to form giant cells (Wooding & Wathes 1980). Thus, there is no direct contact of the embryo with maternal vessels or even with the maternal blood stream (Moffett & Loke 2006). Recently, first evidence was given for an enhanced IFNT dependent gene expression in ruminant peripheral blood (Yankey *et al.* 2001). It was shown that the uterine vein releases IFNT, which induces ISG in extra uterine tissues such as CL and PBL as early as day 15 of pregnancy through an endocrine mechanism of action (Yankey *et al.* 2001). Moreover, some ISG as ubiquitin-like modifier (ISG15), previously known as ubiquitin cross-reactive protein (UCRP), 2',5'-oligoadenylate synthetase 1 (OAS1), myxovirus resistance 1 (MX1) and myxovirus resistance 2 (MX2) were shown to be up-regulated in PBL during early pregnancy in cattle on days 16, 18 and 20 after estrus (Gifford *et al.* 2007, Green *et al.* 2010, Han *et al.* 2006).

Synthesis of ISG15 in the bovine endometrium is correlated with IFNT up-regulation (Austin *et al.* 1996). ISG15 is covalently linked to a variety of cellular proteins, suggesting regulation of different cellular processes like DNA repair, transcription, cell-cycle control and signal transduction (Kerscher *et al.* 2006, Kim & Zhang 2003). In response to IFNT, ISG15 conjugates to endometrial cytosolic proteins during early pregnancy and is involved in establishment and maintenance of pregnancy (Johnson *et al.* 1998).

OAS1 is induced by IFNT and is involved in innate immune response to viral infection as well as cell growth, differentiation and apoptosis (Kumar *et al.* 2000, Schmitt *et al.* 1993). Treatment with IFNT in different endometrial bovine cells types increases OAS1 expression (Schmitt *et al.* 1993). In the bovine endometrium differential gene expression of OAS1 was a consequence of IFNT production by the conceptus (Forde *et al.* 2011).

Messenger RNA up-regulation of myxovirus resistance genes (MX) during pregnancy is found in uteri of cows and sheep (Hicks *et al.* 2003). Interferon-induced protein with tetratricopeptide repeats 2 (IFIT2) and interferon-induced protein 44 (IFI44) was shown to be up-regulated in endometrium tissue from pregnant animals at day 16 of pregnancy (Forde *et al.* 2011). Interferon-induced protein 16 (IFI16) and IFIT2 show higher messenger ribonucleic acid (mRNA) expression in bovine endometrium of pregnant animals at day 18 (Klein *et al.* 2006).

In vivo intra-uterine infusion of IFNT on day 14 of the estrous cycle induced the expression of IFT2, IF44 and MX2 in non-pregnant cows (Forde *et al.* 2011). Many of the proteins encoded by these genes contribute to the antiviral response of cells targeted by IFN and are considered to be classical ISG (Stark *et al.* 1998, Horisberger & Gunst 1991). Although IFNT released by the trophoblast regulate gene expression of different factors in the bovine endometrium and PBL, few of these proteins have been characterized, with their functions largely remaining unknown.

In addition, studies in mammals have shown that cytokines and growth factors are produced by the pre-implantation embryo and are implicated in embryo-endometrium interaction during early pregnancy (Imakawa *et al.* 1997, Tartakovsky & Ben Yair 1991). Gene expression investigation showed that leukemia inhibitory factor (LIF) and macrophage colony stimulating factor (M-CSF) were produced in bovine endometrium and may play different roles in early and mid-pregnancy (Oshima *et al.* 2003). The cytokine group of interleukins may also be involved in maintenance of pregnancy in the human endometrium (Dimitriadis *et al.* 2005, Lee & Demayo 2004, Castro-Rendon *et al.* 2006), in the human peripheral blood cells (Fischer *et al.* 1999) and in the bovine endometrium (Muneta *et al.* 2005, Paula-Lopes *et al.* 1998, Paula-Lopes *et al.* 1999, Groebner *et al.* 2011). Tumor necrosis factor alpha (TNFa) play a role during early pregnancy in mice (Castro-Rendon *et al.* 2006) and in bovine (Okuda *et al.* 2002).

Different studies have analyzed signaling responses due to pregnancy in humans (Kao *et al.* 2002, Lappin *et al.* 1992, Hasty *et al.* 1994), however less is known about the bovine embryo-maternal interface. The diversities of implantation and placentation between species may result in different gene regulation. It was demonstrated that the expression of cytokines in bovine PBL is stimulated by IFNT showing the capacity of the conceptus to influence the maternal immune system in the periphery (Emond *et al.* 1998, Emond *et al.* 2000). The physiological implications of these influences are yet mostly unknown, but the changes provide the opportunity to identify a pregnancy gene expression signature in bovine leukocytes.

1.4 Diapedesis: leukocytes migration from blood into milk

The cellular blood components include erythrocytes (red blood cells = RBCs), leukocytes (white blood cells = WBC) and thrombocytes (platelets). Erythrocytes represent the most abundant cell population and are responsible for the transport of oxygen and CO_2, while thrombocytes are important in blood clotting. Innate immune responses depend on the activity of leukocytes, which are classified into two main groups: granulocytes belonging to the innate immune system which include neutrophils, eosinophils, and basophils, and agranulocytes belonging to the adaptive immune system which include B- and T-lymphocytes (Janeway 2005). In order to build the immune system, leucocytes also produce, transport and distribute antibodies (Janeway 2005).

The movement of leukocyte out of the peripheral circulatory system is called extravasation. This process forms part of the innate immune response and involves the recruitment of leukocytes from peripheral blood into sites of damage or infection as part of the inflammatory response as for example in the case of mastitis (Prin-Mathieu *et al.* 2002). It is supposed that through complex interactions the leukocytes from blood migrate to milk.

A blood-milk-barrier is established to control which cells, substances and molecules can move from blood into milk (Smits *et al.* 1999). A particle to cross from blood into milk, has to pass three barriers: the vessel endothelium, the intralobular collagen and the alveolar epithelium (figure 3) (Smits *et al.* 1999). To maintain constant defense mechanism in milk, blood cells can pass the barrier through a process called diapedesis (Smits *et al.* 1999).

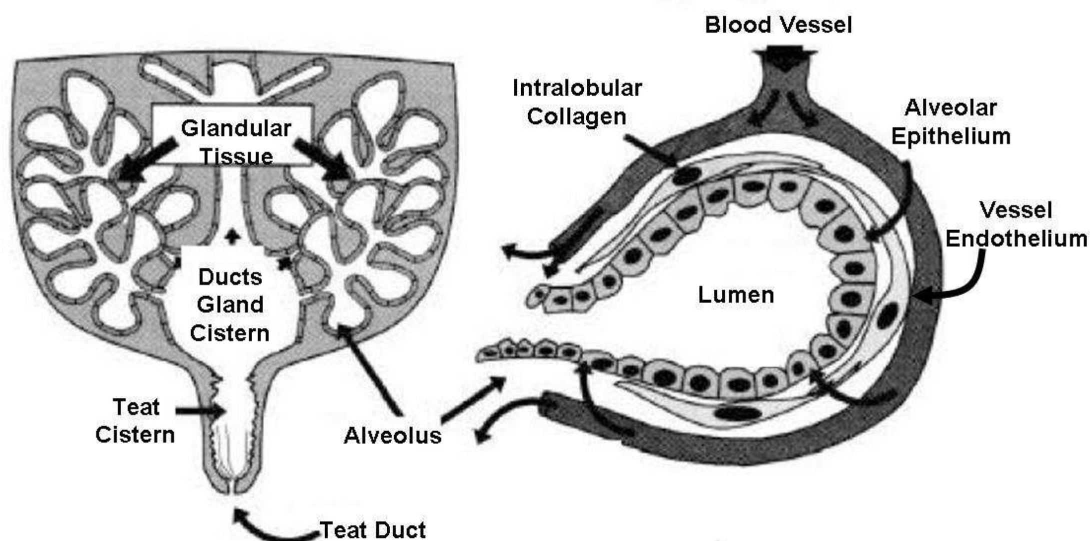


Figure 3: Structure of the mammary gland highlighting the blood-milk barrier (following (Schroeder 1997)).

Milk somatic cells are primarily leukocytes and some epithelial cells from the mammary gland (Pfaffl *et al.* 2003). Epithelial cells that are released from alveoli or ducts contribute with around 2% to the somatic cell count (SCC), representing only a minor part of the somatic cells (Sarikaya *et al.* 2006). The SCC is traditionally a parameter to assess the health of the udder (Leitner *et al.* 2000).

Blood is usually used for diagnostic tests, as it displays an accumulation of cellular information from all sections of the body and is easy to access. As dairy cows have a daily milking routine, it is faster, easier and less invasive to sample milk instead of blood for scientific investigations on living animals. Gene expression analysis of leukocytes in milk thus seems to be a promising tool for early pregnancy diagnosis in cattle.

1.5 Gene expression analysis as a potential method for pregnancy diagnosis in cattle

In cattle, embryonic mortality has a major impact on reproductive efficiency. Pregnancy diagnosis is an important tool for reproductive management. By pregnancy diagnosis using common methods as rectal palpation, the interval between first and second insemination ranges from 1 to 2 months. An early diagnosis of pregnancy as well as non-pregnancy could enable an earlier second insemination and thus significantly increase the profitability of cattle operations (De Vries 2006, Lucy 2001).

Rectal palpation is probably the most commonly used method for pregnancy diagnosis because it is accurate, fast, and cheap. Unfavorably, much practice is necessary and it cannot be performed before 40 - 50 days after fertilization (Fricke 2002). Another method is the transrectal ultrasound, which has a high reliability as a pregnancy diagnosis method and can be conducted relatively early; namely 27 days after insemination (Fricke 2002). However, an ultrasound device is expensive and it takes more time to perform the diagnosis than by rectal palpation. By detecting P4 concentration it is possible to diagnose cattle as non-pregnant around 21 - 24 days after insemination (Dobson & Fitzpatrick 1976, Han *et al.* 2006). Nevertheles, by determination of P4 concentration pregnant animals cannot be assured confidently. A pregnancy diagnosis prior to day 21 could enable a resynchronization in the following estrous cycle after the first insemination.

Recent studies have considered the analysis of ISG in PBL with the objective to determine the pregnancy status as early as day 17 after insemination (Gifford *et al.* 2007, Han *et al.* 2006). Low expression of ISG15 mRNA in PBL combined with low P4 concentration were shown to be indicative of cows that are not pregnant at day 17 after artificial insemination (AI) (Han *et al.* 2006). Stevenson et al. demonstrated that high expression of MX2 mRNA levels in PBL was indicative of pregnancy 21 days after AI (Stevenson *et al.* 2007). Green et al. showed that high expression of ISG15 and MX2 mRNA levels in PBL was indicative of

pregnancy 18 and 20 days after AI (Green *et al.* 2010). However, predicting pregnancy status according to expression changes based on one ISG in PBL has been shown to be an inaccurate method (Gifford *et al.* 2007, Han *et al.* 2006, Spencer *et al.* 2008).

Based on these findings, we hypothesize herein that a gene expression signature of a large number of informative genes may allow pregnancy determination in bovine PBL and also in milk somatic cells with high confidence. Besides the analysis of mRNA expression changes, to improve accuracy of results, another possibility would be to detect microRNA (miRNA) expression changes. MicroRNA are small non-coding single stranded RNA molecules, about 21 - 25 nucleotides (nt) in length. It is believed that due to their small size they may tend less to degradation and modification than mRNA (Cannell *et al.* 2008, Finnegan & Matzke 2003, Shivdasani 2006). Gene expression changes measured by RT-qPCR during early pregnancy submitted to biostatistical tools, like PCA or hierarchical cluster analysis could be helpful to verify, pregnancy status with accuracy.

1.6 Importance of sample processing for gene expression analysis

Quantitative RT-PCR has improved the accuracy of gene expression results revolutionizing molecular diagnosis (Bustin *et al.* 2005, Pfaffl 2001). The benefit and precision of molecular diagnosis by RT-qPCR is limited by sampling procedures and RNA extraction methods. Many impurities found in RNA preparations can interfere in the RT-qPCR reaction resulting in poor sensitivity. It has been shown that results of gene expression analysis are directly affected by mRNA and miRNA quantity and quality (Becker *et al.* 2010, Fleige & Pfaffl 2006). Therefore, to guarantee a stable gene expression signature, the most valid technique for sample collection, RNA stabilization and RNA isolation has to be validated.

For quantitative assays not just mRNA but also RNA with low-molecular weight, such as miRNA, are of emerging importance in molecular diagnostic (Shivdasani 2006, Stefani & Slack 2008). MicroRNA are complementary to one or more messenger RNA molecules (Finnegan & Matzke 2003). These highly conserved small RNA molecules regulate gene expression in a variety of manners, including translational repression, mRNA cleavage, and deadenylation (Cannell *et al.* 2008, Shivdasani 2006).

Gene regulation occurs by interaction of mature miRNA with the complementary sites in the 3’ UTR of the target mRNA by RNA induced silencing complex (RISC) inhibiting translation. Mature miRNA are removed from stem loop precursors, which are transcribed as part of longer primary transcripts (pri-miRNA). Pri-miRNA is processed by the RNAse Drosha and the double-stranded RNA binding protein Pasha in the cell nucleus resulting in the precursor miRNA (pre-miRNA), which is then exported to the cytoplasm (Exportin 5). Pre-miRNA is processed by an RNAse Dicer enzyme to remove the loop sequence resulting in a short asymmetric duplex intermediate. Within this process one strand becomes the active mature

miRNA and is integrated into the RISC complex. This complex is responsible for gene expression down-regulation of multiple genes (Shivdasani 2006, Cannell *et al.* 2008).
To date, thousands of miRNA have been identified in various organisms, mostly by random cloning of small RNA but also by computational prediction. All published miRNA sequences, genomic locations and associated annotation can be found at the miRBase database (http://www.mirbase.org/) (Griffiths-Jones 2010). The database contains over 15000 sequences of 142 species organized into biological classification clusters. For example, in the current version (November 2011, V18.0) 662 sequences from bovine are registered.
In the past, small RNA was considered as degraded RNA product with no importance and extraction methods were optimized to exclude this fraction. The first description of miRNA was in 1993, and in 2001 the term microRNA was established (Lee *et al.* 1993, Ruvkun 2003). Since then the interest in these small molecules has exponentially increased. By now, commercial extraction kits to extract mRNA in conjunction with miRNA are available (table 1).

Table 1: Summary table of four extraction methods which can recover mRNA and miRNA for RT-qPCR analysis.

Commercially kits	Extraction System	Samples	Purification	Nucleic acid obtained
Organic extraction	Trifast	Cell, tissues	Phenol	Total RNA
Qiagen	miRNeasy	Cell, tissues, blood, paraffin	Phenol and Column	Total RNA
Applied Biosystems	mirVana	Cells, tissues, bacteria, yeast, virus	Phenol and Column	mRNA and miRNA
Invitrogen	PureLink	Cells, tissue, bacteria, yeast	Precipitation and column	miRNA

Similar to mRNA extraction, the two main methods used for miRNA extraction are the phenol-based extraction and the column-based nucleic acid purification. Using phenol-based methods total RNA including small RNA are recovered. Using column-based methods, RNA molecules such as 28S ribosomal RNA (28S rRNA), 18S ribosomal RNA (18S rRNA), and mRNA that can inhibit expression analysis of small RNA molecules may be removed.
Phenol-based isolation procedures can isolate small RNA up to 10 nucleotides and are suitable for studies of mRNA, miRNA and other small RNA. Considering that not all isolation methods are specifically adapted for retaining small RNA, for column-based procedure special commercial kits for miRNA extraction are needed. Some of the existing kits combine the advantages of organic extraction followed by immobilization of RNA on glass-fiber filters, as for example the miRNeasy (Qiagen, Hilden, Germany) and the mirVana (Applied Biosystems, Darmstadt, Germany). Others employ a precipitation followed by membrane

immobilization like the PureLink (Invitrogen Corporation, Carlsbad, CA, USA). By changing the alcohol concentration used in the extraction protocol also small RNA are able to bind to the solid phase of the column and can be eluted at the end of the procedure.

After extraction of mRNA and miRNA and evaluation of gene expression data, suitable biostatistical methods are needed to analyze the results obtained. Different methods which allow the study of many variables simultaneously like PCA or hierarchical cluster analysis can be used. These variables may originate from gene expression profiling determined by RT-qPCR (Kubista *et al.* 2006).

PCA reduces the multidimensional data from a subset of variables to two dimensions facilitating analysis of the results. Although PCA cannot be considered a confirmative statistical method, within this mathematical tool it is possible to describe correlations between different groups and therefore, falls in the group of description statistical methods. By applying PCA it is possible to visualize many genes simultaneously and classify samples into groups according to their similarity of the gene expression patterns. With the objective to distinguish control and treatment samples, this method has been used successfully to analyze biomarkers data (Kubista *et al.* 2006, Borovecki *et al.* 2005, Riedmaier *et al.* 2009).

1.7 Aim of the thesis

The aim of this thesis was to study gene expression changes induced locally by the preimplantation embryo in the endometrium and in the periphery in blood and milk leukocytes (figure 4). Therefore the effect of the conceptus on the expression of key factors of the complement system in the bovine endometrium was measured. Additionally it was analyzed whether IFNT was able to induce the activation of complement factors and regulatory factors in an *in vitro* co-culture system.

To explore the change in mRNA expression during the preimplantation period in response to the developing conceptus, gene expression in blood leukocytes and milk leukocytes were analyzed prior to day 20 after insemination. An important aspect of this work was to optimize and validate sampling of blood and milk, extraction of leukocytes and milk somatic cells and RNA extraction methods.

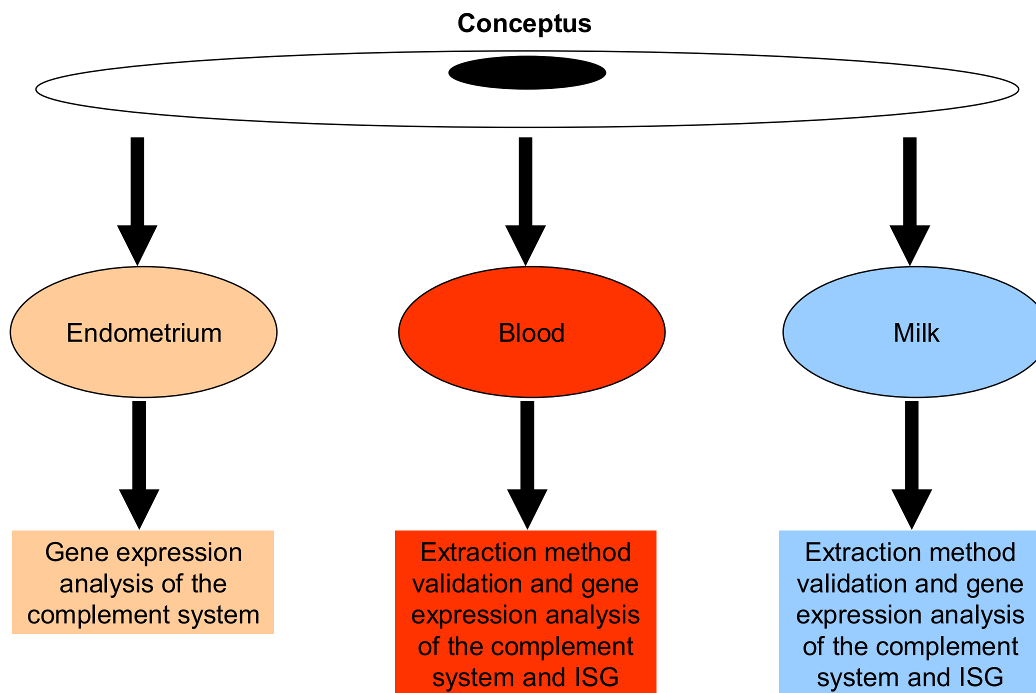


Figure 4: Schematic representation of the study aims.

2 Material and methods

All experiments were performed with permission from the local veterinary authorities and in accordance with the International Guiding Principles for Biomedical Research Involving Animals, as proposed by the Society for the Study of Reproduction and with the European Convention on Animal Experimentation.

2.1 Study design of pregnancy recognition regarding the complement system activation in endometrium

2.1.1 Monozygotic twin animals study

At day 18 after insemination, endometrial samples were taken from five monozygotic twin nonlactating Simmental cow pairs generated by embryo splitting. Embryos produced by *in vitro* fertilization were transferred into the ipsilateral uterus horn of one twin of each pair on day 7 of the estrous cycle. The corresponding control twin (n = 5) received a sham transfer of the same amount of transfer medium without embryos as described previously (Klein *et al.* 2006). These samples were kindly provided by the Institute of Animal Breeding and Institute of Molecular Animal Breeding and Biotechnology, Dr. S. Bauersachs, Munich.

2.1.2 Cyclic animals study

Study A

For the cyclic study A, eight non-related Simmental heifers received an artificial insemination. The pregnant group (n = 4) received an insemination with cryo-conserved sperm and the non-pregnant (n = 4) group received a supernatant of centrifuged sperm (seminal plasma) from the identical bull as already described (Bauersachs *et al.* 2006). These samples were kindly provided by the Institute of Animal Breeding and Institute of Molecular Animal Breeding and Biotechnology, Dr. S. Bauersachs, Munich.

Study B

For validation of results obtained for study A a second animal study was performed, namely study B. For the cyclic study B similar procedure as for study A was used, where eight different animals were estrus synchronized and inseminated as already described (Ulbrich *et al.* 2009b). These samples were kindly provided by Dipl-Biol. A. E. Groebner, Physiology Weihenstephan.

2.1.3 *In vitro* co-culture of glandular epithelial and stroma cells study

Bovine endometrial GE were seeded in cell culture inserts (Anopore membrane, 0.2 Im; Nunc) coated with Growth Factor Reduced Matrigel (BD Biosciences, San Jose, CA). Coculture with stromal cells (SC) were performed in a six-well cell culture plate (Nunclon Δ Surface; Nunc, Wiesbaden, Germany) placed underneath the inserts. In this system co-culture of GE and SC was performed as described earlier (Ulbrich *et al.* 2009a). Cells were stimulated with recombinant bovine IFNT (antiviral activity, 4.8 x 103 U/ml medium; PBL Biomedical Laboratories, Piscataway, NJ) and medium with the respective diluents served as control. The cells were harvested after 4 h of stimulation, suspended on 500 µl of TRIzol reagent (Invitrogen) and stored at -80 °C until further analysis. These samples were kindly provided by M. Sc. E. Englberger, Physiology Weihenstephan.

2.1.4 Tissue sampling and RNA extraction

For all experimental models at day 18 after insemination, animals were slaughtered and intercaruncular endometrial samples were taken from the uterine horn ipsilateral to the ovary bearing the CL as described previously (Bauersachs *et al.* 2006). Only animals where pregnancy was confirmed by the presence of a conceptus in the uterine lumen were included in the analysis. Total RNA was extracted from samples using TRIzol reagent (Invitrogen) according to the manufacturer’s instructions.

2.2 Study design of pregnancy recognition in peripheral blood leukocytes

2.2.1 Validation of blood sampling techniques

Sample collection and storage

Blood samples were drawn from the jugular vein of 5 healthy, 24 month old Brown Swiss cyclic heifers as already described (Hammerle-Fickinger *et al.* 2010). Animals belonged to the same herd and obtained the same feeding regime. PAXgene blood RNA tubes (BD, Heidelberg, Germany) and VACUETTE® evacuated blood collection tubes (Greiner Bio-one, Frickenhausen, Germany) were used to collect samples at the same day from each animal. For all extraction methods, samples were collected in triplicates at the same time point from each animal. All blood collection tubes were gently inverted five times for mixing directly after collection. Tubes spray dried with ethylenediaminetetraacetic acid (EDTA) were immediately stored on ice until further processing and PAXgene RNA tubes were incubated at room temperature for two hours and afterwards stored at -20 °C.

Sample Processing and RNA extraction

For each RNA extraction method, blood samples were collected in triplicates from 5 animals (n = 15) (Hammerle-Fickinger *et al.* 2010). First a cost and time-effective method was used which extracted total RNA from whole blood (WB). The second method extracted total RNA from leukocytes after alkaline lyses of erythrocytes (LY). The following three methods were performed using commercially available kits for the extraction of mRNA and miRNA into separated fractions from different blood cells. A summary of all extraction methods is shown in table 2.

Table 2: Summary table of the five blood extraction methods evaluated for validation**.**

Method applied	Equipment necessary before RNA extraction	Length of procedure before RNA extraction or storage	RNA stabilization	Cell types isolated	RNA extraction methodology	Obtained mRNA and miRNA fractions
WB	Vortex mixer	5 minutes	NO	RBC WBC	Phenol based extraction	1 fraction
LY	Swinging bucket centrifuge	40-50 minutes	NO	WBC	Phenol based extraction	1 fraction
LL	-	5 minutes	NO	WBC	mirVana isolation kit	2 fractions
PI	Swinging bucket centrifuge	20-30 minutes	NO	RBC WBC	miRNeasy Mini isolation kit	2 fractions
PAX	-	120 minutes	Yes	RBC WBC	PAXgene blood RNA isolation kit	2 fractions

Extraction of total RNA from whole blood (WB)

In a 2 ml centrifuge tube (Eppendorf, Hamburg, Germany) 350 µl of whole blood were added to 700 µl of TriFast reagent (Peqlab Biotechnologie), mixed for 10 seconds and stored at -80 °C. Total RNA was isolated by a standardized phenol-based extraction method according to the manufacturer's instructions.

Extraction of total RNA from leukocytes after lysis of erythrocytes (LY)

The isolation of leukocytes from 9 ml whole blood collected in EDTA tubes was done by alkaline lysis of erythrocytes. The whole blood was diluted 1:1 (v/v) with lysis buffer (8.3 g NH_4Cl; 0.037 g Na-EDTA; 1 g KCl in 100 ml H2O pH 7.4) and centrifuged for 10 min in 50 ml reaction tubes at 1000 revolutions per minute (rpm) and 4 °C. Supernatants were discarded,

the cell pellet was resuspended twice in lysis buffer and centrifugation was repeated (Prgomet *et al.* 2005). The leukocytes were suspended in a vial containing 1 ml TriFast reagent and ceramic beads, subjected to mechanical homogenization in the MagNA Lyzer instrument (Roche Diagnostics, Mannheim, Germany) for 20 s at 7.000 rpm and stored at -80 °C. Total RNA was extracted from samples according to the manufacturer's instructions.

