Voor Noémi en Senne

IMMUNOMODULATION OF VETERINARY DRUGS ON LIPOPOLYSACCHARIDE-INDUCED INFLAMMATION IN PIGS:

INFLUENCE OF GAMITHROMYCIN AND KETOPROFEN ON THE ACUTE PHASE RESPONSE

Heidi Wyns

Thesis submitted in fulfilment of the requirements for the degree of Doctor of Philosophy in Veterinary Science (PhD), Faculty of Veterinary Medicine, Ghent University, 2014

Promoters

Prof. dr. S. Croubels

Prof. dr. P. De Backer

Prof. dr. E. Meyer

Department of Pharmacology, Toxicology and Biochemistry

Faculty of Veterinary Medicine, Ghent University

Printed by Alfa Print Solutions, Lovendegem

Immunomodulation of veterinary drugs on lipopolysaccharide-induced inflammation in pigs: influence of gamithromycin and ketoprofen on the acute phase response

ISBN 978-90-5864-401-5

Heidi Wyns (2014)

Department of Pharmacology, Toxicology and Biochemistry
Faculty of Veterinary Medicine - Ghent University, Belgium

"Quod me non necat me fortiorem facit"

TABLE OF CONTENTS

LIST OF ABBREVIATIONS **1**

GENERAL INTRODUCTION **7**

1. The acute phase response **9**
2. Lipopolysaccharide **10**
    - 2.1. Molecular structure of lipopolysaccharide **10**
    - 2.2. Extracellular lipopolysaccharide recognition **12**
    - 2.3. Intracellular lipopolysaccharide signaling **14**
    - 2.4. Inflammasome activation and IL-1β secretion **16**
3. Lipopolysaccharide inflammation in pigs **18**
    - 3.1. Lipopolysaccharide and septic shock **18**
    - 3.2. *In vivo* porcine lipopolysaccharide inflammation models **19**
4. Immunomodulation in pigs **27**
    - 4.1. Nonsteroidal anti-inflammatory drugs **29**
    - 4.2. Steroidal anti-inflammatory drugs **32**
    - 4.3. Antimicrobial drugs **35**

SCIENTIFIC AIMS **39**

EXPERIMENTAL STUDIES **41**

Chapter 1. Pharmacokinetics of gamithromycin and dexamethasone **43**

1.1. Pharmacokinetics of gamithromycin after intravenous and subcutaneous administration in pigs **44**

1.2. Pharmacokinetics of dexamethasone after intravenous and intramuscular administration in pigs **56**

Chapter 2. Immunomodulatory properties of veterinary drugs **67**

2.1. *In vitro* effect of gamithromycin, ketoprofen and dexamethasone on pro-inflammatory cytokine and prostaglandin E_2 production in LPS-stimulated porcine peripheral blood mononuclear cells **68**

2.2. *In vivo* influence of gamithromycin and ketoprofen on the acute phase response in lipopolysaccharide-challenged pigs **82**

Chapter 3. Comparative study of immuno-assays for cytokine detection: ELISA vs flow cytometry **107**

3.1. Development of a cytometric bead array screening tool for the simultaneous detection of pro-inflammatory cytokines in porcine plasma **108**

3.2. Multiplex analysis of pro-inflammatory cytokines in serum of *Actinobacillus pleuropneumoniae*-infected pigs **134**

GENERAL DISCUSSION **145**

REFERENCES **157**

SUMMARY **181**

SAMENVATTING **185**

CURRICULUM VITAE **189**

BIBLIOGRAPHY **191**

DANKWOORD **197**

µg	microgram
µL	microliter
µm	micrometer
Ab	antibody
ACN	acetonitrile
ANOVA	analysis of variance
APP	acute phase protein
APR	acute phase response
AUC	area under the plasma concentration-time curve
BCL	protein B-cell leukemia/lymphoma
BRD	bovine respiratory disease
BW	body weight
C5a	complement factor 5a
CBA	cytometric bead array
CD	cluster of differentiation
CFU	colony forming unit
Cl	total body clearance
cm	centimeter
C_{max}	maximum plasma concentration
COX	cyclooxygenase
CRI	constant rate infusion
CRP	C-reactive protein
CV	coefficient of variation
d	days
DAMP	danger-associated molecular pattern
DEX	dexamethasone
dp	particle size
DTT	DL-Dithiothreitol
EDTA	ethylenediaminetetraacetic acid

ELISA	enzyme-linked immuno sorbent assay
EMA	European Medicines Agency
EtOH	ethanol
F	bioavailability
FBS	fetal bovine serum
g	gravity
G	gauge
GAM	gamithromycin
GPI	glycosylphosphatidylinositol
h	hour
h-ESI	heated electrospray ionisation
Hp	haptoglobin
HPLC	high-performance liquid chromatography
Hsp	heat shock protein
i.d.	internal diameter
IFN	interferon
IKK	IκB kinase
IL	interleukin
IM	intramuscular
IND	indomethacin
IP	intraperitoneal
IRAK	IL-1 receptor-associated kinase
IRF	IFN regulatory factor
IS	internal standard
IU	international units
IV	intravenous
JNK	c-jun N-terminal kinase
k_{abs}	absorption rate constant
kDa	kilodalton
k_{el}; λz	elimination rate constant
KETO	ketoprofen

kg	kilogram
LBP	lipopolysaccharide binding protein
LC-MS/MS	liquid chromatography-tandem mass spectrometry
LOD	limit of detection
LOQ	limit of quantification
LOX	lipoxygenase
LPS	lipopolysaccharide
LT	leukotriene
Mal	MyD88 adaptor like protein
MALT	mucosa-associated lymphoid tissue
MD	myeloid differentiation protein
mCD14	membrane-bound CD14
MeOH	methanol
MFI	median fluorescence intensity
mg	milligram
MIC	minimum inhibitory concentration
min	minute
mL	milliliter
mm	millimeter
MRM	multiple reaction monitoring
MyD88	myeloid differentiation primary response protein 88
m/z	mass-to-charge ratio
N/A	not applicable
ND	not detected
NEM	N-ethylmaleimide
NF-κB	nuclear factor-κB
ng	nanogram
NI	no increase
(N)SAID	(non)steroidal anti-inflammatory drug
p.a.	post administration
p.i.	post infection

PAMP	pathogen-associated molecular pattern
PBMC	peripheral blood mononuclear cell
PBS	phosphate buffered saline
PD	pharmacodynamic
PE	phycoerythrin
PG	prostaglandin
PI	propidium iodide
pig-MAP	pig major acute phase protein
PIM	pulmonary intravascular macrophage
PK	pharmacokinetic
PL	phospholipase
PO	per os
RIP	receptor-interacting protein
ROA	route of administration
RSD	relative standard deviation
RT	rectal body temperature
s	second
SAA	serum amyloid A
SC	subcutaneous
SD	standard deviation
sCD14	soluble CD14
SRD	swine respiratory disease
SRM	selected reaction monitoring
sulfo-SMCC	sulfosuccinimidyl-4-(N-maleimidomethyl)cyclohexane-1-carboxylate
$t_{1/2abs}$	half-life of absorption
$t_{1/2el}$	half-life of elimination
TAK	transforming growth factor β-activated kinase
TIR	toll/interleukin-1 receptor
TIRAP	TIR domain containing adaptor protein
TLR	toll-like receptor
t_{max}	time of maximum plasma concentration

TNF	tumor necrosis factor
TOA	time of administration
Tollip	Toll-interacting protein
TRAF	TNF receptor-associated factor
TRAM	TRIF-related adaptor molecule
TRIF	TIR-domain-containing adapter-inducing interferon-β
TSA	trypticase soy agar
TX	thromboxane
ubc	ubiquitin-conjugating enzyme complex
UPLC	ultra-performance liquid chromatography
V_d	volume of distribution
V_{ss}	volume of distribution at steady state

GENERAL INTRODUCTION

Adapted from

Wyns, H., Plessers, E., De Backer, P., Meyer E., Croubels, S. (2014) *In vivo* porcine lipopolysaccharide inflammation models to study immunomodulation of drugs. *Veterinary Immunology and Immunopathology (under revision).*

1. The acute phase response

In mammals, the acute phase response (APR) is a well-coordinated sequence of processes, initiated at the site of inflammation upon infection or trauma, and is characterized by the production and release of a variety of inflammatory mediators, cellular interactions, vascular and metabolic changes, all eventually attempting to restore homeostasis (Baumann and Gauldie, 1994; Gruys et al., 2005).

While bacteria have developed mechanisms to improve their adhesion and subsequent colonization in host cells, the latter have developed mechanisms to recognise bacterial cell surface components ultimately leading to phagocytosis and elimination of micro-organisms (Heumann and Roger, 2002). In this respect, innate immunity is the first, non-specific line of defence against invading micro-organisms based on pattern-recognition systems, including bacterial lipopolysaccharide (LPS) (Heumann and Roger, 2002; Brown et al., 2011). Both monocytes and neutrophils are important cells in the innate immune response. While neutrophils efficiently initiate degranulation, phagocytosis and killing of invading micro-organisms without new synthesis of proteins, monocytes are a major source of inflammatory mediators. Furthermore, macrophages considerably contribute to the adaptive immune response as antigen-presenting cells (Baumann and Gauldie, 1994; Cavaillon and Adib-Conquy, 2005; Sanz-Santos et al., 2011). In general, the blood monocyte or tissue macrophage is the leukocyte which triggers the APR cascade, by initially releasing early or alarm cytokines, such as tumor necrosis factor α (TNF-α) and interleukin (IL)-1, which subsequently provoke the release of a secondary wave of cytokines, including IL-6. As a result, chemotactic molecules lead to the recruitment and migration of immune cells, such as neutrophils and mononuclear cells, to the target tissue. TNF-α, IL-1 and IL-6 are also considered to be involved in the regulation of the febrile response, mediating fever through the upregulation of cyclooxygenase (COX), mainly COX-2, and the subsequent induction of prostaglandin (PG) E_2, which is finally responsible for the increase of the body temperature. Meanwhile, IL-1 and IL-6 further stimulate the adrenal pituitary axis and the subsequent production of adrenocorticotropic hormone providing a negative feedback and inhibiting further cytokine gene expression. Following cytokine stimulation, hepatic protein synthesis is modified resulting in a dramatic increase or decrease in the concentration of several plasma proteins, the so called positive and negative acute phase proteins (APPs),

respectively (Heinrich et al., 1990; Baumann and Gauldie, 1994; Petersen et al., 2004; Gruys et al., 2005). Major porcine APPs are C-reactive protein (CRP), serum amyloid A (SAA) and haptoglobin (Hp) (Heinrich et al., 1990; Baumann and Gauldie, 1994; Petersen et al., 2004; Gruys et al., 2005).

The vascular tone is altered subsequent to the release of low-molecular-weight mediators from the inflamed tissue, including reactive oxygen species, nitrous oxide and several metabolites of the arachidonic acid cascade. The latter includes thromboxane A_2 (TXA_2), (PGs) and leukotrienes (LTs) which are responsible for tissue vasoconstriction and vasodilatation as well as bronchoconstriction and bronchodilatation. Dilatation and leakage of the blood vessels, particularly at the post-capillary venules, result in extravasation of erythrocytes and apparent redness (Baumann and Gauldie, 1994).

2. Lipopolysaccharide

LPS (Figure 1) is a structural part of the outer membrane of Gram-negative bacteria. The LPS component is crucial for outer membrane integrity and bacterial viability, since it plays a key role in nutrient transport and the access of toxic compounds, including antibiotics. When bacteria multiply or die and lyse, LPS is released from the surface. Released LPS has been identified as a very potent bacterial toxin and to distinguish LPS from the actively secreted exotoxins, it was defined endotoxin (Rietschel et al., 1993 and 1994). However, strictly, LPS refers to the purified glycolipid, whereas endotoxins may also contain small amounts of cell wall proteins, lipids, lipoproteins and polysaccharides in addition to LPS (Fink and Heard, 1990). Bacterial LPS is one of the most effective stimulators of the immune system in mammals (Dinarello, 1997; Tobias et al., 1999, Seydel et al., 2003). In contrast to bacterial exotoxins being toxic by killing host cells, endotoxins evoke an active response of host cells (Rietschel et al., 1994). Conversely, if LPS remains membranar, activation of the innate immune system is weak (Miyake, 2004; Jerala, 2007).

2.1. Molecular structure of lipopolysaccharide

LPS is an amphiphilic molecule, consisting of a hydrophilic heteropolysaccharide and a covalently bound lipid component, lipid A. In the case of *Enterobacteriaceae*, the

heteropolysaccharide can be subdivided into the O-polysaccharide (O-antigen or O-specific chain) and the core oligosaccharide (R-core). This core region on the other hand, can be further subdivided into the O-chain proximal outer core and the lipid A inner core region as illustrated in Figure 1 (Wright and Kanegasaki, 1971; Rietschel et al., 1994).

