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Review article

Novel insights into host responses and reproductive pathophysiology of porcine reproductive and respiratory syndrome caused by PRRSV-2

John C.S. Harding[a,*], Andrea Ladinig[a,1], Predrag Novakovic[a], Susan E. Detmer[a], Jamie M. Wilkinson[b,2], Tianfu Yang[b], Joan K. Lunney[c], Graham S. Plastow[b]

[a] Western College of Veterinary Medicine, University of Saskatchewan, 52 Campus Dr., Saskatoon, Saskatchewan S7N 5B4, Canada
[b] Department of Agricultural, Food, and Nutritional Science, University of Alberta, Edmonton, Alberta T6G 2P5, Canada
[c] Animal Parasitic Diseases Laboratory, Beltsville Agricultural Research Center, Agricultural Research Service, U.S. Department of Agriculture, Beltsville, MD, USA

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ABSTRACT

A large challenge experiment using North American porcine reproductive and respiratory virus (PRRSV-2) provided new insights into the pathophysiology of reproductive PRRS. Deep phenotyping of dams and fetuses identified maternal and fetal predictors of PRRS severity and resilience. PRRSV infection resulted in dramatic decreases in all leukocyte subsets by 2 days post inoculation. Apoptosis in the interface region was positively related to endometrial vasculitis, viral load in endometrium and fetal thymus, and odds of meconium staining. Viral load at the maternal-fetal interface was a strong predictor of viral load in fetal thymus and odds of fetal death. However, interferon-alpha suppression, a consequence of PRRSV infection, was protective against fetal death. Although the prevalence of fetal lesions was low, their presence in fetal organs and umbilical cord was strongly associated with fetal compromise. Fetal death and viral load clustered in litters suggesting inter-fetal transmission starting from a limited number of index fetuses. Factors associated with index fetal infection are unclear, but large fetuses appear at greater risk. Disease progression in fetuses was associated with an up-regulation of genes associated with inflammation, innate immunity, and cell death signaling, and down-regulation of genes associated with cell cycle and lymphocyte quality. A number of maternal transcriptomic responses were associated with PRRS resilience including higher basal gene expression correlated with platelet function, interferon and pro-inflammatory responses. Twenty-one genomic regions across 10 chromosomes were associated with important traits including fetal viral load, fetal death and viability suggesting that selection for reproductive PRRS resilience may be possible.

Contents

* Corresponding author.
E-mail addresses: john.harding@usask.ca (J.C.S. Harding), andrea.ladinig@vetmeduni.ac.at (A. Ladinig), predrag.novakovic@usask.ca (P. Novakovic), susan.detmer@usask.ca (S.E. Detmer), jamie.wilkinson@ualberta.ca (J.M. Wilkinson), tianfu@ualberta.ca (T. Yang), Joan.Lunney@ARS.USDA.GOV (J.K. Lunney), plastow@ualberta.ca (G.S. Plastow).
[1] Permanent address: University Clinic for Swine, Department for Farm Animals and Veterinary Public Health, University of Veterinary Medicine Vienna, Vienna 1210, Austria.
[2] Illumina UK, Chesterford Research Park, Little Chesterford, Essex, CB10 1XL, UK.

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1. Introduction

Porcine reproductive and respiratory syndrome virus (PRRSV) infection causes reproductive failure in pregnant females and respiratory disease in any age of pig; both of which have global significance. An enveloped virus belonging to the genus Arteriviridae, two distinct species of PRRSV are now recognized: PRRSV-1 containing European isolates and formerly known as genotype I; and PRRSV-2, containing North American isolates and formerly known as genotype II (Adams et al., 2016; Van Doorsselaere et al., 2012). Members of the genus Arteriviridae are characterized by asymptomatic infections, severe fatal disease, predilection for macrophages, and remarkable genome plasticity (Snijder and Meulenberg, 1998; Zimmerman et al., 2012). While in early gestation the virus can cause embryonic death (Prieto et al., 1996, 1997), the clinical manifestation of PRRSV infection mainly occurs in late gestation and is characterized by abortions, early farrowing, fetal death, and the birth of weak, congenitally infected piglets resulting in elevated preweaning mortality (Mengeling et al., 1994; Terpstra et al., 1991). Transplacental PRRSV infection mainly occurs in late gestation (Christianson et al., 1993; Kranker et al., 1998) but the exact mechanisms by which PRRSV transmits from the dam to her fetuses have yet to be determined (Karniychuk and Nauwynck, 2013). It has been suggested that fetal death may not be a direct result of PRRSV infection of fetal tissues since severe microscopic lesions are not observed in infected fetuses (Lager and Halbur, 1996; Rossow et al., 1996). It has been shown that the number of sialoadhesin positive ($CD169^+$) and $CD163^+$ macrophages in endometrium and placenta (Karniychuk and Nauwynck, 2009), and virus replication in fetal implantation sites, which causes apoptosis of infected and surrounding cells (Karniychuk et al., 2011), play a role in fetal death. Thus, it is postulated that fetal demise is mainly associated with events occurring at the maternal-fetal interface leading to the separation of fetal placenta from the uterus. The purpose of this review is to summarize published literature pertaining to the pathophysiology of reproductive PRRS, in order to provide new insights on the mechanism(s) of fetal death. In an attempt to avoid duplicating the excellent review on the same subject (Karniychuk and Nauwynck, 2013), the present review places special emphasis on host responses (phenotypic, pathologic, transcriptomic, genomic) following PRRSV infection recently published in association with the Pregnant Gilt Model.

2. The Pregnant Gilt Model (PGM)

The Pregnant Gilt Model (PGM) was a large-scale, multidisciplinary project conducted at the University of Saskatchewan beginning in 2011, with the overarching objectives of advancing the understanding of the pathophysiology of reproductive PRRS and factors associated with PRRS resilience. Undertaken in collaboration with a number of domestic and international research partners, the PGM included a pilot experiment (n = 15 gilts) to develop laboratory methods and evaluate the relative virulence of three North American PRRSV-2 strains that originated from reproductive field cases (generously provide by R. R. R. Rowland, Kansas State University) (Ladinig et al., 2015b). The main experiment used 133 purebred pregnant Landrace gilts (Ladinig et al., 2014d) that were selected on the basis of their average litter birth weight; half from low, and half from high birth weight litters (Ladinig et al., 2014a). At gestation day 85 (±1), 114 gilts were experimentally infected with PRRSV-2 (1×10^5 $TCID_{50}$ of NVSL 97-7895). Nineteen control gilts were similarly mock-infected, and all gilts and fetuses were humanely euthanized at 21 days post inoculation (dpi) at gestation day 106 ± 1. Laboratory and statistical analyses of the estimated 50,000 samples collected enabled the assessment of fetal and maternal host responses to PRRSV infection, genome- and transcriptome-wide association studies, and identification of potential predictors of PRRS resilience.