<u>Extraction of mRNA and miRNA from leukocytes stabilized by LeukoLOCK (LL)</u>

The isolation of the leukocyte population was done immediately after blood collection of 9 ml whole blood in an EDTA tube using the LeukoLOCK™ Total RNA Isolation System (Applied Biosystems). To stabilize the total RNA of cells captured in the filter, RNAlater® was used (Applied Biosystems). The filters were kept on ice and then frozen at -20 ºC within one hour after collection. The isolation of mRNA and miRNA in two different fractions was performed using the mirVana miRNA isolation Kit (Applied Biosystems) following the manufacturer's instructions.

<u>Extraction of mRNA and miRNA from leukocytes after blood fractionation (PI)</u>

RNA was isolated from leukocytes obtained from the plasma interphase of coagulated centrifuged blood. EDTA stabilized blood (9 ml) was centrifuged for 10 min at 3000 rpm and 4 °C. The plasma was removed and the leukocytes were collected with a pipette (~500 µl), suspended in a vial containing 700 µl Qiazol reagent and stored at -80 °C (Qiagen). The isolation of mRNA and miRNA in two different length fractions (shorter than 200 nt and longer than 200 nt, respectively) was performed using the miRNeasy Mini kit (Qiagen) following the manufacturer's instructions.

<u>Extraction of mRNA and miRNA from whole blood collected in PAXgene tubes (PAX)</u>

The isolation of miRNA and mRNA was performed in two different fractions from blood samples collected in PAXgene blood RNA tubes (2.5 ml) (BD). For the extraction the PAXgene Blood RNA Kit (Qiagen) was used employing an amended version of the manufacturer's guidelines as previously described by Kruhoffer et al (2007).

2.2.2 Blood leukocytes study

Pretreatment of animals and sample collection

Cyclic brown Swiss heifers between 20 - 24 months of age were cycle synchronized by a single dose of 2.0 ml Estrumate (Essex Tierarznei, Munich, Germany) injected intramuscularly two times with an interval of 11 days as described in detail elsewhere (Hammerle-Fickinger et. al, in review). Ovulation was synchronized in animals detected in heat by an intramuscular injection of gonadotropin-realizing hormone (Receptal®, Intervet,

Unterschleißheim, Germany) 42 h after the last Estrumate injection. Animals, belonging to the same herd and obtaining the same feeding regime, were divided into NP and XP group. Animals in the XP group were artificially inseminated with cryo-conserved sperm, while NP animals received a seminal plasma insemination derived from the identical bull as from the XP animals. Cows were inseminated on the day of gonadotropin-realizing hormone injection (D0). For transcriptome analysis pair-wise samples were taken. For this, the non-pregnant cycle was followed by an interval of one undisturbed ovulation prior to the ovulation synchronization of the pregnant cycle. In addition, 7 non-pregnant animals and 4 pregnant animals were sampled separately. The sampling scheme is depicted in figure 5.

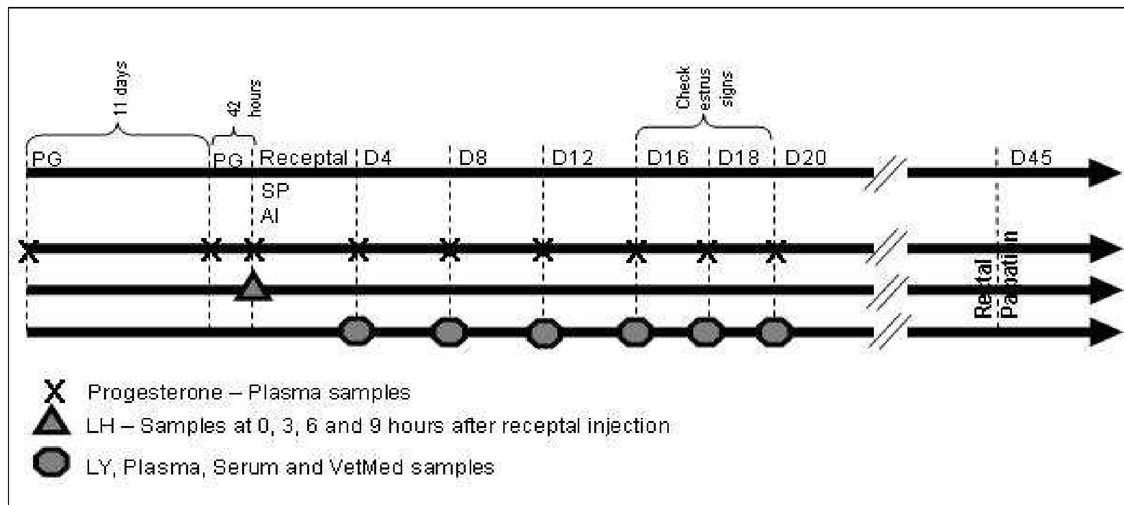


Figure 5: Sampling scheme of the blood leukocytes study.

Blood samples were drawn from the jugular vein and were taken from each animal on days 4, 8, 12, 16, 18 and 20 after insemination. Evacuated EDTA blood tubes (Greiner Bio-one) were used to collect samples from each animal and were immediately stored on ice until further processing. Leukocytes were extracted from whole blood samples after lysis as previously described (Hammerle-Fickinger *et al.* 2010). Pregnancy status for the XP group was confirmed 45 days after fertilization by rectal palpation. Animals not confirmed pregnant were excluded from the study. In total, samples from 10 non-pregnant animals and 8 pregnant animals could be used for further analysis.

Hormone profile and complete blood count

Plasma P4 amount was determined for each animal at the day of sampling using an enzyme immunoassay as previously described (Meyer *et al.* 1990, Prakash *et al.* 1987). Moreover, a complete blood count was performed to gain an appreciation of the health status and the function of organs of each animal at each sampling day (IDEXX Vet Med Labor, Ludwigsburg, Germany). Significant changes of the amount of the different blood cells between the NP and XP groups were determined with an unpaired Student t-test using Sigma-Stat v. 3.0 software (SPSS Inc.,Chicago, IL, USA). Results with $p < 0.05$ were considered as statistically significant.

Extraction of total RNA from leukocytes after lysis of erythrocytes (LY)

Total RNA was extracted from leukocytes cells by using the LY method as described above.

2.3 Study design of pregnancy recognition in milk somatic cells

2.3.1 Validation of milk sampling techniques

Sample collection and analysis of somatic cell count

Milk samples were collected during routine milking as total quarter milk from randomly chosen, healthy, lactating, non-pregnant cows of the Brown Swiss breed. Analysis of the SCC was performed in an accredited milk laboratory (Milchprüfring Bayern e.V., Wolnzach, Germany). The milk samples were stored at 4 °C after collection until further processing within the next three to six hours.

Sample Processing and RNA extraction

To obtain milk somatic cells, cell pellet was isolated by centrifugation and washed with PBS. Therefore, a pre-validation method for somatic milk cells extraction was performed, to optimize following parameters: PBS volume (1.42 g $Na2HPO4*2H2O$; 0.2 g KCL; 0.2 $KH2PO4$; 8 g NaCl in 1000 ml $H2O$ pH 7.4), number of cell pellet wash steps, milk storage time and extraction method of total RNA. For RNA extraction two different methods were tested, the miRNeasy Mini Kit (Qiagen) and peqGOLD TriFast (Peqlab Biotechnologie). They are both commercially available kits and were used according to manufactures protocol.

2.3.2 Milk somatic cells study

Pretreatment of animals and sample collection

Dairy Brown Swiss animals between the first and fourth lactation, belonging to the same herd and obtaining the same feeding regime, were divided into non-pregnant and pregnant group. XP animals were artificially inseminated with cryo-conserved sperm, while NP animals were not inseminated. Cows were inseminated on the day of estrus sign (D0), the sampling scheme is depicted in figure 6.

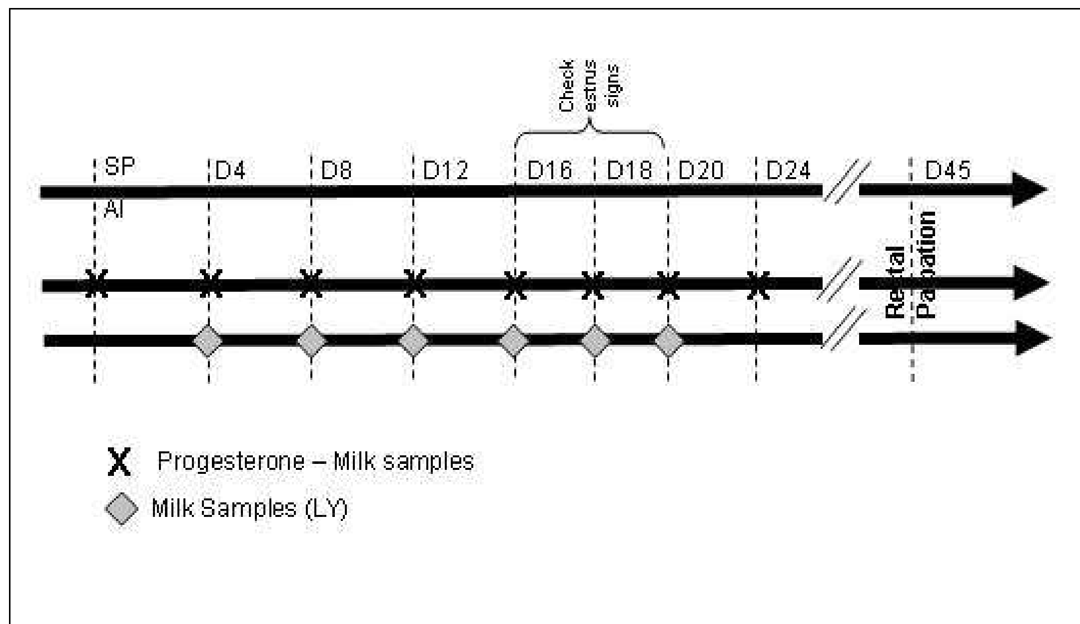


Figure 6: Sampling scheme of the milk somatic cells study.

Milk samples were collected during routine milking as total quarter milk on days 4, 8, 12, 16, 18 and 20 after estrus. Cleaned plastic bottles were used to collect samples from each animal and were immediately stored on ice until further analysis. Somatic cells were extracted from milk as described above. Pregnancy status for the XP group was confirmed 45 days after fertilization by rectal palpation. Animals not confirmed pregnant were excluded from the study. In total, samples from 5 non-pregnant and 5 pregnant animals could be used for further analysis.

Hormone profile

Hormone profile (P4) was generated for each animal at the day of sampling from fat free milk. Fat was separated from milk by centrifugation at 3.000 rpm for 15 min and frozen at -20 °C until further analysis. The P4 concentration was determined by an enzyme immunoassay

as published previously (Meyer *et al.* 1990, Prakash *et al.* 1987). Animals with an abnormal P4 profile from D0 to D24 were excluded from the study.

Extraction of RNA from milk somatic cells

The isolation of somatic cells from 450 ml fresh milk was done by centrifugation at 1500 g at 4 °C for 30 min to obtain a cell pellet. The supernatant fat layer was discarded; the cell pellet was resuspended in 5 ml PBS and transferred to a 50 ml Falcon tube (Greiner Bio-one) to be washed with a total of 50 ml 4 °C cold PBS buffer. After cell pellet resuspension centrifugation was performed with 1500 g at 4 °C for 15 min.
Afterwards, the supernatant was discarded carefully and the remaining cell pellet suspended in a vial containing 700 µl Qiazol reagent (Qiagen) and ceramic beads, subjected to mechanical homogenization in the MagNA Lyzer instrument (Roche) for 20 s at 7.000 rpm and stored at -80 °C. Total RNA was extracted from milk somatic cells using miRNeasy Mini Kit (Qiagen) according to the manufacturer's instructions.

2.4 RNA concentration and quality determination

Extracted amounts of total RNA, mRNA and miRNA were quantified using the NanoDrop 1000 (Peqlab Biotechnologie). RNA purity was estimated based on the OD 260/280 ratio. RNA integrity and quality control was additionally measured for all samples via automated capillary electrophoresis using the Eukaryotic total RNA Nano assay on the 2100 Bioanalyzer (Agilent Technologies). The 2100 Bioanalyzer calculates the RIN ranging from 1 being the most degraded profile to 10 being the most intact. In addition RNA integrity was also evaluated by analysis of the electropherogram obtained (Fleige & Pfaffl 2006).
For the small RNA fraction the integrity of a sample can not be evaluated by a RIN number. In this case an analysis of the miRNA percentage in the small RNA fraction can be measured using the Small RNA Assay (Agilent Technologies) on the 2100 Bioanalyzer. The software calculates the miRNA percentage as a ratio of the miRNA concentration existent in total small RNA (Becker *et al.* 2010).

2.5 Target gene selection and primer design

Target genes (TG) were selected by screening the actual literature for candidate genes which are know to be related with maternal recognition of pregnancy (table 3).

Table 3: Primer sequences of all investigated TG in alphabetically order with their product length in base pair [bp] and accession number.

Gene		Sequenz (5´-3´)	Acc. number	Product length [bp]
18s rRNA	for	AAGTCTTTTGGGTTCCGG	AF176811	488
	rev	GGACATCTAAGGGCATCACA		
ACTB	for	AACTCCATCATGAAGTGTGAC	AY141970	202
	rev	GATCCACATCTGCTGGAAGG		
BOLA	for	ATGGGAACCTCCTCAGACCT	DQ121134	105
	rev	CGCTTCTTCCTCCAGATCAC		
BST2	for	ACACTGAACCGTTGTCCTCC	NC_007305.3	145
	rev	GGGCAGTGGACTCTCTGAAG		
C1Q	for	ATTGAAAGGCACCAAAGGC	NM_001014945	144
	rev	TTCTGGTACACGTTCTCCTGG		
C1R	for	AGGGGATAGTGGAGCGGTC	NM_001733	117
	rev	GGACATTGGTGTAAAACCCG		
C1S	for	AACAGGAGTGGGTCATCCAG	NM_001734	149
	rev	CGGCTGTGTTGGTCTTTCAG		
C2	for	ATGGAACTGGGACCAACATC	NM_001034492	148
	rev	CCCATGTTGGACTTTCCATC		
C3	for	AAGTTCATCACCCACATCAAG	NM_001040469	191
	rev	CACTGTTTCTGGTTCTCCTC		
C3AR	for	TTTCTCCATCTCACCTTGGC	Alignment	111
	rev	GAGCTTGCATAGGAACCAGC		
C4	for	AATCCAGGGTGCAGTACACTG	U16749	128
	rev	GGTCAGCTTCTCCAGGTCAG		
C5	for	AACGCAAACGCAGATGACACC	NM_001166616.1	238
	rev	CGATGGCACAACAGCTCTTG		
C5AR	for	ATACCGTCCTTTGTGTTCCG	NM_001007810	158
	rev	ATTGTAAGCGTGACCAGCG		
C9	for	AGTTGGCTGCTGAGGCTAAG	BC105174	147
	rev	CCCGACTTCTCATCACAAATC		
CD46	for	TTTGTGTCAACCACCTCCAG	NM_183080	118
	rev	TTCATCTGGTCCATTTGAAGG		
CD55	for	ATTTTGTGTGGTCCAAACCTG	NM_001030303	187
	rev	GTATTTCCCACAGGAGCACAG		
CFB	for	TTGAAGGCAGGGACCAATAC	XM_591873	130
	rev	GCAAACCGTCAGTCATGATG		
CFD	for	AGCTCTCTGAGAAAGCCGTG	NM_001034255	159
	rev	GCAGGAGTAGGTGCTGCAG		
CFH	for	AGAACCATGCCTCAGACAATG	X98697	160
	rev	GCCACTCTCTGTGCAGGTC		
CFI	for	AATGGGAAACGCATTCCTC	NM_001038096	159
	rev	GTCCAGCTCACCATTGCAC		
CPN1	for	TGTCCTTTCAGCCAATCTCC	NM_001076080	124
	rev	AAGCTTGTCATCAGGCGTG		

Table 3 (continued)

Gene		Sequenz (5´-3´)	Acc. number	Product length [bp]
CR1	for	CCATTTGCTGTTTGGTTGG	Alignment	111
	rev	CTATGGAATCTGGATTTAGGCG		
CR3	for	GGGAGAGCAGGAGAACCAG	AY841169	125
	rev	GCCCGAAATACTGAAGCTTG		
CSF1	for	GATGATCCCGTGTGCTACCT	NM_174026	143
	rev	AAGCAGCTCTTCAGCCTCAG		
CXCL17	for	TCAGTCAACAAAGCCGTGAG	BC134719.1	96
	rev	TGCTTTGGGGATGATTTAGG		
GAPDH	for	GTCTTCACTACCATGGAGAAGG	U85042	197
	rev	TCATGGATGACCTTGGCCAG		
HIS3	for	ACTGCTACAAAAGCCGCTC	AF469469	233
	rev	ACTTGCCTCCTGCAAAGCAC		
IDO	for	GGGCCCATGACTTATGAGAA	BC151535	107
	rev	GAGGCAGCTGCTATTTCCAC		
IFI16	for	GCCTGACTGTGTTGACAAGC	BC111608.1	217
	rev	CACAGGTGTATCCTTGACTG		
IFI16	for	CAGTTCCCCTTTTCCCTGTT	BC111608.1	191
	rev	GTGCCTCAGTGGGATGTCTT		
IFI44	for	GGCTTGTTGAAATGGAAGATG	XM_872122.3	208
	rev	ATCACCATAGGCCTTGAGC		
IFIT2	for	TTCTTCCGTATCGGCTCC	BT025389.1	196
	rev	CCTGTATGAAATGGTGGATGG		
IGF2	for	ACCCTCCAGTTTGTCTGTGG	BC126514	166
	rev	ACACATCCCTCTCGGACTTG		
IL18	for	GAAGTGTTCCAGGACATGATG	EU574909.1	222
	rev	CTAGTTCTGGTTTTGAACAGTGAAC		
IL1B	for	TTCTCTCCAGCCAACCTTCATT	M37211	198
	rev	ATCTGCAGCTGGATGTTTCCAT		
ISG15	for	ACTCCATGACGGTATCCGAG	NM_174366	203
	rev	ACCCTTGTCGTTCCTCAC		
MX1	for	GTACGAGCCGAGTTCTCCAA	AF047692	197
	rev	ATGTCCACAGCAGGCTCTTC		
MX2	for	CTTCAGAGACGCCTCAGTCG	NM_173941	232
	rev	TGAAGCAGCCAGGAATAGTG		
OAS1	for	GATGAGGCTCTTCAGCTTGG	NM_178108.2	201
	rev	GATGAGGCTCTTCAGCTTGG		
OOSP1	for	TGCCAAGATTAAACCCACAC	NR_024196.1	86
	rev	CGTAGGTTACAGGGCAGTCA		
SERPING1	for	ACCAACCTCAGGATCAGGC	NM_174821	149
	rev	CTATCTTCCACTTGGCGCTC		
STAT1	for	CTCATTAGTTCTGGCACCAGC	NM_001077900.1	108
	rev	CACACGAAGGTGATGAACATG		
TNFa	for	CCACGTTGTAGCCGACATC	AF348421	108
	rev	ACCACCAGCTGGTTGTCTTC		
UBQ	for	AGATCCAGGATAAGGAAGGCAT	Z18245	198
	rev	GCTCCACCTCCAGGGTGAT		
XAF1	for	GAGGAGGCTCTGAGCTTGC	BT021626	143
	rev	GCAGAGAAAGATGTCCGTCC		
YWHAZ	for	CAGGCTGAGCGATATGATGAC	NM_174814	141
	rev	GACCCTCCAAGATGACCTAC		

Primer pairs were designed using published bovine nucleic acid sequences of GenBank (http://www.ncbi.nlm.nih.gov/entrez/query.fcgi) or Ensemble (http://www.ensembl.org/index.html). Designed primers were ordered and synthesized at Eurofins MWG (Ebersberg, Germany).

2.6 Quantitative RT-PCR

2.6.1 Gene expression analysis in endometrium

A two-step RT-qPCR was performed as 1.0 µg of total RNA were reverse transcribed to cDNA as described earlier (Ulbrich *et al.* 2004). Gene expression was quantified using the LightCycler DNA Master SYBR Green I protocol (Roche) as already described (Ulbrich *et al.* 2009b). Sequences of commercially synthesized PCR primer pairs (Eurofins MWG), accession number and product length [bp] are depicted in table 3. The quantitative cycle (Cq) required achieving a definite fluorescence signal was calculated by the "Second Derivative Maximum" method (LightCycler software, version 4.05). The Cq is inversely proportional to the logarithm of the initial template concentration.

2.6.2 Gene expression analysis for validation of blood sampling

mRNA expression analysis

A two-step RT-qPCR was performed as 0.5 µg of total RNA were reverse transcribed to cDNA as already described (Ulbrich et al. 2004). Gene expression was quantified using the Mastercycler ep realplex (Eppendorf). Quantitative RT-PCR was performed using the 2.5 x 5 PRIME qPCR MasterMix Kit (5 PRIME, Hamburg, Germany). A master mix was prepared as follows: 6.75 µl RealMasterMix, 0.75 µl forward primer (10 pmol/µl), 0.75 µl reverse primer (10 pmol/µl) and 4.75 µl RNase free water. For RT-qPCR analysis 2.0 µl cDNA were added to 13.0 µl Master Mix. Sequences of commercially synthesized PCR primer pairs (Eurofins MWG), accession number and product length [bp] are depicted in table 3.
The following general RT-qPCR protocol was employed: denaturation (95 °C, 2 min), cycling program [40 cycles: 95 °C denaturation (5 s); 60 °C annealing (10 s); 68 °C elongation (20 s)] and finally a melting curve analysis. The Cq value of a sample was set on the cycle number at which the fluorescence signal intersected with the threshold. This value was determined automatically by the CalQplex realplex software (Version 1.5, Eppendorf) and was significantly above the noise of the baseline.

miRNA expression analysis

A two-step RT-qPCR was performed as either 0.125 µg purified small RNA (from LL, PI, PAX isolation methods) or 0.5 µg total RNA (from WB and LY isolation methods) were first reverse transcribed to cDNA in a 10 µl reaction volume using the miScript Reverse Transcription Kit (Qiagen). Gene expression was quantified using the Mastercycler ep realplex (Eppendorf). Quantitative RT-PCR was performed using the miScript SYBR® Green PCR kit (Qiagen) according to the manufacturer's instructions. A master mix was prepared as follows: 7.5 µl QuantiTect SYBR Green PCR mastermix, 1.5 µl 10 x universal primer, 1.5 µl 10 x miScript primer assay and 2.5 µl RNase free water. For RT-qPCR analysis 2.0 µl cDNA was added to 13.0 µl Master Mix.

The following general RT-qPCR protocol was employed: denaturation (95 °C, 15 min), cycling program [40 cycles: 94 °C denaturation (15 s); 55 °C annealing (30 s); 70 °C elongation (30 s)], and melting curve analysis. Following miRNA were quantified: MIR let-7a, MIR 27b, MIR 101, MIR 145, MIR 142, MIR 181a, and MIR 16. The sequences of these miRNA in various species are published at miRBase (http://microrna.sanger.ac.uk/sequences/). The primer homology to bovine was controlled and confirmed with the "Basic Local Alignment Search Tool" (http://blast.ncbi.nlm.nih.gov/Blast.cgi) and miRNA primer were commercially synthesized (Qiagen). Pipetting was done by the epMotion 5075 robot system (Eppendorf) and measurements by the Mastercycler ep realplex (Eppendorf). The Cq value of each sample was determined automatically by the CalQplex realplex software (Eppendorf). The predicted size of RT-qPCR products was assessed via capillary electrophoresis in the Experion system using the Experion RNA StdSens kit (Bio-Rad Laboratories, Munich, Germany).

2.6.3 Gene expression analysis on blood leukocytes and on milk somatic cells

A two-step RT-qPCR was performed as 0.5 µg of total RNA were reverse transcribed to cDNA as described earlier (Ulbrich *et al.* 2004). Gene expression was quantified by using the iQ5 (Bio-Rad). Quantitative RT-PCR was performed by using the MESA GREEN qPCR MasterMix Plus for SYBR® Assay w/ fluorescein Kit (Eurogentec, Cologne, Germany). A master mix was prepared as follows: 7.5 µl MESA GREEN 2 x PCR Master Mix, 0.5 µl forward primer (10 pmol/µl), 0.5 µl reverse primer (10 pmol/µl) and 4.5 µl RNase free water. For RT-qPCR analysis 2.0 µl cDNA was added to 13.0 µl Master Mix.

The following general RT-qPCR protocol was employed for all investigated factors: denaturation for 5 min at 95 °C, 40 cycles of a two segmented amplification and quantification program (denaturation for 3 s at 95 °C, annealing for 10 s at primer specific

annealing temperature) and a melting curve analysis. The melting curves and Cq values were acquired by using the iQ5 Optical System software 2.0. A negative control was included by measuring water instead of cDNA. All amplified PCR fragments were commercially sequenced to verify the resulting PCR product (Eurofins MWG). Sequences of commercially synthesized PCR primer pairs (Eurofins MWG), accession number and product length [bp] are depicted in table 3.