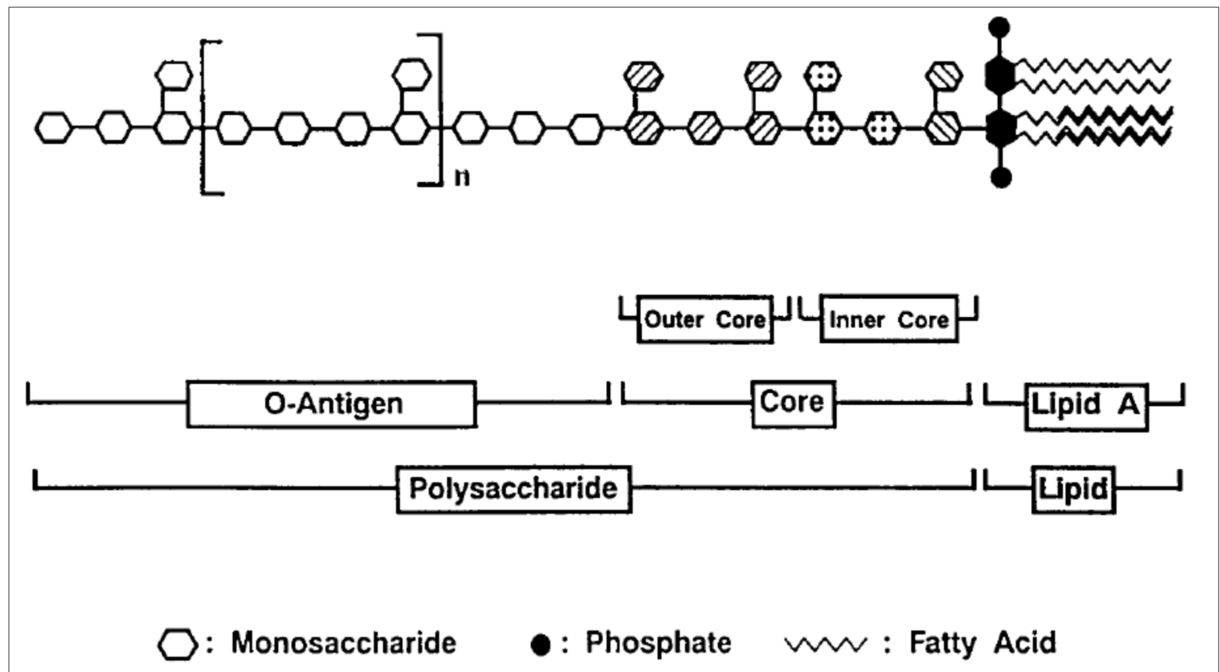


Figure 1. Schematic diagram of the LPS structure (Tobias et al., 1999)

The O-specific chain is a polymer of repeating oligosaccharide units, composed of either identical or different monosaccharide residues. The structure of these repeating units (nature, ring form, anomeric configuration, substitution, sequence and type of linkage of the monosaccharide residues) is characteristic for a given LPS molecule and its parental bacterial strain within a serotype. The R-core oligosaccharide is a saccharide portion, composed of up to 15 monosaccharide residues (Holst, 2007). The lipid A component is a fatty acid acylated and phosphorylated disaccharide (Tobias et al., 1999). The structures of lipid A and of the R-core oligosaccharide appear to be constant for a given genus. Even in different genera, the lipid A/R-core does not vary significantly (Wright and Kanegasaki, 1971). In contrast, the O-polysaccharide shows a high degree of variability amongst bacteria and determines the LPS antigenic specificity (Miyake, 2004). Variability in the R-core and O-polysaccharide can significantly affect the response as well as the type of the signaling pathway (Jerala, 2007).

The lipid A component has been proven to constitute the endotoxic and immunomodulatory principle of LPS (Wright and Kanegasaki, 1971; Rietschel et al., 1994). Recently, also the covalently linked core region has been suggested to possess immunogenic properties (Holst, 2007).

2.2. Extracellular lipopolysaccharide recognition

The extracellular recognition of LPS requires the sequential cooperation of three extracellular LPS-binding proteins, lipopolysaccharide binding protein (LBP), cluster of differentiation 14 (CD 14) and the glycoprotein myeloid differentiation (MD)-2, which chaperone LPS from the bacterial membrane to the transmembranar toll-like receptor (TLR) 4 (Jerala, 2007).

2.2.1. Lipopolysaccharide binding protein

Lipopolysaccharide binding protein (LBP) is a glycoprotein of approximately 60 kDa which is normally circulating in plasma (Martin et al., 1992). Here it recognizes and forms a high-affinity complex with the LPS lipid A component presented as either fragments, free molecules, or even still bound to the outer membrane of intact bacteria (Pålsson-McDermott and O'Neill, 2004). Due to its amphiphilic nature, LPS isolates typically form aggregates in a solution. One of the main functions of LBP is the disaggregation of LPS for subsequent presentation to cells (Tobias et al., 1999). Thus, LBP rapidly catalyzes the transfer of LPS to either membrane-bound CD14 (mCD14) or soluble CD14 (sCD14) (Triantafilou and Triantafilou, 2002). However, it should be remarked that LBP has a concentration-dependent dual role: at low concentrations it stimulates LPS signalling by extracting LPS from the bacterial membranes and transferring LPS monomers to CD14, while at high concentrations, it inhibits LPS signaling by shuttling the LPS to serum lipoproteins and by forming aggregates with LPS. As systemic LBP levels increase dramatically after induction of an acute phase reaction, LBP serves as an inhibitor of the excessive response to LPS (Gutsmann et al., 2001; Jerala, 2007).

2.2.2. CD14

The CD14 molecule exists in two forms. While mCD14 is attached to the surface of myeloid cells via a glycosylphosphatidylinositol (GPI) tail, sCD14 occurs in plasma where it helps to convey LPS signaling in cells lacking the membrane-bound CD14 form, including endothelial and epithelial cells (Pålsson-McDermott and O'Neill, 2004). Although CD14 has been identified as an LPS receptor, it is a GPI-anchored protein lacking transmembrane and intracellular domains. Therefore, the role of CD14 in LPS signaling appears to be binding of LPS and subsequent presentation and transfer of LPS to MD-2 (Triantafilou and Triantafilou, 2002; Pålsson-McDermott and O'Neill, 2004; Peri et al., 2010).

2.2.3. TLR4-MD-2 complex

TLRs are a family of cell-surface and endosomally expressed receptors, recognizing a variety of pathogen-associated molecular patterns (PAMPs), including lipids, proteins, lipoproteins and nucleic acids, as well as endogenous mediators released upon tissue damage (danger-associated molecular patterns or DAMPs) (Ulevitch, 2004; Lorne et al., 2010; Wittebole et al., 2010; Brown et al., 2011). TLRs are expressed on innate immune cells, including monocytes, macrophages, neutrophils, dendritic cells and mucosal epithelial cells (Beinke and Ley, 2004). More specifically, TLR4 has been established as the receptor for LPS (Pålsson-McDermott and O'Neill, 2004).

The discovery of TLR4 unravelled the missing link between LPS recognition by LBP and then CD14 on the one hand, and the intracellular signaling pathway, leading to the production of pro-inflammatory cytokines on the other hand (Wittebole et al., 2010). TLRs consist of extracellular leucine-rich repeats, a transmembrane region and an intracellular Toll/Interleukin-1 receptor (TIR) domain. For effective LPS recognition, TLR4 additionally requires MD-2, which forms a complex with its extracellular domain (Triantafilou and Triantafilou, 2002; Jerala, 2007). The secreted glycoprotein MD-2 acts as an extracellular adaptor protein in the activation of TLR4 by firstly binding LPS and secondly associating with TLR4 via the extracellular leucine-rich repeats inducing TLR4 aggregation (Pålsson-McDermott and O'Neill, 2004). Dimerization of the TLR4-MD-2-LPS-complex leads to the recruitment of adaptor proteins to the intracellular domain of TLR4, initiating the intracellular signaling cascade (Peri et al., 2010).

2.3. Intracellular lipopolysaccharide signaling

2.3.1. Nuclear factor-κB activation

Nuclear factor-κB (NF-κB) represents a family of transcription factors regulating the expression of diverse genes involved in immune and inflammatory responses. In unstimulated cells, NF-κB is kept inactive in the cytoplasm, where its nuclear import is blocked (Verstrepen et al., 2008).

Apart from LPS, NF-κB transcription factors can be activated in response to various stimuli including cytokines, infectious agents, injury and other stressful conditions requiring rapid reprogramming of gene expression. Following a coordinated cooperation of membrane-bound or cytosolic adaptor proteins and kinases, NF-κB is finally released and translocates to the nucleus to bind the promoters of responsive genes. These include pro-inflammatory cytokines, such as TNF-α, IL-1 and IL-6, type I interferons (IFNs), COX-2, chemokines and adhesion molecules, which collectively regulate the recruitment of immune cells to infection sites. In addition, TNF-α and IL-1 stimulation of their respective receptors also strongly activates NF-κB, which plays an important role in amplifying and extending the innate immune response (Beinke and Ley, 2004; Brown et al., 2011).

Theoretically, activation of TLR4 with subsequent cytokine production is beneficial for the host, but dysregulation of this process can lead to life-threatening syndromes such as sepsis and septic shock (Peri et al., 2010).

Figure 2 provides an overview of the LPS signaling pathways (Verstrepen et al., 2008).

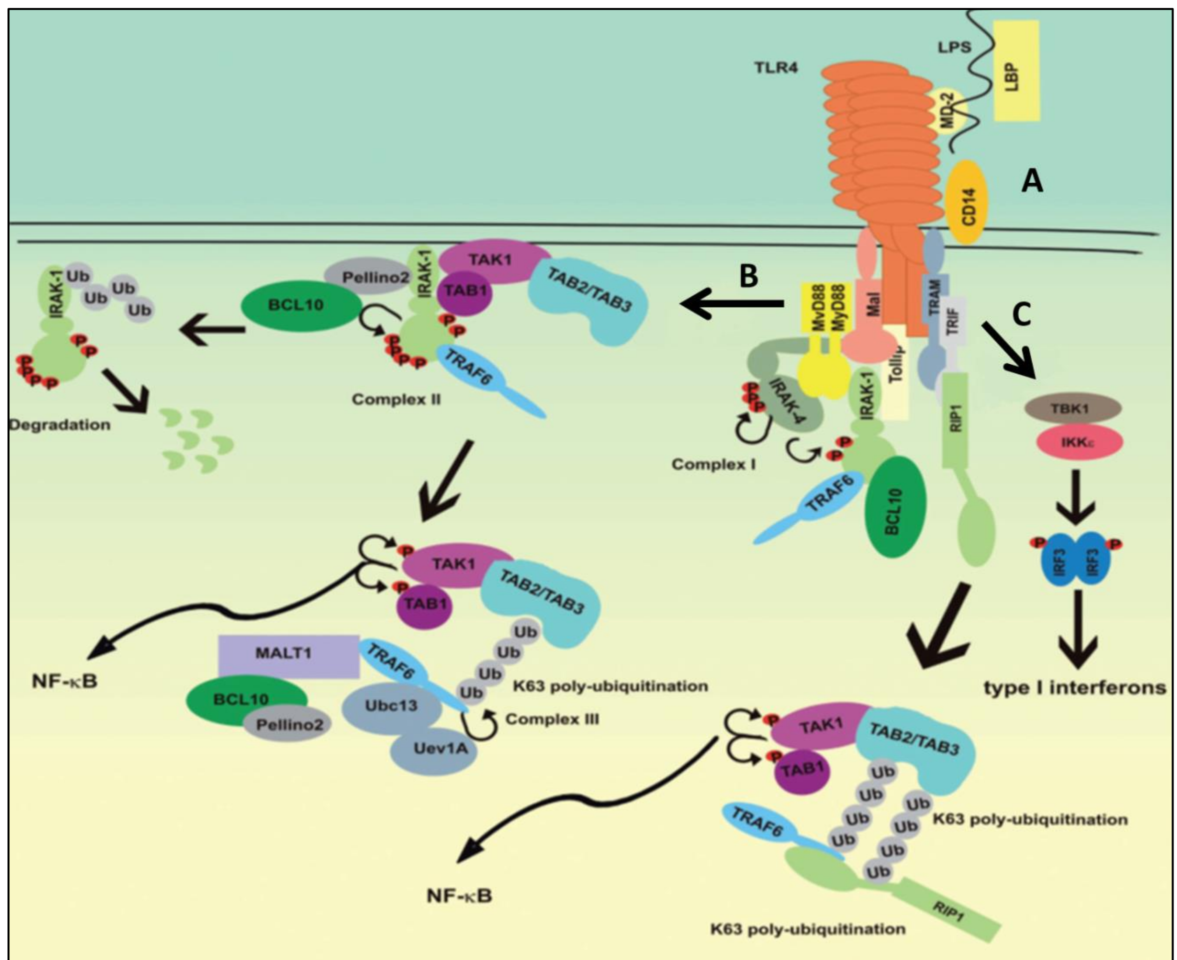


Figure 2. Lipopolysaccharide (LPS) signaling pathways to NF-κB activation. (A) Extracellular LPS recognition. (B) MyD88-dependent intracellular LPS signaling. (C) MyD88-independent intracellular signaling (adapted from Verstrepen et al., 2008).

2.3.2. MyD88-dependent and -independent pathways

The TLR4-mediated response to LPS can be divided into an early myeloid differentiation factor 88 (MyD88)-dependent response and a delayed MyD88-independent response (Pålsson-McDermott and O'Neill, 2004). Activation of both signaling pathways occurs sequentially since TLR4 initially induces MyD88-dependent signaling at the plasma membrane and after endocytosis of the receptor complex, the MyD88-independent signaling is activated in the endosomes. TLR4 is the only TLR, signaling through both MyD88-dependent and -independent pathways (Jerala, 2007).