3. Responses to PRRSV-2 infection in the dam

3.1. Clinical responses and viral load

The clinical presentation of PRRS varies greatly between herds from asymptomatic to devastating, depending on herd-specific factors, stage of gestation and immune status of individual animals, and the species (genotype) and strain of PRRSV. The three PRRSV-2 strains compared in the PGM pilot experiment varied considerably in terms of inducing fever, decreased feed intake, cytokine responses and reproductive failure (Ladinig et al., 2015b). Although the most virulent strain, NVSL 97–7895 isolated from a farm experiencing severe reproductive disease (Allende et al., 1999; Osorio et al., 2002) was used to inoculate 114 third trimester gilts in the main experiment, only one gilt died (11 dpi) and two gilts aborted (17 dpi, 20 dpi) within 20 days of experimental PRRSV inoculation. PRRSV infection resulted in reduced daily feed intake, and the presence of fever that occurred in biphasic pattern peaking at D2/3 and D8 post inoculation (Ladinig et al., 2014d). Reproductive signs due to PRRSV infection largely depend on the stage of gestation and mainly occur in late-gestation. In our NVSL 97–7895 inoculated gilts, fetal mortality rate ranged from 0% to 94.4% (mean 41.0, SD ±22.8%) compared to 1.4 ±3.4% in sham-inoculated gilts.

Following combined intranasal/intramuscular inoculation, viremia occurred rapidly; evident in 100% of gilts by 2 dpi and peaking at 6 dpi. At 21 dpi, PRRSV RNA could be detected in 85% and

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90% of gilt sera and lung samples, respectively, and in 100% of dams' reproductive lymph node (*Lnn. uterini*). However, viral load (target PRRSV RNA concentration per mg or μl) in the dam's sera and tissues was strain dependent (Ladinig et al., 2015b) and except for levels at the maternal-fetal interface, was unrelated to the severity of reproductive disease (Ladinig et al., 2015a). The PGM provided no substantive evidence that severity of reproductive failure is affected by dam birth weight (Ladinig et al., 2014a) or litter size (Ladinig et al., 2015a). It was concluded that the concentration of PRRSV RNA in non-reproductive systemic or lymphoid tissues of dams is of little importance for the reproductive outcome of PRRS, except that it may help to "seed" the uterus with virus.

3.2. Systemic immune responses in pregnant gilts

The immune responses following PRRSV infection have been recently reviewed in detail (Loving et al., 2015); most immunologic data has been garnished from *in vitro* or *in vivo* studies of young pigs using respiratory models of PRRS. By contrast, reports on immune responses of pregnant females are sparse. One such study demonstrated significant decreases in total leukocyte counts, and absolute numbers of CD172a$^+$ cells, CD1$^+$ cells, CD4$^+$ and CD8α$^+$ T cells at 3 and 7 dpi in sows experimentally infected with PRRSV in mid-gestation (Christianson et al., 1993). PGM results confirmed and expanded these findings (Ladinig et al., 2014b) by demonstrating a massive, acute drop (~45%) in total white blood cell numbers affecting all immune cell subpopulations by 2 dpi. Subsets involved in cytotoxic and killer activities (NK cells, cytotoxic T lymphocytes [CTLs]) were most severely affected, as were naïve B cells, T-helper cells, and γδT cells compared to their respective effector or memory counterparts. All immune cell types, except antibody producing B cells, started to rebound by 6 dpi and returned to pre-inoculation levels by 19 dpi. Although it is not entirely certain if these acute and dramatic reductions in cell numbers are associated with cellular trafficking or destruction (apoptosis or necrosis), changes in the whole blood transcriptome related to apoptotic, T cell signaling, and mitotic pathways between 0 and 2 dpi, which are subsequently reversed between 2 and 6 dpi, provide some support for the latter (Wilkinson et al., 2016b). Of all leukocyte subsets investigated, absolute numbers of T-helper between 0 and 6 dpi and myeloid cells between 0 and 19 dpi, may be most clinically relevant; with higher numbers significantly associated with decreased odds of fetal death and decreased concentration of PRRSV RNA in fetal thymus, respectively (Fig. 1) (Ladinig et al., 2015a).

The ability of PRRSV infection to suppress interferon-alpha (IFNα) secretion from macrophages and plasmacytoid dendritic cells (pDC) is widely understood (as reviewed by Loving et al., 2015), and on first glance appears to contradict the findings of the PGM. In gilt serum, levels of IFNα rose sharply by 2 dpi before falling to baseline by 6 dpi. High serum levels of IFNα were