2.6.4 Statistical analysis of gene expression data

A statistical analysis of the gene expression data was conducted using Sigma-Stat v. 3.0 software (SPSS Inc.). For each target the gene identical amplification efficiency of 100% was assumed, allowing a more simple quantification model (Livak & Schmittgen 2001). Suitable reference genes RG for normalization of the gene expression data were evaluated individually for each study using the geNorm algorithm in GenEx version 5.0 (MultiD Analyses AB, Gothenburg, Sweden). The optimal RG was selected using pairwise variation analysis integrated in geNorm algorithm implemented in GenEx (MultiD Analyses AB). The most suitable RG is classified by the M-value, which is defined by calculating the mean pairwise variation between a particular gene and all the others used (Vandesompele *et al.* 2002). The highest M-value corresponds to the least stable expression in a set of samples.
If more than one RG was determined, the RG arithmetic mean was used as RG index. Target genes were normalized against the RG index (Livak & Schmittgen 2001) and presented as ΔCq. For relative quantification of mRNA, ΔCq values of the NP group was compared to the XP group, according to the the ΔΔCq-model as following:

$$\Delta Cq = Cq_{\text{(target gene)}} - Cq_{\text{(reference gene index)}}$$

$$\Delta\Delta Cq = \text{mean } \Delta Cq_{\text{(treatment)}} - \text{mean } \Delta Cq_{\text{(control)}}$$

$$\text{Expression ratio} = 2^{-\Delta\Delta Cq}$$

The expression ratio of the treatment group compared to the control group is represented as mRNA expression ratio or X -fold regulation, where a value of 1.0 indicates no expression change after treatment. Significant differences between both groups were calculated using Student t-test. Results were considered as statistically significant at $p < 0.05$. Graphs were plotted using Sigma-Plot 8.0 (SPSS Inc.). Values were presented as (20 - ΔCq) to allow a relative comparison between different genes.
Hierarchical cluster analysis (visualized via a heat map) based on mean centered values (ΔCq value of a gene minus mean of all ΔCq values of this gene) was performed using GenEx version 5.0 (MultiD Analyses AB). To analyze the relationship between treatment and

control group PCA was employed using GenEx version 5.0. For mRNA and miRNA gene expression results means were compared by t-test, two-way and three-way ANOVA using Sigma-Stat v. 3.0 software (SPSS Inc.). All pair wise multiple comparison procedures were done by the Holm-Sidak method.

2.7 Microarray analysis

2.7.1 Gene expression analysis in endometrium

Endometrial tissue samples from day 18 pregnant (n = 4) and corresponding control samples (n = 4) were analyzed with Affymetrix Bovine Genome Arrays (Affymetrix, Santa Clara, California). The Affymetrix cell intensity files (CEL) derived from this analysis were processed with Robust Multi-array Analysis (RMA) and the probe sets were in addition filtered based on present calls. RMA is a popular R package used to preprocess Affymetrix oligonucleotide array data (Irizarry *et al.* 2003). The intensity values of this filtered probe sets were then analyzed using Significance Analysis of Microarrays (SAM) Excel Add-in in order to find differentially expressed genes (Tusher *et al.* 2001). These analyses were performed by Dr. S. Bauersachs, Institute of Molecular Animal Breeding and Biotechnology, Munich.

2.7.2 Gene expression analysis on blood leukocytes

Blood samples collected from pair-wise non-pregnant (n = 3) and pregnant (n = 3) Brown Swiss heifers on days 4, 8 and 12 after estrus and insemination were submitted to hybridization analysis. Microarray analysis was performed using 4 x 44k Custom Bovine Gene Expression microarrays (Agilent Technologies). Cy3-labeled cRNA was produced using the Quick Amp Labeling Kit, one-color (Agilent Technologies) and hybridized to the microarrays according the manufacturer's instructions. Hybridized and washed slides were scanned at 3 µm resolution with an Agilent DNA Microarray Scanner (G2505C, Agilent Technologies). Image processing was performed with Feature Extraction Software 10.5.1.1 (Agilent Technologies). Processed signals were filtered based on “Well above background” flags (detection in all 3 samples in either one of the experimental groups) and subsequently normalized with the BioConductor package VSN (Huber *et al.* 2002). For quality control normalized data was analyzed with a distance matrix and a heatmap based on pair-wise distances (BioConducter package geneplotter). The statistics analysis was performed using first Microsoft Excel add-in SAM, two-class (Tusher *et al.* 2001) as well as the BioConductor package paired local pooled error method (PLPE) for comparison between NP and XP animals (n = 3) within samples taken at days 4, 8, and 12. These analyses were performed by Dr. S. Bauersachs, Institute of Molecular Animal Breeding and Biotechnology, Munich.

The correlation of RT-qPCR and cDNA array hybridization results was calculated by linear regression. Linear regression is a mathematical approach which models the relationship between two set of data. The accuracy of the data to the linear equation is represented by R^2, where $R^2 = 0$ represents no correlation and $R^2 = 1$ represents perfect correlation between the two set of data.

2.8 Determination of C1Q concentration by ELISA

The complement C1Q concentration was evaluated in bovine serum comparing both the NP and XP group at days 8 (n = 8), 18 (n = 3) and 20 (n = 3). The serum was separated by centrifugation at 3.000 rpm for 10 min and frozen at -20 °C until analysis. The determination of C1Q concentration was performed by using a commercially available ELISA kit for bovine complement 1q (Uscn Life Science Inc., Wuhan, China) according to the manufacturer's instructions.

3 Results

3.1 Analysis of pregnancy recognition regarding the complement system activation in endometrium

3.1.1 Expression of complement system factors by RT-qPCR

Selected factors from the complement system were analyzed by RT-qPCR on endometrial tissue samples obtained from paired animals (monozygotic twin study). From the classical pathway, five selected genes (C1R, C1Q, C1S, C2 and C4) were measured. As reference genes, Polyubiquitin 3 (UBQ) and Histone H3 (HIS3) were considered. RNA integrity numbers (RIN) ranged between 7 and 10 and showed intact RNA ready for downstream RT-qPCR studies. The differential gene expression ranged from 2.0 to 16.0 -fold ($p < 0.05$) (table 4). To verify the results obtained, one selected gene (C1Q) was analyzed on endometrial non-paired samples taken from cyclic animals in study A, showing a significant up-regulation of 2.31 -fold ($p < 0.05$) in correlation with results obtained from the Twin study.

Table 4: Gene expression results on endometrial tissue. Bold values represent significant regulated genes. For microarray analysis results, ND stands for not detectable.

	RT-qPCR								Microarray	
	Twin study				***Cyclic study A***				***Cyclic study B***	
	20-ΔCq ± SEM				20-ΔCq ± SEM					
	Control	Treatment	fold	*p* -value	Control	Treatment	fold	*p* -value	fold	*p* -value
Classical pathway										
C1R	26.71 ± 0.31	28.95 ± 0.62	**4.79**	**< 0.05**					**2.10**	**< 0.001**
C1Q	22.31 ± 0.17	23.50 ± 0.27	**2.30**	**< 0.01**	21.62 ± 0.30	22.82 ± 0.35	**2.31**	**< 0.05**	**2.20**	**< 0.001**
C1S	26.76 ± 0.17	29.11 ± 0.46	**5.10**	**< 0.01**					**1.86**	**< 0.001**
C2	21.82 ± 0.41	25.81 ± 0.58	**15.89**	**< 0.001**					**4.06**	**< 0.001**
C4	25.55 ± 0.45	28.56 ± 0.37	**8.06**	**< 0.001**					**3.89**	**< 0.001**
Alternative pathway										
C3	25.12 ± 0.35	26.40 ± 0.32	**2.43**	**< 0.05**	24.14 ± 0.29	25.14 ± 0.52	2.00	0.22	**2.28**	**< 0.001**
CFB	19.67 ± 0.16	21.23 ± 0.25	**2.97**	**< 0.001**	21.21 ± 0.21	21.80 ± 0.47	1.50	0.38	**5.04**	**< 0.001**
CFD	21.37 ± 0.28	20.75 ± 0.37	0.65	0.216					**0.38**	**< 0.05**
Inhibitor proteins										
CFH	22.77 ± 0.45	22.38 ± 0.31	0.76	0.494					0.13	> 0.05
CFI	25.05 ± 0.62	24.85 ± 0.39	0.87	0.792	21.25 ± 0.23	23.18 ± 0.58	**2.92**	**< 0.05**	**2.15**	**< 0.001**
CPN1	21.41 ± 0.23	21.63 0.17	1.20	0.467					0.12	> 0.05
CD46	29.09 ± 0.11	29.17 ± 0.23	1.06	0.736	28.10 ± 0.25	27.77 ± 0.18	0.79	0.45	0.04	> 0.05
CD55	27.92 ± 0.29	28.13 ± 0.30	1.16	0.632	27.71 ± 0.44	26.84 ± 0.17	0.55	0.11	0.03	> 0.05
SERPING1	27.47 ± 0.22	29.58 ± 0.24	**4.30**	**< 0.01**	26.87 ± 0.27	28.97 ± 0.16	**4.27**	**< 0.05**	**2.19**	**< 0.001**
Activator proteins										
C3AR	22.62 ± 0.21	24.25 ± 0.44	**3.10**	**< 0.01**	21.89 ± 0.04	23.83 ± 0.22	**3.82**	**< 0.01**	ND	
C5AR	17.06 ± 0.51	18.15 ± 0.53	2.13	0.179					**2.34**	**< 0.001**
CR1	19.96 ± 0.67	18.68 ± 0.44	0.41	0.148					ND	
CR3	19.88 ± 0.33	20.52 ± 0.59	1.56	0.376					ND	
Terminal Components										
C5	20.08 ± 1.15	19.12 ± 0.71	1.95	0.497	18.86 ± 0.59	18.61 ± 0.38	0.84	0.53	ND	
C9	13.99 ± 0.51	15.37 ± 0.61	2.60	0.112					ND	

Two selected genes (C3 and CFB) from the alternative pathway measured on samples taken from the twin study showed a significant up-regulation of 2.43 to 2.97 -fold ($p < 0.05$) respectively (table 4). No significant changes for C3 and CFB were measured for samples taken from cyclic animals in study A (table 4). In addition, inhibitor protein factors were analyzed and significant up-regulation of 4.30 and 4.27 -fold ($p < 0.05$) for SERPING1 was measured for the twin study and the cyclic study A respectively (table 4). For the inhibitory factor of the alternative pathway (CFI), a significant up-regulation of 2.92 -fold ($p < 0.05$) was measured only for the non-paired samples from study A (table 4).

The activator protein complement component 3a receptor (C3AR) demonstrated 3.10 and 3.82 -fold ($p < 0.01$) gene expression increase for samples taken from twin and cyclic animals in study A respectively (table 4). Comparing pregnant animals vs. non-pregnant animals at day 18 after insemination, no gene expression variation for the terminal components was measured at any study model (table 4).

3.1.2 Expression of complement system factors by microarray analysis

A microarray analysis was performed within samples taken from cyclic animals in study B. The results concerning the classical pathway showed significant up-regulation for factors C1R, C1Q, C2 and C4 between 2.0 to 4.0 -fold ($p < 0.001$), supporting results obtained by RT-qPCR analysis (table 4). The direction of regulation between both methods was in agreement in all cases.

For the alternative pathway, a significant increase of factors C3 and CFB of 2.28 and 5.04 -fold ($p < 0.001$) was measured respectively, correlating within results obtained from the twin study (table 4). Results for inhibitory protein sustain results obtained from the twin study and the cyclic study A where a significant up-regulation of 2.19 -fold ($p < 0.001$) for SERPING1 was also detected (table 4). Microarray CFI measurements revealed a significant 2.15 -fold change in gene expression ($p < 0.001$) as measured from cyclic animals in study A (table 4). In summary, the expression ratios obtained by array hybridization and RT-qPCR for significantly expressed genes correlated well.

3.1.3 Expression of complement system factors in an *in vitro* co-culture system

A RT-qPCR analysis was performed for GE after IFNT stimulation (table 5). The expression of C1R and C2 showed significant up-regulation of 2.08 and 3.95 -fold ($p < 0.05$) respectively, after IFNT treatment in GE. In addition, for the activator factor C3 from the alternative pathway a significant increase of 1.63 -fold ($p < 0.05$) after IFNT treatment in GE was measured.

Table 5: Gene expression results in GE after stimulation with IFNT. Bold values represent significant regulated genes.

	mRNA expression Glandular epithelial cells (GE)			
	20-ΔCq ± SEM		fold regulation	*p* -value
	Control	Treatment		
Classical pathway				
C1R	**18.33 ± 0.84**	**19.39 ± 0.80**	**2.08**	**< 0.05**
C2	**19.45 ± 0.74**	**21.43 ± 0.46**	**3.95**	**< 0.01**
Alternative pathway				
C3	**20.72 ± 0.63**	**21.42 ± 0.50**	**1.63**	**< 0.05**
CFB	26.28 ± 0.51	26.26 ± 0.40	0.99	0.94
Inhibitor proteins				
CFI	23.02 ± 0.52	23.30 ± 0.36	1.21	0.3556
CD46	**28.52 ± 0.11**	**28.91 ± 0.14**	**1.31**	**< 0.01**
CD55	**26.12 ± 0.23**	**26.21 ± 0.19**	**1.63**	**< 0.05**
SERPING1	**21.45 ± 0.32**	**22.00 ± 0.34**	**1.46**	**< 0.001**
Activator proteins				
C3AR	**17.11 ± 0.20**	**18.31 ± 0.29**	**2.04**	**< 0.01**
Terminal Components				
C5	**12.95 ± 0.40**	**17.97 ± 0.34**	**32.48**	**< 0.001**
C9	18.64 ± 0.33	18.71 ± 0.29	1.05	0.72

The inhibitory proteins CD46, CD55 and SERPING1 expressed in GE were also influenced by IFNT stimulation generating significant up-regulation of 1.31, 1.63 and 1.46 -fold ($p < 0.05$) respectively (table 5). Measurements of factor C3AR in GE also showed a significant 2.04 -fold increase ($p < 0.01$) following IFNT treatment. For the terminal factor C5 a high up-regulation of 32.48 -fold ($p < 0.05$) for GE was detected (table 5). In summary, gene expression for C1R, C2, C3 and C3AR obtained for GE after IFNT treatment correlated well with the results described above from the *in vivo* experiments. Interestingly, the inhibitory protein CD46 and CD55 and the terminal component C5 were significantly up-regulated in GE after IFNT treatment, which was not the case for the *in vivo* studies measured in endometrium tissue after 18 days of insemination.

3.2 Analysis of pregnancy recognition in peripheral blood leukocytes

3.2.1 Validation of blood sampling techniques

RNA quantity and quality

The amounts of total RNA, mRNA and miRNA measured varied with respect to the different extraction approach. The results are depicted in table 6.

Table 6: RNA quantity, RNA purity, RIN of large RNA (> 200 nt) and small RNA (< 200 nt).

		RNA (> 200nt)		
Extraction method	**Total volume (ml)**	**µg/ml blood**	**OD 260/280**	**RIN**
LY	9	4.44 ± 0.30	2.02 ± 0.01	9.45 ± 0.04
PI	9	4.53 ± 0.58	1.95 ± 0.01	7.37 ± 0.90
WB	0.35	14.32 ± 1.10	1.71 ± 0.01	4.96 ± 0.58
PAX	2.5	1.68 ± 0.14	2.01 ± 0.02	8.87 ± 0.14
LL	9	3.10 ± 0.23	2.10 ± 0.01	7.04 ± 0.53
		RNA (< 200nt)		
		µg/ml blood	**OD 260/280**	**miRNA %**
LY	9	---	---	6.13 ± 0.27
PI	9	0.34 ± 0.04	1.23 ± 0.03	29.13 ± 5.48
WB	0.35	---	---	NA
PAX	2.5	1.51 ± 0.18	1.18 ± 0.10	6.33 ± 1.74
LL	9	8.68 ± 0.73	1.57 ± 0.04	NA

For the two phenol-based extraction methods (LY and WB) the total RNA was isolated and therefore no absolute quantification of small RNA using the NanoDrop 1000 could be evaluated. Only the relative estimation by the Bioanalyzer analysis was possible. In the case of WB and LL extraction methods the miRNA percentage in the small RNA fraction measured was not available (NA). Results are presented as the mean values ± standard error of mean (SEM) for triplicate samples from 5 animals. Significant differences in the mean OD 260/280 ratios and in the mean RIN values among the different methods (LY, PI, WB, PAX and LL) were observed and shown in table 6 and 7 (Hammerle-Fickinger *et al.* 2010). Considering all five extraction methods of total RNA / mRNA a three-way ANOVA test was evaluated (table 7).

Electropherograms of total RNA from all samples of LY, PI, PAX, and LL extraction methods had a comparable profile without undefined peaks. By contrast, all samples of WB extraction method resulted in electropherograms with unexpected peaks. In addition to these results, the analysis of each small RNA electropherogram showed that samples extracted by the LY and the PAX methods had an uniform profile with no unexpected peaks. For the other methods considered, the electropherograms showed unexpected peaks for all samples.

Table 7: Three-way ANOVA results of RNA characteristics. DF stands for degrees of freedom, SS for sum of squares, MS for mean square, F for F-statistic test and P for the probability value. Significant differences are considered if P < 0.05.

Source of Variation	ANOVA statistics				
	df	SS	MS	F	P
RNA Yield					
Extraction Methods	4	1502.09	375.52	76.33	<0.001
Animal	4	74.2	18.55	3.77	0.013
Replicates	2	1.85	0.92	0.18	0.829
RNA Integrity Number (RIN)					
Extraction Methods	4	185.35	46.34	15.84	<0.001
Animal	4	24.56	6.14	2.1	0.104
Replicates	2	8.11	4.05	1.38	0.265
RNA Purity ($A_{260/280}$)					
Extraction Methods	4	1.5	0.37	250.51	<0.001
Animal	4	0.015	0.0039	2.61	0.053
Replicates	2	0.00039	0.00019	0.13	0.877
miRNA Yield					
Extraction Methods	2	1165685.68	582842.84	185.52	<0.001
Animal	4	92742.58	23185.64	7.38	0.001
Replicates	2	7950.95	3975.47	1.26	0.309
% miRNA					
Extraction Methods	2	5244.4	2622.2	12.25	<0.001
Animal	4	626.97	156.74	0.73	0.583
Replicates	2	140.93	70.46	0.32	0.724

The results obtained for the determination of the mRNA quality and miRNA percentage as mean RIN number ± SEM versus mean miRNA% ± SEM for each extraction method are summarized in figure 7. To obtain a high quality of mRNA and high miRNA percentage in the same sample, the LY extraction method gives the best results with the lowest variability.

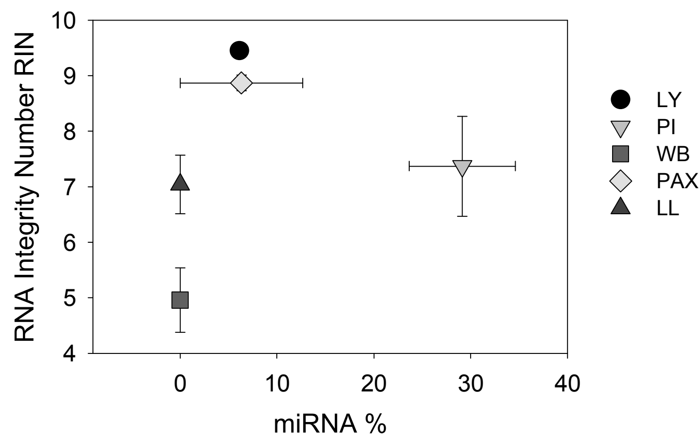


Figure 7: Quality of mRNA versus miRNA percentage.

mRNA quantification and data analysis

Different gene transcript abundances of mRNA were quantified showing the best mRNA RT-qPCR performance for samples extracted by the LY and PAX methods (Hammerle-Fickinger *et al.* 2010). The results shown in table 8 compare the methods PI, WB, PAX, and LL to the LY method statistically, which was considered to give the lowest Cq with the least SEM.

Table 8: Gene expression was determined for each mRNA transcript. Numbers in bold are equivalent to the lowest mean Cq ± SEM value considering each gene and the different extractions. Numbers in italic are equivalent to the highest Cq ± SEM value. Significance comparing all methods in relation to the best method (LY) is given as: * for P < 0.05, ** for P < 0.01 and *** for P < 0.001.

	Mean expression levels				
	LY	**PI**	**WB**	**PAX**	**LL**
18S rRNA	**17.28±0.16**	20.37±0.51***	21.22±0.35***	18.15±0.18	*22.24±0.95****
ACTB	18.98±0.10	19.28±0.20	20.59±0.18**	**18.85±0.11**	*22.33±0.80****
UBQ3	**22.26±0.20**	23.29±0.22**	24.84±0.24***	22.52±0.12	*24.97±0.50****
HIS3	30.00±0.21	**27.98±0.53****	30.79±0.36	28.92±0.47	*33.13±0.62****
IL1B	**22.34±0.09**	*27.67±0.41****	25.42±0.12***	23.15±0.11	25.96±0.70***
CD14	25.74±0.20	**25.37±0.13**	27.11±0.14***	26.34±0.14*	*27.75±0.40****
C3	**26.36±0.22**	27.80±0.23***	28.59±0.18***	27.29±0.17*	*28.63±0.50****
C1Q	25.9±0.13	**24.99±0.14*****	26.34±0.19*	25.99±0.11	*27.44±0.30****
CSF1	28.26±0.14	**25.53±0.19*****	26.94±0.24***	28.17±0.14	*29.3±0.31****
NFKB1	**24.77±0.11**	25.61±0.11*	26.09±0.07***	25.19±0.21	*26.78±0.48****
PTGS2	28.13±0.61	**25.43±0.96****	27.47±0.79	*31.04±0.55***	30.29±0.87*

For each extraction method a PCA was employed using the expression values (Cq) of all quantified genes as the initial variable. Results are shown in figure 8. Tight cluster in the scatter plot showed that the relationship between the samples highly correlated among themselves and spread clusters showed a high variance between the samples. Therefore LY showed the best results followed by PAX, PI, WB, and LL which shows the most spread cluster respectively.

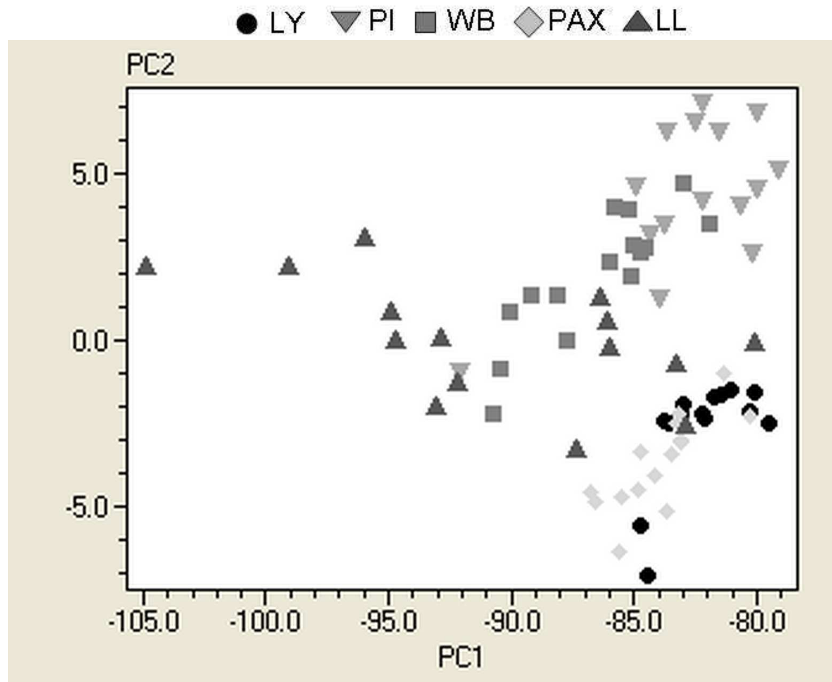
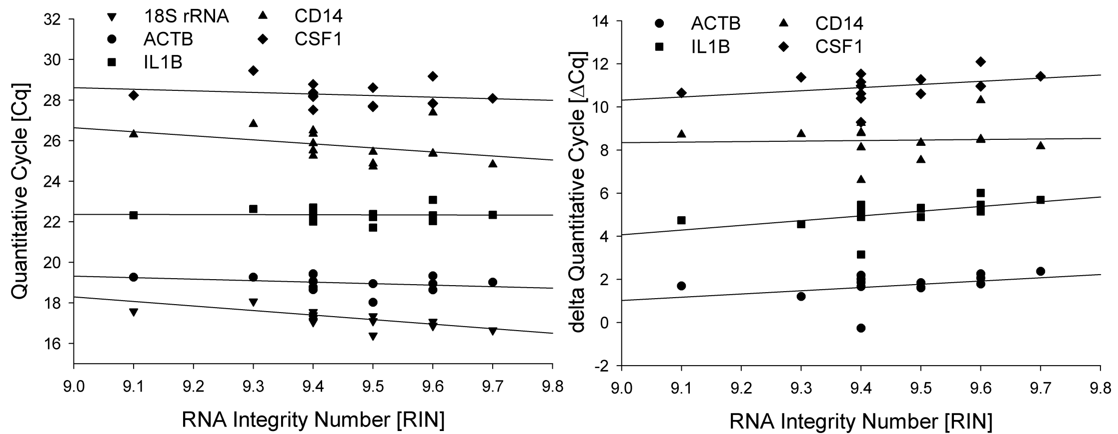


Figure 8: Principal component analysis performed for mRNA results.

Expression data were analyzed by relative quantification (ΔCq) for each extraction method, the mRNA quality expressed as RIN values showed minor influence on both Cq and ΔCq of the samples extracted by LY, PAX, and WB methods.

A



B

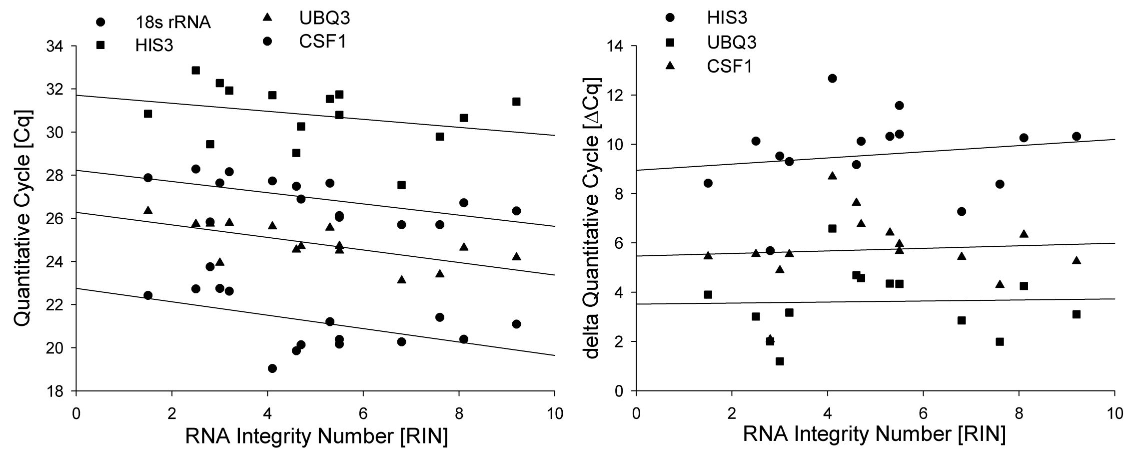


Figure 9: Influence of the RNA integrity (RIN) on the quantitative cycle (Cq) and on the ΔCq.

