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HUMORAL AND CELL-MEDIATED IMMUNITY TO PORCINE CIRCOVIRUS TYPE 2 (PCV2) VACCINATION AND NATURAL INFECTION

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To my family,

To Dario

Abstract

Porcine circovirus type 2 (PCV2) has been identified as the main causative agent of the postweaning multisystemic wasting syndrome (PMWS), one of the major swine diseases worldwide that is commonly referred, together with other relevant porcine diseases related to PCV2, as belonging to the porcine circovirus associated diseases (PCVD).

The most important strategy to prevent and control PCV2 associated diseases, apart from management procedures and control of coinfections, is the vaccination of piglets or sows and gilts. Nowadays there are three commercial PCV2 vaccines available; even if their efficacy in reducing the viremia burden and viral-induced specific lymphoid lesions has been proved, the mechanisms by which they are able to elicit protective immunity have not been thoroughly clarified. Besides the development of humoral immunity that is generally characterised by the detection of total anti-PCV2 and virus-neutralizing antibodies, the mechanisms that allow the adaptive cell-mediated immune response to control PCV2 infection and the related diseases have not been clearly elucidated, particularly under field conditions.

The present Thesis investigated the efficacy of a one-dose porcine circovirus 2 (PCV2) subunit vaccine based on the PCV2 Cap protein expressed in a baculovirus system in two different farms (farm1 and 2) at which a history of porcine circovirus-associated disease (PCVD) was present. Morbidity, mortality, average daily weight gain, carcass weight, PCV2 load in serum and vaccine immunogenicity, in terms of PCV2-specific antibodies, PCV2-specific IFN- γ secreting cell frequencies and mRNA expression profiles of relevant pro-inflammatory and immune cytokines, were assessed. Serology to potential coinfections due to porcine reproductive and respiratory syndrome virus (PRRSV) and *Mycoplasma hyopneumoniae* (*M. hyo.*) was also carried out.

A double-blind, randomised, and controlled field trial was performed distributing 818 piglets in two treatment groups. At inclusion (weaning at 21 ± 3 days of age), 408 animals received a 2-ml intramuscular dose of Porcilis PCV[®] (vaccinated group) suspended in a tocopherol-based adjuvant (Diluvac Forte[®]). Controls (410 piglets) received 2 ml of the same adjuvant alone intramuscularly. Weights were recorded at inclusion and at 12 and 26 weeks of age, and the average daily weight gain (ADWG) was calculated. The carcass weights of the pigs from farm 2 were recorded at slaughter (274 day-old pigs). All dead animals (died or culled) underwent autopsy to classify them as PMWS-affected or not. At each farm, blood samples were collected for serologic and cellular studies aimed at investigating the humoral (ELISA determination of PCV2-antibody titres in serum) and cell-mediated (ELISpot assay

for the measurement of the PCV2-specific IFN- γ secreting cell frequencies in PBMC) immune response of pigs.

The analyses of the present Thesis showed that vaccination with a single dose of a PCV2 Cap vaccine had beneficial effects against the PCVD, and especially PMWS. The vaccination reduced the mortality rate and morbidity, PCV2 viremia and viral load, and improved productive performances (e.g. ADWG: +70 g/day between 12 and 26 weeks of age when viremia and the specific disease occurred) as well as carcass weight at slaughter age (+4.5 kg). These effects were associated with virologic and clinical protection derived from the immunogenicity of the vaccine measured as activation of both humoral and a cellular immune responses. In this regard, ELISA quantification of PCV2-specific antibodies showed seroconversion (with exception of pigs with a titre of maternally derived antibodies $>8 \log_2$) and long lasting protective immunity in all vaccinated pigs. Furthermore, the increased frequency of IFN- γ secreting cells that was detected by the ELISpot assay during the post-vaccination period demonstrated the capability of a single dose of the PCV2 Cap-based vaccine to induce a virus-specific cell-mediated immune response. During the post-exposure period, vaccinated animals rapidly and efficiently counteracted virus spread since both humoral and cell-mediated immunity were associated with absent or low viremia and less severe clinical signs.

In addition, in order to obtain more thorough information about the mechanisms of cellular immune reactivity, the evaluation of expression patterns of relevant pro-inflammatory (IL-8, TNF- α , IL-1 β) and immune (IFN- γ , IL-10) cytokines was carried out.

Cytokine modulation and course of viremia were assessed in 10 PCV2-vaccinated and 20 non-vaccinated pigs from farm 1. These analyses were performed by reverse transcriptional-quantitative PCR (RT-qPCR) before the onset of PCV2 viremia (16 weeks of age), upon PCV2 infection and after the onset of PMWS clinical signs (19 and 22 weeks of age, respectively). The cytokine response was evaluated with regards to evident clinical signs related to PMWS and course of viremia, grouping the animals into three groups: 1) vaccinated (PCV2-vac) pigs; 2) unvaccinated spontaneously infected/non-PMWS-affected (Ctrl) pigs; 3) unvaccinated spontaneously infected/PMWS-affected (Ctrl-PMWS+) pigs.

Moreover, in order to establish an association between cytokine expression and viremia burden, each of the above mentioned groups was analysed dividing the animals in three different subgroups based on viremia: non-viremic pigs (NV), pigs with viremia $<10^6$ ($V < 10^6$) and pigs with viremia $\geq 10^6$ ($V \geq 10^6$) viral genome copy number / ml of serum.

Higher IL-8, TNF- α and IFN- γ levels were detected in the PCV2-vac group, testifying a more efficient immune responsiveness, especially when compared to the Ctrl-PMWS+ group.

In Ctrl-PMWS+ pigs, lower IFN- γ at 19 weeks of age was associated with high IL-10 at 19 weeks of age and low levels of pro-inflammatory cytokines at 22 weeks of age, namely IL-8 and TNF- α , a condition likely correlated with the onset of the disease.

Contrarily, at 19 weeks of age, PCV2-vac and Ctrl pigs showed lower IL-10 expression, together with higher IFN- γ levels than the Ctrl-PMWS+ animals. At 22 weeks of age, vaccinated animals maintained higher levels of the pro-inflammatory cytokines.

These evidences support that the outcome of PMWS could be associated with a reduction of the innate/pro-inflammatory response. Overall, the results show a different cytokine modulation in vaccinated and unvaccinated-infected pigs also developing PMWS. Vaccinated pigs coped with infection showing low or absent viremia burden, absence of PMWS disease and stronger inflammatory response and cellular IFN- γ -related reactivity.

Riassunto

Il Circovirus suino di tipo 2 (PCV2) è stato identificato come il principale agente eziologico della sindrome da deperimento post-svezzamento del suino (PMWS), una delle patologie del suino maggiormente diffuse in tutto il mondo, comunemente indicata, insieme ad altre patologie legate all'infezione da PCV2, come facente parte delle malattie associate al circovirus suino (PCVD).

La strategia più importante per controllare e prevenire le malattie associate a PCV2, oltre alle procedure di gestione e controllo delle coinfezioni, è la vaccinazione dei suinetti o delle scrofe e delle scrofette. Tre sono i vaccini commerciali oggi disponibili; anche se è stata dimostrata la loro efficacia nel ridurre la viremia e le lesioni presenti nei tessuti linfoidi indotte dal virus, i meccanismi attraverso i quali questi vaccini sono in grado di indurre un'immunità protettiva non sono ancora stati completamente chiariti.

A parte lo sviluppo di una risposta immunitaria di tipo umorale generalmente caratterizzata dalla presenza di anticorpi totali e anticorpi virus neutralizzanti PCV2-specifici, i meccanismi che permettono all'immunità adattativa cellulo-mediata di controllare l'infezione data da PCV2 e le malattie ad essa associate non sono stati completamente compresi, specialmente in condizioni di campo.

In questo lavoro di Tesi è stata valutata l'efficacia di un vaccino monodose verso PCV2, basato sulla proteina capsidica Cap del virus espressa in un sistema baculovirus, somministrato in due diversi allevamenti (allevamento 1 e 2) con anamnesi di malattia associata a circovirus suino di tipo 2 (PCVD).

Sono stati considerati parametri quali morbilità, mortalità, incremento ponderale giornaliero, peso della carcassa, titolo di PCV2 nel siero e immunogenicità del vaccino in termini di anticorpi specifici verso PCV2, di frequenza di cellule secernenti IFN- γ PCV2-specifiche e di livelli d'espressione genica di importanti citochine pro-infiammatorie e immunitarie. Sono state inoltre effettuate analisi sierologiche verso potenziali coinfezioni sostenute da virus della sindrome riproduttiva e respiratoria del suino (PRRSV) e *Mycoplasma hyopneumoniae* (*M. hyo.*).

È stata condotta una prova di campo in doppio-cieco, randomizzata e con gruppo di controllo distribuendo 818 suinetti in due gruppi di trattamento. All'inizio della prova (giorno dello svezzamento: 21 ± 3 giorni di età), 408 suinetti (gruppo vaccinato) hanno ricevuto una dose di vaccino Porcilis PCV[®], risospeso in adiuvante a base di tocoferolo (Diluvac Forte[®]), per via intramuscolare (2 ml). Gli animali controllo (410 suinetti) hanno ricevuto 2 ml di solo

adiuvante per via intramuscolare. L'aumento ponderale giornaliero (ADWG) è stato calcolato misurando il peso degli animali all'inizio della prova, a 12 e a 16 settimane di età. I pesi delle carcasse dei suini dell'allevamento 2 sono stati registrati al momento della macellazione (274 giorni di vita). Tutti gli animali morti (morti o abbattuti) sono stati sottoposti ad autopsia per essere classificati come animali affetti o non affetti da PMWS.

In ciascun allevamento sono stati prelevati campioni di sangue per effettuare indagini sierologiche e della componente immunitaria cellulare. Le risposte immunitarie umorali e cellulo-mediate dei suini sono state valutate rispettivamente mediante ELISA, per rilevare il titolo degli anticorpi PCV2-specifici nel siero, e tecnica ELISpot, per misurare la frequenza delle cellule secernenti IFN- γ PCV2-specifiche nelle PBMC.

I risultati riportati nella presente Tesi suggeriscono che una singola dose di vaccino basato sulla proteina Cap di PCV2 sia efficace contro l'insorgenza delle PCVD e in particolare della PMWS.

La vaccinazione ha ridotto il tasso di mortalità e morbilità, la viremia specifica per PCV2 e la carica virale, portando a un miglioramento delle performance produttive (es. ADWG: 70 g/giorno tra 12 e 26 settimane di età, quando si registra l'insorgenza di viremia e malattia ad essa associata) e del peso della carcassa alla macellazione (+4,5 kg). Questi effetti sono stati associati alla protezione dall'infezione e dal manifestarsi di sintomatologia clinica determinata dall'immunogenicità del vaccino, misurata come attivazione della risposta immunitaria umorale e cellulo-mediata. A questo proposito, la quantificazione mediante tecnica ELISA degli anticorpi PCV2-specifici ha dimostrato sieroconversione (fatta eccezione per i suini con titolo di anticorpi di derivazione materna $>8 \log_2$) e immunità protettiva di lunga durata in tutti i suini vaccinati.

Inoltre, l'aumentata frequenza delle cellule secernenti IFN- γ PCV2-specifiche, quantificata mediante tecnica ELISpot durante il periodo post-vaccinazione, ha dimostrato la capacità di una singola dose di vaccino basato sulla proteina Cap di PCV2 di indurre una risposta immunitaria cellulo-mediata virus-specifica.

Durante il periodo post-esposizione gli animali vaccinati hanno contrastato efficacemente e rapidamente la replicazione virale; l'immunità umorale e cellulo-mediata sono risultate infatti associate ad una bassa o assente viremia e segni clinici di minor gravità.

Inoltre, al fine di ottenere informazioni più approfondite sui meccanismi di reattività immunitaria cellulare, sono stati valutati i profili d'espressione di importanti citochine pro-infiammatorie (IL-8, TNF- α , IL-1 β) e immunitarie (IFN- γ , IL-10). La modulazione

dell'espressione citochinica e il corso della viremia sono state valutate in 10 animali vaccinati contro PCV2 e 20 animali non vaccinati dell'allevamento 1. Queste analisi sono state effettuate mediante PCR quantitativa Retro Trascrizionale (RT-qPCR) prima dell'insorgenza della viremia associata a PCV2 (16 settimane di età) e a seguito dell'infezione da PCV2 e la comparsa di sintomatologia clinica da PMWS (19 e 22 settimane di età).

La risposta citochinica è stata valutata tenendo in considerazione gli evidenti segni clinici relativi alla PMWS e al corso della viremia, suddividendo gli animali in tre gruppi: 1) suini vaccinati (PCV2-vac); 2) suini non vaccinati spontaneamente infettati/non affetti da PMWS (Ctrl); 3) suini non vaccinati spontaneamente infettati/affetti da PMWS (Ctrl-PMWS+).

Inoltre, per determinare un'associazione tra l'espressione delle citochine e l'andamento della viremia, ciascuno dei gruppi sopracitati è stato analizzato dividendo gli animali in tre diversi sottogruppi definiti sulla base del titolo virale (numero di copie di genoma virale/ ml di siero): suini non viremici (NV), suini con viremia $<10^6$ ($V < 10^6$) e suini con viremia $\geq 10^6$ ($V \geq 10^6$).

Gli alti livelli di IL-8, TNF- α e IFN- γ rilevati nel gruppo PCV2-vac indicano che questi animali hanno mostrato una responsività immunitaria più efficiente, specialmente se confrontati al gruppo di animali Ctrl-PMWS+.

Nei suini Ctrl-PMWS+, rispetto agli altri gruppi sperimentali, sono stati osservati livelli più ridotti di IFN- γ a 19 settimane di età, associati a più elevati livelli di IL-10 a 19 settimane di età e ad una più ridotta espressione di citochine pro-infiammatorie a 22 settimane di età, in particolare IL-8 e TNF- α , condizione probabilmente correlata all'insorgenza della malattia. Al contrario, a 19 settimane di età, i suini dei gruppi PCV2-vac e Ctrl hanno mostrato una più bassa espressione di IL-10 e maggiori livelli di IFN- γ rispetto agli animali Ctrl-PMWS+. A 22 settimane di età gli animali vaccinati hanno mantenuto livelli di citochine pro-infiammatorie più elevati.

Questi dati supportano l'ipotesi che l'esito della PMWS potrebbe essere associato ad una riduzione della risposta immunitaria innata/pro-infiammatoria. Complessivamente, i risultati mostrano una diversa modulazione citochinica tra i suini vaccinati e non vaccinati infetti da PCV2 che sviluppano PMWS. I suini vaccinati combattono l'infezione mostrando ridotta o assente viremia, assenza di PMWS e una risposta infiammatoria e reattività cellulare associata all'IFN- γ più intensa.

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List of abbreviations

ADV	Aujeszky's disease virus
ADWG	average daily weight gain
AM	alveolar macrophages
BMDC	bone-marrow derived dendritic cells
Ctrl	controls (unvaccinated spontaneously infected/non-PMWS-affected pigs)
Ctrl-PMWS+	controls-PMWS+ (unvaccinated spontaneously infected/PMWS-affected pigs)
DC	dendritic cells
GAPDH	glyceraldehyde 3-phosphate dehydrogenase
HI	haemoagglutination inhibition
ICTV	International Committee for the Taxonomy of Viruses
IFN	interferon
Ig	immunoglobulin
IL	interleukin
MDA	maternally derived antibodies
M. hyo	Mycoplasma hyopneumoniae
MdM	monocyte-derived macrophages
MOI	multiplicity of infection
NA	neutralising antibodies
ODN	oligodeoxynucleotides
PCV	porcine circovirus
PCV1	porcine circovirus type 1
PCV2	porcine circovirus type 2
PCVAD	porcine circovirus associated diseases
PCVD	porcine circovirus diseases
PMWS	postweaning multisystemic wasting syndrome
PPV	porcine parvovirus
PCV2-vac	PCV2-vaccinated pigs
PRRSV	porcine reproductive and respiratory syndrome virus
RT-qPCR	reverse transcription-quantitative PCR (polimersase chain reaction)
SC	secreting cells
SIV	swine influenza virus

CHAPTER 1.

INTRODUCTION

1.1 Porcine circovirus type 2 (PCV2)

1.1.1 PCV2 history

Porcine circovirus (PCV) was first detected in 1974 as a virus morphologically similar to a picornavirus, contaminating the porcine kidney cell line PK-15 (ATTC-CCL33) (Tischer et al., 1974). This contaminant agent was subsequently demonstrated to be a circular single-stranded DNA (ssDNA) virus that was accordingly named porcine circovirus (PCV) (Tischer et al., 1982). Since the virus induced an antibody response but no disease in the pig population, it was defined as non-pathogenic (Tischer et al., 1986; Dulac and Afshar, 1989; Allan et al., 1994).

Postweaning multisystemic wasting syndrome (PMWS) is a multifactorial disease that was first reported in North America in 1991 (Clark, 1997; Harding, 1997); since then, this disease has affected the swine industry worldwide. The clinical signs of this syndrome include weight loss, severe growth retardation and death in weaned piglets; PMWS is also characterized by a multiorgan disease including lymphadenopathy, respiratory dysfunction, hepatitis, splenomegaly and gastric ulcers (Clark, 1997; Harding, 1997), lymphocyte depletion, monocytic infiltration in lymphoid tissues and high amounts of viruses in these lesions (Segalés et al. 2002).

After the isolation of a PCV-like agent from tissues of PMWS-affected pigs, both in North America and Europe (Allan et al., 1998b; Ellis et al., 1998), the non-pathogenic PK-15 cell culture-derived virus and the circovirus isolated from PMWS-affected pigs were compared. Nucleotide sequence analyses revealed significant genetic differences between viruses (Allan et al., 1998a), less than 80% of sequence identity (Meehan et al., 1998); because of that they were divided into two types: the non pathogenic PCV type 1 (PCV1) and the virus associated with clinical disease, that is PCV type 2 (PCV2) (Hamel et al., 1998; Meehan et al., 1998).

1.1.2 Taxonomy

Both PCV1 and PCV2 belong to the Circoviridae family (Todd et al. 2005; Opriessnig et al. 2007) that is composed of icosahedral, small, non-enveloped ssDNA viruses infecting vertebrates (Lukert et al., 1995). Viruses within the Circoviridae family, based of their morphology and genomic organization, are divided in two genera: Circovirus and Gyrovirus (Todd et al. 2005; Opriessnig et al. 2007).

Circovirus genus includes Porcine circovirus type 1 and type 2 and, according to the International Committee for the Taxonomy of Viruses (ICTV), other known avian viruses such as Beak and feather disease virus (Ritchie et al., 1989), Canary circovirus (Phenix et al., 2001), Duck circovirus (Hattermann et al., 2003), Finch circovirus (Shivaprasad et al., 2004), Goose circovirus (Todd et al., 2001), Gull circovirus (Smyth et al., 2006), Pigeon circovirus (Woods et al., 1993), Starling circovirus (Johne et al., 2006) and Swan circovirus (Halami et al., 2008).

The genus Gyrovirus, that differs from circovirus for its negative sense genome and its large virions (Gelderblom et al., 1989; Gillespie et al., 2009), contains only Chicken anaemia virus (CAV) (Todd et al., 2005; Opriessnig et al., 2007).

1.1.3 Genotypes

Several phylogenetic analyses have demonstrated that PCV2 isolates from different geographical origins can be divided into 2 distinctive genogroups (Larochelle et al., 2002; Mankertz et al., 2000; Olvera et al., 2007). In some studies a stronger association of certain PCV2 genogroups with the PCVD onset (Grau- Roma et al., 2008; Timmusk et al., 2008) has been described whereas other reports have stated that there is no direct relationship between the development of PMWS and the infection by a specific genogroup of PCV2 (Allan et al., 2007; Olvera et al., 2007). The difficulty to identify pathogenic differences between genotypes has been recently attributed to the presence of a conserved decoy epitope in the C-terminal region of the PCV2 capsid protein (Trible and Rowland, 2011).

The two phylogenetic groups, depending on the author, have been commonly referred as PCV2a and PCV2b in North America and PCV2 group 1 (included in the PCVb group) and PCV2 group 2 (included in the PCVa group) in Europe. In addition, some North American laboratories, based on predicted restriction fragment length polymorphism (RFLP) patterns,

grouped the virus into two RFLP patterns designated as 422 and 321. Isolates with the RFLP pattern 422 typically cluster into PCV2a (PCV2 group 2), whereas isolates with a 321 RFLP pattern can be either PCV2a (PCV2 group 2) or PCV2b (PCV2 group 1) (Olvera et al., 2007; Opriessnig T. et al., 2007).

Nowadays the North American nomenclature is officially accepted and the two clusters are designated as PCV2a and PCV2b (Gagnon et al., 2007; Segalés et al., 2008); PCV2a contains a genome of 1.767 nucleotides (nt) and can be divided into 3 clusters (1A–1C), while PCV2b is characterised by a 1.768 nt genome and can be divided into 5 clusters (2A–2E) (Olvera et al., 2007; Gillespie et al., 2009). The existence of discrete antigenic differences between different PCV2 genetic clusters has been described in a recent study that performed epitopes' competition analysis using a panel of universal and cluster-specific mAbs (Saha et al., 2011).

Several epidemiological studies worldwide have reported that PCV2b is becoming predominant in many countries, underling a genotype switch of virus from PCV2a (circulating with prevalence in the 1990's) to this major group (Trible and Rowland, 2011). A new PCV2 genogroup, PCV2c has been recently detected in Denmark in archived serum samples from non-clinical pigs collected in 1980, 1987 and 1990; furthermore, it has been demonstrated that this genogroup is more closely related to PCV2b (95%) than PCV2a (91-93.6%) as sequence homology (Dupont et al. 2008; Opriessing et al. 2010).

1.1.4 Molecular characteristics

Porcine circoviruses (PCVs) are the smallest viruses infecting mammalian cells, being characterised by an icosahedral, non-enveloped virion particle of 17 ± 1.3 nm of diameter (Tischer et al., 1982) that contains a covalently closed circular ssDNA genome with a size of 1759 bp and 1768 bp for PCV1 and PCV2 respectively (Meehan et al., 1998). PCVs, as the other Circoviruses, replicate via rolling circle replication (RCR) so, after the infection of the cells, their ssDNA is converted into a intermediate dsDNA, called replicative form (RT). During this phase of the viral life cycle, the genome has an ambisense organization and genes encoded by both the positive and negative strands (Cheung 2006; Meehan et al., 1997) (Fig.1).

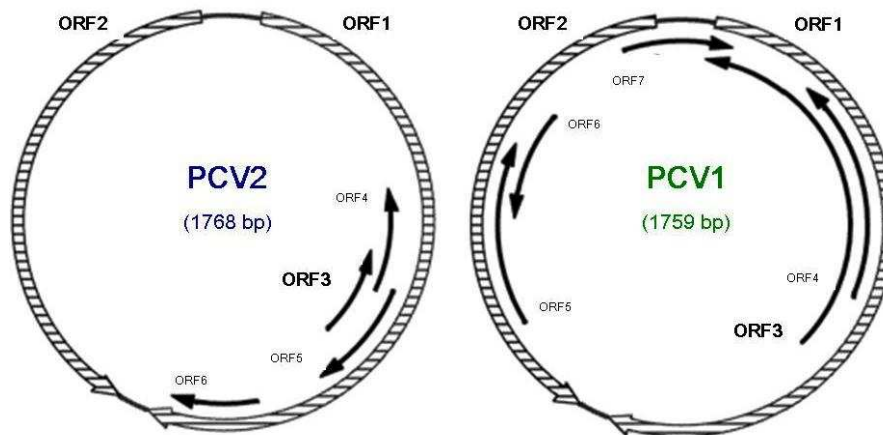


Figure 1. PCV2 and PCV1 genomes (adapted from Gillespie J. et al., 2009)

This arrangement creates two intergenic regions, a shorter one between the 3'-ends of the Rep and Cap gene and a larger one between their 5'-ends, the latter comprising the origin of viral genome replication (Finsterbusch T. and Mankertz A., 2009). The origin of replication (OR) is characterized by a putative stem-loop structure with a nonamer in its apex and hexamer motifs, contiguous to the stem-loop, which serve as binding site for the replicases (Mankertz et al., 1997; Finsterbusch T. and Mankertz A., 2009) (Fig. 2).

PCV2 replication starts when Rep protein binds these hexamer repeats (Mankertz et al., 2004) and, since PCV2 is dependent on cellular DNA polymerases, meanwhile the Rep proteins nick and join the nucleotide segments at the initiation and termination of the cycle, the cellular polymerase synthesizes DNA (Cheung 2006; Steinfeldt et al., 2006).

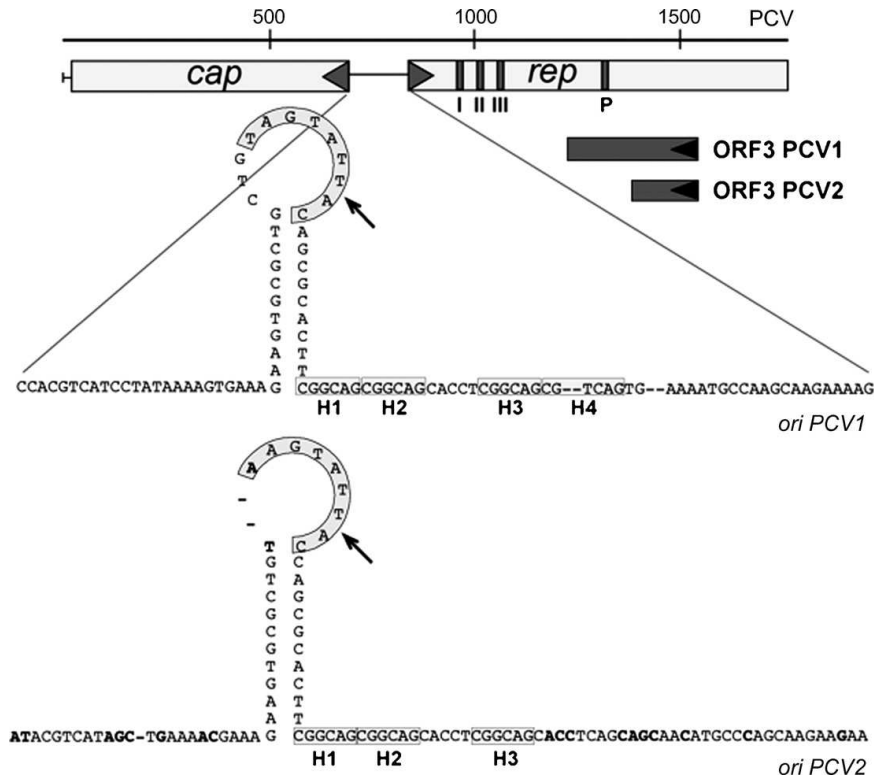


Figure 2. A linear representation of the PCV genome (upper part); the two major ORF, *rep* and *cap*, three motifs conserved in RCR enzymes (I–III), a dNTP-binding domain (P) within the *rep* gene and the ORF3 (grey boxes) are indicated. The lower part shows a comparison of the two origin regions of PCV1 and PCV2; the hexamer repeats 1–4 (open boxes), the conserved nonamer sequence within the single-stranded loop of the hairpin (grey box) and the nicking site (arrow) are indicated (Finsterbusch T. and Mankertz A., 2009).