The TIR domain containing adaptor protein MyD88 is recruited to the TLR4 complex via interaction with the TIR domain containing adaptor protein (TIRAP), also known as MyD88 adaptor like protein (Mal). As a result, IL-1 receptor-associated kinase 1 (IRAK-1),

the adaptor protein Toll-interacting protein (Tollip), TNF receptor-associated factor 6 (TRAF6), as well as the IRAK-1 related kinase IRAK-4 all are recruited to the activated receptor complex in order to form complex I. In addition, protein B-cell leukemia/lymphoma 10 (BCL10) interacts with IRAK-1. Activation of IRAK-4 by intramolecular autophosphorylation leads to the subsequent phosphorylation and activation of IRAK-1. Subsequently, hyperphosphorylation of IRAK-1 results in the dissociation from MyD88 and Tollip, whereupon the IRAK-1-TRAF6 complex interacts with a transforming growth factor β-activated kinase 1 (TAK1)-TAK1-binding protein (TAB1)-TAB2 (or TAB3) membrane-bound complex, forming complex II. Upon LPS stimulation, BCL10-interacting protein mucosa-associated lymphoid tissue 1 (MALT1) interacts with BCL10 as well as TRAF6, and pellino-2 interacts with BCL10 in both the membrane-bound and cytosolic TAK1 complex. While IRAK-1 remains at the membrane and becomes poly-ubiquitinated, the TRAF6-TAK1-TAB1-TAB2/3 complex translocates to the cytoplasm, where TRAF6 interacts with the E2 ubiquitin-conjugating enzyme complex Ubc13/Uev1A, forming complex III. Consequently, poly-ubiquitination of TRAF6 results in the final activation of the IKK complex and c-jun N-terminal kinase (JNK). IKKβ is responsible for the phosphorylation, poly-ubiquitination and proteolytical degradation of IκBα. Finally, NF-κB is released and translocates to the nucleus to bind the promoters of responsive genes (Verstrepen et al., 2008).

The MyD88-independent signaling pathway, on the other hand, concerns the recruitement of the TIR domain containing adaptor-inducing IFN-β (TRIF) and the TRIF-related adaptor molecule (TRAM). TRIF associates with TRAF6 and receptor-interacting protein 1 (RIP1) to induce NF-κB activation. Moreover, activation of the IKK-related kinases TANK-binding kinase 1 (TBK1) and IKKε by TRIF results in dimerization and phosphorylation of the transcription factor IFN regulatory factor 3 (IRF3), which binds and activates type I IFN promoters in the nucleus (Verstrepen et al. 2008).

2.4. Inflammasome activation and IL-1β secretion

The production and secretion of biologically active IL-1β involves the combined effect of at least 2 activation cascades: first, the gene transcription and cytosolic synthesis of an inactive 31-kDa precursor, pro-IL-1β, are induced upon exposure to typically

extracellular (pro-inflammatory) stimuli (vitaPAMPs) pattern recognition receptors (PRRs), including TLR4, and down-stream NF-κB signaling; secondly, this pro-IL-1β is proteolytically cleaved into mature 17-kDa IL-1β by cysteine-aspartic proteases (caspases), which are activated by the inflammasome (Underhill and Goodridge, 2012; Bi et al., 2014).

The inflammasome is a large multimolecular intracellular protein complex which is typically composed of 3 components: a receptor protein part, the apoptosis associated speck-like protein (ASC) and caspase 1. The receptor part of inflammasomes is categorized by the domain structure of the protein. Similar to TLRs, cytoplasmic nucleotide-binding domain and leucin-rich repeat containing receptors (NLRs) are a family of PRRs, which can contain a pyrin domain (NLRP) (Horvath et al., 2011; Lamkanfi and Dixit, 2014). The canonical inflammasome protein NLRP3 engages ASC and activates caspase 1 to finally promote cleavage and secretion of IL-1β, as well as (pyroptotic) cell death.

To date, inflammasome activation by LPS is not yet fully elucidated and this subject is out of the scope of this thesis. Nevertheless, it has been observed in murine macrophages that LPS from only one *E. coli* serotype, more specifically O111:B4, was able to elicit caspase 1 activation and secretion of active IL-1β albeit only in combination with another toxin as secondary stimulus (Kayagaki et al., 2013). Recently, Gram-negative bacteria, including *E. coli*, also elicit non-canonical inflammasome activation, in which caspase 11 was required in addition to NLRP3, ASC and caspase 1 to induce pyroptosis (Kayagaki et al., 2013). This *E. coli*-induced caspase 11 activation plays an important role in murine endotoxic shock and sepsis, as cytoplasmic LPS is directly recognized by caspase 11 and induces the oligomerization and activation of this proteolytic enzyme (Shi et al., 2014).

Of relevance for this PhD thesis, porcine inflammasome activation, however, remains largely unknown till date. It has been suggested that this signaling is expected not to differ considerably from other mammalian species i.e. man or mice (Kim et al., 2014). Corroborating this suggestion, very recently one group described that the porcine reproductive and respiratory syndrome virus (PRRSV) infection significantly induced IL-1β production and processing in primary porcine alveolar macrophages in an on TLR/MyD88 signaling- and NLRP3 inflammasome activation-dependent manner, respectively (Bi et al., 2014).

3. Lipopolysaccharide inflammation in pigs

3.1. Lipopolysaccharide and septic shock

LPS challenge has been commonly used as a model for Gram-negative bacterial inflammation and sepsis (Schrauwen et al. 1988; Schmidhammer et al., 2006). LPS is hypothesized to play a major role in sepsis, since the antibiotic-induced disintegration of bacteria may lead to massive and biologically active LPS release followed by a severe acute hemodynamic deterioration (Røkke et al., 1988; Lepper et al., 2002; Poli-de-Figueiredo et al., 2008). Sepsis is defined as a complex dysregulation of inflammation, ultimately affecting multiple organ systems and leading to irreversible damage (Buras et al., 2005). Sepsis is often, but not always, associated with the presence of a severe bacterial, fungal or viral infection and remains a major cause of morbidity and mortality (Fink and Heard, 1990; Dickneite and Leithäuser, 1999; Poli-de-Figueiredo et al., 2008). Accordingly, septic shock is a circulatory decompensation following sepsis and is often the result of vascular invasion by bacteria (Fink and Heard, 1990).

Porcine Gram-negative bacterial shock is characterized by an initial hyperdynamic phase with an increased cardiac output, a decreased total peripheral vascular resistance and a normal to high arterial blood pressure. Subsequently, when bacteria invade the vascular system, the hyperdynamic phase can be followed by a hypodynamic phase with a decreased cardiac output, increased total peripheral vascular resistance and a low arterial blood pressure (Schrauwen and Houvenaghel, 1985; Holger et al., 2010). Furthermore, similar hemodynamic and clinical changes were observed following intravenous (IV) administration of living *E. coli* bacteria and a high or low dose of LPS in pigs, suggesting the activation of identical pathophysiological mechanisms. More specifically, porcine *E. coli* sepsis and endotoxemia are hemodynamically characterized by a profound pulmonary hypertension, systemic arterial hypotension, a decrease in cardiac output and a reflex increase in heart rate (Schrauwen et al. 1988; Olson et al., 1985; Klosterhalfen et al., 1992; Schmidhammer et al., 2006).

However, the suitability of the LPS model to study sepsis is controversial (Fink and Heard, 1990; Poli-de-Figueiredo et al., 2008). Experimental endotoxemia as well as bacteremia mainly represent a systemic challenge lacking a preceding infectious focus and

the characteristic sepsis-induced immune reaction (Poli-de-Figueiredo et al., 2008). Moreover, killed *E. coli* bacteria are more lethal than LPS, suggesting that other components of the Gram-negative bacterial cell wall likely also contribute to the systemic inflammatory response (Fink and Heard, 1990; Poli-de-Figueiredo et al., 2008).

In conclusion, LPS injection is generally accepted as a model to study the hypodynamic phase of Gram-negative bacterial shock, rather than sepsis (Fink and Heard, 1990).

3.2. *In vivo* porcine lipopolysaccharide inflammation models

3.2.1. LPS challenge

Table 1 provides a chronological overview of porcine *E. coli* LPS inflammation models used to study the APR-induced cytokines and, if available, APPs. The techniques used for determination of these mediators were also included. In addition to the selected *E. coli* serotype and LPS dose, the duration and route of administration (ROA) are additional important variables between these studies.

Table 1. *In vivo E. coli* LPS inflammation models in pigs

LPS		Animals			Time to maximal cytokine levels (h p.a.)			Acute phase proteins (APP)			Technique for cytokine / APP analysis	Reference
Serotype	**Dose (µg/kg BW)**	**N°**	**Age (d)**	**BW (kg)**	**TNF-α**	**IL-1β**	**IL-6**	**CRP**	**SAA**	**Hp**		
Intravenous LPS administration												
O111:B4	0.5 over 0.5h	10*	-	28-32	1	-	3	-	-	-	Radioimmunoassay Bioassay	Klosterhalfen et al., 1992
O111:B4	200	3*	60-90	-	1	-	-	-	-	-	Bioassay	Nakajima et al., 1995
O111:B4	75	32	±28	-	1	Increase	2.5	Increase	-	Decrease	ELISA/ELISA	Frank et al., 2003
O111:B4	4µg/kg/h (0.5h) 1 µg/kg/h (5.5h)	4	84-98	20-26	1	-	2-3	-	-	-	ELISA	Goscinski et al., 2004
O111:B4	25	20	27-30	-	1	-	2-2.5	-	-	-	ELISA	Carroll et al., 2005
O111:B4	0.06	5	7-10	2.4±0.17	1	1	-	-	-	-	ELISA	John et al., 2008
O111:B4	25	26	35-42	-	1	3	2.5 3.5	Increase	Increase	Increase	ELISA/ELISA	Williams et al., 2009
O111:B4	0.625-5 µg/kg/h (0.75h) 5 µg/kg/h (1.25h) 0.625 µg/kg/h (3h)	7	-	12-16	2.5	Increase to 3.5	Increase to 3.5	-	-	-	ELISA	Levenbrown et al., 2013
O26:B6	2.5-15 µg/kg/h (0.5h) 2.5 µg/kg/h (4.5h)	9	-	30	(1) 2	-	4	-	-	-	In-house fluorometric assay	Nielsen et al., 2007
O26:B6	4 (8h)	4*	-	25-35	2	ND	4	-	-	-	ELISA	Ruud et al., 2007
O26:B6	2.5-15 µg/kg/h (0.5h) 2.5 µg/kg/h (5.5h)	12*	96±4	40±3	1	-	4	Increase	-	-	In-house fluorometric assay Spectrophotometry	Ebdrup et al., 2008
O55:B5	2 or 20	24	-	35 or 85	1-2	-	1-2	-	-	-	ELISA; Bioassay	Myers et al., 1999
O55:B5	2	6*	90-120	25-35	1	NI	3	-	-	-	ELISA; Bioassay	Myers et al., 2003
O55:B5	2	8		70-75	1	-	3	-	-	-	ELISA	Peters et al., 2012
Intraperitoneal LPS administration												
K-235	0.5, 5 or 50	15*	-	15-25	1.5	-	-	-	-	-	ELISA	Warren et al., 1997
K-235	0.5, 5	48*	-	11.6 ± 0.19	2	-	4	-	-	-	ELISA; Bioassay	Webel et al., 1997
K-235	5	48*	42	11±1.05	2	NI	-	Increase	Increase	NI	ELISA	Llamas Moya et al., 2006
O111:B4	100	45	12, 28, 30	-	1	-	-	-	-	-	ELISA	Kanitz et al., 2002
O111:B4	100	72	12 or 56		1	ND	-	-	-	-	ELISA	Tuchscherer et al., 2004
O55:B5	100	6*	±26	10.2 ± 0.9	2	-	-	-	-	Increase	ELISA / Colorimetric, enzymatic assay	Wright et al., 2000
Intramuscular LPS administration												
O111:B4	25 or 50	24*	-	21.3±0.48	-	-	-	NI	Increase	NI	ELISA; Peroxidase activity test (Hp)	Frank et al., 2005
O55:B5	25	20	126	85-100	1	-	-	-	-	Increase	ELISA / Immunoturbidimetric method	Leininger et al., 2000
Intramammar LPS administration												
O111:B4	2	5*	-	235	3	-	3	-	-	-	ELISA	Wang et al. 2006

BW: body weight; d: days; h p.a.: hours post administration; ND: not detectable; NI: no increase; -: not investigated; *studies including negative control pigs

The majority of the studies (over 50 %) used *E. coli* serotype O111:B4, whereas *E. coli* serotypes O55:B5, O26:B6 and K-235 are used in > 20, > 10 and > 10 % of these studies, respectively. LPS challenge can be performed either as a single IV, intraperitoneal (IP) or intramuscular (IM) bolus administration, or as a continuous IV infusion. One study, however, provided LPS intramammarily. While a single LPS bolus administration provides a better perception of the sequence of events following LPS challenge, a continuous LPS infusion would imitate more accurately a clinical endotoxemia/septicemia, since endotoxin remains in circulation for a longer period (Fink and Heard, 1990; Olson et al., 1995; John et al., 2008). As previously mentioned, endotoxin is continuously produced and released by surviving bacteria in Gram-negative bacterial sepsis (Schrauwen et al., 1988). In 65 % of the reported studies, LPS was IV administered. Moreover, 2/3 of these studies used a bolus injection and in 1/3 of the studies an infusion was applied. Conversely, IP and IM administration are far less frequently applied, i.e. only in ± 25 and ± 10 % of the studies, respectively.