The mRNA quality of the samples extracted by PI and LL showed a high influence on the Cq and the ΔCq of specific TG. Figure 9 shows normalized expression results obtained from the LY method (figure 9A) and from the WB method (figure 9B). On the left side graph the relation between RNA degradation to the amplified product can be observed (Cq). On the right side graph by normalizing the TG to a RG (18S rRNA) the effect of RNA integrity was decreased (ΔCq).

miRNA quantification and data analysis

The RT-qPCR results of miRNA quantification showed a tendency to obtain lower Cq for the samples extracted by the LY and the WB method followed by PI, PAX, and LL (table 9). Based on the reproducibility (SEM values) the best results were obtained from samples extracted by the LY method and the worst from samples extracted by the LL method (Hammerle-Fickinger *et al.* 2010).

Table 9: The gene expression was determined for each miRNA transcript. The numbers in bold are equivalent to the lowest mean Cq ± SEM value considering each gene and the different extractions. The numbers in italic are equivalent to the highest Cq ± SEM value. The significance comparing all methods in relation to the best method (LY) is given as: * for $P < 0.05$, ** for $P < 0.01$ and *** for $P < 0.001$.

Mean expression levels					
	LY	PI	WB	PAX	LL
miRNA 16	18.14±0.15	19.98±1.26	15.59±0.24*	18.11±0.59	24.81±0.85***
miRNA let7a	**18.07±0.15**	21.16±1.21**	18.19±0.15	21.48±0.40***	29.67±0.61***
miRNA 142	23.94±0.13	25.96±0.61**	25.77±0.30**	26.15±0.64**	**23.24±0.56**
miRNA 181	22.59±0.11	25.02±1.24*	**21.41±0.23**	26.19±0.56***	28.50±0.72***
miRNA 27b	**22.56±0.18**	25.55±0.67***	22.88±0.32	26.01±0.80***	29.67±0.75***
miRNA 101	26.39±0.16	24.33±0.67***	23.91±0.33***	26.28±0.91	27.57±0.62*
miRNA 145	**28.41±0.22**	30.72±0.66***	28.85±0.26	30.10±0.63*	36.85±0.56***

Additionally, for each extraction method the relationship between all genes measured was determined by PCA (figure 10). For the samples obtained by LY it can be observed that the group arranges tightly showing a better clustering than WB, PAX, PI, and LL respectively (Hammerle-Fickinger *et al.* 2010).

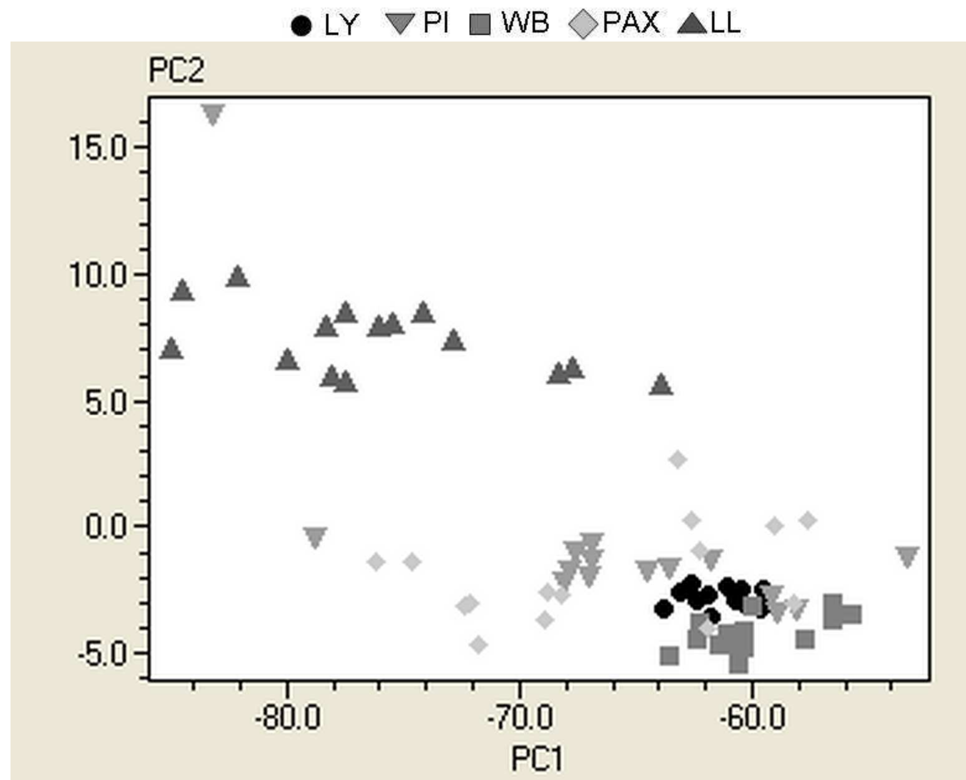


Figure 10: Principal component analysis performed for miRNA results.

3.2.2 Identification of a gene expression signature in bovine blood leukocytes

Hormone profile and complete blood count

The hormone profile of P4 was generated to affirm the endocrine state of the animals at days of sampling (figure 11). The hormone profiles of all animals studied indicate the normal estrous cycle of cows since P4 decreases for the NP group and remains high for the XP group at day 18.

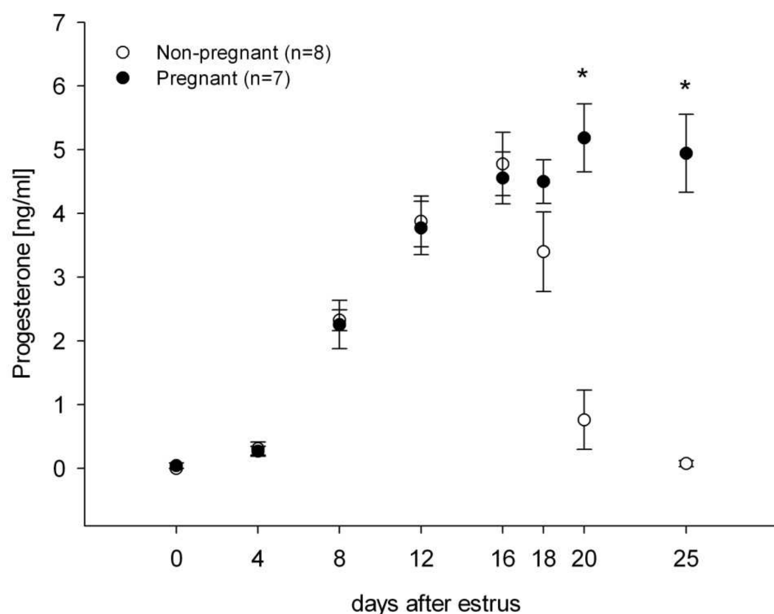


Figure 11: Hormone profile of P4 measured in plasma. Pregnant animals are represented by black dots and non-pregnant animals by white dots. Significant differences per day comparing NP versus XP groups are indicated (*, $P < 0.05$).

Blood parameters related to kidney, liver, pancreas and muscle metabolism indicated that all animals analyzed were healthy. Haemograms showed that leukocytes, monocytes, eosinophils and lymphocytes cells count ranged in physiological levels with no significant differences between pregnant and non-pregnant animals (table 10). Normal physiological levels for leukocytes, monocytes, eosinophils and lymphocytes range from 5 to 10 G/l, 2 to 6 %, 1 to 10 % and 45 to 65 % respectively (IDEXX Vet Med Labor).

Table 10: List of p-values obtained for the amount of the different blood cells comparing pregnant and non-pregnant animals.

	Leukocytes (G/l)			Monocytes (%)		
	NP (n=8)	XP (n=7)	*p*-value	NP (n=8)	XP (n=7)	*p*-value
Day 4	7.99 ± 0.50	8.31 ± 0.66	0.51	4.63 ± 1.13	3.57 ± 1.00	0.66
Day 8	8.65 ± 0.63	8.97 ± 0.63	0.72	3.75 ± 0.77	2.71 ± 1.08	0.45
Day 12	8.65 ± 1.07	9.09 ± 0.69	0.74	6.13 ± 1.30	5.43 ± 0.95	0.67
Day 16	8.10 ± 0.70	9.29 ± 0.44	0.18	4.25 ± 0.37	2.57 ± 0.75	0.08
Day 18	9.11 ± 0.82	8.23 ± 0.51	0.38	2.75 ± 0.62	2.86 ± 0.83	0.92
Day 20	8.48 ± 0.51	9.17 ± 0.45	0.32	6.13 ± 0.81	4.00 ± 0.98	0.12
	Eosinophils (%)			Lymphocytes (%)		
	NP (n=8)	XP (n=7)	*p*-value	NP (n=8)	XP (n=7)	*p*-value
Day 4	6.50 ± 1.90	6.57 ± 1.67	0.71	53.38 ± 5.13	63.14 ± 2.33	0.06
Day 8	4.25 ± 1.31	4.57 ± 0.75	0.83	55.88 ± 2.51	61.43 ± 2.79	0.16
Day 12	4.38 ± 1.24	3.29 ± 1.39	0.57	48.63 ± 5.18	56.00 ± 5.46	0.35
Day 16	2.50 ± 0.93	3.57 ± 1.04	0.46	46.88 ± 3.55	42.86 ± 6.24	0.59
Day 18	2.38 ± 0.94	3.43 ± 1.15	0.49	48.50 ± 5.58	56.00 ± 4.69	0.32
Day 20	2.75 ± 1.00	4.86 ± 1.44	0.25	49.88 ± 6.08	58.71 ± 7.89	0.39

Analysis of selected genes by RT-qPCR

Different genes related to early pregnancy in cattle were analyzed by RT-qPCR comparing NP (n = 8) and XP (n = 7) animals within samples taken at days 4, 8, 12, 16, 18 and 20. As reference genes, glyceraldehyde 3 phosphate dehydrogenase (GAPDH), actin beta (ACTB) and tyrosine 3-monooxygenase/tryptophan 5-monooxygenase activation protein, zeta polypeptide (YWHAZ) were considered. To determine the most stable RG, the Cq values of 10 different factors were applied to the geNorm algorithm (GenEx) and ACTB was chosen as the most suitable RG (figure 12).

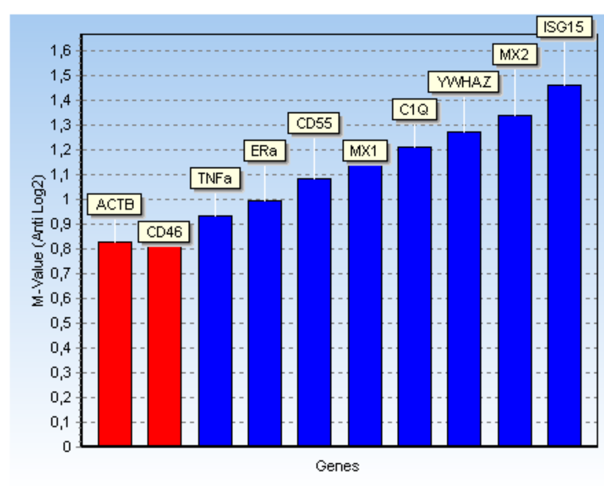


Figure 12: Reference gene determined by applying geNorm algorithm (GenEx).

The RIN mean values of all analyzed samples were 8.7 ± 0.07, indicating fully integer total RNA. The absorbance mean values of all analyzed samples were 1.9 ± 0.006 and 1.8 ± 0.03 for OD 260/280 and OD 260/230 respectively, indicating pure total RNA. In table 11, statistically significant expressed genes are shown and bold values represent significant regulated genes at days 8, 16, 18 and 20 of pregnancy.

Table 11: Gene expression signature in PBL at different days of pregnancy measured by RT-qPCR.

	Day 4				***Day 8***			
	20-ΔCq ± SEM				20-ΔCq ± SEM			
	NP (n=8)	XP (n=7)	fold regulation	*p*-value	NP (n=8)	XP (n=7)	fold regulation	*p*-value
BOLA	19.4 ± 0.1	19.4 ± 0.1	1.0	0.88	19.3 ± 0.1	19.2 ± 0.1	1.0	0.81
C1Q	11.9 ± 0.4	11.1 ± 0.4	0.6	0.20	**12.6 ± 0.3**	**11.4 ± 0.4**	**0.4**	**0.04**
C1S	2.3 ± 0.6	3.1 ± 0.5	1.7	0.35	**3.5 ± 0.3**	**4.9 ± 0.5**	**2.6**	**0.05**
C4	7.3 ± 0.1	7.2 ± 0.1	1.0	0.75	7.0 ± 0.2	7.2 ± 0.2	1.2	0.38
CD46	17.8 ± 0.3	18.0 ± 0.3	1.1	0.66	17.5 ± 0.3	17.7 ± 0.3	1.1	0.69
CD55	18.0 ± 0.3	18.4 ± 0.1	1.5	0.10	**17.0 ± 0.4**	**18.6 ± 0.1**	**3.1**	**0.00**
CFB	10.2 ± 0.2	10.8 ± 0.3	1.5	0.17	10.6 ± 0.2	10.9 ± 0.3	1.2	0.39
CSF1	12.0 ± 0.4	12.0 ± 0.5	1.0	0.95	**10.8 ± 0.4**	**11.9 ± 0.2**	**2.1**	**0.03**
CXCL17	6.5 ± 0.3	7.2 ± 0.7	1.6	0.38	**7.4 ± 0.2**	**6.4 ± 0.3**	**0.5**	**0.03**
IFI16	14.5 ± 0.2	15.2 ± 0.4	1.7	0.15	**13.4 ± 0.3**	**14.5 ± 0.2**	**2.1**	**0.01**
IFI44	19.0 ± 0.4	20.0 ± 0.2	2.0	0.07	**18.3 ± 0.5**	**20.0 ± 0.4**	**3.3**	**0.03**
IFIT2	16.3 ± 0.2	16.8 ± 0.3	1.5	0.17	**15.8 ± 0.3**	**16.7 ± 0.2**	**1.9**	**0.03**
IGF2	2.8 ± 0.2	2.8 ± 0.1	1.0	0.94	**2.3 ± 0.3**	**1.1 ± 0.3**	**0.5**	**0.01**
IL18	19.3 ± 0.3	20.2 ± 0.3	1.9	0.07	**18.5 ± 0.4**	**20.0 ± 0.5**	**2.8**	**0.04**
ISG15	11.0 ± 0.4	11.5 ± 0.6	1.4	0.55	10.6 ± 0.4	11.5 ± 0.6	2.0	0.23
MX1	14.2 ± 0.3	13.8 ± 0.5	0.7	0.53	13.7 ± 0.3	14.1 ± 0.5	1.3	0.54
MX2	14.0 ± 0.5	15.4 ± 0.5	2.7	0.07	**13.2 ± 0.3**	**14.6 ± 0.4**	**2.6**	**0.03**
OAS1	15.5 ± 0.2	15.7 ± 0.4	1.1	0.71	15.0 ± 0.3	15.3 ± 0.3	1.2	0.64
TNFa	12.7 ± 0.2	12.4 ± 0.3	0.8	0.40	**12.6 ± 0.3**	**11.7 ± 0.2**	**0.5**	**0.02**

Table 11 (continued)

	Day 12				***Day 16***			
	20-ΔCq ± SEM				20-ΔCq ± SEM			
	NP (n=8)	XP (n=7)	fold regulation	*p*-value	NP (n=8)	XP (n=7)	fold regulation	*p*-value
BOLA	19.0 ± 0.2	19.4 ± 0.3	1.3	0.34	**19.2 ± 0.2**	**19.9 ± 0.2**	**1.6**	**0.04**
C1Q	12.0 ± 0.3	12.0 ± 0.2	0.9	0.84	12.4 ± 0.4	11.8 ± 0.1	0.7	0.99
C1S	2.3 ± 0.7	3.9 ± 0.7	2.9	0.15	3.2 ± 0.7	3.9 ± 0.7	1.6	0.52
C4	7.1 ± 0.2	7.4 ± 0.1	1.3	0.18	7.1 ± 0.3	7.7 ± 0.2	1.5	0.11
CD46	17.8 ± 0.2	17.9 ± 0.3	1.1	0.77	17.1 ± 0.4	17.4 ± 0.1	1.3	0.38
CD55	17.5 ± 0.4	18.0 ± 0.4	1.3	0.52	16.9 ± 0.4	18.1 ± 0.3	2.3	0.134
CFB	10.3 ± 0.3	11.0 ± 0.2	1.6	0.11	**10.4 ± 0.2**	**11.1 ± 0.3**	**1.7**	**0.01**
CSF1	10.8 ± 0.5	12.0 ± 0.5	2.4	0.10	11.3 ± 0.4	12.1 ± 0.4	1.8	0.17
CXCL17	6.2 ± 0.4	6.8 ± 0.4	1.5	0.30	7.4 ± 0.4	6.6 ± 0.4	0.6	0.24
IFI16	14.3 ± 0.4	14.6 ± 0.2	1.2	0.64	13.6 ± 0.4	13.7 ± 0.3	1.1	0.72
IFI44	18.8 ± 0.5	19.2 ± 0.5	1.4	0.55	18.0 ± 0.5	19.0 ± 0.5	2.0	0.21
IFIT2	15.5 ± 0.5	16.2 ± 0.4	1.6	0.31	15.4 ± 0.5	15.3 ± 0.3	0.9	0.88
IGF2	1.7 ± 0.3	2.5 ± 0.4	1.7	0.19	1.6 ± 0.4	2.6 ± 0.5	2.0	0.15
IL18	18.4 ± 0.3	18.5 ± 0.5	1.1	0.86	17.9 ± 0.5	18.2 ± 0.6	1.2	0.74
ISG15	10.2 ± 0.4	11.9 ± 0.7	3.1	0.08	**10.2 ± 0.4**	**11.5 ± 0.3**	**2.5**	**0.00**
MX1	13.8 ± 0.4	14.7 ± 0.5	1.9	0.18	13.8 ± 0.3	14.5 ± 0.5	1.6	0.20
MX2	13.6 ± 0.7	14.6 ± 0.6	2.0	0.27	**12.2 ± 0.2**	**13.6 ± 0.3**	**2.6**	**0.01**
OAS1	13.8 ± 0.3	15.2 ± 0.6	2.7	0.07	**13.2 ± 0.5**	**15.6 ± 0.6**	**5.5**	**0.01**
TNFa	12.3 ± 0.3	12.5 ± 0.3	1.1	0.65	**12.1 ± 0.2**	**13.0 ± 0.4**	**1.8**	**0.02**

	Day 18				***Day 20***			
	20-ΔCq ± SEM				20-ΔCq ± SEM			
	NP (n=8)	XP (n=7)	fold regulation	*p*-value	NP (n=8)	XP (n=7)	fold regulation	*p*-value
BOLA	**18.8 ± 0.2**	**19.5 ± 0.2**	**1.6**	**0.04**	**19.0 ± 0.2**	**19.7 ± 0.2**	**1.6**	**0.03**
C1Q	11.8 ± 0.4	12.5 ± 0.3	1.7	0.17	**11.8 ± 0.3**	**12.8 ± 0.3**	**2.0**	**0.04**
C1S	3.7 ± 0.8	4.4 ± 0.4	1.5	0.49	**3.3 ± 0.5**	**4.7 ± 0.3**	**2.6**	**0.04**
C4	**7.1 ± 0.1**	**7.6 ± 0.2**	**1.4**	**0.05**	**6.8 ± 0.2**	**8.0 ± 0.3**	**2.4**	**0.00**
CD46	17.5 ± 0.3	17.9 ± 0.3	1.3	0.42	**17.0 ± 0.2**	**18.2 ± 0.2**	**2.3**	**0.00**
CD55	17.4 ± 0.4	18.2 ± 0.5	1.7	0.21	16.9 ± 0.4	17.9 ± 0.3	2.1	0.06
CFB	10.8 ± 0.2	11.3 ± 0.2	1.4	0.10	11.0 ± 0.1	10.9 ± 0.3	0.9	0.79
CSF1	11.4 ± 0.5	12.2 ± 0.3	1.8	0.15	11.5 ± 0.4	12.2 ± 0.5	1.7	0.30
CXCL17	7.3 ± 0.3	7.0 ± 0.4	0.9	0.65	6.9 ± 0.5	6.5 ± 0.6	0.8	0.64
IFI16	14.4 ± 0.3	14.4 ± 0.2	1.0	0.97	14.2 ± 0.3	14.7 ± 0.3	1.4	0.33
IFI44	**18.2 ± 0.4**	**19.7 ± 0.2**	**2.7**	**0.01**	**18.1 ± 0.5**	**21.0 ± 0.3**	**7.2**	**0.00**
IFIT2	**15.7 ± 0.3**	**17.2 ± 0.2**	**2.8**	**0.00**	15.8 ± 0.4	15.7 ± 0.2	1.0	0.95
IGF2	2.0 ± 0.4	2.5 ± 0.4	1.4	0.48	**1.3 ± 0.3**	**2.6 ± 0.3**	**2.5**	**0.02**
IL18	18.5 ± 0.3	18.9 ± 0.3	1.4	0.31	**17.5 ± 0.3**	**19.9 ± 0.2**	**5.1**	**0.00**
ISG15	**10.5 ± 0.6**	**13.4 ± 0.4**	**7.4**	**0.00**	**10.9 ± 0.5**	**13.4 ± 0.3**	**5.9**	**0.00**
MX1	**13.6 ± 0.5**	**15.0 ± 0.2**	**2.7**	**0.03**	**13.7 ± 0.4**	**15.0 ± 0.3**	**2.5**	**0.02**
MX2	**13.4 ± 0.5**	**15.5 ± 0.2**	**4.4**	**0.00**	**13.6 ± 0.5**	**15.6 ± 0.5**	**3.8**	**0.03**
OAS1	**13.4 ± 0.4**	**16.3 ± 0.4**	**7.2**	**0.00**	**14.4 ± 0.4**	**16.6 ± 0.4**	**4.7**	**0.00**
TNFa	**12.1 ± 0.2**	**13.0 ± 0.3**	**2.0**	**0.02**	**12.4 ± 0.2**	**13.4 ± 0.3**	**2.0**	**0.01**

PCA was conducted at days 8, 16, 18 and 20 using significant differential expressed genes shown in table 11. The raw data used for PCA were the Cq values of the differential expressed genes, which were normalized against the RG and the result values were mean centered. Afterwards, P4 values were added to the matrix and PCA was performed. Results in figure 13 show a distinct separation for days 8, 18 and 20 of non-pregnant and pregnant animals.

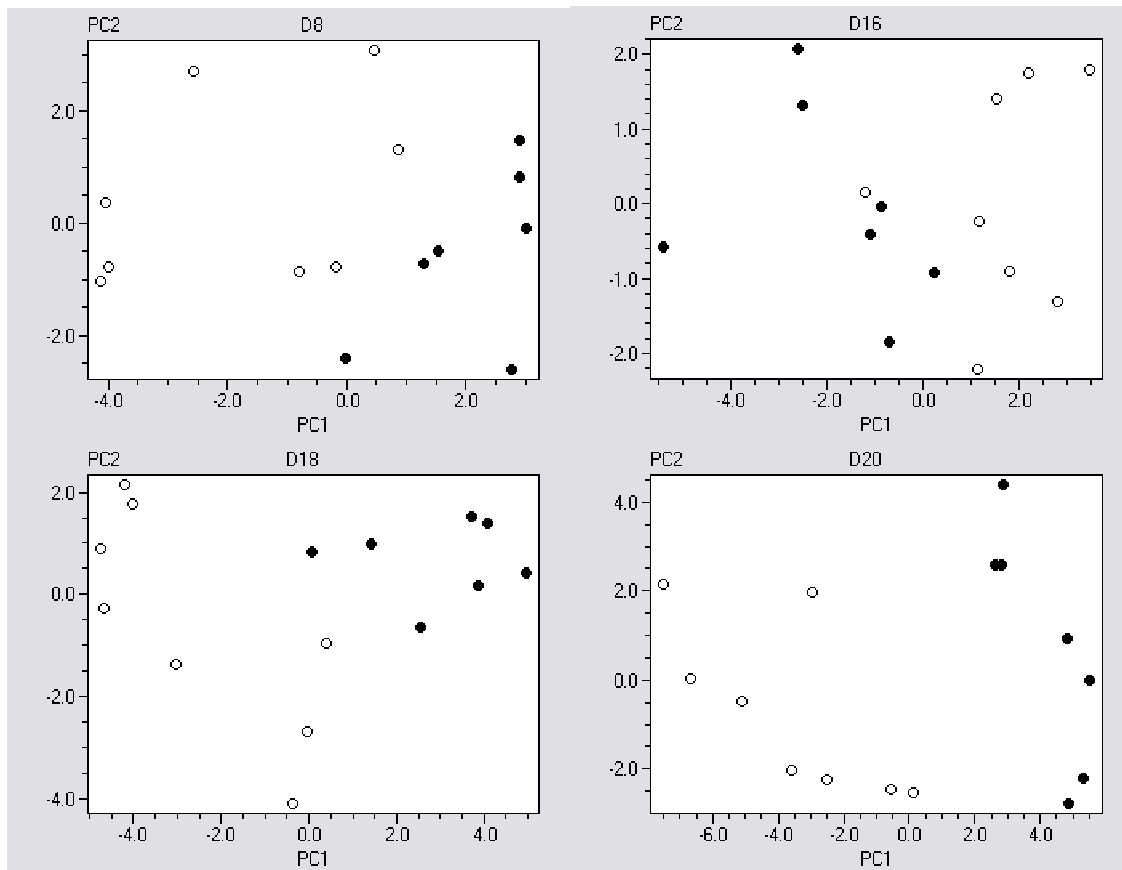


Figure 13: Principal component analysis. PCA was conducted using at days 8, 16, 18 and 20 respectively 12, 6, 9 and 14 differential expressed genes. Non-pregnant animals are represented by white dots and pregnant animals by black dots.

