PATHOLOGIC EVALUATION OF TYPE 2 PORCINE REPRODUCTIVE AND RESPIRATORY SYNDROME VIRUS INFECTION OF PREGNANT GILTS DURING LATE GESTATION

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ABSTRACT

Despite nearly 25 years of research, pathogenesis of fetal death in PRRS still remains unclear. This large-scale study is aimed to provide overarching insight into the process of infection within uterus and fetus. Hypotheses of this thesis are that PRRSV-specific pathological lesions, their severity, replication in susceptible cells, and cell death are significantly associated with PRRSV infection both on maternal and fetal side, and fetal death. The objectives of this thesis were to assess potential associations between specific histopathological lesions, numbers of CD163, CD169, apoptotic cells and areolae at the maternal-fetal interface (MFI) and PRRSV RNA concentration in the fetuses and the MFI. Dams and their litters were humanely euthanized and necropsied 21 days later. Samples from the maternal-fetal interface (uterus with fully attached placenta) and fetal thymus were collected for analysis by RT-qPCR to quantify PRRSV RNA concentration. The corresponding paraffin-embedded uterine section was subjected to immunohistochemistry for PRRSV nucleocapsid N protein, CD163, CD169, cathepsin and TUNEL assay for apoptosis. Our findings confirm type 2 PRRSV infection in pregnant gilts induces significant histopathological lesions at MFI, but they are not associated with the presence of PRRSV in the MFI and fetal thymus at 21 days post infection. Fetal pathological lesions are associated with the presence of PRRSV in the MFI and fetal thymus, and meconium staining of fetuses is significantly associated with the presence of both fetal and umbilical lesions observed 21 days post infection. Studies presented in this thesis confirm that although type 2 PRRSV infection in pregnant gilts in the third trimester of gestation induces significant pathological lesions at the maternal-fetal interface, fetal and umbilical pathology together with PRRSV viral load in the fetus and increased cell death at the MFI contribute to fetal compromise and death. This study also found that only an increase in numbers of CD163 positive

macrophages in the endometrium in concert with the decrease in the numbers of CD163 positive macrophages in the fetal placenta significantly increased the probability of PRRSV infection of the fetus.

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LIST OF ABBREVIATIONS

AEC	3-amino-9-ethylcarbazole
AIAO	all-in/all-out
AREB	animal research ethics board
BVDV	bovine viral diarrhea virus
CD	cluster of differentiation
DNA	deoxyribonucleic acid
DPI	days post inoculation
HE	hematoxylin and eosin
HIP	humane intervention point
HP-PRRSV	highly pathogenic porcine reproductive and respiratory syndrome virus
IFN	interferon
Ig	immunoglobulin
IL	interleukin
McREBEL	management changes to reduce exposure to bacteria to eliminate losses
MELOGIT	mixed effects logistic regression model
MEOLOGIT	mixed effects proportional odds model
MIXED	mixed effects linear regression models
MLV	modified-live vaccine
Nsp	nonstructural proteins

NVSL	national veterinary services laboratories
OR	odds ratio
ORFs	open reading frames
PAM	pulmonary alveolar macrophages
PCR	polymerase chain reaction
PEE	porcine endometrial endothelial cells
PIM	intravascular macrophages
PDNS	porcine dermatitis and nephropathy syndrome
PRDC	porcine respiratory disease complex
PRRS	porcine respiratory and reproductive syndrome
PRRSV	porcine respiratory and reproductive syndrome virus
qRT-PCR	quantitative reverse transcription PCR assay
RNA	ribonucleic acid
$TCID_{50}$	50% Tissue Culture Infective Dose
TdT	terminal deoxynucleotidyl transferase
TNF	tumor necrosis factor
TUNEL	terminal deoxynucleotidyl transferase-mediated dUTP nick end labeling
UTP	uridine-5'-triphosphate

1 LITERATURE REVIEW

1.1 Female reproduction of swine

1.1.1 Normal reproductive cycle and gestational physiology

Pigs are polytocous species of domestic animals, which means that, in a reproductive cycle, can give birth to a larger number of offspring (piglets), which, in modern swine production is typical between 10 and 16 piglets (Soede 2011). The ovarian activity of pigs is cyclical during the whole year and manifested by 18-25 day long estrus cycle with ovulation happening approximately 44 hours after the onset of the luteinizing hormone (LH) peak (Soede 2011). In pigs, sexual maturity (puberty) is reached between 150 and 220 days of age and the gestation lasts an average of 114 days (Senger 2012). Pregnancy is a complex physiological process in which the whole body (and particularly reproductive organs) undergo numerous morphological, histological, and hormonal changes which are a direct consequence of growth and development of the conceptus and strong maternal organ responses (Senger 2012).

The success of the pregnancy depends on proper attaching of the embryo to the uterus epithelium in the four-step process comprised of the development of the embryo, hatching of the blastocyst, maternal recognition of gestation and formation of extraembryonic membranes (placenta) (Bazer and Johnson 2014). The unique property of gilts and sows in comparison to other mammalian species is the ability of pig fetus to produce estradiol as a signal for maternal recognition of pregnancy (Bazer 2013).

Another specificity of pig gestational physiology is the secretion of $PGF_{2\alpha}$ under the influence of estradiol which is rerouted from the uterine endometrial vasculature into the uterine lumen preventing the luteolysis of the *corpus luteum* which secretes sufficient amounts of progesterone and termination of the pregnancy (Bazer 2013). Porcine fetuses also produce PGE_2

to stimulate contractions of the uterine myometrium which has the goal to distribute fetuses equally in the uterine horns attaining at least two fetuses per uterine horn (Senger 2012). Conversely, PGE_2 will be secreted to the endometrial vasculature and cause luteolysis (Senger 2012). In comparison to other species, the pig placenta do not produce progesterone but secrete the relaxin and estrogen which are essential for initiation and facilitation of the parturition (Bazer 2009).

1.1.2 Placentation

In pigs, the placenta is diffuse epitheliochorial type characterized by direct attachment of intact uterine epithelium and placental trophectoderm during whole pregnancy (Friess 1981). Through the attachment of these epithelial cells, maternal hematotroph and histotroph provide the metabolites for the development and growth of the fetus (Bazer and Johnson 2014). Furthermore, chorionic folds and endometrial folds form invaginations that increase the area and size of uterine-placental attachment and reduces the distance between maternal and fetal blood creating short diffusion distances for nutrients (Enders and Blankenship 1999). In addition to the extensive interdigitation of microvilli on the trophectoderm and uterine epithelium, there are also specialized structures called areolae that support the fetus (Friess 1981). These are dome-shaped structures that form at the openings of uterine glands, where the trophectoderm, and then chorion, never fuses with uterine epithelium (Friess 1981). In the areolae, secretions from superficial and deep uterine glandular epithelium together with selective transudation from maternal serum create histotroph which is absorbed by fluid phase pinocytosis into tall columnar cells of chorionic epithelium and further released into the placental capillaries and fetal circulation (Bazer and Johnson 2014). This mechanism is similar to the absorption of

immunoglobulins in colostrum across the gut and directly into the blood of neonatal mammals (Bazer and Johnson 2014).

The histotroph contains many essential molecules necessary for the development of the fetus, such as uteroferrin, plasmin/trypsin inhibitor, lysozyme, hexosaminidase, phospholipases, prostaglandin synthases, insulin-like growth factors 1 and 2, glucose, amino acids, prostaglandins, calcium, sodium, potassium, and some hormones (Bazer 2009). Uteroferrin is known to have an essential role in the transport of the iron to the fetus for the synthesis of hemoglobin (Saunders 1985). A significant correlation exists between porcine fetal weight and placental length, placental surface area, and total areolae surface area per placenta suggesting the importance of areolae in the development of the fetus (Knight 1977). During gestation, a fully functional placenta supporting the fetus with the adequate placental surface area and numbers of areolae (e.g. 2500) are formed by 60 to 70 days (Knight 1977). In the reproductive form of PRRS, associated lesions are frequently located in the uterus and placenta of pregnant gilts and sows, both in cases of natural and experimental PRRSV infection (Christianson 1993; Lager and Halbur 1996).

1.1.3 Cathepsin

Cathepsins are a group of serine proteases present in the lysosomes of cells (Turk 2012). Due to their importance in the terminal degradation of protein in the lysosomes, they play numerous roles in physiological processes such as angiogenesis, apoptosis, and remodeling of the tissue (Turk 2012). In mammalian species, epithelium of the endometrium, placental areolae, and fetal intestine are confirmed to express cathepsins B and L (Salamonsen and Nie 2002). The role of cathepsins in the remodeling of endometrial and placental tissues is to reduce the distance between maternal and fetal blood vessels, and in that way enhance transplacental transport of

gasses and nutrients required for the development of the fetus (Salamonsen and Nie 2002). In pigs, cathepsin L1 is particularly highly expressed during gestation in the chorionic epithelium that forms areolae (Song 2010).

1.2 Overview of PRRS

1.2.1 Disease background and impact

When porcine reproductive and respiratory syndrome (PRRS) was initially named "Mystery Swine Disease," in the late ninteen-eighties after several catastrophic clinical outbreaks of abortions and respiratory disease (Keffaber 1989), no one in the veterinary scientific community was aware that many years after that, many aspects of this disease would remain mysterious and unresolved. Since the original emergence of PRRS, it has become one of the most economically devastating diseases of pigs. Losses are currently estimated to be $664 million per year, in the United States (U.S.) alone (Holtkamp 2013). Initial research work related to PRRS was focused on determining the etiology of the disease characterized by reproductive failure, interstitial pneumonia, and increased mortality in nursing pigs (Hill 1990). In 1991, the etiology of European PRRS was discovered to be a previously unknown RNA virus and classified into genus Arterivirus of family Arteriviridae (Terpstra 1991; Wensvoort 1991). Shortly thereafter, a similar virus was isolated from outbreaks in the U.S. (Collins 1992) and Canada (Dea 1992). However, retrospective studies demonstrated that the virus had been circulating in North America prior to the initial outbreaks. The earliest evidence of porcine reproductive and respiratory syndrome virus (PRRSV) infection in North America used archived pig serum from 1979 in Ontario, Canada (Brar 2011).

PRRS is currently an endemic disease (OIE 2015) and represents a substantial economic burden for pork producers in most swine producing regions of the world. Although the accurate

reports on the prevalence of disease in the North America are not available, most concerning is the high seroprevalence (49.8%) of unvaccinated herds (APHIS 2009). In Canada, two studies confirmed higher PRRSV antibody prevalence in market pigs in Quebec (89.9%) than in serum samples from Manitoba (54.3%) (Magar and Larochelle 2004). PRRSV seroprevalence in Quebec breeding herds was estimated to be 74% (Lambert 2012).

Direct financial losses of the clinical effect of disease are due to increased mortality, reduced average daily gain, increased treatment cost per pig, reduced feed efficiency, and a decrease in reproductive health (Dee 2003). Epidemiological features of PRRSV, characterized by many routes of transmission between and within herds, its persistence in herds and rate of mutation make current PRRS control efforts challenging, suggesting that PRRS-associated economic losses will continue in the future (Holtkamp 2013). Future research work that will improve our understanding of epidemiologic characteristics of the PRRSV and pathogenesis of the disease is required to help develop new control measures such as vaccines and diagnostic tests.

1.2.2 Reproductive PRRS

Initial work examining the reproductive form of PRRS described the pathological lesions affecting the various fetal organs and the pregnant uterus but did not reveal specific lesions or mechanisms that could explain fetal death (Christianson 1993; Lager and Mengeling 1995; Rossow 1996b). Furthermore, determining pathogenetic mechanisms of the PRRSV-induced disease has proved to be much more challenging than determining the etiology. Although the majority of research work has been focused on the respiratory form of the disease in neonatal, nursing and growing pigs (Rossow 1995), the breakthrough discovery from these early studies was cell-specific tropism of PRRSV to a limited group of blood monocytes and porcine alveolar

macrophages (Plagemann and Moennig 1992; Mardassi 1994). Recent studies identifying specific receptors on susceptible cells for PRRSV infection (Calvert 2007; Van Gorp 2008; Zhang and Yoo 2015) and mechanism of cell death (Sur 1998; Karniychuk 2011; Gómez-Laguna 2013) have resulted in a renewed interest in the pathogenesis of fetal death in the reproductive form of PRRS.

1.2.3 PRRS transmission

Horizontal transmission is accomplished through contact between infected and naïve animals (Wills 1997a). During the infection PRRSV has been detected in blood, semen, saliva, feces, milk and colostrum (Wills 1997a; Christopher-Hennings 1998; Wagstrom 2001; Batista 2002; Rowland 2003). For example, PRRSV-infected boars shed virus in the semen for an extended period after inoculation (92 days) (Christopher-Hennings 1998). When contaminated semen enters the uterus, infection apparently begins from the endometrial tissues and regional lymph nodes resulting in the haematogenous or lymphoid dissemination of PRRSV (Christopher-Hennings 1998).

Vertical transmission of PRRSV has been confirmed, particularly during mid to late gestation (Christianson 1993). The exact route of PRRSV transmission from dam to the fetus is still not completely understood. One hypothesis is that PRRSV after its primary replication in the tonsils and respiratory tract causes viremia through the infection of susceptible blood monocytes. Movement of monocytes through the endometrial vessel walls and differentiation into tissue macrophages leads to the final migration of the virus to the fetal placenta and fetus (Karniychuk and Nauwynck 2013). Part of this hypothesis has been confirmed by identifying PRRSV-positive cells between endothelial cells of endometrial blood vessels (Karniychuk 2011).

However, following routes of PRRSV transmission from the endometrial tissue across the maternal-fetal interface into the fetal placenta during early infection has not been done.

Indirect transmission can involve inanimate objects (equipment, boots, coveralls) as potential sources of PRRSV to naïve pigs (Dee 2002). Needles were also confirmed to be a significant source of indirect PRRSV transmission between pigs (Otake 2002b). Also, under experimental conditions, PRRSV can be mechanically transmitted on fomites particularly in the cold and to a lesser degree warm weather (Dee 2002). Transmission via arthropods such as house flies and mosquitos is also confirmed (Otake 2002a; Otake 2003). Finally, aerosol transmission is considered as a potentially significant route of PRRSV spread between farms (Dee 2005a). Several studies confirmed that aerosol contaminated with PRRSV can infect naïve pigs over distances of 0.5 to 150 meters (Kristensen 2004; Dee 2005a; Dee 2005b).

1.2.4 PRRS immune response

Innate immunity is the first line of defense against viral infection. Unlike other viruses, PRRSV has poor ability to induce secretion of INF-α, which is critical cytokine for the efficient antiviral innate immune response in pigs (Lee 2004; Miller 2004). Although IFN-α is induced by PRRSV infection (Ladinig 2014c), the overall secretion of INF-α and INF-β cytokines are suppressed *in vivo* (Albina 1998). On the other hand, the early PRRSV infection is dominated by up-regulation of INF-γ (Loving 2008) that is positively associated with PRRSV load (Ladinig 2014c).

After the weak innate immune response of infected macrophages, the humoral immune response first appears 5-7 days post-infection (DPI) with PRRSV-specific IgM in the serum (Díaz 2005). However, at this time the most abundant antibodies are non-neutralizing (Ostrowski 2002). Neutralizing antibodies are detected in the serum as early as three weeks post infection,

but at the low levels (Loemba 1996). GP5 protein of PRRSV is confirmed to incite majority of neutralizing antibodies (Mateu and Diaz 2008). M and N proteins are also responsible for induction of smaller portion of neutralizing antibodies (Mulupuri 2008). In some animals, during the PRRSV infection, viremia can occur in the presence of neutralizing antibodies which suggests that humoral immune response is not a critical factor for viral elimination (Yoon 1996).

The cellular-mediated immune response is primarily composed of T-cells specific for structural proteins of the virus, but it is transient and is not correlated with the PRRSV load in the tissue and the resolution of the infection (Xiao 2004; Rascón-Castelo 2015). Early PRRSV infection (2 DPI) was confirmed to cause marked leukopenia affecting primarily lymphocytes (Ladinig 2014b). However, after 6 DPI the numbers of inflammatory cells starts to recover and marked lymphocyte proliferation develops especially after four weeks post-infection (López Fuertes 1999).

Poor innate immunity due to alteration of cytokine secretion in cooperation with weak and delayed acquired immunity both humoral and cellular-mediated leads to prolonged acute and subsequent development of persistent infection (Wills 2003; Mateu and Diaz 2008).

1.2.5 Strategies for control and eradication of PRRS

Identification of risk factors for PRRSV infection at the herd level is essential and the first step in determining efficient control, eradication measures and cost effective surveillance strategies (Velasova 2012). It has been found that increased herd size, distance to the neighboring pig herds and exposure to infected semen are significantly associated with increased risk of PRRSV infection (Mortensen 2002). Additionally, total confinement housing, pig density, a greater number of purchases of gilts and boars, and poor management and biosecurity practices are found to be significant risk factors (Weigel 2000). Recently to the list of risk factors is added

the application of the live virus vaccine at the farm (Velasova 2012). Present identification of carrier animals is not fast, accurate or economical, particularly because viremia or seroconversion are not reliable criteria for identification of persistently infected animals (Wills 1997b). Hence, the presence of carrier animals in a swine population represents an additional risk factor.

The ultimate goal of every control strategy is the production of PRRSV-negative weaned piglets (Holtkamp 2011). In other words, it is required to prevent both vertical and horizontal transmission of the PRRSV in the breeding herd and to establish so-called "stable" herd (Corzo 2010). A subcommittee was formed by the American Association of Swine Veterinarians and the United States Department of Agriculture in order to classify herds by PRRSV infection/shedding and exposure status of the breeding herd (Holtkamp 2011). The classifications are listed here:

Positive unstable (I)	Breeding herds actively shed virus and are exposed to the virus (undergoing acute PRRS outbreak or repeated chronic)
Positive stable (II)	Absence of clinical signs of PRRS in the breeding herd and absence of viremia in weaning-age pigs for 90 days *II-B are undergoing elimination and II-A are not*
Provisional negative (III)	Negative shedding, but positive exposure status
Negative (IV)	both negative shedding and exposure status

Numerous control measures can be used to stabilize breeding herd. In this review, only a few reported to be the most effective are described below.

Strict implementation of biosecurity measures for prevention of reinfection of the herds is still essential control measure (Corzo 2010). Systematic cleaning and disinfection between groups of pigs combined with separation of production units into individual rooms are effective control measures to reduce the horizontal transmission between infected and newly moved animals (Dee, 2003). This control strategy is known as “all-in/all-out” (AIAO) (Pitkin 2015).

"Management Changes to Reduce Exposure to Bacteria to Eliminate Losses" (McREBEL) is simple and low-cost control strategy consisting of a systematic series of measure with a goal to decrease the spread of PRRSV and secondary bacteria among piglets (McCaw 1995). It can repeatedly be applied to unstable herds and includes the reduction of cross-fostering and elimination of weak, thin or "poor doing" piglets (Dee 2003).

Obtaining efficient protective immunity against PRRSV is another possible strategy for control of the disease. Vaccinated pigs can develop sufficient active immunity to be protected against reproductive effects of PRRSV infection after the exposure to homologous PRRSV (Yoon 1996). First commercially available vaccines came out in the early nineteen-nineties. They were modified-live vaccine (MLV) designed for type 2 PRRSV strains (Renukaradhya 2012). PRRSV-MLV vaccines have been successfully used for many years in North America. However, disadvantages of these vaccines are concerns for potential MLV virus replication in a herd, reversion of the MLV strain to pathogenicity and lack of cross-protection for many field strains (Charerntantanakul 2012). Antigenic and genetic variation of PRRSV is the most responsible factor in repeated outbreaks of this disease and the main difficulty for the development of an effective vaccine against PRRSV infection (Renukaradhya 2015). On the other hand, inactivated vaccines are safe, but there are concerns about their efficacy (Geldhof 2012).

A promising strategy to control PRRS is the development of genetically resistant breeds of pigs (Lunney and Chen 2010). It was observed that some breeds of pigs, when infected with PRRSV, develop more severe clinical signs than others (Halbur 1998). Numerous studies in this research area have found that multiple immunological factors play a major role in different breed response to PRRSV infection. For example, different levels of expression of CD169 receptors on

the PRRSV susceptible population of macrophages and cytokines such as TNF-α, IL8 (Ait-Ali 2007) or in the reproductive form of the disease, INF- γ (Lowe 2005). Furthermore, it was found in the nursery pigs that selection for a specific trait can also be an important factor for the outcome of PRRSV infection (Petry 2007). Hence, animals that were growing faster have been experiencing a more acute form of PRRSV infection (Doeschl-Wilson 2009).

A recent study of the role the host genetics during the PRRSV infection has confirmed that nursing pigs can be categorized based on the PRRSV viral load into resistant and tolerant phenotypic groups (Boddicker 2012). The tolerant phenotype in this study gained weight (close to non-infected control pigs) even though these pigs had acute outbreak levels of PRRSV load (Boddicker 2012). This finding has led to the identification of a region on chromosome 4 (SSC4) which was correlated with this resistance (Boddicker 2014). Recently, the putative causal mutation was confirmed in the region of Guanylate Binding Protein 5 (GBP5) at the major quantitative trait locus (QTL) of SSC4 (Koltes 2015). The GBP5 protein is an important mediator of inflammatory immune response in mammals and plays an important role in inflammasome assembly (Shenoy 2012). GBP5 host genetic variation is responsible for the host response to PRRSV infection (Koltes 2015). These results may serve as new opportunities for development of breeding programs for production of pigs with increased resistance to PRRSV infection and high growth rate at the same time (Rowland 2012).

Eradication of PRRSV can be accomplished through many methods such as whole herd depopulation and repopulation, test and removal, and herd closure and rollover (Corzo 2010). Whole herd depopulation and repopulation is characterized by the elimination of all animals from the farm, disinfection of the facilities and introduction into the farm PRRSV-negative pigs (Rowland and Morrison 2012). This method is very effective, but very costly and rarely is used

in the big production systems (Rowland and Morrison 2012). Test and removal elimination method include determining exposure status of the breeding herd and subsequent removing of all seropositive animals (Dee 2001; Dee 2003). The weakness of this approach is the cost of diagnostics and early removal of PRRSV exposed animals from production system (Dee 1996). Herd Closure and Rollover is the most widely used method for eliminating PRRS from sow herds (FitzSimmons and Daniels 2003). This method is characterized by the gradual extinction of PRRSV in herds that are closed for 200 days. Then seropositive sows are removed and replaced with negative pigs (FitzSimmons and Daniels 2003). It is reported that this elimination method is the least expensive (Fraile 2012). However, the biggest risk factor and the weakness of all elimination programs is still potential reinfection of the herd with new and unrelated PRRS strain to developed immunity.

Finally, PRRSV regional elimination has been shown to be a promising and potentially effective way to control the disease at a regional or national level (Corzo 2010). Successful examples including Chile and Sweden on the national level, and Stevens County, Minnesota on regional level suggest that control and elimination programs can be only accomplished through close communication and coordinated cooperation between government, industry and swine veterinarians (Corzo 2010).

1.3 Mechanisms of reproductive PRRS

1.3.1 PRRSV infection

The process of PRRSV infection is still not completely understood. However, the ability of PRRSV to replicate in a permissive subpopulation of local tissue macrophages before its subsequent spreads to lymphoid organs, lungs, and other tissues, is the first and critical step in this process (Thanawongnuwech 1997b). The primary cell population for viral replication is a

well-differentiated subset of monocyte-derived cells, such as PAM and PIM in the lung (Thanawongnuwech 1997a; Thanawongnuwech 2000b) and macrophages in lymphoid tissue (Rossow 1998). It is also described in the literature that PRRSV can also replicate in microglia cells (Rossow 1996a). Also, acute PRRSV infection can over time become a persistent infection since the virus can continue to replicate in susceptible cells even when significant humoral and cellular immunity has been developed (Horter 2002). An example of this was PRRSV-infected 35-day-old pigs which remained persistently infected for several months (Wills 1997b). Additionally, since PRRSV replicates in the macrophages, a significant loss in macrophage numbers during infection can predispose the host to potential infections with secondary pathogens (Costers 2008). Pigs infected with PRRSV often experience more severe secondary infections with bacterial or viral pathogens such as *Mycoplasma hyopneumoniae, Salmonella choleraesuis, Haemophilus parasuis, Streptococcus suis, Pasteurella multocida,* and influenza A virus (Thanawongnuwech 2000a; Brockmeier 2002; Zimmerman 2012).

Several studies have confirmed that PRRSV can cause transplacental infection of a fetus after experimental and natural infection of the sow (Lager and Mengeling 1995; Mengeling 1996; Nielsen 2002; Karniychuk and Nauwynck 2013). The absence of microscopic lesions in the internal organs of stillborn piglets suggests that fetal death may not be a consequence of PRRSV replication in the internal organs of fetuses (Rossow 1996b; Karniychuk and Nauwynck 2013). More recent research findings suggest that the maternal-fetal interface plays a significant role in the pathogenesis of transplacental PRRSV infection (Karniychuk 2011).

1.3.2 Properties of virus

There are two genetically distinct types of PRRSV: Type 1 (European genotype) and Type 2 (North American genotype). Both types are found in North America, Europe, and Asia

(Plagemann 2003; Dietze 2011). Differences between Type 1 and Type 2 are estimated to be 40% of the whole genome (Hanada 2005). Presently, Type 2 PRRSV consists of 9 distinct genetic lineages, and Type 1 has at least four subtypes described as pan-European subtype 1 "Lelystad virus-like", Eastern European subtypes 2 and 3, and subtype 4 from Belarus and Latvia (Salguero 2015). A recently discovered highly pathogenic East European subtype 3 PRRSV isolate, named "Lena," was confirmed to be antigenically and genetically different from other Type 1 strains (Karniychuk 2010). In addition to their genetic differences, Type 1 and Type 2 PRRSV strains also demonstrate differences in pathogenicity *in vitro* and *in vivo* (Choi 2015). Type 2 PRRSV grow easily on MARC-145 cells while type 1 PRRSV have to be adapted for *in vitro* growth on cells other than myeloid pig cells (PAM) (Salguero 2015). *In vivo* evidence suggest that respiratory disease caused by type 1 PRRSV is less severe than by type 2 (Choi 2015). Furthermore, outbreaks of more severe disease were more often linked to Type 2 than to Type 1 PRRSV infection (Tian 2007). Examples are the emergence of Highly Pathogenic Porcine Reproductive and Respiratory Syndrome (HP-PRRSV) related to a novel Type 2 PRRSV strain, observed in China in 2005 (Tian 2007).

The PRRSV genome is long 15 kb and possesses eight open reading frames (ORFs) (Dea 2000). In the past, restriction fragment length polymorphism (RFLP) of ORF5 was used for PRRSV typing confirming numerous RFLP cut patterns of PRRSV. Although RFLP demonstrated to be useful in differentiating vaccine from field strains, sequencing revealed that genetically diverse viruses shared the same cut patterns and *vice versa* (Brar 2011).

ORF 1a and ORF 1b compromise most of the viral genome, and encode RNA replicase required for virus replication and are translated into 12 smaller nonstructural proteins (nsp) (Snijder and Spaan 2007). Beside the role of nsp in PRRSV replication, they also have a role in

host immune response to PRRSV (Oleksiewicz 2001). Interestingly, both HP-PRRSV and Lena strain have a similar deletion in the nsp2 part of ORF1, a mutation which may be responsible for the observed higher pathogenicity of these strains (Weesendorp 2013). Structural proteins are GP5, M, and N protein (Dea 2000). GP5 is the protein of the virus envelope which during infection is responsible for recognition of specific cell receptors (Vanderheijden 2003). Transmembrane M is the genetically conserved protein and has a function in virus assembly and budding (Mardassi 1996). This protein forms disulfide-linked heterodimers that are essential for interaction with cellular receptors and virus infectivity (Mardassi 1996). N protein interacts with viral RNA in the assembly of infectious particles, and it is the most abundant protein (40%) protein in the virion (Yoo 2003).

A unique property of PRRSV along with other Arteriviruses is a relatively high mutation rate, which is responsible for continuous antigenic and genetic variation of field strains (Murtaugh 2010; Kappes and Faaberg 2015). Mutation and recombination are responsible for genetic variations, but the viral diversity is also a result of factors such as transmission, management and vaccination practices and pig genetic variations (Shi 2010). These features of PRRSV make the design of effective vaccines and diagnostic methods challenging (Renukaradhya 2015).

1.3.3 Cell susceptibility

In addition to the susceptible subpopulation of local tissue macrophages such as pulmonary alveolar macrophages (PAM) and intravascular macrophages (PIM) in the lung (Thanawongnuwech 1997a; Thanawongnuwech 2000b) and macrophages in lymphoid tissue (Rossow 1998), there are rare reports of PRRSV ability to infect other cell types. Various immortalized and primary cell lines have been able to support PRRSV growth: primary pig

macrophages (PAM and PIM), porcine endometrial endothelial cells (PEE) expressing sialoadhesin (CD169) and CD151, but not CD163 (Feng 2013), MARC-145 (green monkey kidney) cells, CL 2621, and PK-15 expressing CD163 and sialoadhesin (Delrue 2010; Provost 2012). PRRSV infection of nasal, bronchiolar, alveolar epithelium and endothelial cells has been reported, although the mechanism of the PRRSV infection of these cell types remains unexplained (Rossow 1996a; Thanawongnuwech 1997a; Rossow 1998).

1.3.4 Receptors

The PRRSV enters susceptible macrophages through receptor-mediated endocytosis (Van Gorp 2008). Several cell receptors are mentioned in the literature as necessary for PRRSV attachment and entry into the permissive cell types (Zhang and Yoo 2015).

Attachment of Type 1 PRRSV to target cells is believed to be accomplished by primary interaction with the sialoadhesin (CD169) receptor (Vanderheijden 2003). CD169 receptors can be found in the subgroup of macrophages in the splenic, hepatic, lymphatic, pulmonary hematopoietic tissue (Hartnell 2001). CD169 is responsible for interactions between cells through the binding of sialic acid ligands on monocytes, NK cells, B cells, and some cytotoxic T-cells (Martinez-Pomares and Gordon 2012). Also, it participates in the adaptive immunity, processing of the antigen, presentation to T cells, activation of B cells and CD8+ T cells (O'Neill 2013). Recent studies with porcine CD169 suggest it has a role as an endocytic receptor in "targeted delivery" of antigens to macrophages (Delputte 2011).

A molecule found to facilitate the attachment of PRRSV to susceptible cells is heparan sulfate. It was confirmed that heparan sulfate *in vitro* can bind to M and N proteins of the PRRSV (Delputte 2002). One study hypothesized that heparan sulfate is required for initial weak attachment of the virus to the susceptible cells leads to stronger interaction with the CD169

receptor (Zhang and Yoo 2015). Unfortunately, no subsequent studies have confirmed an essential role for heparan sulfate in PRRSV infection (Delputte 2005).

Another important receptor ligand for PRRSV infection of susceptible macrophages is the CD163 entry mediator (Van Gorp 2008). CD163 is a membrane protein present primarily on some types of macrophages and monocytes (Møller 2012). CD163 is so-called macrophage scavenger receptor with numerous roles in processes such as uptake of haptoglobin-hemoglobin complexes, erythroblast adhesion, innate immunity of bacteria, and apoptosis (Etzerodt and Moestrup 2013).