PCV2 genome contains several potential open reading frames (ORFs) larger than 200 nt, however only three of them have been demonstrated to be functionally expressed: ORF1 (*Rep*), ORF2 (*Cap*) and ORF3 (Hamel et al., 1998; Segalés et al., 2005; Liu et al., 2005). ORF1 is located on the positive strand, clockwise oriented and encodes for the viral replication proteins *Rep* and *Rep'* which are 314 and 178 amino acids (aa) in length, respectively (Mankertz et al., 1998; Cheung, 2003b). ORF2 is situated on the negative strand, counterclockwise oriented, and encodes for the capsid protein *Cap* which is the major structural protein and the main antigenic determinant of the virus (Mankertz et al., 2000; Nawagitgul et al., 2000). ORF3 is completely overlapped with the ORF1 gene, located in the negative strand and counterclockwise oriented, it encodes for a protein that has been characterized as an inducer of apoptosis (Liu et al., 2005; Liu et al., 2007). Several recent studies have shown that the ORF3 protein might play a role in viral replication and induction

of immunosuppression (Karuppanan et al., 2009); indeed it has been proved, both in BALB/c mice (Liu J. et al., 2006) and in specific-pathogen-free (SPF) piglets, that the knock-out of ORF3 reduces PCV2 pathogenicity (Karuppanan, A. K., 2009). The non pathogenic PCV1 also has a third open reading frame but its function has still to be characterised (Chaiyakul M. et al., 2010).

1.1.5 PCV2-associated diseases

All the recognized syndromes associated with PCV2 infection can be nowadays designated by two similar terms: PCVD (*porcine circovirus diseases*), proposed in 2002 by Allan and co-workers and still predominantly used in Europe, and PCVAD (*porcine circovirus associated diseases*), introduced by the American Association of Swine Veterinarians (AASV) in 2006 and used mainly in North America. At present there is still no consensus with regard to the disease nomenclature and both acronyms are accepted.

PCV2 is the primary causative agent of the syndromes included in PCVD but many other common pathogens are involved in their onset; the different forms can be accomplished by observation of characteristic lesions in the intestines, lungs, and lymphoid tissue (Opriessnig et al., 2007).

PCV2 has been associated with subclinical diseases or other clinical manifestations such as postweaning multisystemic wasting syndrome (PMWS), PCV2-Associated Enteritis, PCV2-Associated Pneumonia, PCV2-Associated Reproductive Failure, Porcine Dermatitis and Nephropathy Syndrome (PDNS) and PCV2-Associated Neuropathy (Gillespie et al., 2009).

The syndromes associated with PCV2, besides PMWS, are the following:

- **Subclinical infections** characterised by the absence of evidence of clinical disease although PCV2 is present. *In vivo* study on PCV2-inoculated pigs have shown that PCV2 lesions can be limited to 1 or 2 lymph nodes without causing any apparent clinical problems (Opriessnig et al., 2004; Opriessnig et al., 2006a); cases of necrotising lymphadenitis (Opriessnig et al., 2006a; Kim et al., 2005) or decrease in vaccine efficacy have also been reported in healthy PCV2-infected pigs (Opriessnig et al., 2006b).

- **PCV2-Associated Enteritis** affects piglets from 8 to 16 weeks of age inducing increased mortality, diarrhea and severe growth retardation: this syndrome is similar in clinical signs to ileitis associated with *Lawsonia intracellularis* infection but an histopathological study can distinguish between the two diseases; animals affected by PCV2-Associated Enteritis are indeed characterised at necroscopy by an enlargement of mesenteric lymph nodes, thickening of intestinal mucosa (Jensen et al., 2006), distinctive PCV2 lesions in Peyer's patches but not in other lymph nodes and granulomatous enteritis detectable by means of microscopical analysis.

- **PCV2-Associated Pneumonia** occurs in pigs from 8 to 26 weeks of age and its symptomatology includes reduced feed efficiency and growth rate, anorexia, fever, cough, and dyspnea (Gillespie et al., 2009); histopathological studies on diseased pigs show lymphohistiocytic to granulomatous bronchointerstitial pneumonia with necrotizing and ulcerative bronchiolitis and bronchiolar fibrosis characterised by abundant PCV2 antigen in the lesions. This evidence suggest that PCV2 may play a role in the Porcine Respiratory Disease Complex (PRDC) in which also Porcine Reproductive and Respiratory Syndrome Virus (PRRSV) are involved (Sorden, 1999; Sorden et al., 2000; Gillespie et al., 2009).

- **PCV2-Associated Reproductive Failure** damage herds of gilt startups or new populations (Mikami et al., 2005) inducing clinical manifestations as increased abortion, still births, foetal mummies, and pre-weaning mortalities; a non-suppurative to necrotizing or fibrosing myocarditis has been also found in histopathological lesions of stillborn and neonatal pigs (Mikami et al., 2005; Opriessnig et al., 2007). It has been proved that the time of infection determines the clinical course of the disease: several studies have demonstrated that fetuses experimentally intrauterine infected in an earlier phase of gestation (57 weeks of gestation) present higher viral load and lesions as edema, enlarged liver and congestion, than those infected in a later phase (75 and 92 days of gestation) (Sanchez et al., 2001); it has been also shown that late term infections (86, 92, and 93 days of gestation) can cause an increase in reproductive abnormalities (Johnson et al., 2002; Gillespie et al., 2009).

- **Porcine Dermatitis and Nephropathy Syndrome (PDNS)** was first described in the United Kingdom in 1993 (Smith et al., 1993) and was associated with PCV2 only later, in 2000 (Rossel et al., 2000). Many pathogens including PRRSV and bacteria such as

Pasteurella multocida, *Streptococcus suis* type 1 and 2, among others, have been implicated in the etiology of disease (Lainson et al., 2002; Thomson et al., 2002). PDNS is not always associated with PCV2, in several studies it was indeed experimentally reproduced with PRRSV and TTV in PCV2-free pigs (Krakowka et al., 2008).

This disease mostly affects growing pigs but can also occur in recently weaned and feeder pigs from 1.5 to 4 months of age (Smith et al., 1993; Thibault et al., 1998); PDNS is clinically characterized by an acute onset of multifocal and well circumscribed skin lesions (raised purple progressing to multifocal raised red scabs with black centers most prominent on the rear legs), fever, and lethargy and is often fatal within 3 days of development (Done et al., 2001; Duran et al., 1997; Chae, 2005). Macroscopically, the kidney appears enlarged and having pale cortex with multiple red circular haemorrhagic cortical foci (Ramos-Vara et al., 1997). Microscopically, the most significant lesion is the severe, fibrinoid, necrotizing vasculitis in the dermis, subcutis, lymph nodes, stomach, spleen, liver and kidney which can be associated with dermal and epidermal necrosis and necrotizing and fibrinous glomerulonephritis.

- **PCV2-Associated Neuropathy** causes in pigs congenital tremors and a nonsuppurative meningoencephalitis associated with demyelination of the brain and spinal cord (Laroche et al., 2002; Pensaert et al., 2004; Correa et al., 2007). PCV2 infection has also been associated with cerebellar lymphohistiocytic vasculitis or with lymphohistiocytic meningitis (Correa et al., 2007) but even today the role played by this virus in this kind of diseases has still to be clarified.

1.2 Postweaning multisystemic wasting syndrome (PMWS)

The most significant manifestation of PCVAD is the postweaning multisystemic wasting syndrome (PMWS). Since the 1990s, porcine circovirus type 2 (PCV2) has been considered the causative agent of this disease, even if the majority of infections caused by PCV2 are sub-clinical, and only a small proportion of PCV2-infected pigs develops the clinical form of disease. PMWS is considered a multifactorial disease and the fully clinical expression of this syndrome is indeed due to the co-presence of PCV2 and pathogens such as porcine reproductive and respiratory syndrome virus (PRRSV), swine influenza virus (SIV), porcine parvovirus (PPV), *Haemophilus parasuis*, *Actinobacillus pleuropneumoniae*, *Streptococcus suis* and *Mycoplasma hyopneumoniae* (Chae, 2004).

1.2.1 Epidemiology

PMWS was described for the first time in a Canadian high-health-status herd in 1991 and was later recognized worldwide (Allan and Ellis, 2000) being associated with major losses in Europe (Harding et al., 2000; Opriessing et al., 2008). In 1996, both in British and French farms, there were cases of wasting and high losses in growing pigs (Madec et al., 2004) and PCV2 was isolated from the animals and the disease was later defined as PMWS.

Cases of PMWS were retrospectively identified in archived serum and tissues samples from 1962 in Germany (Jacobsen et al., 2009), 1969 in Belgium (Sanchez et al., 2001a), 1970 in the United Kingdom (Grierson et al., 2004), 1973 in Ireland (Walker et al., 2000) and 1985 in Canada and Spain (Magar et al., 2000; Rodríguez-Arrijoja et al., 2003).

During the following decade, PMWS spread over the world becoming a considerable economic problem in many pig-producing countries. In fact, PCV2 infection is so widespread today that it is almost impossible to find seronegative farms in epidemiology studies (Grau-Roma et al., 2009). PMWS morbidity is associated with the development of clinical manifestations of disease and ranges between 4% and 30%, being (although) up to 60% in some herds (Segalés and Domingo, 2002). PMWS prevalence generally ranges from 4% to 30% and mortality ranges from 4% to 20%, but can reach 50% (Allan and Ellis 2000; Harding and Clark, 1997; Segalés and Domingo, 2002).

1.2.2 Clinical features

PCV2 infection can occur during the whole pig productive life, but PMWS usually affects animals from 8 to 16 weeks of age (Sibila et al., 2004; Grau-Roma et al., 2009).

However, PMWS has been shown to occur at different ages in the United States (from 7 to 16 weeks of age) and Europe (from 5 to 12 weeks of age) (Allan and Ellis 2000; Segalés and Domingo, 2002) due to different management and vaccination practices.

Clinical signs of this disease include wasting with progressive weight loss (Fig. 3), lethargy, dark-colored diarrhea, and paleness or jaundice that may occur at a different degree (Segalés et al., 2005; Allan and Ellis 2000; Gillespie J. et al., 2009). The earliest symptoms are weight loss, ill-thrift, pale skin, and rough hair; dyspnea, tachypnea, anemia, diarrhea, and jaundice generally appear in the latest phases of the disease, in some cases coming with coughing and gastric ulceration (Opriessnig et al., 2007; Gillespie J. et al., 2009).

Gross lesions of PMWS commonly include pale and enlarged lymph nodes (superficial inguinal, submandibular, mesenteric and mediastinal), mottled and firm lungs that fail to collapse (Allan and Ellis, 2000) and, in chronic cases, kidneys with white streaks or spots (Rosell et al., 1999). The histopathological analysis of PMWS lesions displays a generalised lymphadenopathy with infiltration of histiocytic cells and multinucleated giant cells and characterised by pronounced depletion of lymphocytes. These findings are unique and allow to distinguish this syndrome from other wasting manifestations.

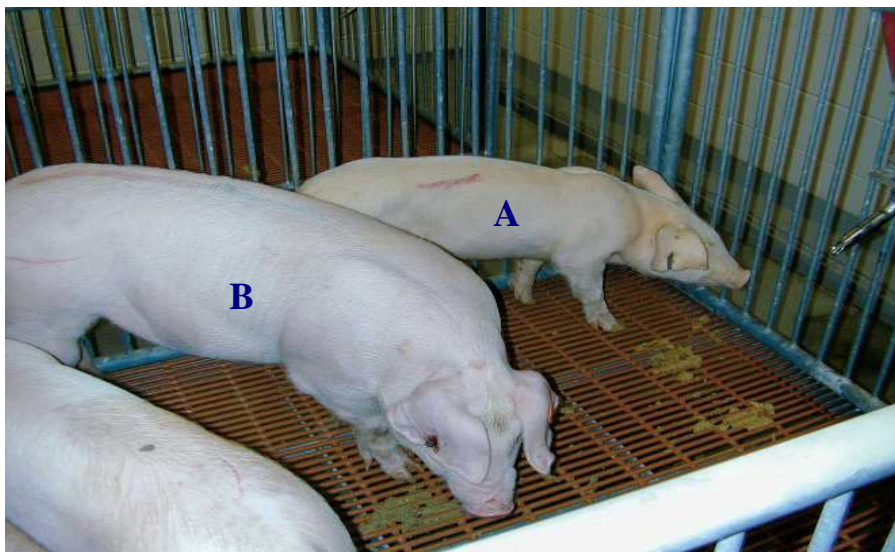


Figure 3. Pigs suffering from PMWS (A) compared to a healthy pig of the same age (B) (Opriessnig et al., 2007).

The lymphatic system can be involved at different levels by the disease. Based on that, a scoring system has been defined to evaluate the severity of the disease. This estimation system allow to assign scores from 0 to 9 ranking the severity of lesions, the amount of PCV2 antigen and the distribution of lesions in seven indicative lymphoid tissues such as the tracheobronchial lymph nodes, the mesenteric lymph nodes, the mediastinal lymph nodes, the superficial inguinal lymph nodes, the external iliac lymph nodes, the tonsils, and the spleen (Opriessnig et al., 2004; Gillespie et al., 2009).

In case of PMWS the immune system of pigs can be strongly compromised and this can increase the probability to be subjected to secondary infections (Segalés et al., 2005).

1.2.3 Diagnosis

PCV2 induces several clinical signs that are also shared by other pig diseases; for this reason a diagnosis of a specific syndrome is not easy to define. The presence of PCV2 genome in serum and the observation of a diseased status of pigs is not enough to define a PCVD case.

It has been established that to make a diagnosis of PCVD, in addition to clinical signs, PCV2 antigen has to be necessarily found in more than one lymphoid tissue, or one lymphoid tissue and one other organ such as the lungs, liver, kidney or intestine, or in two organs (Gillespie et al., 2009).

More specifically, to categorise PMWS cases, Segalés and co-workers suggested in 2005 the following criteria:

- 1) clinical signs compatible with PMWS (growth retardation and wasting);
- 2) moderate to severe histopathological lesions characterized by lymphocyte depletion together with granulomatous inflammation;
- 3) moderate to high amount of PCV2 genome/antigen within lesions.

Tests such as polymerase chain reaction, in situ hybridization (ISH) and immunohistochemistry (IHC) are considered the optimal techniques to detect PCV2 antigen or nucleic acid and make a diagnosis of PMWS (Opriessnig et al., 2007). Serological tests such as IPMA (immunoperoxidase monolayer assay) or SN (seroneutralisation) are useful to identify an infectious state but not enough to substitute histopathological evaluations and PCR analysis (Allan et al., 1998b; Grierson et al., 2004; Allan et al., 2000; Gillespie et al., 2009).

Seropositivity to PCV2 can be found in many clinically healthy pigs and the status of subclinical infection is commonly set when low amounts of PCV2 are detected in blood and/or tissues, associated with no or minimal lesions. The cut-off generally considered to distinguish between PMWS-diseased pigs and sub-clinically infected pigs is 10^7 PCV2 DNA copies/ml of serum (Opriessnig et al., 2007).

Currently, there is no field test for the diagnosis of PMWS, but to identify and manage its outbreaks within a herd it is important to determine whether the disease is a significant or a sporadic problem. It has been defined that there is an important herd problem if the following conditions are observed (Segales J., 2006; Gillespie et al., 2009):

- 1) significant increased postweaning mortality that is equal or higher than the mean historical mortality plus 1.66 times the standard deviation. If historical data are not available, a herd problem can be described when the postweaning mortality exceeds the national or regional level by 50% or more;
- 2) confirmation of PMWS in individual cases.

1.2.4 Pathogenesis

In case of PCV2 infection there are significant differences between sub-clinical and PMWS-affected pigs; current evidence support a central role for immunodepression in the pathogenesis of PMWS.

In pigs that develop PMWS, the highest amount of PCV2 is found in the cytoplasm of monocyte and macrophage lineage cells (Rosell et al., 1999; Sanchez et al., 2004).

The virus can infect these cells without an active replication for a long period of time (Gilpin et al., 2003; Vincent et al., 2003). The capability of PCV2 to induce functional impairment of *in vitro* cultured dendritic cells (DC) has been also described (Vincent et al., 2005); this underlines the ability of the virus to interfere with innate and virus-specific immune responses.

It has been displayed that PCV2 is not able to encode for its own polymerase and its replication depends on host's nuclear polymerases (Tischer et al., 1987). For this reason it is possible to identify cells that support replication of PCV2 by evaluating the presence of Rep, the PCV2 replication associated protein, in the nucleus of the cell (Rovira et al., 2002). Earlier studies have demonstrated the presence of nuclear PCV2 in epithelial cells of PMWS-affected pigs, proposing these cells as candidate for primary PCV2 infection (Rosell et al., 1999).

Studies on experimentally PCV2-inoculated pigs have proven viral replication in lymphocytes from PBMC and bronchial lymph nodes by measuring Cap mRNA levels in the cells (Yu et al., 2007a). The subpopulations of leukocytes that support PCV2 replication are prevalently circulating T cells, both CD4+ and CD8+, and in a lower proportion B lymphocytes; PBMC-derived monocytes do not seem to sustain viral replication (Yu et al., 2007b; Lefebvre et al., 2008b; Lin et al., 2008).

PMWS is characterised by lymphocyte and follicular dendritic cell depletion from follicular regions, together with increased numbers of histiocytic cells (Chianini et al., 2003).

It is still unknown whether the lymphocyte depletion is due to reduced production in the bone marrow, reduced proliferation in secondary lymphoid tissues, or increased loss of lymphocytes in the bone marrow, peripheral blood, or secondary lymphoid tissues via virus-induced necrosis or apoptosis (Opriessnig et al., 2007). A reduction of B and T lymphocytes, especially CD8+ cells, has been also reported in blood circulation; at the same time, an increased number of circulating neutrophils and monocytes determine a reversal of the normal ratio of lymphocytes/neutrophils (Nielsen et al., 2003; Segales et al., 2001). Infection studies have not clarified yet this phenomenon.

An experimental PCV2 infection study showed that at 7 days post-PCV2 infection the lymphocyte depletion has already started, whereas maximal depletion of both B and T cell subsets, followed by a huge or total loss of NK cells, occurs later, at 21 days post-infection (Nielsen et al., 2003).

Humoral immunity seems to play a very important role in controlling and resolving viremia (Fort et al., 2007; Opriessnig et al., 2008b). In sub-clinical animals, an efficient humoral response is frequently associated with long-lasting viremia, low concentration of virus at lymphoid tissue, and no significant changes in the status of the immune system (Allan et al., 1999a; Resendes et al., 2004a). On the contrary, weak humoral responses can be related to increased viral replication, resulting in the severe lymphoid lesions and immunosuppressive status characteristic of PMWS (Bolin et al., 2001).

Several experimental and field studies supported the multifactorial nature of PMWS and highlighted that not all pigs that are infected by PCV2 develop clinical PCVAD.

The outcome of this syndrome can be influenced by several factors that can be grouped in four main areas: virus, host, coinfections, and immune modulation (Opriessnig et al., 2007). The accurate mechanism by which these factors cooperate determining the onset of the

disease in PCV2-infected pigs are not completely been elucidated yet. A schematic diagram involving factors influencing the onset of PMWS and PCV2 infection progression/outcome is shown in Figure 4.

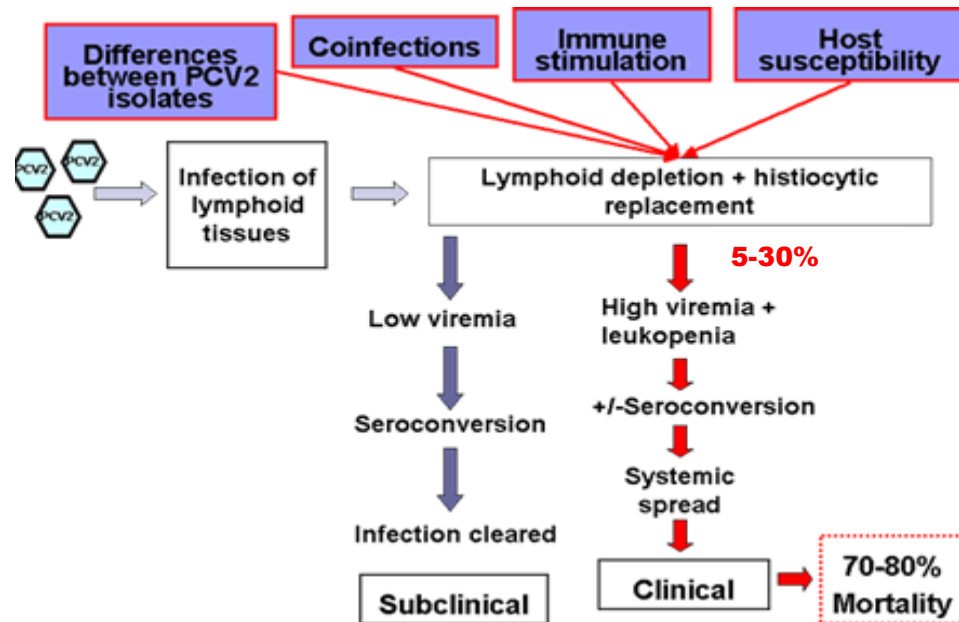


Figure 4. Scheme of the current understanding of the progression of porcine circovirus type 2 (PCV2) infection and clinical outcome (Opriessnig et al., 2007).

1.2.5 Intervention strategies

Since the first economically significant appearance of PMWS in the 1990s, control measures have been focused on the control of risk factors involved in the progression of the disease, but have been accomplished with several problems mostly related to the lack of knowledge of the full aetiology and epidemiology of the disease and the absence of commercial vaccines.

The first strategy to control PMWS by adjustments of housing and management routines in affected farms were proposed by Madec and co-workers in 2001; they elaborated a 20-point plan of recommendations essentially focused to reduce overall stress and improve hygiene and infection status within the herd. Due to difficulties in application on large commercial units, the Madec's principles have been later refined into four rules: 1) limiting pig-pig contact, 2) reduction of physiological stress, 3) good hygiene conditions improving disinfection and cleaning procedures, and 4) good nutrition (Muirhead, 2002).

The implementation of Madec's plan as a guideline for the control of postweaning mortality in PMWS affected farms proved to be efficacious in reducing the PMWS-associated losses (Allan and McNeilly, 2006; Segalés et al., 2005).

1.2.6 Vaccine development and vaccination

Vaccination against PCV2 represents an important strategy to control PMWS in pig herds. For this reason there is active interest in the development of vaccines able to prevent or limit the PCV2-associated diseases. Nowadays, several commercial products are used in the herds and all of them are based on PCV2a genotypes that have been demonstrated to provide cross-protection also to PCV2b (Fort et al., 2008, 2009; Segalés et al., 2009).

Nowadays, three commercially vaccines are available against PCV2:

- **CIRCOVAC**[®] (Merial) was the first vaccine on the market; it has been extensively used in Europe but it has been also available in Canada. It is an inactivated PCV2, oil-adjuvanted vaccine for use in sows and gilts 2-4 weeks prior to farrowing (Charreyre et al., 2005; Gillespie et al., 2009) that is given as two injections IM (intramuscular administration) 3-4 weeks apart and completed at least 2 weeks before breeding and once at each subsequent gestation (Opriessnig et al., 2007).

The active immunisation on these breeding-aged animals is used to induce passive immunisation to the offspring by means of colostrum transfer.

The other two commercial products use different approaches for establishing protective immunity against PCV2. They are recombinant vaccines designed for use in growing pigs, at about 3-4 weeks of age.

- **Ingelvac CircoFLEX**[®] is a capsid-based subunit vaccine based on the product of the ORF2 expressed in a baculovirus system; it is administered as a single dose IM in piglets from 2 weeks of age. The immunity of the treated animals starts about 2 weeks after vaccination remaining protective at least for further 17 weeks. A significant decrease in mortality in vaccinated pigs compared to unvaccinated pigs was reported on 4 different Canadian finishing sites (Desrosier et al., 2007; Gillespie et al., 2009).

- The vaccine from Intervet/Schering-Plough/Merck is also a capsid-based subunit vaccine expressed in a baculovirus, it is designated as **Porcilis PCV**[®] in Europe and Asia and **Circumvent PCV**[®] in the United States and Canada (Gillespie et al., 2009).

Porcilis PCV[®] can be administered to 3-day-old and older piglets, following a double dose IM protocol (at 3-5 days and 3 weeks of age) or given as a single dose IM at 3 weeks of age; **Circumvent**[®] is given to 3-week-old and older piglets and is administered as a single dose at 3 weeks of age. Both of them induce protective immunity that remains active from 2 to 22 weeks after vaccination; studies including 35,000 pigs on 21 different farms showed that mortality of vaccinated pigs is reduced by 77.5% when compared to unvaccinated pigs (Grau et al., 2007; Gillespie et al., 2009).