A heat map analysis was performed considering the mean ΔCq values of the significant regulated genes for comparison between NP and XP group (figure 14). Interestingly, although not clustering directly in the same subgroup, the heat map pattern with respect to ISG at day 8 XP was quite similar to days 18 XP and 20 XP.

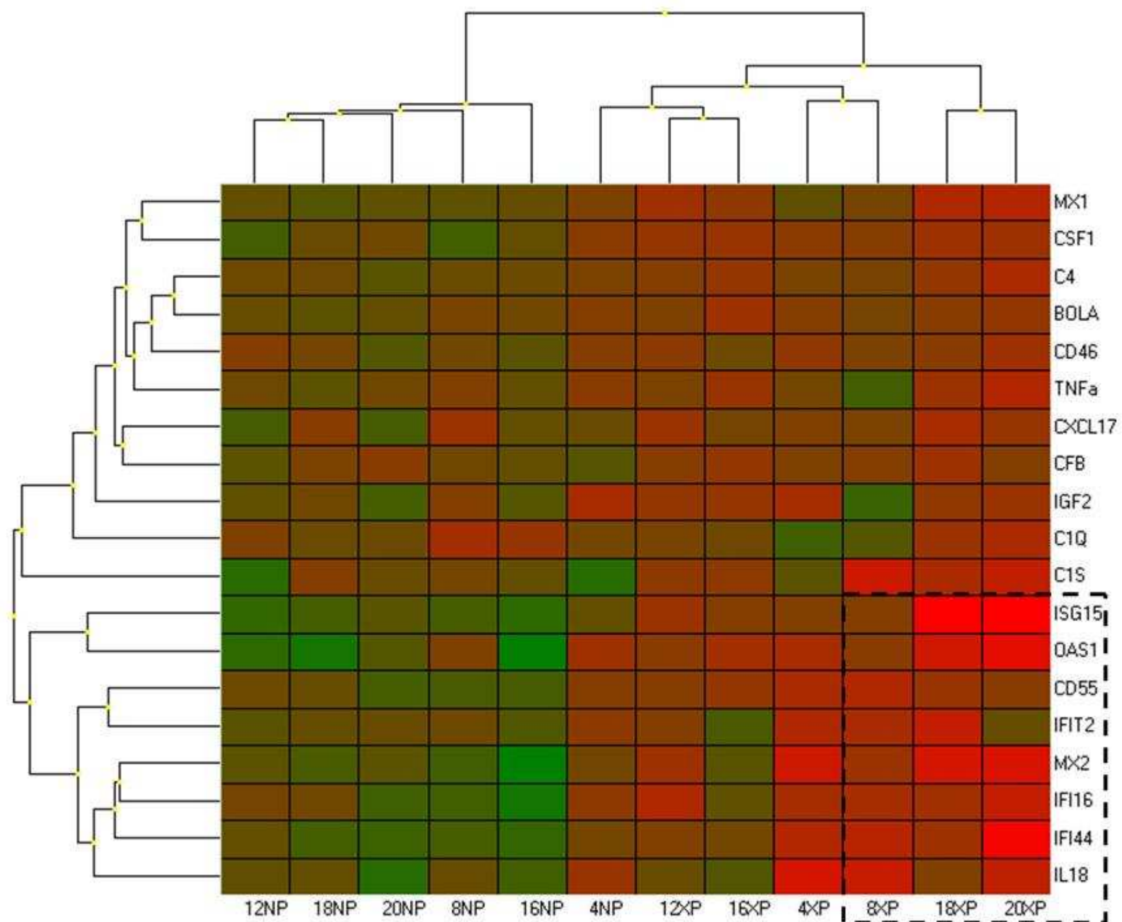


Figure 14: Cluster analysis based on significantly regulated genes. Red fields are equivalent to low ΔCq (high gene expression) and green fields to high ΔCq (low gene expression). Although not clustering directly in the same subgroup, the heat map pattern at day 8 XP with respect to ISG is quite similar to days 18 XP and 20 XP as highlighted.

Microarray analysis and validation

Pair-wise samples comparing NP and XP groups taken from 3 animals at days 4, 8, and 12 were analyzed by SAM analysis and did not reveal any significant differences between any time point analyzed. A PLPE test revealed 73 differentially expressed genes at day 4, 71 at day 8 and 38 at day 12 of pregnancy with a false discovery rate (FDR) less than 0.05 or p-values less than 0.05 with at least 1.5 -fold signal difference between NP and XP animals (see appendix table 1). Within the differentially expressed genes of the PLPE analysis, PCA was applied for days 4, 8 and 12 resulting in no clustering distinction between the NP and the XP groups (figure 15).

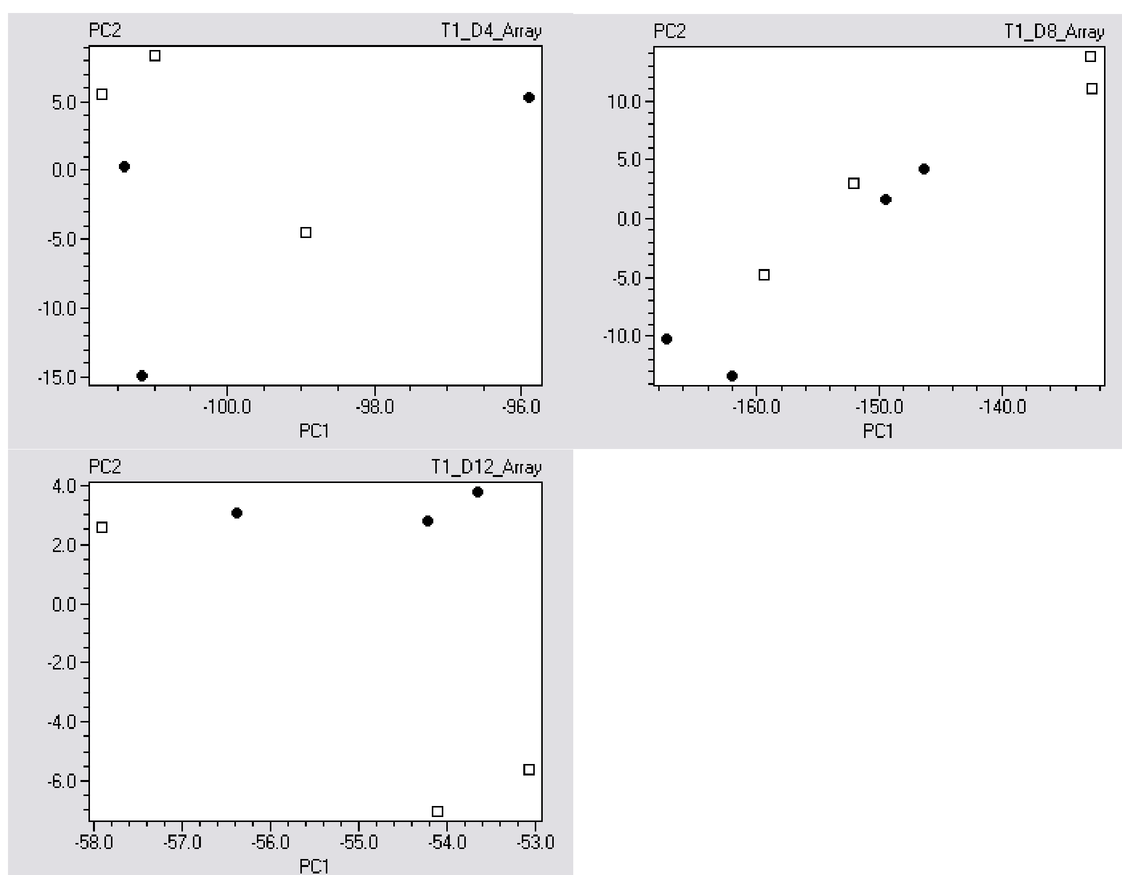


Figure 15: Principal component analysis. PCA was conducted using at days 4, 8, 12 respectively 73, 71 and 38 differential expressed genes measured with the microarray analysis. Non-pregnant animals are represented by white dots and pregnant animals by black dots.

The transcript abundance of selected genes from the microarray results at day 4 (IL1B), day 8 (IL18), and day 12 (OOSP1) were validated by RT-qPCR. While analyzing identical biological samples (n = 3), the RT-qPCR did not indicate significant differences between groups in any case. Significance was obtained when additional animals, the non-paired 5 NP and 4 XP animals were considered (table 12).

Table 12: Array validation by RT-qPCR.

		Microarray (n=3)				**RT-qPCR (n=3)**		**RT-qPCR (n=8)**	
	Day 4 of pregnancy	*Fold*	*P-value t*	*P-value L*	*FDR*	*Fold*	*P-value*	*Fold*	*P-value*
IL1B	interleukin 1 beta	1.40	0.52	0.04	0.04	2.70	0.34	3.10	0.03
	Day 8 of pregnancy								
IL18	interleukin 18	2.50	0.46	0.01	0.03	4.80	0.03	2.80	0.04
	Day 12 of pregnancy								
OOSP1	oocyte-secreted protein 1	0.50	0.01	0.22	0.05	0.30	0.10	0.40	0.03

The linear regression relating the microarray and RT-qPCR expression values were calculated along with the coefficient of determination (R^2) and the p-value. For the optimal visualization of data in one diagram, the RT-qPCR data is shown as 20 – ΔCq (figure 16). The microarray results showed up-regulation of 1.30 -fold (p = 1.09) for MX2 and 2.59 -fold (p = 1.09) for IFIT2 without reaching any significance when n = 3. RT-qPCR results showed up-regulation of 2.80 -fold (p = 0.14) for MX2 and 2.20 -fold (p = 0.13) for IFIT2 without reaching any significance when n = 3. By increasing the number of samples (n = 8), the RT-qPCR results showed significant up-regulation of 2.60 -fold ($p < 0.05$) for MX2 and 1.90 -fold ($p < 0.05$) for IFIT2.

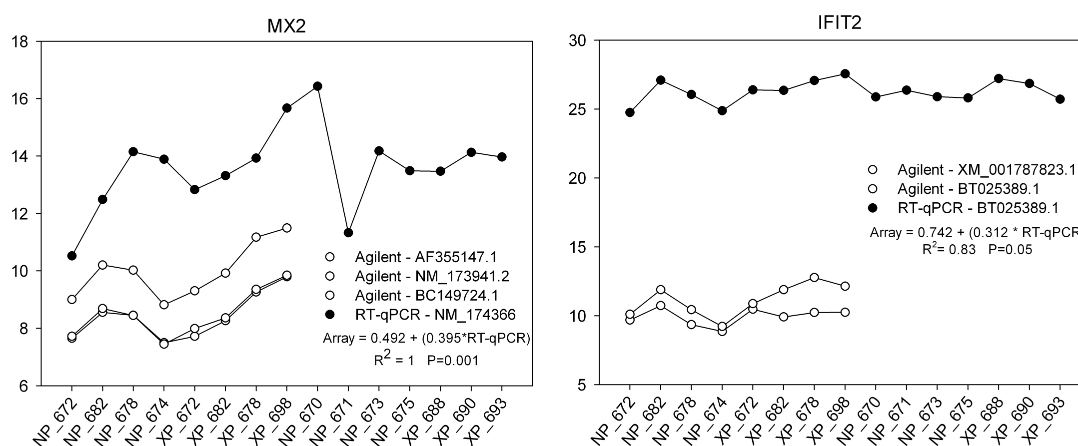


Figure 16: Expression profiles for microarray and RT-qPCR analysis. RT-qPCR data is represented by black dots (n = 7/8) and microarray data by white dots (n = 3).

Determination of C1Q concentration by ELISA

The C1Q protein concentration of bovine serum determined at day 8 (18.1 ± 3.2 µg/ml and 23.0 ± 4.1 µg/ml for NP and XP, respectively), day 18 (22.7 ± 5.0 µg/ml and 20.2 ± 6.6 µg/ml for NP and XP, respectively) and day 20 (20.2 ± 2.3 µg/ml and 17.9 ± 4.3 µg/ml for NP and XP, respectively) was not significantly different ($P > 0.05$) between NP and XP animals.

3.3 Analysis of pregnancy recognition in milk somatic cells

3.3.1 Validation of milk sampling techniques

Evaluation of sample volume

For the validation of milk sampling, animals in different stages of lactation were randomly selected, resulting in SCC variation of 80 to 128 x10^3 cells/ml. Results showed an average of total RNA yield from 2.50 µg to 9.81 µg for 100 ml and 450 ml of collected milk, respectively. For downstream processing at least 5 µg of total RNA is required, therefore 450ml of milk was used per sample for further studies.

Evaluation of PBS buffer volume

The cell pellet obtained after centrifugation of 450 ml of milk was submitted to a PBS washing step considering different volumes. The results show that RNA yield did not change significantly in respect to the different PBS volumes (table 13). Quality and purity of RNA showed a significant change, thus for further studies the washing of the cell pellet was done using 50 ml of PBS.

Table 13: Effect of PBS volume.

	RNA [µg]	**OD 260/280**	**Quality [RIN]**
50 ml	9.9	2.04	7.3
SEM	0.5	0.02	0.13
150 ml	9.21	1.98	6.65
SEM	0.73	0.03	0.31
ANOVA	P>0.05	P<0.05	P<0.05

Evaluation of wash steps

To verify whether the purity and quality could be improved, the cell pellet was washed one and two times after the establishment of the PBS volume (table 14).

Table 14: Effect of wash steps on sample quantity and quality.

	RNA [µg]	OD 260/280	Quality [RIN]
1 Wash Step	14.58	1.84	6.8
SEM	2.76	0.09	0.95
2 Wash Steps	4.39	1.93	6.08
SEM	1.76	0.05	0.77
ANOVA	**P<0.05**	P>0.05	**P<0.05**

The results in table 14 demonstrate that a higher RNA yield was extracted from milk somatic cells after only one wash step of the cell pellet. The performance of two wash steps showed no significant difference in purity, but a significant influence on the RNA quality. Hence, for further studies 1 wash step of the cell pellet was applied.

Storage Time

After establishing the sample volume, buffer volume and wash steps, different milk storage times were tested. The samples were stored at 4 °C and the extraction of milk somatic cells and RNA was performed at 4 time points, namely at the time of sampling (0 h) and 3 h, 6 h and 24 h after time of sampling (table 15). Milk samples were collected from one animal and processed in triplicate for each time point of extraction.

Table 15: Effect of storage time on RNA.

	RNA [µg]	OD 260/280	Quality [RIN]	miRNA %
0 h	11.8	2.06	7	34.67
SEM	1.06	0.02	0.35	0.33
3 h	10.85	2.07	7.37	35
SEM	0.58	0.01	0.13	1
6 h	11.88	2.07	7.77	39
SEM	0.09	0	0.12	1.15
24 h	9.13	2.07	7.2	42
SEM	0.32	0.01	0	1
ANOVA	**P<0.05**	P>0.05	P>0.05	**P<0.05**

From the starting time point (0 h) until 6 h after sampling nearly no difference in the RNA yield was measured. Only after 24 h a decrease in the RNA yield could be observed. An

increase on miRNA percentage was obtained from samples stored above six hours. During the period of 24h, total RNA purity and quality were stable with no significant differences.
To analyze whether the time of storage has an effect on the gene expression, a RT-qPCR was performed (figure 17) within the same samples. In this case, some of the differentially expressed candidate genes found in blood leukocytes were measured. Additionally, UBQ was investigated as it is known to be involved in cell apoptosis.

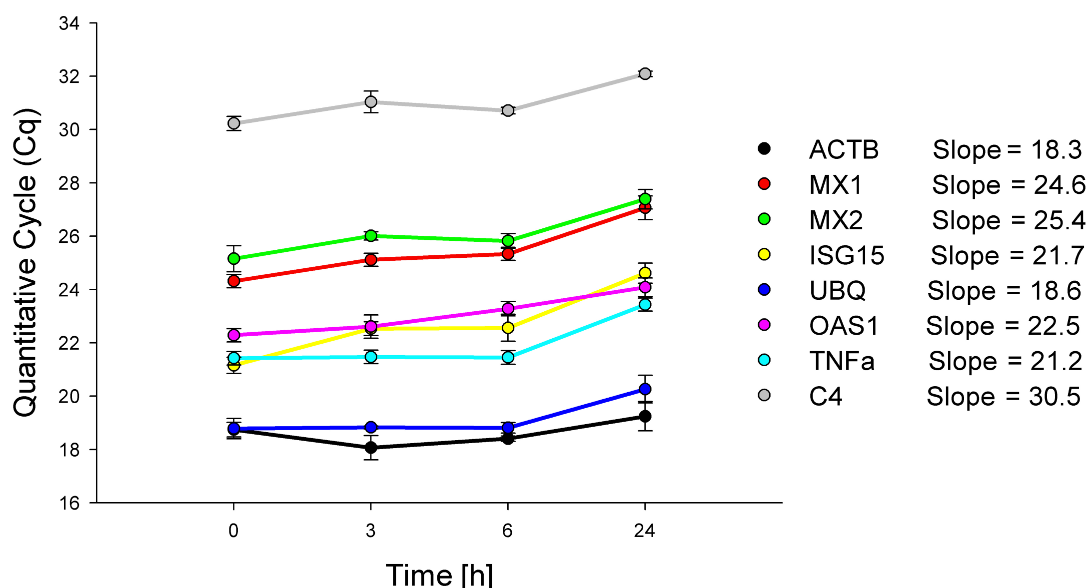


Figure 17: Effect of milk storage time on mRNA gene expression of selected target genes.

As it can be seen in figure 17, all genes measured had a higher Cq after 24 hours of storage and were therefore less expressed. TNFa and UBQ had almost the same Cq in the first six hours. For MX1, MX2, ISG15 and C4 the highest increase in Cq occurred in the first three hours and were continuously down-regulated over the remaining time. OAS1 was most down-regulated between hour three and six. Only ACTB was higher expressed in the first three hours, but afterwards ACTB also showed decrease in gene expression.

Evaluation of SCC

To assure sufficient RNA quantity for downstream processes, RNA was extracted from samples with different SCC (figure 18).

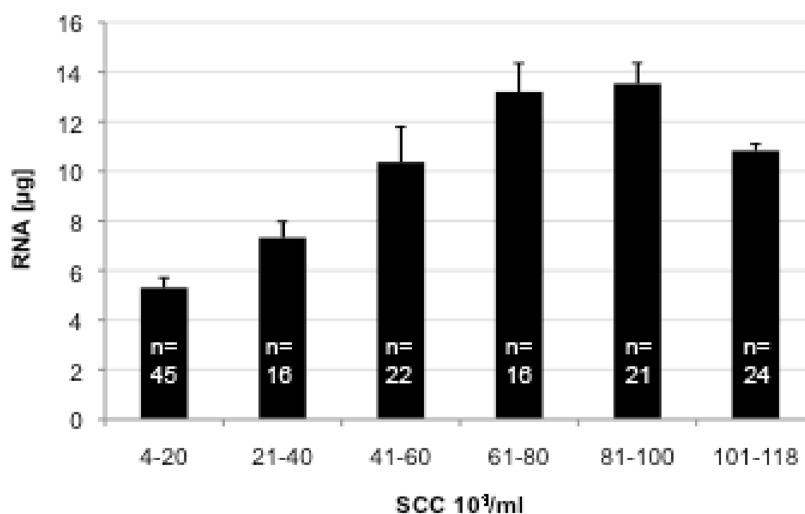


Figure 18: Effect of SCC on total RNA yield.

As expected, the RNA yield rose as more cells were present in milk. Figure 18 showed that the highest possible RNA yield was achieved when the extraction was done for milk samples with a range of 60 x 103 to 100 x 10^3 cells/ml. A SCC of more than 100 x 10^3 cells/ml resulted in lower amount of RNA.

Extraction method

Two different extraction methods were evaluated with the objective to provide mRNA and miRNA from bovine somatic milk cells with good quality and purity (table 16).

Table 16: Effect of extraction method on mRNA quality and miRNA percentage.

	RNA [µg]	**OD 260/280**	**Quality [RIN]**	**miRNA %**
Trifast	8.03	1.96	3.23	46.61
SEM	2.02	0.03	0.66	1.78
miRNeasy	10.74	2.03	7.73	44.15
SEM	2.07	0.02	0.16	6.48
ANOVA	P>0.05	**P<0.05**	**P<0.05**	**P<0.05**

The results represent the mean values ± SEM for duplicate samples from 3 different animals. The total RNA yield showed no significant differences between both extraction methods, for which the total RNA was isolated. Therefore no absolute quantification of the small RNA

could be evaluated using the NanoDrop 1000 but only the relative estimation by the Bioanalyzer analysis. The percentage of miRNA in total RNA was higher ($P < 0.05$) for samples extracted with the Trifast than with the miRNeasy kit. For the total RNA, significant differences in the mean OD 260/280 ratios and in the mean RIN values among the two methods were observed (table 16) showing that the better quality and purity was obtained by using the miRNeasy kit. Hence, for further studies the extraction with miRNeasy kit was applied.

Evaluation of sampling time point

After the establishment of milk somatic cells and the RNA extraction method from milk cells, the variation of the results due to sampling at different days was performed. The validation was carried out for the yield, purity, quality and miRNA percentage. Therefore a two way ANOVA was applied for the data obtained from three animals with samples taken in quadruplicate at three different days (table 17).

Table 17: Two Way ANOVA on RNA constitution.

Source of Variation	ANOVA statistics				
	df	**SS**	**MS**	**F**	**P**
RNA Yield					
Animal	2	124293.8	62146.898	64.552	<0.001
Replicates	3	2389.17	796.39	0.845	0.484
Date	2	12915.35	5760.376	5.983	0.002
RNA Integrity Number (RIN)					
Animal	2	0.582	0.291	1.129	0.339
Replicates	3	0.536	0.179	0.696	0.564
Date	2	4.292	2.146	8.326	0.002
RNA Purity ($A_{260/280}$)					
Animal	2	0.0111	0.00554	1.343	0.279
Replicates	3	0.00591	0.00197	0.463	0.711
Date	2	0.00377	0.00189	0.457	0.812
% miRNA					
Animal	2	221.118	110.559	5.043	0.014
Replicates	3	74.975	24.992	1.077	0.379
Date	2	39.66	19.83	0.905	0.418

The two way ANOVA was performed on: degrees of freedom (DF), sum of square (SS), mean square (MS), F-statistic test (F) and probability (P). The variation in the RNA yield and miRNA percentage within different animals was measured. Different sampling time points influence the total RNA yield and quality significantly. The replicates obtained from same animal did not vary much in any of the analyzed parameters.

mRNA quantification and data analysis

To test the precision of the results, the Cq of 11 different genes which were measured in samples from three different animals collected in quadruplicate were examined (table 18).

Table 18: Two Way ANOVA on gene analysis.

	DF	SS	MS	F	P		DF	SS	MS	F	P
ACTB						**MX1**					
Animal	2	20.634	10.317	40.384	**<0.001**	Animal	2	14.016	7.008	2.571	0.097
Replicates	3	0.232	0.0772	0.063	0.979	Replicates	3	8.863	2.954	0.649	0.592
CD45						**MX2**					
Animal	2	17.268	8.634	19.379	**<0.001**	Animal	2	8.7	4.35	11.541	**<0.001**
Replicates	3	2.232	0.744	0.698	0.563	Replicates	3	0.843	0.281	0.32	0.811
CK8						**TNFα**					
Animal	2	111.312	55.656	77.104	**<0.001**	Animal	2	7.557	3.778	18.034	**<0.001**
Replicates	3	1.227	0.409	0.0741	0.973	Replicates	3	0.153	0.0509	0.108	0.955
CD55						**UBQ**					
Animal	2	13.434	6.717	13.159	**<0.001**	Animal	2	0.293	0.147	0.814	0.454
Replicates	3	3.149	1.05	1.016	0.405	Replicates	3	0.366	0.122	0.486	0.695
HIS						**YWAHZ**					
Animal	2	10.434	5.217	2.848	0.077	Animal	2	9.452	4.726	8.74	**0.001**
Replicates	3	6.012	2.004	1.139	0.355	Replicates	3	1.86	0.62	0.612	0.614
ISG15											
Animal	2	3.173	1.587	2.125	0.141						
Replicates	3	1.07	0.357	0.485	0.696						

As a positive control Cytokeratin 8 (CK8) was measured. CK8 is known to be expressed in bovine mammary epithelial cells and can be quantified in milk samples (Boutinaud *et al.* 2008). Additionaly, CD45 a leukocyte common antigen was measured (Boutinaud *et al.* 2002). CD55, ISG15, MX1, MX2 and TNFa were measured based on the candidate genes considered for blood leukocytes. In table 18, the two way ANOVA performed for gene expression changes demonstrated that regulation was significantly different among different animals for most factors measured, namely ACTB, CD45, CK8, CD55, MX2, TNFa and YWHAZ. No difference could be seen for HIS3, ISG15, MX1 and UBQ. Like for the constitution of RNA, no significant difference between replicates at any time was measured.

3.3.2 Identification of a gene expression signature in bovine milk somatic cells

Hormone profile

The hormone profile for P4 of all animals used in the studied is depicted in figure 19 and showed expected non-pregnant and pregnant profiles indicating the presence of an endocrine active CL graviditate in the pregnant group.

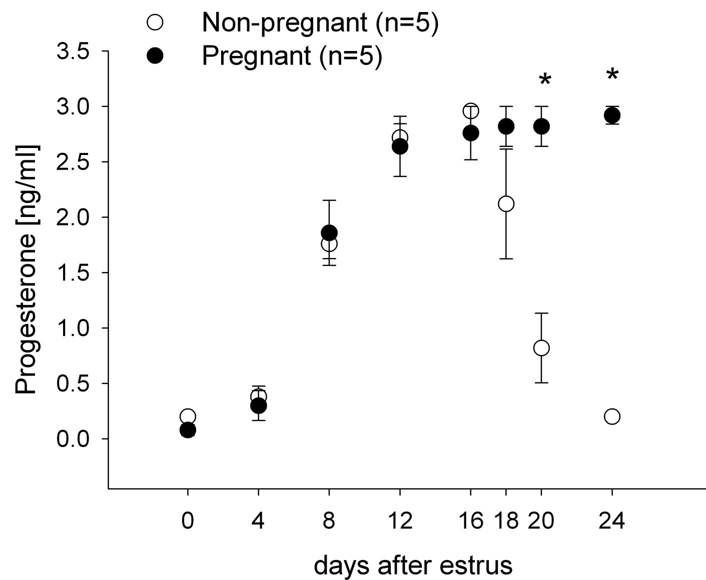


Figure 19: Hormone profile for P4 measured in fat free milk.