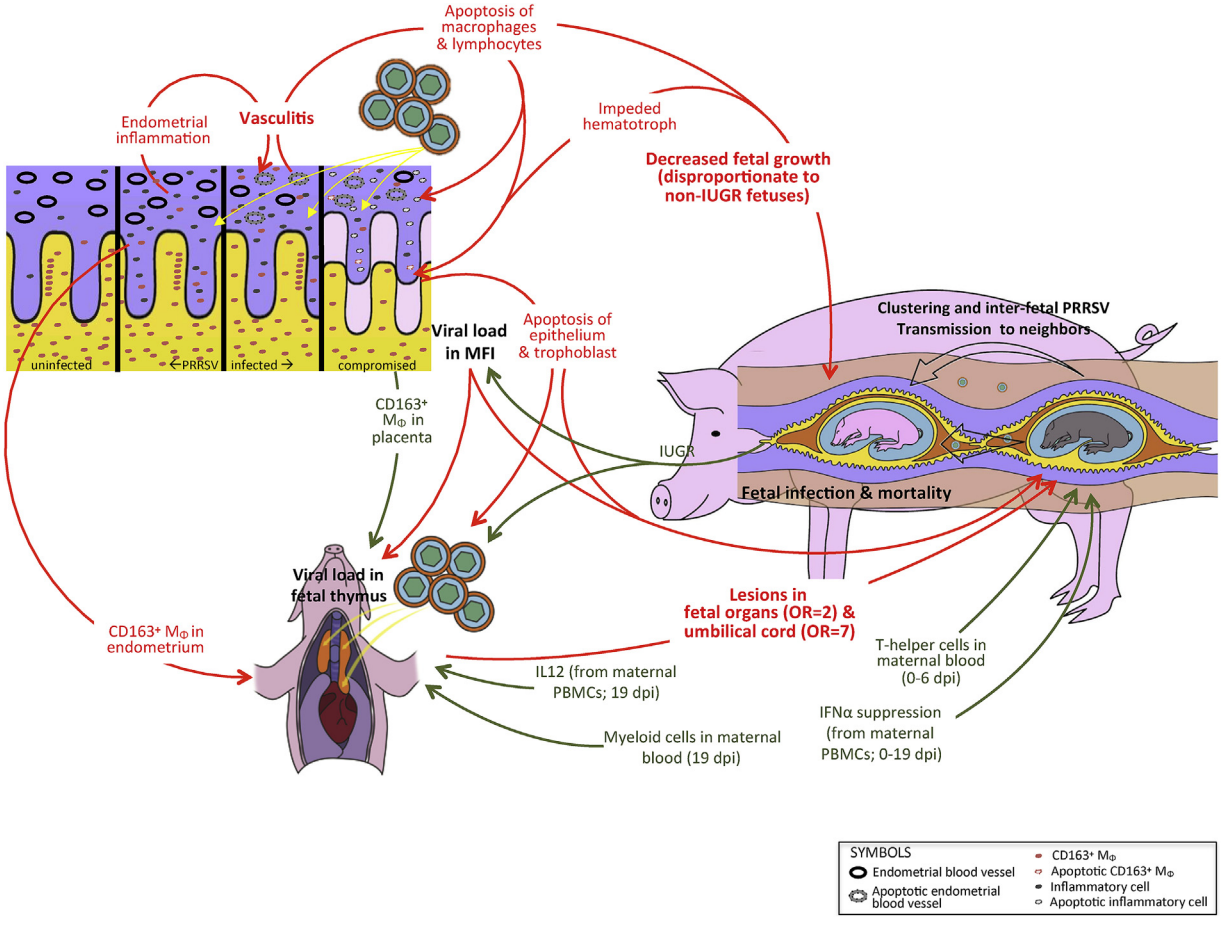

Fig. 1. Phenotypic responses associated with the pathophysiology of reproductive PRRS. Phenotypic responses (physiologic, immunologic, pathologic, virologic) beneficially (green arrows) or adversely (red arrows) associated with events in the maternal-fetal interface (top left; endometrium = purple, fetal placenta = gold), fetal thymus (bottom left), and fetal infection and/or mortality (right). References: Ladinig et al., 2014a,d, 2015a; Novakovic et al., 2016a,b, 2017.

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significantly associated with high viral load in serum and tonsil, but were unrelated to viral load in lung and reproductive lymph node (Ladinig et al., 2014c). In whole blood, IFNα gene expression was profoundly up-regulated by 2 dpi, but profoundly down-regulated by 6 dpi, mirroring IFNα protein levels in serum (Wilkinson et al., 2016b). Similarly, the supernatants of peripheral blood mononuclear cells (PBMC) collected from PRRSV-inoculated dams on 2, 6, and 19 dpi showed profound suppression of IFNα secretion following re-stimulation with PRRSV (Ladinig et al., 2014c). Thus, the rise in IFNα levels appears to be short lived in sera of pregnant gilts, and occurs during the very initial stages of viral replication, prior to peak viral load and the full expression of IFNα-suppressive mechanisms. Even though PRRSV infection-induced IFNα suppression may be one of the mechanisms PRRSV employs to modulate the host immune response, suppression may be beneficial to the fetus. Higher levels of IFNα suppression from PBMCs was associated with decreased fetal mortality rate and lowered odds of fetal death (Fig. 1) (Ladinig et al., 2015a) possibly because IFNα up-regulated the expression of sialoadhesin and therefore enhanced PRRSV infection of monocytes (Delputte et al., 2007).

Serum levels of two other cytokines differed between PRRSV-infected and non-infected dams. Chemokine ligand 2 (CCL2), involved in the recruitment of monocytes from blood to tissue macrophages increased on 2 and 6 dpi following PRRSV infection, whereas IFN-gamma (IFNγ), an inducer of Th1 responses, showed a bimodal response; being significantly elevated on 2 dpi and numerically lower than in controls on 21 dpi (Ladinig et al., 2014c). However, neither of these cytokines was related to reproductive outcome (Ladinig et al., 2015a).

3.3. Whole blood transcriptome in acute PRRS

RNA obtained from whole blood collected on 0, 2, and 6 dpi from a subset of gilts was subjected to genome-wide analyses of transcriptomic responses using RNA-sequencing (Wilkinson et al., 2016b). The analyses provided the opportunity to assess differentially expressed genes (DEGs) in early infection, and to contrast transcripts in PRRS 'resilient' (RES; n = 8) and 'susceptible' (SUS; n = 8) gilts with mean fetal mortality rates of 5% (range: 0–13%) and 76% (range: 67–94%) respectively. In addition to the profound DEGs associated with type I and II interferon responses discussed above, the blood transcriptome at 2 dpi reflected increased cytokine, apoptosis and inflammatory responses, and decreased protein synthesis and T cell receptor signaling compared to 0 dpi.

Fifteen genes were up-regulated in RES gilts across all three time-points, of which seven encoded proteins with known functions in platelets (Fig. 2). In addition to important functions related to the repair of vascular damage, an important initiating lesion of PRRSV infection, platelets are effector cells of