Considerable species differences are reported with respect to endotoxin sensitivity. Unlike poultry and rodents, pigs and cattle are very sensitive to LPS administration (Olson et al., 1995; Schmidhammer et al., 2006; Poli-de-Figueiredo et al., 2008). While in pigs and cattle LPS doses of 25 and 2.5 µg/kg body weight (BW), respectively, can be considered as high; in broiler chickens and rodents doses as high as 2500 and 20000 µg/kg BW have been applied, respectively (Gerros et al., 1993; De Boever et al., 2010; Purswani et al., 2002; Zhang et al., 2008; Plessers et al., manuscript submitted). Doses of 500 and 2500 µg/kg BW have been proven to be lethal in pigs (Schrauwen et al., 1984, 1986; Schrauwen and Houvenaghel, 1985). In pigs, 80 % of the reported studies used an LPS dose ≤ 25 µg/kg BW (Table 1). In marked contrast, the IV administration of 0.004 µg/kg BW LPS to humans already causes fever and a hyperdynamic cardiovascular response (Martich et al., 1993).

In neonatal calves, Gerros et al. (1993) established that neither the dose nor the route of LPS administration affects the sequence of mediator release. To date, the influence of LPS serotype, dose and ROA have not been investigated thoroughly in pigs.

3.2.2. Clinical symptoms in endotoxemic pigs

As fast as within 15 min after LPS challenge, the first clinical signs occur in pigs. Symptoms such as intermittent coughing, salivation, chewing movements, retching and vomiting are recurrently observed. Subsequently, pigs become depressed for several hours, as manifested by general sickness, lethargy, somnolence and sternal or lateral decubitus. Respiratory difficulties develop varying from panting to severe dyspnea. Occasionally, shivering, generalized rubor, cyanosis and even necrotic lesions have been described (Schrauwen and Houvenaghel, 1985; Schrauwen et al., 1988; Johnson and von Borell, 1994; Leininger et al., 2000; Kanitz et al., 2002; Myers et al., 2003; Peters et al., 2012).

Reduced feed intake and anorexia are also indisputably associated with the administration of LPS (Warren et al., 1997; Wright et al., 2000; Myers et al., 2003). Additionally, a dose-dependent reduction in feed consumption and activity after an LPS bolus administration in pigs was described (Johnson and von Borell, 1994; Frank et al., 2005). Following intratracheal LPS administration, only moderate clinical signs were observed (Villarino et al., 2013).

3.2.3. Pro-inflammatory cytokines

Over the years, enzyme-linked immuno sorbent assay (ELISA) remains the most popular immuno-assay for the determination of porcine TNF-α, IL-1 and IL-6 in serum or plasma. In initial reports, researchers mostly relied on bioassays for measurement of TNF-α and IL-6 using specific murine cell lines. While the presence of IL-6 was commonly established by IL-6 dependent proliferation assays, TNF-α activity was quantified by measurement of its cytotoxicity to cells in culture (Table 1). Currently, ELISA is still considered the gold standard for analysis of systemic cytokines as well as APPs, yet multiplex, flow cytometric assays for the simultaneous measurement of multiple porcine inflammatory parameters are gaining popularity (Johannisson et al., 2006; Lawson et al., 2010).

It should be remarked that the use of different techniques may yield different results, i.e. a bioassay measuring biologically active TNF-α will generate different results

compared to an ELISA measuring immunoreactive (free and receptor-bound) TNF-α (Myers et al., 1999).

A wide variation in the time points of sampling can be observed within the porcine LPS inflammation studies. With the exception of Warren et al. (1997) and Levenbrown et al. (2013), who surprisingly determined a maximal plasma concentration of TNF-α only at 1.5 and 2.5 h post LPS administration (p.a.), respectively, the peak concentration of TNF-α is unarguably established at 1 h in pigs after IV, IP and IM LPS administration. A number of papers describing a peak concentration of TNF-α at 2 h p.a. simply did not include a sampling point at 1h p.a. (Webel et al., 1997; Wright et al., 2000; Llamas Moya et al., 2006; Ruud et al., 2007). Notwithstanding John et al. (2008), who observed a 9-fold increase in TNF-α levels p.a. following an IV LPS dose as low as 0.06 µg/kg BW, it was repeatedly reported that LPS doses < 1 µg/kg failed to provoke a systemic TNF-α response indicating a dose-dependent effect on TNF-α levels. Additionally, the ROA is also partially responsible for inconsistent results (Warren et al., 1997; Webel et al., 1997; Myers et al., 1999).

As for IL-6, maximal concentrations were measured between 2.5 and 4 h p.a. Only Myers et al. (1999), described a maximal IL-6 concentration already between 1 and 2 h p.a. Noteworthy, in set-ups utilizing frequent blood sampling, maximal levels of IL-6 were repetitively established at 2.5 h p.a. (Frank et al., 2003; Carroll et al., 2005; Williams et al., 2009). Although IL-6 is a potent inducer of the APR, it has notable anti-inflammatory properties as well. Accordingly, it has been increasingly suggested to classify IL-6 as an anti-inflammatory cytokine (Opal and DePalo, 2000; Philippart and Cavaillon, 2007).

Although IL-1 is recognized to be of major importance in the inflammatory process, this cytokine is rather scarcely included in the characterisation of the APR of porcine LPS inflammation models. IL-1 consists of two related proteins, IL-1α and IL-1β (Murtaugh et al., 1996), of which only the latter is investigated in pigs (Table 1). Conversely to TNF-α and IL-6, these data presented on porcine IL-1β are not straightforward. More specifically, IL-1β has repeatedly been reported either not detectable or not increasing in plasma p.a. of LPS in pigs (Myers et al., 2003; Tuchscherer et al., 2004; Llamas Moya et al., 2006; Ruud et al., 2007). In this respect, it has been suggested that IL-1β production is less sensitive to the effects of LPS compared to TNF-α and IL-6 (Myers et al., 2003). Nevertheless, John et al. (2008) determined a 5-fold increase in the concentration of IL-1β at 1 h p.a. of an LPS dose as low as 0.06 µg/kg BW. Frank et al. (2003) observed a significant increase of this pro-

inflammatory cytokine between 1 and 2 h p.a. In another study, maximal IL-1β levels were only seen at 3 h p.a. (Williams et al., 2009). Interestingly, the studies describing a noticeable increase or maximal concentration of IL-1β p.a., all applied *E. coli* serotype O111:B4 (Table 1).

3.2.4. Eicosanoids

3.2.4.1. PGE_2 and the febrile response

It is generally accepted that PGE_2 is responsible for the increase of the body temperature in mammals. PGE_2 exerts its pyrogenic action by binding to receptors on thermoregulatory neurons in the hypothalamus (Baumann and Gauldie, 1994; Netea et al., 2000; Blatteis et al., 2005). However, the role of PGE_2 in the induction of the febrile response has been considered both crucial and controversial (Coceani et al., 1986; Blatteis et al., 2005). These PGE_2 inconsistencies have been reviewed in detail by Blatteis et al. (2005). Briefly, the IV administration of both pyrogenic cytokines and PGE_2 to pigs undeniably induces fever.

Treatment with a COX-inhibitor, on the other hand, completely prevents this LPS-induced fever and sickness in pigs, confirming an important role for PGE_2 in the induction of fever (Johnson and von Borell, 1994; Vellucci and Parrott, 1994; Parrott et al., 1995; Peters et al., 2012) (Figure 5). The synthesis of PGE_2 is catalysed by the COX-2 enzyme following its transcription and translation. The systemic appearance of PGE_2 is therefore expected to occur later (Blatteis et al., 2005).

However, in pigs, maximal plasma concentrations of PGE_2 are already observed within 1 or 2 h p.a. of LPS (Wright et al., 2000; Peters et al., 2012). The porcine febrile response commonly peaks at approximately 4 h p.a. of LPS, with a body temperature rising above 40 °C (Johnson and von Borell, 1994; Warren et al., 1997; Leininger et al., 2000; Wright et al., 2000; Mustonen et al., 2012a). The duration of the febrile response has been determined dose-dependent in LPS-challenged pigs (Johnson and von Borell, 1994).

3.2.4.2. TXA_2 and pulmonary hypertension

In marked contrast to other animal species, a dramatic increase in pulmonary arterial pressure is noticed in pigs in response to an endotoxin challenge (Olson et al., 1985; Schrauwen et al. 1988; Klosterhalfen et al., 1992; Schmidhammer et al., 2006). Remarkably, differentiated macrophages are observed within porcine lung capillary vessels (Winkler and Cheville, 1985). It is hypothesized that those pulmonary intravascular macrophages (PIMs) respond to LPS with a rapid and massive release of TXA_2, resulting in a severe pulmonary hypertension and possibly right heart dysfunction (Klosterhalfen et al., 1992; Dickneite and Leithäuser, 1999; Schmidhammer et al., 2006). The concentration of TXB_2, a stable metabolite of TXA_2, rapidly increases following LPS challenge in pigs, attaining peak plasma concentrations as early as 30 to 45 min p.a. (Klosterhalfen et al., 1992; Mustonen et al., 2012a). However, Friton et al. (2006) established a maximal concentration of TXB_2 only at 2 h p.a., while Peters et al. (2012) detected no increase at all in pigs. High concentrations of TXB_2 are suggested to be associated with more severe clinical symptoms (Mustonen et al., 2012a).

As for the cytokines, commercial ELISA kits are popular for the determination of eicosanoids (Friton et al., 2006; Cao et al., 2006; Mustonen et al., 2012a; Peters et al., 2012). Although these methods are sensitive, they are less specific. The development of highly specific analytical methods for the detection and quantification of multiple arachidonic acid metabolites by liquid chromatography-tandem mass spectrometry (LC-MS/MS) is therefore of growing interest (Araujo et al., 2013).

3.2.5. Acute phase proteins

To study APPs in pigs, inflammation was often aseptically induced by a single subcutaneous injection of turpentine (Lampreave et al., 1994; González-Ramón et al., 1995; Eckersall et al., 1996). The maximal plasma concentrations of these APPs are typically reached within 24 to 48 h after the initiation of the APR (Lampreave et al., 1994; Petersen et al., 2004). Additionally, the APP profile has been comprehensively characterized after experimental infections with *Actinobacillus (A.) pleuropneumoniae* and *Streptococcus suis* (Heegaard et al., 1998; Sorensen et al., 2006).

In contrast, data on APPs in LPS inflammation models are rather scarce, especially in pigs (Table 1). Williams et al. (2009) determined an increase in SAA, CRP as well as Hp after an IV LPS bolus injection of 25 µg/kg BW. Apart from a significant increase in SAA, Frank et al. (2005) surprisingly observed no increase in CRP nor in Hp after an IM LPS challenge of 25 or 50 µg/kg BW. The authors considered the possibilities that both CRP and Hp were already elevated prior to LPS challenge, or that the blood sampling points (i.e. 0 and 48 h p.a.) were likely inappropriate to detect changes in both the APPs. On the other hand, Leininger et al. (2000) and Wright et al. (2000) demonstrated increased levels of Hp after an IM LPS bolus of 25 µg/kg BW and an IP LPS bolus of 100 µg/kg BW, respectively. Llamas Moya et al. (2006) detected an increase in both SAA and CRP after an IP LPS injection of only 5 µg/kg BW, yet levels of Hp were not elevated. Finally, Ebdrup et al. (2008) established a rise in CRP levels after LPS infusion. Although pig-MAP has been recurrently suggested as an excellent biomarker for the indication of porcine stress conditions and pathologies (Alava et al., 1997; Piñeiro et al., 2007; Heegaard et al., 2011), to date only our group has reported the increase of this interesting APP in an IV LPS inflammation model.

Apart from a few exceptions, concentrations of APPs were, as for cytokines and eicosanoids, determined using commercial ELISA kits (Table 1), which yielded diverse and sometimes contradictory results between studies. As CRP is a major APP in humans and numerous animal species, different species-specific CRP ELISA kits are commercially available. To illustrate the need for careful interpretation, it should be remarked that Slagman et al. (2011) considered the results of one CRP ELISA highly controversial.

In contrast to most human clinical laboratories, where automated immunonephelometric and immunoturbidimetric assays are routinely used to quantify concentrations of CRP, in clinical veterinary medicine, determination of CRP has not been routinely used (Tugirimana et al., 2011; Algarra et al., 2013). Recently, sensitive turbidimetric and nephelometric methods, relying on the calcium-mediated, but species-independent binding of CRP to phosphocholine have been described, which offer further interesting perspectives for application in veterinary medicine (Tugirimana et al., 2011; Drieghe et al., 2014).

In conclusion, it can be stated that research on APPs is not as well-established as cytokine research in porcine inflammation. A current lack of appropriate and reliable detection methods of these APPs in general and CRP in specific is definitely a major hurdle.

3.2.6. Endotoxin tolerance

Endotoxin tolerance is defined as a reduced responsiveness to an LPS challenge following a first encounter. This phenomenon is regarded as a pathophysiological adaptation to protect the host from extensive tissue damage and the manifestation of septic shock following uncontrolled inflammation. In this respect, cells or organisms exposed to LPS concentrations become temporally unable to respond to further LPS challenges (Cavaillon and Adib-Conquy, 2005; Biswas et al., 2009).

TNF-α has been reported to be the best marker for endotoxin tolerance (Cavaillon and Adib-Conquy, 2005). Indeed, TNF-α levels increase both rapidly and dramatically following a first LPS administration. After repetitive LPS administrations, TNF-α still peaked at 1 h p.a., yet decreasing peak plasma levels were observed in pigs (Klosterhalfen et al., 1992; Nakajima et al., 1995). Likewise, the release of TXB_2 and the marked elevation of the pulmonary arterial pressure was greatly reduced (Klosterhalfen et al., 1992). Additionally, also CRP levels in porcine saliva are suggested to be tolerance subjective (Escribano et al., 2014).