Analysis of selected genes by RT-qPCR

Candidate genes determined in previous studies as differentially expressed during early pregnancy in blood cells were analyzed in milk SC cells by RT-qPCR for comparison between NP (n = 5) and XP (n = 5) animals within samples taken at days 4, 8, 12, 16, 18 and 20. As reference genes GAPDH, ACTB, HIS, 18s rRNA and UBQ were considered for analysis. By applying geNorm algorithm GAPDH and UBQ were chosen as the most suitable RG (figure 20).

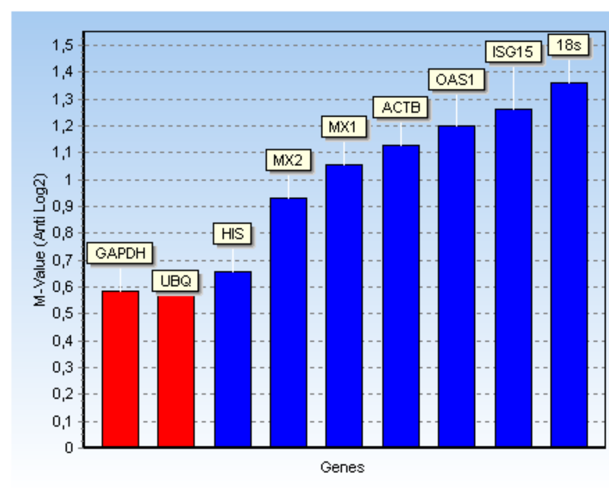


Figure 20: Reference gene determined by applying geNorm algorithm (GenEx).

RIN mean values of all analyzed samples were 6.9 ± 0.17 indicating fully integer total RNA. Absorbance mean values of all analyzed samples were 2.0 ± 0.01 and 1.4 ± 0.05 for OD 260/280 and OD 260/230 respectively, indicating pure total RNA. In table 19, statistically significant expressed genes are shown and bold values represent significant regulated genes at days 4, 8, 12, 16, 18 and 20 of pregnancy.

Table 19: Gene expression signature in milk SC at different days of pregnancy measured by RT-qPCR.

	Day 4				***Day 8***			
	20-ΔCq ± SEM				20-ΔCq ± SEM			
	NP (n=5)	XP (n=5)	fold regulation	*p*-value	NP (n=5)	XP (n=5)	fold regulation	*p*-value
MX2	14.6 ± 0.1	14.8 ± 0.6	1.2	0.78	**15.1 ± 0.2**	**16.0 ± 0.2**	**1.8**	**0.05**
MX1	13.3 ± 0.4	13.8 ± 0.7	1.5	0.57	**13.9 ± 0.2**	**15.0 ± 0.3**	**2.2**	**0.03**
OAS1	14.7 ± 0.4	16.2 ± 0.8	2.7	0.20	**15.1 ± 0.6**	**16.8 ± 0.4**	**3.3**	**0.05**
TNFa	15.4 ± 0.6	16.3 ± 0.5	1.9	0.33	15.9 ± 0.4	17.1 ± 0.5	2.4	0.14
IFI16	14.7 ± 0.5	14.6 ± 0.3	0.9	0.84	15.3 ± 0.4	15.7 ± 0.4	1.3	0.60
IDO	13.7 ± 0.2	13.4 ± 0.3	0.8	0.57	14.6 ± 0.2	14.4 ± 0.7	0.8	0.77
STAT1	**15.2 ± 0.2**	**16.0 ± 0.3**	**1.8**	**0.09**	**15.2 ± 0.1**	**16.6 ± 0.4**	**2.7**	**0.02**
BST2	10.2 ± 0.3	11.0 ± 0.4	1.7	0.18	**10.3 ± 0.4**	**11.7 ± 0.4**	**2.6**	**0.04**

	Day 12				***Day 16***			
	20-ΔCq ± SEM				20-ΔCq ± SEM			
	NP (n=5)	XP (n=5)	fold regulation	*p*-value	NP (n=5)	XP (n=5)	fold regulation	*p*-value
MX2	**15.3 ± 0.1**	**16.5 ± 0.3**	**2.3**	**0.04**	**14.4 ± 0.3**	**16.1 ± 0.5**	**3.3**	**0.03**
MX1	**14.5 ± 0.2**	**15.7 ± 0.4**	**2.3**	**0.05**	**13.7 ± 0.5**	**15.6 ± 0.4**	**3.7**	**0.03**
OAS1	**15.7 ± 0.5**	**17.7 ± 0.5**	**4.1**	**0.04**	**14.9 ± 0.7**	**17.8 ± 0.3**	**7.4**	**0.01**
TNFa	16.8 ± 0.5	17.5 ± 0.5	1.6	0.40	16.1 ± 0.9	17.7 ± 0.5	3.1	0.17
IFI16	14.9 ± 0.4	14.9 ± 0.3	1.0	1.00	14.5 ± 0.5	15.4 ± 0.4	1.9	0.25
IDO	14.7 ± 0.3	14.1 ± 0.2	0.7	0.21	14.3 ± 0.6	14.6 ± 0.3	1.2	0.71
STAT1	**15.8 ± 0.2**	**16.8 ± 0.2**	**2.1**	**0.01**	**15.3 ± 0.5**	**17.1 ± 0.3**	**3.4**	**0.04**
BST2	**11.2 ± 0.3**	**13.6 ± 0.5**	**5.4**	**0.01**	**10.6 ± 0.5**	**12.5 ± 0.3**	**3.9**	**0.01**

	Day 18				***Day 20***			
	20-ΔCq ± SEM				20-ΔCq ± SEM			
	NP (n=5)	XP (n=5)	fold regulation	*p*-value	NP (n=5)	XP (n=5)	fold regulation	*p*-value
MX2	**14.5 ± 0.4**	**16.4 ± 0.6**	**3.8**	**0.05**	**14.9 ± 0.4**	**16.6 ± 0.5**	**3.2**	**0.03**
MX1	**13.1 ± 0.4**	**15.3 ± 0.7**	**4.5**	**0.05**	**13.7 ± 0.4**	**15.7 ± 0.6**	**3.9**	**0.05**
OAS1	**15.0 ± 0.8**	**17.6 ± 0.8**	**6.5**	**0.05**	15.2 ± 0.6	17.8 ± 0.8	6.1	0.06
TNFa	15.4 ± 0.8	17.8 ± 0.7	5.5	0.06	**15.2 ± 0.6**	**18.2 ± 0.6**	**8.1**	**0.01**
IFI16	**13.7 ± 0.4**	**15.2 ± 0.3**	**2.9**	**0.02**	**13.5 ± 0.3**	**15.3 ± 0.5**	**3.4**	**0.03**
IDO	**13.9 ± 0.5**	**15.3 ± 0.3**	**2.6**	**0.05**	**13.7 ± 0.4**	**15.5 ± 0.4**	**3.6**	**0.02**
STAT1	**14.7 ± 0.4**	**17.1 ± 0.7**	**5.4**	**0.04**	**15.1 ± 0.3**	**17.3 ± 0.7**	**4.4**	**0.05**
BST2	**10.1 ± 0.7**	**12.3 ± 0.3**	**4.7**	**0.04**	**10.9 ± 0.6**	**12.9 ± 0.5**	**4.1**	**0.04**

PCA was conducted for days 8, 12, 16, 18 and 20 using the significantly differential expressed genes shown in table 19. The Cq values where used as raw data which was normalized and mean centered for analysis, after that P4 values were added to the matrix

and PCA was performed. The results showed a distinct separation of non-pregnant and pregnant animals at days 12 and 18 of pregnancy (figure 21).

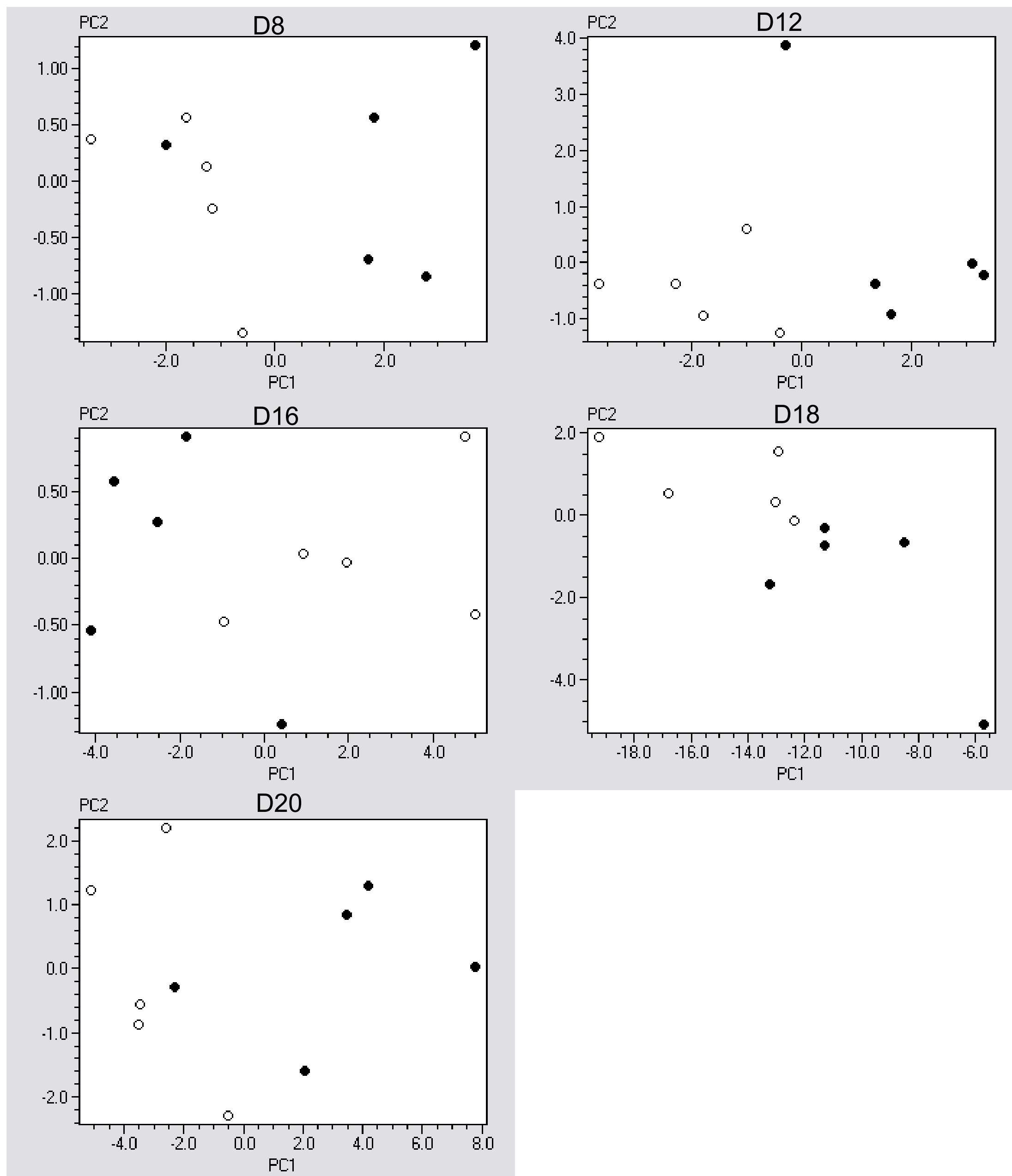


Figure 21: Principal component analysis. PCA was conducted using at days 8, 12, 16, 18 and 20 respectively 5, 5, 5, 7 and 7 differential expressed genes. Non-pregnant animals are represented by white dots and pregnant animals by black dots.

A heat map analysis performed using mean ∆Cq values of each day comparing NP group versus XP group with the significant regulated genes is illustrated in figure 22. A clear separation between NP and XP animals for all days besides day 4 of pregnancy could be observed.

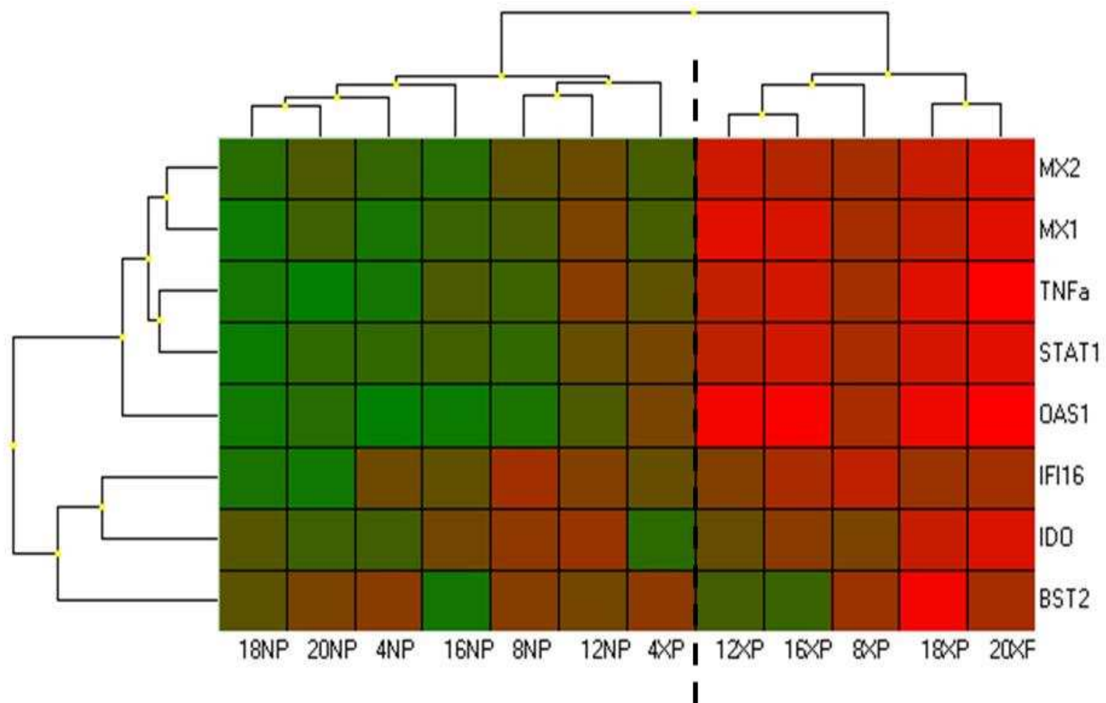


Figure 22: Cluster analysis based on significantly regulated genes**.** Red fields are equivalent to low ΔCq (high gene expression) and green fields to high ΔCq (low gene expression).

4 Discussion

4.1 Analysis of pregnancy recognition regarding the complement system activation in endometrium

It has been shown that the activation of complement system factors in humans and mice take place during pregnancy (Rampersad *et al.* 2008, Richani *et al.* 2005b, Richani *et al.* 2005a, Girardi *et al.* 2003). Despite of the fact that increased activation of specific complement components as C3a, C4a and C5a is related to normal pregnancy in humans, an excessive activation may contribute to sub-optimal pregnancy outcomes (Richani *et al.* 2005b, Richani *et al.* 2005a). In humans, pregnancy disorders such as the anti-phospholipid syndrome (APS) and preeclampsia in association with an unrestrained activation of the complement system can lead to preterm abortion (Derzsy *et al.* 2010, Girardi *et al.* 2003). We demonstrated that the activation of the complement system might exist during bovine pregnancy. Hence, we show that the complement system pathway answers to the conceptus presence and that there is a direct response to the IFNT release in the bovine endometrium.
Factors of the classical pathway as C1S, C1R and SERPING1 were significantly differentially expressed in the bovine endometrium at day 18 of pregnancy (Klein *et al.* 2006). In humans C1R, C2 and CFD were higher expressed in the endometrium during the time of implantantion (Kao *et al.* 2002). Results from all three *in vivo* studies support these hypotheses showing not only an up-regulation of C1S, C1R and SERPING1 but also an up-regulation of C1Q, C2, C4, C3, CFB and C3AR components at day 18 of pregnancy in the bovine endometrium.
To examine the effect of IFNT release on the gene expression of endometrial GE and stroma cells an *in vitro* system of co-cultivated cells was used. Experimental results *in vitro* showed a significant up-regulation of C1R, C2 and SERPING1 in GE due to IFNT, correlating to results obtained for the *in vivo* studies. An increase of C2 and SERPING1 transcription was previously demonstrated to be stimulated by IFN-alpha, IFN-beta and IFN-gamma in human peripheral blood monocytes (Lappin *et al.* 1990, Lappin *et al.* 1992). Hence, these results suggest that IFNT is responsible for the up-regulation of complement components *in vitro* and probably also during bovine pregnancy.
In addition to the increased regulation of the C1 complex, the inhibitor protein SERPING1 is also up-regulated in the bovine endometrium. The C1 complex activation dependent on the presence of C1S, C1R and C1Q and is controlled by the inhibiting factor SERPING1 (figure 23). This inhibitory factor binds to the C1 complex preventing the cleavage of C4 and the formation of C4b2b (C3 convertase). Its main function is the inhibition of the complement

system by binding to C1 and tightly regulation of the classical pathway. In addition, SERPING1 is a progesterone induced glycoprotein which may help to prevent maternal rejection of allografts (Tekin *et al.* 2006). Thus, during bovine pregnancy SERPING1 is probably not only induced by P4 but further stimulated by IFNT to promote maternal tolerance of the embryo.

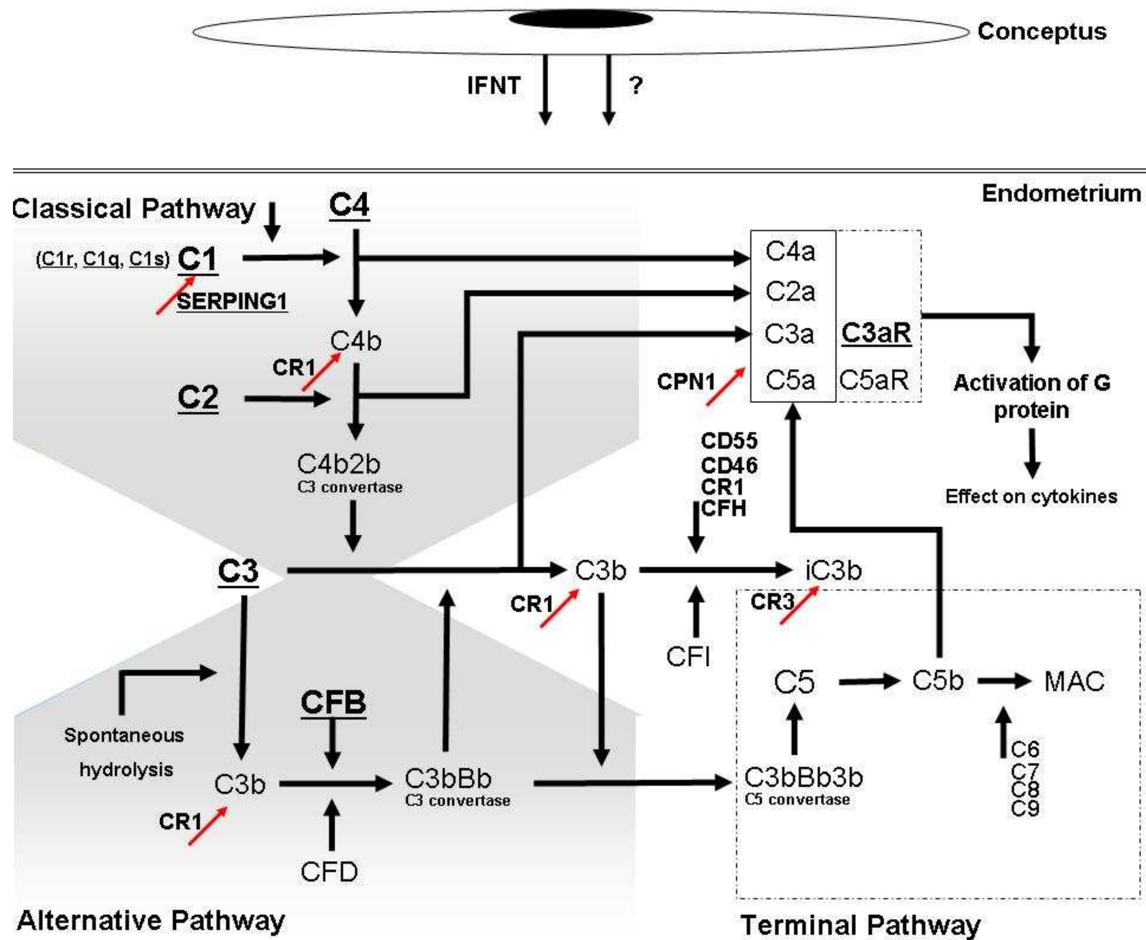


Figure 23: Activation and regulation of the complement system. Underlined components correspond to up-regulated factors at *in vivo* results.

As shown in figure 23 the alternative pathway cascade can be initiated by the spontaneous hydrolysis of C3. However, the deficiency of C3 can compromise early pregnancy in murine and is correlated with longer estrous cycle and higher resorption rates after implantation (Chow *et al.* 2009). A partial activation of the complement system can trigger embryonic development. In murine pregnancies oviductal C3 is converted into iC3b, and may support the development of the trophectoderm (Lee *et al.* 2004, Lee *et al.* 2009). Results of this study

showed a significant up-regulation of C3 in the endometrium of pregnant monozygotic twin cows, which after spontaneous hydrolysis may produce C3b. The regulatory factor CFI is responsible to cleave C3b into iC3b and therefore may be considered as an embryo protective component. It could be dangerous for the embryo if increasing expression of C3 is not compensated with concomitant generation of iC3b. Since the *in vivo* endometrial expression of C3 is not in accordance with CFI when comparing the three studies, in case of C3 activation a basal level of CFI may be enough for inactivation of C3b during early bovine pregnancy.

Additionally, a significant up-regulation of C3 in GE after IFNT treatment was also measured. These results may indicate a potential regulation of C3 by IFNT which is probably not the only mechanism of influence but also P4 may play a role in the regulation of the complement system. It was shown that C3 is present in human endometrial GE and its synthesis and regulation rises in the P4 secretory phase of the menstrual cycle (Hasty *et al.* 1994). In addition, the presence of estrogens triggered the expression of the complement factor C3 in the uterus of rats (Brown, 1990). In this case a possible spontaneous hydrolysis within the presence of a C3 convertase could lead to C3a release.

The production of anaphylatoxins such as C3a can activate further biochemical cascades via interaction with specific receptors (Monsinjon *et al.* 2003). Our results show a significant up-regulation of the C3a specific receptor C3aR during pregnancy and after IFNT treatment. The increased expression of C3aR suggests that C3a might exert an enhanced effect on cytokine production via binding to its G-protein coupled receptor on macrophages. The induction of beneficial cytokines can promote conceptus growth and survival as already discussed (Emond *et al.* 2000). As previously mentioned, an excessive activation of anaphylatoxins may contribute to pregnancy failure (Richani *et al.* 2005a). This indicates that other mechanisms besides IFNT may control the regulation of the complement factors. Possibly, together with IFNT, C3 may be controlled by P4 to support embryo development during bovine pregnancy.

In the *in vitro* experiment C5 component from the terminal pathway showed to be strongly regulated by IFNT and no regulation of this factor was measured in pregnant animals. In the complement cascade the activation of the component C5 could lead to release of the anaphylatoxin C5a which recruits and activates neutrophils. The recruitment of inflammatory cells by C5a during pregnancy has been shown to lead to fetal death in human and mice (Girardi *et al.* 2003, Richani *et al.* 2005a). Presumably during bovine pregnancy any other protective mechanisms are activated to avoid the IFNT effect on C5 release and protect the

embryo. Progesterone or other unknown components that may be produced from the embyo itself may participate in these mechanisms to prevent the activation of the terminal cascade.
Though none of the inhibitory complement factors was regulated in pregnant animals, the *in vitro* experiments show an up-regulation of CD46 and CD55 in GE after IFNT treatment. In humans excessive activation of the complement system is avoided by up-regulation of CD46 and CD55 in the endometrium at the late secretory phase and during pregnancy on the trophoblast cells (Brooimans *et al.* 1992, Cunningham & Tichenor, Jr. 1995, Young *et al.* 2002). Thus, considering that in GE cells C5 is highly expressed, it seems that excessive formation of C5 convertase might also be controlled by IFNT release which increases the expression of regulatory factors, to avoid C5a release.
For the activation of C5 component following the release of C5a, the presence of C3bBb formed after C3 hydrolysis and binding of CFB is necessary (figure 23). During pregnancy, CFB is significantly up-regulated, hence formation of C3bBb might occur. Though, no significant gene expression regulation of CFD component was measured, the basal level of CFD might be able to cleave C3bBb into C3a and C3b with formation of C3bBb3b. In this case, regulatory factors as CFH, CFI, CR1, CD55 and CD46 play an important role by inactivating C3b and preventing complement activation from proceeding on the host cell (Ehrnthaller *et al.* 2010). Since, the terminal component C5 was not differentially expressed in the endometrium of pregnant animals, apart of IFNT regulation different mechanisms may exist avoiding the activation of the terminal pathway and thereby protecting the bovine embryo during early pregnancy.
These data, together with the observation that there is an activation of the C1 complex and a possible activation of the C3 component, support the idea that the embryo is primarily recognized as a non-innate surface due to its immunohistocompatibility, and its survival might be dependent on the expression of regulatory factors for defense from the complement attack of the mother.