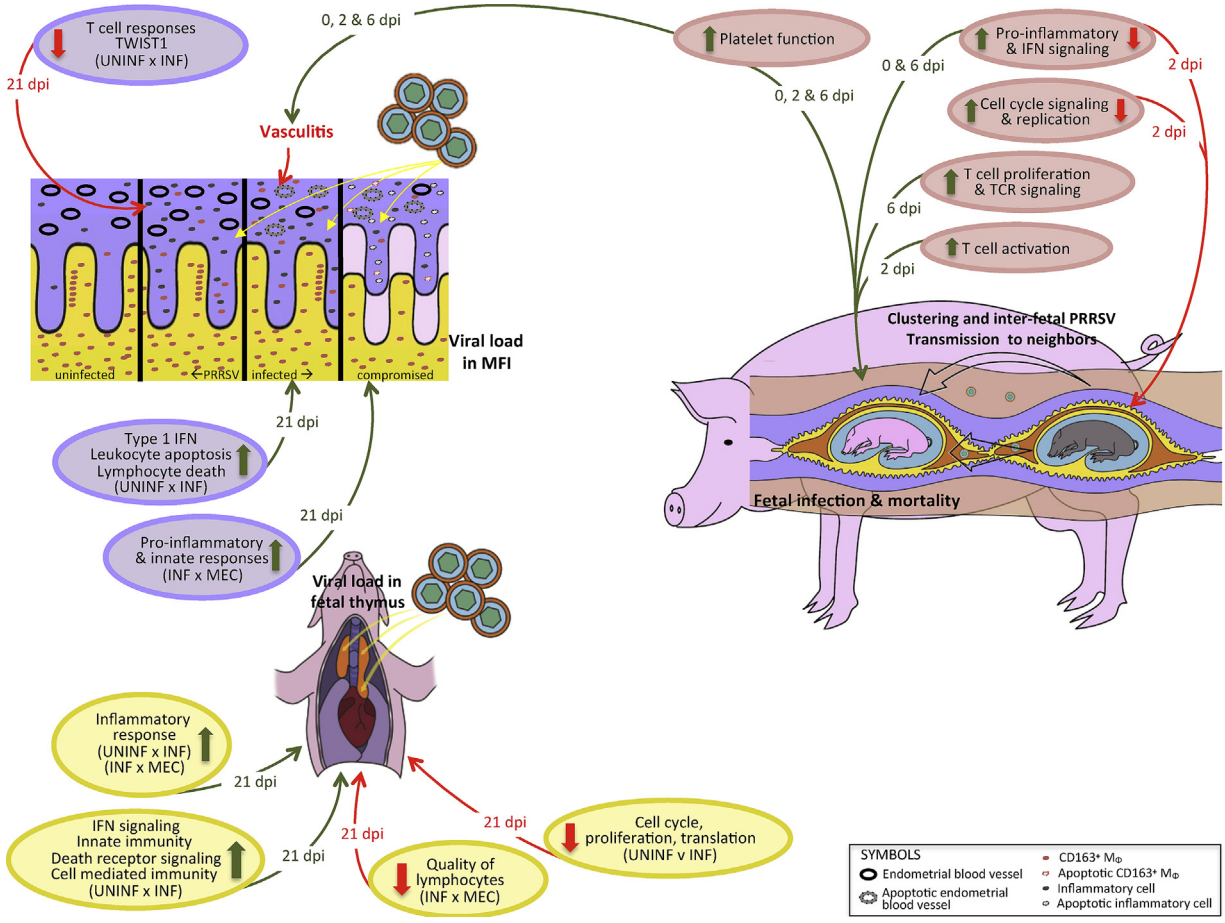

Fig. 2. Transcriptomic responses associated with the pathophysiology of reproductive PRRS. Transcriptomic responses beneficially (green arrows) or adversely (red arrows) associated with events in the maternal-fetal interface (top left; endometrium = purple, fetal placenta = gold), fetal thymus (bottom left), and fetal infection and/or mortality (right). Background color of oval is indicative of the tissue used for the RNA-Seq: purple = maternal-fetal interface (endometrium + fetal placenta); yellow = fetal thymus (uninfected UNINF; infected INF; meconium stained MEC); pink = whole blood of dam. References: Wilkinson et al., 2016a,b.

inflammation with important myeloid-like functions (pro-inflammatory, antimicrobial), and facilitate CTL responses in murine models of viral infection (Elzey et al., 2003; Iannacone et al., 2008). Thus, it is possible that higher baseline platelet function is associated with faster response to vascular injury and innate cellular responses that temper PRRSV-initiated inflammation, vasculitis and potentially apoptosis at the maternal-fetal interface following infection. In addition to greater platelet function, low fetal mortality RES gilts had higher basal IFN and pro-inflammatory gene expression prior to PRRSV inoculation which suggests an opportunity to select animals for PRRS resilience in PRRSV-free herds. By 2 dpi, the expression of pro-inflammatory genes in the RES group was lower than in the high fetal mortality SUS group, while expression of T cell-related transcripts was higher. At day 6, IFNγ signaling was higher in low than high fetal mortality gilts (Fig. 2). Overall, the oscillating expression data suggests that lower fetal mortality may be related to higher basal innate and inflammatory responses followed by faster transition from innate to adaptive responses following PRRSV infection (Wilkinson et al., 2016b). Various researchers have investigated relationships between health and performance traits, and innate and adaptive immune responses in pigs (Clapperton et al., 2005, 2009; Flori et al., 2011a,b; Wilkie and Mallard, 1999); some reporting heritability estimates (h^2) for various immunological traits. Innate responses reported to have high heritability ($0.45 < h^2 < 0.81$) include cytokines interleukin 12 and interferon-alpha, and counts of various cell subsets such as platelets, lymphocytes, neutrophils and natural killer cells (Flori et al., 2011a). Similarly, a number of antibody-mediated and cell-mediated immune response traits were also reported to have high heritability (antibody $0.68 < h^2 < 0.92$; cellular $0.52 < h^2 < 1.0$) (Flori et al., 2011a). More specific to reproductive PRRS, IgG antibody response measured by ELISA following a commercial farm outbreak had moderate heritability ($h^2 = 0.45$ SEM $\pm$ 0.13) (Serao et al., 2014). This data collectively supports our hypothesis that variation in the phenotypic response to PRRS-2 virus infection in pregnant gilts may in part be genetically controlled involving various innate and adaptive immune response traits.

4. Responses to PRRSV-2 PRRSV in the fetus

4.1. Fetal preservation and viral load

It has been proposed that fetal death is the result of apoptosis at the fetal implantation site (Karniychuk et al., 2011) and unrelated to fetal pathology (Rossow et al., 1996). Although the PGM also demonstrated a positive association between apoptosis and PRRSV RNA concentration at the maternal-fetal interface (Fig. 1) (Novakovic et al., 2017) there is unfortunately no conclusive evidence that apoptosis *per se* leads to sufficient placental insufficiency or detachment by itself to cause death of the fetus. Moreover, the presence and/or severity of apoptosis in the fetus, and its relationship to viral load have not been investigated, but could plausibly contribute to fetal demise. In the PGM, fetal preservation at 21 dpi was categorized using a scoring system related to susceptibility and the estimated timing of fetal infection; from most susceptible (autolysed > decomposed) to most resilient (viable > meconium stained) (Ladinig et al., 2014d). Fetuses were allocated into these two broad categories in an approximate 60:40 split. In the early phase of the project, meconium staining (MEC) was identified as phenotype indicative of fetal compromise and imminent death in agreement with previous findings (Lager and Halbur, 1996). Viral load in fetal thymus and serum was significantly related to preservation category; being highest in MEC fetuses and lowest in viable (VIA) fetuses (Ladinig et al., 2014d). Moreover, PRRSV RNA concentration at the maternal-fetal interface was a strong predictor of fetal viral load and the odds of fetal death (Fig. 1). This relationship between PRRSV RNA concentration in the maternal-fetal interface and fetal thymus was linear, with each 1 logarithm base 10 ($\log_{10}$) per mg increase at the maternal-fetal interface associated with a 0.58 $\log_{10}$/mg increase in fetal thymus across the ~1400 PGM fetuses (Ladinig et al., 2015a).