In pigs, CD163 has been reported to be entry mediator into macrophages for PRRSV and African swine fever virus (Møller 2012). *In vitro* studies have shown that both receptors, CD169, and CD163, are required for PRRSV infection (Van Gorp 2008). Also, it has been reported that both CD169 and CD163 positive macrophages demonstrated significant quantitative changes at the maternal-fetal interface, pig embryos, and fetuses during gestational infection with Type 1 PRRSV (Karniychuk and Nauwynck 2009). One model of infection proposes that type 2 PRRSV is using CD169 receptor to attach to susceptible cells and CD163 to enter into the cytoplasm of susceptible cells through the process of endocytosis (Whitworth 2016).

CD151 is a recently described cell-surface protein which was found in the population of newly discovered PRRSV susceptible St-Jude porcine lung (SJPL) cells (Provost 2012). *In vitro* infection of SJPL cells confirmed that CD151 downregulation in the cells decreases the amount of PRRSV replication (Wu 2014). Since this cell line does not have CD163 and CD169 receptors, the role of CD151 *in vivo* is unknown. CD151 was also found on porcine endometrial endothelial (PEE) cells derived from the primary endothelial cells originating from the uterine microcirculation which are susceptible to PRRSV infection *in vitro* (Feng 2013). PEE cells are

CD169 and CD151 positive, but CD163 negative, suggesting that these two receptors may act as possible alternatives for attachment of type 1 PRRSV attachment on some cells (Provost 2012).

DC-SIGN (dendritic cell-specific intercellular adhesion molecule-3-grabbing non-integrin) or CD209 is an adhesion receptor present on macrophages and dendritic cells detected on PRRSV susceptible BHK-21 cell line can enhance the viral transmission from the engineered BHK donor cells to target MARC-145 cells (Huang 2009).

1.3.5 Cellular damage

It was hypothesized that PRRSV induces cellular damage and subsequent pathological lesions by a variety of mechanisms. The most relevant mechanisms of apoptosis and inflammation of the vasculature are discussed in this section.

1.3.5.1 Apoptosis

Apoptosis is a well-described mechanism of cell death in the pathogenesis of viral infections (Roulston 1999). Viruses induce apoptosis of susceptible cells to promote viral spread in the tissue and prevent efficient immune response (Roulston 1999).

Previous studies confirm that PRRSV can induce apoptosis during infection (Sur 1997; Kim 2002; Miller and Fox 2004). Under *in vitro* experimental conditions apoptosis of PRRSV-infected cells has been well described (Kim 2002). More specifically, in the early PRRSV infection of macrophages the infected cells are protected from the apoptosis, but as infection continues, infected cells undergo apoptosis suggesting that PRRSV modulates mechanisms of programmed cells death (Costers 2008). An *in vitro* experiment on MARC-145 cells confirmed that PRRSV regulates apoptosis by activation of extrinsic pathway of apoptosis (Lee and Kleiboeker 2007). *In vivo* studies also revealed that apoptotic cells can be found in the variety of tissues from PRRSV-infected pigs (Sur 1998; Labarque 2003; Chang 2005; Costers 2008).

Additionally, the number of apoptotic cells detected in the lymphatic tissue of experimentally PRRSV infected pigs is higher than a number of infected cells (Gómez-Laguna 2013). The majority of apoptotic cells during the PRRSV infection are macrophages and lymphocytes in the lungs and lymphatic organs (Labarque 2003). Besides apoptosis, PRRSV antigen is often associated with necrosis as well (Miller and Fox 2004; Lee and Kleiboeker 2007).

Increased apoptosis affecting macrophages and lymphocytes is also detected in the fetal placental tissue and endometrium at the maternal-fetal interface of Type 1 PRRSV-infected pregnant sows (Karniychuk 2011). Since many of the apoptotic cells were also negative for PRRSV (Miller, 2004) or not previously described as permissive such as lymphocytes (Gómez-Laguna 2013) , it is hypothesized that PRRSV can induce apoptosis not only directly by infecting susceptible cells, but also by some still unknown indirect mechanism (Flores-Mendoza 2008).

Indirect apoptosis in bystander cells appears to be an important mechanism in PRRSV-induced cells death, but how the virus induces it, still needs to be explained. One of the potential mechanism is up-regulation of secretion of the pro-apoptotic inflammatory cytokines (Lee and Kleiboeker 2007). Nevertheless, numerous studies have confirmed that apoptotic cells are detected in PRRSV-infected cultured cells, tissues and animals and that these apoptotic cells contribute to the pathology (Prieto 1997).

In summary, future studies of PRRSV-induced apoptosis in the reproductive form of PRRS, either through examination of pro-apoptotic gene expression during PRRSV infection or testing apoptotic markers are needed. More specifically, it is necessary to examine the association between PRRSV load and the apoptosis at the maternal-fetal interface and fetus during the transplacental infection of the fetus, because this information could potentially elucidate the role of apoptosis in fetal death.

1.3.5.2 Vasculitis

Vasculitis or inflammation of vasculature can demonstrate as a primary disease with no detectable cause (idiopathic) and/or secondary as a result of inflammation, malignancy or autoimmune disease (Luzina and Handwerger 2000). Vasculitis can affect vasculature at all levels from microvasculature to large caliber blood vessels (Luzina and Handwerger 2000).

In pigs, widespread vasculitis has been described for infections with PRRSV, African swine fever virus, classical swine fever virus, bovine viral diarrhea virus (BVDV), ovine herpesvirus type 2, pseudorabies virus, porcine circovirus type 2, *Salmonella choleraesuis*, *Streptococcus suis*, *Escherichia coli*, and *Erysipelothrix rhusiopathiae* (Langohr 2010).

Pathophysiology of secondary vasculitis is complex and involves several mechanisms (Guillevin and Dörner 2007). Only the mechanisms of vasculitis that are relevant to PRRSV infection are described herein.

Many infectious agents can cause direct damage to the endothelial lining of blood vessels and subsequent vasculitis (Belizna 2009). This type of primary vasculitis is accomplished if pathogens have a tropism to infect endothelial cells such as equine arteritis, infectious canine hepatitis, canine distemper, African swine fever, and classical swine fever (Maxie and Robinson 2007). Also, damage to endothelium can also be accomplished through the production of various toxic products secreted by pathogens such as endotoxins of gram-negative bacteria (e.g., *Mannheimia haemolytica)*, or exotoxins of bacteria (e.g., *Actinobacillus pleuropneumoniae*, *Histophilus somni*) (Maxie and Robinson 2007). This first mechanism is suspected to be responsible for PRRSV-induced vasculitis since PRRSV antigen has occasionally been detected in the endothelial cells (Halbur 1995). Although, it remains unclear how the virus enters

endothelial cells, the recently discovered CD151 receptors on PEE cells (Feng 2013) suggest a potential mechanism of entry.

Another potential mechanism of primary vasculitis is immune complex (type III hypersensitivity) reaction (Belizna 2009). In animals, immune complexes can be detected in the inflamed blood vessel walls in many diseases such as systemic lupus erythematosus, Aleutian disease of mink, feline infectious peritonitis and porcine dermatitis and nephropathy syndrome (PDNS) (Maxie and Robinson 2007). The pathogenesis of this type of vasculitis is characterized by precipitation of increased amounts of antigens and specific antibodies within vessel walls (basement membrane), which activates the complement, attract neutrophils and incite necrotizing vasculitis (Millikan and Flynn 1999). Since it has been confirmed in the past that PRRSV infection causes delayed humoral immune response characterized by low titer of neutralizing antibodies (Labarque 2000), this mechanism is less likely to be a cause of PRRSV-induced vasculitis.

Other mechanisms of primary vasculitis include cell-mediated (type IV hypersensitivity) reaction, abnormal immune regulation due to abnormal expression of adhesion molecules on pathogen infected endothelial cells and overproduction of cytokines by direct stimulation from the infectious agents (Manders 1998; Weyand and Goronzy 2003; Belizna 2009). All of these are even less likely to play a role in PRRSV infection.

PRRSV-induced vasculitis can vary in its distribution and severity, and affect all sizes of blood vessels (Rossow 1995). PRRSV-associated vasculitis was initially described as severe necrotizing and leukocytoclastic with thrombosis, affecting chiefly post-capillary venules of the skin and kidneys (Thibault 1998). In PRRSV-infected, gnotobiotic pigs between 3 and 21 days post-inoculation, lymphohistiocytic inflammation was described in the thoracic aortas, as well as,

numerous arterioles and veins (Rossow 1995). The damage to the endothelium of the inflamed blood vessels along with fibrinoid vascular necrosis of the tunica media was occasionally reported as well (Cooper 1997).

1.3.6 Fetal and maternal pathology

Reproductive PRRS is principally characterized by acute viremia and transplacental transmission resulting in reproductive failure (Rossow 1998). In sows, mortality reaches 1-4% during acute illness (Hopper 1992). Acute PRRSV infection in sows and gilts can range from no apparent clinical signs of fever, anorexia, lethargy, and severe respiratory clinical signs (Christopher-Hennings 1998).

Gross pathological lesions of the reproductive form of PRRS in the sows are commonly demonstrated by variable degrees of endometrial and myometrial edema, endometritis, and myometritis (Caswell and Williams 2007). "Paintbrush" hemorrhages have been observed in the placenta and petechial hemorrhages, and necrosis occurs in umbilical vessels, skin, and renal cortex (Rossow 1998). Microscopic pathological lesions present in the myometrium and particularly endometrium consist of edema and lymphoplasmacytic and lymphohistiocytic myometritis and endometritis (Lager and Halbur 1996). Lymphoplasmacytic segmental vasculitis and marked microseparations between endometrial epithelium and placental trophoblasts can occasionally be observed, as well (Lager and Halbur 1996).

Lesions in aborted fetuses, dead fetuses, and stillborn piglets are uncommon, but the absence of lesions in fetuses does not rule out PRRSV (Schlafer and Miller 2007). Dead fetuses are commonly covered with a thick brown mixture composed of meconium and amniotic fluid. This finding may non-specifically suggest fetal stress and hypoxia (Lager and Halbur 1996). Most of the lesions found in the fetuses are variable, non-specific, often obscured by autolysis

and inconsistent for PRRSV infection (Rossow 1998). Grossly, they include perirenal edema, edema of the splenic ligament, mesenteric edema, ascites, hydrothorax, and hydroperitoneum (Lager and Halbur 1996).

Microscopic lesions in live piglets may include mild to moderate, multifocal to diffuse, histiocytic and proliferative interstitial pneumonia characterized by mononuclear cell septal infiltration (Caswell and Williams 2007). Hypertrophy and hyperplasia of type 2 pneumocytes are occasionally present (Rossow 1998). Mild segmental necrotizing and lymphohistiocytic arteritis has been seen in lungs (Caswell and Williams 2007). Mild lesions in the heart and liver are also multifocally present composed of lymphohistiocytic inflammation and rare hemorrhage (Lager and Halbur 1996).

Finally, an important diagnostic lesion that can occur in some cases is a segmental hemorrhage of umbilical cord characterized by segmental lymphoplasmacytic and sometimes necrosupparative vasculitis (Lager and Halbur 1996).

2 Rationale, hypotheses and objectives

Despite nearly 25 years of research work, the underlying mechanisms of fetal death in the reproductive form of PRRS are still undetermined. The main body of literature regarding the critical factors of PRRSV infection and PRRSV-induced mechanisms of cellular death represent experiments done either *in vitro* or for the postnatal respiratory form of the disease. Similarly, studies of the reproductive form of PRRS are sparse, often investigating single pathogenetic factors and having contradictory conclusions between investigations. Recent studies begin to challenge the previous understanding of fetal death as a result of PRRSV replication in the fetal tissues, proposing conversely that pathological processes at the maternal-fetal interface are responsible for fetal death (Rowland 2010; Karniychuk 2011).

Additionally, scientific knowledge regarding essential factors of transplacental infection of the fetus are still in the unproven hypothesis stage (Karniychuk and Nauwynck 2013). Therefore, there is a need for large-scale studies on the reproductive form of PRRS that will provide more insight into the process of infection within the uterus and fetus. Critical information is needed to understand the relationships between specific pathological lesions, their severity and PRRSV infection, along with the role of PRRSV replication, PRRSV susceptible cells and cell death on both the maternal and fetal sides of the maternal-fetal interface. Once we understand all critical factors of fetal disease and death, we can develop new treatments and vaccines that will be effective in preventing them.

The following hypotheses were developed to examine the mechanisms of PRRSV fetal infection:

Hypothesis 1: There is a significant association between PRRSV load in the MFI and the fetus of the PRRSV-infected gilts in the third trimester of gestation, and PRRSV-associated microscopic lesions in the fetal tissues, uterus and fetal placenta.

Hypothesis 2: There is a significant association between PRRSV load in the MFI and the fetus and the numbers of PRRSV, CD163 and CD169 positive PRRSV susceptible cells, areolae and apoptotic cells at the MFI.

Hypothesis 3: There is a significant association between degree of cellular death (apoptosis) at the MFI and PRRSV-associated microscopic lesions in the fetal tissues, uterus and fetal placenta during type 2 PRRSV infection of late gestation pregnant gilts.

Hypothesis 3: There is a significant relationship between fetal preservation status and PRRSV-associated microscopic lesions in the fetal tissues, uterus, fetal placenta, and the degree of cellular death at MFI.

The following specific objectives for this study were developed to test these hypotheses:

Objective 1. To qualitatively assess the PRRSV-associated microscopic lesions in the fetal tissues, uterus and placenta.

Objective 2. To develop a grading scheme and assess the severity of microscopic lesions associated with PRRSV infection at the MFI.

Objective 3. To determine relationships between the severity and distribution of microscopic lesions, PRRSV RNA concentration in the MFI and the fetus, and fetal preservation status.

Objective 4. To identify, localize and quantify PRRSV antigen, CD163 and CD169, TUNEL positive cells, and cathepsin-L positive areolae in the MFI.

Objective 5. To evaluate the relationship between numbers of CD163, CD169 positive cells, TUNEL positive cells in the endometrium and fetal placenta, and cathepsin positive areolae at the MFI and PRRSV load in the MFI and fetal thymus.

Objective 6. To evaluate the relationship between histopathological grades of inflammation in the endometrium and blood vessels, and the numbers of TUNEL positive cells at the MFI in type 2 PRRSV-infected pregnant gilts.

Objective 7. To evaluate the relationship between numbers of TUNEL positive cells at the MFI and odds of the fetus being meconium stained or viable (preservation status of the fetus).

3 Pathologic evaluation of type 2 porcine reproductive and respiratory syndrome virus infection at the maternal-fetal interface of late gestation pregnant gilts

This chapter represents the initial microscopic pathology assessments during the type 2 PRRSV infection of pregnant gilts in the late gestation. The initial assessments were for the absence or presence of a lesion in the uterine and fetal samples. Furthermore, histological scoring schemes were developed for the purpose of evaluation the relationships between the severity of pathological lesions and PRRS virus load in the maternal-fetal interface.

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Author contributions: Novakovic P., Ladinig A., Harding J.S.C. and Detmer S.E., conducted the sample collection and laboratory testing for this study. Novakovic P., Harding J.S.C., Al-Dissi A.and Detmer S.E., contributed to conception and design of the study, acquisition, analysis and interpretation of the data, and writing of the manuscript. Ladinig A. critically reviewed the manuscript.

3.1 Abstract

The pathogenesis of fetal death caused by porcine reproductive and respiratory syndrome virus (PRRSV) remains unclear. The objective of this study was to improve our understanding of the pathogenesis by assessing potential relationships between specific histopathological lesions and PRRSV RNA concentration in the fetuses and the maternal-fetal interface. Pregnant gilts were inoculated with PRRSV (n=114) or sham inoculated (n=19) at 85±1 days of gestation. Dams and their litters were humanely euthanized and necropsied 21 days later. PRRSV RNA concentration was measured by qRT-PCR in the maternal-fetal interface and fetal thymus (n=1391). The presence of fetal lesions was positively related to PRRSV RNA concentration in the maternal-fetal interface and fetal thymus ($P<0.05$ for both), but not to the distribution or severity of vasculitis, or the severity of endometrial inflammation. The presence of fetal and umbilical lesions was associated with greater odds of meconium staining ($P<0.05$ for both). The distribution and severity of vasculitis in endometrium were not significantly related to PRRSV RNA concentration in the maternal-fetal interface or fetal thymus. Endometrial inflammation severity was positively related to distribution and severity of vasculitis in the endometrium ($P<0.001$ for both). Conclusions from this study suggest that type 2 PRRSV infection in pregnant gilts induces significant histopathological lesions at maternal-fetal interface, but they are not associated with the presence of PRRSV in the maternal-fetal interface at 21 days post infection. Conversely, fetal pathological lesions are associated with the presence of PRRSV in the maternal-fetal interface and fetal thymus, and meconium staining is significantly associated with the presence of both fetal and umbilical lesions observed 21 days post infection.

3.2 Introduction

Porcine reproductive and respiratory syndrome virus (PRRSV) is one of the most important viral pathogens in swine production worldwide, causing one of the most costly diseases facing the North American swine industry with production losses estimated to be $664 million USD every year in the USA (Holtkamp 2013). PRRSV is an enveloped, positive-stranded RNA virus, belonging to the genus Arterivirus, family Arteriviridae, in the order of Nidovirales (Done 1996; Rossow 1998). All arteriviruses feature cytopathic replication in macrophages, the capacity to develop and preserve an asymptomatic infection, and cause severe and fatal disease (Plagemann 2003). Clinical presentation of the syndrome caused by PRRSV varies considerably between herds, ranging from asymptomatic to devastating disease (Zimmerman 2012). The severity of PRRS clinical signs is influenced by virus strain involved, host immune status, genetic susceptibility (Boddicker 2014), concurrent infections and other management factors (Done 1996). Clinical disease in pregnant females is characterized by acute viremia and transplacental transmission in mainly third trimester resulting in reproductive failure (Rossow 1998; Karniychuk and Nauwynck 2013).

In the reproductive form of PRRS in late gestation gilts or sows, gross and microscopic lesions are frequently located in the uterus, both in cases of natural and experimental PRRSV infection (Christianson 1993; Lager and Halbur 1996). The myometrium and particularly endometrium are characterized by marked edema with lymphoplasmacytic and histiocytic endometritis with perivascular cuffing. Occasionally, segmental lymphoplasmacytic vasculitis and marked microseparations between endometrial epithelium and placental trophoblasts can be observed (Rossow 1998). Infected sows and gilts farrow between 100-118 days of gestation with litters composed of variably sized normal, weak, and dead piglets that can be fresh stillborn

(intrapartum death), autolytic, partially mummified or completely mummified (Christianson 1993; Lager and Mengeling 1995; Rowland 2003). Some dead and viable fetuses are covered with a brown mixture composed of meconium and amniotic fluid. Gross and microscopical lesions are mostly present in the umbilical cord and less often in the internal organs of fetuses (Rossow 1998). Meconium staining of the fetuses indicates the occurrence of fetal stress during gestation, however,the specific pathogenic mechanism is unclear. Hypoxia was hypothesized to play an important role, but whether it is caused by disruption of the blood flow through the umbilical cord or due to lesions at the maternal-fetal interface still needs to be revealed (Lager and Halbur 1996).

Despite nearly 25 years of PRRS research, the primary mechanism of PRRSV-induced reproductive failure is poorly understood. The absence of significant microscopic lesions in the internal organs of stillborn piglets suggests that the fetal death might not be a sequela of PRRSV replication in the fetal tissues (Karniychuk 2011). Recent studies propose that type-1 PRRSV replication within the fetal placental mesenchyme causing severe histopathological lesions at maternal-fetal interface in the third trimester of gestation is responsible for PRRSV-related reproductive disease (Karniychuk and Nauwynck 2013). One recent study with type 2 PRRSV infection found a lack of correlation between the presence of gross abnormalities in the fetus and productive fetal infection suggesting the source of reproductive pathology is potentially infection of tissues on the maternal side, damage to maternal tissues, or production of maternal factors that affect the fetus (Rowland 2010). However, no published studies to date have investigated the relationship between the presence of the PRRSV in the uterus and the fetus and the severity and distribution of lesions at maternal-fetal interface, or evaluated potential involvement of maternal-fetal interface lesions in fetal death. The pathologic evaluation of a large dataset of the maternal-

fetal interface may cast some new information on the pathogenesis of transplacental PRRSV infection.

The objectives of this study were to: 1) qualitatively assess the PRRSV-associated microscopic lesions in the fetal tissues, uterus and placenta; 2) develop a grading scheme and assess the severity of microscopic lesions associated with PRRSV infection at maternal-fetal interface and fetuses; and 3) determine relationships between the severity and distribution of microscopic lesions, PRRSV RNA concentration in the maternal-fetal interface and the fetus, and fetal preservation status.

3.3 Material and methods

3.3.1 Ethics statement

Inoculation of gilts or sows in the last trimester of gestation is a widely accepted and commonly used model for studying reproductive PRRS (Cheon and Chae 2001; Cano 2009; Rowland 2010; Karniychuk 2011; Han 2012; Karniychuk 2012). Although we recognize that some fetuses die after inoculation, death can occur unpredictably at any time after inoculation and no alternative models are available to study the reproductive effects of PRRSV infection. Monitoring fetal stress and discomfort is not feasible in a litter-bearing species like swine. Dams were monitored daily according to a humane intervention point (HIP) checklist developed specifically for this project as is presented in detail (S1 File). Animal numbers were carefully considered and the number of inoculated gilts was selected to enable both deep phenotyping and genotyping of gilts and fetuses. Gilts were housed in Biosecurity Level 2 rooms, 3 to 5 animals per room, in 2' x 7' gestation pens on perforated flooring. Room temperature was maintained at 10-20^0 C using mechanically controlled ventilation, and gilts were individually fed a non-medicated, commercial gestation diet once daily, and had *ad libitum* access to water. Given that

fetal death was an outcome, the experimental protocol was considered carefully before approval by the University of Saskatchewan's Animal Research Ethics Board. It adhered to the Canadian Council on Animal Care guidelines for humane animal use (permit #20110102).

3.3.2 Experimental procedures

Detailed experimental protocol for this study has been published (Ladinig 2014d). Briefly, on gestation day 85±1, 114 PRRSV-naïve pregnant gilts, approximately 10 months of age, were intramuscularly and intranasally inoculated with PRRSV (10^5 $TCID_{50}$ total dose, NVSL 97–7895, Gen Bank Accession No. AF325691) and 19 negative control pregnant gilts were similarly sham inoculated with the minimum essential medium. NVSL 97-7895 is highly abortifacient strain isolated from cases of severe reproductive failure by the Diagnostic Virology Unit of NVSL USDA/APHIS in a farm located in southeast Iowa in December 1996 (Allende 2000). The decision to use this particular strain in the present study was based on the results of our pilot study during which NVSL 97-7895 demonstrated higher virulence than two other type 2 strains (Ladinig 2015b). During the course of the present study, two gilts aborted, one died, but no other gilts demonstrated severe clinically illness as previously described in detail (Ladinig 2014d). At 21days post inoculation (DPI), dams and their litters were humanely euthanized for necropsy examination. Gilts were sedated with intravenous barbiturate (30 mL Euthanyl Forte supplying 16,200 mg pentobarbital sodium, Vetoquinol, Lavaltrie, QC) and humanely euthanized by cranial captive bolt followed by pithing. The gilt reproductive tract was removed intact and opened starting at the tip of each horn. A sample of the uterus with adherent placental layers (herein; maternal-fetal interface (MFI)), 10 cm in circumference and surrounding the umbilical attachment was collected from each fetus. From this sample, a full thickness, 4 cm^2 section was collected in 10% buffered formalin for histology, and a 0.5 cm^2 section was snap frozen in liquid

nitrogen for PRRSV RNA quantification using an in-house quantitative real-time PCR assay (qRT-PCR) as previously described (Ladinig 2014d). The preservation status was assigned for each fetus (Ladinig 2014d) based on the external gross appearance of their skin and umbilical cord as: viable (normal, white to purple skin with visible hair and regular umbilical cord), meconium-stained (skin covered with inspissated, brownish amniotic fluid and regular umbilical cord with edema), decomposed (<50% of skin discoloured, no blood in the umbilical cord), autolysed (>50% of skin discoloured), and mummified fetuses (dehydrated and small with crown-rump length <20 cm). Fetal samples of lung, liver, heart, thymus, mesenteric lymph node and cerebellum were collected in 10% buffered formalin for histology. A section of fetal thymus was also snap frozen in liquid nitrogen for PRRSV RNA quantification by real-time qRT-PCR. Fetal samples for histopathology were not collected from autolyzed or mummified fetuses.

The formalin fixed tissues were processed within 48 hours of collection, paraffin embedded, and 4μm microsections were hematoxylin and eosin (HE) stained. Upon removal from liquid nitrogen, snap frozen gilt and fetal tissue samples were stored at -80°C pending RNA extraction from 10–20 mg using the RNeasy extraction kit (Qiagen Inc., Toronto, ON) according to the manufacturer's instructions. PRRSV RNA concentration was measured using an in-house probe-based real-time qRT-PCR as previously described (Ladinig 2014d).

3.3.3 Histopathology

Uterine and fetal samples (1452 infected and 231 negative controls) were assessed by pathologists (SED and PN) blinded to qRT-PCR results and inoculation status. Histopathological evaluation of the fetal organs, placenta, endometrium, myometrium, and umbilical cords was based on a qualitative (presence/absence) assessment of potentially significant pathological lesions associated with PRRSV. Assessment of fetal lesions included those previously described

such as segmental lymphoplasmacytic arteritis and periarteritis in heart and lung (Lager and Halbur 1996), interstitial pneumonia (Rossow 1998), periportal hepatitis, lymphoplasmacytic myocarditis with the loss of myocardial fibers, and leukoencephalitis particularly affecting cerebellum (Rossow 1996b). Also, fetal thymuses and mesenteric lymph nodes were assessed for previously described lesions such as thymic cortical atrophy and lymph node necrosis, polycystic degeneration, polykaryocytes, germinal center hypertrophy, and hyperplasia (Rossow 1994a; Rossow 1994b). Umbilical cords were evaluated for the presence of necrotizing umbilical arteritis and severe periarterial hemorrhage (Lager and Halbur 1996). Uterine tissues with fetal placentas were assessed for previously described lesions in the myometrium, endometrium and placenta such as lymphohistiocytic vasculitis, endometritis, and placental microseparations (Christianson 1993; Lager and Halbur 1996).

3.3.4 Histologic grading

A grading scheme was developed to score the endometrial inflammation, vasculitis severity, and vasculitis distribution. Only uterine tissue sections with fully attached fetal placenta were selected for grading of lesions (n=679 from 110 infected gilts). Endometrial inflammation was scored by assessing the total area of the endometrium tissue present on the microscope slide at 200X field of magnification. The inflammation in the endometrium was categorized as follows: grade 0 (normal) = very rare inflammatory cells present; grade 1 (minimal) = inflammatory cells multifocally present in < 10% of the tissue section; grade 2 (mild) = multifocal to coalescing inflammatory cell infiltrate in 10-25% of the tissue; grade 3 (moderate) = diffuse inflammatory cell infiltrate in 25-50% of the tissue, and grade 4 (severe) = inflammatory cells diffusely present in >50% of the tissue section.

To score vasculitis severity, three 200X microscopic fields of the endometrium per uterine tissue section were selected randomly by relocating the slide to a new field of view whilst withdrawing eyes from the eyepiece (ocular lenses). Within each of those three fields, three blood vessels (at 3, 9 and 12 o'clock in the field) were scored at 400X magnification as: grade 1 = presence of inflammatory cells only within the blood vessel wall; grade 2 = presence of the inflammatory cells together with degeneration (vacuolation and splitting of smooth muscles) or necrosis of the blood vessel wall layers; grade 3 = presence of inflammatory cells together with degeneration and necrosis in the blood vessel wall layers. The nine individual vessel scores were averaged providing a single severity score per fetus.

The vasculitis distribution score was adapted from the previous work (Cribier 1999; Gross 2000) and based on the distribution of vasculitis in the endometrium present in a 200X microscopic field as follows: grade 0 (normal) = no blood vessels affected; grade 1 = from 0 to <30% of vessels affected by vasculitis; grade 2 = from 30% to 70% of vessels affected by vasculitis and grade 3 = >70% of blood vessels affected by vasculitis in the tissue section.

3.3.5 Statistical Analyses

All statistical analyses were performed using two-level, mixed-effects regression models that controlled for litter of origin as a random effect (Stata 13, StataCorp LP, TX, USA). A mixed effects logistic regression model (MELOGIT) was used to assess potential relationships between fetal lesions (presence/absence) and vasculitis distribution score, average vasculitis severity score, endometrial inflammation score, and PRRSV RNA concentration (logarithm_{10} target genomic copies/gram) in MFI and fetal thymus. MELOGIT was also used to assess potential relationships between fetal preservation status (viable/meconium-stained) and the presence of fetal lesions, umbilical lesions, distribution and severity of vasculitis and endometrial

inflammation. Separate unconditional mixed effects linear regression models (MIXED) were used to assess potential relationships between average vasculitis severity score and PRRSV RNA concentration in the uterus or fetal thymus. For these models, average vasculitis severity score was natural log (ln) transformed to ensure the model assumptions of linearity and homogeneity were not violated. Separate unconditional, proportional odds models (MEOLOGIT) were used to assess potential relationships between vasculitis distribution score and PRRSV RNA concentration in the uterus or fetal thymus. MEOLOGIT models were also used to assess relationships between endothelial inflammation score and PRRSV RNA concentration in uterus or thymus. Potential violation of proportional odds assumption was evaluated by comparing the coefficients with those generated with an equivalent generalized ordered logistical (GOLR) model in which the RNA concentration variable was unconstrained. In all cases, the proportional odds assumptions were not violated (coefficients were similar in both the PO and GOLR models), so results of the proportional odds models are reported herein. Finally, the potential association between endothelial inflammation score and vasculitis distribution and severity was assessed in a single proportional odds model that contained both vasculitis variables as fixed effects. To visualize statistically significant relationships between dependent and independent variables in proportional odds models, probability plots (GLLAPRED) were generated. For all models, litter of origin was included as a random effect and all models were verified for normality and homogeneity of residuals. Statistical significance was assigned at the $P < 0.05$ level *a priori*.

3.4 Results

Of the 114 gilts PRRSV-inoculated, two aborted, 1 died, and 1 had all uterine tissue samples with completely detached fetal placentas. All were removed from the analyses. There

were 1452 fetuses from the remaining 110 PRRSV-infected gilts and 231 fetuses from the 19 control gilts (total = 1683). Of these, tissues were not collected from 585 autolysed and 35 mummified fetuses. Microscopic examination was performed on all available fetuses including 1069 lungs, 1077 livers, 1080 hearts, 1037 thymuses, 983 mesenteric lymph nodes and 1072 cerebella (Table 3.1). Some organs from some fetuses were inadvertently not collected.