4.2 Analysis of pregnancy recognition in peripheral blood leukocytes

The quantification of gene expression faces numerous challenges concerning the handling and preparation of the samples, which can negatively impact RT-qPCR sensitivity and accuracy (Rainen *et al.* 2002). With the purpose to evaluate blood sampling, handling, and preparation effects, this study compared RNA yield, quality profiles, and gene expression performances of different extraction methods. Regarding RNA yield no significant differences between the sampling replicates taken from the same animal were measured, assuring optimal reproducibility. However, the mean yields obtained significantly varied from animal to

animal. This can be explained by the individual leukocyte number which changes during the estrous cycle and exposure to antigenic stimuli (Ahmadi *et al.* 2006).
Small RNA yield quantification did not show reproducible results for the samples extracted by the PAX, PI, and LL methods. A reason for that may be the extraction protocol used to isolate total RNA in two fractions (mRNA + miRNA). Probably the conditions of the filter membrane were not perfectly optimized for binding small RNA resulting in the high variance.
RNA quality determination showed the best results for the samples extracted by the LY method, followed by PAX, PI, LL and WB. Thus, with the LY method it is possible to extract the most intact RNA for further expression analysis (Hammerle-Fickinger *et al.* 2010). It should be noted that WB and LY extraction methods differ with respect to the presence of RBCs in the samples. This may contribute substantially to the lowest RNA quality of WB, as RBCs could interfere in gene expression quantification assays (Feezor *et al.* 2004, Fleige & Pfaffl 2006, Fleige *et al.* 2006, Wright *et al.* 2008).
In addition miRNA percentage was evaluated and a comparison of miRNA percentage versus mRNA quality for each extraction was drawn. The results suggest that the LY method is the best option to get a high mRNA quality with high miRNA percentage from the same extraction. Likewise for miRNA quantification the LY method often showed the most reproducible data. To our knowledge, we are the first who compared different extraction methods correlating mRNA quality and miRNA amount extracted from leukocytes. Besides the LY method, the WB method showed accurate results and the other three methods PI, PAX and LL did not show reproducible results for miRNA quantification. Although most methods tested worked well for mRNA extraction, some adjustments in the protocols may be done to improve extracted miRNA quality. There is a tendency to obtain the best miRNA results for the samples where total RNA was extracted in one fraction. For extraction performed in one fraction there is no need to separate the miRNA by applying filter membrane. Thus, a low variance in the results was obtained. To improve the results, one possibility would be to adjust the PAX, PI and LL protocols to enable extraction of total RNA in one fraction.
The precision of molecular assays depends in large part on the quality of the extracted RNA and on the reproducibility of the sample collection. To test the precision of the results, we examined the SEM evaluated for the Cq values of the different extraction methods. Therefore differentially abundant genes were measured and results of mRNA quantification show a tendency to obtain the lowest Cq within the most reproducible data for samples extracted by the LY and PAX methods (Hammerle-Fickinger *et al.* 2010).

Even though in the PAX method all blood cells are included in RNA extraction, no statistically significant difference was obtained for most of the TG measured comparing to LY method containing only WBC. For the LY method, blood handling or processing procedures could improve the risk of inducing changes in gene expression. It was shown that storage time of blood in EDTA tubes influence induction of gene expression (Kruhoffer *et al.* 2007). This induction could result in gene expression activation and for this reason the LY method could have shown the lowest Cq values. Since there was no significant difference between LY and PAX methods, it is possible to assume that the length of LY procedure before RNA extraction is not relevant for the genes measured in this study.

The WB method does not show high RNA quality, but its gene expression results can be improved by normalizing the TG to a RG (18S rRNA). It has been shown that normalization by an internal RG can partly decrease sample preparation derived effects on the quantitative results for mRNA (Fleige & Pfaffl 2006, Wittwer *et al.* 1997). For all variables considered in this study the PI and the LL extraction methods showed poor reproducibility. A reason can be the inaccurate form of WBC collection from the interphase between plasma and RBCs in the PI extraction method. In addition, it has to be considered that the LL system is optimized to stabilize and extract RNA from human blood which differs from bovine blood with respect to its cellular composition. Thus, the results obtained showed that this system may be improved prior to the use with bovine blood.

In summary, the results obtained offer an overview of practical concerns of different mRNA and miRNA extraction methods from bovine blood. As superior quality parameters were obtained with different extraction protocols, the method of choice has to be based on the necessity of the study conditions. For our study conditions the LY protocol has shown to be the best extraction method enabling a straightforward approach to handle blood samples. With the objective to guarantee a stable gene expression signature in PBL samples, the LY method was applied in the following trial.

An enhanced expression of ISG in PBL has been shown between days 15 and 20 of bovine pregnancy, in correlation with the increasing release of IFNT by the elongating embryo (Yankey et al. 2001, Han et al. 2006, Gifford et al. 2007). In this study ISG15, OAS1, MX1 and MX2 were confirmed by RT-qPCR to be up-regulated on days 16, 18 and 20 of pregnancy in PBL. Together with other significantly expressed candidate genes these data formed the basis of a PCA, clustering NP and XP groups during the time of embryo elongation.

Using PCA, we were able to clearly discriminate the pregnant from the non-pregnant group of heifers at days 18 and 20. At day 16, the samples of the two groups clustered all except

for one cow. This is in line with previous findings which showed that an endocrine response to the IFNT released by the trophoblast in the uterus enables the discrimination of pregnant versus non-pregnant cows as early as 15 days after fertilization in PBL (Oliveira *et al.* 2008).
Differential gene expression prior to the massive IFNT secretion by the conceptus has not been reported in PBL to date. As the results show, differential gene expression in maternal PBL as early as day 8 was estimated. IFNT has been detected in small amounts around day 8 of pregnancy in sheep (Farin *et al.* 1989). In bovine the hatching of the blastocyst from the zona pellucida occurs around day 8 to 10.
Other studies have quantified the expression of different genes that are prominently expressed in bovine early blastocysts and hatched blastocysts produced *in vitro* (Mohan *et al.* 2002, Hwang *et al.* 2004). Recent studies showed that bovine hatched blastocysts express genes implicated in attachment, cell adhesion, and extracellular matrix digestion (Rekik *et al.* 2011). Thus, gene expression changes at day 8 of pregnancy may correspond to an early recognition of the hatched blastocyst by the maternal immune system.
Although a maternal response to IFNT is likely to occur only locally where the blastocyst is adjacent to the endometrium (Ulbrich *et al.* 2010), it seems feasible that a maternal recognition might occur as early as the blastocyst stage if only the signal is specific enough to be encountered and amplified due to IFNT or other trophoblast secreted factors. Consequently, a number of genes in PBL possibly indicative of pregnancy as early as day 8 were estimated. The present results demonstrate for the first time that maternal PBL shows differential gene expression at day 8 of pregnancy. A PCA of these genes clearly clustered the pregnant and the non-pregnant animals in different groups.
To broaden the candidate gene approach and to follow the initial RT-qPCR findings at day 8, microarray hybridization for pair-wise samples (n = 3) taken on days 4, 8 and 12 was conducted. Microarray hybridization data for pair-wise samples was submitted to SAM analysis and did not reveal any significantly differentially expressed gene; indicating that differences in expression signals between XP and NP samples were not that consistent to reach significance after multiple testing corrections with this limited number of replicates. Therefore, a PLPE method especially useful when the number of replicates is low (Jain *et al.* 2003, Arnold *et al.* 2009, Wathes *et al.* 2009), was applied revealing differentially expressed genes at days 4, 8 and 12 of pregnancy.
The differentially expressed genes obtained by microarray analysis however did not show a distinct clustering by applying PCA at day 8. This was probably due to either low magnitude of expression differences not coherent in the number of replicates used or the number of false (positive or negative) identified. False negatives are actually differentially expressed

genes, but with a p-value larger than the rejection level (Pounds & Morris 2003), resulting in the omission of this gene as a potential candidate gene, as we have shown for MX2 and IFIT2 (figure 16). In this case, by increasing the number of samples a significant up-regulation was measured. Therefore, instead of increasing the number of animals for additional microarray hybridization, within the candidate gene approach obtained with PLPE method, further RT-qPCR gene expression analysis with a larger number of samples (n = 8) was performed.

Interestingly, IFI44, IFIT2 and IFI16 were not significantly up-regulated at day 12, and displayed inconsistent differences in expression at later days, as shown in table 11. How these gene expression changes are induced in blood remains speculative. It is feasible that antigen presenting cells such as dentritic, macrophages and B cells might migrate from the uterus to the draining lymph node to induce an immune response, which in turn can be measured in the periphery (Hey-Cunningham *et al.* 2011). Circulating nucleic acids may as well serve as messengers and are present in small amounts in serum, plasma and other body fluids (Swarup & Rajeswari 2007, Wright & Burton 2009). It is possible that immune cells or nucleic acids under the effect of IFN release, derive from the endometrium and circulate to the periphery to induce ISG in PBL (Oliveira *et al.* 2008).

A plausible explanation for the regulation of IFI44, IFIT2 and IFI16 as an IFNT mediated effect is however complicated. The expression of IFNT is fairly evident in the blastocyst stage in cattle before hatching (Hernandez-Ledezma *et al.* 1992) but increases promptly between days 14 to 21 of pregnancy (Farin *et al.* 1990). Following the pregnancy time course, if an endocrine mechanism has taken place, it is to expect that with ongoing conceptus development a consistent IFNT signal until elongation starting day 13/14 would cause a consistent receivable signal. In the endometrium spefic changes due to IFNT are measured primary at day 16 of pregnancy (Forde *et al.* 2011). Thus, gene expression changes at day 8 of pregnancy may not only be due to an endocrine response to IFNT. Rather, we hypothesize that these changes may be caused by other secreted factors from the hatching blastocyst or by an immune system response due to presence of the conceptus allograft.

It has been shown that leukocyte populations vary during late pregnancy (Oliveira & Hansen 2008). In our study, the number of total leukocytes showed no statistical differences between NP and XP animals. Hence, gene expression changes measured in leukocytes were not caused by the different number of leukocytes cells and can be considered as real gene expression regulation due to pregnancy status. To our knowledge, there is no study relating the variation of leukocyte subpopulation during early pregnancy in PBL. If changes in leukocyte subpopulations had occurred during early pregnancy causing the gene expression

differences at day 8 of pregnancy, these changes on the maternal immune function would as well indicate that the embryo can signalize its presence. Thus, even thought we have quantified the total number of leukocytes, changes in leukocyte subpopulation could lead to a differential gene expression pattern which could be used as biomarker and would nevertheless be promising. Further studies need to confirm this aspect thoroughly.

Studies in the endometrium of mammals have shown that cytokines and growth factors produced by the pre-implantation embryo are important for the maintenance of pregnancy. Studies in humans and other mammals have shown that TNFa and colony stimulating factor 1 (CSF1) are implicated in embryo-endometrium interaction during early pregnancy (Castro-Rendon *et al.* 2006, Dimitriadis *et al.* 2005, Fischer *et al.* 1999, Imakawa *et al.* 1997, Lee & Demayo 2004, Paula-Lopes *et al.* 1998, Tartakovsky & Ben Yair 1991).

Our results showed that TNFa was significantly down-regulated at day 8 of pregnancy, possibly involved in preventing the induction of luteolysis (Meidan *et al.* 1999). Other studies suggest that IFNT plays a luteoprotective role by suppressing the action of TNFa on $PGF_{2\alpha}$ synthesis in early pregnancy (Okuda *et al.* 2002). Following the time course of this factor during the pre-implantation time, a significant increase of TNFa gene expression was observed.

RT-qPCR analysis also showed a significant regulation of complement system factors at day 8 of pregnancy. The down-regulation of C1Q and up-regulation of the CD55 factor measured at day 8 of pregnancy may indicate an inhibition of the classical pathway. We sought to follow the differential gene expression of C1Q on protein level, but failed to find concomitant differences. Hence, we consider it likely that an mRNA signaling occurs in PBL, which may not be perceived as subsequent peripheral protein response.

In summary, these results show for the first time differential expression in PBL between pregnant and non-pregnant heifers as early as day 8 after insemination. The present findings may be of specific interest to develop a screening method via gene expression analysis to observe pregnancy status at day 8 after insemination in bovine PBL. The set of informative genes seems to be of critical importance. By applying PCA to this set of genes, it was possible to distinguish pregnant and non-pregnant animals, showing the correlation of this gene expression signature to early pregnancy status.

The results obtained offer an overview of differential gene expression present in maternal blood related to early pregnancy. To verify if gene expression signature can also be determined in milk cells, the same factors were measured in samples obtained from dairy cows.

4.3 Analysis of pregnancy recognition in milk somatic cells

To assure perfect sampling of milk somatic cells for the RT-qPCR analysis, the sample processing was evaluated based on previous studies (Feng et al. 2007, Sarikaya & Bruckmaier 2006). The viability of milk cells is directly correlated to the storage temperature and centrifugation conditions (Feng et al. 2007). In our studies, 450ml of milk sample were required to obtain a sufficient amount of RNA for the downstream processing. By rising the centrifugation force results in increasing the viable and non-viable cell number (Sarikaya & Bruckmaier 2006). After centrifugation, around 80% of the SC were discarded with the skim milk and the fat layer and therefore a large sample volume was necessary (Sarikaya et al. 2006).

With the objective to verify whether the volume of PBS buffer used for washing the cell pellet has an effect on yield, purity or quality of the mRNA, the cell pellet was submitted to different PBS washing steps. When comparing the results a significantly decrease of RNA quantity extracted from the final cell pellet was measured. One plausible reason might be that due to the second wash step cells were unnecessarily washed away, resulting in a lower final RNA concentration.

Moreover, results showed that a different storage time of milk samples did not significantly influence RNA purity and quality, but RNA yield. Other studies confirm these results, showing a slight decrease of the cell viability when stored at the same temperature over 24 h (Feng et al. 2007). Furthermore, a long time storage of samples can have an influence on gene expression changes (Kruhoffer et al. 2007). As expected, the difference of storage time resulted in gene expression changes for all 8 factors analyzed. Therefore, to assure results with confidence, a normalization of gene expression data has to be applied. These results enforce the importance of processing samples as fast as possible and always at the same time point after collection.

Another point to be considered when planning gene expression analysis is to ensure the necessary RNA quantity for the downstream processing. RNA quantity is related to the number of SC present in milk which can vary due to breed, age, health and lactation state of each animal (Sarikaya & Bruckmaier 2006). Due to the fact that more cells produce more mRNA, a proportional influence of SCC on RNA yield is demonstrated in this study for SCC less than 100 x 10^3 cells/ml. For SCC over 100 x 10^3 cells/ml the RNA yield decreased. Perhaps in animals with a moderate SCC inhibition between the somatic cells occurs because no further immune response was needed. Probably in case of an infection, like mastitis where SCC is above 400 x 10^3 cells/ml, the mRNA yield would rise again. Nevertheless, the objective was to assure enough RNA quantity for the downstream

processing. The results for samples with a SCC less than 10 x 10^3 cells/ml show a yield of 5 µg which could be problematic for the downstream processing, but enough for RT-qPCR analysis.
The best suitable RNA extraction method for milk SC was evaluated by comparing peqGOLD TriFast (Peqlab Biotechnologie) to the miRNeasy Mini Kit (Qiagen). Results suggest that the miRNeasy Mini Kit (Qiagen) is the most suitable extraction method. By applying this method it is possible to get a high RNA yield, purity and quality. In addition, a higher percentage of miRNA for samples extracted with miRNeasy Mini Kit (Qiagen) was measured. Considering that the integrity obtained for total RNA was satisfactory (Fleige & Pfaffl 2006) the detected miRNA should not have been contaminated with degraded long RNA (Becker et al. 2010). Hence, future studies to evaluate mRNA and miRNA gene expression of somatic milk cells can be performed using the extraction method established in this study.
After the optimization of the extraction method, a validation of the method was carried out to evaluate if the established protocol produced stable results. Therefore, the samples obtained from three animals were taken in quadruplicate at three different days and an extraction of RNA out of milk SC and a gene expression analysis was performed. As expected, the mean values obtained for RNA yield and miRNA percentage significantly varied from animal to animal. As shown for PBL also for milk somatic cells RNA yield and miRNA ratio changed significantly between different animals (Hammerle-Fickinger et al. 2010). This can be explained by the individual SCC which changes from animal to animal, resulting in different RNA yield at different days. Regarding all 4 parameters analyzed (yield, purity, quality miRNA%) no significant differences between replicates were found assuring an optimal reproducibility for the established method.
Since physiologically and immunological constitutions vary between individuals, almost all genes measured show a significant difference in gene expression. Sample replicates had not significantly differed for any of the investigated parameters, neither in RNA constitution nor in mRNA expression, assuring an optimal reproducibility. In summary, with the results obtained, a reliable and reproducible methodology for milk somatic cells and RNA extraction was established. With the objective to guarantee a stable gene expression signature in milk samples, the established protocol was applied in the following trial.
The presence of the embryo has been shown to change gene expression locally in the endometrium and in PBL (Bauersachs et al. 2006, Gifford et al. 2007, Groebner et al. 2010, Han et al. 2006, Klein et al. 2006). An analysis performed in maternal blood offered an overview of differential gene expression due to pregnancy status in the time window between days 8 to 20 (Hammerle-Fickinger et. al, in review). The same group of factors was analyzed

in milk somatic cells to find out whether gene expression information due to pregnancy status can also be measured in milk samples. Similar to gene expression in maternal blood, MX2, MX1 and OAS1 were differentially expressed in milk SC. MX2, MX1 and OAS1 were significantly up-regulated on days 8, 12, 16, 18 and 20 of pregnancy in milk somatic cells. For days 16, 18 and 20 of pregnancy similar results were measured in PBL (Hammerle-Fickinger et. al, in review).

The gene regulation in milk SC for OAS1 ranged from 3.3 -fold at day 8 to 6.5 -fold at day 18 of pregnancy. Considering the IFNT released during this period of pregnancy, these results suggest that IFNT stimulates gene expression of ISG as MX2, MX1 and OAS1 in milk somatic cells. However, as already shown, the treatment of co-cultivated endometrial GE and stroma cells with IFNT resulted in a high stimulation of classical ISG as ISG15, exceeding the gene expression obtained in untreated cells collected at day 18 of pregnancy from the uterine lumen (Groebner et al. 2011). These indicate that besides the IFNT release, other mechanisms may be responsible for the change of the differential gene expression of MX2, MX1 and OAS1 in milk SC during pregnancy.

One possible mechanism is the posttranscriptional regulation of gene expression by miRNA. The down regulation of target mRNA by miRNA has already been observed in different studies (Bagga et al. 2005, Wu et al. 2006). Mature miRNA can bind to the 3´ untranslated region (UTR) of genes by complementary base pairing (Bartels & Tsongalis 2009). Base pairing of miRNA to mRNA may mediate the translation repression or mRNA degradation. In addition, mature miRNA can alter gene expression by binding to the 5´ UTR (Tay et al. 2008).

Different factors related to early bovine pregnancy, such as bone marrow stromal cell antigen 2 (BST2), signal transducer and activator of transcriptions 1 (STAT1) and Indoleamine 2,3-dioxygenase (IDO) were additionally measured in milk SC (Bauersachs et al. 2006, Groebner et al. 2011). BST2 and STAT1 gene expression was significantly up-regulated in milk SC between days 8 to 20 of pregnancy. In line with these results, for BST2 and STAT1 higher expression differences in endometrium samples from day 18 pregnant vs. non-pregnant were measured by RT-qPCR and array analyses (Bauersachs et al. 2006). In addition, the localization of BST2 mRNA expression in endometrium tissue at day 18 of pregnancy was shown by in situ hybridization (Bauersachs et al. 2006). Exact information regarding the function of these factors during early pregnancy is to date not clear.

According to reported results in the endometrium of pregnant animals at day 18 after fertilization (Groebner et al. 2011), IDO was significantly higher expressed in this study at days 18 and 20 of pregnancy in milk SC. The increase of IDO mRNA in bovine endometrium

due to trophoblast derived IFNT as shown *in vitro* is accompanied by elevated enzyme activity, indicating a protection mechanism for the embryo against maternal rejection (Groebner et al. 2011). Within these results, a number of genes in milk SC possibly indicative of pregnancy were estimated. Using PCA, it was possible to clearly discriminate the pregnant from the non-pregnant group of dairy cows at days 12 and 18 of pregnancy. At days 8, 16 and 20 samples of the two groups clustered all except for one cow.

Previous results estimate differential gene expression in maternal PBL as early as day 8 which enables the discrimination of pregnant from the non-pregnant group of heifers by using PCA (Hammerle-Fickinger et. al, in review). In milk somatic cells 5 differential expressed genes were measured at day 8 of pregnancy but PCA was not able to clearly discriminate the non-pregnant from the pregnant group of dairy cows. Interestingly, the same group of differential expressed genes was measured at day 12 after pregnancy in milk SC with PCA showing a good separation of both groups.

The raw data used for PCA were the ΔCq values of the differential expressed genes and the P4 values. The ΔCq values of the 5 differential expressed genes used for PCA at day 12 were slightly higher than at day 8 for most genes measured. In particular, BST2 fold regulation was two times higher at day 12 than at day 8 of pregnancy in milk SC. Other studies have demonstrated that no differences of milk progesterone concentrations were found until day 12 when comparing pregnant versus non-pregnant animals (Bulman & Lamming 1979, Lamming & Bulman 1976). Considering that before day 21 after insemination bovine pregnancy cannot solely be determined by P4 concentration (Dobson & Fitzpatrick 1976), it is reasonable to use P4 values in the PCA until day 12. It seems that due to differences in gene expression, PCA was able to clearly discriminate the pregnant animals from the non-pregnant animals at day 12 and not at day 8.

How exactly these gene expression changes are induced in milk SC remains speculative, but it is feasible to consider that these factors were influenced due to conceptus IFNT secretion. It is possible that movement of the leukocytes carrying IFNT response occurs out of the peripheral circulatory system by diapedesis into the milk cells (Smits et al. 1999). Therefore, it can be assumed that the underlying factors involved in regulatory mechanisms causing differential gene expression during pregnancy in PBL also diffuse to the mammary gland making it possible to measure gene expression differences in milk somatic cells during early pregnancy.

The understanding of IFNT expression regulation in ruminants during pregnancy is far from being complete. Most studies of IFNT influence in gene expression due to pregnancy in bovine have been focused on the endometrium and blood (Bauersachs et al. 2007, Forde et

al. 2011, Gifford et al. 2007, Yankey et al. 2001). The present results demonstrate for the first time that milk somatic cells show differential gene expression of ISG in the time window of days 8 to 20 of pregnancy. The number of quantified genes is yet too less to draw conclusions on its biological mechanisms, but it is possible to determine genes that might act as potential biomarkers. Within these set of genes, PCA shows that it is possible to distinguish pregnant and non-pregnant animals showing its correlation to early pregnancy.
The identified genes may be useful to determine the pregnancy status in milk somatic cells which is a non invasive sample to be obtained from living animals. The benefit for the dairy industry of this test is the possibility to diagnose pregnant cows before day 21 after insemination. Consequently, within that it is possible to have more cows with optimum calving intervals. Nevertheless, statistically relevant conclusions will be possible if more samples are investigated.

5 Conclusions and perspectives

The understanding of the immune signaling pathways in the pre-implantation period is of clinical and economical significance. In order to achieve advances in bovine reproductive technology, a deep knowledge of cellular, molecular and genomic processes is required. Thus, by interpreting gene expression changes that characterize the pre-implantation period, it may be possible to understand the immunological response to the embryo. This is the first study with focus on the gene expression analysis on complement system factors in the endometrium and on different factors stimulated in blood and milk cells during the pre-implantation period.

Regarding the complement system activation, the results of this thesis indicate a partial activation of the complement system cascade during early pregnancy in cattle. It is assumable, that activation of the complement cascade until a certain level might support the development of the bovine embryo and might avoid resorption after implantation. Nevertheless, excessive activation of the complement system has to be avoided by regulation of inhibitory factors in order to prevent the semi-allogenic embryo from rejection.

We presume that during bovine pregnancy the regulation of the complement system responds not only to the paternal antigen presented by the embryo but also to the IFNT stimulation. The *in vitro* experiments demonstrate that the complement system is triggered by IFNT and possibly by other signaling factors expressed by the mother and the embryo. This response to IFNT shows the possibility of the conceptus to influence the maternal immune system by adjusting regulatory factors in order to avoid rejection.

The physiological implications of the pathways involved in the modulation of the immune system induced by the embryo during early pregnancy are yet mostly unknown. A massive IFNT secretion by the conceptus takes place after day 15 of pregnancy; prior to this time point, a plausible explanation for the regulation of ISG as an endocrine signal is however complicated. Nevertheless, the changes provide the opportunity to identify a pregnancy gene expression signature in peripheral tissues as PBL and milk somatic cells. So far, only studies considering days 15 to 20 of pregnancy have been accomplished in PBL.

To ensure a sensitive and specific detection of the target genes in blood and milk samples, sampling and extraction methods were successfully established, enabling a straightforward approach to handle blood and milk samples in the main trials. To test the potential to use a gene expression signature in the bovine periphery as indicative of early pregnancy, animal trials with heifers and dairy cows were carried out. Though not lactating, heifers instead of dairy cows were used for the trial with blood cells. That way, with a reasonable effort the metabolic state could be kept highly homogenous and mammary gland inflammation

activating the immune system under investigation could be excluded. Candidate genes found to be differentially expressed in PBL were then analyzed in milk samples obtained from dairy cows.

The results of this thesis show changes in gene expression due to early pregnancy in blood leukocytes and in milk somatic cells. Comparing the results obtained from both studies, it could be observed that 5 differentially regulated genes in PBL (MX1, MX2, OAS1, IFI16, and TNFa) show similar expression regulation in the milk somatic cells. Regulation of fold change was similar between both studies indicating that gene expression in the circulating system might be detected in milk somatic cells showing a distinct gene regulation concerning the state of pregnancy.

Although the number of quantified genes in both studies is yet too small to draw conclusions regarding biological pathways, gene expression data in combination with PCA show promising results for the development of potential gene expression biomarkers. This study demonstrates that a specific set of genes showed differential gene expression between pregnant and non-pregnant heifers not only at days 16, 18 and 20 of pregnancy, but also at day 8 of pregnancy in blood leukocytes and at day 12 in milk somatic cells. A PCA clearly clustered the NP and the XP group, thus showing that it is possible to consider this set of informative genes as pregnancy biomarkers. For a more precise and reliable pregnancy prediction, further parameters such as breed, nutritional status, age and immunological status of the animal have to be taken into consideration.