At 21 dpi, the body weight of live fetuses (VIA, MEC) was significantly lower in PRRSV-inoculated *versus* sham-inoculated gilts by about 17% (Ladinig et al., 2014d) (Fig. 1). Although low experimental power limited statistical inference, our comparison of three PRRSV-2 strains demonstrated numeric differences with KS06-483 infection having the least effect on fetal body weights and mortality, even though viral load did not differ when compared to the KS06-72109 or NVSL97-7895 infected groups (Ladinig et al., 2015b).

Fetal viral load and death cluster within litter (Fig. 1) with the first two adjacent fetuses (either to right, left, or both) being more influential than the next two (third and fourth) adjacent fetuses. This finding, along with spatial patterns within litters, indicate that horizontal infection spreads laterally (fetus to fetus) starting from a selected number of "index" fetuses (Ladinig et al., 2015a). While there is an incomplete understanding of factors explaining why index fetuses are at the highest risk of infection, it may be related to their placentation. During periods of nutrient restriction or placental inefficiency, fetuses are subjected to intra-uterine growth retardation (IUGR), characterized by the disproportionate growth of brain tissue at the expense of all other fetal organs. This phenomenon is common to all mammals and results from numerous genetic, epigenetic, endocrine and environmental factors (Wu et al., 2007). Although it is intuitive to think that IUGR fetuses would have higher viral load associated with placental compromise, the opposite appears to occur. PGM results demonstrated that PRRSV RNA concentration in fetal thymus was over 3 logs lower in IUGR fetuses compared to their non-IUGR siblings (Ladinig et al., 2014a), indicating small (IUGR) fetuses have a possible advantage in terms of PRRSV resilience. It is not clear if this is related to fundamental differences in the metabolism of IUGR and non-IUGR fetuses, or if differences in placental size or efficiency place non-IUGR fetuses at greater risk of transplacental transmission, although the same trends are noted with viral concentration at the maternal fetal interface. This is further supported by PGM results demonstrating that crown-rump length of autolysed and viable fetuses did not differ, even though autolysed fetuses died an estimated 7 or more days prior to termination of the dam, whereas viable fetuses lived until the end of the experiment.

4.2. Fetal pathology

While several previous reports have surmised that the absence of fetal lesions in stillborn pigs or the lack of correlation between fetal pathology and productive infection provides evidence that fetal death is not a direct consequence of PRRSV replications in fetal tissues (Karniychuk et al., 2011; Rossow et al., 1996) these previous studies have had insufficient sample sizes to detect subtle relationships. Although lesions in PGM fetuses were infrequent (present in only 11.4% of mesenteric lymph node, 5.9% of umbilical cord, 3.2% of heart, 2.5% of liver, and 2.2% of cerebellar samples), the odds ratio of meconium-staining was 2.1 times higher in fetuses with one or more fetal lesions, and 6.9 times higher in fetuses with umbilical lesions compared to fetuses with no lesions (Fig. 1). The presence of fetal lesions was also positively associated with viral load in fetal thymus, but unrelated to the severity of vasculitis and inflammation at the maternal fetal interface (Novakovic et al., 2016a). Fetal infection doubled the odds of fetal death (Ladinig

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et al., 2015a). Together, these results support our conclusion that events in the fetal compartment, in addition to those in the maternal-fetal interface, are associated with fetal demise.

4.3. Fetal transcriptome association with disease progression

A detailed understanding of the fetal transcriptome associated with endometrial and/or fetal infection is poor. Rowland (2010) demonstrated mRNA transcription of TNFα (from lung) and interleukin 10 (IL-10)(from lung and mandibular lymph node) in randomly selected, near term fetuses sampled ~3 weeks after infection with PRRSV-2. Moreover, TNFα and IFNγ protein were detectable in fetal serum, but not amniotic fluid, indicating the fetus is "immunocompetent and capable of initiating an antiviral response" (Rowland, 2010). We attempted to measure cytokine proteins in the serum of PRRSV-infected and non-infected PGM fetuses using a fluorescent microsphere immunoassay (IL-1β, IL-4, IL-8, IL-10, IL-12, IFNα, CCL2) and ELISA (IFNγ) but failed to detect group differences in any cytokine (Ladinig, unpublished).

To provide a more detailed understanding of how the fetal transcriptome changes in relation to disease progression, we performed RNA-Seq on fetal thymus of four strategically selected PGM groups: uninfected fetuses from sham-inoculated gilts, uninfected fetuses from PRRSV-infected gilts, infected viable fetuses from PRRSV-infected gilts, and infected meconium-stained fetuses from PRRSV-infected gilts (Wilkinson et al., 2016a). The expression profile of infected, viable fetuses (compared to uninfected fetuses from the same dams) was characterized by up-regulation of genes involved in innate immunity, inflammation (including pro-inflammatory and antiviral cytokines, complement, acute phase proteins), pathogen recognition receptors and cell death signaling (apoptosis), and down-regulation of genes involved in cell cycle, proliferation and mRNA translation. There was a further up-regulation of genes involved in pro-inflammatory responses and down-regulation of genes involved in T cell signaling and lymphocyte quality as disease progressed from viable to meconium-staining. However, progression to meconium-staining did not involve the up-regulation of genes involved in type I interferon responses, which might be related to the concurrent PRRSV-induced IFNα suppression.

The difference in gene expression profile of uninfected fetuses from PRRSV-infected gilts compared with control fetuses from sham-inoculated gilts was remarkable, and demonstrated that fetuses are affected by PRRSV-infection at the maternal-fetal interface even prior to their own infection. In this contrast, the gene expression profile is primarily inflammatory in nature containing genes associated with activation of leukocytes, calcium signaling, agranulocyte and granulocyte adhesion and diapedesis, and atherosclerosis. However, there is an absence of expression differences in genes associated with antiviral (interferon) responses. These responses are likely induced by inflammatory processes underway in the maternal-fetal interface, and may be indicative of early stages of fetal hypoxia, however, hypoxia *per se*, has never been directly quantified in PRRSV-infected fetuses.

5. Responses to PRRSV-2 infection in the maternal-fetal interface

5.1. Pathology and viral load

The maternal-fetal interface is comprised of endometrial tissue layers (lamina propria, vasculature, mesenchymal and glandular areas), interface (interdigitations of maternal uterine epithelium and fetal trophoblastic epithelium), and fetal allantochorion (mesenchyme and vasculature). In the diffuse, epitheliochorial placentation, nutrients pass from maternal to fetal vessels across six cell layers (hematotroph). The two intact epithelial layers (maternal epithelium, fetal trophoblast) prevent the transmission of large molecules such as immunoglobulins, which are considerably smaller than PRRS viral particles, from the dam to fetus (Karniychuk and Nauwynck, 2013). In addition, maternal uterine glands supply nutrients in the form of uterine milk to specialized fetal structures (areolae) by way of pinocytosis (histotroph) (Bazer and Johnson, 2014). Three mechanisms of transplacental PRRS viral transmission have been proposed (Karniychuk and Nauwynck, 2013): (a) direct movement of free virus, (b) movement of virus into and through intact epithelial cell layers, and (c) movement by way of infected maternal macrophages that migrate from maternal to fetal tissues.