3.4.1 Histopathology of the fetus

The most prevalent lesions in fetuses from infected gilts were observed in mesenteric lymph nodes and umbilical cords. In 11.4% of mesenteric lymph nodes, there was mild to moderate follicular atrophy with the replacement of lymphocytes by macrophages. In one fetus, multinucleated cells with cytological features resembling polykaryocytes were found. Rarely, fetal mesenteric lymph nodes demonstrated focal areas of lymphoid hyperplasia. Lesions in 5.9% of umbilical cords were characterized by focal to multifocal, mild lymphocytic perivascular cuffing. Occasionally, mild to moderate hemorrhage surrounding the umbilical arteries and vein was observed. For one fetus, there was diffuse and severe hemorrhage that markedly distended umbilicus. The presence of inflammatory cells in vessel walls (vasculitis) of the umbilical arteries or veins was not observed. In 3.2% of fetal hearts, there was mild, focal to multifocal, lymphocytic myocarditis and perivascular cuffing. Occasionally, myocardiocyte degeneration and necrosis were observed. In 2.5% of fetal livers, small infiltrates of lymphocytes were focally present in the portal areas (mild periportal hepatitis). In 2.2% of fetal cerebella, there was perivascular cuffing and gliosis. In one fetus, there was severe meningoencephalitis. In 1.3% of fetal lungs there was multifocal, mild thickening of the alveolar septa by small numbers of macrophages and lymphocytes (mild interstitial pneumonia). In 0.7% fetal thymuses mild to moderate atrophy was observed. No microscopic evidence of vasculitis was seen in any fetal

tissue and no microscopic lesions were found in tissues obtained from fetuses of negative control pregnant gilts.

3.4.2 Histopathology of the maternal-fetal interface

The most prevalent lesion observed at the MFI was a lymphohistiocytic endometritis, ranging from mild to severe. This was observed in 99.6% of PRRSV-infected MFI tissue samples (uterus with fetal placenta) corresponding to each fetus. Less prevalent were lymphocytic myometritis in 58.5% and lymphohistiocytic placentitis in 6.6% of PRRSV-infected gilts (Table 3.2). Most of the samples of MFI from the negative control gilts had no lesions. However, mild lymphocytic inflammation was observed in one myometrium (adjacent viable fetus without lesions) and two endometria (adjacent one viable fetus without fetal lesions, and one autolysed fetus) out of 230 negative control fetuses examined (Table 3.2). Vasculitis in the endometrium was primarily observed within the small caliber vessels, but occasionally arteries and veins were affected. The lymphocytes were found in all layers of the blood vessels, along with various degrees of degeneration of the tunica intima and media. Fibrinoid vascular degeneration was rarely observed.

3.4.3 Relationship between fetal lesions and PRRSV RNA concentration

The PRRSV RNA concentration (target copies/mg) was measured in fetal thymus and sections of MFI adjacent the umbilical stump of each fetus. Across all PRRSV-infected gilts and fetuses, the average PRRSV RNA concentration (log10 copies/mg) in the MFI and fetal thymus was 2.97 (±2.23) and 3.17 (±2.72), respectively. The proportion of the litter and MFI samples that tested PRRSV qRT-PCR positive were very similar (S1 Fig. and S2 Fig.). Of the 679 fetuses with fully attached fetal placenta that were used in the histological analyses below, the average PRRSV RNA concentration (log10 copies/mg) in MFI and fetal thymus was 3.32 (±2.36) and

3.05 (±3.22), respectively. One or more lesions were present in the tissues of 96 of 679 of fetuses (14%). Similarly, 95 of 679 fetuses (14%) were meconium stained in this dataset. The presence of fetal lesions was positively related to PRRSV RNA concentration in the maternal-fetal interface (odds ratio [OR], 1.4; 95% CI 1.2 to 1.6; $P<0.001$) as well as in the fetal thymus (OR, 1.3; 95% CI 1.2 to 1.4; $P<0.001$). There was no significant relationship between the presence of fetal lesions and vasculitis distribution, vasculitis severity, or endometrial inflammation severity. The presence of fetal and umbilical lesions significantly increased the odds of a fetus being meconium stained compared to being viable (OR 2.1, 95% CI 1.1 to 4.1, $P<0.05$ for fetal lesions; OR 6.9, 95% CI 3.2 to 14.9, $P<0.001$ for umbilical lesions). However, fetal preservation status was not associated with vasculitis distribution or severity, or endometrial inflammation severity.

3.4.4 Assessment of endometrial inflammation and vasculitis and relationship to viral load

A total number of 679 uterine tissue sections from 110 PRRSV-infected pregnant gilts were scored for endometrial inflammation, vasculitis severity and vasculitis distribution (Figure 3.1). Results revealed that 72.9% of the uterine tissues demonstrated moderate (grade 3) lymphohistiocytic endometritis, while minimal evidence of lymphohistiocytic endometritis was found in only 2.5% tissue sections (grade 1). We found that in 67.3% of the tissue sections the vasculitis affected <30% of vessels (grade 1), and only in 2.4% of tissue sections, the vasculitis affected more than 70% of blood vessels (grade 3). Regarding the severity of the vasculitis in the endometrium, we found that 62.8% of tissue sections demonstrated grade 1, 33.1% grade 2, and 4.1% grade 3 severity score (Table 3.3). No evidence of vasculitis was found in 1.0% (7/679) of the endometrial tissues from PRRSV-infected pregnant gilts, and they were graded as normal (grade 0). Uterine tissue sections from the negative control gilts had no significant inflammatory lesions, and they were all graded as normal (grade 0).

There was no significant relationship between endometrial inflammation score and PRRSV RNA concentration in the uterus, and neither vasculitis distribution nor average vasculitis severity score was significantly related to PRRSV RNA concentration in the uterus or in fetal thymus. Endometrial inflammation severity was negatively related to PRRSV RNA concentration in the fetal thymus ($P<0.05$; Figure 3.2). Endometrial inflammation score was positively related to both vasculitis distribution ($P<0.001$) and severity of vasculitis in the endometrium ($P<0.001$; Figures 3.3 and 3.4).

3.5 Discussion

The present study is part of a large-scale reproductive PRRS project investigating the phenotypic and genotypic factors associated with viral load and fetal death. The decision to inoculate at 85 days of gestation was based on the results of previous studies that confirm that late gestation infection between 72 to 93 days consistently results in transplacental infection and reproductive failure that is similar to field observations (Karniychuk and Nauwynck 2013). While the decision to terminate at 21 DPI was potentially past peak viral load and acute-stage uterine histopathology, it was required to fulfill the overarching project objectives including the identification of factors associated with fetal death and viral load, and to advance the understanding of factors involved in the pathogenesis of fetal death. In spite of the single collection time point well past the acute stage in the dam, the present histopathological study enabled the unprecedented assessment of a large number of fetal and maternal tissues and determined potential relationships amongst lesions and viral load.

The objectives of this present study were to evaluate the microscopic lesions in the uterus, placenta, and fetal tissues, histologically grade lesion severity associated with PRRSV infection at the maternal-fetal interface, and determine potential associations between severity of

lesions at maternal-fetal interface, fetal lesions, PRRSV viral load in the uterine tissue and the fetus, and fetal preservation status.

The most prominent and consistent lesions were observed in the uterus and the fetal placenta while fetal histopathological lesions were less frequent and more variable in severity and histological presentation. The majority of uteri and fetal placental samples obtained from PRRSV-infected pregnant gilts demonstrated significant inflammatory lesions affecting mostly endometrium, fetal placenta, and blood vessels. Furthermore, when severity and distribution of inflammation lesions were histologically graded the majority of uterine tissue samples exhibited moderate (73%) or severe (10%) lymphohistiocytic endometritis, and almost one-third of samples demonstrated severe vasculitis in more than 30% of blood vessels. These findings indicate that inflammation in the uterus, fetal placenta, and endometrial blood vessels are the most prominent pathological characteristics of type 2 PRRSV infection in pregnant gilts at 21 days post inoculation. This finding is also in agreement with previously published studies (Lager and Halbur 1996). In the present study, we found a significant relationship between the distribution and the severity of the vasculitis in the lamina propria, and the severity of endometrial inflammation in the uterus. This finding supports the important role of endometrial blood vessels in PRRSV transmission into the uterus of viremic pregnant gilts. The exact mechanism of transplacental viral transmission is still unknown, but it has been hypothesized that it is mediated either by the infection of blood monocytes (Karniychuk and Nauwynck 2013) and/or endothelial cell of the endometrial blood vessels (Halbur 1995). However, there was no significant relationship between the concentration of PRRSV RNA in the maternal-fetal interface and the severity of endometrial inflammation or the distribution and severity of endometrial vasculitis. Also, no significant association was found between the concentration of PRRSV RNA

in the fetal thymus and the distribution and severity of endometrial vasculitis. A statistically significant negative relationship between endometrial inflammation and PRRSV RNA concentration in fetal thymus was, in our opinion, most likely related to collecting samples at 21 DPI in our study. It is known that viremia in the sows and postnatally infected pigs reaches peak at 7-9 days post inoculation. However, the duration of viremia and presence of PRRSV in the fetus or congenitally infected pigs is slightly longer (Rowland 2003). The reason for the longer viremia in the fetus is due to PRRSV replication in the primary lymphoid organs such as thymus (Cheon and Chae 2001). It is possible that the severity of endometrial inflammation in some infected gilts had started to subside before 21 DPI, while PRRSV in fetus continued to replicate unabated until termination. However, the statistically significant relationship between PRRSV RNA concentration in the fetal thymus and the severity of endometrial inflammation found in this study (Figure 3.2) may be too weak to be biologically relevant based on the value of the regression coefficient (β=0.08).

The most important finding in this study was a significant, positive relationship between the presence of fetal lesions and PRRSV RNA concentration, both in the MFI and the fetal thymus. In other words, the odds of a fetus having PRRSV-associated lesions increased as did PRRSV viral load in both tissues. Even more, meconium staining, an early sign of fetal compromise, was significantly associated with the presence of histological fetal and umbilical lesions. In this study, we determined that fetuses with umbilical lesions were seven times more likely to be meconium stained than those fetuses without lesional cords. Fetuses with lymphoid and/or systemic lesions were two times more likely to be meconium stained than fetuses without these lesions. These results revealed that not only umbilical lesions as previously suspected

(Lager and Halbur 1996) but also fetal lesions, play a significant role in the pathogenesis of fetal demise following type 2 *in utero* PRRSV infection.

In retrospect, the ability to quantify viral load in the maternal (endometrium) and fetal (adherent placenta) portions of the MFI would have been very beneficial, but it was not possible based on the volume of samples processed on a given sample collection day. That being said, pathological processes observed at 21 DPI at the MFI have no significant relationship on the occurrence of fetal lesions or fetal preservation status, likely because the inflammatory process is well past its peak by this time. As these results are in contradiction with some previous reports (Karniychuk 2011; Karniychuk and Nauwynck 2013) that suggested that fetal death in the reproductive PRRSV infection is primarily a consequence of the pathological processes at the MFI and not in the fetus itself, we have additional experiments planned using early termination points to help clarify this issue. However, our present results indicate that pathological processes associated with fetuses are essential predictors of the fetal preservation status. This finding is in accordance with some of the most recent studies indicating that presence of PRRSV in fetuses, particularly in the thymus, increased the likelihood of fetal death (Ladinig 2015a).

3.6 Conclusions

This large-scale, multicenter, challenge experiment enabled an extensive evaluation of fetal and uterine lesions caused by type 2 PRRSV infection in third-trimester pregnant gilts at 21 DPI, and provided new insights into the pathogenesis of fetal death. Severe microscopic lesions were observed in the uterus and fetal placenta, but not in the fetus. Lymphohistiocytic endometritis and lymphocytic myometritis were observed in nearly 100% and 60% of uterine samples, respectively, whereas the most common fetal lesions, follicular atrophy of mesenteric lymph nodes and lymphocytic perivascular cuffing in umbilical vessels, were markedly less prevalent.

The presence of fetal lesions was positively associated with PRRSV viral load in the uterus and fetal thymus, but not with severity of uterine pathology. Moreover, the presence of fetal and umbilical lesions, but not uterine pathology, increased the likelihood of fetal meconium staining. While the distribution and severity of inflammation in endometrial blood vessels were positively associated with inflammation in the lamina propria, the severity and distribution of vasculitis, and severity of endometrial inflammation were not associated with PRRSV RNA concentration at the maternal-fetal interface or in fetal thymus at 21 DPI. The overall conclusion of this study is that although type 2 PRRSV infection in pregnant gilts induces significant pathological lesions at the maternal-fetal interface, fetal and umbilical pathology and PRRSV viral load all likely contribute to fetal compromise and death.

3.7 Acknowledgments

The authors wish to acknowledge the numerous technicians and students from the Western College of Veterinary Medicine, Vaccine and Infectious Disease Organization, Prairie Diagnostic Services, Inc. and University of Alberta who assisted with this project. Pregnant gilts were provided and bred by Fast Genetics Inc., Spiritwood.

Table 3.1. Numbers of fetal tissues with histopathological lesions in type 2 PRRSV-infected and negative control pregnant gilts inoculated at gestation day 85 (± 1d).

	Fetal tissues						
	Lung	Liver	Heart	Thymus	Mesenteric Lymph Node	Cerebellum	Umbilical cord
Negative control	0/221*	0/223	0/224	0/222	0/204	0/224	1/228
PRRSV-infected	11/848	21/854	27/856	6/815	89/779	19/848	78/1321

* Number of tissues with lesions/number of tissues examined.

Table 3.2. Number of uterine and placental tissues demonstrating histopathological lesions in type 2 PRRSV-infected and negative control pregnant gilts inoculated at gestation day 85 (± 1d).

	Uterus		
	Myometrium	Endometrium	Placenta
Negative control	1/230*	2/230	0/230
PRRSV-infected	822/1404	1399/1404	91/1360

* Number of tissues with lesions/number of tissues examined. One section of maternal fetal interface (uterus with adherent fetal trophoblast) was collected at the umbilical stump of each fetuses examined.

Table 3.3. Numbers of uterine tissue sections scored for endometrial inflammation, distribution of vasculitis and severity of vasculitis distribution.

	Uterine tissues				
	Grade 0	Grade 1	Grade 2	Grade 3	Grade 4
Endometritis inflammation score	0/679*	17/679	97/679	495/679	70/679
Vasculitis distribution score	7/679	457/679	199/679	16/679	0
Severity vasculitis score	157/679	269/679	225/679	28/679	0

* Number of tissues with score/number of tissues examined.

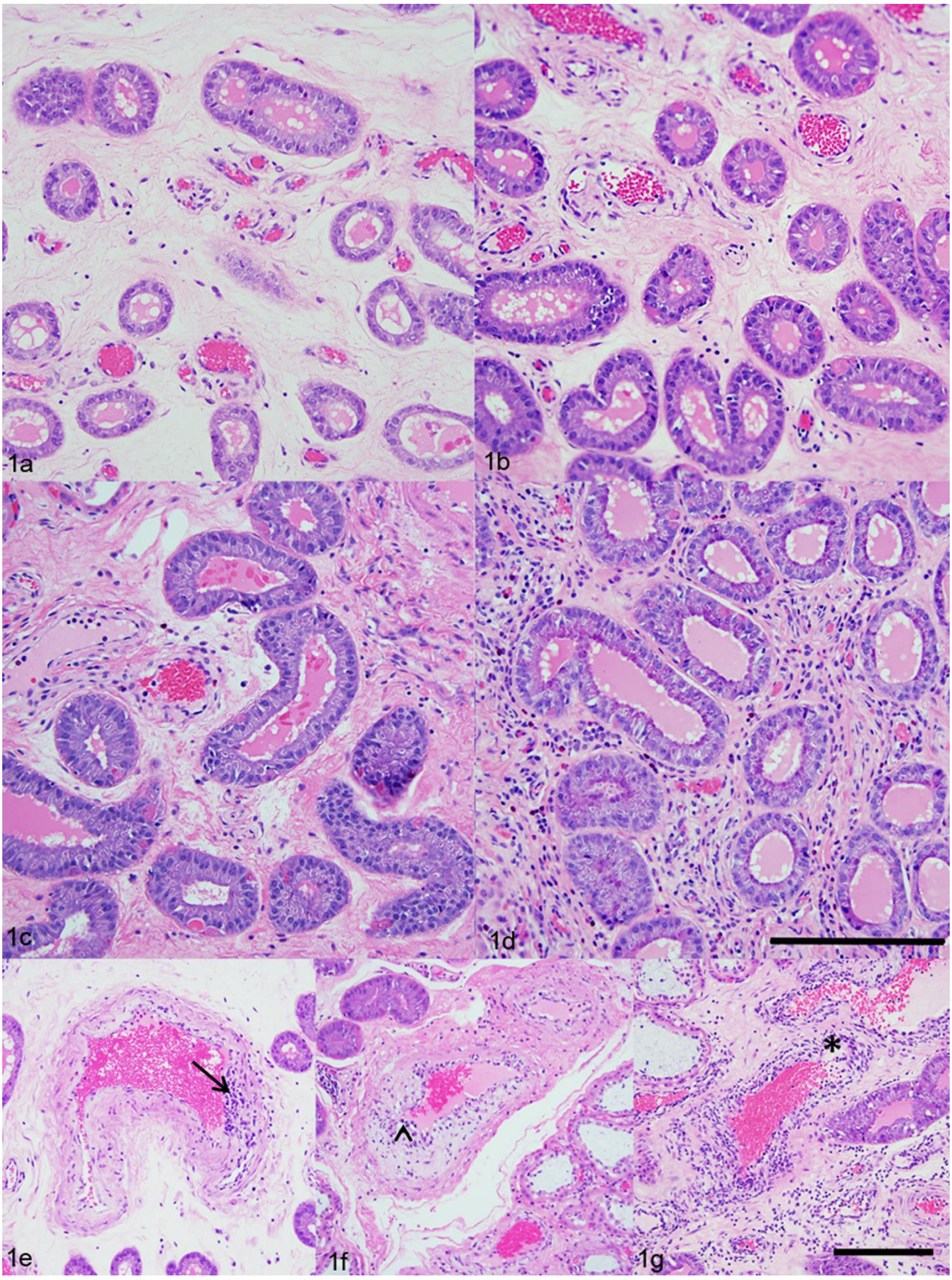
1a
1b
1c
1d
1e
1f
1g
*

Figure 3.1. Histologic scores of endometrial inflammation and severity of vasculitis. **(a)** Uterus, endometrium, lamina propria; PRRSV-infected pregnant gilt; Hematoxylin and Eosin (HE). Minimal lymphohistiocytic endometritis (grade 1.). **(b)** Uterus, endometrium, lamina propria; PRRSV-infected pregnant gilt; HE. Mild lymphohistiocytic endometritis (grade 2.). **(c)** Uterus, endometrium, lamina propria; PRRSV-infected pregnant gilt; HE. Moderate lymphohistiocytic endometritis (grade 3.). **(d)** Uterus, endometrium, lamina propria; PRRSV-infected pregnant gilt; HE. Severe lymphohistiocytic endometritis (grade 4.) (Scale bar = 200 μm). **(e)** Uterus, endometrium, blood vessel; PRRSV-infected pregnant gilt; HE. Lymphocytic vasculitis (severity grade 1.) (arrow). (**f)** Uterus, endometrium, blood vessel; PRRSV-infected pregnant gilt; HE. Lymphocytic vasculitis with vacuolar degeneration of the cells in the *tunica intima* (severity grade 2.) (arrowhead). **(g)** Uterus, endometrium, blood vessel; PRRSV-infected pregnant gilt; HE. Severe lymphocytic vasculitis with necrosis (severity grade 3.) (asterisk) (Scale bar = 200 μm).

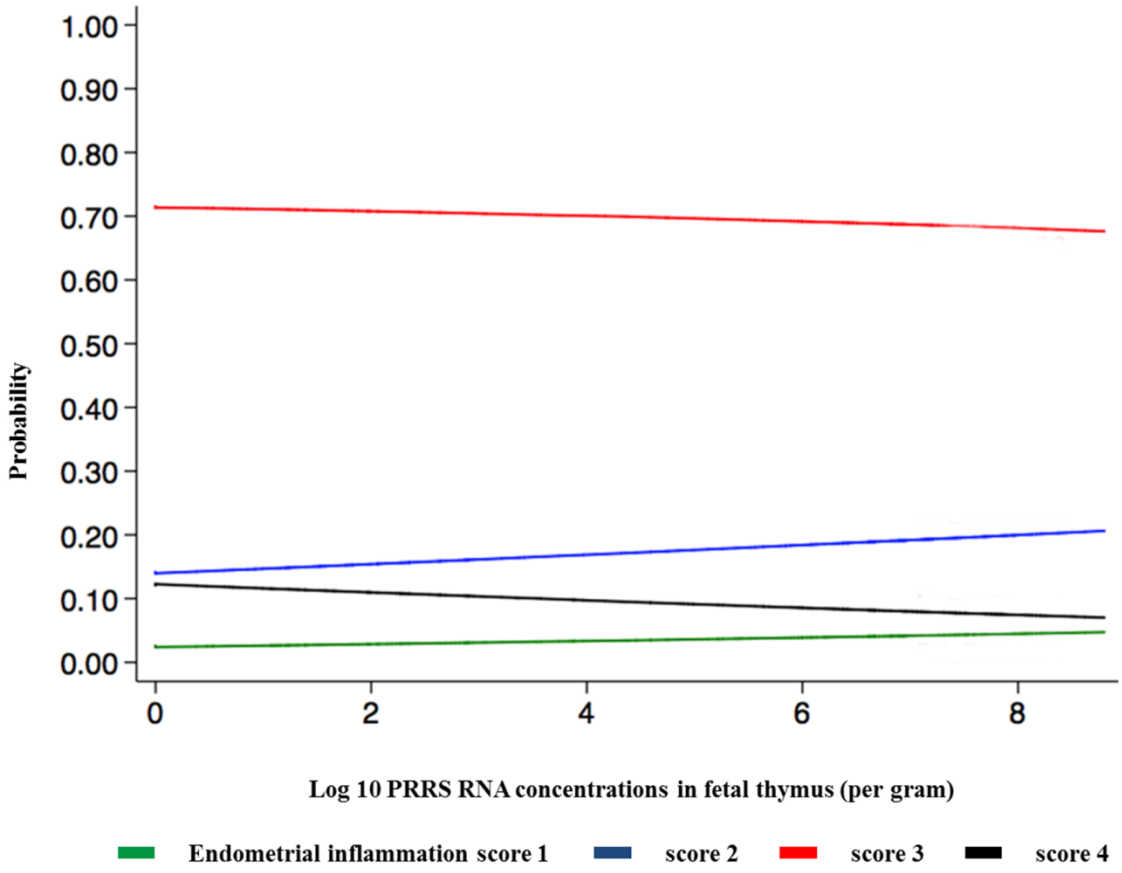

Figure 3.2. Relationship between endometrial inflammation and PRRSV RNA concentration (log_{10}/gram) in fetal thymus.

Endometrial inflammation severity is indicated by 4 coloured lines: minimal (green) = inflammatory cells multifocally present in < 10% of the tissue section; mild (blue) = multifocal to coalescing inflammatory cells infiltrate in 10-25% of the tissue; moderate (red) = diffuse inflammatory cell infiltrate in 25-50% of the tissue; severe (black) = inflammatory cells diffusely present in >50% of the tissue section. Results indicate increased viral load in fetal thymus is associated with decreased probability of moderate and severe endometrial inflammation observed at 21 DPI.

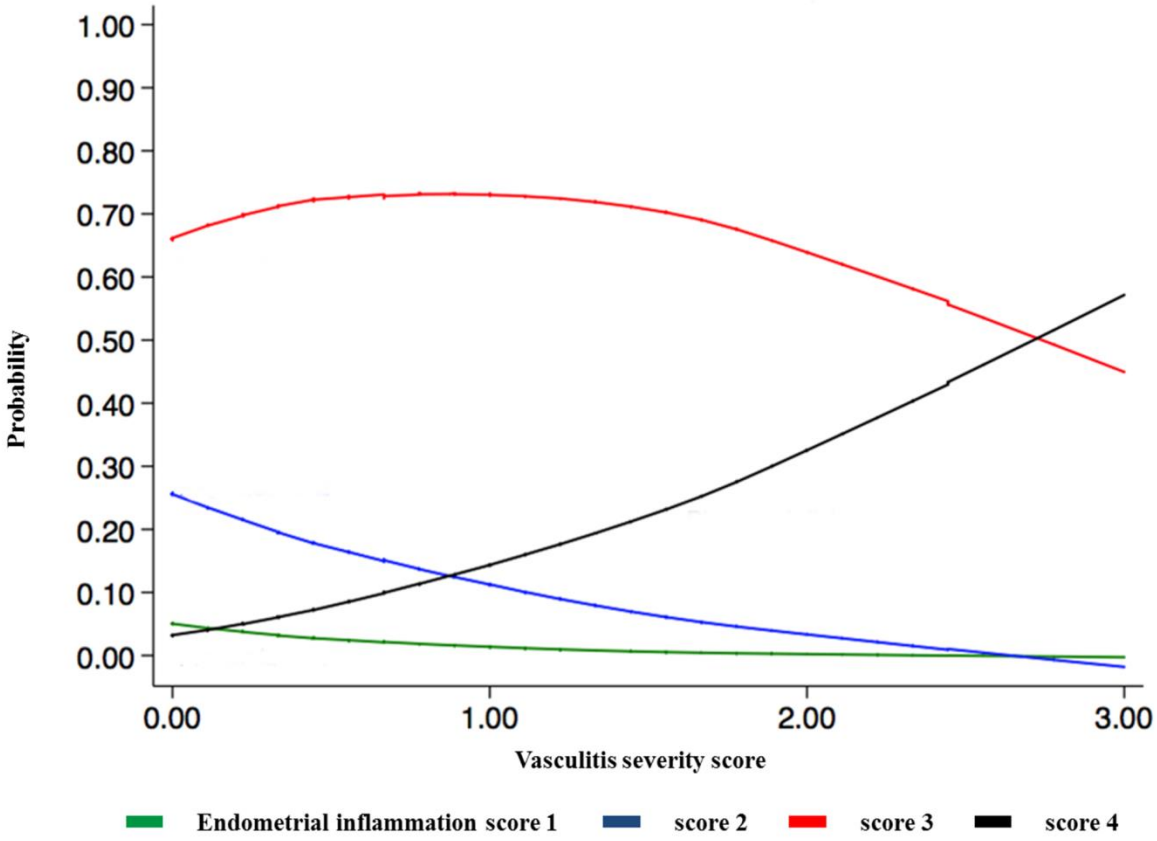

Figure 3.3. Relationship between endometrial inflammation and severity of vasculitis. Endometrial inflammation severity is indicated by 4 colored lines: minimal (green) = inflammatory cells multifocally present in < 10% of the tissue section; mild (blue) = multifocal to coalescing inflammatory cells infiltrate in 10-25% of the tissue; moderate (red) = diffuse inflammatory cell infiltrate in 25-50% of the tissue; severe (black) = inflammatory cells diffusely present in >50% of the tissue section. Results indicate the probability of observing severe endometrial inflammation at 21 DPI increases with vasculitis severity.

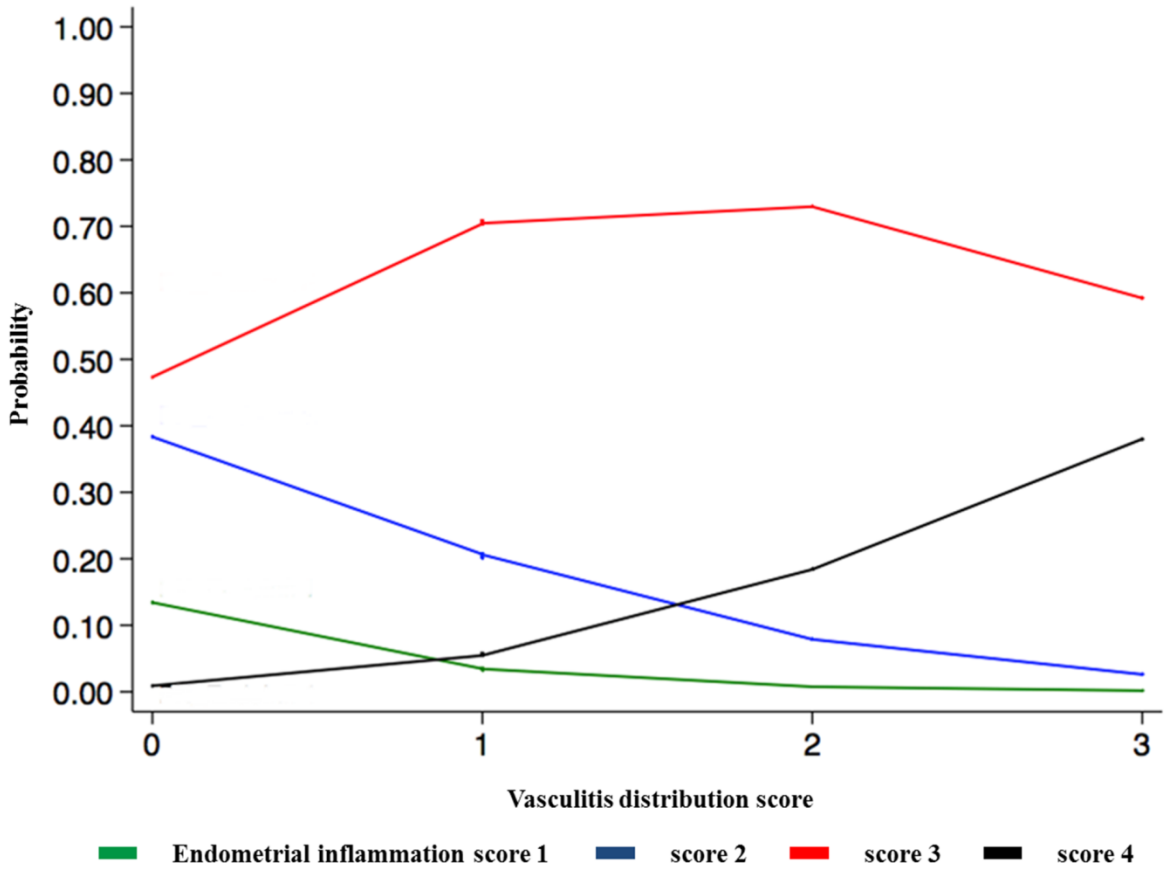

Figure 3.4. Relationship between endometrial inflammation and distribution of vasculitis. Endometrial inflammation severity is indicated by 4 coloured lines: minimal (green) = inflammatory cells multifocally present in < 10% of the tissue section; mild (blue) = multifocal to coalescing inflammatory cells infiltrate in 10-25% of the tissue; moderate (red) = diffuse inflammatory cell infiltrate in 25-50% of the tissue; severe (black) = inflammatory cells diffusely present in >50% of the tissue section. Results indicate the probability of observing severe endometrial inflammation at 21 DPI increases with more extensive vasculitis distribution.

4 Relationships of CD163 and CD169 positive cell numbers in the endometrium and fetal placenta with type 2 PRRSV RNA concentration in fetal thymus

In chapter 3, we confirmed significant pathological lesions affecting MFI of PRRSV-infected pregnant gilts. This chapter represents immunohistochemical detection and assessment of the numbers of PRRSV, CD163, CD169 positive cells, and cathepsin positive areolae at the MFI, and evaluation of their potential relationship with PRRSV viral load in the MFI and fetal thymus.

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Author contributions: Novakovic P, Ladinig A, Harding JCS and Detmer SE, conducted the sample collection and laboratory testing for this study. Novakovic P, Harding JCS, Al-Dissi AN, MacPhee DJ and Detmer SE, contributed to conception and design of the study, acquisition, analysis and interpretation of the data, and writing of the manuscript.