1.2.7 Effectiveness of vaccination

The effectiveness of the vaccines available against PCV2 has been widely evaluated in several studies. Due to the poor clinical manifestation in piglets infected by PCV2 only, some studies have also evaluated the responses in animals co-infected by two or three porcine pathogens. Experimental co-infection try to reproduce herd field conditions in which numerous pathogens, more frequently PRRSV and *Mycoplasma Hyopneumoniae*, contribute to PCVD outbreak (Beach and Meng, 2011). In this regard it has been found that the presence of PRRSV can increase the severity of PCV2-related clinical signs, inducing a wide spread of the virus by oronasal and faecal excretions (Allan et al., 2000; Rovira et al., 2002; Sinha et al., 2011). Contrarily, a large number of vaccines against PCV2 have demonstrated to induce neutralising antibody (NA) secretion, reduced viral load and lymphoid lesions in cases of PCV2 infection but also in the presence of SIV (swine influenza virus) or PRRSV (Opriessnig et al., 2009) coinfections.

The vaccination of boars by using Suvaxyn PCV2 one dose, followed by infection with PCV2 or *Mycoplasma*, seems to prevent serious clinical manifestation, reduce viral titres in the blood and virus excretion by faeces and semen with respect to an unvaccinated control group (Opriessnig et al., 2011). Boars vaccination does not alter semen characteristics and is proved to be a good practice to reduce vertical PCV2 transmission.

In addition, sow and gilt vaccination has been reported to increase the number of live born pigs and the number of pigs per sow per year, reducing the number of mummies per sow (Thacker et al., 2008). It is relevant to highlight that the vaccination of sows is not able to completely eliminate viral transmission by colostrum or prevent intrauterine infection but is however important to increase productive parameters of the herds (Beach and Meng, 2011).

Several studies have demonstrated the efficacy of PCV2 vaccination; in a infection study, vaccine treatment of piglets from a PRDC group reduced the mean viral titre from 83% to 55% and its duration overtime, improving average daily weight gain (ADWG) (Fachinger et al., 2008). In another study, vaccination of piglets from a PMWS/PCVD farm showed a 50% reduction of mortality and a 9.3 % increase of ADWG (Horlen et al., 2008).

The efficacy of a subunit vaccine containing PCV2 capsid protein has been also proven after experimental infection with four different PCV2 isolates of the two genotypes (PCV2a and PCV2b) (Fort et al., 2008).

Therefore, after vaccination of growing pigs, under experimental conditions, it has been observed a reduction of viremia, lymphoid lesions and amount of PCV2 DNA in tissues, oronasal and fecal PCV2 excretion and specific IgM, IgG and NA production, as well as cross-protection to both PCV2a and PCV2b (Fenaux et al., 2004; Fort et al., 2008; Opriessnig et al., 2008c). Under field conditions, increases of ADWG and percentage of lean meat, improvement of feed conversion index and reductions of mortality have been observed (Fachinger et al., 2008; Horlen et al., 2008; Kixmoller et al., 2008; Cline et al., 2008; King et al., 2008; Opriessnig et al., 2008a, 2008b; Tacker et al., 2008; Desrosiers et al., 2009; Segalés et al., 2009; Martelli et al., 2011), together with increased numbers of PCV2-specific IFN- γ secreting cells (IFN- γ SC) suggesting the presence of effector and/or memory T cells (Fort et al., 2009; Lyoo et al., 2011).

1.3. PCV2 and the immune system

The specific immune response that develops in pigs after infection by PCV2 is crucial and strongly influences the outcome of infection. The animals that develop PMWS often appear to be immunosuppressed and unable to eliminate the virus from the blood circulation.

In the final phase of the clinical manifestation, infected-diseased pigs show extensive lymphoid lesions and altered cytokine expression patterns in PBMC and lymphoid organs due to their ineffective immune response (Kekarainen et al., 2010).

Not always PCV2 infection determines the outbreak of clinical signs and immunological disorders; in fact, there are asymptomatic animals that show higher virus-specific and neutralising antibody titres than PMWS-affected animals. The mechanisms by which PCV2 can affect the immune responses have not been completely elucidated but recent studies have pointed out virus interaction with macrophages and plasmacytoid dendritic cells and the role of viral DNA in regulation of immune cell functions.

1.3.1 Interaction between PCV2 and immune cells

In infected animals, cells of the monocytic lineage, including monocytes, macrophages and dendritic cells (DC), are most frequently associated to intracellular detection of PCV2 which however does not seem to replicate in such cells (Gilpin et al., 2003; Vincent et al., 2003). These cells accumulate viral antigen and DNA for extended periods of time, but since viral replication is inefficient, they are thought to play a major role in viral persistence and transmission (Gilpin et al., 2003; Vincent et al., 2003; Pérez-Martín et al., 2007).

Dendritic cells

In dendritic cells (DC) the presence of live PCV2 particles leads to different effects depending on the cell subpopulation.

In myeloid dendritic cells (mDC) the virus does not appear to be detrimental to cell survival and does not interfere with their maturation; *in vitro* studies on mDC infected with PCV2 have indeed proven that the cell expression of major histocompatibility complex (MHC) class I and II or cluster of differentiation (CD) 80/86 is not altered by the virus, even after exposure to IFN- α and tumor necrosis factor TNF- α (Vincent et al., 2005; Vincent et al., 2003).

In the same studies the antigen presenting and processing ability of mDC was reported not to be compromised by the infection.

On the contrary, the interaction of PCV2 with plasmocitoid dendritic cells (pDC), also called Natural Interferon Producing cells (NIPC), induces impaired responsiveness to danger signals, inhibits interferon (IFN)- α and tumor necrosis factor (TNF)- α production and interferes with NIPC maturation as well as paracrine maturation of mDC (Vincent et al., 2005).

The virus seems not to be transmitted from DC to lymphocytes; this may be a viral strategy to escape the host's immune defence and disseminate exploiting the circulation of DC (Vincent et al., 2003). Since asymptomatic piglets produce anti-PCV2 antibodies (Krakowka et al., 2002; Ladekjaer-Mikkelsen et al., 2002; Nielsen et al., 2003; Steiner et al., 2009) and cytotoxic responses (Steiner et al., 2009), the presence of PCV2 in DC does not always impair their immunobiological interaction with lymphocytes (Kekarainen et al., 2010).

Monocytes and macrophages

In vitro and *ex vivo* studies on the early immune responses following PCV2 infection have determined that also monocytes, monocyte-derived macrophages (M ϕ M) and alveolar macrophages (AM) are able to internalize PCV2 (Gilpin et al., 2003; Kekarainen et al., 2010); in particular, AM phagocyte the virus but, as for DC, viral replication is not strongly detectable (Chang et al., 2006). The microbicidal and phagocytic functions of macrophages seem to be influenced by PCV2; in addition, increased production of pro-inflammatory cytokines such as interleukin (IL)-8 and TNF- α as well as the up-regulation of macrophage-derived chemotactic factor-II (AMCF-II), granulocyte colony-stimulating factor (G-CSF) and monocyte chemotactic protein-1 (MCP-1) have been reported (Chang et al., 2006; Kekarainen et al., 2010).

It was also suggested that the PCV2-mediated alteration of AM functionality can support opportunistic and secondary pulmonary infections.

Peripheral Blood Mononuclear Cells (PBMC)

The lymphopenia observed in PMWS-affected pigs is likely due to an indirect effect promoted by PCV2 infection in DC and macrophages (Kekarainen et al., 2010). In response to recall antigen (PCV2), PBMC from diseased pigs can respond by an increased production of IL-10 and IFN- γ compared to PBMC from infected healthy pigs, and display an impaired ability to produce IL-4, IL-2 and IFN- γ upon stimulation with antigen (porcine pseudorabies

virus; PRV), mitogen (phytohaemagglutinin; PHA) or superantigen (staphylococcal enterotoxin B; SEB) (Darwich et al., 2003a; Kekarainen et al., 2008b). Moreover, Kekarainen and co-workers (2008b) reported that PCV2 is able to modulate the specific immune responses developed by pigs to other pathogens. They showed that IL-12, IFN- α , IFN- γ and IL-2 recall responses of PBMC after pseudorabies virus stimulation were down-regulated by PCV2, underling that the decreased production of IL-12, IFN- α and IFN- γ could be due to the release of PCV2-induced IL-10 by PBMC, CD172a⁺ cells and bone-marrow derived DC (BMDC).

Immune regulatory role for PCV2 DNA

An immune regulatory role for PCV2 DNA was described for the first time in a study focused on the evaluation of the ability of various subpopulations of porcine DC to endocytose PCV2 from infected PK15A cell lysates (Vincent et al., 2003). Viral genomic ssDNA (Vincent et al., 2005) and dsDNA replicative intermediates endocytosed from the infected cell lysates (Vincent et al., 2007) were found in DC. Vincent et al. (2007) theorised that these DNA elements can interact with endosomal toll-like receptors (TLR) or cytosolic helicases inducing impairment of cytokines produced by pDC. These findings suggested the presence of DNA sequences in the PCV2 genome capable to interfere with DC and immune defences.

Several CpG motifs have been characterised in PCV2 genome revealing synthetic oligodeoxynucleotides (ODN) sequences able to modulate cytokine production of porcine PBMC cultures by induction or inhibition (Hasslung Wikström et al., 2003; Kekarainen et al., 2008a). The effect of these ODN was also analysed on porcine PBMC recall responses and cytokine production by BMDC (Kekarainen et al., 2008a; Kekarainen et al., 2010).

It has been found that some ODN induce a decrease in the IFN- α response of BMDC upon PRV stimulation. The majority of the inhibitory ODN is located within the Rep region of the PCV2 genome but none of them, conversely to the whole virus, have been demonstrated to induce IL-10 production in PBMC (Kekarainen et al., 2008b). However, the presence of ODN in PBMC cultures appeared to decrease the IFN- γ or IL-2 responses to recall antigens (Kekarainen et al., 2010).

1.3.2 Humoral response

Immunity against PCV2 in piglets can be transmitted as maternal antibodies by the sow. This passive immunity has been demonstrated to protect from PMWS onset in a dose-dependent manner (Rose et al., 2007). Colostrum administration is an important practice to induce protection of the offspring but also the PCV2 infection status of the sow has been demonstrated to be crucial. With regards to these evidence Calsamiglia et al. (2007) showed that, from sows with low levels of PCV2 antibodies, there is a higher percentage of piglets that result PMWS-affected. Nevertheless, it is important to remind that the presence of PCV2 antibodies is not necessarily protective because not all antibodies are neutralizing upon PCV2 infection.

In experimentally infected pigs, seroconversion commonly occurs between 14 and 28 days post-inoculation (dpi) (Allan et al., 1999a; Balasch et al., 1999; Krakowka et al., 2001; Meerts et al., 2005; Segalés et al., 2005). More specifically, several studies have observed that PMWS-affected pigs seroconvert later (Fort et al., 2007; Meerts et al., 2006; Bolin et al., 2001; Okuda et al., 2003), or show a weak response characterised by lower antibody titres at 21 dpi than in subclinically PCV2-infected animals (Meerts et al., 2006; Kekarainen et al., 2010).

The different immunoglobulin isotypes follow the course of total antibody titres (Meerts et al., 2006); in PCV2-subclinically infected pigs also the titres of virus neutralising antibodies (NA) follow this course (Meerts et al., 2006; Fort et al., 2007). Contrarily, the impaired humoral response of diseased pigs is characterised by decreased production of total antibodies and low levels of NA that subsequently result in a higher viral load (Fort et al., 2007; Meerts et al., 2005).

Under field conditions, the first protection against PCV2 is represented by passive immunity transferred by the sow; this protection lasts during the lactating and nursery periods and is depleted by the end of nursery and the beginning of fattening periods (Rodriguez-Arrioja et al., 2002; Rose et al., 2002; Larochelle et al., 2003). Active seroconversion to PCV2 usually occurs between 7-12 weeks of age (Segalés et al., 2005) and anti-PCV2 antibodies generally last until at least 28 weeks of age (Rodriguez-Arrioja et al., 2002). An impaired humoral response may extend the period between the decline of maternal immunity and the onset of

active seroconversion in piglets, thus increasing the probability to develop PMWS upon PCV2 infection.

It has also been showed that the titres of anti-PCV2 IgM overtime, in contrast with the course of IgG₁, IgG₂ and IgA antibodies, remain lower in diseased pigs than in subclinical infected animals (Meerts et al., 2006).

In case of PCV2 infection, the adaptive humoral immune responses play a crucial role in determining whether or not the outbreak of PMWS occurs despite other immune mechanisms are required to obtain complete viral clearance.

1.3.3 Cell-mediated immune responses

The role of adaptive cell-mediated responses in controlling PCV2 infection is still less studied compared to humoral responses (Kekarainen et al., 2010). However, the few studies that have already been performed provide relevant information. On gnotobiotic experimentally infected pigs, it has been demonstrated that the treatment with cyclosporine A (CyA) (i.e. an immunosuppressing agent), before PCV2 inoculation determines an increase of viral replication (Krakowka et al., 2002; Meerts et al., 2005). The IFN- γ mRNA expression levels in PBMC from these PCV2-inoculated animals resulted to be correlated with viral replication and immunosuppressed status induced by CyA, suggesting that a higher expression of IFN- γ can help pigs be less susceptible to PCV2 replication (Meerts et al., 2005).

Other studies have analysed the development of IFN- γ secreting cells (IFN- γ SC) in either conventional colostrum-fed pigs infected with PCV2 alone (Fort et al., 2009a) or in colostrum-fed specific pathogen free (SPF) pigs infected with PCV2 together with Porcine parvovirus as a potential triggering factor for PMWS development (Steiner et al., 2009). Caesarean-derived colostrum deprived (CD/CD) pigs infected with PCV2 along with lipopolysaccharide (LPS) (Fort et al., 2009a) have also been analysed (Kekarainen et al., 2010). The results of these three studies underline the key role of IFN- γ SC in developing the anti-PCV2 adaptive cellular response.

It has been found that vaccination treatments are effective in reducing PCV2 load in the blood, concomitantly with the development of a virus-specific humoral response, especially mediated by NA both in field (Kixmoller et al., 2008) and experimental (Opriessnig et al.,

2008c) infection models. In addition, it has been shown the onset of a cellular response in terms of PCV2-specific IFN- γ SC in pigs vaccinated with a PCV2 sub-unit vaccine, experimentally infected (Fort et al., 2009b). Thus, we can assume that if at least one of these responses fails, viral clearance will be impaired, and the risk of developing PMWS can increase. However, the cell-mediated responses of pigs to PCV2 infection, although in cases of previous vaccination, have still to be thoroughly investigated.

1.3.4 PCV2 modulation of cytokine profiles

Cytokine mRNA expression profiles can be important to characterise the host's immune responses that occur upon viral infections, therefore pro-inflammatory and immune cytokine production has been recently investigated in different PMWS and infection models (Chae et al., 2011; Kekarainen et al., 2008a, 2010). With regards to lymphoid tissues, increased levels of IL-10 mRNA expression were found in the thymus and decreased levels of IL-4 and IL-2 were detected in tonsils and spleen from PMWS-affected pigs (Darwich et al., 2003b).

Opposed results were described for IFN- γ : low mRNA expression levels were detected in inguinal and tracheo-bronchial lymph nodes whereas high expression was shown in tonsils (Darwich et al., 2003b; Zhang et al., 2010).

An increase of IL-10, together with slight increases in IL-8, IFN- γ and TNF- α and a decrease in IL-2 and IL-4 mRNA levels, were also detected in PBMC from PCV2 naturally infected pigs (Sipos et al., 2004).

Significantly higher expression of IL-10 was found in PCV2-infected lymphoid tissues compared to uninfected control tissues (Doster et al., 2010); furthermore, serum IL-10 was detected in pigs developing severe PMWS and the increased expression of this cytokine has been reported to be correlated with viremia in subclinically PCV2-infected pigs (Stevenson et al., 2006).

Increased serum levels of the acute phase proteins (APP) haptoglobin, pig major acute phase protein (pig-MAP), C-reactive protein (CRP), serum amyloid A (SAA) and albumin were also reported in PMWS-affected pigs (Parra et al., 2006; Stevenson et al., 2006; Segalés et al., 2004).

The production of cytokines and the balance between pro-inflammatory, pro-immune and regulatory cytokines play a pivotal role in eliciting the innate response as well as in priming and coordinating the adaptive immune response.

For this reason the study of cytokines appears to be an important tool to evaluate the cellular immune response against PCV2. The above mentioned evidence suggest a severe immunosuppression in PMWS-affected pigs but the mechanisms determining the immunological impairment, that is not detectable in subclinically infected animals, are still poorly understood (Kekarainen et al., 2010).

CHAPTER 2.

OBJECTIVES OF THE RESEARCH

Management strategies, control of coinfections and vaccination are at present the measures by which PMWS and Porcine Circovirus Diseases (PCVD) are controlled.

Nowadays PCV2 vaccination represents one of the major strategies to overcome PCV2 infections in the herds and therefore several commercial vaccines are available.

All PCV2 vaccines currently available on the market have been tested under field conditions resulting to be effective and helpful in decreasing mortality and cull rates and significantly improving the average daily weight gain (ADWG), concomitantly with decreasing the frequency of coinfections in herds affected with PMWS (Cline et al., 2008; Desrosiers et al., 2009; Fachinger et al., 2008; Horlen et al., 2008; King et al., 2008; Kixmoller et al., 2008; Opriessnig et al., 2008b,c; Tacker et al., 2008; Segalés et al., 2009; Pérez-Martin, 2010).

Even if PCV2 vaccines are effective in reducing the viremia burden and viral-induced specific lymphoid lesions, the mechanisms by which they are able to elicit protective immunity are not thoroughly known (Fachinger et al., 2008; Fort et al., 2008; Horlen et al., 2008).

However, since PCV2 can evade immune surveillance and PCV2 infection is often associated with co-infections in the field (e.g. porcine reproductive and respiratory syndrome virus - PRRSV, *Mycoplasma hyopneumoniae* - *M. hyo.*), it is not easy to obtain a complete resolution of the disease.

Therefore, the improvement of vaccine formulations and administration strategies represents one of the main areas of interest in PCV2 vaccinology.

The activation of the host's immune response has been proved to be one of the primary factors modulating the progression of the disease. Several studies have shown the importance of the antibody response, especially that mediated by neutralizing antibodies (NA), in coping with infection. Also the cell-mediated immune response seems to play a key role in preventing viral replication and counteract viral diffusion, despite many immune mechanisms which sustain virus clearance and the resolution of infection are still unclear (Fenaux et al., 2004b; Kixmoller et al., 2008; Fort et al., 2009a, 2009b; Steiner et al., 2009; Pérez-Martin et al., 2010).

For these reasons, several studies have been performed to investigate the mechanisms by which PCV2 can elude the immune defences in pigs and to develop new vaccination strategies aimed at inducing efficient immune activation and immune protection upon infection. The effects of vaccination treatments are related to total and neutralising antibody responses as well as to cell-mediated immunity (Larochelle et al., 2003; Fort et al., 2008, 2009a; Kixmoller et al., 2008; Opriessnig et al., 2008b, 2008c).

The humoral immunity to PCV2 infection were characterised, by most of the published reports, through the detection of total anti-PCV2 antibodies, showing seroconversion that occurs either in subclinically or non-PMWS infected and PMWS-affected pigs (Rodriguez-Arrijo et al., 2000; Sibila et al., 2004; Grau-Roma et al., 2009).

On the other hand, the role and mechanisms of the adaptive cell-mediated immune response in controlling PCV2 infection and the related diseases have not been clearly elucidated, particularly under field conditions. Previous reports based on laboratory trials describe that viral clearance may be mediated by cell-mediated immunity, measured by the number of PCV2-specific interferon- γ (IFN- γ) secreting cells (SC), together with neutralising antibodies (Fort et al., 2009a, 2009b) and that the load and the extent of viral replication may influence the intensity of the cell-mediated immune response.

Specifically PCV2 vaccines showed to induce an intense antibody and cellular responses but data regarding the immune responses under field conditions and underlying vaccine-induced protection are still incomplete. These aspects are worth investigating under field conditions both in diseased pigs naturally infected by PCV2 in the presence of coinfections and in vaccinated animals showing no or few clinical signs.

Furthermore, the modulation of cytokine patterns are important to categorize the host immune responses that occur during viral infections; for successful resolution of infection, efficient activation of innate/inflammatory and acquired immunity is required to block pathogen replication and invasion, as well as to promote tissue clearance of the pathogens and/or infected cells. The production of pro-inflammatory cytokines (IL-1 β , TNF- α , IL-8) and the balance between pro-immune (IFN- γ) and regulatory (IL-10) cytokines play a pivotal role in eliciting the innate response as well as in priming and coordinating the adaptive immune response. However, if production is impaired, the innate response will be delayed and inefficient in clearing the pathogen (Borghetti et al., 2010).

Therefore, pro-inflammatory and immune cytokine production has been investigated in different PMWS and infection models. Increased levels of IL-10 mRNA expression were found in the thymus and decreased levels of IL-4 and IL-2 were detected in the tonsils and spleen from PMWS-affected pigs (Sipos et al., 2004). Contrary results were described for IFN- γ : low mRNA expression levels were detected in inguinal and tracheobronchial lymph nodes whereas high expression was shown in the tonsils (Darwich et al., 2003b; Zhang et al., 2010). An increase of IL-10, together with slight increases of IL-8, IFN- γ and TNF- α and a decrease of IL-2 and IL-4 mRNA levels, were also detected in PBMC from naturally

PCV2-infected pigs (Sipos et al., 2004). Furthermore, serum IL-10 was associated with subclinically PCV2-infected pigs or pigs developing severe PMWS (Stevenson et al., 2006). A significantly higher expression of IL-10 was also found in PCV2-infected lymphoid tissues compared to uninfected control tissues (Doster et al. 2010).

Cytokine secretion may play a key role in evaluating the cellular immune response against PCV2, but the cytokine modulation investigated so far in different organs has not provided univocal results likely due to different working conditions.

Taking into account the above mentioned features, the aim of the present thesis is to investigate the efficacy of PCV2 vaccination under field conditions in vaccinated and unvaccinated pigs upon PCV2 natural infection and associated PCVD in terms of clinical protection and development of the humoral and cell-mediated immune response.

The specific objectives of the present study are the following:

1. the evaluation of the efficacy of a one-dose PCV2 subunit vaccine based on the PCV2 Cap protein expressed in a baculovirus system (Porcilis PCV[®]) at two different farms where PCVD was present, in terms of clinical protection;
2. the assessment of the vaccine immunogenicity in vaccinated and unvaccinated pigs exposed to PCV2 natural infection in terms of development of humoral (total PCV2-specific antibodies) and cell-mediated (PCV2-specific IFN- γ secreting cells) immune responses;
3. the quantitative modulation of pro-inflammatory and immune cytokines in vaccinated and unvaccinated pigs exposed to natural PCV2 infection, in relation to the onset of PCV2 viremia burden and PMWS clinical signs.

The results regarding the evaluation of PCV2 vaccine efficacy as clinical protection and humoral and cell-mediated immune responses were published in Martelli P., Ferrari L., Morganti M., De Angelis E., Bonilauri P., Guazzetti S., Caleffi A., Borghetti P. *Veterinary Microbiology*. 2011; 149(3-4): 339-351.

The data concerning the study of cytokine immune modulation were submitted to the journal "Comparative Immunology, Microbiology and Infectious Diseases" as Borghetti P., Morganti M., Ferrari L., De Angelis E., Saleri R., Cavalli V., Corradi A., Martelli P. Modulation of pro-inflammatory and immune cytokines in PBMC of vaccinated and unvaccinated pigs exposed to porcine circovirus type 2 (PCV2) natural infection (submitted, 2011).

CHAPTER 3.

EXPERIMENTAL STUDY UNDER FIELD CONDITIONS

3.1. Materials and methods

3.1.1. Selection of farms

The study was conducted in the Northern part of Italy at two farms with history of **PMWS**. In both herds, at approximately 15-20 weeks of age, clinical signs of **PWMS** characterised by wasting, respiratory signs and growth retardation were mainly associated with a marked increase in the mortality rate. The diagnosis fulfilled the internationally accepted disease case definition, including clinical signs, gross lesions, histopathologic findings, and presence of PCV2 in lymphoid lesions (Segale' s et al., 2005). Seropositivity to PCV2 in all categories of animals (replacement gilts, sows, nursery pigs, growers, and fatteners) was demonstrated. Before the start of the study, 5 wasted pigs were autopsied at each farm and the diagnosis was reconfirmed.

FARM 1 was a 900-sow farrow-to-finish herd that, in the previous year, experienced a 6% and an 8% mortality rate in the nursery and fattening periods, respectively. This farm was seronegative for Aujeszky's disease virus (ADV) and seropositive for PRRSV, *M. hyopneumoniae* and *A. pleuropneumoniae*. Low titres of antibodies at haemagglutination inhibition (HI) to SIV were obtained from some samples and were not informative.

FARM 2 was a three-site farm with 1850 sows experiencing a 2% and a 10% mortality rate in the nursery and fattening periods, respectively. Seropositivity to PRRSV and *M. hyopneumoniae* and seronegativity to ADV were found.

Sows of both herds were vaccinated for Aujeszky's disease (3 times/year), porcine parvovirus and erysipelas (both at mid-lactation). Piglets were vaccinated for Aujeszky's disease according to the National Control Program.

The protocols and results of the evaluation of PCV2 vaccine efficacy as clinical protection and humoral and cell-mediated immune responses are published in Martelli P., Ferrari L., Morganti M., De Angelis E., Bonilauri P., Guazzetti S., Caleffi A., Borghetti P. *Veterinary Microbiology*. 2011; 149(3-4): 339-351.

3.1.2. Animals and experimental design

This **study** was a **double-blind, randomised**, controlled field trial performed according to the principles of ‘‘Good Clinical Practice’’ and included 818 piglets (males and females). The day before inclusion, piglets were identified, double ear-tagged and assigned to two treatment groups [unvaccinated = placebo/control (group A) and PCV2-vaccinated (group B)] as they came to hand sequentially (A–B–A–B–A–B...). The sequential allocation was continued over the litters. The identification of the sow and the date of birth of the piglets were recorded.

At inclusion (weaning: 21 ± 3 days of age), vaccinated animals (group B) received one dose of a commercial PCV2a-based subunit vaccine (Porcilis PCV1 - Intervet/Schering-Plough Animal Health, Boxmeer, The Netherlands) containing the PCV2 capsid (Cap) protein expressed in a baculovirus system suspended in an α -tocopherol(+ liquid paraffin)-based adjuvant administered intramuscularly (2 ml) in the right neck muscle according to the manufacturer’s recommendations. The same amount of adjuvant was injected into the same anatomic location in control unvaccinated pigs (group A).