4. Immunomodulation in pigs

Immunomodulation is defined as a reorganization of the inflammatory response by modifying or regulating functions of the host's immune system. Immunomodulation differs from immunosuppression and anti-inflammation, as it involves both suppression and stimulation of the immune response (Blecha, 1988; Kanoh and Rubin, 2010).

The immunomodulatory properties of drugs have been studied after either *in vitro* or *in vivo* LPS challenge in different animal species. Notwithstanding *in vivo* LPS inflammation models are extensively applied in porcine research, reports on the possible immunomodulatory properties of drugs in such models in pigs are relatively scarce. The most studied classes of drugs are (N)SAIDs, while the study of antimicrobial drugs, as well as *in vitro* research is very limited in pigs. Conversely, in humans and rodents, such studies are commonly performed and well-established.

Table 2. Immunomodulatory effects of drugs in *in vivo* LPS inflammation models in pigs

Class of drugs	Drug	DRUG DOSE		ROA	TOA (h)	EFFECTS					REFERENCE
		Bolus mg/kg BW	Infusion $mg.kg^{-1}.h^{-1}$			Body temperature	Pulmonary response	Total clinical score	TNF-α	IL-6	
NSAIDs	Acetylsalicylic acid	100 or 35		PO	-2; 2	Decrease	-	-	-	-	Salichs et al., 2012
	Flunixin meglumine	2	1	IV	-0.5	-	Effect	-	-	-	Olson et al., 1985
	Flunixin meglumine	2.2		IM	-24; -0.5	-	-	Decrease	No influence	No influence	Peters et al., 2012
	Flurbiprofen	1		IV	-0.5	-	Effect	-	-	-	Schrauwen et al., 1984
	Indomethacin	5	3	IV	-0.5	-	Effect	-	-	-	Olson et al.,1985
	Indomethacin	5		IP	0	-	-	Decrease	-	-	Johnson and von Borell, 1994
	Ketoprofen	2 or 4		PO	1	Decrease	-	Decrease	-	-	Mustonen et al., 2012a
	Ketoprofen	1.5		PO	-2; 2	Decrease	-	-	-	-	Salichs et al., 2012
	Meloxicam	0.4		IM	-1	-	-	Decrease	-	-	Friton et al., 2006
	Paracetamol	30		PO	-2; 2	Decrease	-	-	-	-	Salichs et al., 2012
SAIDs	Dexamethasone	5	1	IV	-18; -1	-	Effect	-	-	-	Olson et al., 1985
	Dexamethasone	5		IV	-1.33	Decrease	-	-	-	-	Parrott et al., 1997
	Dexamethasone	0.5		IV	-18; 0; 12	-	-	-	Decrease	Decrease	Myers et al., 2003
	Prednisolone	10		IV	-0.42	-	Effect	-	-	-	Schrauwen and Houvenaghel, 1984
	Prednisolone	10		IV	0.25; 1.25; 4.5	No influence	No influence	No influence	-	-	Schrauwen and Houvenaghel,1985
Antimicrobial drugs	Ceftazidime	41.7		IV	-0.08	-	No influence	-	No influence	Decrease	Goscinski et al., 2004
	Tobramycin		17.5 (0.33h)	IV	-0.17	-	No influence	-	No influence	No influence	Goscinski et al., 2004

BW: body weight; ROA: route of administration; TOA: time of administration relative to LPS administration; IV: intravenous; IM: intramuscular; PO: oral administration; IP: intraperitoneal; NSAIDs: non-steroidal anti-inflammatory drugs; SAIDs: steroidal anti-inflammatory drugs; - : not investigated;

Table 2 provides a comprehensive overview of the immunomodulatory properties of drugs on the inflammatory parameters earlier described in *in vivo* porcine LPS inflammation models. Additionally, in this paragraph, a brief impression of similar research in human and rodents is offered.

4.1. Nonsteroidal anti-inflammatory drugs

4.1.1. Mechanism of action

Prostaglandins (PGs), TXs and LTs are all synthesized from cell membrane phospholipids and collectively referred to as eicosanoids (Adams, 2001). Arachidonic acid, which is incorporated into membrane phospholipids, is the main source of eicosanoids. In response to phospholipase (PL) A_2, arachidonic acid is released from the cell membrane after which it is subject to a rapid oxidative catabolism by two separate enzymatic pathways, a COX and a lipoxygenase (LOX) pathway (Adams, 2001) (Figure 5).

Nonsteroidal anti-inflammatory drugs (NSAIDs) possess anti-inflammatory, antipyretic and analgesic properties which are believed to be the result of blocking the PG production by binding to and blocking COX (Vane, 1971; Curry et al., 2005). COX exists in two major isoforms, COX-1 and COX-2. COX-1 is a constitutively expressed, housekeeping enzyme, responsible for the production of PGs and TXs involved in the maintenance of physiological processes, including protection of the gastrointestinal mucosa and platelet aggregation. COX-2, on the other hand, is not constitutively present, but is induced when cells are exposed to bacterial LPS or cytokines and is mainly responsible for the PGs involved in inflammation and pain (Vane, 1971; Hersh et al., 2000; Adams, 2001). Blocking COX-1 would be associated with more side effects and consequently NSAIDs preferentially blocking COX-2 are clinically desirable (Curry et al., 2005). In this respect, the COX-2:COX-1 ratio represents the amount of drug necessary to inhibit the respective COX isoforms (Curry et al., 2005). However, recently, also COX-1 was suggested to be involved in inflammatory processes (Teeling et al., 2010). The *E. coli* LPS-induced fever model has been accepted for the evaluation of the antipyretic effect of nonsteroidal anti-inflammatory drugs (NSAIDs; EMA, 2001).

In contrast to COX, LOX is not as ubiquitous and is predominantly found in the lungs, leukocytes and platelets. LTA_4 is a potent mediator of (local) inflammation, whereas LTB_4 is a potent chemoattractant. NSAIDs are not capable of inhibiting LTs synthesis. Paradoxically, the use of a COX-inhibitor might result in an overpresentation of arachidonic acid to the LOX pathway and following increase in LTs production (Adams, 2011; Curry, 2005) (Figure 5).

4.1.2. Immunomodulatory effects of nonsteroidal anti-inflammatory drugs

4.1.2.1. Effects on clinical symptoms and febrile response

In LPS-challenged mice, indomethacin and ibuprofen significantly influenced behavioural changes, including burrowing, while paracetamol did not. Additionally, indomethacin completely blocked PGE_2 production and both indomethacin and paracetamol reduced changes in body temperature (Teeling et al., 2010).

In LPS-challenged pigs, treatment with indomethacin completely inhibited the anorexia as well as the reduction in activity. Remarkably, when provided alone (without LPS administration), indomethacin increased feed intake at 1 to 2 h (Johnson and von Borell, 1994). Treatment of pigs with meloxicam or flunixin meglumine before LPS challenge significantly decreased the clinical symptoms, including lethargy, skin flushing, vomiting, coughing and labored breathing (Friton et al., 2006; Peters et al., 2012). Different oral doses of ketoprofen p.a. of LPS showed similar effects (Mustonen et al., 2012a). Flurbiprofen pretreatment significantly prolonged the survival time in endotoxemic pigs and completely abolished the production of arachidonic acid metabolites (Schrauwen et al., 1984).

An oral bolus of ketoprofen (Figure 3) administered after LPS administration prominently decreased the rise in rectal body temperature. Ketoprofen drinking water medication started before LPS administration, on the other hand, completely blocked the rise in rectal body temperature (Mustonen et al., 2012a; Salichs et al., 2012). Furthermore, Salichs et al. (2012) reported the superiority of ketoprofen to acetylsalicylic acid and paracetamol in antipyretic activity after oral administration to LPS-challenged pigs.

Treatment with meloxicam before LPS challenge reduced the rectal body temperature (Friton et al., 2006). Treatment of pigs with flunixin meglumine before LPS challenge prevented the increase in PGE_2 production, and effected the body temperature (Peters et al., 2012). In contrast, in pigs experimentally infected with *A. pleuropneumoniae*, flunixin meglumine administration after the challenge showed no antipyretic effect, whereas ketoprofen was again proven to be an effective antipyretic drug (Swinkels et al., 1994).

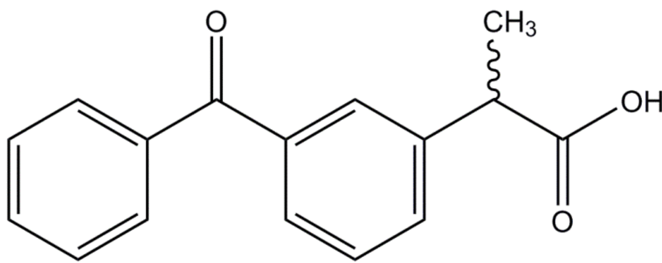


Figure 3. Chemical structure of ketoprofen

4.1.2.2. Effects on pro-inflammatory cytokines and acute phase proteins

In LPS-challenged mice, indomethacin had no inhibiting effect on the serum levels of pro-inflammatory cytokines TNF-α, IL-1β and IL-6 p.a. Surprisingly, even increased levels of TNF-α were observed (Teeling et al., 2010). In this context, it has been reported that NSAIDs stimulate, rather than inhibit, the production of TNF-α and thereby even increase mortality in murine endotoxic shock models (Pettipher and Wimberly, 1994; Ghezzi et al., 1998).

In pigs, treatment with flunixin meglumine had no influence on the production of TNF-α and IL-6 p.a. of LPS (Peters et al., 2012), whereas meloxicam had no effect on the levels of CRP and haptoglobin (Friton et al., 2006).

4.1.2.3. Effects on pulmonary response

In LPS-challenged pigs, ketoprofen, meloxicam and flurbiprofen significantly reduced the concentrations of TXB_2 (Schrauwen et al., 1984; Friton et al., 2006; Mustonen et al., 2012a). Flurbiprofen significantly inhibited the early increase in pulmonary arterial

pressure and pulmonary vascular resistence (Schrauwen et al., 1984). Likewise, flunixin meglumine and indomethacin blocked the initial increase in pulmonary arterial pressure and pulmonary vascular resistance, as well as the decrease in cardiac output in LPS-challenged pigs. However, both drugs did not modify the subsequent increase in pulmonary vascular resistance and decrease in cardiac output (Olson et al., 1985).

4.2. Steroidal anti-inflammatory drugs

4.2.1. Mechanism of action

SAIDs or glucocorticoids, are potent anti-inflammatory, yet immunosuppressive drugs, having a profound suppressive effect on the endogenous glucocorticoid regulation. This class of drugs exerts its main anti-inflammatory effects by the inhibition of PLA_2 after first enhancing the production of lipocortin. Glucocorticoids are used in veterinary medicine to treat inflammatory, immunological and allergic disorders (Ferguson et al., 2009). However, glucocorticoids, and some NSAIDs, are known to exert additional anti-inflammatory effects by modulation of COX-independent signal transduction, involving inhibition of transcription factors such as NF-κB (Tegeder et al., 2001). In this respect, glucocorticoids have been reported to inhibit LPS-induced TNF-α, IL-1β and IL-6 levels both *in vitro* and *in vivo* in different animal species (Morikawa et al., 1996, Bessler et al., 1999; Teeling et al., 2010; Myers et al., 2003).

Since LPS-induced fever is mainly PG-dependent, both NSAIDs and glucocorticoids are expected to have antipyretic properties (Figure 5). PGE_2 is a major inflammatory mediator, involved in the development of fever (Ivanov and Romanovsky, 2004). The *de novo* formation of PGE_2 comprises a three-step cascade reaction catalyzed by PLA_2, liberating arachidonic acid from the membrane; COX, converting arachidonic acid to PGH_2; and PGE synthase, finally isomerizing PGH_2 to PGE_2. Subsequently, PGE_2 is rapidly converted to its major inactive metabolite 13,14-dihydro-15-keto PGE_2, and can be subject to further metabolism to 13,14-dihydro-15-keto PGA_2 and finally bicyclic PGE_2 (Fitzpatrick et al., 1980; Granström et al., 1980; Ivanov and Romanovsky, 2004).

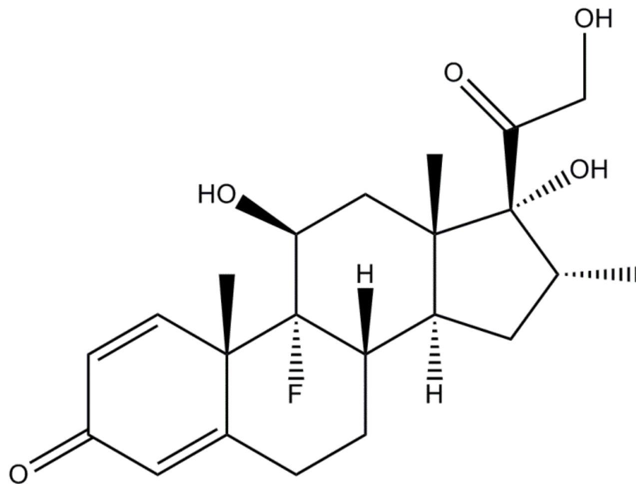


Figure 4. Chemical structure of dexamethasone

4.2.2. Immunomodulatory effects of steroidal anti-inflammatory drugs

4.2.2.1. Effects on clinical symptoms and febrile response

While no effect of dexamethasone was reported on the behavioural changes in LPS-challenged mice, PGE_2 production was significantly reduced and changes in body temperature were completely blocked (Teeling et al., 2010).