4.1 Abstract

The pathogenesis of transplacental infection associated with porcine reproductive and respiratory virus (PRRSV) is still poorly understood. Several routes of PRRSV transmission across the porcine intact diffuse epitheliochorial placentation have been proposed, but none have been proven. The objectives of this study were to investigate associations between numbers of CD163 and CD169 positive macrophages, cathepsin positive areolae, and PRRSV load at the maternal-fetal interface in order to examine important factors of PRRSV transplacental transmission. On gestation day 85±1, naïve pregnant gilts were inoculated with PRRSV (n=114) or were sham inoculated (n=19). At 21 days post-inoculation, dams and their litters were humanely euthanized and necropsied. Samples of the maternal-fetal interface (uterus with fully attached placenta) and fetal thymus were collected for analysis by RT-qPCR to quantify PRRSV RNA concentration. The corresponding paraffin-embedded uterine tissue sections were subjected to immunohistochemistry for PRRSV nucleocapsid N protein, CD163, CD169, and cathepsin. Our findings confirm significant increases in the numbers of PRRSV, CD163 and CD169 positive cells at the maternal-fetal interface during type 2 PRRSV infection in pregnant gilts. An increase in the ratio of $CD163^{+}$ cells in endometrium versus placenta was associated with an increase in PRRSV load in fetal thymus, and a decrease in odds of the fetus being PRRSV-negative. This finding suggests an important role for placental $CD163^{+}$ cells during trans-placental PRRSV transmission.

Key words: PRRSV, uterus, placenta, CD163, CD169, areolae, cathepsin-L

4.2 Introduction

Porcine reproductive and respiratory syndrome (PRRS) continues to be the most costly disease of the North American swine industry resulting in economic losses estimated to be $664 million annually (Holtkamp 2013). PRRS is caused by an enveloped positive-stranded RNA virus, in the genus of Arterivirus and family *Arteriviridae* (Done 1996; Rossow 1998; Zimmerman 2012). Viral replication of porcine reproductive and respiratory syndrome virus (PRRSV) initially occurs in local permissive macrophages and then rapidly spreads to well-differentiated monocyte-derived cells, such as pulmonary alveolar macrophages (PAM), intravascular macrophages (PIM) in the lung (Thanawongnuwech 1997a; Thanawongnuwech 2000a; Delputte and Nauwynck 2004), and macrophages in lymphoid tissue (Rossow 1998).

Two markers identified on the surface of permissive macrophages are CD163 entry mediator and sialoadhesin (CD169) receptor (Van Gorp 2008). CD163 is a glycosylated membrane protein expressed almost exclusively on macrophages and monocytes (Møller 2012). As a macrophage scavenger receptor, CD163 is involved in taking up haptoglobin-hemoglobin complexes, erythroblast adhesion, innate immunity of bacteria, and binding of TNF-like inducers of apoptosis (Møller 2012). CD163 is an essential receptor for the entry and uncoating of PRRSV from the early endosomes of permissive cells (Zhang and Yoo 2015).

Attachment of PRRSV to target cells is believed to occur through the interaction of the viral ligand GP5 and M heterodimer complex with CD169 receptor (Vanderheijden 2003; Zhang and Yoo 2015). CD169 receptors belong to the family of sialic acid-binding immunoglobulin-like lectins that are expressed on specific subsets of tissue macrophages found in the spleen, lymph nodes, bone marrow, liver, colon, and lungs (Hartnell 2001). Previously, CD169 was reported to be involved in the attachment and internalization of viruses (Delputte and Nauwynck

2004) and bacteria (Jones 2003), but recent studies with porcine CD169 suggest its role as an endocytic receptor in targeted delivery of toxins and antigens to macrophages (Delputte 2011).

Both CD169 and CD163 have been shown to be required for type 1 PRRSV infection *in vitro* (Van Gorp 2008). It has also been demonstrated that both CD169 and CD163 positive macrophages are increased within the implantation sites and organs of porcine embryos and fetuses during gestation (Karniychuk and Nauwynck 2009). However, CD169 negative transgenic pigs infected with type 2 PRRSV have demonstrated no difference in virus replication compared to infected wildtype pigs (Prather 2013), while CD163 negative transgenic pigs did not develop type 2 PRRSV infection (Whitworth 2016). Experiments with type 1 PRRSV infection of pregnant sows have confirmed that all PRRSV-infected cells at the maternal-fetal interface were also CD163 and CD169 positive (Karniychuk 2011).

While CD163 and CD169 macrophages have been explored, other potential mechanisms of transplacental infection of PRRSV have been largely uninvestigated. In pigs, the transplacental exchange of gases, micronutrients and macromolecules is accomplished by maternal hematotrophic and histotrophic nourishment of the fetus. Histotroph is a source of nutrition for the fetus provided by secretions of uterine epithelia in the dome-shaped structures over the openings of uterine glands called areolae (Bazer and Johnson 2014). In the areolae, secretions from superficial and deep uterine glandular epithelium, and selective transudation from maternal serum are absorbed and transported across the chorioallantois by fluid phase pinocytosis into the fetal circulation (Friess 1981). During gestation, lysosomal cysteine protease cathepsin-L, is highly expressed in the chorionic epithelium of the areolae (Song 2010). Therefore, a potential site of trans-placental infection may be areolae.

Recent studies revealed a significant association between type 1 PRRSV infection and the numbers of CD169 positive cells in the maternal-fetal interface (MFI) (Karniychuk 2013). Additionally, results of our histopathological evaluation of type 2 PRRSV infection in pregnant gilts in the third trimester of pregnancy confirmed marked inflammatory changes at MFI (Novakovic 2016a) suggesting potential cell-associated PRRSV transmission from the mother to the fetus (Karniychuk and Nauwynck 2013). In order to further test this hypothesis, we developed two objectives for the present study. The first objective was to evaluate the numbers of PRRSV, CD163 and CD169 positive cells in the endometrium and fetal placenta, and to test if the numbers of cathepsin positive areolae at the MFI differed between groups selected on the basis of PRRSV viral load (negative, low, high). The second objective was to assess the relationship between PRRSV viral load in the fetal thymus compared to the numbers of CD163 and CD169 positive cells in the endometrium and fetal placenta, and cathepsin positive areolae at the MFI.

4.3 Material and Methods

4.3.1 Experimental design and selection of samples

The animal use protocol was reviewed and approved by the Animal Research Ethics Board (AREB) at the University of Saskatchewan and followed the principles established by the Canadian Council on Animal Care (permit #20110102). The experimental protocol for this study has been described in detail (Ladinig 2014d). Briefly, on 85±1 gestation day, 114 PRRSV-naïve pregnant gilts were intramuscularly and intranasally inoculated with PRRSV (1×10^5 $TCID_{50}$ total dose, NVSL 97–7895, Gen Bank Accession No. AF325691) and 19 negative control pregnant gilts were sham inoculated with minimum essential medium (Life Technologies, Burlington, Canada). At 21days post-inoculation (DPI), dams and their litters were humanely

euthanized for necropsy examination. Samples of fetal thymus and MFI (endometrium with adherent placental layers) adjacent to the umbilical stump of each fetus were collected for histology and an in-house quantitative RT-PCR analysis of PRRSV RNA concentration, as previously described (Ladinig 2014d). From a total of 679 available MFI samples collected from live fetuses with intact uterine-placental tissue, 120 paraffin-embedded MFI samples were selected, based on the PRRSV RNA concentration in the MFI, for PRRSV, CD163, CD169, and cathepsin immunohistochemistry (IHC).

Three viral load groups were designated negative, low and high. Negative samples were RT-qPCR negative samples from non-infected gilts (mean 0.7 ±0.8 sd; n=40). Low viral load samples from infected gilts had PRRSV RNA concentration less than 2.3 $\log_{10}$ copies per mg MFI (mean 5.2 ±1.2 sd; n=40). High viral load samples from infected gilts had PRRSV RNA concentration greater than 2.8 $\log_{10}$ copies per mg MFI (n=40). PRRSV RNA concentration in the low and high viral load groups averaged 0.7 ± 0.8 and 5.2 ± 1.2 $\log_{10}$ copies/mg, respectively. The low and high samples were matched pairs from 40 infected gilts. The negative samples came from five gilts, eight samples per gilt.

4.3.2 PRRSV Immunohistochemistry

Five-micrometer tissue sections were prepared for IHC using proteinase K (Dako, Carpinteria, USA) antigen retrieval and Background Punisher blocking reagent (Biocare Medical, Concord, USA). The wash buffer was 0.05M Tris-buffered saline (Sigma-Aldrich, Oakville, Canada), pH 7.6 with 0.05% Tween-20 (TBST; Fisher Scientific, Markham, Canada). Primary monoclonal antibody against PRRSV nucleocapsid N protein (SDOW17, Rural Technologies Inc., Brookings, USA) was diluted 1:200 in antibody diluent (Dako) and placed on tissue sections overnight at 4^{o}C in a humidified chamber. Envision+System-HRP (Dako) anti-

mouse secondary antibody containing 2% normal swine serum (Life Technologies) was applied to sections for 45 min at RT. The signal was revealed using 3-Amino-9-Ethylcarbazole (AEC) chromogen (Dako) for 15 min and sections were counterstained with Mayer's hematoxylin (Fisher Scientific). Coverslips were applied to slides using Glycergel (Dako) mounting medium. Negative controls consisted of uterine tissues obtained from non-infected gilts.

4.3.3 CD163, CD169 and Cathepsin Immunohistochemistry

IHC for CD163 and CD169 was performed as previously described (Lau 2004; Detmer 2013) using rabbit polyclonal antibodies directed against human CD163 at dilution 1:100 (ab87099, Abcam, Toronto, Canada) and mouse monoclonal antibodies directed against human CD169 (clone HSn 7D2) at dilution 1:50 (NB600-534, Novus Biologicals, Oakville, Canada). IHC for cathepsin was performed using mouse monoclonal antibodies directed against human cathepsin L + V at dilution 1:100 (ab6314, Abcam). Antigen retrieval was performed in 10mM citrate buffer (pH 6.0) at 100°C for 10 min. Using a Dako Automated Immunostainer and TBST for washing buffer, the antigen signals were amplified using Envision+System-HRP with 2% normal swine serum for 45 minutes and AEC chromogen for 10 min. Slides were counterstained with Mayer's hematoxylin. Pig fetal lung and lymph node were used for the positive controls for CD163 and CD169 IHC. A normal pig uterus with placenta was used for the cathepsin positive control. Normal rabbit serum was used in place of primary antisera for the negative control.

4.3.4 Image Analysis

Quantitative analyses of immunohistochemical staining for PRRSV, CD163, CD169, and cathepsin positive areolae were performed using Image-Pro Plus version 7 software (Media Cybernetics, Inc., Rockville, USA). Ten microscopic fields of the endometrium, captured using a 20X microscope objective lens, each representing 1 mm^2 area (10 mm^2 total/slide) were

randomly selected from each image of the uterine-placental tissue. Thereafter, multiple polygonal fields of fetal placenta comprising 3 to 4 mm^2 in total were randomly selected from the same image at the same magnification. Inside these chosen fields, the total numbers of PRRSV, CD163 and CD169 immunopositive cells were manually counted. The total number of areolae was determined by manually counting the regions of cathepsin immunopositive staining across the entire maternal-fetal interface. All counts were expressed as a number per 1 mm^2 area for statistical analyses.

4.3.5 Statistical Analysis

Separate statistical analyses were performed for each of the objectives of this study using Stata 13 (StataCorp LP, College Station, USA). To determine if numbers of CD163 and CD169 positive macrophages and cathepsin positive areolae in the endometrium and fetal placenta differed among PRRS viral load groups (negative, low, high), separate two-level, linear mixed-effects regression models were developed. For these models, numbers of CD163 and CD169 positive macrophages in the endometrium and fetal placenta were zero-skewness log (lnskew0) transformed to ensure that model assumptions of linearity and homogeneity were not violated. Secondly, the potential relationships among numbers of CD163 and CD169 positive macrophages in the endometrium and the fetal placenta, numbers of cathepsin positive areolae at the MFI, and PRRS viral load in the fetal thymus were determined by using a two-level, zero-inflated Poisson regression model. For this model, PRRSV RNA concentration in fetal thymus (per mg) was converted into a count variable, with each successive count representing a one $\log_{10}$ increase in RNA concentration. Based on these results, the relationship between viral load in fetal thymus and the numbers of $CD163^+$ and $CD169^+$ macrophages in endometrium and fetal placenta was further explored using a single level proportional odds model (a two-level model

was not required) for which PRRSV RNA concentration in fetal thymus was categorized as negative (not detected), low ($0 < \log_{10}$ copies per mg < 5) and high ($\log_{10}$ copies per mg ≥ 5), and new variables representing the ratio of $CD163^{+}_{endo}$:$CD163^{+}_{plc}$ and $CD169^{+}_{endo}$:$CD169^{+}_{plc}$ were created. For this, only fetuses from PRRSV infected gilts were included. All two-level models accounted for clustering by litter of origin by including gilt as a random effect. Linear mixed models were assessed for normality and homogeneity of residuals. The proportional odds model was assessed for non-violation of the proportional odds assumption using the Brant test. Count models were assessed by evaluating how well the models predicted raw data, and where possible, evaluating the degree of over-dispersion. *P* value was deemed significant at 0.05 *a priori*.

4.4 Results

4.4.1 Distribution of immunopositive cells within the maternal-fetal interface

PRRSV immunopositive cells were detected in all sections of MFI obtained from PRRSV infected gilts. The majority of samples demonstrated strong PRRSV immunopositivity of cells located primarily at the endometrial placental junction (mean 8.6 ± 10.8 cells per mm^2). PRRSV immunopositive cells at these histological sites were closely associated with inflammatory cell infiltrates and had cytological features suggestive of tissue macrophages. Occasionally PRRSV antigen was clearly present in the uterine superficial glandular epithelial cells of the areolae (Figure 4.1A). In the endometrium, PRRSV immunopositive macrophages were rare and located away from blood vessels in the *lamina propria* (mean 1.7 ± 2.2 cells per mm^2) (Figure 4.1B). Additionally, a remarkable finding in the endometrium was strong, albeit occasional, PRRSV immunopositive staining of epithelial cells of uterine glands. Rare PRRSV-infected macrophages were found in the fetal placenta residing in proximity to the MFI of PRRSV-infected gilts. PRRSV-infected cells were not detected in the MFI of sham inoculated gilts.

IHC for CD163 and CD169 revealed the largest number of CD163 immunopositive macrophage-like cells (mean 212.3 ± 82.6 per mm^2) resided in the fetal placenta in close proximity to maternal and fetal microvilli interdigitation (Figure 4.1C), while the number of CD169 immunopositive cells observed in the fetal placenta were significantly lower (mean 69.8 ± 71.6 per mm^2). In the endometrium, markedly lower numbers of CD163 immunopositive cells (mean 34.2 ± 27.2 per mm^2) and CD169 immunopositive cells (mean 15.6 ± 15.7 per mm^2) were observed multifocally in the *lamina propria*, occasionally in the blood vessel walls, and always interspersed throughout inflammatory cell infiltrate (Figures 4.1D and 4.1E). IHC of cathepsin revealed strong positive cytoplasmic staining of collections of trophoblastic cells forming distinct placental structures at the maternal-fetal interface most consistent with areolae (Figure 4.1F). Regardless of the infection status of pregnant gilts, numbers of areolae counted at the MFI of each uterine tissue section were relatively stable (mean 3.5 ± 1.9).

4.4.2 Relationship of cell counts to viral load

Statistical analyses of the results of IHC experiments revealed significantly higher numbers of immunopositive cells in the endometrium and fetal placenta obtained from PRRSV infected gilts than negative control gilts for all markers, except for the numbers of CD163 positive cells in the fetal placenta (Figures 4.2A-C). Additionally, statistical differences were found when low and high viral load groups were compared in terms of the numbers of PRRSV-infected cells present in the endometrium and the fetal placenta at the MFI ($P<0.05$) (Figure 4.2A). High PRRSV load group was characterized by significantly higher numbers of CD163 positive cells in the endometrium, compared to the low PRRSV load group (Figure 4.2B). No significant differences were found in the numbers of $CD169^+$ cells in the endometrium and fetal placenta between low and high PRRSV load groups (Figure 4.2C). Numbers of cathepsin-L

immunostained areolae across the MFI were also not statistically different between PRRS viral load groups.

The numbers of $CD163^+$ and $CD169^+$ macrophages in the endometrium and fetal placenta and cathepsin positive areolae were assessed as potential predictors of PRRSV viral load in fetal thymus. After a backwards-stepwise elimination, only numbers of $CD163^+$ macrophages in endometrium and fetal placenta were related to PRRS viral load in the fetal thymus (P=0.01). More specifically, increased PRRSV RNA concentration (log_{10} copies/mg) in the fetal thymus was associated with increased numbers of $CD163^+$ macrophages in the endometrium and decreased numbers of CD163 macrophages in placenta (Table 1). In the proportional odds model used to further explore these relationships, viral load in fetal thymus (categorized as negative, low and high) was found to be very strongly related to $CD163^+_{endo}$:$CD163^+_{plc}$ ratio (coef 7.1 ±2.1, 95% CI: 3.1, 11.2; P=0.001) (Figure 4.3). In fact, all fetuses with thymic PRRSV RNA concentration less than 6 log_{10} copies per mg had ratios less than 0.4 (Figure 4.4).

4.5 Discussion

The goal of this study was to evaluate the associations between the numbers of CD163 and CD169 positive cells in the endometrium and fetal placenta, cathepsin positive areolae at the MFI, and PRRSV viral load in the MFI and fetal thymus. These results provide improved insights into the events occurring at the maternal-fetal interface during type 2 PRRSV infection and help to clarify the pathogenesis of PRRSV transplacental infection and induced reproductive failure. Previous studies (Lager and Halbur 1996; Rossow 1996b) as well as our observations in a related histopathology study of type 2 PRRSV infection in pregnant gilts (Novakovic 2016a) confirmed that type 2 PRRSV infection causes significant microscopic lesions in the uterus and fetal placenta at the maternal-fetal interface. Consequently, we hypothesized that in addition to

factors such as CD169 positive cells, *in utero* infection is influenced by other susceptible cell populations at the MFI.

The findings of our first objective confirmed that there was a significant increase in the numbers of $CD163^+$ and $CD169^+$ macrophages in the endometrium and the fetal placenta in PRRSV-infected versus non-infected pregnant gilts. This finding was not unexpected because large numbers of these cells represent a significant portion of the cellular infiltrate in the highly inflamed areas of *lamina propria* suggesting a role in the innate immune response to PRRSV infection. On the other hand, differences in these cell numbers were much less consistent when the low and high PRRSV groups were compared. For example, only the numbers of $CD163^+$ cells in endometrium were significantly increased in high versus low viral load groups. Nevertheless, this inconsistent relationship between cell counts and viral load reported herein is in the agreement with the results of our previously reported PRRS histopathologic evaluation (Novakovic 2016a) in which it was demonstrated that the severity of endometrial inflammation was not associated with PRRSV viral load in the uterus at 21 DPI.

Not only did cell counts in fetal placenta not differ between high and low PRRS viral load groups, they markedly diverged from the results obtained from the endometrium. In particular, this applied to the numbers of $CD163^+$ cells in placenta, which did not significantly differ in non-infected gilts compared to gilts with high PRRS viral load at the MFI. A possible explanation for this finding could be the presence of a residential population of fetal tissue macrophages, which in human placenta are known as Hofbauer cells. This population of histiocytes found in human villous mesenchyme and amniochorion on the fetal side of the uteroplacental unit is relatively constant, and can constitute nearly all macrophages in this region (Bulmer and Johnson 1984). It is believed these macrophages are involved in the prevention of

the transmission of the pathogens from the mother to the fetus (vertical transmission) and early placental development (Tang 2011). Importantly, Hofbauer cells strongly express CD163, CD68, and CD206 (Joerink 2011; Tang 2011; Sivakumar 2013).

The presence of Hofbauer cells in the porcine fetal chorioallantois has not been reported to date, but in this present study, we observed large numbers of histiocytes with cytological features resembling Hofbauer cells and expressing strong cytoplasmic CD163 immunopositivity residing along the chorionic villi of the fetal placenta in both infected and non-infected gilts. Moreover, the number of $CD163^+$ cells in placenta was negatively associated with viral load in fetal thymus, and increased numbers were significantly related to increased odds of a fetus being virus negative. This finding implies that $CD163^+$ tissue macrophages in the placenta have a potentially significant role in transplacental PRRSV transmission. While numbers of $CD163^+$ tissue macrophages in the endometrium are concomitant with uterine viral load, the numbers of CD163 positive cells in the fetal placenta might decrease subsequent to transplacental PRRSV infection.

To explore this potential dichotomy further, we assessed the relationship between viral load in fetal thymus and the ratio of $CD163^+$ cells present in endometrium and fetal placental ($CD163_{endo}$:$CD163_{plc}$), based on our hypothesis that a high ratio (reflecting increased macrophage numbers and infection pressure in uterus and low placental immune surveillance) would be associated with high fetal viral load. As anticipated, viral load in fetal thymus increased very substantially as the $CD163^+$ endo:plc ratio increased. In addition, the odds of fetal thymus being PRRSV virus negative decreased dramatically in a nearly linear manner as $CD163_{endo}$:$CD163_{plc}$ increased (Figure 4.3). Thus, large numbers of $CD163^+$ macrophages in

placenta, particularly in the presence of low $CD163^+$ macrophages in the endometrium, may represent an exciting, yet unexplored mechanism of PRRSV resistance in late gestation fetuses.

However, in order to draw this conclusion, at least one criterion would have to be met which is that porcine analogs of Hofbauer cells at the fetal placenta are not susceptible to PRRSV infection and subsequent viral replication. Unfortunately, current knowledge on the properties of this cell population regarding the PRRSV susceptibility is poor; therefore, future studies in this area are highly warranted. On the other hand, fetal placenta (chorioallantois) is confirmed to be highly susceptible to type 1 PRRSV infection demonstrating markedly larger numbers of PRRSV-positive cells than the endometrium at 10 DPI (Karniychuk 2011). Although type 1 and type 2 PRRS viruses are genetically distinct with differences in pathogenicity *in vitro* and *in vivo* (Choi 2015), it can be hypothesized that by collecting the fetal placenta 21 DPI in our study we detected a decrease in the numbers of CD163 positive cells as a result of the cytopathic effect of PRRSV on a highly susceptible population of cells. Furthermore, this finding could suggest that instead of a protective role, placental $CD163^+$ cells may play a role in transplacental infection of the fetus or in viral replication in the fetal compartment following infection. In other words, once PRRSV transmits transplacentally, the resident CD163 positive cells become "fertile soil" for viral propagation and spread to the fetal organs. Therefore, it is essential to conduct future studies aimed at determining the susceptibility of porcine placental macrophages to PRRSV infection.

Unlike for the $CD163^+$ cells, there was no significant difference between high and low viral groups. Only a difference between infected and uninfected was statistically significant for CD169 and both cell types demonstrated an increase in the endometrium and placenta with infection. A few previously published reports indicate the importance of CD169 as a receptor

mediating cell entry for type 1 PRRSV (Van Gorp 2008; Karniychuk and Nauwynck 2009). However, some of the more recent studies in the transgenic pigs (Prather 2013) confirmed that intact CD169 receptor is not required for the productive type 2 PRRSV infection. The importance of CD163 rather than CD169 for type 2 PRRSV viral replication was corroborated in pigs with an edited (non-functional) CD163 where viral replication did not occur (Whitworth 2016). As our results suggest, $CD163^{+}$ macrophages play a potentially important role in PRRSV transmission across the MFI and possibly in viral propagation in the fetus thereafter. More research is clearly needed to determine the exact role of $CD163^{+}$ placental macrophages, if they are also relevant for other reproductive pathogens in pigs and other animals (including humans), and the potential effects of $CD163^{-/-}$ gene editing in a pregnant animal.

Since the PRRSV antigen has been occasionally detected in the uterine epithelial cells of the areolae and considering active role of the areolae in the nourishment of the fetus, we also evaluated the potential association of the numbers of cathepsin positive placental areolae and PRRSV load in the fetus. However, numbers of areolae were not significantly associated with PRRSV load in the fetal thymus.

PRRSV IHC studies on the uterine tissue with fully attached fetal chorioallantois have been rarely reported in the past. In the present study, the presence of the PRRS virus antigen in uterine tissues and fetal placentae was infrequent and rarely localized in the inflammatory cells of the *lamina propria*. This unexpected finding could be due to the time point used for the collection of samples which at 21 DPI was past peak. Previous experiments also confirmed lesser numbers of type 1 PRRSV immunopositive cells in the endometrium of the sows euthanized at 20 DPI than in those euthanized at 10 DPI (Karniychuk 2011; Karniychuk and Nauwynck 2013). Therefore, using IHC for detection of PRRSV antigen in the MFI is optimal in the early time

points of infection, but in our study other experimental activities necessitated collection at 21 DPI. On the other hand, the PRRSV replication in the fetal lymphoid organs can continue and persist after birth (Rowland 2003).

The largest numbers of cells staining with PRRSV antigen in the cytoplasm were found at the MFI resembling histiocytes, and to a lesser degree but surprising, in uterine epithelial cells and the rare fetal trophoblastic cells. PRRSV immunopositivity of uterine and trophoblastic epithelial cells along with occasional moderate PRRSV immunostaining of the glandular epithelium of the uterine glands were novel findings in this study. PRRSV infection of nasal, bronchiolar and alveolar epithelium has been reported before, but the mechanism of the PRRS virus infection of these cell types remains unexplained (Rossow 1996a; Thanawongnuwech 1997a; Rossow 1998). Nevertheless, it has been confirmed that some epithelial cells such as St-Jude porcine lung cells are susceptible to *in vitro* PRRSV infection due to the expression of receptor CD151 (Provost 2012). CD151 receptor has been also implicated in PRRSV infection of porcine endometrial endothelial cells, where it is believed to act as alternative receptor along with CD169 (Feng 2013). Additionally, syndecan-4, which is heparan sulfate proteoglycan, is confirmed to be required in the PRRSV attachment to MARC-145 cells (Wang 2016). Heparan sulfate proteoglycans are present on the epithelial and endothelial cells and are confirmed to bind to M and N proteins of the PRRSV (Delputte 2002). Another important finding from our PRRSV immunohistochemical analysis was the detection of PRRSV antigen in the smaller numbers of macrophage-looking cells present in the fetal chorioallantois in the proximity of MFI suggesting potential cell-associated virus spread from the endometrium to the fetal membranes.

In summary, the results of this study have confirmed significant increases in the numbers of PRRSV, $CD163^+$ and $CD169^+$ cells at the MFI during late gestation, type 2 PRRSV infection

in pregnant gilts. The relationship between numbers of $CD163^+$ cells in the endometrium and fetal placenta, and PRRSV viral load in the fetal thymus confirmed by this study indicates the essential role of CD163 expressing cells in the endometrium and fetal placenta, which provides additional evidence of their potential role in the mechanism of transplacental transmission of type 2 PRRSV during late gestation.

4.6 Acknowledgments

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Table 4.1. Association of CD163 positive cells in the endometrium and placenta and PRRSV RNA concentration in fetal thymus*.

PRRS RNA concentration in fetal thymus count†	**Coefficient (SE)**	**95% CI**	***P* values**
Continuous model (relationship of cell numbers to PRRSV concentration in fetal thymus)			
Numbers of CD163 positive cells per 1mm^2 of endometrium	0.004[a] (0.002)	0.0009, 0.007	0.011
Numbers of CD163 positive cells per 1mm^2 of fetal placenta	-0.002[b] (0.001)	-0.003, -0.0006	0.005
Inflated (logit) model‡ (relationship of cell numbers to likelihood of PRRSV concentration in fetal thymus being equal to zero (PRSV negative)			
Numbers of CD163 positive cells per 1mm^2 of endometrium	-0.044[c] (0.012)	-0.067, -0.021	<0.001
Numbers of CD163 positive cells per 1mm^2 of fetal placenta	0.006[d] (0.003)	0.0004, 0.011	0.033

[*] Results of zero-inflated Poisson regression model.

[†]Based on the distribution of data, a count model was used after categorizing PRRSV RNA concentration into nine 1-$\log_{10}$ replicates, from 0 to 9 $\log_{10}$ copies/mg.

[‡] The logit portion of the model predicts the odds of a fetus being negative (PRRSV RNA concentration = 0) from the population of fetuses from infected and negative control gilts.

[a] For each one unit increase in the numbers of CD163 positive cells/mm in endometrium, PRRSV RNA concentration in fetal thymus increases by 0.004 $\log_{10}$ copies/mg.

[b] For each one unit increase in the numbers of CD163 positive cells/mm in fetal placenta PRRSV RNA concentration in the fetal thymus decreases by 0.002 $\log_{10}$ copies/mg.

[c] For each one unit change in the numbers of CD163 positive cells/mm in endometrium the odds of PRRSV RNA concentration in fetal thymus being equal to 0 (zero) decreases 1.045 times ($e^{-0.044}$).

[d] For each one unit change in the numbers of CD163 positive cells/mm in fetal placenta the odds of PRRSV RNA concentration in the fetal thymus being equal to 0 (zero) increases 1.006 times ($e^{0.006}$).

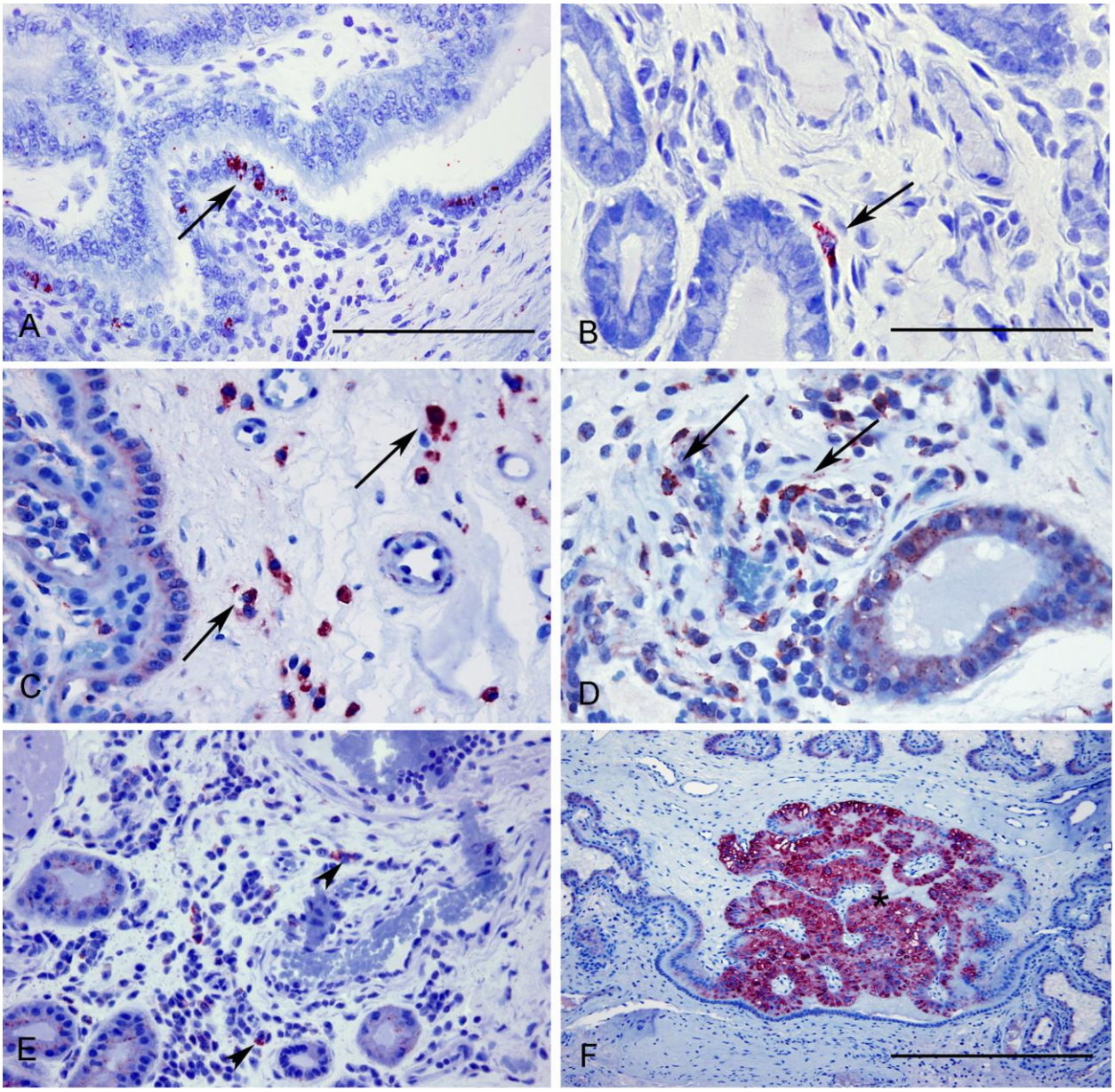

Figure 4.1. IHC for PRRSV, CD163, CD169, and cathepsin in the PRRSV-infected uterine-fetal placental tissues.