The **administration of vaccine and placebo** was performed using a **double-blind fashion system** for both farms. Animals of both groups were injected at weaning and moved to the nursery units. Table 1 lists the details of the studied animals at the time of inclusion.

Table 1. Details of the animals under study at the time of inclusion. Group A: placebo/control pigs; group B: PCV2-vaccinated pigs.

	Farm 1		Farm 2	
	Group A	Group B	Group A	Group B
Pigs at inclusion	206	205	204	203
Males–females	97–109	112–93	109–95	108–95
Total pigs	411		407	
Litters	43		40	
Weighed at inclusion	206	205	204	203
Bled at inclusion	22	22	22	22

After weaning, pigs from both treatment groups were kept in mixed groups until the end of the trial, when animals were sent to the slaughterhouse (at approximately 9 months of age).

Treatments, housing, husbandry, and feeding were conformed to the European Union Guidelines and identical for both experimental groups at each farm. At each accommodation change, pigs were commingled according to usual farm procedures.

Pigs were **clinically monitored on a weekly basis** from the administration of vaccine or placebo until slaughter. From one week post-vaccination, pigs were monitored daily for vaccination reactions. **Individual live body weights** of all animals enrolled in the study were measured the day before inclusion and at 12 and 26 weeks of age. The **average daily weight gain** (ADWG) was calculated based on the ADWG of animals being alive at the end of each weighing period. Carcass weight was recorded at the slaughterhouse in pigs from farm 2.

If concomitant treatments (injections) were used, the number of animals treated within each group and the duration of treatment were recorded. The number of relapsed animals (retreatments) were also considered. The number of injections was used as a parameter to evaluate **morbidity**.

Dead animals or those that had to be euthanised for reasons of animal welfare were recorded daily.

In both herds, at inclusion (day of vaccination, 3 weeks of age), blood samples were collected from 2 piglets of each pluriparous sow litter: one for each treatment group, up to 44 (22 piglets for each treatment group). These piglets were identified by a progressively numbered ear tag.

Moreover, in piglets from **FARM 1**, **blood samples** were taken at 4 [+1 week post-vaccination (PV)], 5 (+2 weeks PV), 6 (+3 weeks PV), 7 (+4 weeks PV), 9 (+6 weeks PV), 12 (+9 weeks PV), 15 (+12 weeks PV), 16 (+13 weeks PV), 17 (+14 weeks PV), 18 (+15 weeks PV), 19 (+16 weeks PV), 20 (+17 weeks PV), 22 (+19 weeks PV), 26 (+23 weeks PV), and 35 (+32 weeks PV) weeks of age.

In **FARM 2**, **blood samples** were collected at vaccination (3 weeks of age), at 4 (+1 week PV), 6 (+3 weeks PV), 12 (+9 weeks PV), 16 (+13 weeks PV), 18 (+15 weeks PV), 20 (+17 weeks PV), 22 (+19 weeks PV), 24 (+21 weeks PV), 26 (+23 weeks PV), and 35 (+32 weeks PV) weeks of age.

3.1.3. Evaluation of gross pathology, histopathology, and immunohistochemistry

The objective of the pathologic studies was to establish the PMWS diagnosis in all pigs from both farms that died, those needing euthanasia, and runts during the entire study period. These pigs underwent gross pathologic examination and histopathology within 24 hours.

Samples from inguinal, mesenteric and mediastinic lymph nodes were removed from necropsied pigs and fixed in 10% buffered formalin. Fixed samples were processed for routine histopathology and 5- μ m thick sections were stained with hematoxylin and eosin to be examined for lesions compatible with PMWS.

The diagnosis of PMWS was made when all three criteria of the accepted international individual case definition for the disease were present, that are: 1) clinical signs, mainly including wasting; 2) moderate-to-severe lymphoid lesions; 3) moderate-to-high amounts of PCV2 in those lesions.

The amount of PCV2 in tissue samples was also assessed by real-time quantitative PCR (qPCR) using the methods reported by Olvera et al. (2004).

Immunohistochemistry for detection of PCV2-specific antigen was performed on formalin-fixed and paraffin embedded sections of inguinal, mediastinal, and mesenteric lymph nodes using a rabbit polyclonal antiserum (Sorden, 2000). PCV2 antigen scoring was performed by a pathologist in a blind manner using the score range in accordance with Opriessnig et al. (2004).

3.1.4. Extraction and qPCR detection of PCV2 DNA from tissue samples and serum

In order to detect and quantify the PCV2 DNA by PCR, DNA was firstly extracted from 200 μ l of serum or 200 μ l of 1:10 phosphate-buffered saline (PBS) homogenate of lymph node tissue, by using TRIzol LS (Invitrogen, San Diego, CA,USA) following the manufacturer's instructions. The DNA obtained was suspended in 50 μ l of diethylpyrocarbonate (DEPC)-treated water. Real-time qPCR was carried out using a LightCycler 1.5 (Roche, Basel-CH). Real-time qPCR was performed using primers and probes according to Olvera et al. (2004). Results of the qPCR were expressed as number of PCV2 genome copies per milliliter of serum or gram of tissue.

Table 2. Properties, sequence and localisation of primers and probes designed for the PCV2 real-time PCR by primer express (Olvera et al., 2004).

	<i>T_m</i> (°C)	%GC	bp	Sequences (5'→3')	Location in PCV2 genome
PCV2 F	60	63	19	CCAGGAGGGCGTTGTGACT	1535 → 1553
PCV2 R	59	55	20	CGCTACCGTTGGAGAAGGAA	1633 → 1614
PCV2 P	68	52	25	AATGGCATCTTCAACACCCGCCTCT	1612 → 1592

PCV2 F = primer forward, PCV2 R = primer reverse, PCV2 P = probe, *T_m* = melting temperature.

3.1.5. Serology

3.1.5.1. PCV2-specific antibody titres

The anti-PCV2 antibody titres in sera were determined using a blocking enzyme-linked immunosorbent assay (ELISA). The wells of microtitre plates were coated overnight at 2-8°C with baculovirus-expressed PCV2 ORF2 antigen. Subsequently, the plates were washed and blocked with casein buffer at 37°C for 1 h. After washing, serial 4-fold dilutions of the test sera were added. An internal standard serum and a positive and negative standard serum were run in parallel in each plate. The sera were incubated for 1 h at 37°C and the plates were then washed before the addition of a PCV2-specific biotinylated monoclonal antibody (mAb). After 1 h incubation at 37°C, plates were washed again and incubated for 45 min at 37°C with avidin-labeled horseradish peroxidase (APO; DAKO A/S, Glostrup, Denmark). After washing, a 3,3',5,5'-tetramethylbenzidine (TMB) substrate solution was added and incubated for 15 min. at room temperature. The reaction was stopped by the addition of 4 N sulphuric acid and the extinction was read in a photometer fitted with a 450-nm filter (Titertek Multiscan Plus MK 11 – Titertek Instruments Inc., Huntsville, AL, USA) within 15 min. after the reaction was stopped. The raw data were processed and titres were calculated using the Multi-calc program with a cut-off extinction value set at 50% blocking. The cut-off extinction was calculated from the positive and negative standard serum and titres were expressed as log₂.

3.1.5.2. Antibody titres to other infectious agents

The presence of antibodies to PRRSV and sample/positive (S/P) ratios were determined using a commercially available ELISA kit (HerdChek1 Porcine Reproductive and Respiratory Syndrome Antibody Test Kit, IDEXX Laboratories, Westbrook, ME, USA) according to the manufacturer's instructions. The Herd Check1 test bases the sample classification on the S/P ratio, which is defined as (sample O.D. - negative control O.D.) / (positive control O.D. - negative control O.D.). Sample to positive control ratios ≥ 0.4 were considered positive. Antibodies to *M. hyopneumoniae* were evaluated by a commercially available ELISA test (Herd Check1 *M. hyopneumoniae*, IDEXX Laboratories). The presence of antibodies to gE glycoprotein of ADV was measured using a commercially available ELISA kit [HerdChek1 PRV g1 (gE) test kit, IDEXX Laboratories] according to the manufacturer's instructions. A commercial ELISA kit (CHEKIT-APP-Apx IV ELISA test kit, IDEXX Laboratories) was used for the detection of antibodies against *A. pleuropneumoniae*. Serology to swine influenza virus (SIV) was performed by using HI.

3.1.6. ELISpot for determination of PCV2-specific IFN- γ secreting cells (SC)

An IFN- γ ELISpot assay was performed according to Martelli et al. (2009) in order to evaluate the frequencies of IFN- γ SC in the peripheral blood of pigs from farm 1.

The assay was performed in MultiScreen[®]_{HTS}-IP plates (MSIPS4510 – Millipore) coated with 10 μ g/ml anti-pig IFN- γ mAb (P2G10, BD, Biosciences, Franklinlakes, NJ, USA) at 4°C overnight. After incubation, plates were washed with sterile PBS and blocked with RPMI-1640 supplemented with 10% foetal bovine serum (FBS) for 2 h at 37°C, 5% CO₂.

Peripheral blood mononuclear cells (PBMC) were isolated by density gradient using a Histopaque-1.077[®] solution and plated at 2×10^5 cells/well in RPMI-1640 + 10% FBS.

For the *ex vivo* antigen recall, a whole PCV2 strain (I12/11) at 0.1 multiplicity of infection (MOI) was used as stimulus, in RPMI-1640 + 10% FBS, for 20 h at 37°C, 5% CO₂; the SC response was also evaluated at 0.05 and 0.25 MOI. In all samples, PBMC were > 98% viable as confirmed by Trypan blue exclusion. Afterward, cells were removed by washing with PBS + 0.05% Tween-20 (PBST) and the plates were incubated for 1 h at 37°C with 0.5 μ g/ml anti-pig IFN- γ biotin-labeled mAb (P2C11, BD). After washing, plates were incubated with 1:750 alkaline phosphatase (AP)-conjugated anti-biotin mAb in PBS + 0.5% BSA for 1 h at 37°C. Plates were finally incubated for 7 min. with a BCIP/NBT solution (BioRad, Hercules,

CA, USA) in order to let spots develop and the reaction was stopped with distilled water. The frequencies of PCV2-specific IFN- γ SC were determined using an AID[®] ELISpot Reader (AID[®] ELISpot Software v.3.5). As a positive control, 1×10^5 PBMC/well were incubated with phytohemagglutinin (PHA, 10 μ g/ml); as a negative control, 2×10^5 PBMC were incubated in the absence of antigen (mock stimulus: supernatant of non-PCV2-infected PK-15 cells). The background values (number of spots in negative control wells) were subtracted from the respective counts of the stimulated cells and the immune responses were expressed as number of IFN- γ SC per million PBMC (IFN- γ SC/ 10^6 PBMC).

The IFN- γ SC responses were also classified as responsiveness categories on the basis of the responses observed in the present study and with regards to categories reported in literature (Fort et al, 2009b; Ferrari et al., 2011).

Specifically the responsiveness categories identified were:

- 0-40 IFN- γ SC/ 10^6 PBMC = no-poor
- 45-100 IFN- γ SC/ 10^6 PBMC = low
- 105-200 IFN- γ SC/ 10^6 PBMC = intermediate
- 205-400 IFN- γ SC/ 10^6 PBMC = high
- ≥ 405 IFN- γ SC/ 10^6 PBMC = very high

Particularly, the range for the “no-poor” category was determined based on the responses observed in unvaccinated animals throughout the post-vaccination period and data in literature (Pérez-Martin et al., 2010).

3.1.7. Evaluation of cytokine gene expression

Blood samples collected from 10 PCV2-vaccinated and 20 non-vaccinated pigs were used as source of swine peripheral blood mononuclear cells (PBMC) in order to determine mRNA expression levels of relevant pro-inflammatory and pro-immune cytokines.

At the end of the trial, taking into consideration the time of the onset of viremia and the appearance of clinical signs, blood samples taken at 16 (before infection and the disease onset), 19 and 22 weeks of age (during PCV2 viremia and disease outcome) were used for the quantification of cytokine expression.

For the purposes of this study, the thirty sampled pigs were attributed to three different groups: 1) non vaccinated and spontaneously PCV2 infected animals (N = 5) that showed

over clinical signs attributable to PMWS (Ctrl-PMWS+); 2) non-vaccinated and spontaneously PCV2 infected animals (N = 15) that did not develop PMWS (Ctrl); 3) vaccinated pigs (N = 10) that were not infected and did not develop PMWS (PCV2-vac). Moreover, to establish an association between cytokine expression and viremia burden, each of the above mentioned groups was analysed dividing the animals in three different subgroups based on viremia: non-viremic pigs (NV), pigs with viremia $<10^6$ ($V < 10^6$) and pigs with viremia $\geq 10^6$ ($V \geq 10^6$) viral genome copy number / ml of serum.

3.1.7.1 Isolation of PBMC and extraction of total RNA

mRNA expression levels of relevant pro-inflammatory (IL-8, TNF- α , IL-1 β) and immune (IFN- γ , IL-10) cytokines were determined in swine peripheral blood mononuclear cells (PBMC) after isolation by density gradient using Histopaque-1077[®] (Sigma) and total RNA extraction by TRI-reagent[®] (Ambion) according to the manufacturer's instructions; purity and concentration were assessed by UV spectrophotometry at 260/280 and 260 nm respectively (GeneQuant Pro[®], Amersham Pharmacia Biotech-GE Healthcare Life Sciences, Little Chalfont, UK).

3.1.7.2. RNA reverse transcription (RT)

Total RNA (1 μ g) was reverse transcribed using a High-capacity cDNA Reverse Transcription kit (Applied Biosystems). The reverse transcription was performed by using a StepOne[™] thermocycler (Applied Biosystems, StepOne software v. 2.1) and, according to the manufacturer's established procedures, under the following thermal conditions: 10 min. at 25°C, 120 min. at 37°C followed by 5 min. at 85°C and a pre-storage at 4°C. All cDNA samples were stored at -20°C until PCR was performed.

3.1.7.3 Quantification of cytokine mRNA by quantitative PCR

The obtained cDNA was used (20 ng) as a template for real-time PCR (qPCR) performed by using a StepOne[™] thermocycler (Applied Biosystems, StepOne software v. 2.1). The cDNA (20 ng/20 μ l) was amplified in duplicate with Fast SYBR[®] Green Master Mix (Applied Biosystems) along with specific sets of primers at optimized concentrations.

The primers were designed based on published gene sequences (Royae et al., 2004; Meissonnier et al., 2008; von der Hardt et al., 2004) or by using Primer Express[®] software for primer design (purchased from MWG). Details of each primer set for detection of cytokine gene expression are reported in Table 3.

The reference gene glyceraldehyde 3-phosphate dehydrogenase (GAPDH) was selected as endogenous control according to Fisher et al. (2006) and Borghetti et al. (2010).

Samples were kept at 95°C for 20 sec. (hold step) to allow DNA polymerase activation and then subjected to 40 cycles constituted of a denaturation step at 95°C for 3 sec. followed by an annealing/extension step at 60°C for 30 sec. (Applied Biosystems “Fast” real-time PCR protocol). Fluorescence due to SYBR[®] Green incorporation was acquired at the end of the extension step. A no-template control was included in each experiment.

A melting curve analysis for specific amplification control was performed (from 60°C to 95°C) at the end of the amplification cycles.

Table 3. Details of the primer sequences of pig pro-inflammatory (IL-8, TNF- α , IL-1 β) and immune (IFN- γ , IL-10) cytokines used for quantitative SYBR[®] Green real-time PCR. GAPDH gene was used as endogenous control gene.

Gene	Primer sequence (forward – reverse)	Concentration (nM)	Amplified product (bp)
IL-8 (Royae et al., 2004)	<i>for</i> 5'-CCGTGTCAACATGACTTCCAA-3' <i>rev</i> 5'-GCCTCACAGAGAGCTGCAGAA-3'	300 300	75
TNF-α (Meissonnier et al., 2008)	<i>for</i> 5'-ACTGCACTTCGAGGTTATCGG-3' <i>rev</i> 5'-GGCGACGGGCTTATCTGA-3'	300 300	118
IL-1β (von der Hardt et al., 2004)	<i>for</i> 5'-ATGCTGAAGGCTCTCCACCTC-3' <i>rev</i> 5'-TTGTTGCTATCATCTCCTTGCAC-3'	300 300	89
IFN-γ (Royae et al., 2004)	<i>for</i> 5'-TGGTAGCTCTGGGAAACTGAATG-3' <i>rev</i> 5'-GGCTTTGCGCTGGATCTG-3'	300 300	79
IL-10 (Royae et al., 2004)	<i>for</i> 5'-TGAGAACAGCTGCATCCACTTC-3' <i>rev</i> 5'-TCTGGTCCTTCGTTTGAAAGAAA-3'	300 300	114
GAPDH (Primer Express)	<i>for</i> 5'-GGTGAAGGTCGGAGTGAACG-3' <i>rev</i> 5'-GCCAGAGTTAAAAGCAGCCCT-3'	300 300	70

Data were analyzed according to the $2^{-\Delta\Delta Ct}$ method; in the present experiment the expression levels of each cytokine, normalized to the GAPDH cDNA amount and expressed as relative quantities (RQ), were calculated with regards to some PCV2-vaccinated uninfected animals

early in the post-exposure (PE) period. In order to apply the $2^{-\Delta\Delta C_t}$ method, because of the required normalisation step, it was necessary to first assess that the efficiency of the cytokine primers was comparable to that of the housekeeping gene. In this regard, a concentration range for each primer pair was tested by serial dilution of a cDNA sample in which both the housekeeping and target genes could be easily amplified. Only primers showing a comparable amplification efficiency to that of GAPDH were used.

3.1.8. Statistical analysis

To estimate the effect of vaccination on the probability of a pig of becoming viremic, a mixed effect logistic regression model was fitted to take into account the non independence of the repeated measures on the same subjects and the effect of the sow (litter effect). These two variables were treated as ‘‘random effects’’ in the model, whereas the effect of the farm (two levels), sex, time, and treatment and their interactions were considered as ‘‘fixed effects’’. The effect of the time x treatment interaction was highly significant ($P = 0.0146$), indicating that these results are not attributable to chance alone. The package ‘‘lme4’’ was used (Bates and Sarkar, 2007; lme4: linear mixed effects models using S4 classes, R package version 0.999375-32). To estimate the effect of vaccination on the probability of a pig suffering from PCVD and being lost, considering the competing risks of dying or being lost from other causes, a stratified Cox proportional hazard model was fitted, according to Putter et al. (2007) and Therneau and Grambsch (2000). The model accounted also for the ‘‘cluster’’ effect of the sow (‘‘litter effect’’) because piglets from the same litter are expected to have similar clinical histories. The effect of the vaccination on weight gain was evaluated within a mixed effects model, given the hierarchical structure of the experiment (Pinheiro and Bates, 2000).

Vaccine efficacy was measured by the proportion of cases that it prevented, comparing disease outcome in the treated versus control groups. Efficacy was presented here as one-risk ratio (the so-called preventive fraction), which gives the risk in the vaccinated group as a proportion of the risk in the control group (Kirkwood and Sterne, 2003).

Humoral and cellular immunity, determined as titres of anti-PCV2 antibodies and frequencies of IFN- γ SC respectively and also quantitative data of cytokine gene expression were statistically evaluated by using ANOVA (analysis of variance) and Dunnett’s test in order to highlight differences between treatment groups and changes over time within the same group throughout the experiment. The significance level was set to $p < 0.05$.

3.2. Results

3.2.1. Morbidity and mortality evaluation: reduction in PCV2-vaccinated animals

In both herds, clinical signs potentially compatible with PMWS were mainly observed during the fourth and fifth month after vaccination (weeks 16-23 PV).

Morbidity was quantified by recording the number of individual antimicrobial treatments (injections) during the duration of the experiment. Unvaccinated animals received more injections than PCV2-vaccinated throughout the study (Fig. 5); according to the statistical analysis using a Poisson model, the differences between groups were significant ($P < 0.0001$). Specifically, animals belonging to group A (placebo/ controls) are expected to receive 30% more injections as compared to vaccinated pigs [95% confidence interval (CI_{95%}): 16-50%]. Pathologic and virologic investigations were carried out to categorise dead pigs, animals needing euthanasia, and runt non-marketable pigs (lost pigs) as PMWS or non-PMWS, using the recognised diagnostic criteria. Before the onset of viremia, the mortality rates in both groups were comparable and all dead animals were not affected by PMWS.

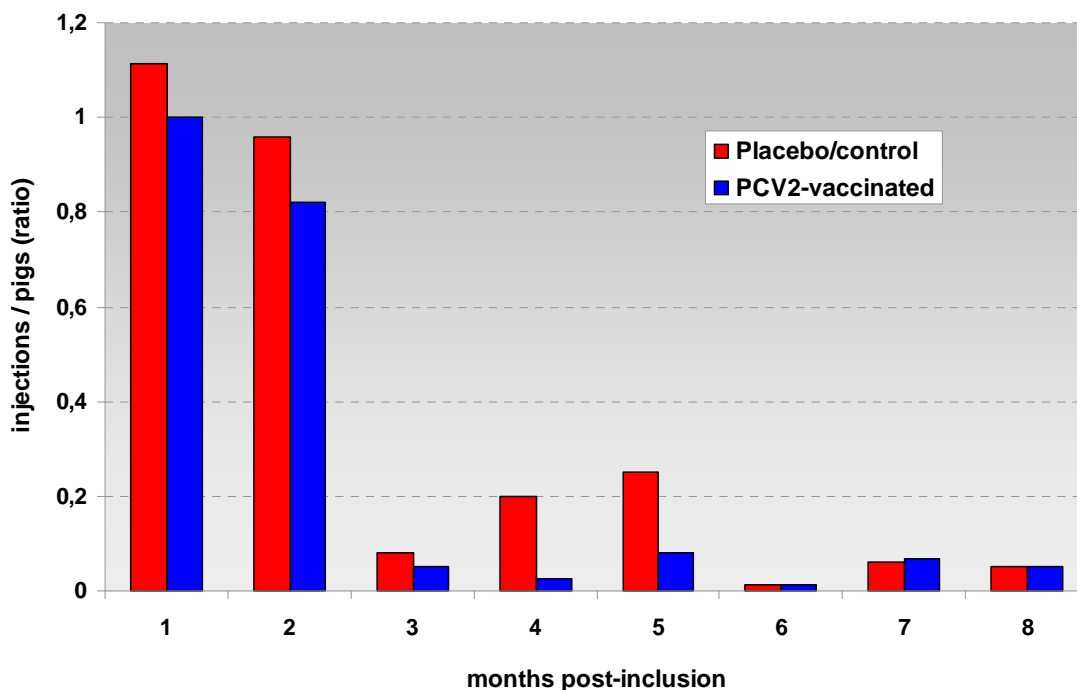


Figure 5. Course of morbidity (injections/pigs) in placebo/control (group A) and PCV2-vaccinated (Group B) animals (Poisson model; $P < 0.0001$).

At 15-16 weeks of age and onward in both herds, mortality was associated with PCR positivity to PCV2 and with macroscopic and microscopic lesions referred as PMWS using the pathologic criteria. The details of the occurrence of mortality categorised as PMWS or non-PMWS on a weekly basis at both farms are shown in figure 6.

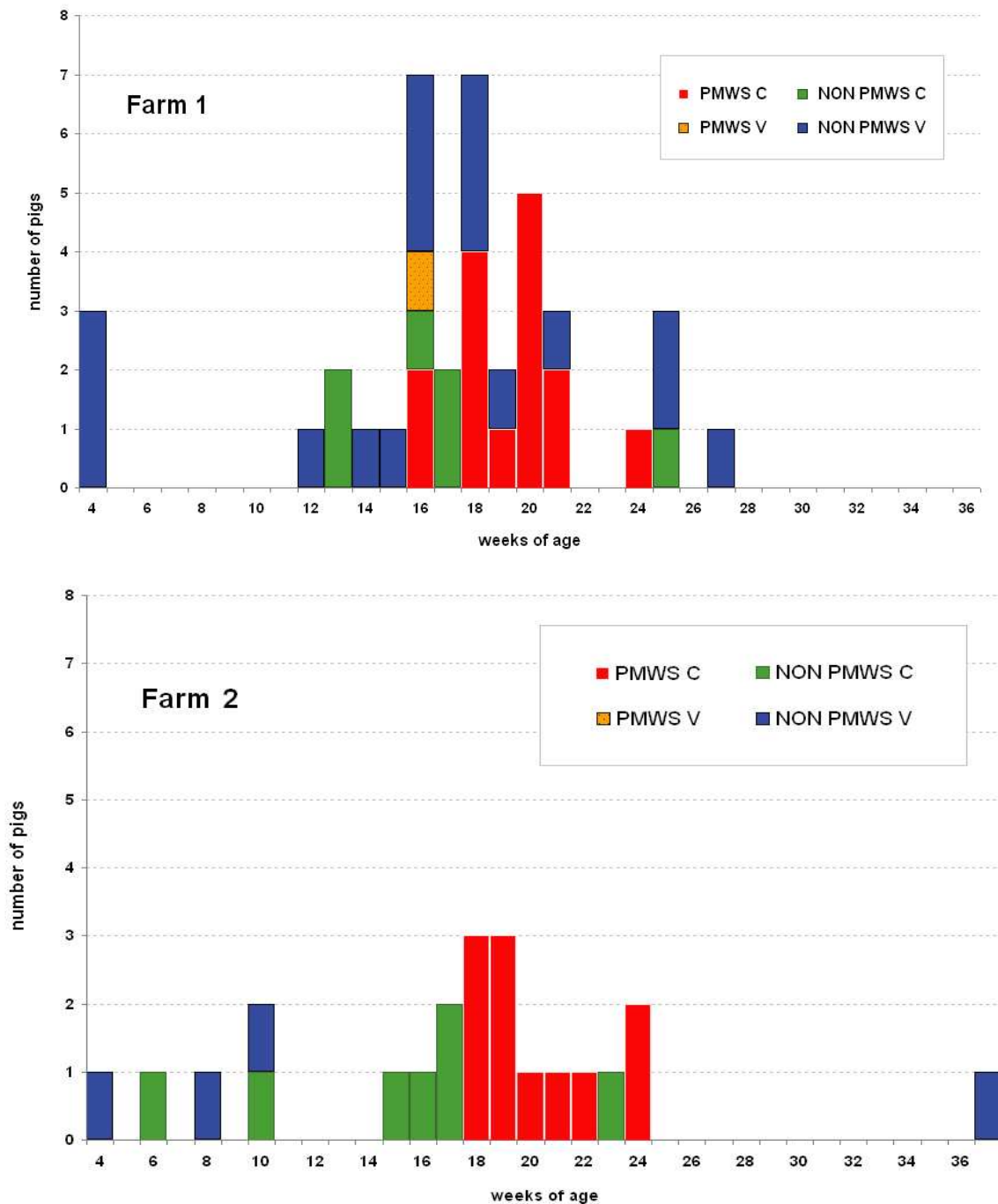


Figure 6. Losses due to PMWS and non-PMWS in PCV2-vaccinated and placebo/control pigs of farm 1 (top) and farm 2 (bottom). Legend: PMWS C: control pigs diagnosed as PMWS-affected; NON PMWS C: control pigs not affected by PMWS; PMWS V: vaccinated pigs diagnosed as PMWS-affected; NON PMWS V: vaccinated pigs not affected by PMWS.