PRRSV-induced lesions in the maternal-fetal interface include mild to severe lymphocytic endometritis, vasculitis and myometritis, and lymphohistiocytic placentitis. At 21 dpi endometritis and vasculitis are common in maternal tissues (affected >99% of PGM samples). Myometritis was less commonly observed (58% of PGM samples) and placentitis rare (6.6% of PGM samples) but in line with the prevalence of lesions in other fetal tissues and umbilical cord (Novakovic et al., 2016a). This low prevalence of fetal and placental lesions is in contrast to the fetal transcriptomic profile which is largely characterized by an up-regulation of genes involved in inflammation and innate responses (Wilkinson et al., 2016a), suggesting the fetus may have insufficient resources to produce an encompassing cellular inflammatory infiltrate in organs and placenta. Similarly, PRRSV immunohistochemical staining (SDOW17) is most intense in the inflammatory cell infiltrates at the maternal-fetal interface mainly involving macrophages and occasionally epithelial cells of maternal uterine glands and fetal areolae, but viral staining is rare in the surrounding fetal mesenchymal cells (Novakovic et al., 2016b).

5.2. Macrophage subsets and viral load

$CD163^+$ is a surface marker of monocytes and macrophages with capacity to exert strong anti-inflammatory function in local tissues. CD163 expression is down-regulated by pro-inflammatory mediators and up-regulated by glucocorticoids and IL-10 (Kowal et al., 2011). Following PRRSV-infection of uterine tissue, the richest population of $CD163^+$ macrophages is present in the interdigitating interface region (212 cells/mm^2) and is markedly lower (34 cells/mm^2) in the endometrium within inflammatory infiltrates in lamina propria and around blood vessels. Sialoadhesin/$CD169^+$ cell counts follow a similar trend but are lower in number overall (69 cells/mm^2 in placenta, 15 cells/mm^2 in endometrium) (Novakovic et al., 2016a). The expression of sialoadhesin on $CD163^+$ cells is up-regulated in endometrium and placenta following PRRSV infection (Karniychuk et al., 2013).

Given that $CD163^+$ are permissive to PRRSV infection, one would expect viral load to increase with $CD163^+$ cell numbers. While cell numbers increase in endometrium following PRRSV infection, increases in cell numbers in placenta are more muted; seen in association with low, but not high viral load in placenta. The relationship between $CD163^+$ cell counts and viral load in fetal thymus is equally interesting (Fig. 1). High levels of thymic viral load are associated with greater $CD163^+$ cell counts in endometrium, but decreased numbers in fetal placenta (Novakovic et al., 2016b). While the exact reason for this dichotomy is not understood, we hypothesized two processes may be involved. Firstly, PRRSV infection may be lytic to a proportion of placental $CD163^+$macrophages, which could explain the modest decline in $CD163^+$ cells associated with high viral load. Secondly, the $CD163^+$ population in placenta may also include a high residential population of Hofbaurer–like cells, which in human placentae, are thought to have a role in preventing transmission of pathogens

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from dam to fetus (Bulmer and Johnson, 1984; Tang et al., 2011). If correct, this may in part explain the high basal numbers of CD163$^+$ in the placenta, and their negative association with viral load in fetal thymus, opposite to that noted in endometrium (Novakovic et al., 2016b).

5.3. Apoptosis at the maternal fetal interface

Apoptosis is an important physiologic process required for proper remodelling of the porcine placenta during all gestational stages (Cristofolini et al., 2013), and it is therefore not unexpected that it is evident in the maternal-fetal interface of both PRRS-infected and non-inoculated control animals (Karniychuk et al., 2011; Novakovic et al.,2017). However, the number of apoptotic cells in endometrium and placenta significantly increases following PRRSV infection (Fig. 1) (Karniychuk et al., 2011; Novakovic et al., 2017). Apoptotic cells in endometrium included inflammatory cells (mainly lymphocytes, macrophages), occasional uterine glandular epithelial cells, and rarely endothelial cells of inflamed maternal vessels (Novakovic et al., 2017, while those in the placenta were distributed in the mesenchyme (Karniychuk et al., 2011). Across the maternal-fetal interface region, apoptosis ranged in severity from individual cells to multifocal areas in fetal or maternal tissues, sometimes associated with placental separation. Apoptosis at the maternal-fetal interface was positively associated with the severity of vasculitis in endometrium and with PRRSV RNA concentration in fetal thymus. Moreover, the odds of meconium staining, an early pathological indicator of fetal compromise, was positively associated with the number of TUNEL positive cells (per mm^2 of interface tissue) (Fig. 1) (Novakovic et al., 2017). Thus, endometrial vasculitis and PRRS-induced apoptosis of maternal uterine and glandular epithelium may all contribute to reduced growth and eventual compromise by impeding transfer of hematotrophic nutrition. This may also explain why larger (non-IUGR) fetuses have higher risk of PRRSV infection, given their expected greater demand for nutrients compared to IUGR fetuses.

5.4. PRRS-induced transcriptomic responses in the maternal-fetal interface

PGM transcriptomic analyses enabled the characterization of endometrial gene expression relative to disease progression in fetuses at 21 dpi. Three contrasts were undertaken comparing uninfected fetuses in control and PRRSV-infected gilts, infected to non-infected fetuses, and infected to meconium-stained fetuses in PRRSV-infected gilts. Genes associated with innate and antiviral responses were up-regulated in all three contrasts demonstrating increasingly stronger innate responses associated with disease