A Strong immunopositive staining for PRRSV of uterine epithelial cells (arrow) of areola from PRRSV-infected pregnant gilt. IHC for SDOW17, bar = 200 μm: Figures 4.1A, and E. **B** PRRSV positive immunostained macrophage-like cell (arrow) in the endometrium of PRRSV-infected pregnant gilt. IHC for SDOW17, bar = 100 μm: Figures 4.1B, C, and D. **C** Strong cytoplasmic

immunopositive staining for CD163 of macrophages (arrows) in the chorioallantois of PRRSV-infected pregnant gilt. IHC for CD163. **D** Increased numbers of CD163 macrophages in highly inflamed areas (arrows) in the *lamina propria* of endometrium from uterus of PRRSV-infected pregnant gilt. IHC for CD163. **E** Positive CD169 cells (arrowheads) in the *lamina propria* of endometrium from the uterus of PRRSV-infected pregnant gilt. IHC for CD169. **F** Cathepsin immunopositive stained areola (asterisk) at the uterus-fetal placenta interface from PRRSV-infected pregnant gilt. IHC for Cathepsin, bar = 500 μm.

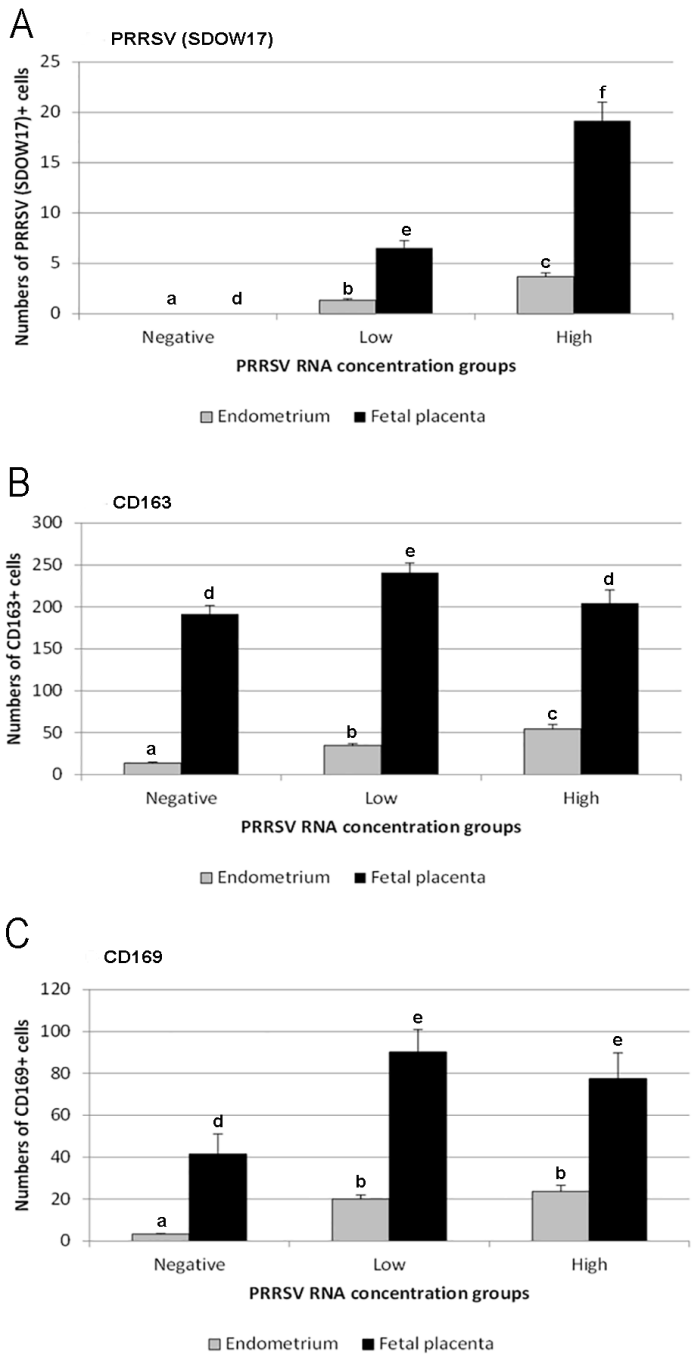

Figure 4.2. Mean numbers of PRRSV, CD163, and CD169 positive cells per 1 mm^2 of endometrium and placenta.

A Mean numbers of PRRSV (SDOW17) positive cells per 1 mm^2 of the endometrium and fetal placenta. **B** Mean numbers of CD163 positive cells per 1 mm^2 of the endometrium and fetal

placenta. **C** Mean numbers of CD169 positive cells per 1 mm^2 of the endometrium and fetal placenta. Superscript letters ($^{a, b, c}$ or $^{d, e, f}$) indicate significant differences ($P < 0.05$) between PRRSV viral load groups. Error bars represent standard error.

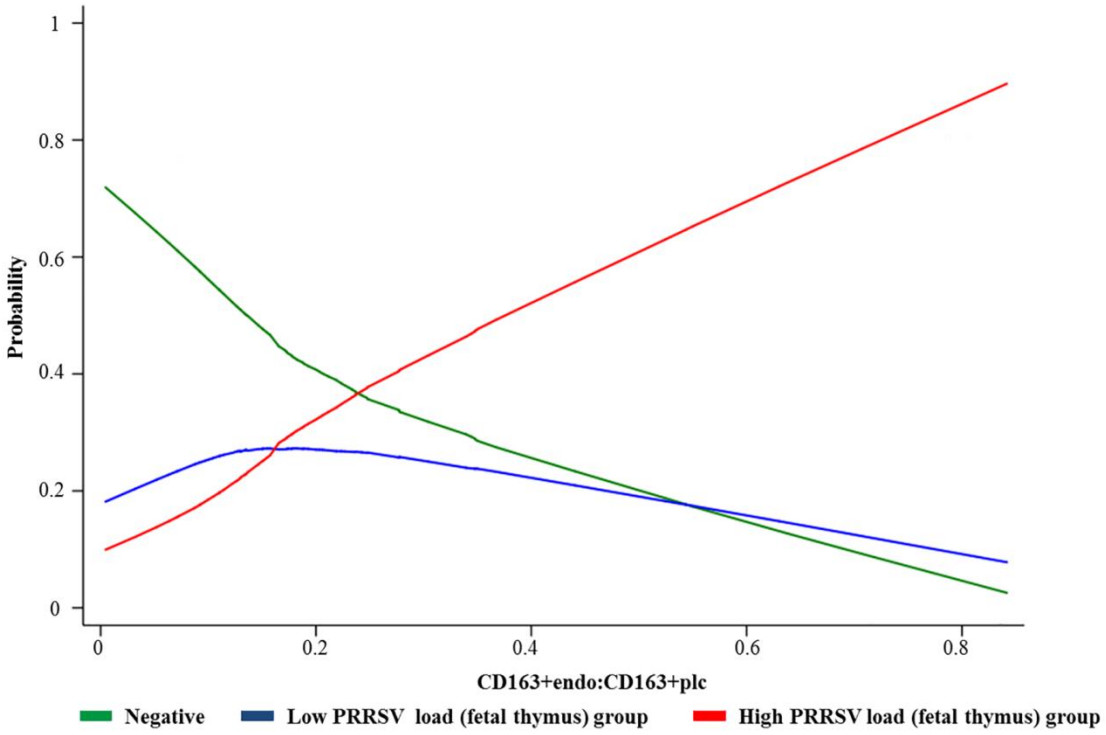

Figure 4.3. Relationship between PRRSV RNA concentration in fetal thymus and the ratio of $CD163^{+}_{endo}$:$CD163^{+}_{plc}$.

PRRSV RNA concentration in fetal thymus groups are indicated by 3 coloured lines: negative (green), low (blue; $0 < \log_{10}$ copies per mg < 5) and high (red; > 5 $\log_{10}$ copies per mg). Results indicate that increased ratio of $CD163^{+}_{endo}$:$CD163^{+}_{plc}$ is associated with decreased probability of a fetus being in the negative and low thymic PRRSV load groups and increased probability of being in the high PRRSV load group. Single level proportional odds model.

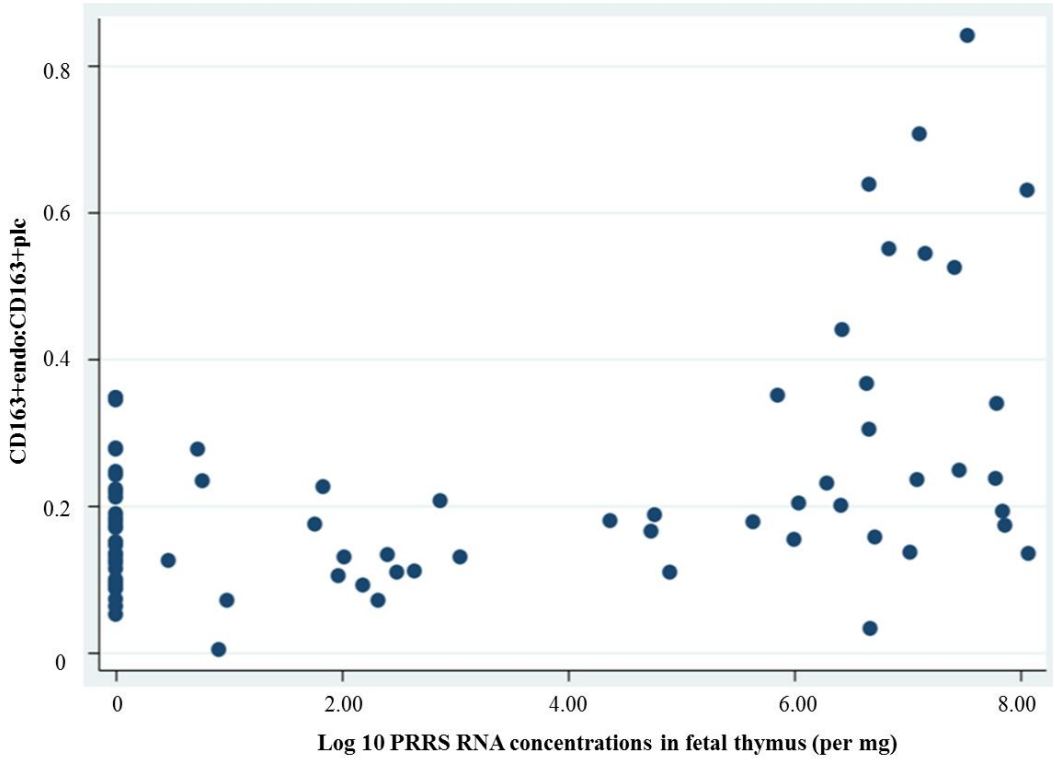

Figure 4.4 Relationship between ratio of $CD163^{+}_{endo}$:$CD163^{+}_{plc}$ and PRRSV RNA concentration in fetal thymus.

Scatter plot of ratio of numbers of $CD163^{+}$ cells in the endometrium and $CD163^{+}$cells in fetal placenta (Y axis), and PRRSV RNA concentration (log_{10} copies/mg) in fetal thymus (X-axis). Each dot represents one fetus. Results indicate that PRRSV RNA concentration in fetal thymus is positively associated with ratio of $CD163^{+}_{endo}$:$CD163^{+}_{plc}$ (all fetuses with high endo:plc ratios (>0.4) had high viral load).

5 Vasculitis, endometrial inflammation and cell death at the maternal-fetal interface during type 2 porcine reproductive and respiratory syndrome virus infection

In chapter 4, we have confirmed significant increase in the numbers of PRRSV, CD163+ and CD169+cells at the MFI during late gestation, type 2 PRRSV infection in pregnant gilts. This chapter presents a further investigation of pathogenesis of fetal by assessing the numbers of apoptotic cells at the MFI, and evaluating their potential relationship with PRRSV viral load in the MFI and fetal thymus, severity of microscopic lesions in the MFI and fetal preservation status.

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Author contributions: P. Novakovic, J.C.S. Harding and S.E. Detmer, conducted the sample collection and laboratory testing for this study. P. Novakovic, J.C.S. Harding, A. N. Al-Dissi and S.E. Detmer contributed to conception and design of the study, acquisition, analysis and interpretation of the data, and writing of the manuscript.

5.1 Abstract

The pathogenesis of fetal death associated with porcine reproductive and respiratory virus (PRRSV) is hypothesized to be a consequence of trans-placental PRRSV infection causing severe lesions at the maternal-fetal interface. The objectives of this study were to determine associations between apoptosis, PRRSV load, PRRSV-induced microscopic lesions at the maternal-fetal interface and fetal preservation status in order to elucidate mechanisms of fetal demise. A total of 114 PRRSV-naïve, high-health pregnant gilts were inoculated with PRRSV on gestation day 85±1 with euthanasia 21 days later; 19 sham-inoculated gilts served as controls. Two hundred and fifty samples of MFI were selected based on negative, low RNA, and high PRRSV RNA concentration (0, < or > 2.7 $\log_{10}$ copies/mg). TUNEL assay was used to detect apoptosis in the endometrium and at the MFI. PRRSV load in the maternal-fetal interface, but not in the fetal thymus, is significantly positively associated with the degree of apoptosis in the endometrium and MFI. The number of TUNEL positive cells at the MFI was also positively associated with the distribution ($P<0.05$) and severity ($P<0.001$) of vasculitis, but not endometrial inflammation severity at 21 days post infection. Increased numbers of TUNEL positive cells at the MFI were associated with greater odds of meconium staining of the fetus at 21 days post infection ($P<0.001$). These findings suggest an important role of apoptosis in the pathogenesis of cell death of uterine epithelial cells and trophoblastic cells at the MFI. Moreover, cell death at the MFI significantly contributes to fetal demise during *in utero* type 2 PRRSV infection.

Keywords: porcine reproductive and respiratory syndrome, PRRS, uterus, apoptosis, TUNEL, vasculitis, endometritis, placenta.

5.2 Introduction

Past studies proposed that PRRSV induces microscopic, gross lesions and subsequently clinical disease by a variety of mechanism such as apoptosis of PRRSV infected cells, non-infected cells (bystander cells), induction of inflammatory cytokines, induction of polyclonal B cell activation and reduction in bacterial phagocytosis (Miller and Fox 2004). Apoptosis has been extensively reported within PRRSV infected cell lines *in vitro* (Kim 2002). Additionally, during the PRRSV infection of nursing and growing pigs, apoptotic cells were found widely distributed in infected tissues such as lungs, testes, lymph nodes, and thymus (Sur 1998; Labarque 2003; Costers 2008). Several studies have investigated the role of apoptosis during PRRSV infection *in vitro* and in the postnatal form of the disease. However, only one study so far has investigated apoptosis in the reproductive form using type 1 PRRSV and focused exclusively on the maternal-fetal interface (MFI) (Karniychuk 2011). Therefore, there is a need to clarify the role of apoptosis at the MFI during type 2 PRRSV infection and also its potential associations with PRRSV infection both in the MFI and the fetus, PRRSV-induced microscopic lesions in the MFI, and fetal preservation status in order to elucidate the pathogenesis of fetal death.

Vasculature of the uterus and fetal placenta has an essential role in the growth and development of the conceptus throughout gestation (Rogers 1996). During the placental development endometrial and placental tissues are remodeled to achieve areas of reduced interhemal distance (Bazer 2009). In pigs, epitheliochorial placentation is characterized by an intact uterine luminal epithelium which is directly attached to fetal placental trophectoderm during pregnancy. Transplacental exchange of gases, micronutrients (amino acids, glucose) and macromolecules (proteins, hormones, cytokines and other regulatory molecules) can only be accomplished by maternal hematotrophic and histotrophic nourishment of the fetus (Bazer 2009).

Hematotroph is the source of nutrition from maternal blood provided by the capillary bed in the endometrial invaginations in close proximity to uterine luminal epithelial cells (Enders and Blankenship 1999). Histotroph is another source of nutrition in pig placentation that is provided through secretions of uterine epithelia into the dome-shaped structures over the openings of uterine glands called areolae (Bazer and Johnson 2014).

Maternal vascular complications in the human uterus and placenta have been associated with preterm delivery, premature rupture of placental membranes and spontaneous preterm labor (Kelly 2009). Additionally, it has been extensively reported that significant intrauterine inflammation (endometritis) is highly associated with the preterm and abnormal term labor (Kemp 2014). Subsequently inflammatory changes in fetal tissues (chorioamnionitis) were negatively related to gestational age of the fetus at delivery (Yoon 2000). Cell death (apoptosis) at the human maternal-fetal interface is the one of the most important underlying mechanisms of infection-associated rupture of human fetal membranes in reproductive failures (Fortunato 2000).

Porcine reproductive and respiratory syndrome virus (PRRSV), together with other members of the virus family Arteriviridae, are known to cause a systemic necrotizing vasculitis affecting predominately small caliber blood vessels (Dal Canto and Virgin Iv 2000) and PRRSV-induced vasculitis can vary in distribution and severity (Rossow 1995). Initial reports of PRRSV-induced vasculitis in weaned and feeder pigs described it as severe, necrotizing and leukocytoclastic (Thibault 1998). In infected gnotobiotic and pathogen-free pigs between 3 and 21 days post inoculation, PRRSV was shown to affect numerous arterioles and veins, and cause lymphohistiocytic arteritis in the thoracic aorta (Rossow 1995; Cooper 1997).

PRRSV infection during gestation induces pathological lesions in the reproductive tract consisting of variable degrees of lymphoplasmacytic and histiocytic endometritis, perivascular

cuffing, lymphoplasmacytic segmental vasculitis, and occasionally marked microseparations between endometrial epithelium and placental trophoblasts (Rossow 1996b). Results from a recent study using a large number of PRRSV-infected uterine tissues 21 days post-infection (DPI) confirmed that severe inflammation in endometrial lamina propria and vasculature at the maternal-fetal interface (MFI) was the most frequent and consistent histopathological lesion in type 2 PRRSV infected pregnant gilts, and was markedly more frequent than any fetal lesion (Novakovic 2016a). Although cell death (apoptosis) was confirmed to be significantly increased at the MFI in the type 1 PRRSV-infected pregnant sows (Karniychuk 2011), it is still unclear whether the type 2 PRRSV induces cells death by same mechanism, or if other potential mechanisms are involved in the pathogenesis of reproductive failure. Thus, a mechanism has been proposed that in addition to PRRSV mediated apoptosis, other pathogenic mechanisms such as PRRSV-induced endometrial infection and/or vasculitis with subsequent vascular dysfunction and hypoperfusion may compromise the fragile maternal-fetal barrier and contribute to the cell death at the MFI and associated placental detachment from the uterus.

The first objective of the present study was designed to evaluate the numbers TUNEL positive cells in the endometrium and MFI, and to test if the numbers of apoptotic cells differed between groups selected on the basis of PRRSV viral load (negative, low, high) in the MFI. The second objective was to evaluate the potential relationships between the numbers of TUNEL positive cells at MFI, PRRSV load at the MFI and severity of histopathological lesions affecting endometrium and related vasculature. Finally this study also examined the relationship between the apoptosis at MFI, PRRSV viral load in the fetal thymus and the preservation status of the corresponding fetus.

5.3 Material and Methods

5.3.1 Experimental design

The animal use protocol was reviewed and approved by the Animal Research Ethics Board (AREB) at the University of Saskatchewan and followed the principles established by the Canadian Council on Animal Care (permit #20110102). The experimental protocol for this study has been described in detail previously (Ladinig 2014d). Briefly, on 85±1 gestation day, 114 PRRSV-naïve pregnant gilts were intramuscularly and intranasally inoculated with PRRSV (10^5 TCID50 total dose, NVSL 97–7895, GenBank Accession No. AF325691) and 19 negative control pregnant gilts were sham inoculated with minimum essential medium. At 21 DPI, dams and their litters were humanely euthanized for necropsy examination. The reproductive tract was removed intact and cut opened from the tip of the horns. Each fetus was removed along with its umbilical cord, placenta and a portion of the uterus adjacent to the umbilical stump. The preservation status of each live fetus was assessed as previously described (Ladinig 2014d) based on the external gross appearance of its skin and umbilical cord as: viable (VIA; normal, white to purple skin with visible hair and regular umbilical cord), and meconium-stained (MEC; skin covered with inspissated, brownish amniotic fluid and regular umbilical cord with edema). All dead fetuses (partially decomposed to autolyzed) were excluded from this study since the MFI was compromised. Samples of MFI tissue (uterine with adherent placental layers) collected 4 cm from the umbilical attachment of each corresponding live fetus were analyzed by in-house RT-qPCR analysis to determine PRRSV RNA concentration (log10 copies/mg) as previously described (Ladinig 2014d).

From a total of 1452 infected and 231 negative control fetuses, only 679 MFI met the criteria of size (≥ 1 cm) and fully attached fetal placenta for microscopic lesion grading of

inflammation and vasculitis (Novakovic 2016a). A subset of 250 MFI samples was selected based on the PRRSV RNA concentration in the MFI. The three groups were negative (50 samples from PRRSV non-infected pregnant gilts, PRRSV RNA not detected), low (100 samples from PRRSV-infected pregnant gilts, PRRSV RNA concentration less than 2.7 log10 copies per mg MFI), and high (100 samples from PRRSV-infected pregnant gilts, PRRSV RNA concentration greater than 2.7 log10 copies per mg MFI). The low and high viral samples of MFI corresponding to individual fetus were matched pairs from 61 PRRSV infected gilts (one pair from 24 gilts and two pairs from 38 gilts). Fifty MFI samples corresponding to different fetuses were selected from six negative control gilts (9 MFI samples from 5 gilts, 5 MFI samples from 1 gilt).

5.3.2 TUNEL assay

The detection of DNA fragmentation due to apoptosis was performed using Terminal deoxynucleotidyl transferase-mediated dUTP nick end labeling (TUNEL) assay as previously described at room temperature (Al-Dissi 2007). Briefly, after deparaffinization and rehydration of 5 μm microsections of FFPE MFI tissue, protein digestion was done using Proteinase K solution (Dako, Carpinteria, CA) for 15 min. Endogenous peroxidase activity was blocked using 3% hydrogen peroxide for 5 min. Equilibration buffer provided with the ApopTag Plus Peroxidase In Situ Apoptosis Detection Kit (Millipore, Etobicoke, Ontario) was immediately applied for 10 sec at room temperature, followed by terminal deoxynucleotidyl transferase (TdT) enzyme at 37°C for 1 hour in a humidity chamber. The slides were washed with stop/wash buffer for 15 sec and incubated for 10 min. After that, they were washed three times with phosphate buffered saline with tween20 (Fisher Scientific, Markham, Ontario) for 1 min and incubated with the anti-digoxigenin conjugate for 30 min. The signal was revealed using 3-Amino-9-

Ethylcarbazole (AEC) chromogen (Dako) for 15 min and sections were counterstained with Mayer's hematoxylin (Fisher Scientific) and cover slipped.

5.3.3 Histopathology and grading schemes

Microsections of formalin-fixed paraffin embedded (FFPE) MFI tissue stained with hematoxylin and eosin were used for histopathological lesion scoring of endometrial inflammation, distribution and severity of the vasculitis by pathologists (SD, AA, and PN) blinded to PRRSV RNA concentration results (Novakovic 2016a). Endometritis was assessed for the total area of tissue on the slide at 200X magnification and defined as normal (grade 0) if there were very rare inflammatory cells present. For minimal (grade 1), mild (grade 2), moderate (grade 3) and severe (grade 4) the percent of total tissue containing inflammatory cells was <10%, 10-25%, 25-50% and >50%, respectively (Novakovic 2016a). Additionally, assessment of the inflammation within the lamina propria of the endometrium was performed by counting the total number of inflammatory cells present in 9 random grid boxes at 600X field magnification which was equivalent to 0.05 mm^2 area size per each microscopic slide (Novakovic 2016a).

Vasculitis was scored based on the presence of inflammatory cells in the blood vessel wall with concomitant vacuolar degeneration and/or necrosis of the cells in the blood vessel wall layers (intima, media, and adventitia) as previously described (Novakovic 2016a). Briefly, vasculitis severity was scored by randomly selecting three 200X microscopic fields of endometrium per uterine tissue section in which three blood vessels were scored at 400X magnification as: grade 1 = only mild inflammation in wall; grade 2 = moderate inflammation in wall with vacuolation and splitting of smooth muscles or necrosis of wall layers; grade 3 = severe inflammation in wall with degeneration and necrosis in the blood vessel wall layers. Vasculitis distribution was scored using the percent of vessels affected by vasculitis within a

random 200X field as follows: grade 0 = none; grade 1 = <30%; grade 2 = 30-70; and grade 3 = >70% (Novakovic 2016a).

5.3.4 Image Analysis

Quantitative analysis of TUNEL staining was performed using an Image-Pro Plus, version 7 software (Media Cybernetics, Inc., Rockville, MD, USA) as previously described (Novakovic 2016b). Briefly, ten microscopic fields of the endometrium, captured using a 20X microscope objective lens, each representing 1 mm^2 area (10 mm^2 total/slide) were randomly selected from each image of the uterine-placental tissue. Multiple polygonal fields (total area size of 3-4 mm^2) were randomly selected at the MFI (uterine and fetal placenta interdigitation area). Inside those selected fields, total numbers of TUNEL positive cells were manually counted and averaged per 1 mm^2 area size for the statistical analyses.

5.3.5 Statistical Analysis

To determine if numbers of TUNEL positive cells in the endometrium and MFI differed among PRRS viral load groups (negative, low, high), separate two-level, linear mixed-effects regression models were developed controlling for litter of origin (gilt identification) as a random effect with Stata 13 (StataCorp LP, TX, USA). For these models, TUNEL positive cells in the endometrium and MFI were zero-skewness log (lnskew0) transformed to ensure that model assumptions of linearity and homogeneity were not violated. The relationship between numbers of TUNEL positive cells at the MFI (outcome variable) and potential endometrial inflammation and/or vasculitis predictors was also assessed using two-level, linear mixed-effects regression models. Firstly, five non-correlated, biologically plausible potential predictor variables related to the severity and distribution of vasculitis, endometrial inflammation severity, and viral load were confirmed to be unconditionally associated with the number of TUNEL positive cells ($P<0.001$

for all). All five variables were subsequently placed in a full model, and a backward, stepwise elimination performed to a parsimonious final model. In all models, the outcome variable (number of TUNEL positive cells) was natural logarithm transformed to avoid violation of the normality and homogeneity model assumptions. Fisher's exact test was used to compare histological scores of endometrial inflammation, distribution, and severity of vasculitis between PRRSV viral load groups.

Finally, a multilevel mixed-effects negative binomial model was used to determine potential relationships between PRRS viral loads fetal thymus, and the numbers of TUNEL positive (apoptotic) cells at the MFI. Generalized estimating equations (GEE) accounting for gilt of origin (family-binomial, link-logit, correlation-exchangeable) were used to determine if the TUNEL positive cell count at the MFI was related to the odds of a fetus being MEC (versus VIA). Firstly, a model including 200 PRRSV-infected fetuses (26 MEC, 174 VIA) was run. Given the small number of MEC (n=26) relative to VIA (n=174) fetuses in this initial model, a series of smaller, balanced GEE models were developed in which the 26 MEC were matched with 26 randomly selected VIA fetuses. Six balanced GEEs were run, each using a different set of 26 randomly selected VIA fetuses; 156 fetuses in total or 90% of the VIA population. The coefficients, SE, P values were compared across all models to ensure consistency and are reported below.

5.4 Results

5.4.1 Distribution of TUNEL staining in the maternal-fetal interface

TUNEL positive (apoptotic) cells were variably distributed throughout the MFI, but primarily in the endometrial-placental junctional areas. TUNEL positive cells at the maternal-fetal interface ranged from focal areas involving single cell apoptosis of trophoblast on the fetal

side of placenta, and uterine epithelium on the maternal side (Figure 5.1A) to multifocal areas of multiple cell apoptosis that were clearly associated with microseparations (Figure 5.1B). In rare instances, more severe separation of the placenta from the uterine epithelium was evident. Single cell apoptosis was also randomly distributed in the endometrium, affecting mostly mild to moderate numbers of inflammatory cells; predominantly lymphocytes. Occasionally individual cells of the uterine glandular epithelium were affected as well.

Analyzed in the subset of 120 out of 250 samples of uterine tissue with fetal placenta, numbers of TUNEL positive (apoptotic) cells in the endometrium and fetal placenta were both positively associated with PRRS viral load groups ($P<0.001$, $P<0.05$, respectively). The number of TUNEL positive (apoptotic) cells was greater in the high viral load compared to low viral load group, in both the endometrium and fetal placenta. While also detected in the MFI of negative control gilts, cell counts were markedly lower in both endometrium and fetal placenta (Figure 5.2) compared to infected gilts.

5.4.2 Relationship of apoptotic cell counts at MFI to severity of microscopic lesions

The most frequent and consistent microscopic finding was lymphohistiocytic endometritis which was characterized by the presence of large numbers of lymphocytes mixed with moderate numbers of histiocytes and lesser numbers of plasma cells infiltrating lamina propria, uterine glands and uterine interdigitation areas (Figure 5.3). In the most severely inflamed areas, uterine glands lesions were characterized by attenuation and marked loss of the columnar glandular epithelium (necrosis), and the presence of infiltrates of large numbers of vacuolated macrophages in their lumina (histiocytic adenitis) (Figure 5.4). Vasculitis in the endometrium was multifocal to occasionally diffuse, primarily lymphocytic and to a lesser degree lymphohistiocytic affecting typically small to medium-sized blood vessels (Figure 5.5).

Both arteries and veins were affected without apparent predilection (arteritis and phlebitis). Occasionally vasculitis was observed in basal arteries that supply basal portion of the endometrium, which consisted of marked lymphocytic infiltration of the tunica intima (endarteritis) (Figure 5.6). Medium arteries were also affected by variable degrees of lymphohistiocytic arteritis accompanied by edema in the tunica intima and cytoplasmic vacuolation (degeneration), nuclear pyknosis and karyorrhexis of tunica media myocytes (necrosis). The presence of a severe lymphohistiocytic inflammation was also a frequent finding in the stroma of the maternal-fetal interdigitation areas (Figure 5.7). These same areas also exhibited strong TUNEL positive staining affecting both uterine epithelial cells and trophectoderm cells of the chorioallantois (Figure 5.8).