After the onset of PCV2 viremia, pigs belonging to the group of animals that had to be euthanised or removed because they were runts, were removed from the study because of wasting (15 vs. 0 pigs; placebo/control vs. vaccinated), growth retardation (12 vs. 3 pigs, respectively), locomotory disorders (4 vs. 5 pigs, respectively), and intestinal torsions (1 vs. 2, respectively).

Before the onset of viremia, total losses were 7.3% and 7.8% and after PCV2 viremia 9.02% and 0.2%, respectively in the placebo/control and vaccinated groups. Overall, considering both herds for the study duration, total losses (dead, euthanised, and runts) were 16.03% and 8.0% in the placebo/control and vaccinated groups, respectively. The estimated hazard ratio for losses related to PMWS in group B (vaccinated animals) compared to group A (placebo/control) was 0.082 (CI_{95%}: 0.030-0.229; P< 0.0001; Fig. 7).

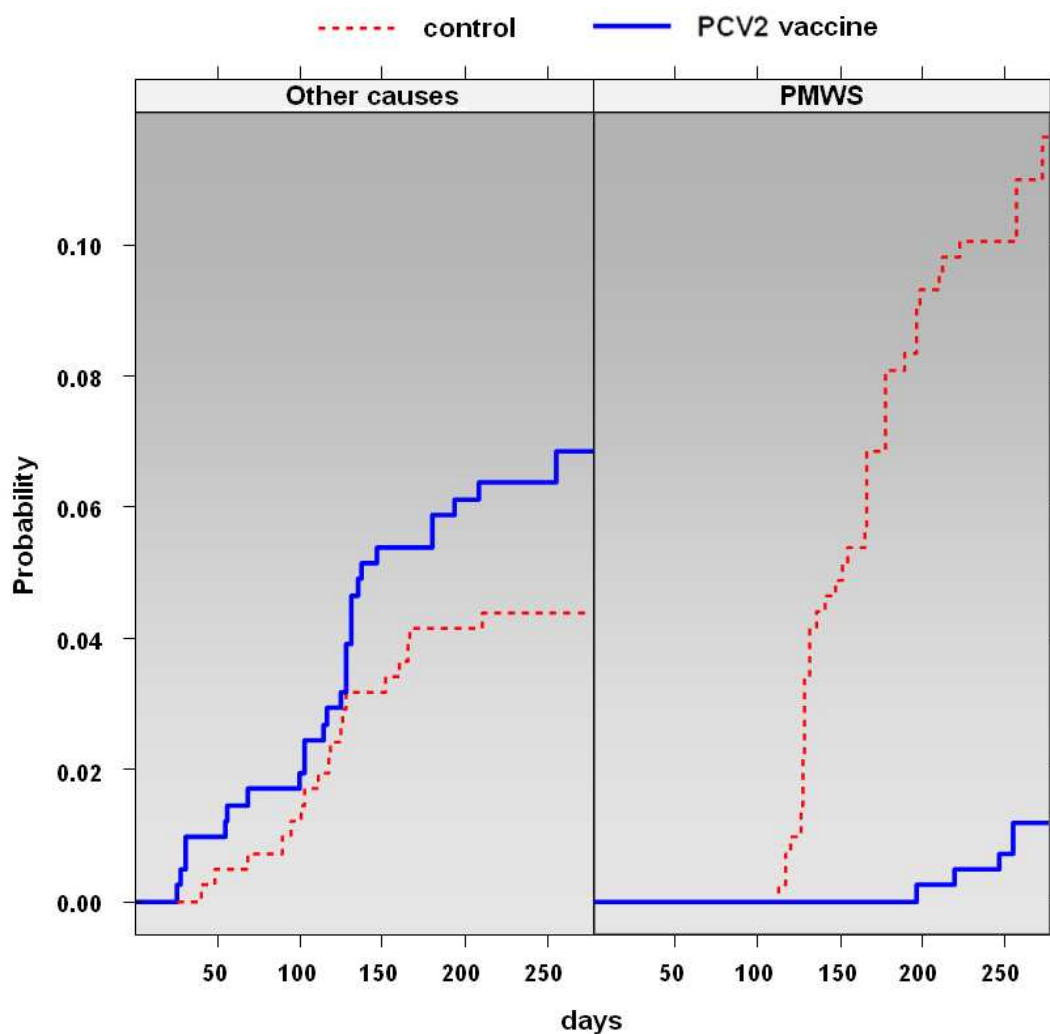


Figure 7. Probability of a pig in the placebo/control (A) and PCV2-vaccinated (B) groups to be lost because of PCVD and other causes according to the estimated hazard risk over time, in both farms.

Under the conditions of this study, according to a stratified Cox proportional hazard model accounting for the non independence of repeated measurement of the same subject and the effect of the sow (litter effect) and for the competing risks of dying from other causes, the probability of a pig vaccinated with a single dose of the test vaccine at 3 weeks of age suffering from PCVD/PMWS was 12 times less than an unvaccinated control pig. The overall efficacy of the vaccine administered, expressed as preventive fraction was 0.918 (CI_{95%}: 0.771-0.970). The preventive fraction represents and provides the proportion of cases that can be prevented by vaccination compared to the unvaccinated population.

3.2.2. ADWG and carcass weight at slaughterhouse

The ADWG is a parameter used to measure the effect of PMWS either in acute or in subclinical cases.

Table 4 shows the ADWG in vaccinated and placebo/control animals for the intervals among the three different weighing time points. Significant differences in the ADWG between the treatment groups were not observed during the first time period (3-12 weeks of age), whereas they were observed during the subsequent time period (12-26 weeks), when the vaccinated animals had 70 g/day higher weight gain than placebo/control animals ($P < 0.001$).

Moreover, the proportion of animals whose body weight was at least 25% lower than the mean body weight of the respective treatment group at 26 weeks of age was 6.5% and 2.6% in placebo/control and vaccinated groups, respectively. Carcass weights in pigs from farm 2 were recorded at the slaughterhouse, as shown in Table 4. In vaccinated animals, the average carcass weight was 4.5 kg higher than in placebo/controls ($P < 0.012$).

Table 4. ADWG in PCV2-vaccinated (group B) and placebo/control (group A) for two intervals between three different weighing time points during two study periods and carcass weight at slaughter.

ADG (g)	A	B	Diff.	P value
3-12 weeks of age	481	478	+3	> 0.05
12-26 weeks of age	811	881	-70	< 0.001
CARCASS (kg) *	140.5	145.0	+4.5	< 0.012

*carcass weight with head left on

3.2.3. PCV2 genome detection and viremia calculation

The course of PCV2 viremia at both farms is shown in figure 8.

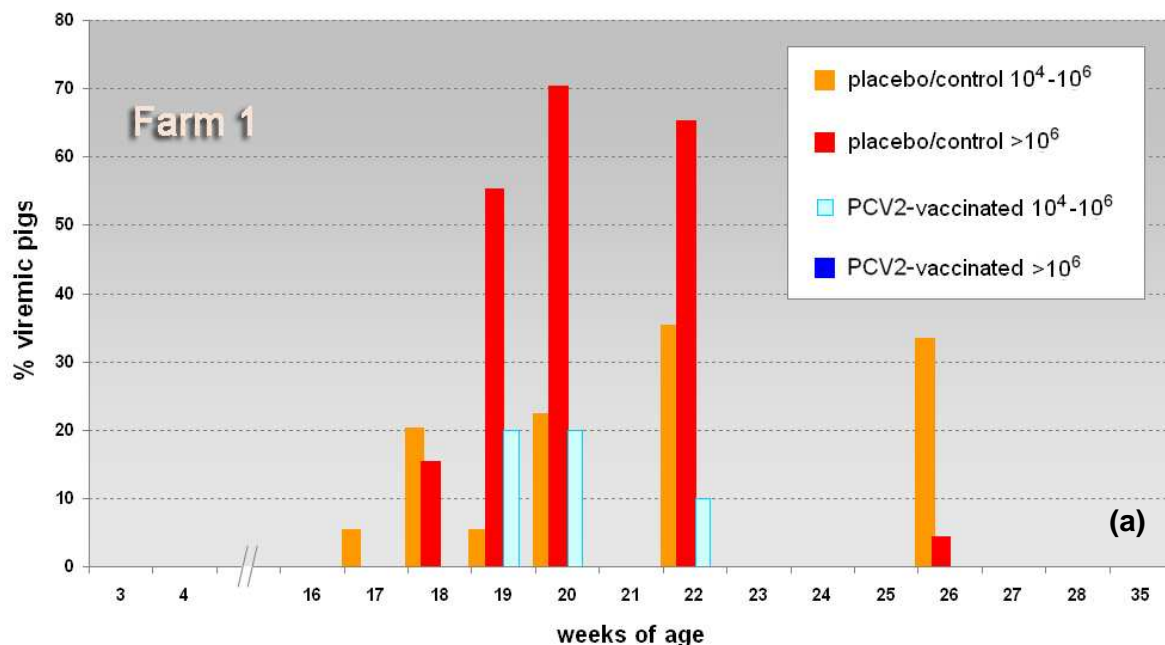
A rapid onset of viremia was observed at 16-17 weeks of age at both farms.

In placebo/control animals of farm 1, peak levels of 95-100% of PCR-positive blood samples were reached when animals were 20-22 weeks old. A decline of PCR positivity was detected at 26 weeks. The majority of samples with high viral loads ($>10^6$ DNA copies/ml of serum) were observed at 19-22 weeks of age (Fig. 8a), and 70% of the animals had at least one blood sample with a viral load $>10^7$ DNA copies/ml.

Contrarily, in the vaccinated group, the proportion of viremic pigs was significantly lower compared to the placebo/control group; between weeks 19 and 22 of age, 40% of the animals were viremic, with a viral load never $>10^6$ DNA copies/ml serum (Fig. 8a).

At farm 2, the peak of viremia was observed at 18-20 weeks of age with 95% PCR-positive pigs; pigs with a high viral load ranged from 55% to 60% (Fig. 8b). A viral burden $>10^7$ DNA copies/ml was detected in 42% of blood samples from the controls.

In the vaccinated group only one blood sample was PCR positive at 18 weeks of age, with a low viral burden ($<10^6$ DNA copies/ml; Fig. 8b).



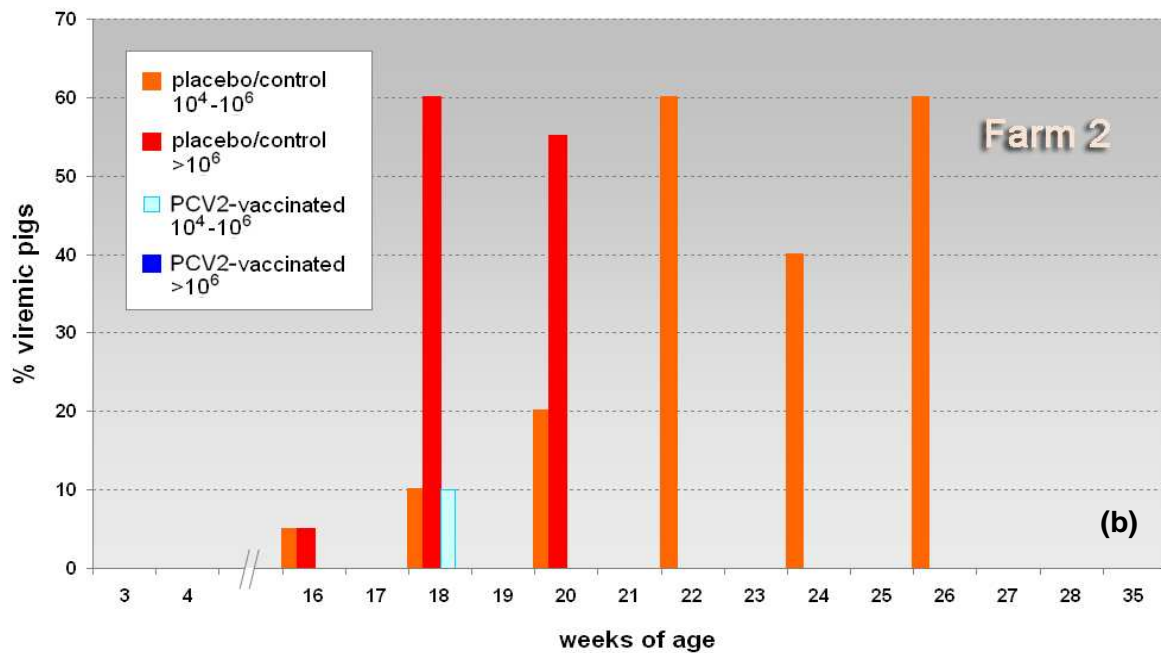


Figure 8. Course of viremia over time in placebo/control and PCV2-vaccinated pigs of farm 1 (a) and farm 2 (b). Data are expressed as number of PCV2 DNA copies/ml of serum.

The present data clearly indicate that vaccination against PCV2 induced a statistically significant reduction of the viral load in the blood and of the proportion of viremic animals for both farms in this study ($P < 0.001$).

3.2.4. Serology

3.2.4.1. Production of specific antibodies following PCV2 vaccination and infection

The course of serology for PCV2 at both farms is shown in figure 9.

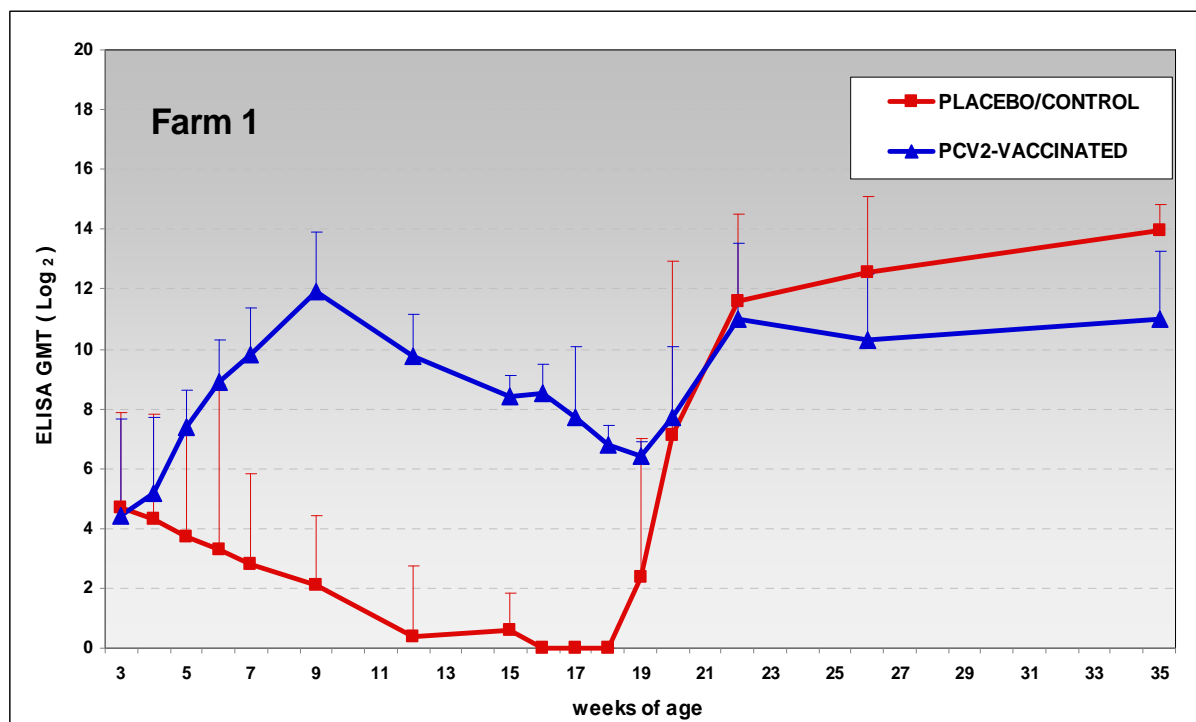
At inclusion (vaccination day), due to residual maternally derived antibodies (MDA), pigs of both placebo/control and PCV2-vaccinated groups showed comparable levels of ELISA antibodies (5.95 and 6.69 \log_2 , respectively). The difference was not statistically significant.

After vaccination, PCV2-specific antibody titres progressively declined in placebo/control animals, whereas a significant increase was observed in vaccinated pigs. Starting at 2 weeks PV, the differences between the two groups were statistically significant. Animals in the vaccinated groups showed a continuous increase of total antibody titres, reaching a peak of ELISA antibodies at 6-9 weeks PV, with an average geometric mean ranging from

12 to 13 \log_2 . From this time point on, the levels of total antibodies in vaccinated groups slightly decreased even if never below a geometric mean of 6 \log_2 .

At the last time point of blood sampling before the onset of viremia, the placebo/control animals from farm 1 showed a geometric mean of total antibodies under the cut-off for positivity (set at 2 \log_2), while this parameter was a little over the limit (3.4 \log_2) in placebo/control pigs of farm 2. In approximately 10% of the sampled population, the antibody titres at inclusion were higher than 8 \log_2 . In vaccinated animals these titres showed neither an increase as a consequence of vaccination nor a decline but maintained a steady course over time.

In the placebo/control group, the decline of maternally derived antibodies in animals with a titre higher than 8 \log_2 reached low levels within 10 weeks approximately (data not shown). At both farms, after the onset of PCV2 viremia, seroconversion occurred within 2-3 weeks in placebo/control or vaccinated groups so that at 20-22 weeks of age the antibody levels in both groups were comparable (11-12 \log_2). At 26 and 35 weeks of age, vaccinated animals had lower geometric mean antibody titres than that in placebo/control pigs because of a continuing increase of antibodies in the latter group of animals. At the last sampling time point of this trial (35 weeks of age), total ELISA antibodies were above the titre of 10 \log_2 in both treatment groups.



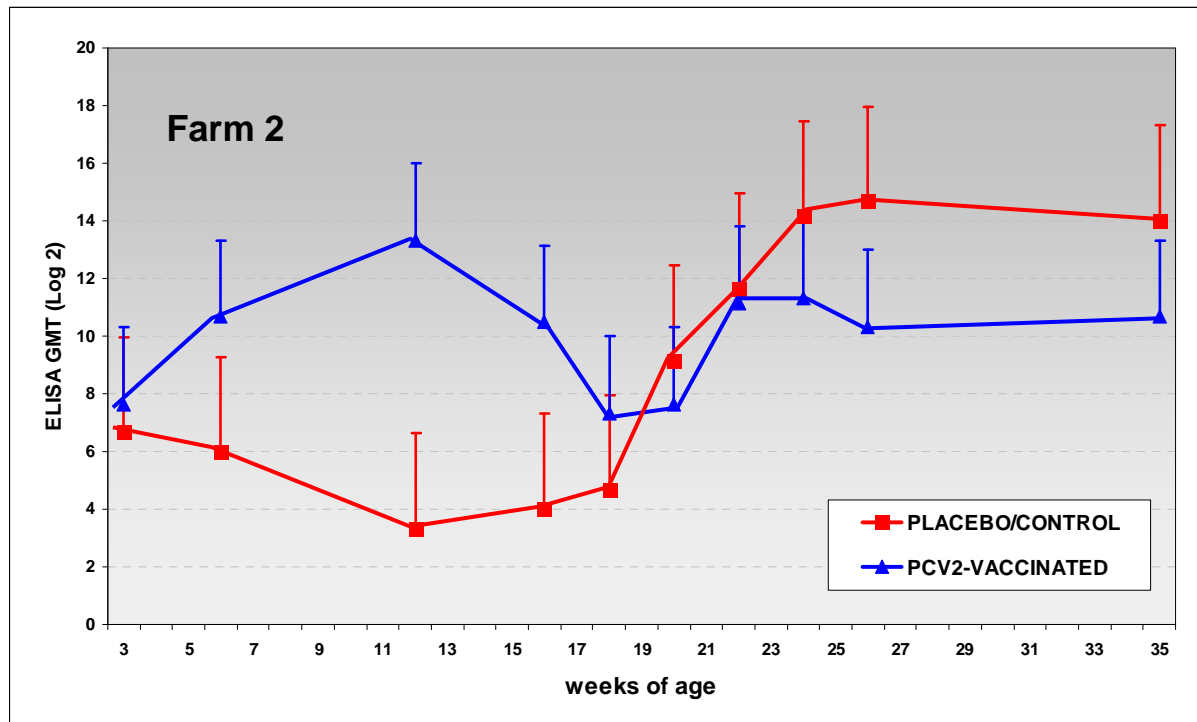


Figure 9. Course of the serologic response to PCV2 (anti-PCV2 antibodies) at farm 1 and farm 2 from inclusion (3 weeks of age) to the end of the trial (data are expressed as \log_2 of the geometric mean titres, GMT).

3.2.4.2. Antibody response to other infections

Serologic investigations performed to monitor the most frequently occurring infections in the herds (PRRSV and *M. hyopneumoniae*) found that the prevalence of PRRSV infection was 100% at 12 weeks of age in pigs from farm 1 concomitantly with *M. hyopneumoniae* seroconversion. For this latter pathogen, seroprevalence continued to increase in the subsequent period.

At farm 2, at 12 weeks of age, pigs of both groups were positive (100%) for PRRSV and still negative for *M. hyopneumoniae*. Seroconversion to *M. hyopneumoniae* started to be detected after 15 weeks of age (data not shown). At both farms, low titres of antibodies at HI to Swine Influenza Virus were detected in some samples and were not informative.

3.2.5. Levels of IFN- γ secreting cells detected by ELISpot assay in response to PCV2-vaccination and infection

3.2.5.1. ELISpot assay: antigen recall with 0.1 MOI of PCV2

After vaccination, PCV2-vaccinated animals showed an increased level of PCV2-specific IFN- γ SC at 2 weeks PV and reached a peak with a mean value of 120 IFN- γ SC/ 10^6 PBMC 1 week later. The mean values remained the same until 9 weeks of age (Fig. 10).

In the same period, at 2-3 weeks after vaccination, the number of animals with a progressively higher individual IFN- γ response increased (Tab. 5).

In pigs from the placebo/control group the number of PCV2-specific IFN- γ SC remained at basal levels (<20 PCV2-specific IFN- γ SC) for the entire post-vaccination period (Fig. 10) and no significant individual differences were found (Fig. 10). After the occurrence of the infection at 15-16 weeks of age, vaccinated animals showed an erratic course in the number of PCV2-specific IFN- γ SC with moderate individual increases ranging from 40 to 60 IFN- γ -SC on average (Fig. 10).