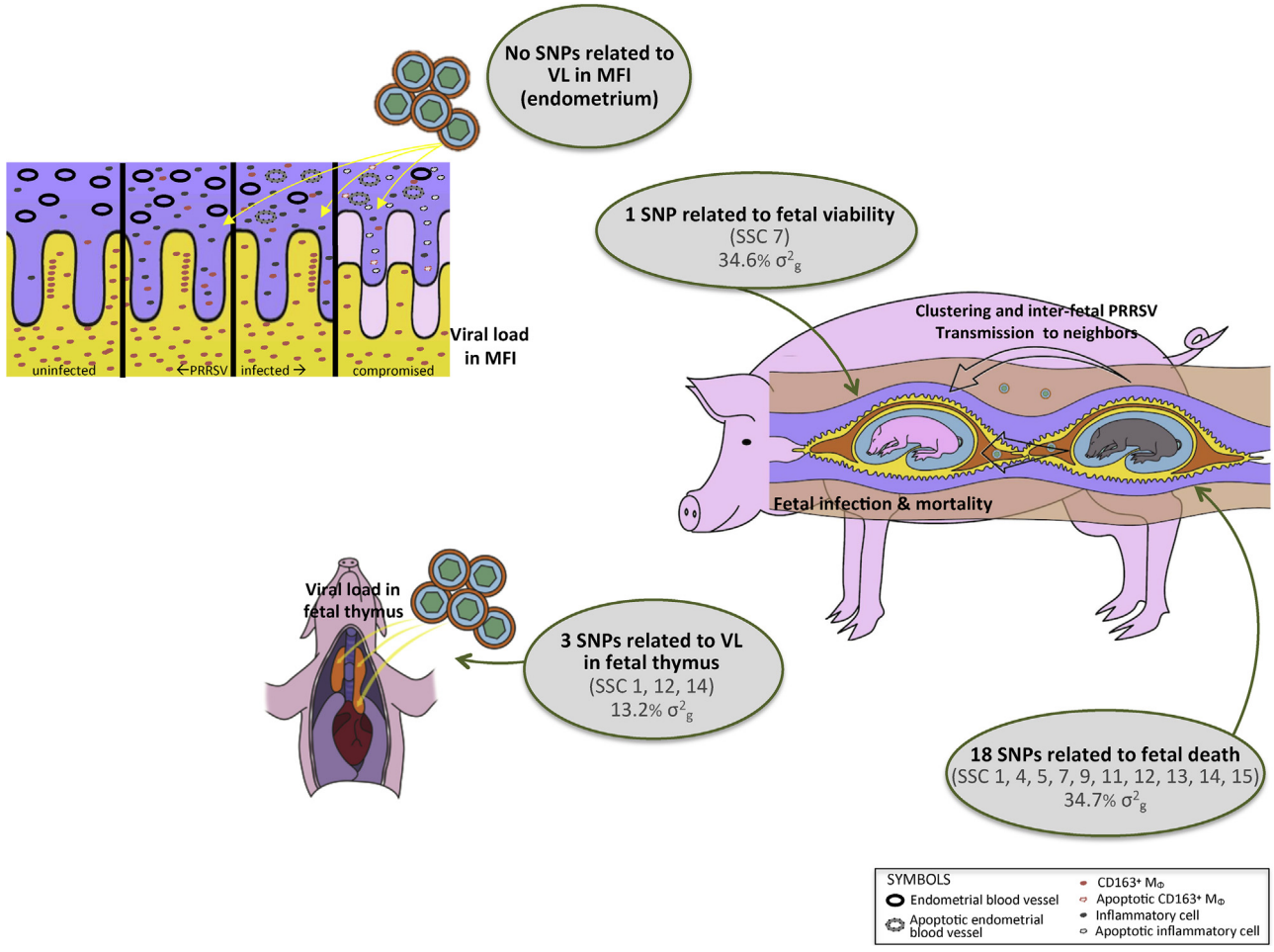

Fig. 3. Genomic responses associated with the pathophysiology of reproductive PRRS. Single nucleotide polymorphisms (SNPs) found to be significantly associated with three fetal outcomes of interest 21 days post inoculation: (a) fetal viability (viable vs. meconium stained + decomposed), b) fetal viral load in thymus, and (c) fetal death (viable + meconium stained vs. decomposed). For each trait, the number of SNPs significantly associated with the trait and their chromosomal location (SSC) is shown. σ^2_g = the total genetic variance explained by the collection of SNPs identified for each trait. VLthy = viral load in fetal thymus. Reference: Yang et al., 2016.

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progression (Wilkinson et al., 2016a). By contrast, TWIST1, a placental transcription factor involved in placental development and maturation was increasingly inhibited as fetal disease progressed from uninfected to meconium-staining (Ng et al., 2012; Pirinen and Soini, 2014; Yamakoshi et al., 2012). Although up-regulated compared to non-infected gilts, fetal infection was associated with a down-regulation of genes involved in adaptive immune response (T cell receptor and T helper cell signaling, CTL activation, B cell development) and a concurrent up-regulation of genes associated with apoptosis and cellular death (Fig. 2) (Wilkinson et al., 2016a).

6. Genome wide association studies (GWAS)

In recent years, several single nucleotide polymorphisms (SNPs) have been discovered associated with PRRS outcome following experimental or natural challenge. The most characterized is located on *Sus scrofa* chromosome (SSC) 4 and susceptibility is associated with the production of a defective Guanylate binding protein 5 (GBP5) which is normally involved in inflammasome assembly during the immune response (Boddicker et al., 2012; Koltes et al., 2015). During a farm outbreak of reproductive PRRS in the field, PRRSV immunoglobulin G level (sample/positive ELISA ratio; S/P) was highly heritable ($h^2 = 0.45$) and had high genetic correlation with litter size traits. Three regions on SSC7 accounted for over 40% of the genetic variation in S/P (Serao et al., 2014).

The large sample sizes of PGM enabled a rare opportunity to analyze a relatively clean experimental dataset to identify new genomic regions associated with reproductive PRRS outcome. A number of GWAS, including 928 fetuses genotyped using the PorcineSNP60 Genotyping BeadChip v2 (Illumina, San Diego, CA, USA), were undertaken to identify SNPs associated with viral load in fetal thymus and endometrium, fetal viability and fetal death. Twenty-one candidate genomic regions across 10 chromosomes were found to be significantly associated with these traits (Fig. 3), seven of which overlapped with previously reported quantitative trait loci (QTLs) for pig health and reproduction (Yang et al., 2016). The three SNPs associated with viral load in fetal thymus and 18 SNPs associated with fetal death were additive, meaning additional favorable alleles were associated with a more positive outcome; *i.e.*, decreased viral load and lower likelihood of being meconium-stained or decomposed, respectively. The one SNP associated with fetal viability explained 34% of the total genetic variation; a very considerable amount. Moreover, several DEGs mapped to candidate genomic regions identified. Many of these candidate regions had QTLs putatively involved in innate and adaptive immune responses (IFN modulation/suppression, NK cell activation, monocyte activation, T cell regulation), apoptosis or response to secondary infections adding evidence to their biologic relevance. Thus, the GWAS results provide new evidence about the genetic basis of fetal response to PRRSV challenge, and may ultimately lead to alternative control strategies to reduce the impact of reproductive PRRS.