Among the 200 PRRSV-infected uterine tissue sections with fully attached fetal placenta examined microscopically, there were no significant differences in endometrial inflammation scores between low and high PRRS virus load groups (Figure 5.9). Grade 3 endometrial inflammation was the most prevalent histological severity score; present in 76% (152/200) of PRRSV-infected samples. The vasculitis severity and vasculitis distribution scores did not differ between low and high PRRS viral load groups (Figures 5.10 and 5.11). Grade 1 vasculitis distribution (66.5%, 133/200) and vasculitis severity (62%, 124/200) were the most prevalent scores among PRRSV-infected samples.

The variables unconditionally associated with the number of TUNEL positive cells at the MFI included: vasculitis distribution and severity score, endometrial inflammation scores, the total number of inflammatory cells in the endometrium, and viral load group. Of these, only distribution and severity of vasculitis, and PRRS viral load at the MFI were confirmed to be significantly associated with the number of TUNEL positive cells. Distribution of vasculitis

(coef. 0.27 ±0.1, 95% CI: 0.075, 0.46; P=0.007) and vasculitis severity score (coef. 0.29 ±0.08, 95% CI: 0.13, 0.45; P<0.001) were both positively related to numbers of TUNEL positive cells at the MFI. Also, the numbers of TUNEL positive cells at the MFI were significantly higher in the low PRRSV load group than in the negative group (coef. 0.885 ±0.16, 95% CI: 0.57, 1.20; P<0.001), and in the high PRRS viral load group compared to the low PRRS viral load group (coef. 0.42 ±0.09, 95% CI: 0.25, 0.6; P<0.001).

5.4.3 Relationship of apoptotic cell counts at MFI to PRRSV load in the fetal thymus and fetal preservation status

In order to understand the association between the severity of apoptosis in fetal placenta and viral load in the MFI and fetal thymus, we have analyzed the subset of 120 out of 250 samples of uterine tissue with fetal placenta. A two-level negative binomial model was developed and after a backwards-stepwise elimination, only viral load in the MFI (not in fetal thymus) was significantly associated with numbers of TUNEL positive (apoptotic) cells in fetal placenta (P<0.001). The number of TUNEL positive (apoptotic) cells in the fetal placenta increased 0.24 ±0.03 (95% CI: 0.18, 0.29) for each 1 $\log_{10}$ increase in PRRSV RNA concentration (copies/mg) in MFI. These results suggest a direct link between viral load at the MFI and apoptosis, which is independent from the degree of viral replication in the fetus.

For the relationship between fetal preservation status and TUNEL positive cells count at the MFI, all GEE models result were consistent in confirming that an increase in TUNEL positive cell count at the MFI significantly increased the odds (1.06 to 1.15, depending on the model) of a fetus being meconium stained compared to being viable (Table 5.1).

5.5 Discussion

A better understanding of the pathological processes at the MFI during type 2 PRRSV infection is needed to understand the pathogenesis of PRRSV-induced fetal death. In the related study we have confirmed that type 2 PRRSV infection causes significant microscopic lesions in the uterus and fetal placenta (Novakovic 2016a). The goal of this study was to evaluate the associations between the numbers of apoptotic cells in the endometrium and fetal placenta, and PRRSV viral load in the MFI and fetal thymus, microscopic lesions and fetal preservation status.

The TUNEL analyses undertaken in the present study confirmed a significant increase in numbers of apoptotic cells in uterine tissue and fetal placenta and their positive association with PRRS viral load in the MFI. Although the apoptosis was very prominent feature in the MFI, it appears that it affects morphologically different cell types. In the *lamina propria* of the endometrium, the majority of the cells undergoing apoptosis were inflammatory cells; mainly lymphocytes. Since lymphocytes have not been reported as PRRSV susceptible cells, and in this study few PRRSV antigen-positive cells were present in the *lamina propria*, it is possible that significant apoptosis of individual lymphocytes may be the indirect effect of PRRSV replication in the susceptible cells (Sirinarumitr 1998) causing secretion of pro-apoptotic cytokines, e.g. TNFα secreted by macrophages (Elmore 2007).

On the other hand, the largest numbers of apoptotic cells at maternal-fetal junction comprised of fetal trophoblastic epithelial cells followed by uterine epithelial cells. This finding, consistently present throughout all MFI samples obtained from PRRSV infected gilts, and rare in negative control gilts, was in agreement with previously published results (Karniychuk 2011). In addition, apoptosis was frequently observed closely associated with areas of detachment of the

chorioallantois from uterine epithelium, and could potentially be indicative of the mechanism of placental separation.

Analysis of PRRSV infection in the statistical model confirmed that only PRRS viral load in the MFI, and not in the fetus, was significantly associated with presence of apoptosis in the fetal chorioallantois. This information is important for the understanding of the pathogenesis of PRRSV induced fetal death, because it suggests that PRRSV present in the MFI has influence on the occurrence of cell death in the fetal placenta. Hence, increased apoptosis in the fetal placenta could potentially compromise the maternal-fetal barrier causing subsequent detachment of the fetal placenta from the uterus, and along with other factors such as PRRSV infection of the fetus (Ladinig 2015a) and fetal lesions and umbilical lesions (Novakovic 2016a) contribute to fetal compromise.

Although PRRS virus can induce cell death in PRRSV-permissive and bystander cells by apoptosis (Miller and Fox 2004), it is not clear whether this is the only pathogenic mechanism associated with severe detachment of chorioallantois from the uterus during type 2 PRRSV infection. Therefore, the additional hypothesis being tested in this study was that MFI apoptosis was also mediated through endometritis, metritis, and vasculitis underlying the MFI, leading to vascular dysfunction and consequently fetal placental damage. Also examined were the associations between type 2 PRRSV-induced uterine lesions and viral load, and the occurrence of cell death at the MFI of the pregnant gilts, as well as the relationship between fetal preservation status and cell death at the MFI.

We found significant lesions of inflammation in the endometrium at 21 DPI. Endometrial lesions consisted of lymphohistiocytic inflammation in the lamina propria, necrosis of uterine glandular epithelium with lymphocytic and lymphohistiocytic adenitis, and variable degrees of

inflammation affecting the maternal-fetal chorionic interdigitation areas. Based on the nature of inflammatory cell infiltrate in the endometrium, we can conclude that innate immune system plays important role in the defense against PRRSV infection at MFI at 21 DPI. Local innate immunity at the MFI is essential for the development and immunoprotection of human fetus (Kemp 2014). A significant release of pro-inflammatory cytokines associated with the cellular innate immune response is characterized by upregulation of the IL-1β, tumor necrosis factor alpha (TNFα) and downregulation of placental interleukin 10 (IL-10). TNFα is also pro-apoptotic cytokine and IL10 has been proven to be involved in pregnancy-associated disorders (Murphy 2009). Although in the present study endometrium exhibited a robust inflammatory response, no association with numbers of apoptotic cell at the MFI has been found during type 2 PRRSV infection of gilts in the third trimester of gestation. It is clear, however, that in future work, cytokine expression within the endometrium needs to be measured to examine this innate response.

Vascular lesions were characterized by lymphocytic and lymphohistiocytic arteritis and phlebitis, affecting primarily microcirculation, and occasionally medium caliber arteries with inflammation of tunica intima (endarteritis). Intimal arterial inflammation with evidence of reactive and degenerated endothelial cells is strong indication of endothelial damage (Maxie and Robinson 2007). This finding is suggestive of potential endothelial cell involvement in the virus transmission from the blood stream into the stromal tissue during PRRSV viremia. Previous studies reported that PRRSV antigen can be occasionally detected in the endothelial cell (Halbur 1995), although the exact mechanism of PRRSV entry in this cell type is still not explained. However, recent study confirmed that porcine endometrial endothelial (PEE) cells derived from the primary endothelial cells originating from the uterine microcirculation are susceptible to

PRRSV infection *in vitro* (Feng 2013). PEE cells are sialoadhesin (CD169) and CD151 positive and CD163 negative, suggesting that these two receptors may act as possible alternatives for PRRSV attachment in some cells (Provost 2012). In this study, we found that some endothelial cell of the inflamed arteries have strong TUNEL positivity, but detection of PRRSV-specific SDOW17 antigen in these same sites could not be confirmed by immunohistochemistry in our related study (Novakovic 2016b). Inflammation of medium caliber arteries was also occasionally characterized by vacuolation of the cytoplasm (degeneration), pyknosis, and karyorrhexis of the nuclei of the smooth myocytes (necrosis) of the tunica media. Rarely tunica adventitia was affected by inflammation accompanied with moderate perivascular edema. Evidence of fibrinoid vascular necrosis, perivascular hemorrhage or thrombi were not observed in this study.

Microcirculation of the uterus and fetal placenta demonstrated the most severe lesions of inflammation with frequent complete disruption of the vessel wall. According to our histological grading results, higher scores (grade 3 and 4) of inflammation affecting lamina propria, uterine glands and interdigitation areas of endometrium were more frequent compared to lower scores (grades 1 and 2) in type 2 PRRSV infected samples. However, no difference was found in the endometrial inflammation scores across PRRS viral load groups. Distribution and severity of vasculitis showed the predominance of lower scores in both PRRSV load groups without significant difference. These findings were in accordance with previous similar observations (Lager and Halbur 1996; Rossow 1998; Zimmerman 2012), and our previous observations (Novakovic 2016a) which confirmed the importance of endometrial inflammation as specific, reproducible lesion in the reproductive form of type 2 PRRSV infection at 21 DPI. However, when the histological grading scores were offered in the statistical model used to predict cell death at the MFI, only PRRSV load, distribution and severity of vasculitis, but not the severity of

endometrial inflammation, were positively related to the numbers of apoptotic cells. These results indicate a potential role of PRRS virus-induced vasculitis in the pathogenesis of the cell death at MFI rather than endometrial inflammation. Furthermore, this suggests that hematotroph, which plays an essential role in the development and growth of the porcine fetus, may be compromised. This may be one reason why fetal death appears to disproportionately affect large fetuses rather than small fetuses and why the body weight of surviving fetuses is decreased following transplacental PRRSV infection (Ladinig 2014a). Vascular dysfunctions causing hypoperfusion and ischemia have already been implicated in human pregnancies complicated by preeclampsia, placental abruption, and fetal growth restriction (Kelly 2009). Whether or not PRRS virus-induced vascular inflammation affecting primarily microcirculation could impair blood perfusion to such degree to lead to severe metabolic derangements of uterine and trophoblastic epithelial cells of the porcine epitheliochorial placenta will require further investigation in the future. Considering the results obtained in the present study, we think that, in addition to the PRRSV mediated apoptosis at the MFI, PRRSV induced vascular lesions may very well act as a supplementary pathogenic mechanism which exacerbates the deleterious effects on maternal-fetal barrier.

Finally, this study confirmed a significant, positive relationship between the degree of cell death (apoptosis) at the MFI and meconium staining, an indicator of intrauterine stress and early pathologic stage of acute fetal PRRSV infection (Ladinig 2014a). More specifically, the odds of a fetus being meconium stained increased as did the numbers of apoptotic cells at the MFI. This result reveal that in addition to the PRRSV infection of the fetus (Ladinig 2015a) and fetal and umbilical lesions (Novakovic 2016a), apoptosis of the uterine epithelial cells and

trophoblastic cells at the MFI also contribute to fetal demise during in utero type 2 PRRSV infection.

5.6 Conclusions

In this experimental study of type 2 PRRSV infection of third trimester pregnant gilts, we confirmed a significant increase in the numbers of apoptotic cells at the MFI, significant, positive association between the apoptosis at the MFI and the severity and distribution of PRRSV-induced vasculitis in the endometrium. We also determined that an increase in the numbers of apoptotic cells at the MFI is significantly associated with odds of fetal meconium staining. This research helps to advance the understanding of the pathogenesis of fetal demise in type 2 reproductive PRRS.

5.7 Acknowledgments

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Table 5.1 Results of Generalized estimating equations (GEE) confirming the association between numbers of TUNEL positive cells at MFI and the odds of a fetus being MEC (versus VIA).

GEE model*	No.[a]	Coefficient	Odds Ratio	SE	*P* values[†]	95% CI
Final[‡]	200	0.08	1.08	0.015	<0.001	0.051, 0.109
1	52	0.074	1.08	0.021	<0.001	0.033, 0.115
2	52	0.136	1.15	0.04	<0.001	0.058, 0.214
3	52	0.093	1.10	0.026	<0.001	0.042, 0.143
4	52	0.066	1.07	0.021	0.001	0.256, 0.106
5	52	0.062	1.06	0.02	0.002	0.023, 0.101
6	52	0.101	1.11	0.029	0.001	0.044, 0.159
AVG of 6 balanced GEE models		0.089	1.09			

[*] A model with 200 fetuses was initially run followed by six balanced GEE models including 26 MEC fetuses and a different set of 26 randomly selected VIA fetuses.

[†] Statistical significance was assigned at the $P < 0.05$ level.

[‡] Initial GEE model with 200 PRRSV-infected fetuses (26 MEC, 174 VIA).

[a] Total number of fetuses analyzed in the model

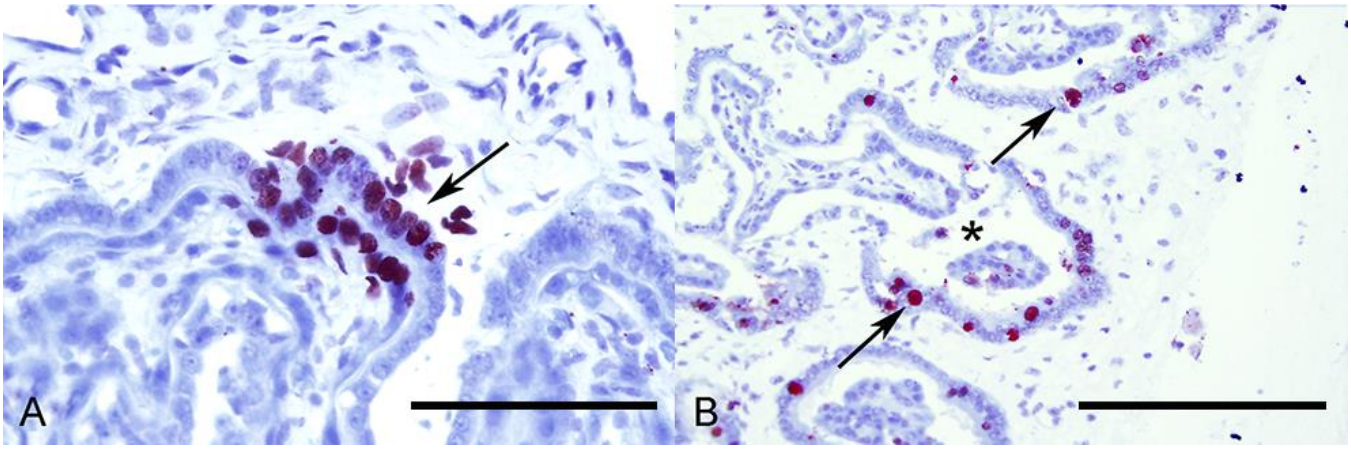


Figure 5.1A-B. TUNEL assay in the uterine-fetal placental tissues from PRRSV-infected pregnant gilts.

A Focal area of apoptosis (arrow) affecting both the trophoblast and uterine epithelial cells at the uterus-fetal placenta interface from PRRSV-infected pregnant gilt. TUNEL, bar = 100 μm. **B** Multiple apoptotic cells (arrows) closely associated with the area of microseparation between chorioallantois and uterus (asterisk) from. TUNEL, bar = 200 μm.

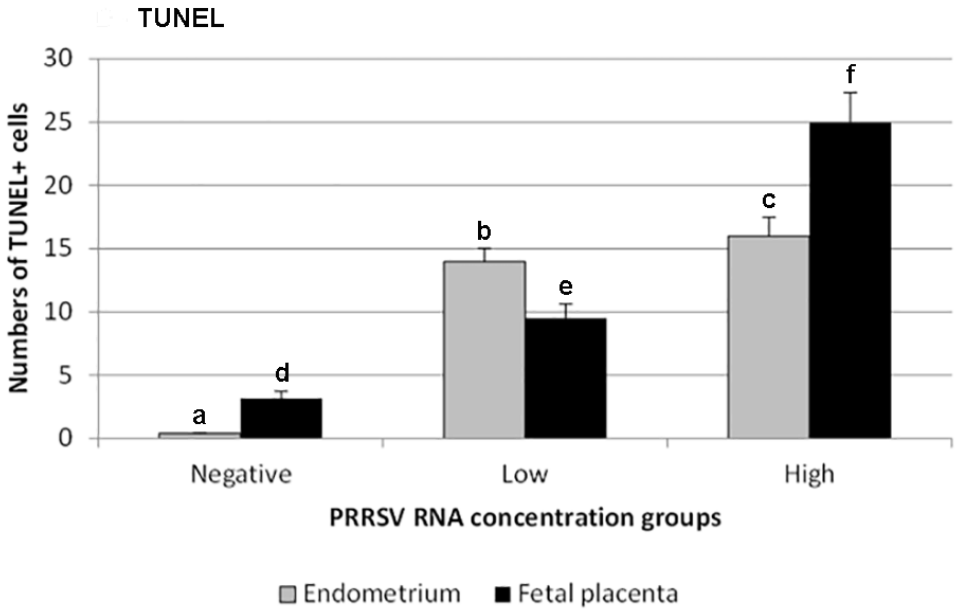
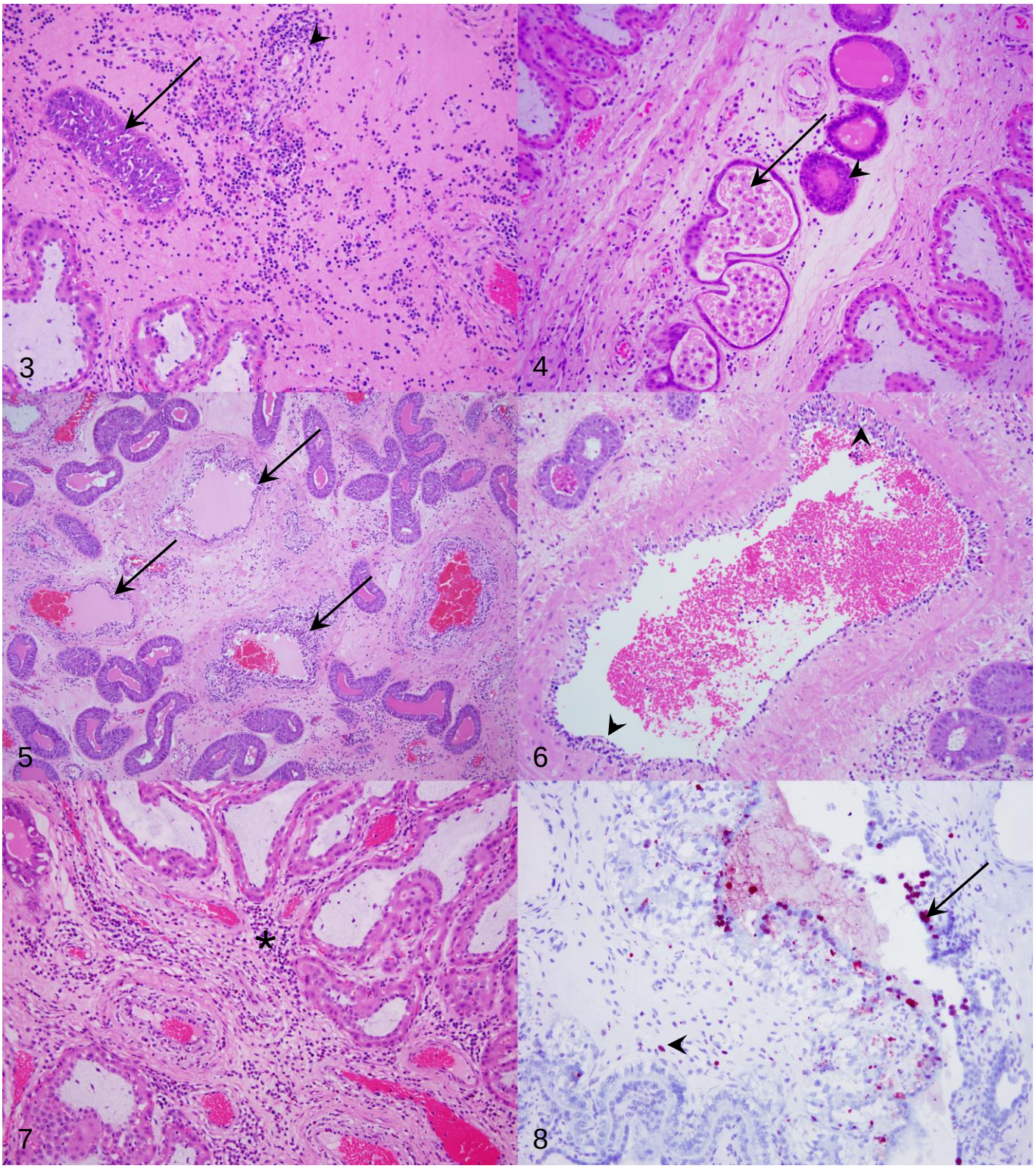


Figure 5.2. Mean numbers of TUNEL (apoptotic) positive cells per 1 mm^2 of the endometrium and fetal placenta.

Superscript letters ($^{a, b, c}$ or $^{d, e, f}$) indicate significant differences ($P < 0.05$) between PRRSV viral load groups. Error bars represent standard error.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Figures 5.3-5.8. Microscopic lesions and apoptosis at MFI during type 2 PRRSV-infection, pregnant gilt, uterus**.**

Figure 5.3. Inflammatory cell infiltrates in the lamina propria (stroma) of the endometrium, surrounding capillaries (arrowhead) and infiltrating uterine glands (arrow). Hematoxylin and eosin (HE). **Figure 5.4.** Uterine glands in the lamina propria have lost glandular epithelium (necrosis) and are dilated by infiltrates of inflammatory cells (arrow); normal uterine glands (arrowhead). HE. **Figure 5.5.** Multiple arteries in the lamina propria affected by lymphohistiocytic vasculitis (arrows). HE. **Figure 5.6.** Basal endometrial artery with clusters of lymphocytes infiltrating tunica intima only (endarteritis) (arrowheads). HE. **Figure 5.7.** Large numbers of inflammatory cells are infiltrating stroma, arterioles and capillaries of uterine-fetal placenta interdigitations (asterisk). HE. **Figure 5.8.** Apoptotic inflammatory cells (arrowhead) closely associated with uterine luminal epithelial and trophoblastic cells undergoing apoptosis in the area of severe separation of the chorioallantois from the uterus (arrow). TUNEL assay.

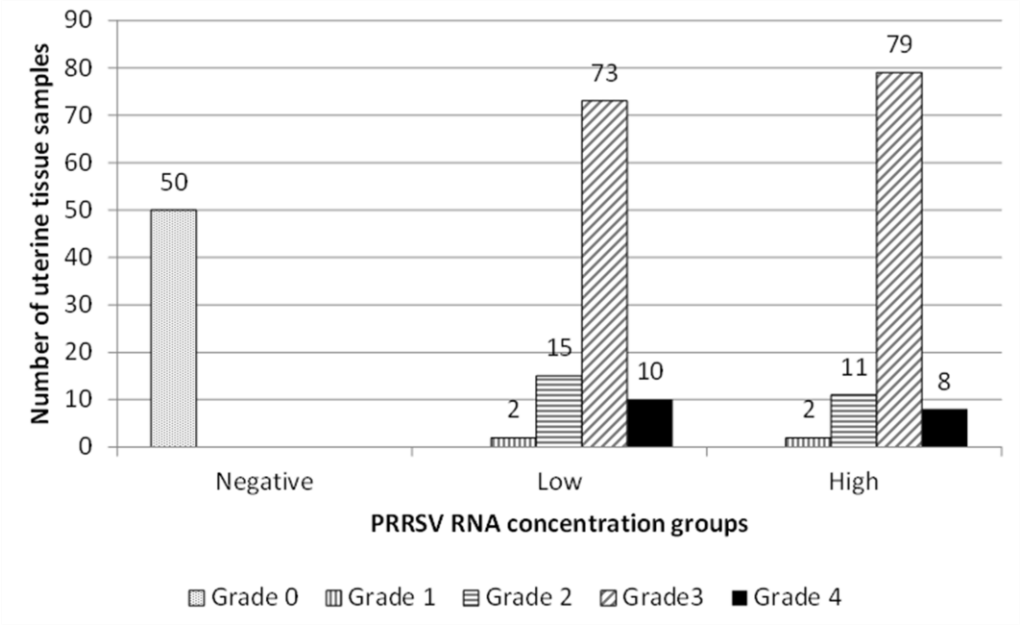

Figure 5.9. Histological grades for endometrial inflammation for each PRRS viral load group.

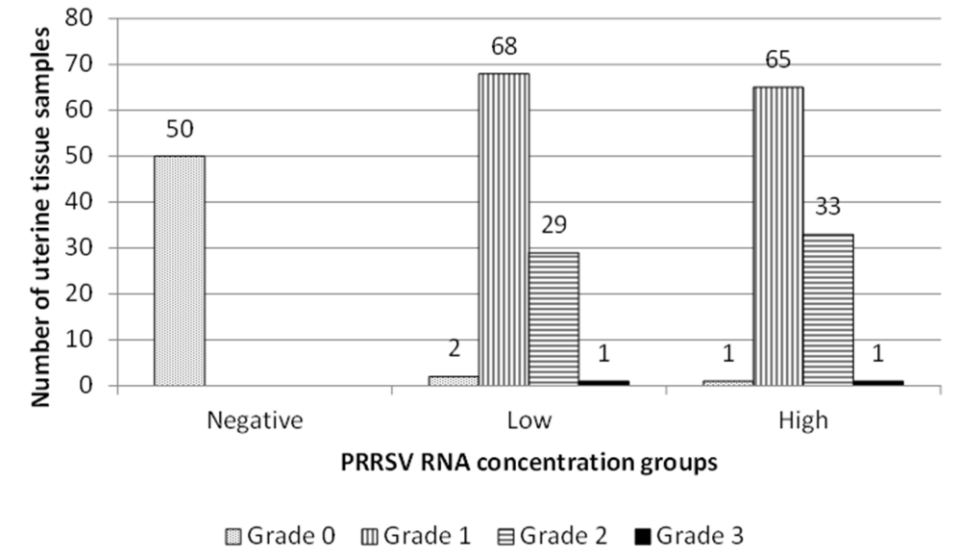

Figure 5.10. Histological grades for vasculitis distribution for each PRRS viral load group.

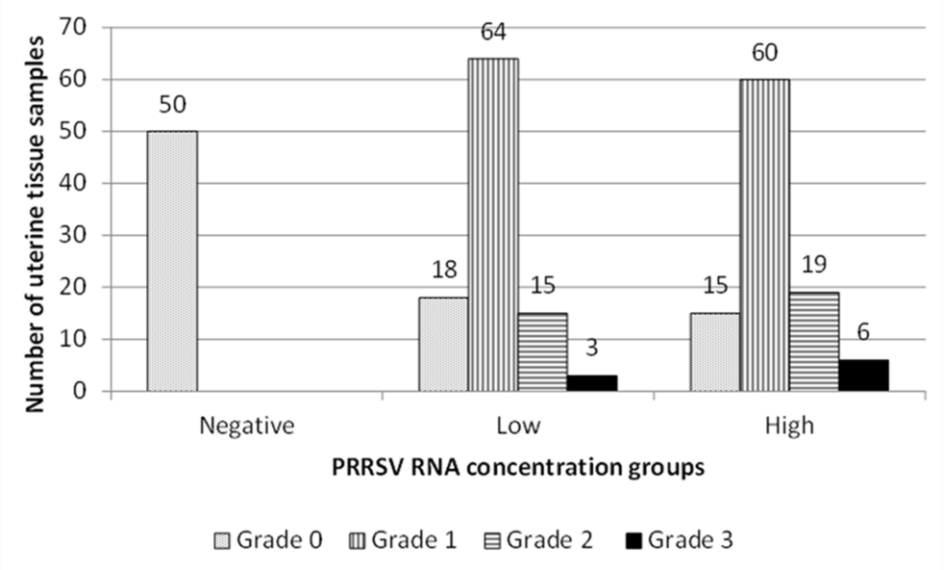

Figure 5.11. Histological grades for vasculitis severity for each PRRS viral load group.

6 General discussion

The overall aims of the studies presented in this thesis were to improve understanding of the pathogenesis of PRRSV fetal infection and death during the type 2 PRRSV infection in the third trimester of pregnant gilts. Therefore, examination and assessment was needed on significant pathological lesions, potential relationships between histological lesions, numbers of CD163, CD169 positive PRRSV susceptible cells, areolae, PRRSV-induced apoptosis at the MFI, and PRRSV RNA concentration in the fetuses and MFI.

The tissues assessed were from a large, multicenter PRRS study with the highest number of pregnant gilts ever used in a single trial, providing an unprecedented opportunity to examine large numbers of PRRSV-infected fetuses and corresponding uterus with the fetal placenta. Results generated by the pathological assessment of tissues from 1683 fetuses and adjacent uterine tissue with the fetal placenta in Chapter 3, demonstrated that the most consistent and prominent lesions were observed at the MFI including endometrium, vasculature, and fetal chorioallantois while fetal histopathological lesions were less frequent and more variable in severity and presentation.

These results showed to be in agreement with previous studies (Lager and Mengeling 1995; Rossow 1996b; Rossow 1998). The unique contribution of this study to other pathological studies of the reproductive form of type 2 PRRSV infection was the development of the specific histological grading scheme which allowed semi-quantitative assessment of the severity and distribution of microscopic lesions and subsequent analyses of their relationship with concentrations of the PRRSV in the uterine and fetal tissues. Additionally, exceptionally large numbers of tissue samples obtained from this experiment provided substantial statistical power to determine complex associations between different factors of infection. Results of the histological

grading revealed that 73% of uterine tissue samples exhibited moderate and 10% of samples severe lymphohistiocytic endometritis while one-third of the samples had severe vasculitis in more than 30% of blood vessels in the endometrium.

These findings confirmed that inflammation in the uterus, fetal placenta, and endometrial blood vessels are major pathological characteristics of type 2 PRRSV infection at late gestation in pregnant gilts. Nonetheless, a "significant relationship between the concentration of PRRSV RNA in the maternal-fetal interface and the severity of endometrial inflammation or the distribution and severity of endometrial vasculitis" could not be confirmed at 21 days post inoculation (Novakovic, 2016a). These findings indicate that the inflammatory process at the maternal-fetal interface 21 DPI was most likely passed its peak and it was no longer significantly associated with PRRSV load in the MFI. This was also partially supported by the finding of a significant negative relationship between endometrial inflammation and PRRSV RNA concentration in fetal thymus, which may indicate that the duration of PRRSV infection in the porcine fetus is longer than in the uterus while the severity of endometrial inflammation starts to decline at 21 DPI. Therefore, the time of collection of MFI samples at 21 DPI represents one of the biggest limitations of this experiment. While this was done to enable the collection of other phenotypic data from dams and fetuses (Ladinig 2014a; Ladinig 2014b; Ladinig 2014c; Ladinig 2014d; Ladinig 2015a), it did limit the number of MFI samples evaluated since many dead and autolyzed samples were excluded.