In pigs, it was demonstrated that a single dose of prednisolone before LPS administration remarkably increased the survival rate, whereas repeated doses of prednisolone during and after LPS administration had no effect (Schrauwen and Houvenaghel, 1984; 1985). A bolus administration of a high IV dose of dexamethasone prevented LPS-induced fever in pigs. When provided alone (without LPS administration), dexamethasone significantly lowered the body temperature (Parrot et al., 1997).

4.2.2.2. Effects on pro-inflammatory cytokines and acute phase proteins

Dexamethasone suppressed and completely abolished the synthesis of TNF-α, IL-1β and IL-6 in human monocytes primed with LPS and in LPS-challenged mice, respectively (Morikawa et al., 1996; Teeling et al., 2010).

In pigs, repeated IV treatments with dexamethasone before and after LPS challenge significantly decreased TNF-α and IL-6 plasma levels by more than 50 and 90 %,

respectively. Surprisingly, neither LPS nor dexamethasone administration had any effect on IL-1β levels, which were constitutively present in pigs (Myers et al., 2003).

4.2.2.3. Effects on pulmonary response

Dexamethasone showed similar effects compared to NSAIDs, it is therefore strongly indicated that the first phase (0 – 2 h) of the porcine pulmonary response after LPS administration is COX-dependent. In contrast, the second phase was only attenuated by dexamethasone, suggesting the involvement of COX-independent mechanisms, such as lipoxygenase products (Olson et al., 1985).

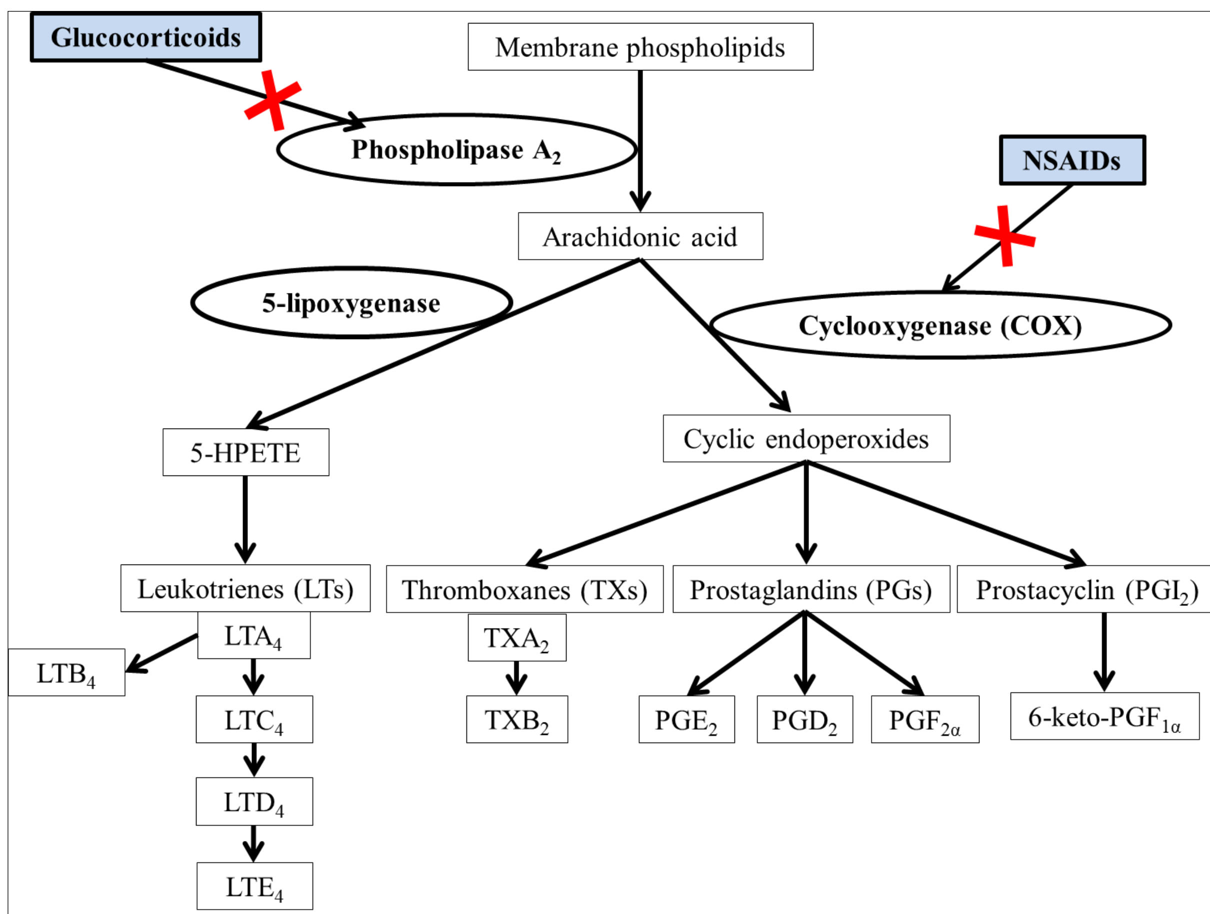


Figure 5. Inhibition of the lipoxygenase- and cyclooxygenase (COX)-catalyzed conversion of arachidonic acid by (non)-steroidal anti-inflammatory drugs ((N)SAIDs)
(Based on Adams, 2001).

4.3. Antimicrobial drugs

4.3.1. Mechanism of action

Besides their antimicrobial activities, which will not be discussed in this thesis, antibiotics also possess possibly clinically important immunomodulatory properties. The effects of (macrolide) antibiotics on inflammation and cytokine release *in vitro*, and to a lesser extent also *in vivo*, have been summarized in detail (Tauber and Nau, 2008). In this respect, mainly 14- and 15-membered, but only few 16-membered macrolides are suggested to exert immunomodulatory properties, varying from the release of cytokines and mediators, neutrophil function, migration, infiltration and accumulation to mucus (hyper)secretion, biofilm formation and bacterial quorum sensing (Čulić et al. 2001; Parnham, 2005; Kanoh and Rubin, 2010).

4.3.2. Immunomodulatory effects of antimicrobial drugs

4.3.2.1. Effects on clinical symptoms and febrile response

The macrolide antibiotics roxithromycin, clarithromycin, erythromycin, azithromycin, tilmicosin and tylosin are all reported to decrease the *in vitro* eicosanoid production in LPS-stimulated murine macrophages and/or peripheral blood mononuclear cells in a concentration-dependent manner (Ianaro et al., 2000; Cao et al., 2006).

Both ciprofloxacin and florfenicol (pre)treatment significantly increased survival rates of LPS-challenged mice by preventing endotoxin-mediated shock and death. In this respect, a crucial role of TNF-α in LPS-induced septic shock has been suggested (Purswani et al., 2002; Zhang et al., 2008).

To date, no reports on the influence of antibiotics on the clinical symptoms and body temperature are available in porcine LPS inflammation models.

4.3.2.2. Effects on pro-inflammatory cytokines and acute phase proteins

In LPS-stimulated human monocytes, fosfomycin inhibited the production of TNF-α and IL-1β, while the production of IL-6 was noticeably enhanced (Morikawa et al., 1996). Quinolones, including ciprofloxacin, pefloxacin and ofloxacin have been reported to inhibit the production of TNF-α, IL-1β and IL-6 in a concentration-dependent manner (Bailly et al., 1990). Morikawa et al. (1996) demonstrated a clear concentration-dependent suppression of the production of TNF-α and IL-1β by clarithromycin, while the suppressive effect on the production of IL-6 was very weak.

In LPS-stimulated murine mononuclear cells, the inhibition of TNF-α, IL-1β and IL-6 production by diverse macrolide antibiotics, including roxithromycin, clarithromycin, erythromycin, azithromycin, tilmicosin and tylosin was reported (Ianaro et al., 2000; Cao et al., 2006). Telithromycin significantly inhibited the production of TNF-α by both LPS-induced murine macrophages and mice (Sugiyama et al., 2007; Leiva et al., 2008). In LPS-stimulated murine dendritic cells, on the other hand, clarithromycin significantly inhibited the production of IL-6, while there was completely no effect on the production of TNF-α observed. Additionally, azythromycin had no effect on the production of TNF-α and IL-6 (Sugiyama et al., 2007). In this respect, also 16-membered macrolide antibiotics, including tylosin and tilmicosin, are proven to decrease the production of TNF-α, IL-1β, IL-6 and PGE_2 in humans and rodents.

Treatment with florfenicol, a broad-spectrum antibiotic and analogue to chloramphenicol family, significantly decreased levels of TNF-α and IL-6 in cell supernatant of LPS-induced murine macrophages. Accordingly, in LPS-challenged mice, similar results were observed. The effect of florfenicol on IL-1β release was negligible both *in vitro* and *in vivo* (Zhang et al., 2008). Ciprofloxacin significantly decreased production of TNF-α in LPS-challenged mice, while only a little or no effect was observed for IL-1β and IL-6 (Purswani et al. 2002).

Finally, in pigs treated with the β-lactam antibiotic ceftazidime, significantly decreased levels of IL-6 were detected at 6 h after the onset of LPS infusion. However, neither ceftazidime nor the aminoglycoside antibiotic tobramycin influenced the levels of IL-1β (Goscinski et al., 2004).

4.3.2.3. Effects on pulmonary response

Neither ceftazidime nor tobramycin affected the pulmonary response in LPS-challenged pigs (Goscinski et al., 2004).

To date, no other *in vivo* reports on the influence of antibiotics on the pulmonary response are available in porcine LPS inflammation models.

In conclusion, LPS inflammation models have been widely applied and demonstrated to be a valuable tool for immune challenge in porcine research. Pigs are very sensitive to the administration of LPS, yet even small doses (≤ 25 µg/kg BW) provoke a profound inflammatory response without causing mortality. After LPS challenge, peak concentrations of TNF-α and IL-6 are expected to occur around 1 h and 2.5 h p.a., respectively. Reports on porcine IL-1β are rather scarce and contradictorily. While maximal concentrations of PGE_2 are already reached within around 1 h p.a., the febrile response peaks no earlier than at 4 h p.a. in pigs. The APP, including CRP, SAA, Hp and pig-MAP generally increase after LPS challenge, yet a lack of appropriate and reliable detection methods can partially explain the discrepancies between different studies.

LPS inflammation models can be used to study possible immunomodulatory capacities of drugs. Single or multiple doses of drugs can be administered before and/or after LPS administration. Ketoprofen was shown to be an effective antipyretic and anti-inflammatory drug when administered both before and after LPS challenge. Numerous macrolide antibiotics, mainly 14 and 15-membered, including erythromycin, clarithromycin and azithromycin; and to a lesser extent also 16-membered, including tylosin and tilmicosin, are proven to decrease the production of TNF-α, IL-1β, IL-6 and PGE_2 in humans and rodents. Murine research has indeed confirmed the suitability of LPS inflammation models to investigate the influence of different (N)SAIDs and even antimicrobial drugs on the clinical changes, production of pro-inflammatory cytokines, APP and the course of the febrile response.

Unfortunately, to date, *in vitro* and *in vivo* reports on the influence of antimicrobial drugs on the APR are largely lacking in pigs, although their well-established use in infectious diseases. Also the effects of a combination therapy of antimicrobial drugs and (N)SAIDs on the APR are poorly studied in pigs. Moreover, the suitability of IL-1β to study immunomodulation of drugs in *in vivo* porcine LPS inflammation models is highly controversial.

Modulation of the host immune response is of growing interest in human and veterinary medicine. To study immunomodulation in different animal species, lipopolysaccharide (LPS) - as a major part of the Gram-negative bacterial outer membrane - has been recurrently applied. While the LPS-induced fever model has been accepted for the evaluation of the antipyretic effect of nonsteroidal anti-inflammatory drugs (NSAIDs), it also offers opportunities for the evaluation of these and other classes of drugs, such as antimicrobials, on pro-inflammatory mediators from the acute phase response (APR).

Recently, the combined use of the antibiotic doxycycline and the NSAID ketoprofen has been proven beneficial in the treatment of swine respiratory disease (SRD). Gamithromycin (GAM) is a 2nd generation macrolide antibiotic which is currently only registered for the treatment and prevention of bovine RD. Similar to cattle, SRD associated with Gram-negative bacteria including *Actinobacillus pleuropneumoniae*, *Pasteurella multocida* and *Haemophilus parasuis* is responsible for considerable economic losses and reduced animal welfare.

Therefore, the **General Aim** of this thesis was to investigate the immunomodulation by GAM, KETO and their combination in appropriate *in vitro* and *in vivo* porcine LPS inflammation models. It is hypothesized that GAM exerts immunomodulatory activities and that its combination with KETO would be beneficial compared to the exclusive administration of each drug with respect to the pigs' clinical condition. Dexamethasone (DEX), a glucocorticoid and well-known cytokine inhibitor, was included as a positive control in this research.

The specific aims of this thesis were:

1) To determine the pharmacokinetic properties of GAM and DEX in pigs.
2) To evaluate the pharmacodynamic effects of GAM, KETO, DEX and their two-drug combinations in appropriate *in vitro* and *in vivo* porcine LPS inflammation models.
3) To develop and validate a cytometric bead array (CBA) screening tool for the simultaneous measurement of porcine pro-inflammatory cytokines in serum and plasma.

EXPERIMENTAL STUDIES

CHAPTER 1.

Pharmacokinetics of gamithromycin and dexamethasone

1.1. Pharmacokinetics of gamithromycin after intravenous and subcutaneous administration in pigs

Adapted from

Wyns, H., Meyer, E., Plessers, E., Watteyn, A., De Baere, S., De Backer, P., Croubels, S. (2014) Pharmacokinetics of gamithromycin after intravenous and subcutaneous administration in pigs. *Research in Veterinary Science* 96, 160-163.