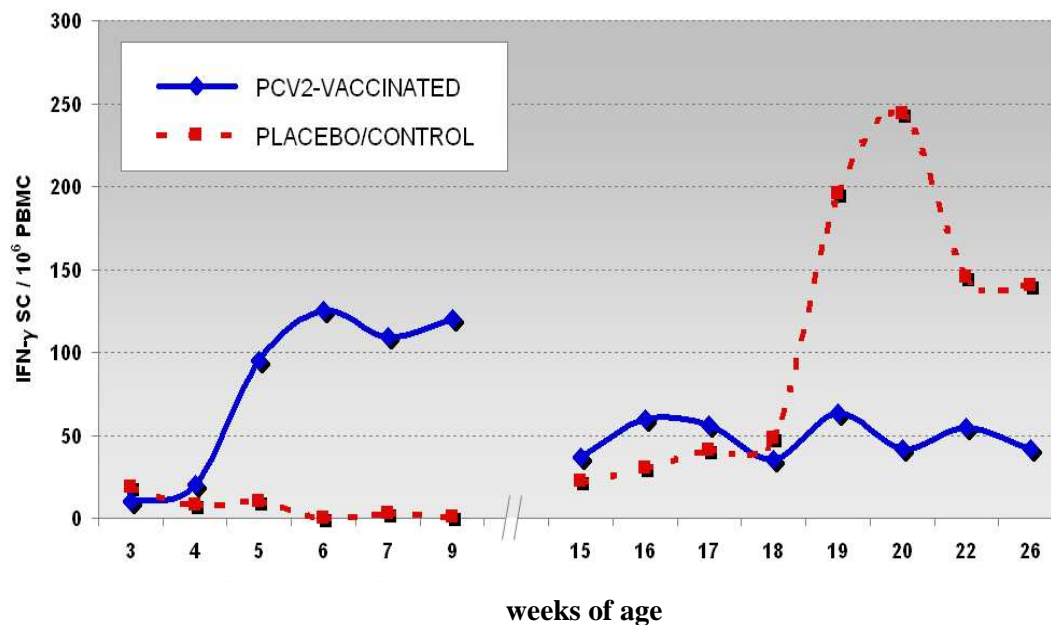
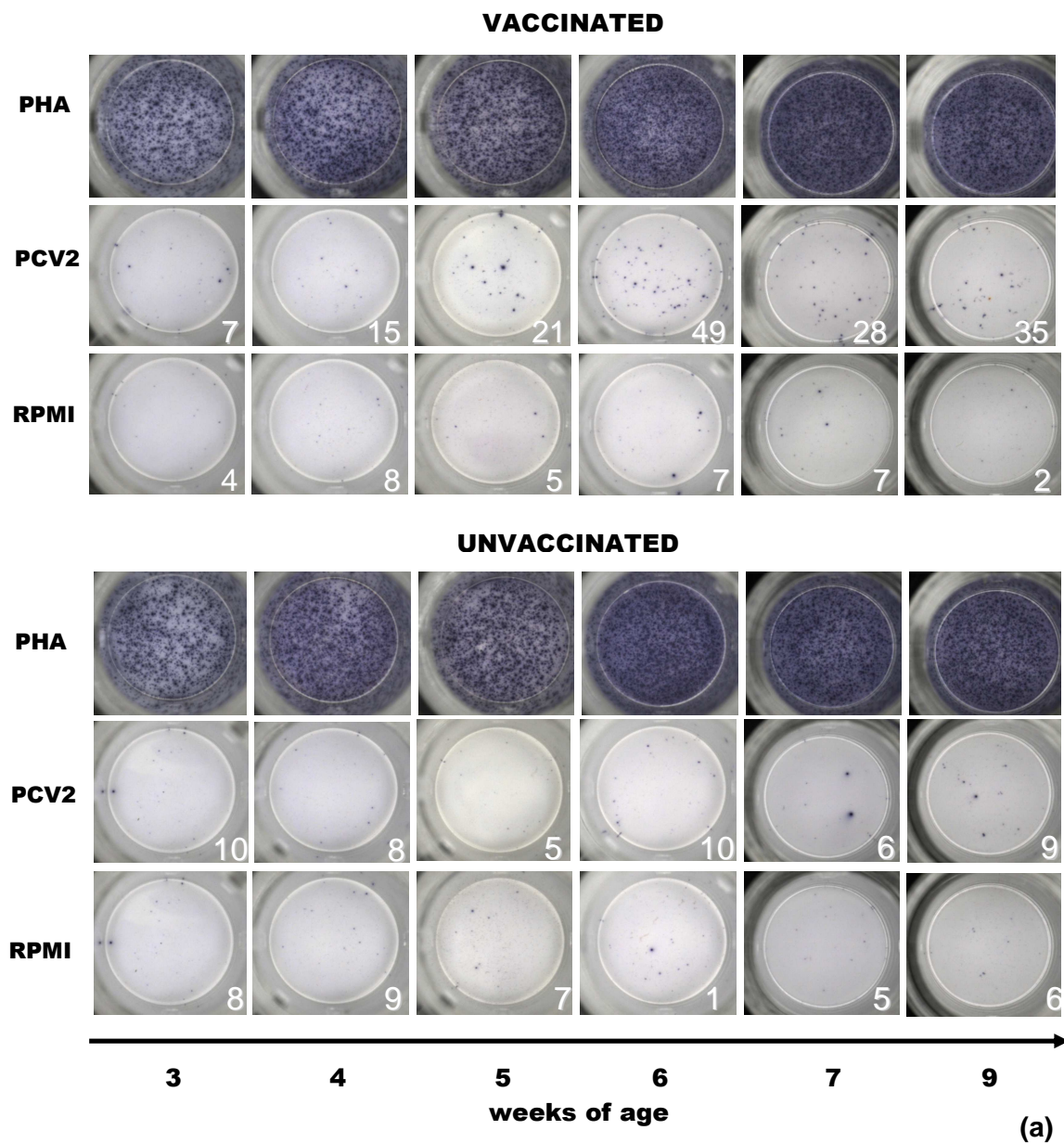


Figure 10. IFN- γ PCV2-specific SC levels in placebo/control and PCV2-vaccinated animals after vaccination at 3 weeks of age (a = placebo/control; b = vaccinated) and after natural exposure to PCV2 (c = placebo/control; d = vaccinated).

Oppositely, in unvaccinated pigs, IFN- γ SC showed a significant increase ($P < 0.01$) with mean values of 196 and 244 IFN- γ SC at 19 and 20 weeks of age, respectively. The mean values remained at high levels (140 IFN- γ SC) until 26 weeks of age (Fig. 10).

The ELISpot IFN- γ SC response after vaccination (PV period) and after exposure to PCV2 natural infection (PE period) of a representative PCV2-vaccinated and an unvaccinated natural infected pig is shown in figure 11.



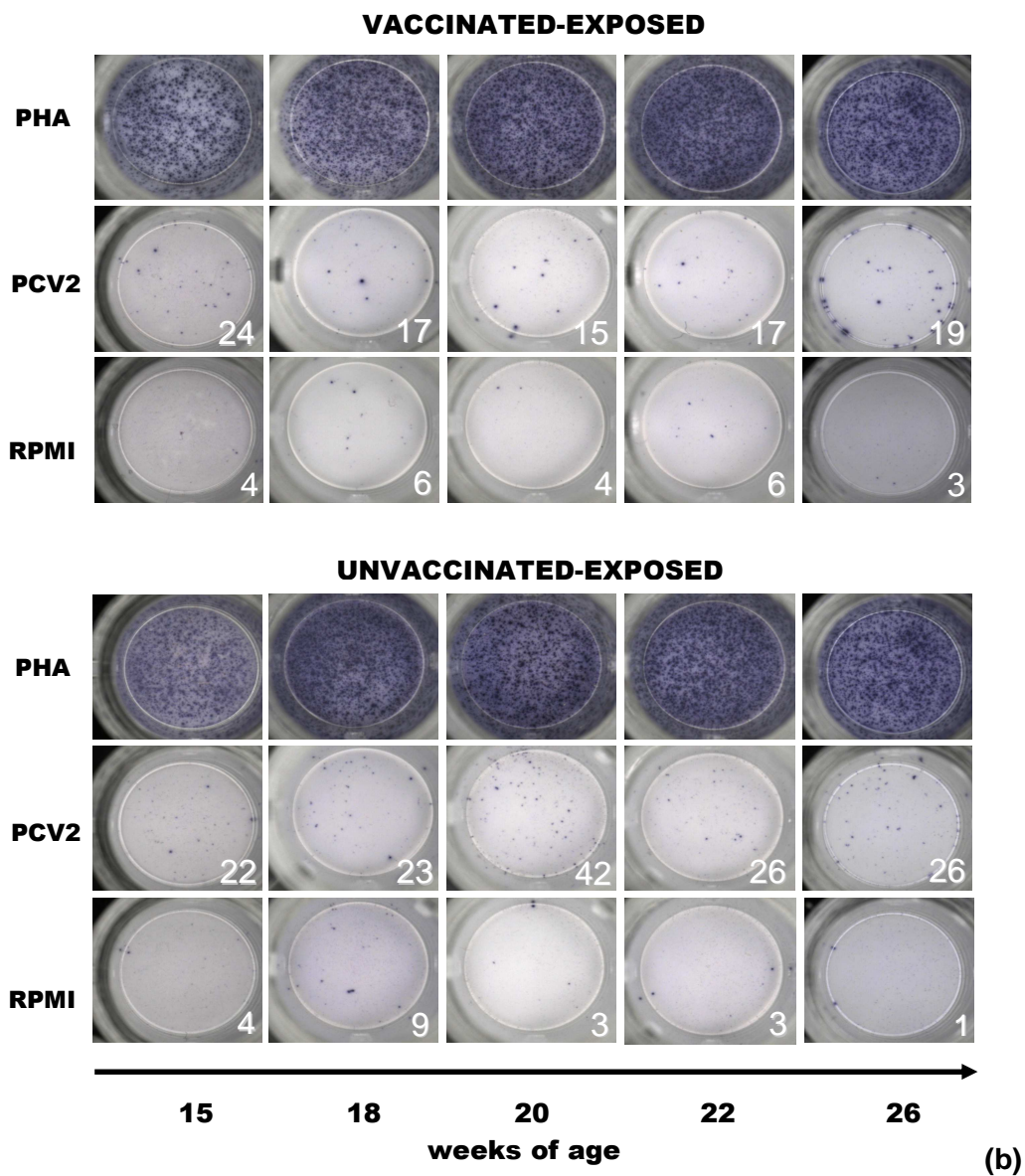


Figure 11. PCV2-specific IFN- γ ELISpot response of a representative PCV2-vaccinated and an unvaccinated pig in the post-vaccination (a) and in the post-exposure period (b). The number of spots corresponds to the number of IFN- γ -secreting cells / 2×10^5 PBMC.

It is worth noting that the IFN- γ -SC response was characterised by a high inter-individual variability, with some pigs showing a very high PCV2-specific secretion (high responders; Table 6).

In order to better evaluate the *ex vivo* PCV2-specific responses within the same experimental group, ELISpot results related to farm 1 were also analysed by grouping animals in responsiveness categories.

As shown in Table 5, the PCV2-vaccinated group showed an increasing percentage of more highly responsive animals to the *ex vivo* recall PCV2 I12/11 strain overtime. In fact, after 5 weeks of age (2 weeks post-vaccination) 30-40% of vaccinated animals responded to the stimulus with more than 105 IFN- γ SC/10⁶ PBMC, mounting responses categorised in this study as intermediate, high and very high. At 7 and 8 weeks of age, 10% of vaccinated pigs showed a higher response to more than 405 IFN- γ SC/10⁶ PBMC. On the contrary, unvaccinated animals showed absent or minimal responses, between 0 and 40 SC/10⁶ PBMC, having however the majority of pigs showing a response between 0 and 20 SC/10⁶ PBMC to the *ex vivo* stimulation at each experimental point.

During the post-exposure period (Table 6), the majority of vaccinated pigs showed a cellular response lower than 100 SC/10⁶ PBMC; only 10% and 20% of animals showed an intermediate response at 17 and 21 weeks of age respectively.

The animals from the placebo/control group responded, instead, increasing the IFN- γ SC frequency. In this group, the highest numbers of IFN- γ SC were reached between 19 and 21 weeks of age, with a maximum (20% of pigs showing more than 405 IFN- γ SC/10⁶ PBMC) at 20 weeks.

Table 5. IFN- γ responsiveness categories in PCV2-vaccinated and placebo/control pigs in the post-vaccination period. Values are reported as percentage of animals within the group. SC: secreting cells.

PCV2-VACCINATED	Responsiveness categories (IFN- γ SC / 10 ⁶ PBMC)				
	0-40 no-poor	45-100 low	105-200 intermediate	205-400 high	≥ 405 very high
weeks of age (PV period)					
3	100	0	0	0	0
4	90	10	0	0	0
5	40	20	30	10	0
6	30	30	20	20	0
7	40	20	30	0	10
9	50	20	10	10	10
PLACEBO/CONTROL	0-40 no-poor	45-100 low	105-200 intermediate	205-400 high	≥ 405 very high
weeks of age (PV period)					
3	100	0	0	0	0
4	100	0	0	0	0
5	100	0	0	0	0
6	100	0	0	0	0
7	100	0	0	0	0
9	100	0	0	0	0

Table 6. IFN- γ responsiveness categories in PCV2-vaccinated and placebo/control pigs in the post-exposure period. Values are reported as percentage of animals within the group. SC: secreting cells.

PCV2-VACCINATED	Responsiveness categories (IFN- γ SC / 10 ⁶ PBMC)				
	0-40 no-poor	45-100 low	105-200 intermediate	205-400 high	≥ 405 very high
weeks of age (PE period)					
15	50	50	0	0	0
16	60	40	0	0	0
17	40	50	10	0	0
18	70	30	0	0	0
19	10	90	0	0	0
20	90	10	0	0	0
22	40	40	20	0	0
26	60	40	0	0	0
PLACEBO/CONTROL	0-40 no-poor	45-100 low	105-200 intermediate	205-400 high	≥ 405 very high
weeks of age (PE period)					
15	75	25	0	0	0
16	80	20	0	0	0
17	45	55	0	0	0
18	50	40	10	0	0
19	30	40	20	0	10
20	35	10	20	15	20
22	10	35	45	5	5
26	5	45	20	30	0

3.2.5.2. ELISpot assay: comparison of three different PCV2-specific stimulations

In order to evaluate the influence of the amount of viral antigen (whole PCV2) used for the *ex vivo* antigenic recall on the identification and extent of the PCV2-specific IFN- γ SC response detected by the ELISpot assays, PBMC were stimulated with also 0.05 and 0.25 MOI, in addition to 0.1 MOI as reported above.

During the post-vaccination period, upon re-stimulation with each virus-to-cell ratio (MOI) tested, vaccinated pigs showed a significant response as IFN- γ SC starting from 2 weeks PV (Fig. 12). Contrarily, unvaccinated pigs did not show any significant response at any MOI throughout the PV period. The course of the IFN- γ secreting cells over time detectable after recall with 0.1 MOI of virus was confirmed in both experimental groups also using a lower (0.05 MOI) and a higher (0.25 MOI) amount of virus. Specifically, re-stimulation with 0.25 MOI showed a peak response at 3 weeks PV (6 weeks of age) with the highest mean value of about 200 IFN- γ SC/10⁶ PBMC. The cellular response in vaccinated animals was clearly influenced by the *ex vivo* antigenic stimulus in a dose-dependent manner.

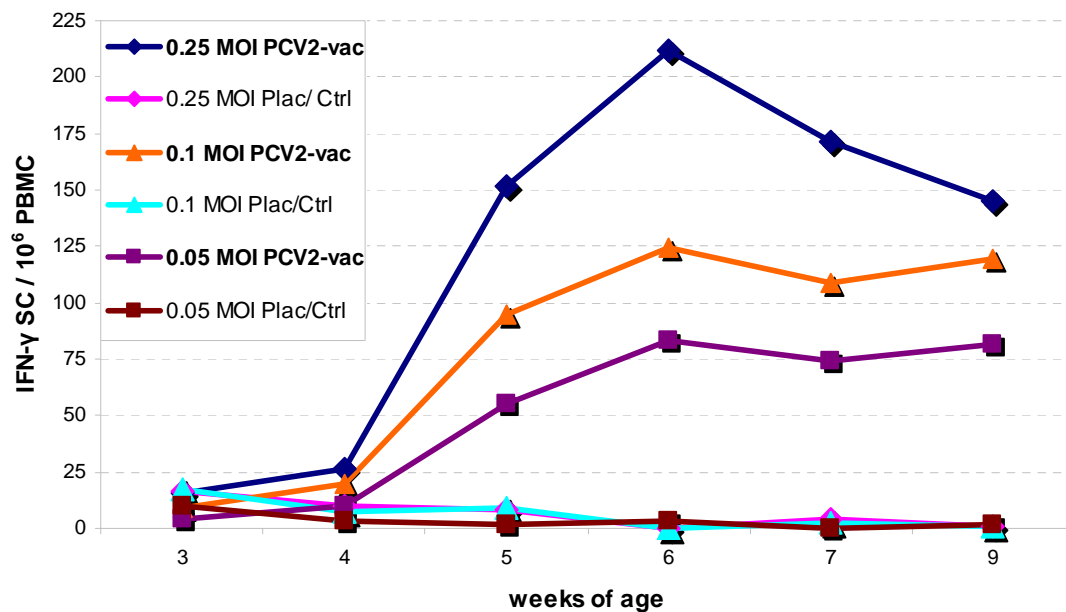


Figure 12. PCV2-specific IFN- γ secreting cell responses of PCV2-vaccinated (PCV2-vac) and placebo/control (Plac/Ctrl) pigs stimulated with 0.05, 0.1 and 0.25 MOI during the post-vaccination (PV) period. Values are expressed as mean values.

In the post-exposure period, the ELISpot results obtained with 0.1 MOI of virus were confirmed both at 0.05 and 0.25 MOI (Fig. 13). In fact, at each MOI used for the *ex vivo* re-stimulation, the placebo/control pigs showed higher frequencies of IFN- γ SC than the PCV2-vaccinated animals between 19 and 26 weeks of age. The most intense cellular response were detected at 19-20 weeks of age.

Despite high individual variability was observed, the response detected only in the unvaccinated group appeared to be influenced by the amount of virus antigen used for re-stimulation in a dose-dependent manner. Vaccinated animals showed a lower and more steady (not influenced by the antigen amount) response throughout the PE period.

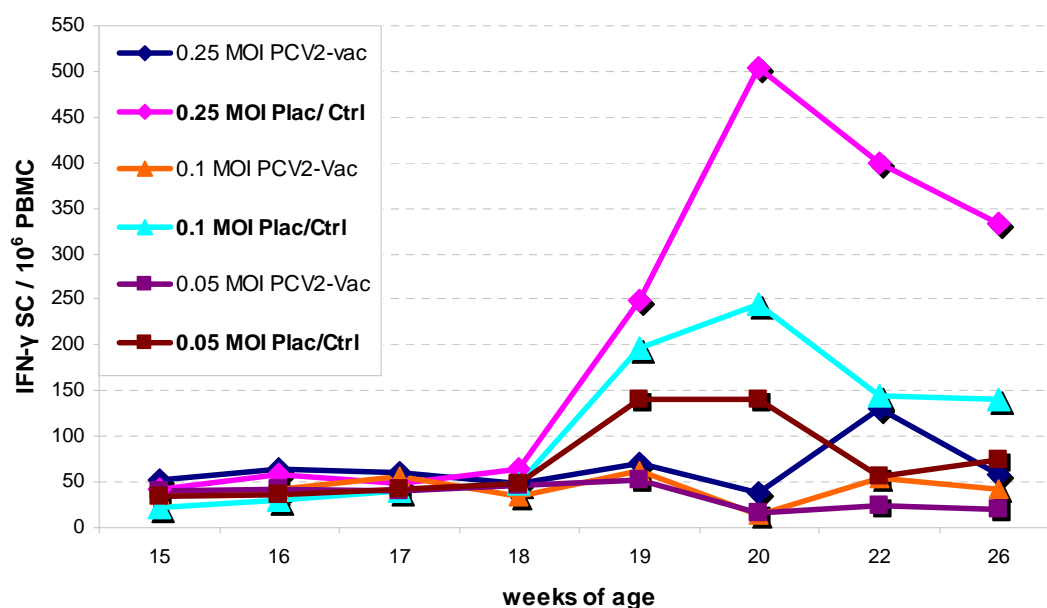


Figure 13. Comparison of the frequencies of PCV2-specific IFN- γ secreting cells of PCV2-vaccinated (PCV2-vac) and placebo/control (Plac/Ctrl) pigs detected upon *ex vivo* re-stimulated with 0.05, 0.1 or 0.25 MOI during the post-exposure (PE) period. Values are expressed as mean values.

3.2.6. Cytokine mRNA expression

The evaluation of pro-inflammatory and immune cytokine mRNA expression was carried out in PBMC from PCV2-vaccinated pigs and from unvaccinated pigs that showed or did not show PCV2 infection and the related disease. During infection, some statistically significant changes of the investigated cytokines were observed between groups. The mRNA cytokine expression was studied by using the RT-qPCR technique. The performed PCR reactions provided target-specific amplification in all samples with minimal, not significant or absent signals related to primer dimer amplification. Each single amplification experiment was evaluated by monitoring amplification curves (Fig. 14) and melting profiles to exclude non-specific amplification (e.g. contamination by genomic DNA) or primer dimer negative effects (Fig. 15).

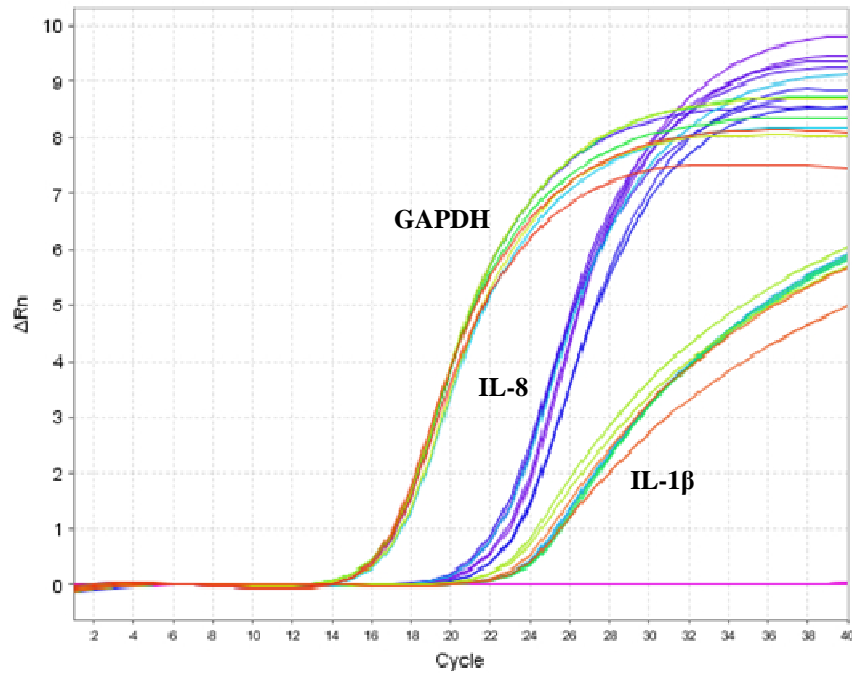


Figure 14. Representative amplification curves of GAPDH, IL-8 and IL-1 β obtained by using Fast SYBR[®] Green Master Mix (Applied Biosystems). Target genes = IL-8 and IL-1 β ; housekeeping gene = GAPDH. Pink curves correspond to no template control (NTC) fluorescence signals.

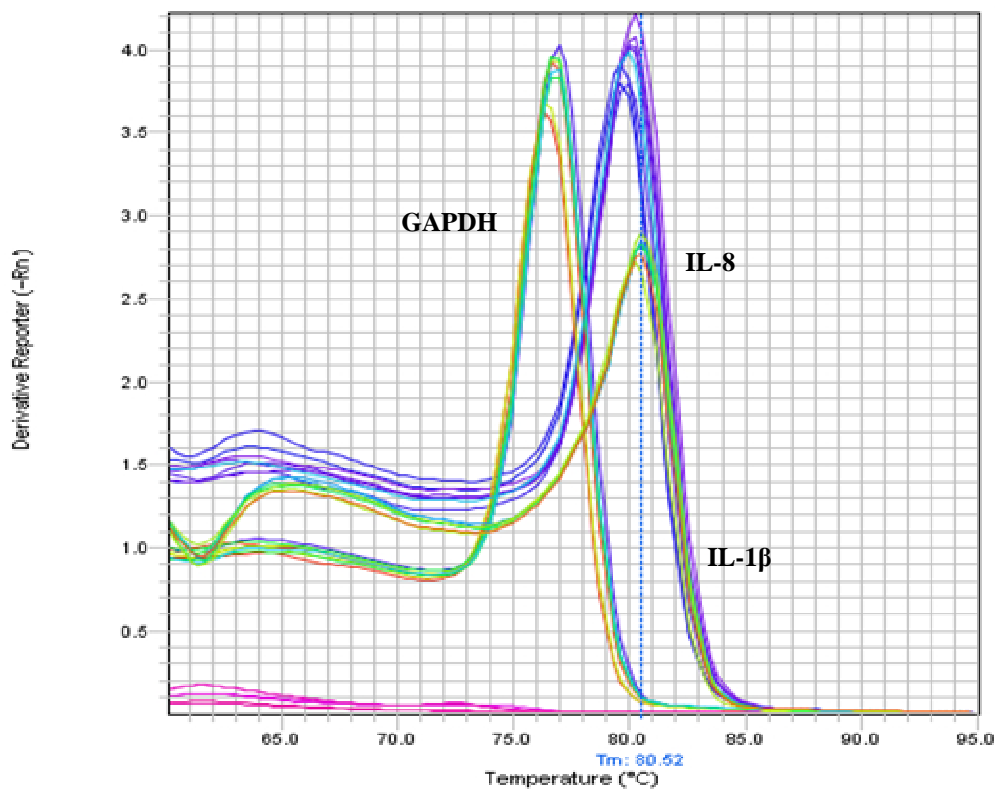


Figure 15. Representative melting curves of GAPDH, IL-8 and IL-1 β obtained by using Fast SYBR[®] Green Master Mix (Applied Biosystems). Target genes = IL-8 and IL-1 β ; housekeeping gene = GAPDH. Pink curves correspond to no template control (NTC).

IL-8 mRNA expression levels showed significantly higher values in vaccinated animals than in the two unvaccinated groups at 22 weeks of age ($p < 0.05$; Fig. 16). TNF- α expression increased in vaccinated animals at 19 and especially at 22 weeks of age ($p < 0.05$; Fig. 17). Conversely, for IL-1 β , the comparison of mRNA expression levels between the three groups did not show any statistically significant difference at any of the considered time points (Fig. 18).

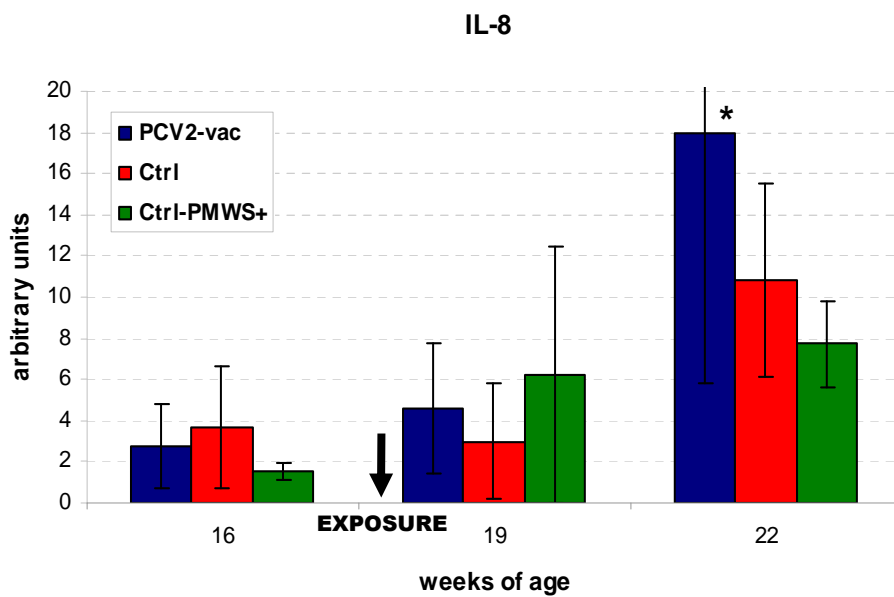


Figure 16. Relative mRNA expression of the pro-inflammatory cytokine IL-8 in PBMC of vaccinated (PCV2-vac) and unvaccinated non-PMWS-affected (Ctrl) or PMWS-affected (Ctrl-PMWS+) pigs exposed to PCV2 natural infection. Data, obtained by RT-qPCR ($2^{-\Delta\Delta C_t}$ method), are shown as mean values \pm standard deviation. (*): $p < 0.05$.