The novel finding in this study was the "significant relationship between the distribution and the severity of the vasculitis in the lamina propria, and the severity of endometrial inflammation" (Novakovic, 2016a) which suggests that endometrial blood vessel inflammation determines the severity of inflammation in the uterus and plays an essential role in the

transmission of PRRSV to the maternal-fetal interface and further to the fetus. Another novel finding was the positive relationship between the concentration of PRRSV at the maternal-fetal interface and the fetal thymus, and fetal lesions indicating that the concentration of PRRSV in the uterus, fetal placenta, and fetus are all related to the occurrence of fetal pathology. Although this finding is in contradiction with some more recent studies (Karniychuk 2011; Karniychuk and Nauwynck 2013), it suggests that fetal lesions are a consequence of a complex set of interactions of virus in fetal and maternal compartments, rather than the result of independent pathological processes at one or the other site. Furthermore, in this study, results confirmed that fetal preservation status is strongly associated with the presence of fetal and umbilical cord lesions indicating their important role in the pathogenesis of fetal death in type 2 *in utero* PRRSV infection.

In the second study (presented in chapter 4), it was confirmed that type 2 PRRSV infection significantly increases numbers of CD163+ and CD169+ macrophages in the endometrium and the fetal placenta in PRRSV-infected versus non-infected pregnant gilts. In particular, the numbers of CD163 and CD169 positive cells in the endometrium were consistently different between PRRSV-infected and negative control pregnant gilts suggesting their role in the innate and cell-mediated immune response to PRRSV infection. The most interesting finding was the discovery of relatively constant numbers of CD163 positive cells in the fetal placenta closely associated with the maternal-fetal interface which was demonstrated by a lack of significant difference in numbers of CD163 positive cells in fetal placenta between negative control and PRRS-infected pregnant gilts. It is possible that this stable population of CD163 positive cells represents fetal tissue-specific macrophages, porcine analogs of human

Hofbauer cells, which are involved in the prevention of the vertical transmission of the pathogens.

Although the numbers of CD169 positive cells were increased both in the endometrium and fetal placenta during the PRRSV infection, only numbers of CD163 positive macrophages in the endometrium and fetal placenta were significantly associated with the PRRSV infection of the fetus. Furthermore, the relationship between viral load in fetal thymus and the ratio of CD163+ cells present in endometrium and fetal placenta, revealed a positive association. In other words, if this ratio is high, such as in situations of increased infection pressure in the uterus and low placental immune surveillance in the fetal placenta, the odds of fetal thymus being PRRSV virus positive increased dramatically, and *vice versa*.

This was an exciting novel finding which suggested a potential, but so far unknown, mechanism of PRRSV resistance in late gestation fetuses and warrants special attention to CD163 positive cells in the fetal placenta in the future studies. Particularly, in the view of the most recent discoveries which have confirmed that knockout pigs (CD163–/–) are resistant to PRRSV challenge, this new piece of information about the importance of residential population of fetal placental macrophages in protection against the transplacental infections has to be carefully considered in planning future genetic selection programs.

The third study (presented in chapter 5) revealed that type 2 PRRSV infection increases significantly the numbers of cells undergoing apoptosis both in the uterine tissue and fetal placenta. However, the type of cells undergoing apoptosis in the endometrium and fetal placenta are quite different morphologically. In the *lamina propria* of the endometrium, the majority of apoptotic cells are inflammatory cells, particularly lymphocytes which may represent the indirect cytopathic effect of PRRSV replication in the susceptible cells, and would be less likely to have

a significant effect on the fetal pathology. However, the significant apoptosis in the MFI affecting the fetal trophoblastic epithelial cells and uterine epithelial cells was a common finding in the PRRSV-infected pregnant gilts and rare in negative control gilts. This degree of apoptosis could potentially compromise the maternal-fetal barrier and have detrimental effects on the fetus.

Additional proof was found in the frequent presence of apoptotic cells in the areas of detachment of the chorioallantois from the uterus, and the significant statistical association between the numbers of apoptotic cells at the MFI and fetal meconium staining. Results from the second study also confirmed that only presence of the PRRSV virus in the uterus and fetal placenta, not in fetal tissues, are significantly positively associated with the extent of apoptosis in the fetal chorioallantois. This suggests the involvement of PRRSV in causing cell death and proposes that cell death at the maternal-fetal interface is a contributing factor in the pathogenesis of fetal death. Figures 6.1 and 6.2 schematically summarize the overall significant pathological factors and their relationships with PRRS viral load in the maternal-fetal interface and fetal thymus confirmed in our first, second and third study.

Finally, the third study revealed that the presence of PRRSV in the uterus and fetal placenta, along with distribution and severity of vasculitis, are positively associated with the numbers of apoptotic cells at the MFI, but not the severity of inflammation in the lamina propria, uterine glands, and interdigitating areas. These results indicate the greater importance of the PRRSV-induced vasculitis than inflammation of the lamina propria, uterine glands and maternal-fetal interdigitation areas in the pathogenesis of the cell death during the type 2 PRSSV infection *in utero*. Therefore, cell death at the MFI could be a result of the combined effects of directly induced and cytokine-mediated PRRSV infection, and vascular lesions causing hypoperfusion and hypoxia. Also, the extent of cell death at the MFI significantly increases the odds of fetal

meconium staining of the fetuses. Figure 6.3 schematically summarizes all the predictors of the odds of fetal meconium staining as indicator of fetal demise confirmed in our first and third study.

In conclusion, this large-scale, multicenter, experiment with pregnant gilts challenged with type 2 PRRSV at third trimester of gestation enabled an unprecedented and extensive evaluation of fetal and uterine lesions at 21 DPI and revealed multiple factors important in the pathogenesis of fetal death. Studies presented in this thesis confirm type 2 PRRSV infection in pregnant gilts in the third trimester of gestation induces significant pathological lesions at the maternal-fetal interface, and that fetal and umbilical pathology together with PRRSV viral load in the fetus and increased cell death at the MFI contribute to fetal compromise and death. In terms of the other important factors of *in utero* PRRSV infection, this study found that only an increase in numbers of CD163 positive macrophages in the endometrium in concert with the decrease in the numbers of CD163 positive macrophages in the fetal placenta significantly increased the probability of PRRSV infection of the fetus.

6.1 Limitations and future directions

Future research work in this area has the potential to evolve into several different directions. One of the biggest limitations of our first study was primarily associated with the time of the sampling. The decision to terminate the study at 21 days post inoculation has resulted in a smaller number of intact MFI samples due to increased numbers of dead and autolyzed fetuses that could not be investigated. Since the sampling also was past the peak viral replication stage (typically 10-14 DPI), a 21 DPI termination also resulted in missing the acute stages of inflammatory reaction and pathological lesions at the maternal-fetal interface. Future studies of PRRSV infection of pregnant gilts must be focused on overcoming that limitation using multiple

and earlier termination points of the experiment. This approach may prove to be a very useful in increasing the numbers of viable and meconium stained fetuses, subsequently increasing the chances of observing more consistent fetal lesions along with immunohistochemical detection of PRRSV antigen in the fetus and chorioallantois, and potentially finding a greater correlation between pathological lesions and the PRRSV presence at the maternal-fetal interface.

Also, future experimental approaches should be focused on the molecular analyses of the pro-inflammatory and pro-apoptotic cytokine profiles in the endometrium of the uterus separately from the fetal chorioallantois. This research work would give new insight into the upregulation and downregulation of inflammatory and apoptotic pathways associated with early type 2 PRRSV infection at the maternal-fetal interface. Another direction for the future research work in this area must include the analyses of the uteroplacental and fetal hypoxia as a potential consequence of the vascular dysfunction by investigating the hypoxia signaling pathways at the maternal-fetal interface and fetal tissue separately.

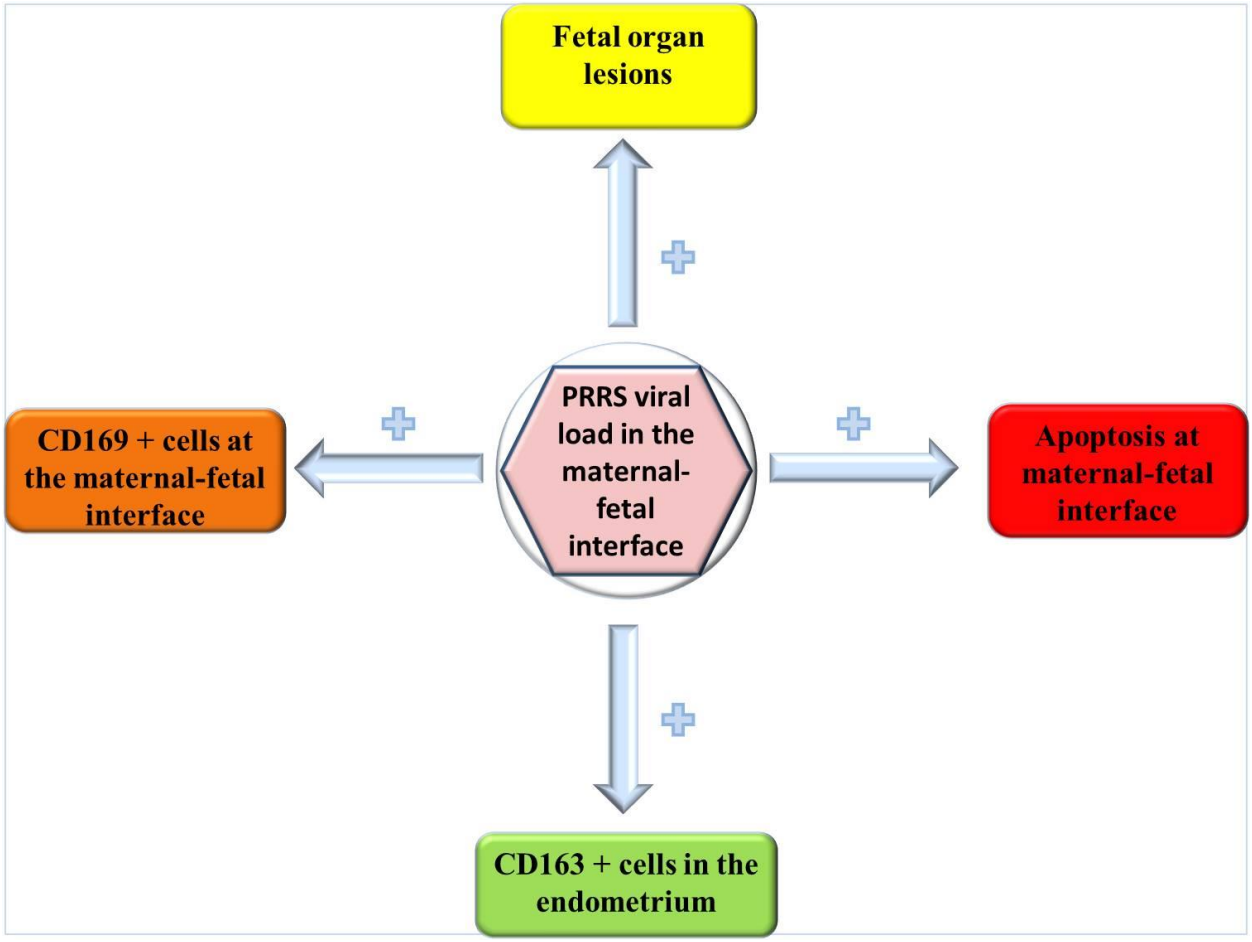

Figure 6.1. Relationship between PRRS viral load in the maternal-fetal interface and various significant variables of PRRSV infection.

Direction of the arrows indicates predictor/outcome relationship, and plus sign is positive association.

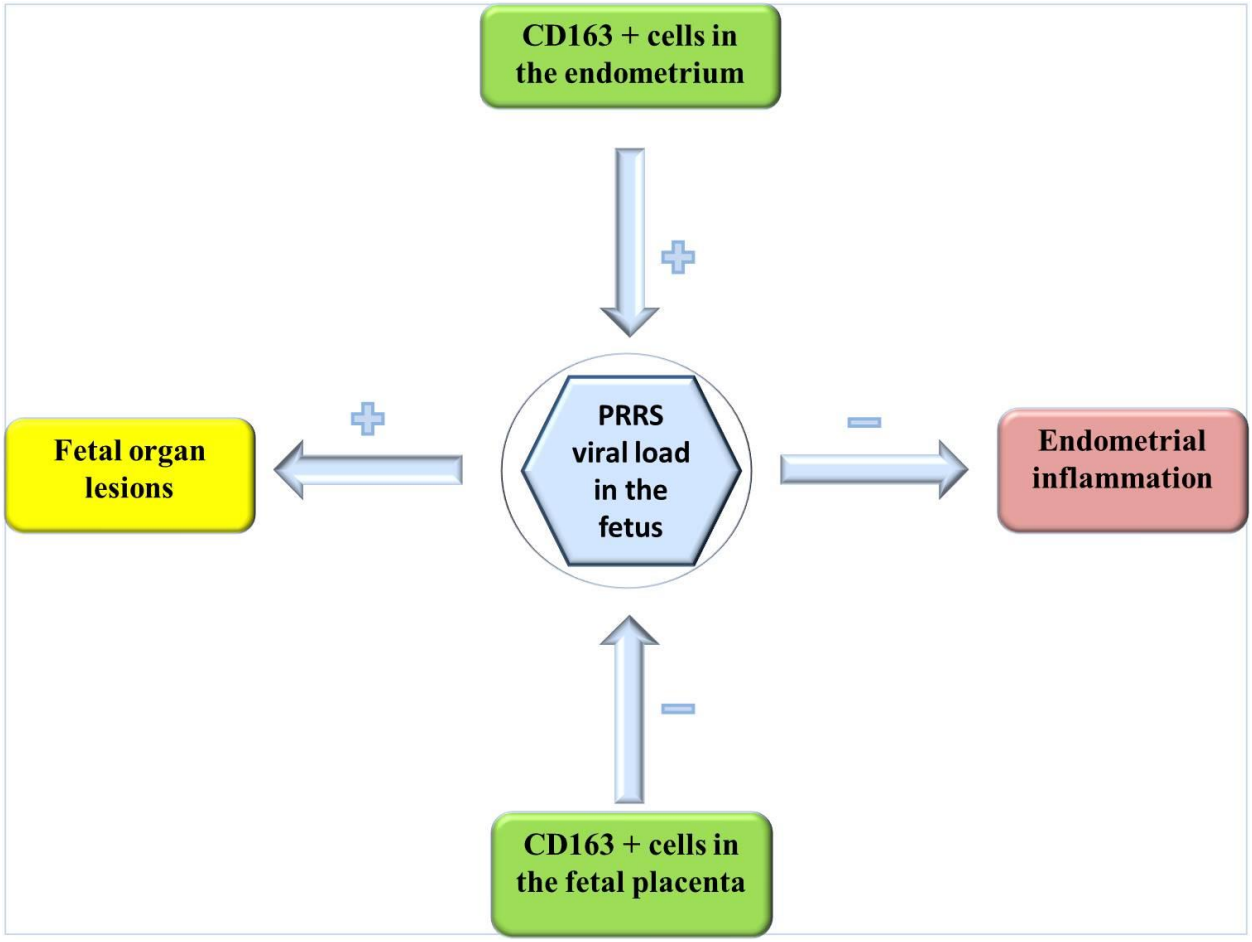

Figure 6.2. Relationship between PRRS viral load in the fetal thymus and various significant variables of PRRSV infection.

Direction of the arrow indicates predictor/outcome relationship, and plus/minus sign represent positive/negative association between variables.

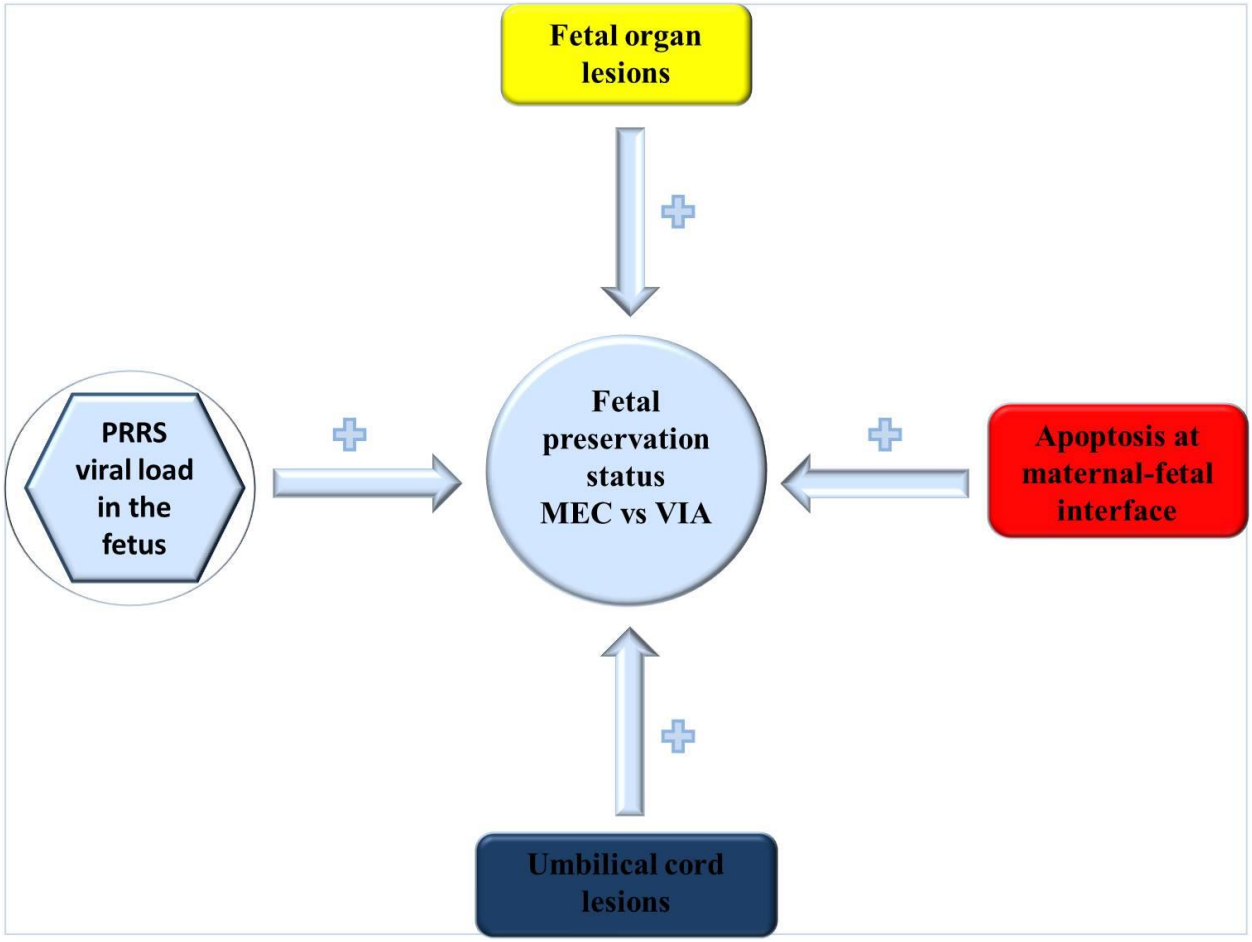

Figure 6.3. Relationship between fetal preservation status (meconium *versus* viable) and various predictor variables of PRRSV infection.

MEC - meconium-stained fetuses with skin covered with inspissated, brownish amniotic fluid and regular umbilical cord with edema. VIA - viable fetuses with normal, white to purple skin with visible hair and regular umbilical cord. Direction of the arrow indicates predictor/outcome relationship, and plus/minus sign represent positive/negative association between variables.

7 References

Ait-Ali, T., Wilson, A. D., Westcott, D. G., et al. (2007) Innate immune responses to replication of porcine reproductive and respiratory syndrome virus in isolated swine alveolar macrophages. Viral Immunol 20, 105-118

Al-Dissi, A. N., Haines, D. M., Singh, B., et al. (2007) Immunohistochemical expression of vascular endothelial growth factor and vascular endothelial growth factor receptor associated with tumor cell proliferation in canine cutaneous squamous cell carcinomas and trichoepitheliomas. Vet Pathol 44, 823-830

Albina, E., Piriou, L., Hutet, E., et al. (1998) Immune responses in pigs infected with porcine reproductive and respiratory syndrome virus (PRRSV). Vet Immunol Immunopathol 61, 49-66

Allende, R., Kutish, G. F., Laegreid, W., et al. (2000) Mutations in the genome of porcine reproductive and respiratory syndrome virus responsible for the attenuation phenotype. Arch Virol 145, 1149-1161

APHIS (2009) PRRS seroprevalence on U.S. swine operations. Veterinary Services, Centers for Epidemiology and Animal Health, https://www.aphis.usda.gov/animal_health/nahms/swine/downloads/swine2006/Swine2006_is_PRRS.pdf, accessed on January 2009

Batista, L., Dee, S. A., Rossow, K. D., et al. (2002) Assessing the duration of persistence and shedding of porcine reproductive and respiratory syndrome virus in a large population of breeding-age gilts. Can J Vet Res 66, 196-200

Bazer, F. W. (2013) Pregnancy recognition signaling mechanisms in ruminants and pigs. Journal of Animal Science and Biotechnology 4, 23-23

Bazer, F. W. & Johnson, G. A. (2014) Pig blastocyst–uterine interactions. Differentiation 87, 52-65

Bazer, F. W., Spencer, T. E., Johnson, G. A., et al. (2009) Comparative aspects of implantation. Reproduction 138, 195-209

Belizna, C. C., Hamidou, M. A., Levesque, H., et al. (2009) Infection and vasculitis. Rheumatol

Boddicker, N., Waide, E. H., Rowland, R. R. R., et al. (2012) Evidence for a major QTL associated with host response to porcine reproductive and respiratory syndrome virus challenge. J Anim Sci 90, 1733-1746

Boddicker, N. J., Garrick, D. J., Rowland, R. R. R., et al. (2014) Validation and further characterization of a major quantitative trait locus associated with host response to experimental infection with porcine reproductive and respiratory syndrome virus. Anim Genet 45, 48-58

Brar, M. S., Shi, M., Ge, L., et al. (2011) Porcine reproductive and respiratory syndrome virus in Ontario, Canada 1999 to 2010: genetic diversity and restriction fragment length polymorphisms. J Gen Virol 92, 1391-1397

Brockmeier, S., Halbur, P. & Thacker, E. (2002) Porcine Respiratory Disease Complex. In Polymicrobial Diseases. Eds K. Brogden, J. Guthmiller. Washington (DC), ASM Press. pp 231-258

Bulmer, J. N. & Johnson, P. M. (1984) Macrophage populations in the human placenta and amniochorion. Clin Exp Immunol 57, 393-403

Calvert, J. G., Slade, D. E., Shields, S. L., et al. (2007) CD163 expression confers susceptibility to porcine reproductive and respiratory syndrome viruses. J Virol 81, 7371-7379

Cano, J. P., Dee, S. A., Murtaugh, M. P., et al. (2009) Infection dynamics and clinical manifestations following experimental inoculation of gilts at 90 days of gestation with a low dose of porcine reproductive and respiratory syndrome virus. Can J Vet Res 73, 303–307

Caswell, J. & Williams, K. (2007) Respiratory system In Jubb, Kennedy, and Palmer's Pathology of Domestic Animals. 5 edn. Ed M. Maxie. Philadelphia, PA, Elsevier Saunders. pp 579-581

Chang, H.-W., Jeng, C.-R., Liu, J. J., et al. (2005) Reduction of porcine reproductive and respiratory syndrome virus (PRRSV) infection in swine alveolar macrophages by porcine circovirus 2 (PCV2)-induced interferon-alpha. Vet Microbiol 108, 167-177

Charerntantanakul, W. (2012) Porcine reproductive and respiratory syndrome virus vaccines: Immunogenicity, efficacy and safety aspects. World Journal of Virology 1, 23-30

Cheon, D. S. & Chae, C. (2001) Distribution of porcine reproductive and respiratory syndrome virus in stillborn and liveborn piglets from experimentally infected sows. J Comp Pathol 124, 231-237

Choi, K., Lee, J., Park, C., et al. (2015) Comparison of the pathogenesis of single or dual infections with type 1 and type 2 porcine reproductive and respiratory syndrome virus. J Comp Pathol 152, 317-324

Christianson, W. T., Choi, C. S., Collins, J. E., et al. (1993) Pathogenesis of porcine reproductive and respiratory syndrome virus infection in mid-gestation sows and fetuses. Can J Vet Res 57, 262–268

Christopher-Hennings, J., Nelson, E. A., Nelson, J. K., et al. (1998) Identification of porcine reproductive and respiratory syndrome virus in semen and tissues from vasectomized and nonvasectomized boars. Vet Pathol 35, 260-267

Collins, J. E., Benfield, D. A., Christianson, W. T., et al. (1992) Isolation of Swine Infertility and Respiratory Syndrome Virus (Isolate ATCC VR-2332) in North America and Experimental Reproduction of the Disease in Gnotobiotic Pigs. J Vet Diagn Invest 4, 117-126

Cooper, V. L., Hesse, R. A. & Doster, A. R. (1997) Renal lesions associated with experimental porcine reproductive and respiratory syndrome virus (PRRSV) Infection. J Vet Diagn Invest 9, 198-201

Corzo, C. A., Mondaca, E., Wayne, S., et al. (2010) Control and elimination of porcine reproductive and respiratory syndrome virus. Virus Res 154, 185-192

Costers, S., Lefebvre, D., Delputte, P., et al. (2008) Porcine reproductive and respiratory syndrome virus modulates apoptosis during replication in alveolar macrophages. Arch Virol 153, 1453-1465

Cribier, B., Couilliet, D., Meyer, P., et al. (1999) The severity of histopathological changes of leukocytoclastic vasculitis is not predictive of extracutaneous involvement. Am J Dermatopathol 21, 532

Dal Canto, A. J. & Virgin Iv, H. W. (2000) Animal models of infection-mediated vasculitis: implications for human disease. Int J Cardiol 75, 37-45

Dea, S., Bilodeau, R., Athanassious, R., et al. (1992) Swine reproductive and respiratory syndrome in Québec: Isolation of an enveloped virus serologically-related to Lelystad virus. Can Vet J 33, 801-808

Dea, S., Gagnon, C., Mardassi, H., et al. (2000) Current knowledge on the structural proteins of porcine reproductive and respiratory syndrome (PRRS) virus: comparison of the North American and European isolates. Arch Virol 145, 659-688

Dee, S. (2003) Approaches to prevention, control, and eradication. In The PRRS Compendium. Eds J. Zimmerman, K. J. Yoon. Des Moines, Iowa, National Pork Board. pp 119–130

Dee, S., Batista, L., Deen, J., et al. (2005a) Evaluation of an air-filtration system for preventing aerosol transmission of Porcine reproductive and respiratory syndrome virus. Can J Vet Res 69, 293-298

Dee, S., Collins, J., Halbur, P., et al. (1996) Control of porcine reproductive and respiratory syndrome (PRRS) virus. J Swine Health Prod 4, 95-98

Dee, S., Deen, J., Jacobson, L., et al. (2005b) Laboratory model to evaluate the role of aerosols in the transport of porcine reproductive and respiratory syndrome virus. Vet Rec 156, 501-504

Dee, S., Deen, J., Rossow, K., et al. (2002) Mechanical transmission of porcine reproductive and respiratory syndrome virus throughout a coordinated sequence of events during cold weather. Can J Vet Res 66, 232-239

Dee, S. A., Bierk, M. D., Deen, J., et al. (2001) An evaluation of test and removal for the elimination of porcine reproductive and respiratory syndrome virus from 5 swine farms. Can J Vet Res 65, 22-27

Delputte, P. L., Costers, S. & Nauwynck, H. J. (2005) Analysis of porcine reproductive and respiratory syndrome virus attachment and internalization: distinctive roles for heparan sulphate and sialoadhesin. J Gen Virol 86, 1441-1445

Delputte, P. L. & Nauwynck, H. J. (2004) Porcine arterivirus infection of alveolar macrophages is mediated by sialic acid on the virus. J Virol 78, 8094-8101

Delputte, P. L., Van Gorp, H., Favoreel, H. W., et al. (2011) Porcine sialoadhesin (CD169/Siglec-1) is an endocytic receptor that allows targeted delivery of toxins and antigens to macrophages. PLoS ONE 6, e16827

Delputte, P. L., Vanderheijden, N., Nauwynck, H. J., et al. (2002) Involvement of the Matrix Protein in Attachment of Porcine Reproductive and Respiratory Syndrome Virus to a Heparinlike Receptor on Porcine Alveolar Macrophages. J Virol 76, 4312-4320

Delrue, I., Van Gorp, H., Van Doorsselaere, J., et al. (2010) Susceptible cell lines for the production of porcine reproductive and respiratory syndrome virus by stable transfection of sialoadhesin and CD163. BMC Biotechnology 10, 48-48

Detmer, S. E., Gramer, M. R., Goyal, S. M., et al. (2013) In vitro characterization of influenza A virus attachment in the upper and lower respiratory tracts of pigs. Vet Pathol 50, 648-658

Díaz, I., Darwich, L., Pappaterra, G., et al. (2005) Immune responses of pigs after experimental infection with a European strain of Porcine reproductive and respiratory syndrome virus. J Gen Virol 86, 1943-1951

Dietze, K., Pinto J., Wainwright, S. Hamilton, C. (2011) Porcine reproductive and respiratory syndrome (PRRS): virulence jumps and persistent circulation in Southeast Asia. In Focus on... Food and Agriculture Organization of the United Nations, 8

Doeschl-Wilson, A. B., Kyriazakis, I., Vincent, A., et al. (2009) Clinical and pathological responses of pigs from two genetically diverse commercial lines to porcine reproductive and respiratory syndrome virus infection. J Anim Sci 87, 1638-1647

Done, S. H., Paton, D. J. & White, M. E. C. (1996) Porcine reproductive and respiratory syndrome (PRRS): A review, with emphasis on pathological, virological and diagnostic aspects. Brit Vet J 152, 153-174

Elmore, S. (2007) Apoptosis: A review of programmed cell death. Toxicol Pathol 35, 495-516

Enders, A. C. & Blankenship, T. N. (1999) Comparative placental structure. Adv Drug Deliv Rev 38, 3-15

Etzerodt, A. & Moestrup, S. K. (2013) CD163 and Inflammation: Biological, Diagnostic, and Therapeutic Aspects. Antioxid Redox Signal 18, 2352-2363

Feng, L., Zhang, X., Xia, X., et al. (2013) Generation and characterization of a porcine endometrial endothelial cell line susceptible to porcine reproductive and respiratory syndrome virus. Virus Res 171, 209-215

FitzSimmons, M. & Daniels, C. (2003) Control in large systems. In The PRRS Compendium. Eds J. Zimmerman, K. J. Yoon. Des Moines, Iowa, National Pork Board. pp 137-142

Flores-Mendoza, L., Silva-Campa, E., Reséndiz, M., et al. (2008) Porcine Reproductive and Respiratory Syndrome Virus Infects Mature Porcine Dendritic Cells and Up-Regulates Interleukin-10 Production. Clin Vaccine Immunol 15, 720-725