The underlying mechanisms causing differential gene expression at day 8 of pregnancy in blood leukocytes point towards an early maternal recognition of pregnancy due to the hatching of the blastocyst and warrant further exploitation. The earliest sampling day possible to distinguish clustering between NP and XP group was day 12 in milk somatic cells. It is possible to hypothesize that this lag time is due to the movement of leukocytes out of the peripheral circulatory system by diapedesis into the milk cells. The physiological regulatory mechanisms remain unclear, however considering that these regulated factors are all ISG they might respond to IFNT release showing the ability of the conceptus to exert a signal on milk somatic cells.

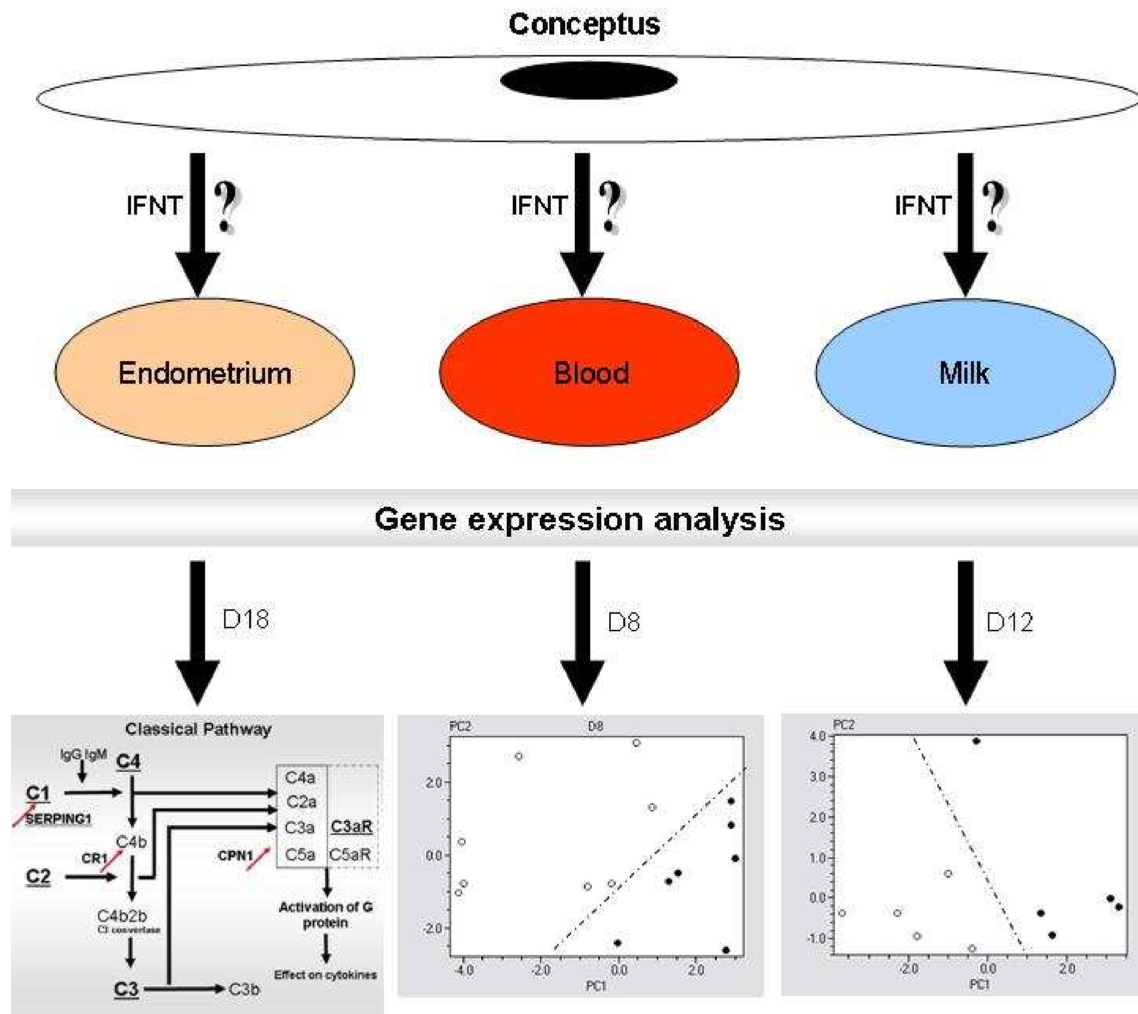


Figure 24: Schematic representation of the results obtained in this study.

By the use of the established methods in this study, miRNA was successfully extracted from blood and milk cells, enabling further investigations. Due to the fact that miRNA are more stable than mRNA, those small molecules are considered an emerging RNA category of biomarkers (Shivdasani 2006, Cannell *et al.* 2008). As a consequence, miRNA are likely to be useful in clinical diagnostic, especially in cancer- and virus diagnostics (Lu *et al.* 2005, Mitchell *et al.* 2008, Esquela-Kerscher & Slack 2006). Considering that few is known about the physiological functionality of miRNA in cattle, future studies in this area may significantly help to understand the role of miRNA in physiological processor function. This as a consequence can contribute to implement a classification of an animal as pregnant or non-pregnant by enlarging the existent data base with the quantification analysis of the miRNA samples of each group. Regarding this for ongoing studies, the correlation results from

mRNA and miRNA analyses do play a key role for a better understanding of physiological modifications during early pregnancy.

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"Evaluation of different extraction protocols of total RNA and microRNA from bovine blood for gene expression analysis"
4th qPCR Symposium, 9-13 March 2009, Freising, Germany

Hammerle-Fickinger A, Bauersach S, Wolf E, Meyer HHD, Pfaffl MW, Ulbrich SE.
"Identification of gene expression indicative of pregnancy in the pre-attachment period in bovine peripheral blood leukocytes"
8th International Ruminant Reproduction Symposium, 3-7 September 2010, Anchorage, Alaska

Curriculum vitae

Andréa Hammerle-Fickinger

Date of birth	July, 11th 1978
Place of birth	Porto Alegre, Brazil

03/2006 - 02/2010	PhD-Thesis at Physiology-Weihenstephan Technical University of Munich
10/2003 - 11/2005	Studies in Chemical and Process Engineering Otto–von–Guericke–Universität–Magdeburg, Germany Degree: “Master of Science”
07/1996 - 01/2003	University studies in Food Engineer, Unisinos São Leopoldo, Brazil Degree: “Food Engineer”
08/1993 - 2/1995	High School Degree “São Francisco de Assis” Porto Alegre, Brazil
10/2002 - 07/2005	Elementary School “Colégio Maria Auxiliadora” Canoas, Brazil

Appendix

Table 1: Microarray results for days 4, 8 and 12 of pregnancy. Statistics analysis was performed using PLPE analysis. Pairwise comparison between NP and XP group was performed within samples taken from 3 animals at days 4 and 12 and 4 animals at day 8 after AI.

Regulated genes on day 4 of pregnancy

Gene Symbol	Gene Name	Systematic Name	Ratio	p value	FDR
ADAMTS1	ADAM metallopeptidase with thrombospondin type 1 motif, 1	BC151273.1	2.22	0.433	0.00
ALOX15	arachidonate 15-lipoxygenase	AY445523.1	0.63	0.012	1.08
ALOX5	arachidonate 5-lipoxygenase	AJ306424.1	0.56	0.041	1.08
ARHGAP10	Rho GTPase activating protein 10	NM_001076830.1	0.63	0.030	1.02
BCAR3	breast cancer anti-estrogen resistance 3	NM_001024483.1	0.67	0.027	1.08
BCAS1	breast carcinoma amplified sequence 1	BC126684.1	2.87	0.239	0.00
BGN	biglycan	BC118460.1	0.62	0.182	0.00
BRB	brain ribonuclease	NM_173891.2	0.65	0.241	0.00
C12H13ORF3	chromosome 13 open reading frame 3 ortholog	NM_001110067.1	1.52	0.023	1.08
C13H10ORF97	chromosome 10 open reading frame 97 ortholog	NM_001076107.1	1.54	0.718	0.00
C16H1ORF14	chromosome 1 open reading frame 14 ortholog	DT857793.1	0.60	0.189	0.00
C3H1ORF212	chromosome 1 open reading frame 212 ortholog	NM_001098950.1	0.63	0.159	0.00
C8H9orf165	chromosome 9 open reading frame 165 ortholog	XM_001252082.2	0.60	0.017	0.05
CD9	CD9 molecule	BC147992.1	0.65	0.071	0.00
CDKL2	cyclin-dependent kinase-like 2 (CDC2-related kinase)	NM_001103274.1	1.54	0.012	1.08
CDKN2B	cyclin-dependent kinase inhibitor 2B (p15, inhibits CDK4)	NM_001075894.1	0.64	0.030	1.08
CFDP2	craniofacial development protein 2	D84514.1	0.66	0.232	0.04
CMA1	chymase 1, mast cell	XM_593156.4	2.55	0.724	0.00
CSF1	colony stimulating factor 1 (macrophage)	AY181987.1	2.02	0.725	0.00
CTNNA3	catenin (cadherin-associated protein), alpha 3	NM_001079638.2	1.53	0.004	1.08
CXCR4	chemokine (C-X-C motif) receptor 4	AF399642.1	1.64	0.873	0.00
DEFB1	defensin, beta 1	NM_175703.3	0.58	0.211	0.00
ESAM	endothelial cell adhesion molecule	BC105434.1	0.64	0.130	0.02
FBP1	fructose-1,6-bisphosphatase 1	DQ520945.1	0.61	0.038	0.20

GATA1	GATA binding protein 1 (globin transcription factor 1)	XM_868355.2	0.56	0.021	0.00
GATA2	GATA binding protein 2	XM_583307.3	2.37	0.940	0.00
GBP6	guanylate binding protein family, member 6	XM_591383.4	1.96	0.218	0.00
GPRIN2	G protein regulated inducer of neurite outgrowth 2	XM_592057.3	3.95	0.305	0.00
GZMB	granzyme B (granzyme 2, cytotoxic T-lymphocyte-associated serine esterase 1)	NM_174296.2	1.69	0.608	0.00
HP	haptoglobin	AJ271156.1	0.62	0.269	0.00
IL15	interleukin 15	EU682380.1	1.62	0.585	0.00
IL1B	interleukin 1, beta	X12498.1	1.50	0.460	0.00
IL5RA	interleukin 5 receptor, alpha	XM_867769.3	0.63	0.048	1.08
ITGA9	integrin, alpha 9	XM_610637.4	0.57	0.003	1.02
KCNJ16	potassium inwardly-rectifying channel, subfamily J, member 16	NM_001076294.1	1.76	0.493	0.00
KPNB1	karyopherin (importin) beta 1	XM_869563.3	2.28	0.164	0.00
KRT23	keratin 23 (histone deacetylase inducible)	BC149166.1	1.87	0.031	1.08
LOC100126815	MHC class I-like family A1	BC113339.1	0.56	0.172	0.00
LOC100139857	similar to ryanodine receptor	XM_001788404.1	0.63	0.013	1.08
LOC100139881	similar to protease-2	XM_001790075.1	2.37	0.808	0.00
LOC511424	hypothetical protein LOC511424	NM_001104973.1	0.58	0.029	1.08
LOC532412	similar to shugoshin-like 2	BC120219.1	2.25	0.351	0.00
LOC538803	similar to ring finger and CCCH-type zinc finger domains 2	XR_042798.1	1.69	0.297	0.02
LOC616153	hypothetical LOC616153	NM_001099391.1	1.62	0.547	0.00
LOC616928	hypothetical LOC616928	XM_869069.2	0.67	0.017	1.08
LOC782827	similar to duodenase	XM_001249716.1	2.12	0.665	0.01
LOC782827	similar to duodenase	XM_001249716.1	2.21	0.652	0.00
LOC785952	similar to duodenase	XM_001252800.2	1.62	0.630	0.00
LOC786126	similar to duodenase	XM_001252848.1	2.17	0.674	0.00
LOC789236	similar to melanoma antigen family B, 4	XM_001256044.2	3.82	0.179	0.00
LOC789628	similar to guanylate binding protein family, member 6	XM_001256326.1	2.00	0.165	0.00
LOC789856	similar to zinc finger CCCH-type containing 13	XM_001256485.1	0.67	0.303	0.00
MPO	myeloperoxidase	XM_001788302.1	0.54	0.023	0.00
N4BP2L2	NEDD4 binding protein 2-like 2	BC120192.1	1.68	0.382	0.00

P2RY12	purinergic receptor P2Y, G-protein coupled, 12	AJ623293.1	1.57	0.049	1.08
PION	pigeon homolog (Drosophila)	XM_866880.3	1.55	0.017	1.08
PPAP2A	phosphatidic acid phosphatase type 2A	NM_001080329.1	0.60	0.125	0.00
PPP1R3C	protein phosphatase 1, regulatory (inhibitor) subunit 3C	BC120042.1	0.60	0.048	1.08
S1PR5	sphingosine-1-phosphate receptor 5	XM_595705.4	0.65	0.012	0.59
SERPINE1	serpin peptidase inhibitor, clade E (nexin, plasminogen activator inhibitor type 1), member 1	BC103451.1	1.56	0.608	0.00
SLU7	SLU7 splicing factor homolog (S. cerevisiae)	BC103394.1	1.84	0.341	0.00
SMPD3	sphingomyelin phosphodiesterase 3, neutral membrane (neutral sphingomyelinase II)	XM_592012.4	0.61	0.054	0.00
SV2B	synaptic vesicle glycoprotein 2B	XM_001787141.1	0.60	0.045	0.48
SYT12	synaptotagmin XII	XM_616964.3	4.70	0.314	0.00
SYT6	synaptotagmin VI	XM_617812.4	5.45	0.185	0.00
TPPP	tubulin polymerization promoting protein	BC133343.1	0.67	0.038	1.08
TTC25	tetratricopeptide repeat domain 25	BC142347.1	1.79	0.264	0.02
TTC30A	tetratricopeptide repeat domain 30A	NM_001081716.1	0.62	0.199	0.00
TTC33	tetratricopeptide repeat domain 33	XM_609769.4	1.53	0.024	1.08
UPF2	UPF2 regulator of nonsense transcripts homolog (yeast)	BC126821.1	1.64	0.574	0.00
UPF3B	UPF3 regulator of nonsense transcripts homolog B (yeast)	NM_001076411.1	1.78	0.108	0.05
WNT2B	wingless-type MMTV integration site family, member 2B	BC146036.1	0.66	0.015	1.08
ZDHHC14	zinc finger, DHHC-type containing 14	XM_595621.3	0.62	0.006	0.92

Regulated genes on day 8 of pregnancy

Gene Symbol	Gene Name	Systematic Name	Ratio	p value	FDR
ACE	angiotensin I converting enzyme (peptidyl-dipeptidase A) 1	XM_865735.1	1.56	0.0296	0.24
ADH6	alcohol dehydrogenase 6 (class V)	NM_001046057.1	2.02	0.0396	0.60
ANGPTL2	angiopoietin-like 2	EU599225.1	3.05	0.0023	0.00
ANKS4B	ankyrin repeat and sterile alpha motif domain containing 4B	NM_001075192.1	1.56	0.0202	0.06
APBA2	amyloid beta (A4) precursor protein-binding, family A, member 2	BC126581.1	0.51	0.0041	0.00
ATAD1	ATPase family, AAA domain containing 1	XM_582436.4	1.53	0.0178	0.07
ATP2B1	ATPase, Ca++ transporting, plasma membrane 1	BC126488.1	1.77	0.0248	0.14

BAZ2B	bromodomain adjacent to zinc finger domain, 2B	BC148866.1	2.71	0.0025	0.00
BBX	bobby sox homolog (Drosophila)	XM_612509.4	1.88	0.0308	0.28
BCAS1	breast carcinoma amplified sequence 1	BC126684.1	2.69	0.0284	0.25
C22H3ORF63	chromosome 3 open reading frame 63 ortholog	XM_587195.4	2.16	0.0320	0.30
C9HXorf21	chromosome X open reading frame 21 ortholog	NM_001038537.1	1.90	0.0567	1.09
CAMK2B	calcium/calmodulin-dependent protein kinase II beta	BC105210.1	1.98	0.0070	0.04
CCPG1	cell cycle progression 1	BC111294.1	1.95	0.0084	0.04
CDH6	cadherin 6, type 2, K-cadherin (fetal kidney)	NM_001034640.1	0.48	0.0072	0.04
CEBPA	CCAAT/enhancer binding protein (C/EBP), alpha	AY621546.1	4.74	0.0485	0.97
CFDP2	craniofacial development protein 2	D84514.1	0.55	0.0034	0.00
CNTN4	contactin 4	XM_600040.4	2.09	0.0578	1.09
CPB2	carboxypeptidase B2 (plasma)	BC112649.1	1.98	0.0331	0.35
CROP	cisplatin resistance-associated overexpressed protein	NM_001034684.1	1.66	0.0514	1.09
DDX58	DEAD (Asp-Glu-Ala-Asp) box polypeptide 58	XM_580928.3	2.08	0.0504	1.07
DHDDS	dehydrodolichyl diphosphate synthase	BC120227.1	1.80	0.0599	1.09
DLGAP5	discs, large (Drosophila) homolog-associated protein 5	BC123614.1	1.75	0.0375	0.49
DLK1	delta-like 1 homolog (Drosophila)	AY360448.1	2.25	0.0288	0.24
EIF2AK2	eukaryotic translation initiation factor 2-alpha kinase 2	AB104655.1	1.88	0.0594	1.09
EIF4E	eukaryotic translation initiation factor 4E	AF257235.1	1.85	0.0056	0.00
FAP	fibroblast activation protein, alpha	NM_001098001.1	1.84	0.0460	0.90
FLYWCH1	FLYWCH-type zinc finger 1	XM_610323.3	2.09	0.0504	1.07
FZD7	frizzled homolog 7 (Drosophila)	XM_602576.4	4.79	0.0289	0.24
GPRIN2	G protein regulated inducer of neurite outgrowth 2	XM_592057.3	3.61	0.0002	0.00
GRIK1	glutamate receptor, ionotropic, kainate 1	BC123877.1	0.60	0.0391	0.60
HERC5	hect domain and RLD 5	BC149081.1	1.91	0.0166	0.07
HLTF	helicase-like transcription factor	BC120459.1	2.10	0.0310	0.27
HSPCA	heat shock 90kD protein 1, alpha	BC102618.1	1.80	0.0137	0.06
HSPCA	heat shock 90kD protein 1, alpha	BC151818.1	1.71	0.0262	0.13
IGF2	insulin-like growth factor 2 (somatomedin A)	AB099052.1	2.42	0.0108	0.04
IGK	Ig kappa chain	BC122795.1	0.65	0.0370	0.50
IL18	interleukin 18 (interferon-gamma-inducing factor)	NM_174091.2	2.50	0.0134	0.03

ISL1	ISL LIM homeobox 1	BC146163.1	3.20	0.0012	0.00
KCNJ1	potassium inwardly-rectifying channel, subfamily J, member 1	XM_585917.4	3.80	0.0001	0.00
KIF15	kinesin family member 15	XM_614710.3	1.85	0.0268	0.19
LPIN1	lipin 1	XM_865119.3	3.27	0.0563	1.09
MAPK6	mitogen-activated protein kinase 6	BC112793.1	2.10	0.0218	0.10
MED24	mediator complex subunit 24	NM_001045892.1	1.61	0.0501	1.09
MORC3	MORC family CW-type zinc finger 3	BC151785.1	2.18	0.0052	0.00
MSR1	macrophage scavenger receptor 1	AM037916.1	1.52	0.0544	1.09
N4BP2L2	NEDD4 binding protein 2-like 2	BC120192.1	2.13	0.0024	0.00
NCAPG	non-SMC condensin I complex, subunit G	BC133564.1	1.56	0.0249	0.13
PACAP	Proapoptotic caspase adapter protein	NM_001098930.1	0.65	0.0586	1.09
PLS3	plastin 3 (T isoform)	BT030679.1	1.58	0.0150	0.05
POPDC2	popeye domain containing 2	BC142098.1	3.02	0.0120	0.03
PPP1R12A	protein phosphatase 1, regulatory (inhibitor) subunit 12A	BC149332.1	2.09	0.0521	1.09
PRKAG3	protein kinase, AMP-activated, gamma 3 non-catalytic subunit	BC109945.1	2.02	0.0580	1.09
RC3H2	ring finger and CCCH-type zinc finger domains 2	XM_001251110.2	1.96	0.0446	0.84
ROBO2	roundabout, axon guidance receptor, homolog 2 (Drosophila)	XM_614756.4	2.29	0.0320	0.30
SAMD9	sterile alpha motif domain containing 9	BC151751.1	2.91	0.0053	0.00
SLC24A6	solute carrier family 24 (sodium/potassium/calcium exchanger), member 6	XM_585733.3	1.79	0.0526	1.09
SLC36A3	solute carrier family 36 (proton/amino acid symporter), member 3	XM_613403.4	2.48	0.0068	0.05
SLC6A2	solute carrier family 6 (neurotransmitter transporter, noradrenalin), member 2	NM_174608.2	1.66	0.0263	1.09
SLU7	SLU7 splicing factor homolog (S. cerevisiae)	BC103394.1	2.43	0.0585	1.09
SYT12	synaptotagmin XII	XM_616964.3	4.99	0.0000	0.00
SYT6	synaptotagmin VI	XM_617812.4	3.07	0.0026	0.00
THUMPD3	THUMP domain containing 3	BT025397.1	2.12	0.0465	0.91
TLN1	talin 1	BC122766.1	1.56	0.0209	0.08
TP53RK	TP53 regulating kinase	BC134447.1	1.62	0.0437	1.09
TTLL3	tubulin tyrosine ligase-like family, member 3	BC120235.1	1.56	0.0525	1.09
UGT2B11	UDP glucuronosyltransferase 2 family, polypeptide B11	XM_001788093.1	1.68	0.0554	1.09
UPF2	UPF2 regulator of nonsense transcripts homolog (yeast)	BC126821.1	1.98	0.0122	0.03

UPF3B	UPF3 regulator of nonsense transcripts homolog B (yeast)	XM_613004.4	2.10	0.0250	0.13
XIAP	X-linked inhibitor of apoptosis	XM_583068.3	1.77	0.0464	0.90
ZEB2	zinc finger E-box binding homeobox 2	NM_001076192.1	1.95	0.0451	0.83

Regulated genes on day 12 of pregnancy

Gene Symbol	Gene Name	Systematic Name	Ratio	p value	FDR
ACTB	actin, beta	NM_173979	0.58	0.008	0.04
ACTL9	actin-like 9	BC111236.1	0.64	0.031	1.10
ANG	angiogenin, ribonuclease, RNase A family, 5	NM_001078144.1	1.81	0.490	0.04
ANG2	angiogenin 2	NM_001099396.1	2.31	0.481	0.00
CRELD1	cysteine-rich with EGF-like domains 1	NM_001014851.2	0.47	0.024	0.51
CTTN	cortactin	NM_001075287.1	0.53	0.040	1.10
E2F2	E2F transcription factor 2	XM_869196.1	0.54	0.254	0.04
FBP1	fructose-1,6-bisphosphatase 1	DQ520945.1	2.33	0.673	0.00
FOXE3	forkhead box E3	XM_001254710.1	0.62	0.315	0.00
GADD45G	growth arrest and DNA-damage-inducible, gamma	BC112476.1	2.01	0.535	0.05
HAMP	hepcidin antimicrobial peptide	NM_001114508.1	2.80	0.302	0.00
HBZ	hemoglobin, zeta	XM_580707.4	0.62	0.011	1.10
HSPB8	heat shock 22kDa protein 8	BC102299.1	1.97	0.034	1.10
ID1	inhibitor of DNA binding 1, dominant negative helix-loop-helix protein	AY830148.1	0.64	0.306	0.00
INHBC	inhibin, beta C	XM_609262.3	0.39	0.148	0.00
IRAK2	interleukin-1 receptor-associated kinase 2	EU528621.1	0.67	0.045	1.10
KCNJ15	potassium inwardly-rectifying channel, subfamily J, member 15	BC142156.1	0.62	0.165	0.00
LOC506672	similar to brain-specific angiogenesis inhibitor 2	XR_028670.2	1.54	0.013	1.10
LOC515676	similar to CG33196-PB	BC149245.1	0.50	0.252	0.00
LOC526739	similar to Glycerol-3-phosphate acyltransferase, mitochondrial precursor (GPAT)	XM_605113.3	1.86	0.017	1.10
LOC527796	similar to Putative serine/threonine-protein kinase F31E3.2	XM_606198.3	0.34	0.072	0.00
LOC616117	similar to Uncharacterized protein C8orf58	XM_868083.3	0.50	0.129	0.00
LOC617296	similar to phospholipase C, gamma 2	XM_869529.2	1.50	0.003	1.10

LOC780933	cationic trypsin	NM_001113727.1	0.55	0.010	1.10
LOC784455	similar to olfactory receptor Olr1615	XM_001252758.1	3.17	0.432	0.00
LOC785561	similar to cyclin B2	XR_028283.2	1.57	0.018	1.10
LOC786537	similar to zinc finger protein 770	XR_042855.1	0.57	0.175	0.00
LOC788205	hypothetical protein LOC788205	BC123905.1	1.61	0.041	1.10
LOC789078	similar to oxysterol-binding protein-like 1A	XM_001255935.2	1.76	0.015	0.77
MAP4	microtubule-associated protein 4	BC126534.1	0.58	0.026	0.52
MRCL	mannose receptor-like precursor	NM_001102034.1	1.96	0.038	1.10
OOSP1	oocyte-secreted protein 1	DQ256127.1	0.51	0.215	0.05
PID1	phosphotyrosine interaction domain containing 1	BC126658.1	1.78	0.007	0.00
SDR42E1	short chain dehydrogenase/reductase family 42E, member 1	NM_001080292.1	1.50	0.045	1.10
SMPD3	sphingomyelin phosphodiesterase 3, neutral membrane (neutral sphingomyelinase II)	XM_592012.4	2.70	0.616	0.00
SRRM2	serine/arginine repetitive matrix 2	XM_587832.4	1.55	0.045	1.10
TAS1R1	taste receptor, type 1, member 1	XM_601773.2	0.45	0.028	0.00
WFDC2	WAP four-disulfide core domain 2	NM_001076490.1	0.37	0.179	0.00