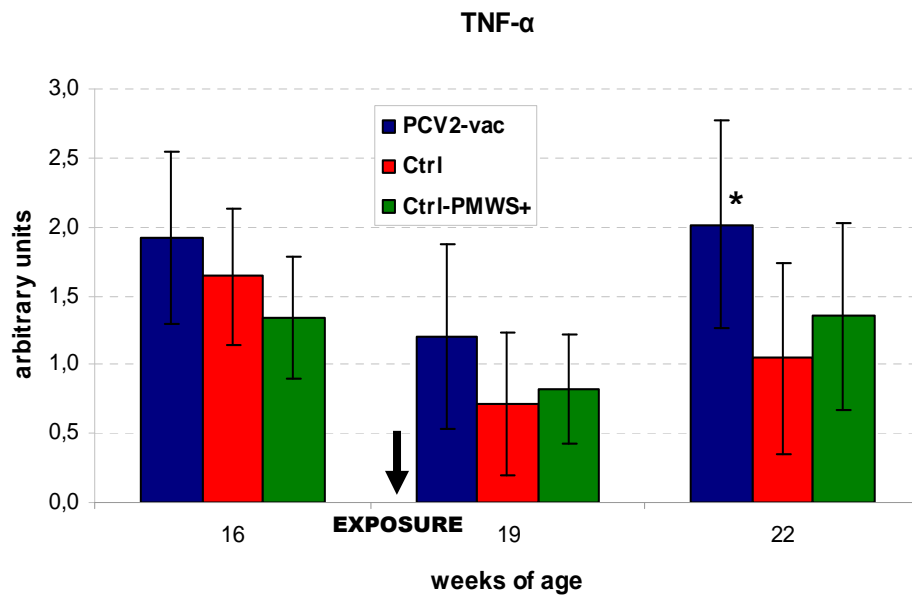


Figure 17. Relative mRNA expression of the pro-inflammatory cytokine TNF- α in PBMC of vaccinated (PCV2-vac) and unvaccinated non-PMWS-affected (Ctrl) or PMWS-affected (Ctrl-PMWS+) pigs exposed to PCV2 natural infection. Data, obtained by RT-qPCR ($2^{-\Delta\Delta C_t}$ method), are shown as mean values \pm standard deviation. (*): $p < 0.05$.

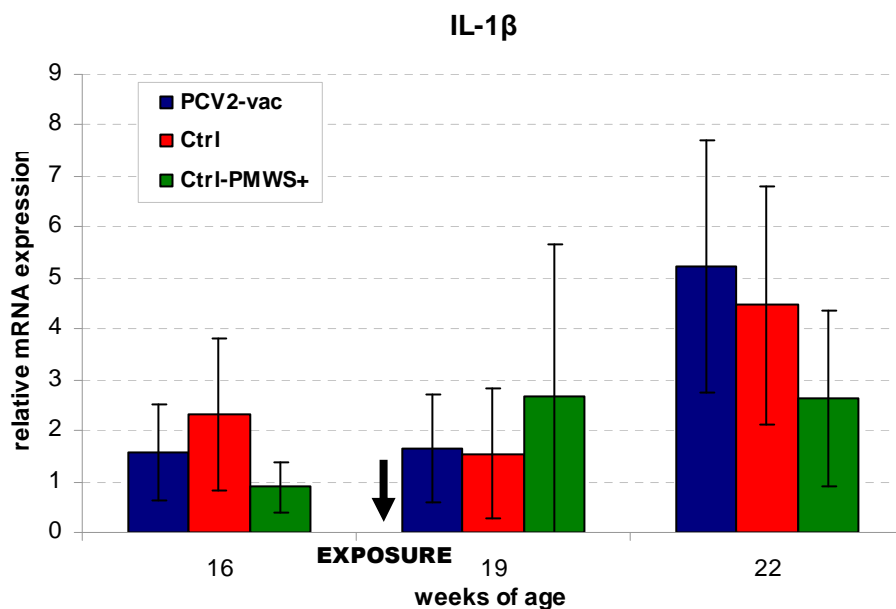


Figure 18. Relative mRNA expression of the pro-inflammatory cytokine IL-1 β in PBMC of vaccinated (PCV2-vac) and unvaccinated non-PMWS-affected (Ctrl) or PMWS-affected (Ctrl-PMWS+) pigs exposed to PCV2 natural infection. Data, obtained by RT-qPCR ($2^{-\Delta\Delta C_t}$ method), are shown as mean values \pm standard deviation. (*): $p < 0.05$.

The expression of IFN- γ was statistically higher in vaccinated animals both at 19 and 22 weeks of age compared to the Ctrl-PMWS+ and Ctrl groups (Fig.19). Interestingly, a progressive decrease of IFN- γ expression was found in all experimental groups concomitantly with PCV2 infection.

At 19 weeks of age, Ctrl-PMWS+ animals had a significantly higher level of IL-10 ($p < 0.05$, Fig. 20) than the other two groups while no relevant differences were found between groups at the end of the study.

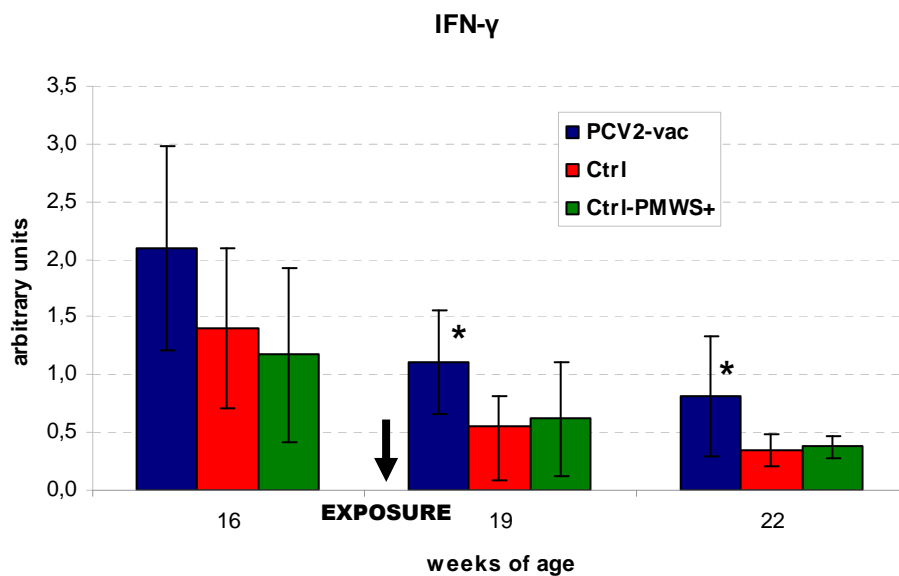


Figure 19. Relative mRNA expression of the pro-immune cytokine IFN- γ in PBMC of vaccinated (PCV2-vac) and unvaccinated non-PMWS-affected (Ctrl) or PMWS-affected (Ctrl-PMWS+) pigs exposed to PCV2 natural infection. Data, obtained by RT-qPCR ($2^{-\Delta\Delta C_t}$ method), are shown as mean values \pm standard deviation. (*): $p < 0.05$.

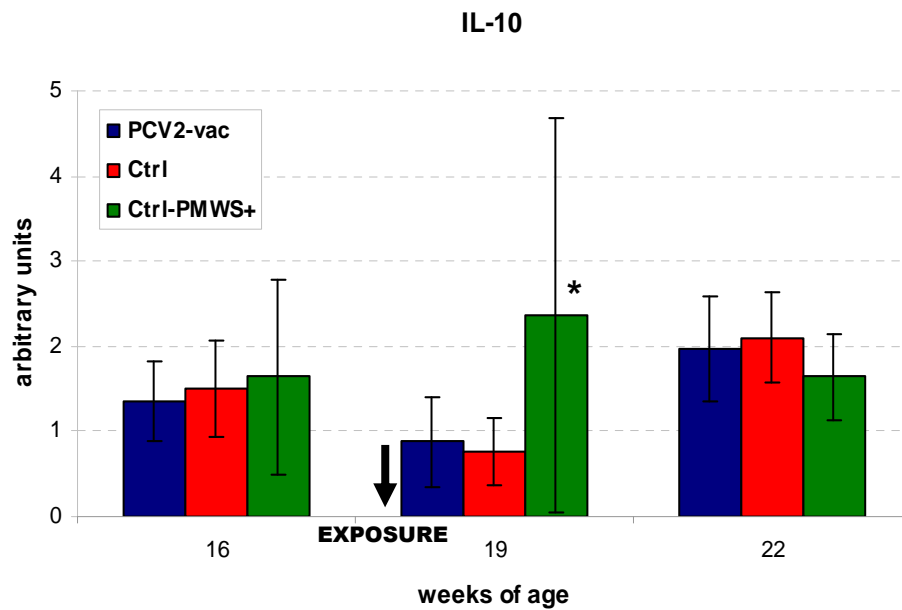


Figure 20. Relative mRNA expression of the regulatory cytokine IL-10 in PBMC of vaccinated (PCV2-vac) and unvaccinated non-PMWS-affected (Ctrl) or PMWS-affected (Ctrl-PMWS+) pigs exposed to PCV2 natural infection. Data, obtained by RT-qPCR ($2^{-\Delta\Delta C_t}$ method), are shown as mean values \pm standard deviation. (*): $p < 0.05$.

3.2.6.2. Course of viremia over time

Serum samples of vaccinated and unvaccinated Ctrl or Ctrl-PMWS+ pigs were analysed by real-time qPCR to detect PCV2 DNA. The course of PCV2 viremia is shown in figure 21.

The onset of viremia was observed in both groups after 16 weeks of age; the highest levels of PCR-positive animals and viral load were reached when animals were 19 weeks old whereas a decrease was found at 22 weeks of age.

The mean values of viremia in vaccinated pigs (Fig. 21) remained significantly lower ($p < 0.01$) than the values of the control groups, both at 19 and 22 weeks of age. Moreover, in vaccinated pigs, a small percentage of animals (20% and 30% at 19 and 22 weeks of age, respectively) resulted viremic and the viral burden always remained $< 10^6$ DNA copies/ml of serum (Fig. 22a).

In the control PMWS+ group, the proportion of viremic animals with high viral load ($\geq 10^6$ DNA copies/ml) was 100% at 19 weeks, and 33.3% became low viremic ($< 10^6$ DNA copies/ml) at 22 weeks of age (Fig. 22c).

In the Ctrl group, the percentage of viremic pigs increased from 19 to 22 weeks of age, showing 43.7% and 62.5% of animals with a high viral burden respectively (Fig. 22b).

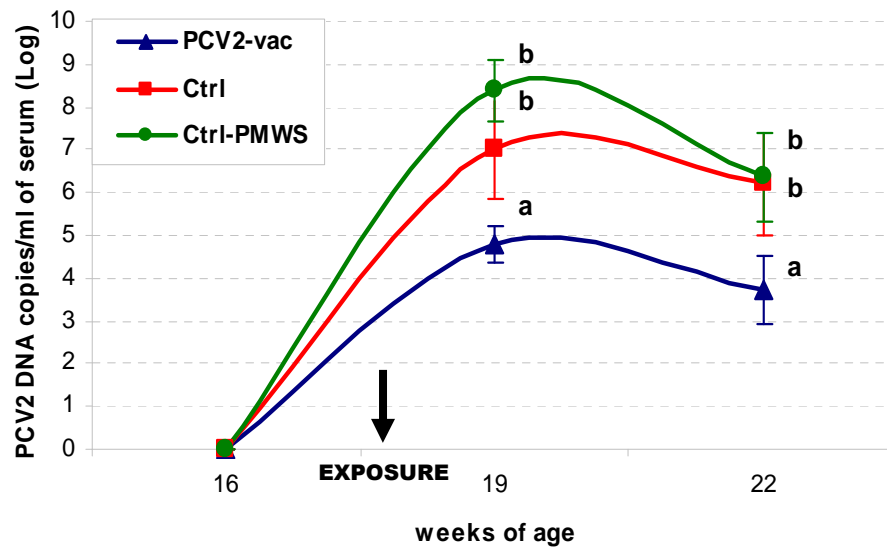


Figure 21. Course of viremia over time vaccinated (PCV2-vac) and unvaccinated PMWS-free (Ctrl) or PMWS-affected (Ctrl-PMWS+) pigs exposed to PCV2 natural infection. Data are expressed as mean values \pm standard deviation of the number of PCV2 DNA copies/ml of serum. Different superscript letters indicate a statistical difference ($p < 0.05$).

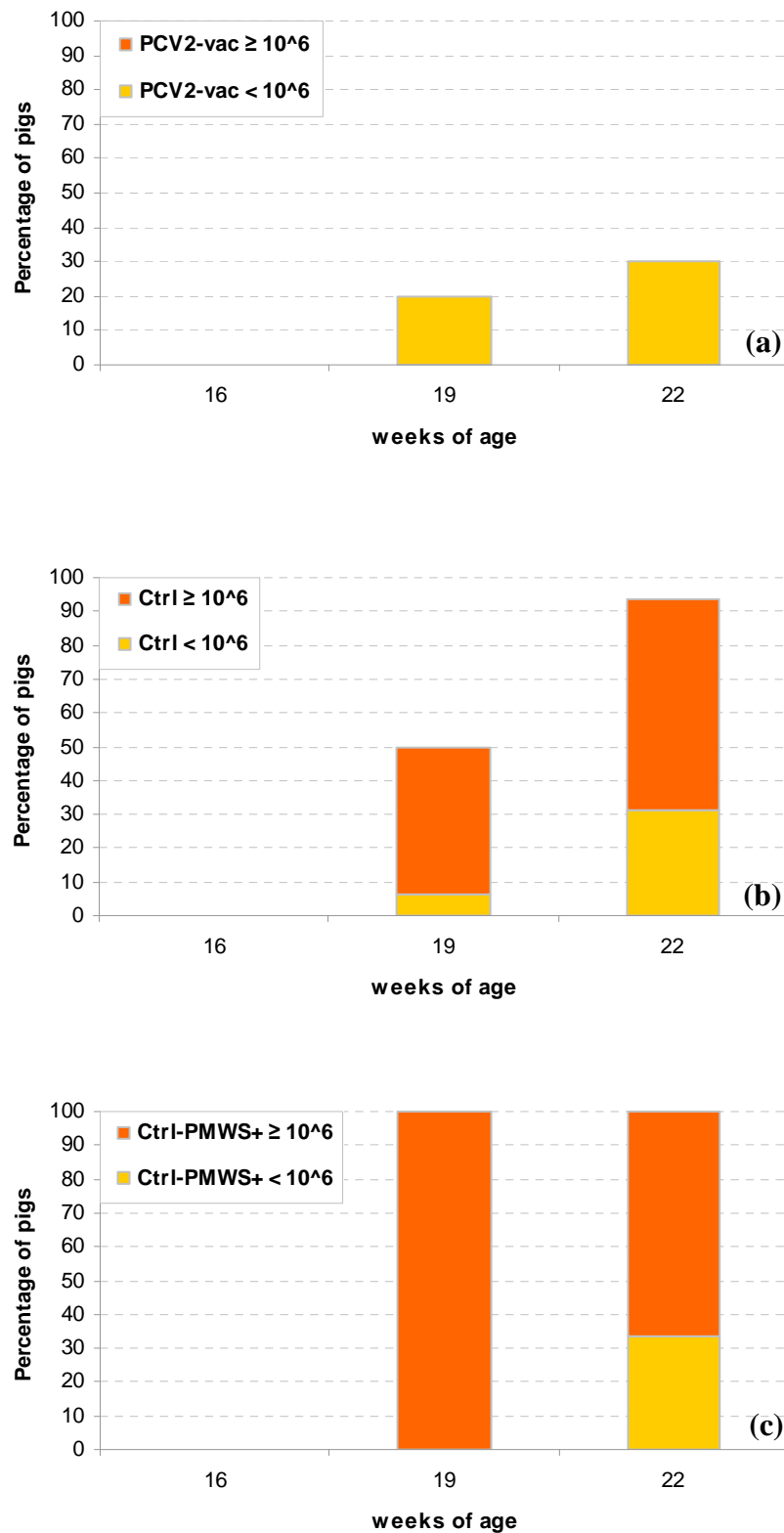


Figure 22. Percentage of PCV2-specific viremic pigs in vaccinated (PCV2-vac) (a) and unvaccinated PMWS-free (Ctrl) (b) or PMWS-affected (Ctrl-PMWS+) animals (c). Data are calculated before the onset (16 weeks of age) and during the outcome of the disease (19 and 22 weeks of age).

3.2.6.3. Association between cytokine mRNA expression and viral load in the blood

Pro-inflammatory and immune cytokine mRNA expression was also evaluated in relation to the viremia burden.

According to the amount of PCV2 in serum, three different subgroups were considered in order to have further information about the cytokine kinetics both in vaccinated and unvaccinated PCV2 groups as previously stated (PCV2-vac, Ctrl-PMWS+ and Ctrl): non-viremic pigs (NV), pigs with viremia $<10^6$ PCV2 DNA copies/ml of serum ($V < 10^6$), and pigs with viremia $\geq 10^6$ PCV2 DNA copies/ml of serum ($V \geq 10^6$).

Table 7 shows the modulation of cytokine levels observed between all subgroups of each experimental group (PCV2-vac, Ctrl, Ctrl-PMWS+) over time.

Comparing IFN- γ expression in relation to the extent of viremia between groups (PCV2-vac; Ctrl-PMWS+ and Ctrl) over time (Table 7), despite a decreasing trend of this cytokine from 16 to 22 weeks of age in all considered groups, in PCV2-vac animals IFN- γ did not show differences between the newly constituted subgroups both at 19 and 22 weeks of age. Nevertheless, these animals maintained higher expression levels compared to the subgroups in Ctrl and Ctrl-PMWS+ animals. Particularly, PCV2-vac animals even with low viremia ($V < 10^6$) had higher IFN- γ expression than highly viremic pigs in both Ctrl groups at 22 weeks of age ($p < 0.05$).

IL-10 mRNA expression appeared to increase in relation to the occurrence of infection and in relation to the extent of viremia (Table 7); particularly, highly viremic ($V \geq 10^6$) animals of the Ctrl-PMWS+ group showed an early increase (19 weeks of age) of this cytokine compared to PCV2-vac animals and Ctrl groups.

Table 7. Levels of immune cytokine mRNA expression in PBMC of vaccinated (PCV2-vac) and unvaccinated PMWS-free (Ctrl) or PMWS-affected (Ctrl-PMWS) pigs exposed to PCV2 natural infection in the post-exposure period.

Cytokines	Groups	Subgroups of viremia	weeks of age		
			16	19	22
IFN- γ	PCV2-Vac	NV	2.1 \pm 0.9 ^a	1.1 \pm 0.4 ^b	0.7 \pm 0.4 ^b
		V<10 ⁶	-	1.0 \pm 0.9 ^a	1.0 \pm 0.8 ^a
		V \geq 10 ⁶	-	-	-
	Ctrl	NV	1.4 \pm 0.7 ^a	0.4 \pm 0.2 ^b	-
		V<10 ⁶	-	1.0	0.4 \pm 0.2 ^b
		V \geq 10 ⁶	-	0.6 \pm 0.2 ^a	0.3 \pm 0.1 ^a
	Ctrl-PMWS+	NV	1.2 \pm 0.7	-	-
		V<10 ⁶	-	-	0.4
		V \geq 10 ⁶	-	0.6 \pm 0.5	0.4 \pm 0.1 ^a
IL-10	PCV2-Vac	NV	1.3 \pm 0.5 ^a	1.0 \pm 0.6 ^a	1.4 \pm 0.5 ^a
		V<10 ⁶	-	0.6 \pm 0.1 ^a	2.5 \pm 0.7 ^b
		V \geq 10 ⁶	-	-	-
	Ctrl	NV	1.5 \pm 0.6 ^a	0.8 \pm 0.5 ^b	-
		V<10 ⁶	-	0.8	2.1 \pm 0.4 ^b
		V \geq 10 ⁶	-	0.7 \pm 0.4 ^a	2.1 \pm 0.6 ^b
	Ctrl-PMWS+	NV	1.6 \pm 1.1	-	-
		V<10 ⁶	-	-	1.8
		V \geq 10 ⁶	-	2.4 \pm 2.3 ^a	1.6 \pm 0.7 ^a

Values are expressed as mean \pm S.D. of the cytokine mRNA expression (arbitrary units were calculated with the $2^{-\Delta\Delta CT}$ method). (-) indicates no value of gene expression since no pigs belonged to the specific subgroup of viremia at that time point. Different superscript letters indicate a statistical difference ($p < 0.05$) between time points within the same subgroups of viremia.

As shown in Table 8, the expression levels of IL-8 and IL-1 β increased between 19 and 22 weeks of age in each subgroup of viremia of both PCV2-vac and Ctrl animals but not in the Ctrl-PMWS+ group.

Table 8. Levels of pro-inflammatory cytokine mRNA expression in PBMC of vaccinated (PCV2-vac) and unvaccinated PMWS-free (Ctrl) or PMWS-affected (Ctrl-PMWS) pigs exposed to PCV2 natural infection in the post-exposure period.

Cytokines	Groups	Subgroups of viremia	weeks of age		
			16	19	22
IL-8	PCV2-Vac	NV	2.8 ± 2.0 ^a	5.5 ± 2.9 ^a	18.9 ± 4.0 ^b
		V<10 ⁶	-	1.0 ± 1.1 ^a	15.7 ± 4.8 ^b
		V≥10 ⁶	-	-	-
	Ctrl	NV	3.7 ± 3.0 ^a	3.7 ± 2.6 ^a	-
		V<10 ⁶	-	9.7	12.7 ± 5.9 ^a
		V≥10 ⁶	-	1.3 ± 0.6 ^a	9.9 ± 4.0 ^b
	Ctrl-PMWS+	NV	1.6 ± 0.4 ^a	-	-
		V<10 ⁶	-	-	8.7
		V≥10 ⁶	-	6.3 ± 6.2 ^a	7.2 ± 2.7 ^a
TNF-α	PCV2-Vac	NV	1.9 ± 0.6 ^a	1.2 ± 0.7 ^a	1.9 ± 0.8 ^a
		V<10 ⁶	-	0.5 ± 0.1 ^a	2.2 ± 0.9 ^a
		V≥10 ⁶	-	-	-
	Ctrl	NV	1.6 ± 0.5 ^a	1.0 ± 0.7 ^a	-
		V<10 ⁶	-	0.7	1.3 ± 1.1 ^a
		V≥10 ⁶	-	0.5 ± 0.1 ^a	1.0 ± 0.5 ^a
	Ctrl-PMWS+	NV	1.3 ± 0.4 ^a	-	-
		V<10 ⁶	-	-	1.8
		V≥10 ⁶	-	0.8 ± 0.4 ^a	1.0 ± 0.2 ^a
IL-1β	PCV2-Vac	NV	1.6 ± 0.9 ^a	1.6 ± 1.1 ^a	5.7 ± 2.9 ^b
		V<10 ⁶	-	0.4 ± 0.5 ^a	4.2 ± 0.5 ^b
		V≥10 ⁶	-	-	-
	Ctrl	NV	2.3 ± 1.5 ^a	2.5 ± 1.3 ^a	-
		V<10 ⁶	-	1.3	6.6 ± 2.4 ^a
		V>10 ⁶	-	0.6 ± 0.5 ^a	3.4 ± 1.5 ^b
	Ctrl-PMWS+	NV	0.9 ± 0.5 ^a	-	-
		V<10 ⁶	-	-	0.7
		V>10 ⁶	-	2.7 ± 2 ^a	3.6 ± 0.4 ^b

Values are expressed as mean ± S.D. of the cytokine mRNA expression (arbitrary units were calculated with the $2^{-\Delta\Delta Ct}$ method). (-) indicates no value of gene expression since no pigs belonged to the specific subgroup of viremia at that time point. Different superscript letters indicate a statistical difference ($p < 0.05$) between time points within the same subgroups of viremia.

In particular, between 19 and 22 weeks, a statistically significant higher levels of IL-8 were detected in non-viremic (NV) PCV2-vaccinated compared to highly viremic ($V \geq 10^6$) control pigs and control PMWS+; in the same period, and particularly at 22 weeks of age, all subgroups of PCV2 vaccinated pigs showed higher levels of IL-1 β compared to the highly viremic ($V \geq 10^6$) pigs of the Ctrl groups, TNF- α showed a similar trend to increase between 19 and 22 weeks of age in PCV2 vaccinated group in respect to control viremic pigs but not if compared to PMWS pigs at 22 weeks.

Overall, in Ctrl-PMWS+ pigs, lower IFN- γ at 19 weeks of age was associated with high IL-10 and subsequently, at 22 weeks of age, is evident a reduction of pro-inflammatory cytokines, namely IL-8 and IL-1 β (Table 7 and 8).

Contrarily, the PCV2-vaccinated pigs, despite an increase of IL-10 from 19 to 22 weeks, showed higher levels of these pro-inflammatory cytokines at 22 weeks of age (Tables 7 and 8).

CHAPTER 4.

DISCUSSION AND CONCLUSIONS

The present Thesis reports a study performed to characterize the humoral and cell-mediated immune response upon PCV2 vaccination and natural infection, evaluating the efficacy of a one-dose of a commercial PCV2a sub-type based subunit vaccine containing the capsid protein expressed in a baculovirus system (Porcilis PCV[®]) in vaccinated and unvaccinated pigs subsequently exposed to PCV2 natural infection.

The trial was performed at two large farms where the exposure of the animals to various pathogens such as PCV, PRRSV, *M. hyopneumoniae*, and commonly detected bacteria was the classical predisposing condition for PCVD (Kawashima et al., 2007). Under the conditions of this study, PCV2 infection was detected during the fifth month of age, a period in which also the onset of the clinical signs related to PCVD occurred in both herds.

The test vaccine, administered intramuscularly with a single dose at 3 weeks of age, consistently reduced clinical signs attributed to PCVD as well as mortality, PCV2 viral load and viremia. Furthermore vaccinated animals showed a different response of pro-inflammatory and immune cytokines upon PCV2-natural infection.

Despite the vaccine used in this field trial and the schedule of administration had been tested under laboratory conditions (Fort et al., 2009a), no peer-reviewed paper has described its efficacy under field conditions.