Fortunato, S. J., Menon, R., Bryant, C., et al. (2000) Programmed cell death (apoptosis) as a possible pathway to metalloproteinase activation and fetal membrane degradation in premature rupture of membranes. Am J Obstet Gynecol 182, 1468-1476

Fraile, L. (2012) Control or eradication? Costs and benefits in the case of PRRSV. Vet Rec 170, 223-224

Friess, A., Sinowatz, F., Skolek-Winnisch, R., et al. (1981) The placenta of the pig. Anatomy and Embryology 163, 43-53

Geldhof, M. F., Vanhee, M., Van Breedam, W., et al. (2012) Comparison of the efficacy of autogenous inactivated Porcine Reproductive and Respiratory Syndrome Virus (PRRSV)

vaccines with that of commercial vaccines against homologous and heterologous challenges. BMC Vet Res 8, 1-16

Gómez-Laguna, J., Salguero, F. J., Fernández de Marco, M., et al. (2013) Type 2 porcine reproductive and respiratory syndrome virus infection mediated apoptosis in B- and T-cell areas in lymphoid organs of experimentally infected pigs. Transbound Emerg Dis 60, 273-278

Gross, W. L., Trabandt, A. & Reinhold-Keller, E. (2000) Diagnosis and evaluation of vasculitis. Rheumatol 39, 245-252

Guillevin, L. & Dörner, T. (2007) Vasculitis: mechanisms involved and clinical manifestations. Arthritis Res Ther 9, S9-S9

Halbur, P. G., Miller, L. D., Paul, P. S., et al. (1995) Immunohistochemical identification of porcine reproductive and respiratory syndrome virus (PRRSV) antigen in the heart and lymphoid system of three-week-old colostrum-deprived pigs. Vet Pathol 32, 200-204

Halbur, P. G., Rothschild, M. F., Thacker, B. J., et al. (1998) Differences in susceptibility of Duroc, Hampshire, and Meishan pigs to infection with a high virulence strain (VR2385) of porcine reproductive and respiratory syndrome virus (PRRSV). J Anim Breed Genet 115, 181-189

Han, K., Seo, H. W., Oh, Y., et al. (2012) Pathogenesis of Korean type 1 (European genotype) porcine reproductive and respiratory syndrome virus in experimentally infected pigs. J Comp Pathol 147, 275-284

Hanada, K., Suzuki, Y., Nakane, T., et al. (2005) The origin and evolution of porcine reproductive and respiratory syndrome viruses. Mol Biol Evol 22, 1024-1031

Hartnell, A., Steel, J., Turley, H., et al. (2001) Characterization of human sialoadhesin, a sialic acid binding receptor expressed by resident and inflammatory macrophage populations. Blood 97, 288-296

Hill, H. (1990) Overview and history of mystery swine disease (swine infertility/respiratory syndrome). Proc Mystery Swine Dis Comm Meet 1, 29-30

Holtkamp, D., Kliebenstein, J., Neumann, E., et al. (2013) Assessment of the economic impact of porcine reproductive and respiratory syndrome virus on United States pork producers. J Swine Health Prod 21, 72-84

Holtkamp, D. J., Polson, D. D., Torremorell, M., et al. (2011) Terminologie zur Klassifizierung des PRRSV-Status von Schweineherden. Tierärztliche Praxis Großtiere 39, 101-112

Hopper, S., White, M. & Twiddy, N. (1992) An outbreak of blue-eared pig disease (porcine reproductive and respiratory syndrome) in four pig herds in Great Britain. Vet Rec 131, 140-144

Horter, D. C., Pogranichniy, R. M., Chang, C.-C., et al. (2002) Characterization of the carrier state in porcine reproductive and respiratory syndrome virus infection. Vet Microbiol 86, 213-228

Huang, Y. W., Dryman, B. A., Li, W., et al. (2009) Porcine DC-SIGN: Molecular cloning, gene structure, tissue distribution and binding characteristics. Dev Comp Immunol 33, 464-480

Joerink, M., Rindsjö, E., van Riel, B., et al. (2011) Placental macrophage (Hofbauer cell) polarization is independent of maternal allergen-sensitization and presence of chorioamnionitis. Placenta 32, 380-385

Jones, C., Virji, M. & Crocker, P. R. (2003) Recognition of sialylated meningococcal lipopolysaccharide by siglecs expressed on myeloid cells leads to enhanced bacterial uptake. Mol Microbiol 49, 1213-1225

Kappes, M. A. & Faaberg, K. S. (2015) PRRSV structure, replication and recombination: Origin of phenotype and genotype diversity. Virology 479–480, 475-486

Karniychuk, U. & Nauwynck, H. (2013) Pathogenesis and prevention of placental and transplacental porcine reproductive and respiratory syndrome virus infection. Vet Res 44, 95-109

Karniychuk, U. U., De Spiegelaere, W. & Nauwynck, H. J. (2013) Porcine reproductive and respiratory syndrome virus infection is associated with an increased number of Sn-positive and CD8-positive cells in the maternal–fetal interface. Virus Research 176, 285-291

Karniychuk, U. U., Geldhof, M., Vanhee, M., et al. (2010) Pathogenesis and antigenic characterization of a new East European subtype 3 porcine reproductive and respiratory syndrome virus isolate. BMC Vet Res 6, 30-30

Karniychuk, U. U. & Nauwynck, H. J. (2009) Quantitative changes of sialoadhesin and CD163 positive macrophages in the implantation sites and organs of porcine embryos/fetuses during gestation. Placenta 30, 497-500

Karniychuk, U. U., Saha, D., Geldhof, M., et al. (2011) Porcine reproductive and respiratory syndrome virus (PRRSV) causes apoptosis during its replication in fetal implantation sites. Microb Pathog 51, 194-202

Karniychuk, U. U., Saha, D., Vanhee, M., et al. (2012) Impact of a novel inactivated PRRS virus vaccine on virus replication and virus-induced pathology in fetal implantation sites and fetuses upon challenge. Theriogenology 78, 1527-1537

Keffaber, K. K. (1989) Reproductive failure of unknown etiology. Am Assoc Swine Pract News l, 1–10

Kelly, R., Holzman, C., Senagore, P., et al. (2009) Placental vascular pathology findings and pathways to preterm delivery. Am J Epidemiol 170, 148-158

Kemp, M. W. (2014) Preterm birth, intrauterine infection, and fetal inflammation. Frontiers in Immunology 5, 574

Kim, T. S., Benfield, D. A. & Rowland, R. R. R. (2002) Porcine reproductive and respiratory syndrome virus-induced cell death exhibits features consistent with a nontypical form of apoptosis. Virus Res 85, 133-140

Knight, J. W., Bazer, F. W., Thatcher, W. W., et al. (1977) Conceptus development in intact and unilaterally hysterectomized-ovariectomized gilts: interrelations among hormonal status, placental development, fetal fluids and fetal growth. J Anim Sci 44, 620-637

Koltes, J. E., Fritz-Waters, E., Eisley, C. J., et al. (2015) Identification of a putative quantitative trait nucleotide in guanylate binding protein 5 for host response to PRRS virus infection. BMC Genomics 16, 1-13

Kristensen, C. S., Bøtner, A., Takai, H., et al. (2004) Experimental airborne transmission of PRRS virus. Vet Microbiol 99, 197-202

Labarque, G., Van Gucht, S., Nauwynck, H., et al. (2003) Apoptosis in the lungs of pigs infected with porcine reproductive and respiratory syndrome virus and associations with the production of apoptogenic cytokines. Vet Res 34, 249-260

Labarque, G. G., Nauwynck, H. J., Van Reeth, K., et al. (2000) Effect of cellular changes and onset of humoral immunity on the replication of porcine reproductive and respiratory syndrome virus in the lungs of pigs. J Gen Virol 81, 1327-1334

Ladinig, A., Ashley, C., Detmer, S. E., et al. (2015a) Maternal and fetal predictors of fetal viral load and death in third trimester, type 2 porcine reproductive and respiratory syndrome virus infected pregnant gilts. Vet Res 46, 107

Ladinig, A., Detmer, S. E., Clarke, K., et al. (2015b) Pathogenicity of three type 2 porcine reproductive and respiratory syndrome virus strains in experimentally inoculated pregnant gilts. Virus Research 203, 24-35

Ladinig, A., Foxcroft, G., Ashley, C., et al. (2014a) Birth Weight, Intrauterine Growth Retardation and Fetal Susceptibility to Porcine Reproductive and Respiratory Syndrome Virus. PLoS ONE 9, e109541

Ladinig, A., Gerner, W., Saalmüller, A., et al. (2014b) Changes in leukocyte subsets of pregnant gilts experimentally infected with porcine reproductive and respiratory syndrome virus and relationships with viral load and fetal outcome. Veterinary Research 45, 128

Ladinig, A., Lunney, J. K., Souza, C. J. H., et al. (2014c) Cytokine profiles in pregnant gilts experimentally infected with porcine reproductive and respiratory syndrome virus and relationships with viral load and fetal outcome. Vet Res 45, 113

Ladinig, A., Wilkinson, J., Ashley, C., et al. (2014d) Variation in fetal outcome, viral load and ORF5 sequence mutations in a large scale study of phenotypic responses to late gestation exposure to type 2 porcine reproductive and respiratory syndrome virus. PLoS ONE 9, 96-104

Lager, K. & Mengeling, W. (1995) Pathogenesis of in utero infection in porcine fetuses with porcine reproductive and respiratory syndrome virus. Can J Vet Res 59, 187–192

Lager, K. M. & Halbur, P. G. (1996) Gross and microscopic lesions in porcine fetuses infected with porcine reproductive and respiratory syndrome virus. J Vet Diagn Invest 8, 275-282

Lambert, M.-È., Arsenault, J., Poljak, Z., et al. (2012) Epidemiological investigations in regard to porcine reproductive and respiratory syndrome (PRRS) in Quebec, Canada. Part 2: Prevalence and risk factors in breeding sites. Prev Vet Med 104, 84-93

Langohr, I. M., Stevenson, G. W., Nelson, E. A., et al. (2010) Vascular lesions in pigs experimentally infected with porcine circovirus type 2 serogroup B. Vet Pathol 47, 140-147

Lau, S. K., Chu, P. G. & Weiss, L. M. (2004) CD163. Am J Clin Pathol 122, 794-801

Lee, S.-M. & Kleiboeker, S. B. (2007) Porcine reproductive and respiratory syndrome virus induces apoptosis through a mitochondria-mediated pathway. Virology 365, 419-434

Lee, S.-M., Schommer, S. K. & Kleiboeker, S. B. (2004) Porcine reproductive and respiratory syndrome virus field isolates differ in in vitro interferon phenotypes. Vet Immunol Immunopathol 102, 217-231

Loemba, H., Mounir, S., Mardassi, H., et al. (1996) Kinetics of humoral immune response to the major structural proteins of the porcine reproductive and respiratory syndrome virus. Arch Virol 141, 751-761

López Fuertes, L., Doménech, N., Alvarez, B., et al. (1999) Analysis of cellular immune response in pigs recovered from porcine respiratory and reproductive syndrome infection. Virus Res 64, 33-42

Loving, C. L., Brockmeier, S. L., Vincent, A. L., et al. (2008) Differences in Clinical Disease and Immune Response of Pigs Challenged with a High-Dose versus Low-Dose Inoculum of Porcine Reproductive and Respiratory Syndrome Virus. Viral Immunol 21, 315-326

Lowe, J., Husmann R, Firkins LD, Zuckermann FA, Goldberg TL (2005) Correlation of cell-mediated immunity against porcine reproductive and respiratory syndrome virus with

protection against reproductive failure in sows during outbreaks of porcine reproductive and respiratory syndrome in commercial herds. J Am Vet Med Assoc 226, 1707-1711

Lunney, J. K. & Chen, H. (2010) Genetic control of host resistance to porcine reproductive and respiratory syndrome virus (PRRSV) infection. Virus Res 154, 161-169

Luzina, I. & Handwerger, B. (2000) Lessons from animal models of vasculitis. Current Rheumatology Reports 2, 369-375

Magar, R. & Larochelle, R. (2004) Evaluation of the presence of porcine reproductive and respiratory syndrome virus in pig meat and experimental transmission following oral exposure. Can J Vet Res 68, 259-266

Manders, S. M. (1998) Toxin-mediated streptococcal and staphylococcal disease. J Am Acad Dermatol 39, 383-398

Mardassi, H., Athanassious, R., Mounir, S., et al. (1994) Porcine reproductive and respiratory syndrome virus: morphological, biochemical and serological characteristics of Quebec isolates associated with acute and chronic outbreaks of porcine reproductive and respiratory syndrome. Can J Vet Res 58, 55-64

Mardassi, H., Massie, B. & Dea, S. (1996) Intracellular synthesis, processing, and transport of proteins encoded by ORFs 5 to 7 of porcine reproductive and respiratory syndrome virus. Virology 221, 98-112

Martinez-Pomares, L. & Gordon, S. (2012) CD169+ macrophages at the crossroads of antigen presentation. Trends Immunol 33, 66-70

Mateu, E. & Diaz, I. (2008) The challenge of PRRS immunology. Vet J 177, 345-351

Maxie, M. & Robinson, W. (2007) Cardiovascular system. In Jubb, Kennedy and Palmer's Pathology of Domestic Animals. 5 edn. Ed M. Maxie. Philadelphia, PA, Elsevier Saunders. pp 69-72

McCaw, M. (1995) MCREBEL PRRS: Management procedures for PRRS control in large herd nurseries. In Proc AD Leman Swine. St Paul, 161–162

Mengeling, W. L., Lager, K. M. & Vorwald, A. C. (1996) Alveolar macrophages as a diagnostic sample for detecting natural infection of pigs with porcine reproductive and respiratory syndrome virus. J Vet Diagn Invest 8, 238-240

Miller, C. L., Laegreid, W. W., Bono, L. J., et al. (2004) Interferon type I response in porcine reproductive and respiratory syndrome virus-infected MARC-145 cells. Arch Virol 149, 2453-2463

Miller, L. C. & Fox, J. M. (2004) Apoptosis and porcine reproductive and respiratory syndrome virus. Vet Immunol Immunopathol 102, 131-142

Millikan, L. E. & Flynn, T. C. (1999) Infectious etiologies of cutaneous vasculitis. Clin Dermatol 17, 509-514

Møller, H. J. (2012) Soluble CD163. Scand J Clin Lab Invest 72, 1-13

Mortensen, S., Stryhn, H., Søgaard, R., et al. (2002) Risk factors for infection of sow herds with porcine reproductive and respiratory syndrome (PRRS) virus. Prev Vet Med 53, 83-101

Mulupuri, P., Zimmerman, J. J., Hermann, J., et al. (2008) Antigen-specific B-cell responses to porcine reproductive and respiratory syndrome virus infection. J Virol 82, 358-370

Murphy, S. P., Hanna, N. N., Fast, L. D., et al. (2009) Evidence for participation of uterine natural killer cells in the mechanisms responsible for spontaneous preterm labor and delivery. American Journal of Obstetrics and Gynecology 200, 308.e301-308.e309

Murtaugh, M. P., Stadejek, T., Abrahante, J. E., et al. (2010) The ever-expanding diversity of porcine reproductive and respiratory syndrome virus. Virus Res 154, 18-30

Nielsen, J., Bøtner, A., Bille-Hansen, V., et al. (2002) Experimental inoculation of late term pregnant sows with a field isolate of porcine reproductive and respiratory syndrome vaccine-derived virus. Vet Microbiol 84, 1-13

Novakovic, P., Harding, J. C. S., Al-Dissi, A. N., et al. (2016a) Pathologic Evaluation of Type 2 Porcine Reproductive and Respiratory Syndrome Virus Infection at the Maternal-Fetal Interface of Late Gestation Pregnant Gilts. PLoS ONE 11, e0151198

Novakovic, P., Harding, J. C. S., Ladinig, A., et al. (2016b) Association of CD163 positive cells in the endometrium and fetal placenta with type 2 PRRSV infection of the fetus. Vet Res (under review)

O'Neill, A. S. G., van den Berg, T. K. & Mullen, G. E. D. (2013) Sialoadhesin – a macrophage-restricted marker of immunoregulation and inflammation. Immunology 138, 198-207

OIE (2015) Porcine reproductive and respiratory syndrome. In 2015 Terrestrial Manual. http://www.oie.int/fileadmin/Home/eng/Health_standards/tahm/2.08.07_PRRS.pdf, accessed on December 10 2015

Oleksiewicz, M. B., Bøtner, A., Toft, P., et al. (2001) Epitope mapping porcine reproductive and respiratory syndrome virus by phage display: the nsp2 fragment of the replicase polyprotein contains a cluster of B-cell epitopes. J Virol 75, 3277-3290

Ostrowski, M., Galeota, J. A., Jar, A. M., et al. (2002) Identification of Neutralizing and Nonneutralizing Epitopes in the Porcine Reproductive and Respiratory Syndrome Virus GP5 Ectodomain. J Virol 76, 4241-4250

Otake, S. (2003) Transmission of porcine reproductive and respiratory syndrome virus by houseflies. Vet Rec 152, 73-76

Otake, S., Dee, S. A., Rossow, K. D., et al. (2002a) Mechanical transmission of porcine reproductive and respiratory syndrome virus by mosquitoes, Aedes vexans (Meigen). Can J Vet Res 66, 191-195

Otake, S., Dee, SA, Rossow, KD, Joo, HS, Deen, J, Molitor, TW, Pijoan, C (2002b) Transmission of porcine reproductive and respiratory syndrome virus by needles. Vet Rec, 114-115

Petry, D. B., Lunney, J., Boyd, P., et al. (2007) Differential immunity in pigs with high and low responses to porcine reproductive and respiratory syndrome virus infection. J Anim Sci 85, 2075-2092

Pitkin, A., Otake, S. & Dee, S. (2015) Biosecurity protocols for the prevention of spread of porcine reproductive and respiratory syndrome virus. Swine Disease Eradication Center, University of Minnesota College of Veterinary Medicine, https://www.aasv.org/aasv/PRRSV_BiosecurityManual.pdf, accessed on April 24 2016

Plagemann, P. & Moennig, V. (1992) Lactate dehydrogenase-elevating virus, equine arteritis virus, and simian hemorrhagic fever virus: a new group of positive-strand RNA viruses. Adv Virus Res 41, 99-192

Plagemann, P. G. (2003) Porcine reproductive and respiratory syndrome virus: origin hypothesis. Emerg Infect Dis 9, 903–908

Prather, R. S., Rowland, R. R. R., Ewen, C., et al. (2013) An intact sialoadhesin (Sn/SIGLEC1/CD169) is not required for attachment/internalization of the porcine reproductive and respiratory syndrome virus. J Virol 87, 9538-9546

Prieto, C., Suárez, P., Simarro, I., et al. (1997) Transplacental infection following exposure of gilts to porcine reproductive and respiratory syndrome virus at the onset of gestation. Vet Microbiol 57, 301-311

Provost, C., Jia, J. J., Music, N., et al. (2012) Identification of a new cell line permissive to porcine reproductive and respiratory syndrome virus infection and replication which is phenotypically distinct from MARC-145 cell line. Virol J 9, 267-267

Rascón-Castelo, E., Burgara-Estrella, A., Mateu, E., et al. (2015) Immunological features of the non-structural proteins of porcine reproductive and respiratory syndrome virus. Viruses 7, 873-886

Renukaradhya, G. J., Dwivedi, V., Manickam, C., et al. (2012) Mucosal vaccines to prevent porcine reproductive and respiratory syndrome: a new perspective. Anim Health Res Rev 13, 21-37

Renukaradhya, G. J., Meng, X.-J., Calvert, J. G., et al. (2015) Inactivated and subunit vaccines against porcine reproductive and respiratory syndrome: Current status and future direction. Vaccine 33, 3065-3072

Rogers, P. (1996) Structure and function of endometrial blood vessels. Hum Reprod Update 2, 57-62

Rossow, K. D. (1998) Porcine reproductive and respiratory syndrome. Vet Pathol 35, 1-20

Rossow, K. D., Bautista, E. M., Goyal, S. M., et al. (1994a) Experimental porcine reproductive and respiratory syndrome virus infection in one-, four-, and 10-week-old pigs. J Vet Diagn Invest 6, 3-12

Rossow, K. D., Benfield, D. A., Goyal, S. M., et al. (1996a) Chronological immunohistochemical detection and localization of porcine reproductive and respiratory syndrome virus in gnotobiotic pigs. Vet Pathol 33, 551-556

Rossow, K. D., Collins, J. E., Goyal, S. M., et al. (1995) Pathogenesis of porcine reproductive and respiratory syndrome virus infection in gnotobiotic pigs. Vet Pathol 32, 361-373

Rossow, K. D., Laube, K. L., Goyal, S. M., et al. (1996b) Fetal microscopic lesions in porcine reproductive and respiratory syndrome virus-induced abortion. Vet Pathol 33, 95-99

Rossow, K. D., Morrison, R. B., Goyal, S. M., et al. (1994b) Lymph node lesions in neonatal pigs congenitally exposed to porcine reproductive and respiratory syndrome virus. J Vet Diagn Invest 6, 368-371

Roulston, A., Marcellus, R. C. & Branton, P. E. (1999) Viruses and apoptosis. Annu Rev Microbiol 53, 577-628

Rowland, R. R. R. (2010) The interaction between PRRSV and the late gestation pig fetus. Virus Res 154, 114-122

Rowland, R. R. R., Lawson, S., Rossow, K., et al. (2003) Lymphoid tissue tropism of porcine reproductive and respiratory syndrome virus replication during persistent infection of pigs originally exposed to virus in utero. Vet Microbiol 96, 219-235

Rowland, R. R. R., Lunney, J. & Dekkers, J. (2012) Control of porcine reproductive and respiratory syndrome (PRRS) through genetic improvements in disease resistance and tolerance. Frontiers in Genetics 3, 260

Rowland, R. R. R. & Morrison, R. B. (2012) Challenges and opportunities for the control and elimination of porcine reproductive and respiratory syndrome virus. Transbound Emerg Dis 59, 55-59

Salamonsen, L. & Nie, G. (2002) Proteases at the endometrial–trophoblast interface: Their role in implantation. Reviews in Endocrine and Metabolic Disorders 3, 133-143

Salguero, F. J., Frossard, J.-P., Rebel, J. M. J., et al. (2015) Host–pathogen interactions during porcine reproductive and respiratory syndrome virus 1 infection of piglets. Virus Res 202, 135-143

Saunders, P. T., Renegar, R. H., Raub, T. J., et al. (1985) The carbohydrate structure of porcine uteroferrin and the role of the high mannose chains in promoting uptake by the reticuloendothelial cells of the fetal liver. J Biol Chem 260, 3658-3665

Schlafer, D. & Miller, R. (2007) Female genital system. In Jubb, Kennedy, and Palmer's Pathology of Domestic Animals. Ed M. G. Maxie. Philadelphia, PA, Elsevier Saunders. pp 518-519

Senger, P. (2012) Pathways to Pregnancy and Parturition. Redmon, OR, USA, Current Conceptions, Inc.

Shenoy, A. R., Wellington, D. A., Kumar, P., et al. (2012) GBP5 Promotes NLRP3 Inflammasome Assembly and Immunity in Mammals. Science 336, 481-485

Shi, M., Lam, T. T.-Y., Hon, C.-C., et al. (2010) Molecular epidemiology of PRRSV: A phylogenetic perspective. Virus Res 154, 7-17

Sirinarumitr, T., Zhang, Y., Kluge, J. P., et al. (1998) A pneumo-virulent United States isolate of porcine reproductive and respiratory syndrome virus induces apoptosis in bystander cells both in vitro and in vivo. J Gen Virol 79, 2989-2995

Sivakumar, K., Bari, M. F., Adaikalakoteswari, A., et al. (2013) Elevated Fetal Adipsin/Acylation-Stimulating Protein (ASP) in Obese Pregnancy: Novel Placental Secretion via Hofbauer Cells. The Journal of Clinical Endocrinology and Metabolism 98, 4113-4122

Snijder, E. & Spaan, W. (2007) Arteriviridae. Philadelphia, Lippincott Williams & Wilkins

Soede, N. M., Langendijk, P. & Kemp, B. (2011) Reproductive cycles in pigs. Animal Reproduction Science 124, 251-258

Song, G., Bailey, D. W., Dunlap, K. A., et al. (2010) Cathepsin B, cathepsin L, and cystatin C in the porcine uterus and placenta: Potential roles in endometrial/placental remodeling and in fluid-phase transport of proteins secreted by uterine epithelia across placental areolae. Biol Reprod 82, 854-864

Sur, J. H., Doster, A. R., Christian, J. S., et al. (1997) Porcine reproductive and respiratory syndrome virus replicates in testicular germ cells, alters spermatogenesis, and induces germ cell death by apoptosis. J Virol 71, 9170-9179

Sur, J. H., Doster, A. R. & Osorio, F. A. (1998) Apoptosis induced in vivo during acute infection by porcine reproductive and respiratory syndrome virus. Vet Pathol 35, 506-514

Tang, Z., Abrahams, V. M., Mor, G., et al. (2011) Placental Hofbauer cells and complications of pregnancy. Ann N Y Acad Sci 1221, 103-108

Terpstra, C., Wensvoort, G. & Pol, J. M. A. (1991) Experimental reproduction of porcine epidemic abortion and respiratory syndrome (mystery swine disease) by infection with Lelystad vims: Koch's postulates fulfilled. Vet Q 13, 131-136

Thanawongnuwech, R., Brown, G. B., Halbur, P. G., et al. (2000a) Pathogenesis of porcine reproductive and respiratory syndrome virus-induced increase in susceptibility to Streptococcus suis infection. Vet Pathol 37, 143-152

Thanawongnuwech, R., Halbur, P. G. & Andrews, J. J. (1997a) Immunohistochemical detection of porcine reproductive and respiratory syndrome virus antigen in neurovascular lesions. J Vet Diagn Invest 9, 334-337

Thanawongnuwech, R., Halbur, P. G. & Thacker, E. L. (2000b) The role of pulmonary intravascular macrophages in porcine reproductive and respiratory syndrome virus infection. Anim Health Res Rev 1, 95-102

Thanawongnuwech, R., Thacker, E. L. & Halbur, P. G. (1997b) Effect of porcine reproductive and respiratory syndrome virus (PRRSV) (isolate ATCC VR-2385) infection on bactericidal activity of porcine pulmonary intravascular macrophages (PIMs): in vitro comparisons with pulmonary alveolar macrophages (PAMs). Vet Immunol Immunopathol 59, 323-335

Thibault, S., Drolet, R., Germain, M. C., et al. (1998) Cutaneous and systemic necrotizing vasculitis in swine. Vet Pathol 35, 108-116

Tian, K., Yu, X., Zhao, T., et al. (2007) Emergence of fatal PRRSV variants: Unparalleled outbreaks of atypical PRRS in China and molecular dissection of the unique hallmark. PLoS ONE 2, e526

Turk, V., Stoka, V., Vasiljeva, O., et al. (2012) Cysteine cathepsins: From structure, function and regulation to new frontiers. Biochimica et Biophysica Acta (BBA) - Proteins and Proteomics 1824, 68-88

Van Gorp, H., Van Breedam, W., Delputte, P. L., et al. (2008) Sialoadhesin and CD163 join forces during entry of the porcine reproductive and respiratory syndrome virus. J Gen Virol 89, 2943-2953

Vanderheijden, N., Delputte, P. L., Favoreel, H. W., et al. (2003) Involvement of sialoadhesin in entry of porcine reproductive and respiratory syndrome virus into porcine alveolar macrophages. J Virol 77, 8207-8215

Velasova, M., Alarcon, P., Williamson, S., et al. (2012) Risk factors for porcine reproductive and respiratory syndrome virus infection and resulting challenges for effective disease surveillance. BMC Vet Res 8, 1-14

Wagstrom, E., Chang, C., Yoon, K., et al. (2001) Shedding of porcine reproductive and respiratory syndrome virus in mammary gland secretions of sows. Am J Vet Res 62, 1876-1880

Wang, R., Wang, X., Ni, B., et al. (2016) Syndecan-4, a PRRSV attachment factor, mediates PRRSV entry through its interaction with EGFR. Biochemical and Biophysical Research Communications 475, 230-237

Weesendorp, E., Morgan, S., Stockhofe-Zurwieden, N., et al. (2013) Comparative analysis of immune responses following experimental infection of pigs with European porcine reproductive and respiratory syndrome virus strains of differing virulence. Vet Microbiol 163, 1-12

Weigel, R., M., Firkins, L., D. & Scherba, G. (2000) Prevalence and risk factors for infection with Porcine Reproductive and Respiratory Syndrome Virus (PRRSV) in swine herds in Illinois (USA). Vet Res 31, 87-88

Wensvoort, G., Terpstra, C., Pol, J. M. A., et al. (1991) Mystery swine disease in the Netherlands: The isolation of Lelystad virus. Vet Q 13, 121-130

Weyand, C. M. & Goronzy, J. J. (2003) Medium- and large-vessel vasculitis. N Engl J Med 349, 160-169

Whitworth, K. M., Rowland, R. R. R., Ewen, C. L., et al. (2016) Gene-edited pigs are protected from porcine reproductive and respiratory syndrome virus. Nat Biotech 34, 20-22

Wills, R. W., Doster, A. R., Galeota, J. A., et al. (2003) Duration of Infection and Proportion of Pigs Persistently Infected with Porcine Reproductive and Respiratory Syndrome Virus. Journal of Clinical Microbiology 41, 58-62

Wills, R. W., Zimmerman, J. J., Yoon, K.-J., et al. (1997a) Porcine reproductive and respiratory syndrome virus: routes of excretion. Vet Microbiol 57, 69-81

Wills, R. W., Zimmerman, J. J., Yoon, K. J., et al. (1997b) Porcine reproductive and respiratory syndrome virus: a persistent infection. Vet Microbiol 55, 231-240

Wu, J., Peng, X., Zhou, A., et al. (2014) MiR-506 inhibits PRRSV replication in MARC-145 cells via CD151. Mol Cell Biochem 394, 275-281

Xiao, Z., Batista, L., Dee, S., et al. (2004) The level of virus-specific T-cell and macrophage recruitment in porcine reproductive and respiratory syndrome virus infection in pigs is independent of virus load. J Virol 78, 5923-5933

Yoo, D., Wootton, S. K., Li, G., et al. (2003) Colocalization and interaction of the porcine arterivirus nucleocapsid protein with the small nucleolar RNA-associated protein fibrillarin. J Virol 77, 12173-12183

Yoon, B. H., Romero, R., Park, J. S., et al. (2000) The relationship among inflammatory lesions of the umbilical cord (funisitis), umbilical cord plasma interleukin 6 concentration, amniotic fluid infection, and neonatal sepsis. Am J Obstet Gynecol 183, 1124-1129

Yoon, K., Wu, L., Zimmerman, J., et al. (1996) Antibody-dependent enhancement (ADE) of porcine reproductive and respiratory syndrome virus (PRRSV) infection in pigs. Viral Immunol 9, 51-63

Zhang, Q. & Yoo, D. (2015) PRRS virus receptors and their role for pathogenesis. Vet Microbiol 177, 229-241

Zimmerman, J., Benfield, D. A., Murtaugh, M. P., et al. (2012) Porcine reproductive and respiratory syndrome virus (porcine arterivirus). In Diseases of Swine. 10 edn. Eds J. Zimmerman, L. Karriker, A. Ramirez, K. Schwartz, G. Stevenson. Ames, Iowa, USA, John Wiley and Sons Ltd. pp 461-486