Abstract

Gamithromycin (GAM) is a 15-membered semi-synthetic macrolide antibiotic of the azalide subclass which has been recently developed for the treatment and prevention of bovine respiratory disease (BRD). In this study, the pharmacokinetic properties and absolute bioavailability of GAM were investigated after an intravenous (IV) or subcutaneous (SC) bolus injection of 6 mg/kg body weight in six male pigs according to a single dose parallel design. The plasma concentrations of gamithromycin were determined using a validated high-performance liquid chromatography-tandem mass spectrometry (LC-MS/MS) method, and the pharmacokinetics were analysed by noncompartmental analysis.

Following IV administration, the mean area under the plasma concentration-time curve extrapolated to infinity (AUC_{inf}) was 3.67 ± 0.75 µg.h/mL. The mean elimination half-life ($t_{1/2\lambda z}$) and plasma clearance (Cl) were 16.03 h and 1.69 ± 0.33 L/h.kg, respectively. The volume of distribution at steady state (V_{ss}) was 31.03 L/kg.

Following SC administration, the mean AUC_{inf} was 4.31 ± 1.14 µg.h/mL. A mean maximal plasma concentration (C_{max}) of 0.41 ± 0.090 µg/mL was reached at 0.63 ± 0.21 h. The mean $t_{1/2\lambda z}$ and Cl were 18.76 h and 1.47 ± 0.40 L/h.kg, respectively.

In conclusion, GAM administered subcutaneously to pigs demonstrated a rapid and complete absorption, with a high absolute bioavailability of 117.6 ± 39.4 %. None of the pharmacokinetic properties significantly differed between both administration routes.

1. Introduction

Macrolide antibiotics are a group of antimicrobials characterized by a similar chemical structure and mechanism of action, but a different spectrum of activity (Jain & Danziger, 2004). The drugs are classified as macrocyclic lactone rings containing 12 to 20 carbon atoms with diverse combinations of deoxy sugars attached to this ring by glycosidic linkages. Erythromycin was the first macrolide isolated from *Streptomyces erythreus*. Numerous other macrolides have been isolated or synthesized from this parent molecule. The antibacterial action comprises the inhibition of protein synthesis by binding to the 50S ribosomal subunit of prokaryotic organisms and consequently inhibiting the translocation. Macrolides in general do not bind to mammalian ribosomes, which makes them a relatively safe group of drugs for veterinary use. Furthermore, erythromycin has been confirmed to stimulate gastrointestinal motility (Papich & Riviere, 2009).

Macrolide antibiotics are mainly active against Gram-positive bacteria. In addition to the anti-infectious properties, these drugs have been reported to influence a variety of inflammatory processes, including the release of cytokines and mediators, the migration of neutrophils and the oxidative burst in phagocytes. A significant decrease of the production of the pro-inflammatory cytokines TNF-α, IL-1β and IL-6 was reported after *in vitro* lipopolysaccharide (LPS) stimulation and treatment with tilmicosin and tylosin. Moreover, macrolides are also characterised by an extensive accumulation into leukocytes and lung tissues, achieving much higher tissue concentrations compared to those observed in plasma (Nightingale, 1997; Ianaro et al., 2000; Cao et al., 2006; Tauber and Nau, 2008; Buret, 2010).

Via introduction of a nitrogen atom into the macrolactone ring, a novel class of macrolide antibiotics, the so-called azalides, was generated. Azythromycin was the first azalide which has been associated with remarkable pharmacokinetics, such as a high tissue distribution, metabolic stability and a high tolerability compared to other macrolide antibiotics (Mutak, 2007).

Gamithromycin (GAM; Figure 1) is a 15-membered semi-synthetic macrolide antibiotic of the azalide subclass with a uniquely positioned alkylated nitrogen at the 7a-position of the lactone ring. GAM has been recently developed for the treatment and

prevention of bovine respiratory disease (BRD) associated with *Mannheimia haemolytica, Pasteurella (P.) multocida* and *Histophilus somni* (Huang et al., 2010). Bacteria, including *Actinobacillus (A.) pleuropneumoniae*, *P. multocida*, *Haemophilus (H.) parasuis* and *Mycoplasma (M.) hyopneumoniae* are major pathogens involved in swine respiratory disease (SRD). In growing pigs, respiratory infections are responsible for severe economic losses and reduced animal welfare. Other second generation macrolide antibiotics, such as tulathromycin (Draxxin®) and tildipirosin (Zuprevo®) have been approved for treatment of SRD in pigs (EMA, 2008a, 2011; Rose et al., 2013).

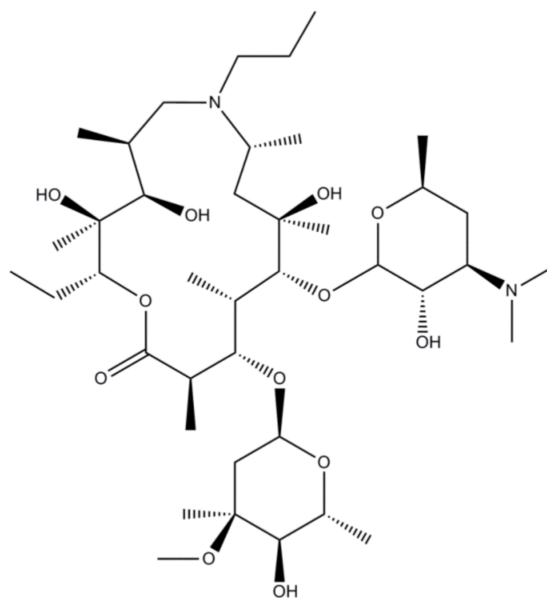


Figure 1. Chemical structure of gamithromycin (GAM)

The pharmacokinetic (PK) properties of GAM have been determined in cattle, foals and broiler chickens (Huang et al., 2010; Berghaus et al., 2011; Watteyn et al., 2013). The aim of this study was to determine the PK properties of GAM in pigs, whereafter the characteristics of the antibiotic can be used in future research to investigate its immunomodulatory properties in a porcine lipopolysaccharide (LPS) inflammation model (Wyns et al., 2013).

2. Materials and methods

2.1. Experimental design

Twelve clinically healthy male pigs with a mean body weight (BW) of 24.81 ± 1.65 kg were randomly divided in two groups. The animals had free access to feed and drinking water. The study was conducted according to a single dose parallel design. The pigs received a bolus injection of 6 mg/kg BW GAM (Zactran®, Merial), either intravenously (IV) in the ear vein (n = 6) or subcutaneously (SC) in the flank region (n = 6). Blood was collected from the jugular vein into ethylenediaminetetraacetic acid (EDTA)-coated tubes (Vacutest Kima) before administration (time 0 h); at 0.25, 0.50, 0.75, 1, 2, 4, 6, 8, 10, 12 h post administration (p.a.) and once daily from day 2 to day 14 p.a. (24 h intervals). Blood samples were centrifuged at 2800 *x g* for 10 min at 4 °C and plasma was stored at ≤ -15 °C until analysis.

The animal experiment was approved by the Ethical Committee of the Faculty of Veterinary Medicine of Ghent University (EC2011/159).

2.2. Veterinary drug, chemicals and solutions

Prior to the administration, the veterinary drug formulation Zactran® of 150 mg/mL (15.0 % w/v) was diluted with *aqua ad injectabilia* (Kela Pharma) to a concentration of 50 mg/mL (5.0 % w/v) in order to avoid possible adverse effects caused by parenteral administration.

High-performance liquid chromatography (HPLC) grade water, methanol (MeOH), acetonitrile (ACN) and analytical grade formic acid were purchased from VWR. Ultra-performance liquid chromatography (UPLC) grade water was purchased from Biosolve. HybridSPE®-Phospholipid cartridges (30 mg/mL) were obtained from Sigma-Aldrich.

Analytical standards of GAM and deuterated GAM (d_5-GAM) were kindly donated by Merial Ltd and stored at 2 – 8 °C. Stock solutions of GAM and d_5-GAM (1 mg/mL) were prepared in HPLC MeOH and stored at ≤ -15 °C for at least one month. Working solutions of 0.010, 0.025, 0.050, 0.10, 0.25, 0.50, 1.0, 2.5, 5.0, 10.0, 25.0, 50.0 and 100 µg/mL of GAM were prepared in HPLC water. For the internal standard (IS, d_5-GAM), a working solution of

1.0 µg/mL was prepared in HPLC water. The working solutions were stored at 2 – 8 °C for at least one month.

2.3. Analysis of gamithromycin

Quantification of GAM in porcine plasma was performed using a validated liquid chromatography-tandem mass spectrometry (LC-MS/MS) method. Prior to LC-MS/MS analysis plasma samples were subjected to a sample preparation by protein precipitation and subsequent phospholipid removal. More specifically, 25 µL of the IS working solution was added to 250 µL of porcine plasma. After a vortex mixing (15 s), 750 µL of 1 % formic acid in ACN was added, followed by a vortex mixing (15 s) and centrifugation step (10 min, 7800 *x g*). The supernatant was transferred to a HybridSPE®-Phospholipid cartridge. The sample was collected into a glass tube and vacuum was applied to remove the remaining liquid drops from the column. Next, 50 µL of the filtrate was transferred to an autosampler vial and 1/2 (v/v) diluted with UPLC water. After vortex mixing, a 2.5 µL aliquot was injected onto the LC-MS/MS instrument.

The LC system consisted of a quaternary, low-pressure mixing pump with vacuum degassing, type Surveyor MSpump Plus and an autosampler with temperature-controlled tray and column oven, type Autosampler Plus, from ThermoFisher Scientific. The temperatures of the column oven and autosampler tray were set at 50 °C and 5 °C, respectively. Chromatographic separation was achieved on a Hypersil Gold (50 mm x 2.1 mm internal diameter (i.d.), particle size (dp): 1.9 µm) column in combination with a pre-column of the same type (10 mm x 2.1 mm i.d., dp: 3 µm), both from ThermoFisher Scientific.

The LC column effluent was interfaced to a TSQ® Quantum Ultra triple quadrupole mass spectrometer, equipped with a heated electrospray ionisation probe operating in the positive ionisation mode (all from ThermoFisher Scientific).

MS Acquisition was performed in the selected reaction monitoring (SRM) mode. For GAM, the two most intense product ions were monitored (i.e. *m/z* 777.45 > 619.35 for quantification and *m/z* 777.45 > 157.80 for identification, respectively). For the IS, only one SRM transition was monitored (*m/z* 782.45 > 624.35).

The method was validated and the following parameters were successfully evaluated: linearity 2.5 - 10.000 ng/mL, within- and between-run accuracy and precision (concentration level 25, 250 and 2500 ng/mL), limit of quantification (LOQ, 5 ng/mL, *n* = 6) and limit of detection (LOD, 0.01 ng/mL, *n* = 6), specificity and carry-over.

2.4. Pharmacokinetic and statistical analysis

The PK properties were determined by means of WinNonlin®, version 6.2.0 software program (Pharsight Corporation) using a noncompartmental model. Values below the LOQ of 5 ng/mL were not included in the analysis. The mean area under the plasma concentration-time curve was calculated using the linear trapezoidal method from time 0 to the last time point with a quantifiable concentration (AUC_{last}). The AUC extrapolated to infinity (AUC_{inf}), the elimination rate constant (λ_z), volume of distribution (V_z), volume of distribution at steady state (V_{ss}), plasma clearance (Cl), maximum plasma concentration (C_{max}), and time to maximum plasma concentration (t_{max}) were also determined. The terminal plasma half-life ($t_{1/2\lambda z}$) was expressed using harmonic mean values. The absolute bioavailability (F) was calculated from the following equation:

$$F(\%) = \frac{AUC_{0\to\infty SC}}{AUC_{0\to\infty IV}} \text{ x } 100$$

The data are presented as mean ± standard deviation (SD) and were statistically analysed by means of single-factor analysis of variance (ANOVA), using SPSS Version 20.0 software for Windows. The level of significance was α = 0.05.

3. Results

No adverse effects following IV or SC administration were observed during the course of the study. The PK properties of GAM (mean ± SD) based on noncompartmental analysis are summarized in Table 1. The mean plasma concentration-time curves following IV and SC administration are plotted on semi-logaritmic graphs in Figure 2.

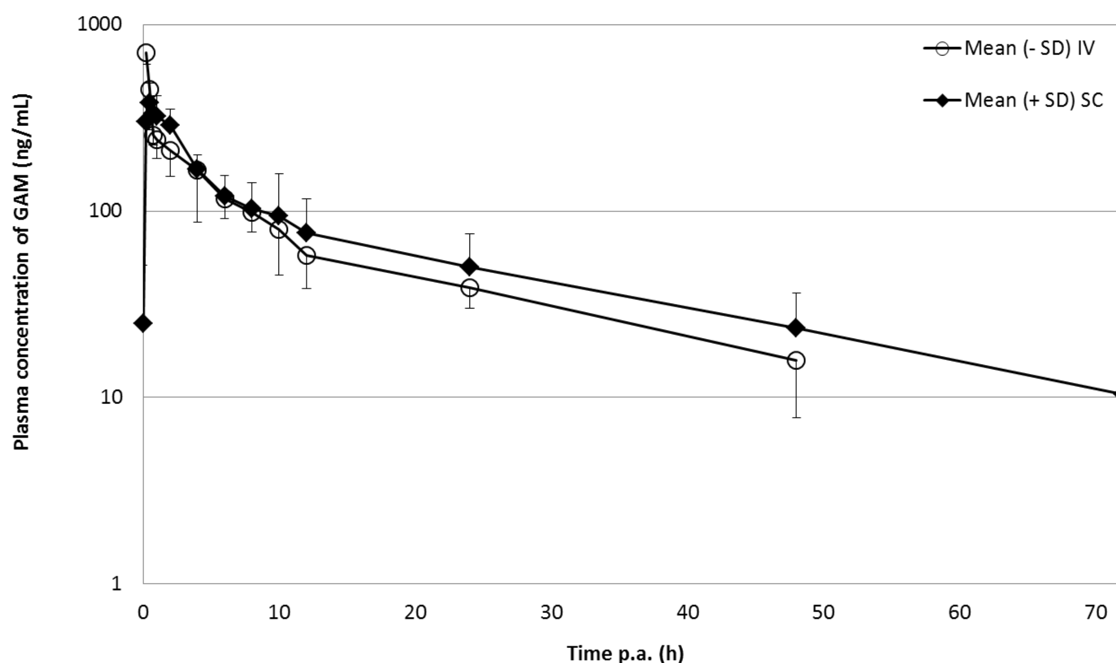


Figure 2. Mean (± SD) plasma concentration-time profiles of gamithromycin (GAM) after IV (*n* = 6) and SC (*n* = 6) bolus administration of 6 mg/kg BW in pigs.