This study pointed out the effects of a PCV2 vaccine on farms with the presence of PCVD during the fattening period in animals aged 4-5 months, in line with the disease history in both farms. The effects of vaccination involved clinical signs and productivity (decreased numbers of pigs needing intramuscular therapy, improvement of ADWG, and decreased mortality) and PCV2 viral load in target organs of dead animals and in the blood of sampled pigs in amounts and duration of viremia.

Serology demonstrated that PCV2 infection occurred in combination with other pathogens, namely, PRRSV and *M. hyopneumoniae*, in both herds. Morbidity was measured recording, throughout the study, the number of individual treatments in unvaccinated and vaccinated animals and was significantly higher in the placebo/control group. According to the statistical model applied (Poisson model), unvaccinated animals were expected to receive on average 30% more injections than vaccinated pigs.

The efficacy of the vaccine under investigation was also evaluated by the comparison of ADWG. Before the onset of PCV2 viremia and associated diseases (from 3 to 12 weeks of age) no differences in ADWG were recorded. Conversely, when PCV2 viremia and PCVD occurred (from 12 to 26 weeks of age), the ADWG in vaccinated pigs was 70 g/day higher

than in controls as a result of the protective effect induced by vaccination. This result is improved compared to evidences reported in previous studies (Fachinger et al., 2008; Horlen et al., 2008; Kixmoller et al., 2008; Segale's et al., 2009). At 26 weeks of age 6.5% and 2.6% of animals from placebo/control and vaccinated groups, respectively, had a body weight at least 25% lower than the mean body weight of the respective treatment group.

The overall mortality at both farms was reduced by vaccination; the statistically significant differences were related to a decreased number of pigs suffering from PMWS and showing specific lesions in target tissues. In fact, PMWS was the cause of death only for 1 of 408 vaccinated pigs. The results show that a pig vaccinated with a single dose of Porcilis PCV[®] at 3 weeks of age had a probability of dying from PMWS 12 times less than that of an unvaccinated control pig.

Furthermore, the similar ADWG and mortality rates in both treatment groups within the first 9 weeks after vaccination indicate that the vaccine does not negatively influence the health status of the animals and suggests that the vaccine is well tolerated. All the improved parameters are associated with a significant reduction in the proportion of infected pigs and in the viral load in the blood.

To categorise pigs as subclinically infected, suspected, and diseased for PCV2-associated diseases, was used a classification based on the amount of PCV2 load in the blood, namely $<10^6$, between 10^6 and 10^7 , and $>10^7$ DNA copies/ml, respectively (Olvera et al., 2004; Opriessniget al., 2007). During the period of PCV2 infection that occurs between 16 and 26 weeks of age, high viral loads in serum ($>10^7$ DNA copies/ml) were detected in a high number of placebo/control pigs, whereas in vaccinated pigs the duration of viremia and the viral load were markedly lower. In fact, in the placebo/control group the 70% and 42% of pigs, for farms 1 and 2 respectively, showed an amount of PCV2 in the blood $\geq 10^7$ DNA copies/ml. Conversely, none of the vaccinated pigs had a viral load in the blood as high as this level; it always was $<10^6$ DNA copies/ml. The ability to diminish both the proportion of infected pigs as well as the viral load of infected animals is one of the better-documented feature of PCV2 piglet vaccination under field conditions (Fachinger et al., 2008; Horlen et al., 2008; Kixmoller et al., 2008).

The reduced number of PCV2-positive pigs and of the viral load in vaccinated pigs is associated with the improved ADWG and reduced mortality. qPCR can be performed on samples from live animals; for this reason it is the most practical tool to monitor the efficacy of a vaccine treatment (Segales et al., 2010).

Quantitative PCR analysis showed that all tested animals were PCV2 negative at the time of vaccination so that the antibody titres detected were most likely of maternal origin.

However, a prompt seroconversion is induced by the use of this PCV2-specific vaccine on piglets, independently of the level of MDA when the titres of ELISA antibodies are below an observed threshold of $8 \log_2$ (approximately 90% of vaccinated pigs). Over this level, observed in approximately 10% of the vaccinated population, the antibody titres showed neither an increase as a consequence of vaccination nor a decline, but maintained a steady course over time. Thus, at the time of infection (>16-17 weeks of age) all vaccinated pigs, independently from the level of antibodies at vaccination, were protected showing an ELISA titre $>6 \log_2$.

Conversely, placebo/control pigs presented either MDA $<8 \log_2$ or high levels ($>8 \log_2$) at inclusion, and showed a gradually declining course that reached low levels within a few weeks. The high proportion of viremic pigs (100% and 95%, respectively, from farms 1 and 2) and the incidence of clinical signs and mortality supported that, during the late infection phase, none of the control animals was protected.

Using the conditions of this study, we suggest that it is possible to set two different thresholds of ELISA antibodies. In pigs with titre of MDA is $>8 \log_2$ seroconversion to vaccination does not occur; however, when infection occurred, these animals resulted protected and showed ELISA titres $>6 \log_2$. Conversely, in controls, the highest titres of MDA decline similarly so that, at later exposure to PCV2, the ELISA titres are $<6 \log_2$ and the animals are completely susceptible to infection with a high viral load.

These results confirm that high MDA titres do not interfere with the effect of a single dose of the vaccine under investigation (Fort et al., 2008, 2009b; Opriessnig et al., 2008b) and that the vaccine is suitable for immunization of seropositive piglets, conferring clinical and virologic protection even if infection occurs very late after vaccination (≥ 4 -5 months).

In contrast to the results reported by Kixmoller et al. (2008), the significant increase of ELISA antibody titres in vaccinated animals from 3 weeks PV allows the use of this serologic investigation, and particularly seroconversion at two subsequent sampling time points, as a reliable tool to evaluate vaccine compliance differentiating vaccinated and controls before infection. However, the very low number of vaccinated animals becoming infected does not allow to assess a correlation between ELISA titres and clinical and virological protection. It is important to point out that under the conditions of this study, PCVD lasted at least until 26 weeks of age and accordingly, the high increase of weight gain in vaccinated animals

compared to placebo/controls indicates that: a) PCVD have devastating effects if they occur at late time points (which is more and more often the case under field conditions), b) passively acquired maternal derived antibodies are not protecting at this age, c) this vaccine provides a long lasting protective immunity.

The protective immunity induced by commercial PCV2 vaccines has been already investigated in terms of development of an effective humoral response, whereas the role of cell-mediated immunity has not been yet clarified. Fort and co-workers demonstrated, in some laboratory studies (2008, 2009b), that during the course of PCV2 infection pigs develop cell-mediated immunity specific to the virus and suggested that the development of PCV2-specific IFN- γ SC might contribute together with neutralising antibodies to viral clearance.

In the present Thesis, we described disease under field conditions where coinfections are present and investigated the cell-mediated immune response after PCV2 vaccination and subsequent infection. Under laboratory conditions, experimentally challenged pigs do not show the specific PCV2-associated diseases, whereas under field conditions coinfections as well as other intrinsic or extrinsic factors exacerbate the disease.

In this study the cell-mediated immunity specific for PCV2 vaccination/infection was determined as PCV2 specific IFN- γ Secreting Cells (SC) by means ELISpot assay.

ELISpot assays detect a significant increase of the frequency of IFN- γ SC already at 2 weeks after vaccination, demonstrating that a single dose of the PCV2 Cap-based vaccine induces a virus-specific cell-mediated immune response.

This evidence also suggests that cell-mediated responses play an important role in vaccine-induced protection, since the protective effect of PCV2 antibodies is titre dependent and the induction of a humoral response alone might not assure full protection against PCV2 infection (Blanchard et al., 2003; Opriessnig et al., 2009; Fort et al., 2009b).

A marked increase of IFN- γ SC was shown by control pigs after the onset of infection, and at the end of the observation period, their high levels of IFN- γ SC were also associated with a reduction of viremia. This demonstrates that cell-mediated responses is involved in the adaptive immunity that pigs develop over the course of the spontaneous PCV2 infection.

According to Fort et al. (2009b), our result suggest that the IFN- γ SC response may be related to viral replication. Thus, in vaccinated pigs, as a consequence of the primary activation by vaccination, the number of these cells was rather low or at residual levels (ranging from

40 to 60 IFN- γ SC/ 10^6 PBMC on average) and the PCV2 viral load remained low or absent overtime.

Conversely, controls pigs had a higher frequency of IFN- γ SC, concomitantly with the increase of PCV2 antibodies and the exhibition of high levels of viremia and disease consistent with PCV2 replication. During the post-exposure period, the animals of the placebo/control group that showed the highest numbers of IFN- γ secreting cells (≥ 405 IFN- γ SC/ 10^6 PBMC at 20 weeks of age) were the ones that showed the highest levels of viremia.

This association was observed in the majority of unvaccinated animals; an exception was shown by unvaccinated animals that died in which the constantly low levels of IFN- γ SC testify that the cell-mediated response was not efficiently triggered by antigen recognition and PCV2 replication (together with secondary co-infections) was not efficiently counteracted by the IFN- γ response.

Contrarily, vaccinated pigs presented low levels of IFN- γ SC paralleled by absent or low viremia; these results suggest that the IFN- γ SC responses of these animals may act more efficiently and/or other efficient immune mechanisms, including virus-neutralizing antibodies, fast counteract virus spread before the need of further clonal proliferation of virus-specific IFN- γ secreting memory cells.

The observed high response in controls with high viral load can be explained by the fact that the IFN- γ SC response develops against both Cap and Rep proteins, in fact, the intensity of the generated response differs using either the Cap protein or the whole PCV2 as *ex vivo* stimulus. The stimulation of PBMC with the whole virus can induce higher IFN- γ responses than with the Cap protein, suggesting that infected animals in which PCV2 is replicating might respond strongly to other viral components different from the Cap protein (Fort et al., 2009a). It is worth noting that the cellular responses detected by using the ELISpot assay proved to be increased by using increasing amounts of virus for the *ex vivo* stimulation.

Under the conditions of the present study, we cannot exclude that the interaction of PCV2 and the adaptive immunity might be modulated by coinfections, with effects on viral replication and load *in vivo* and on the onset of the clinical evidence *in vivo*.

Based on these observations, even if further studies are required to elucidate the inner mechanisms used by cell-mediated immunity to complete viral clearance and its major antigenic target proteins, it seems that the development of the PCV2-specific cell-mediated response might help contrasting progression of PCV2 infection.

The knowledge on the activity of immune mediators such as cytokines and of lymphocyte cell subpopulations is the basis of vaccine strategies and protocols aimed at controlling the related diseases. The production of pro-inflammatory cytokines and the balance between pro-immune and regulatory cytokines play a pivotal role in stimulating the innate response and priming and sustaining the adaptive immune response.

This study investigated also the changes in the expression of pro-inflammatory (IL-8, TNF- α , IL-1 β) and immune (IFN- γ , IL-10) cytokines in vaccinated and unvaccinated pigs, at specific time points of the post-exposure period. The obtained results were analysed in relation to the appearance of PMWS clinical signs and PCV2 specific viremia.

As it is a field study and there are other infections, namely PRRS, it is worth to note that PRRV infection has occurred before the onset of PCV2 viremia as demonstrated by the high seroprevalence (100%) of PRRSV antibodies observed at 12 weeks of age and by the negative results obtained by PCR performed on the frozen samples at 16 and 19 weeks of age. Consequently, it's rational to consider that between 19 and 22 weeks of age the probability to have a PRRSV infected animal is very reduced and that the major/unique infecting agent able to modulate the cytokine expression is PCV2.

IL-8 is a chemokine involved in early inflammation and its primary function is the recruitment of inflammatory cells, especially neutrophils, by chemotaxis.

At the early phase of infection, the mRNA expression of this pro-inflammatory cytokine was not significantly different between animals that subsequently developed PMWS (Ctrl-PMWS+) and PCV2-vac or Ctrl group, whereas its up-regulation was observed in PCV2-vaccinated pigs later after PCV2 natural exposure (22 weeks of age). This could sustain a more efficient innate response against infection in immunized animals compared to controls: in fact, the IL-8 higher levels were associated with absent or minimal viral load. Conversely, in unvaccinated animals IL-8 expression showed a lower increase during infection, particularly in high viremic and diseased pigs. The values obtained in the late phase of infection (22 weeks of age) could parallel with the findings of lower IL-8 levels in lymphoid tissues at more advanced stages of the disease and increased lesions (Darwich et al., 2003a, 2003b).

TNF- α is a crucial pro-inflammatory cytokine that mediate local and systemic effects and play a crucial role in the innate immune response.

The level of TNF- α remained lower in the two unvaccinated groups (Ctrl and Ctrl-PMWS+) especially at 22 weeks, thus supporting a reduced efficiency of the inflammatory/innate immune response.

With regards to the pro-inflammatory cytokine IL-1 β , both the results in the vaccinated group over time and in animals categorized on the basis of PCV2 viremia burden, showed a trend to increase. As previously demonstrated by *in vitro* stimulation experiments (Darwich et al., 2003a), the exposure to PCV2 can stimulate the secretion of IL-1 β in vaccinated and healthy control pigs confirming their capability to react against PCV2 infection through effective inflammatory mechanisms.

IFN- γ is a pro-immune cytokine mainly produced by Th1, cytotoxic T lymphocytes (CTL) and natural killer (NK) cells which plays a key role in the immune response against virus infections involving both innate and specific mechanisms; this cytokine sustains the activation of macrophages and stimulation of MHC type I and co-stimulatory molecule production in the differentiation of CD4+ naïve T helper cells into Th1 cells and also in the inhibition of Th2 cell proliferation.

The evaluation of IFN- γ expression plays a very important role in the study of the immune response because it is responsible for the regulation and amplification of the antiviral response. The higher levels of IFN- γ in the vaccinated animals could support a higher immune reactivity to the infection able to rapidly and efficiently contrast viremia and the related occurrence of the disease.

Indeed, the association between viral titre in serum samples and IFN- γ increase, as well as IFN- γ secreting cell frequency, underlined the relevance of cellular activation and antibody humoral reaction against PCV2 replication (Fort et al., 2009a, 2009b; Martelli et al., 2011; Meerts et al., 2005). Contrarily, reduced IFN- γ expression levels were found both in PBMC (Sipos et al., 2004; Shi et al., 2010; Darwich et al., 2003a) and in lymphoid tissues (Darwich et al., 2003b) of PMWS-affected animals.

IL-10 is a regulatory cytokine, produced primarily by monocytes and to a lesser extent by lymphocytes, as immunosuppressive and anti-inflammatory molecule. It negatively inhibits macrophages and can inhibit the synthesis of pro-inflammatory cytokines such as IFN- γ , IL-2, TNF- α and GM-CSF (Meerts et al., 2005; Crisci et al., 2010). The antagonist immune cytokines, IFN- γ and IL-10, showed opposite levels in the early phase of PCV2 infection.

In accordance with Darwich et al. (2008), control PMWS-affected animals (Ctrl-PMWS+) showed the highest IL-10 levels at the peak of viremia (19 weeks of age), likely supporting a

potential inhibitory effect on the production of Th1 cytokines, such as IFN- γ (Shi et al., 2010). Indeed, the increased expression and production of IL-10 in tissues and PBMC were found both in the study of single infection and in PCV2-PRRSV coinfection cases (Doster et al., 2010; Shi et al., 2010; Darwich et al., 2003a). During PCV2 infection and the PCVD, IL-10 is mainly secreted by macrophage-monocyte and dendritic cell lineage but also by T cell clones, which have been impaired by a prolonged antigen stimulation; indeed PCV2 is able to stimulate the IL-10 release from PBMC and tissues (Darwich et al., 2003b; Kekarainen et al., 2008b; Crisci et al., 2010).

In our study, Ctrl-PMWS+ animals showed significantly higher IL-10 expression levels compared to the other two groups at 19 weeks. On the contrary, in vaccinated-exposed animals, IFN- γ expression remained at higher levels at both post-exposure time points (19 and 22 weeks of age), and this could testify a protective effect of vaccination. Because of the present data, is not possible to define a statistical correlation between IL-10 and IFN- γ changes, we cannot assume that IL-10 had a clear immunosuppressive role influencing the pathogenesis of PCV2 infection and the PMWS outcome.

Anyway, taken together, all these information highlight that a different modulation of the cytokine profiles between animals vaccinated with a PCV2 Cap protein-based vaccine and unvaccinated animals occurs. Particularly, in the case of these study, under natural conditions of PCV2 infection and PMWS outcome, cytokine modulation can indicate some immunoregulatory mechanism involving a reduction of early proinflammatory response as a condition that could influence the outcome of the PMWS disease.

Besides, vaccinated pigs, in addition to low viremia burden and absence of PMWS disease, showed stronger cellular IFN- γ -related reactivity as well as an effective expression of pro-inflammatory/innate cytokines (namely IL-8 and IL-1 β) after the occurrence of the natural infection (week 19-20 to 22) contrarily to what is shown in PMWS-affected animals.

In conclusion, the study reported in the present thesis demonstrates that the vaccination with a single dose of a PCV2 Cap vaccine against PCVD induces beneficial effect under field conditions.

- The similarity between ADWG and mortality rates observed in both experimental groups within the first 9 weeks after vaccination, considered together with the absence of local reactions and the reduced number of injections in vaccinated animals as compared to placebo/controls in the same time period, support the hypothesis that the

vaccine does not negatively influence the health status of the animals and is well tolerated.

- The vaccination reduces the mortality rate, morbidity, PCV2 viremia and viral load, and improves productive performances, namely, daily weight gain as well as carcass weight at the time of slaughter.
- The immunogenicity of the tested vaccine is mainly determined by induction of either humoral (PCV2-specific total antibodies) or cellular immune response (PCV2-specific IFN- γ SC and some pro-inflammatory and immune cytokines patterns in PBMC) and results in virologic and clinical protection.

Particularly, the evaluation of time-related expression changes of proinflammatory and immune cytokines showed some interesting results but their assessment as markers of infection and outcome of the PCV2 related diseases needs further studies.

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CHAPTER 5.

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CHAPTER 6.

RESEARCH ACTIVITIES AND PUBLICATIONS

In 2009-2011, **Dr. MARINA MORGANTI** attended the doctoral course in “Experimental and Comparative Immunology and Immunopathology” of University of Parma (Parma, Italy), performing research activities at the Unit of General Pathology and Veterinary Pathological Anatomy of the Department of Animal Health, Faculty of Veterinary Medicine, coordinated by Prof. Attilio Corradi.

She worked as a Ph.D. student in the group headed by Prof. Paolo Borghetti, supervised by Dr. Luca Ferrari and Dr. Elena de Angelis, in collaboration with Prof. Paolo Martelli, Coordinator of the Unit of Internal Medicine, Department of Animal Health, especially with regards to the evaluation of vaccine efficacy and clinical signs upon PCV2 natural infection.

In 2009 (**first year** of the Ph.D. course), Dr. Morganti dealt with the isolation, immunophenotyping and study of the cell-mediated immune response (after *in vitro* stimulation) of lymphocytes from peripheral blood of pigs PCV2-vaccinated.

She also collaborated with the research group headed by Prof. Angelo Borghetti (Unit of Molecular Pathology and Immunology, Department of Experimental Medicine, University of Parma), dealing with the following topics:

- development of chitosan tubular prosthesis and evaluation of their potential application in replacement surgery of the biliary tract;
- study of the pro-apoptotic activity of bisphosphonate drugs on cells of cholangiocarcinoma;
- biological characterization of ozonated oils in models derived from epithelial and mesenchymal cells.

In 2010 (**second year**), Dr. Morganti worked especially on the cell-mediated immune response in PCV2-vaccinated and unvaccinated pigs, subsequently exposed to PCV2 natural infection. The evaluation of pro-inflammatory and immune cytokine expression levels was performed in mononuclear cells isolated from peripheral blood (PBMC) by using quantitative RT-PCR. Intracellular staining coupled with flow cytometry was performed to determine the fraction of IFN- γ -positive cells in PBMC, after *in vitro* stimulation with PCV2.

From 01/08/2010 to 31/01/2011 Dr. Morganti attended the Laboratory of Immunology, Faculty of Veterinary Medicine of University of Ghent (Ghent, Belgium), coordinated by Prof. Eric Cox whose work has long been focused on the study of the immune response to infection by enterotoxigenic *Escherichia coli* F4+ (ETEC F4+) in swine.

During this period of internship she was mainly involved in:

- Isolation of pigs immune cells from blood and jejunal lamina propria followed analysis of aminopeptidase-N (receptor for ETEC F4 fimbriae) expression and activation status of antigen-presenting cells by flow cytometry directly or after *in vitro* stimulation.
- Study of the endocytosis mechanisms of aminopeptidase N in an experimental model using ST (Swine Testis) cells grown in trans-well systems by ELISA and immunocytochemistry.
- Transfection experiments to obtain a porcine cell line stably expressing aminopeptidase-N to use as experimental model.

In 2011 (**third year**) Dr. Marina Morganti worked on the following research projects:

- Study of the humoral and cell-mediated immune response in pigs simultaneously vaccinated against PCV2 and PRRSV and subsequently exposed to natural infection by both viruses. Comparison of double vaccination with single vaccinations (PCV2 or PRRSV alone) and unvaccinated animals.
 - ELISPOT assays were performed to evaluate the number of virus-specific IFN- γ secreting cells in PBMC;
 - quantitative RT-PCR experiments were performed to study the expression levels of pro-inflammatory and immune cytokines such as TNF- α and IL-10, respectively.
 - ELISA were performed to evaluate seroconversion to PCV2 and PRRSV and the levels of virus-specific IgG and IgM antibodies over time.
 - Viremia burden was determined by quantitative real-time PCR in serum and fecal samples.
- Study of the cell-mediated immune response in PCV2-vaccinated and unvaccinated pigs, subsequently exposed to PCV2 natural infection. The expression levels of several pro-inflammatory and immune cytokines were evaluated by using quantitative RT-PCR; the number of PCV2-specific IFN- γ producing cells in PBMC was determined after *in vitro-ex vivo* stimulation by intracellular staining and ELISpot assay.
- Assessment of cytokine patterns in PBMC of pigs vaccinated and unvaccinated against PRRSV and subsequently exposed to natural infection by a heterologous strain, after

in vitro stimulation with different PRRSV isolates. Following the *in vitro* stimulation, cytokine expression was assessed in PBMC by semi-quantitative RT-PCR and cytokine release was quantified by ELISA in the supernatants of cultured cells. The number of IFN- γ secreting cells in PBMC *ex vivo* re-stimulated with PRRSV strains was also measured.

Congresses

- 1) “Modulazione di citochine pro-infiammatorie e immunitarie in PBMC di suini vaccinati e non vaccinati esposti a infezione naturale da circovirus tipo 2 del suino (PCV2)”. Ferrari L., Morganti M., Borghetti P., De Angelis E., Martelli P. Proceedings of the Società Italiana di Patologia ed Allevamento dei Suini (SIPAS), XXXVII Annual Meeting – Piacenza, 24-25 March 2011, 266-276 (ISBN 978-88-903311-3-8).
- 2) “Pro-inflammatory and immune cytokines in PBMC of vaccinated and unvaccinated pigs exposed to porcine circovirus type 2 (PCV2) natural infection”. Ferrari L., Morganti M., Borghetti P., De Angelis E., Martelli P. Proceedings of the 3rd European Symposium on Porcine Health Management (ESPHM) – Espoo, Finland, 25-27 May 2011, 21.
- 3) “Evaluation of cytokines and immunomodulatory hormones in pigs vaccinated against PRRSV and naturally exposed to a heterologous field isolate”. Ferrari L., Borghetti P., Morganti M., Martelli P. International PRRS Symposium (IPRRS Symposium) – Chicago, USA, 2-3 December 2011, 31.
- 4) “Immune reactivity is associated with clinical protection upon vaccination with a modified-live PRRSV-1 vaccine and subsequent exposure to natural infection by a field strain”. Ferrari L., Borghetti P., Morganti M., Martelli P. International PRRS Symposium (IPRRS Symposium) – Chicago, USA, 2-3 December 2011, 32.
- 5) “Different cytokine patterns in *ex vivo* stimulated PBMC are related to the PRRSV isolate”. Ferrari L., Borghetti P., De Angelis E., Morganti M., Saleri R., Martelli P. 2011 International PRRS Symposium (IPRRS Symposium) – Chicago, USA, 2-3 December 2011, 33.

Publications

- 1) Romani A.A., Desenzani S., **Morganti M.M.**, La Monica S., Borghetti A.F., Soliani P., 2009. Zoledronic acid determines S-phase arrest but fails to induce apoptosis in cholangiocarcinoma cells. *Biochem Pharmacol.* 78(2): 133-141.
- 2) Romani A.A., Desenzani S., **Morganti M.M.**, Baroni M.C., Borghetti A.F., Soliani P., 2010. The BH3-mimetic ABT-737 targets the apoptotic machinery in cholangiocarcinoma cell lines resulting in synergistic interactions with zoledronic acid. *Cancer Chemotherapy and Pharmacology.* 67(3): 557-567.
- 3) Borghetti P., Saleri R., Ferrari L., **Morganti M.**, De Angelis E., Franceschi V., Bottarelli E., Martelli P., 2010. Cytokine expression, glucocorticoid and growth hormone changes after porcine reproductive and respiratory syndrome virus (PRRSV-1) infection in vaccinated and unvaccinated naturally exposed pigs. *Comparative immunology, microbiology and infectious diseases.* 34(2): 143-155.
- 4) Martelli P., Ferrari L., **Morganti M.**, De Angelis E., Bonilauri P., Guazzetti S., Caleffi A., Borghetti P., 2011. One dose of a porcine circovirus 2 subunit vaccine induces humoral and cell-mediated immunity and protects against porcine circovirus-associated disease under field conditions. *Vet. Microbiol.* 149(3-4): 339-351.

Articles on revision

- 1) Borghetti P., **Morganti M.**, Ferrari L., De Angelis E., Saleri R., Cavalli V., Corradi A., Martelli P., 2011. Modulation of pro-inflammatory and immune cytokines in PBMC of vaccinated and unvaccinated pigs exposed to porcine circovirus type 2 (PCV2) natural infection. *Comparative Immunology, Microbiology and Infectious Diseases (CIMID)* (2011, submitted).

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