7. Novel insights associated with the pathophysiology of reproductive PRRS

In spite of the fact that the PGM was a single experiment using one line of Landrace gilts, a single strain of PRRSV-2, and a single termination point, it contributed considerable new knowledge regarding the pathophysiology of reproductive PRRS. Prior to the PGM, the importance of apoptosis, and primary sites of viral replication were reported, however, it is concerning that for a disease with such devastating economic consequences, this historic knowledge is based on few studies each using a small number of sows. One of the most important features of the PGM was the large number of deeply phenotyped animals (150 pregnant gilts, ~1670 fetuses) and the multidisciplinary approach, including traditional and several 'omic technologies, used to advance knowledge related to reproductive PRRS pathophysiology.

As graphically illustrated in Figs. 1–3, the outcome of PRRSV-2 infection during late gestation is dependent on events occurring at the maternal-fetal interface, individual fetuses, uterine compartment, and to a lesser degree on systemic responses in the dam. Based on the results of the PGM and historic knowledge, we are now able to report a number of new insights. Following systemic infection, the virus transmits to endometrium, sometimes within two days (Malgarin et al., 2017), inducing endometrial inflammation and vasculitis (Novakovic et al., 2016a). The virus replicates in $CD163^+$ macrophages within the inflammatory cell infiltrate, and induces apoptosis of macrophages and surrounding bystander inflammatory cells, as well as in the interdigitating interface region, uterine epithelium and fetal trophoblast associated with varying degrees of placental separation (Karniychuk et al., 2011; Novakovic et al., 2017). Suppression of genes associated with angiogenesis and placental implantation (TWIST) (Wilkinson et al., 2016a) may contribute to a progressive loss of placental attachment. Vasculitis, inflammatory infiltrates, and apoptosis of the uterine/fetal epithelium may all adversely affect hematotrophic nutrient transfer to the fetus. In addition, apoptosis of uterine gland epithelium may potentially affect histotrophic nutrient transfer. The adverse effect on both histotrophic and hemotrophic nutrition is a possible explanation for the decreased weight gain of infected, viable fetuses observed in the PGM (Ladinig et al., 2014d).

The mechanism(s) by which PRRSV crosses the uterine epithelium and fetal trophoblast is presently unknown. In addition to the three mechanisms proposed by Karniychuk and Nauwynck (2013), transmission by way of histotroph or hematotroph is a possible mechanism worthy of exploration in our opinion. Microchimerism, the trafficking of a small number of cells from one individual to a relative (sibling and dam) during gestation occurs naturally in pigs, regardless of PRRSV-infection status, and suggests a possible mechanism of transplacental and inter-fetal viral transmission (Karniychuk et al., 2012). However, for this to occur transplacentally, the cells that traffic between the dam and fetus must be PRRSV-permissive and must pass through two intact epithelial barriers (uterine epithelium and fetal trophoblast) which to date has not been conclusively demonstrated. Moreover, it must occur rapidly, because PRRSV RNA is abundant in placental tissue by 2 dpi (Malgarin et al., 2017; Suleman et al., 2017). Present evidence suggests that the virus infects individual "index" fetuses first, then transmits laterally to adjacent neighboring fetuses (Ladinig et al., 2015a), most likely by way of the necrotic ends of the allantochorion which commonly overlap between adjacent fetuses (Karniychuk et al., 2012). It is not presently understood if inter-fetal transmission is associated with free virus or infected fetal cells of any type. Viral load and fetal death cluster within the litter, but are not associated with sex or fetal position within the uterine horn (Ladinig et al., 2015a). Large (non-IUGR) fetuses appear to be more PRRSV susceptible than fetuses experiencing IUGR (Ladinig et al., 2014a), possibly due to their higher nutrient requirements or larger placentae. Fetal compromise is associated with apoptosis and high levels of virus in the maternal-fetal interface (Ladinig et al., 2015a; Novakovic et al., 2017). PRRSV also is associated with increased numbers of $CD8^+CD3^-$ cells (putatively uterine NK cells) in the endometrium; in connective tissue and in close proximity to blood vessels and uterine epithelium. It is proposed that these cells may disrupt the delicate feto-maternal immunological balance, or be directly responsible for tissue damage within the maternal-fetal interface (Karniychuk et al., 2013). PRRSV-specific lesions in fetal organs and umbilical cord rarely occur, but are associated with increased viral load and meconium staining, but not with lesions in

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endometrium (Novakovic et al., 2016a); clear evidence that the virus is directly affecting the fetus.

Several phenotypic responses may be protective, although the mechanisms are not fully understood. Firstly, numbers of T helper cells and myeloid cells in peripheral circulation in early and late infection, respectively, may be beneficial. While IFNα suppression is a hallmark consequence of PRRSV infection, higher levels of suppression appear to be advantageous for fetal survival (Ladinig et al., 2015a). Finally, $CD163^{+}$ cell numbers in fetal placenta are associated with lower viral load in fetal thymus (Novakovic et al., 2016b).

The use of multiple functional genomic technologies in the PGM provided new insights on the underlying host response to reproductive PRRSV infection, and may lead to novel control strategies in the future. The transcription profile detectable in blood of dams in acute infection; characterized by changes in expression levels of inflammatory and innate immune responses, T cell signaling and activation, and cell cycle signaling and replication, suggests that low fetal mortality is associated with a higher basal and faster anti-viral immune responses (Wilkinson et al., 2016b). Interestingly, higher expression levels related to platelet function featured prominently in the profile of resilient (low fetal mortality) gilts. In fetuses and the maternal-fetal interface, disease progression is associated with up-regulated gene expression profiles related to inflammatory, interferon, innate immunity, death receptor signaling and induction of adaptive immunity, but down-regulated expression of genes associated with lymphocyte quality and cell cycling (Wilkinson et al., 2016a). Finally, a number of candidate genomic regions are associated with reproductive outcome, including viral load in fetal thymus, fetal viability and fetal death. Together, these regions account for a biologically relevant portion of the overall genetic variation, and overlap with other known QTLs related to swine health and immunological response (Yang et al., 2016).

8. Conclusions

The PGM dramatically expanded our understanding of the pathophysiology of reproductive PRRS, and for the first time, provided clear evidence that, in addition to events at the maternal-fetal interface, events occurring in fetuses and the uterine compartment are essential in the pathogenesis of reproductive failure in late gestation gilts. Continued research in this area will explore some of the many remaining knowledge gaps with the ultimate goal of identifying and exploiting resilient genotypes.

Animal ethics statement

The experiment was approved by the University of Saskatchewan' s Animal Research Ethics Board, and adhered to the Canadian Council on Animal Care guidelines for humane animal use (protocol #20110102). Readers are referred to Ladinig et al. (2014d) or specific details of the PGM animal experiment.

Conflict of interest

The authors have no conflicts of interest to declare.

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