Quantifiable concentrations of GAM in plasma were present for 48 h and 72 h after IV and SC administration, respectively.

Following an IV bolus injection of 6 mg/kg BW GAM, the AUC_{inf} was 3.67 ± 0.75 µg.h/mL. The $t_{1/2\lambda z}$ was 16.03 h. The V_z and Cl were 40.47 ± 8.73 L/kg and 1.69 ± 0.33 L/h.kg, respectively. The V_{ss} was 31.03 ± 6.68 L/kg.

Following a SC bolus injection of 6 mg/kg BW gamithromycin, the AUC_{inf} was 4.31 ± 1.14 µg.h/mL. A C_{max} of 0.41 ± 0.090 µg/mL was reached at 0.63 ± 0.21 h (t_{max}). The $t_{1/2\lambda z}$ was 18.76 h. The V_z and Cl were 42.70 ± 16.16 L/kg and 1.47 ± 0.40 L/h.kg, respectively. The absolute bioavailability of GAM was 117.6 ± 39.4 %.

None of the PK properties significantly differed between both administration routes.

Table 1. Pharmacokinetic properties of GAM in pigs after IV (*n* = 6) and SC (*n* = 6) bolus administration of 6 mg/kg BW (mean ± SD)

Parameter	Unit	IV	SC
AUC_{last}	µg.h/mL	3.24 ± 0.56	3.48 ±1.90
AUC_{inf}	µg.h/mL	3.67 ± 0.75	4.31 ± 1.14
λz	/h	0.043 ± 0.011	0.037 ± 0.009
$t_{1/2\lambda z}$	h	16.03^{A}	18.76^{A}
MRT_{inf}	h	18.84 ± 4.94	24.41 ± 9.17
V_{ss}	L/kg	31.03 ± 6.68	-
Cl	L/h.kg	1.69 ± 0.33	1.47 ± 0.40
t_{max}	h	-	0.63 ± 0.21
C_{max}	µg/mL	-	0.41 ± 0.090
F	%	-	117.6 ± 39.4

AUC_{last}, area under the plasma concentration-time curve from time 0 to the last time point with a quantifiable concentration; $AUC_{inf,}$ AUC extrapolated to infinity; λz, elimination rate constant; $t_{1/2\lambda z}$, half-life of elimination; V_z, volume of distribution; V_{ss}, volume of distribution at steady state; Cl, clearance; t_{max}, time to maximum plasma concentration; C_{max}, maximum plasma concentration; F, absolute bioavailability

^{A}: harmonic mean

4. Discussion

GAM is a novel semi-synthetic azalide which has been approved for the treatment of BRD caused by *M. haemolytica*, *P. multocida* and *H. somni* in cattle. Following a SC bolus injection of 6 mg/kg BW to cattle, GAM was well absorbed, reaching a mean C_{max} at 1 h p.a. A corresponding absolute bioavailability of 110 % was assessed. Furthermore, the antibiotic was extensively and rapidly distributed to lung tissue which was demonstrated by the high V_{ss} of 24.9 L/kg (Huang et al., 2010). Similarly, in foals, a t_{max} of 1 h was observed after an intramuscular (IM) injection of 6 mg/kg BW GAM, after which the drug largely distributed to pulmonary epithelial lining fluid, brochoalveolar lavage cells and neutrophils (Berghaus et al., 2011).

In the current study in pigs, a SC bolus injection of 6 mg/kg BW GAM resulted in a relatively fast absorption reaching a C_{max} at 0.63 h. The absolute bioavailability was 118 %. Compared to cattle, a higher clearance of GAM was observed in pigs (1.69 and 0.71 L/h.kg in pigs and cattle, respectively), resulting in a shorter $t_{1/2\lambda z}$ after IV as well as SC administration (16.03 and 18.76 h, respectively in pigs and 44.9 and 50.8 h, respectively in cattle). Notwithstanding a generally more pronounced metabolic rate in birds, similar values for clearance and $t_{1/2\lambda z}$ (1.61 L/h.kg and 14.12 h, respectively) were recently observed in broiler chickens. In addition, the V_{ss} was high in both animal species (31.03 L/kg

in pigs and 29.16 L/kg in broiler chickens, respectively), indicating a distinct tissue penetration and corresponding intracellular levels (Watteyn et al., 2013).

Macrolide antibiotics are lipophilic molecules which are well absorbed and extensively distributed in body fluids and tissues (Zuckerman et al., 2011). Conversely, binding to plasma proteins can considerably restrict this extravascular distribution (Huang et al., 2010). In pigs, the mean plasma protein binding of GAM was only 23 % (EMA, 2008b), which contributed to the large V_{ss} of 31.03 L/kg.

In comparison with other macrolide antibiotics, GAM showed a relatively short $t_{1/2\lambda z}$ after IV and SC injection in pigs. The clearance of GAM was considerably higher than that of tulathromycin (0.58 L/h.kg, Benchaoui et al., 2004 and 0.18 L/h.kg, Wang et al., 2012). In this respect, tulathromycin, approved for treatment of bacterial porcine as well as bovine respiratory disease, showed a $t_{1/2\lambda z}$ of 67.5-76.5, 75.6 and 78.7 h after an IV, IM and oral bolus of 2.5 mg/kg BW, respectively (Benchaoui et al., 2004; Wang et al., 2012). Likewise, tildipirosin, a semi-synthetic tylosin analogue also approved for swine respiratory disease, revealed a very slow elimination with a $t_{1/2\lambda z}$ of 97 h following a single IM injection of 6 mg/kg BW (Rose et al., 2013). On the other hand, tylosin, a first generation macrolide antibiotic mainly used to treat pneumonia and dysentery in pigs, has a $t_{1/2\lambda z}$ of 4.52 and 24.5 h after IV and IM administration of 10 mg/kg BW, respectively. GAM has a comparable total body clearance as tylosin (1.88 L/h.kg, Prats et al., 2002). While the short $t_{1/2\lambda z}$ after IV administration can be attributed to the smaller V_{ss} (9.7 L/kg), the extended $t_{1/2\lambda z}$ after IM administration is a reflection of a continuous, but slow release from the injection site (Prats et al., 2002). Tilmicosin is a macrolide antibiotic synthesized from tylosin which has been recommended for the treatment and prevention of bacterial pneumonia in pigs as a feed formulation. It has a $t_{1/2\lambda z}$ of 25.3 and 20.7 h after oral bolus administration of 20 mg/kg and 40 mg/kg, respectively (Shen et al., 2005).

GAM shows a very high absolute bioavailability of 118 % after SC administration in pigs. Similarly, tylosin has a bioavailability of around 95 % after an IM injection (Prats et al., 2002). The bioavailability of tulathromycin after IM administration was > 87 %, whereas the oral bioavailability was only 51 % (Benchaoui et al., 2004; Wang et al., 2012). Since parenteral administration of tilmicosin in pigs is associated with considerable adverse cardiovascular effects, the bioavailability has not yet been determined (Shen et al., 2005).

For GAM, minimum inhibitory concentration (MIC) values of 0.25 and 2 µg/mL were established in pigs for *M. hyopneumoniae* (J-strain) and *A. pleuropneumoniae* (ATCC 27090 reference strain), respectively (unpublished data). Of each pathogen, only one isolate was included. Therefore, these values can only be regarded as a first indication of the susceptibility. For tulathromycin, a MIC_{90} of 0.06 and 16 µg/mL was described for *M. hyopneumoniae* and *A. pleuropneumoniae*, respectively, in pigs (Benchaoui et al., 2004; Godinho, 2008). For tildipirosin, on the other hand, a MIC_{90} value of 8 µg/mL was reported for *A. pleuropneumoniae* (EMA, 2011), yet recent research of Rose et al. (2013) indicated that a MIC_{90} value of 2 µg/mL would be more plausible.

In general, time above the MIC (t > MIC) is the PK/PD index considered to be best correlated with clinical efficacy of conventional macrolides. However, for newer advanced generation macrolide antibiotics such as azithromycin, the plasma AUC/MIC ratio would also be an appropriate index (Nightingale, 1997; Van Bambeke and Tulkens, 2001). Taking the MIC of 0.25 µg/mL for *M. hyopneumoniae* into account, it can be suggested that plasma levels are indeed higher for a certain period of time. In contrast, but in accordance with other macrolide antibiotics, plasma concentrations of GAM never exceed the MIC value of 2 µg/mL of *A. pleuropneumoniae* in this study.

Nevertheless, it is generally accepted that plasma PK and plasma concentrations of macrolides are poor predictors of the antimicrobial activity and clinical efficacy of these drugs. Tissue concentrations at the site of infection would rather be more significant to comprehend the PD properties for this class of antibiotics (Nightingale, 1997). In this respect, it can be stated that for tulathromycin, concentrations were 24.9 – 181 times higher in the lung than those measured in plasma of pigs (Benchaoui et al., 2004). In addition, it was observed that bovine lung concentrations of GAM were 247 – 410 times higher than in plasma over the time course from 1 to 15 days after treatment (Huang et al., 2010).

In accordance with other macrolide antibiotics, the V_{ss} of GAM is also very high and consequently much higher concentrations can be expected in the pig's lung compared to those in plasma.

In conclusion, GAM administered subcutaneously to pigs demonstrated a fast and complete absorption. However, plasma pharmacokinetics of macrolides are poor predictors of antimicrobial activity and clinical efficacy of these drugs. Therefore, tissue

concentrations at the site of infection would be more significant to comprehend the pharmacodynamic properties (Nightingale, 1997). The high V_{ss} indicates a distinct tissue penetration. Although no lung concentrations are available, we still would like to suggest that, in analogy to other second generation macrolides, GAM might be advantageous for the treatment of bacterial respiratory diseases in pigs.

Acknowledgements

The authors would like to thank G. Antonissen, M. Devreese, J. Goossens, J. Lambrecht, V. Vandenbroucke, A. Van den Bussche for the technical assistance during the animal experiment. Special thanks to Merial for providing the analytical standards of GAM and d5-GAM.

1.2. Pharmacokinetics of dexamethasone after intravenous and intramuscular administration in pigs

Adapted from

Wyns, H., Meyer, E., Watteyn, A., Plessers, E., De Baere, S., De Backer, P., Croubels, S. (2013) Pharmacokinetics of dexamethasone after intravenous and intramuscular administration in pigs. *The Veterinary Journal* 198, 286-288.

Abstract

The pharmacokinetics of dexamethasone (DEX) were investigated after an intravenous (IV) or intramuscular (IM) bolus injection of 0.3 mg/kg body weight DEX sodium phosphate in pigs. The plasma concentrations of DEX were determined using a validated high-performance liquid chromatography-tandem mass spectrometry (LC-MS/MS) method, and the pharmacokinetics were determined by one-compartmental analysis.

The mean area under the plasma concentration-time curve and the mean elimination half-life were 133.07 ± 39.59 ng.h/mL and 0.77 h, and 173.24 ± 53.59 ng.h/mL and 1.06 h following IV and IM administration, respectively. The volume of distribution and clearance recorded after IV administration were 2.78 ± 0.88 L/kg and 2.39 ± 0.57 L/h.kg, respectively.

An IM bolus injection of DEX sodium phosphate in pigs resulted in a fast and complete absorption, with a mean maximal plasma concentration of 80.94 ± 21.29 ng/mL at 0.35 ± 0.21 h and a high absolute bioavailability of 131.06 ± 26.05 %.

1. Introduction

Dexamethasone (DEX; Figure 1A) is a long-acting synthetic hydrocortisone analogue and is one of the most potent glucocorticoid drugs. It is extensively used in veterinary medicine to treat inflammatory, immunological and allergic disorders (Toutain et al., 1982; EMA, 2004; Ferguson et al., 2009). In livestock, the use of DEX is indicated in inflammation processes such as mastitis, aseptic laminitis, arthritis and primary ketosis. In contrast, DEX is also reported to be used as an illegal growth promoter (Courtheyn et al., 2002; Ferguson et al., 2009; Vincenti et al., 2009).

In order to improve water solubility, glucocorticoids are often formulated as a phosphate ester prodrug (Figure 1B), which rapidly hydrolyses and releases free and active alcohols (Rohdewald et al., 1987; EMA, 2004). The pharmacokinetic (PK) properties of DEX have been previously described in humans and diverse animal species, but not in pigs. Nonetheless, DEX has been approved for treatment of inflammatory and allergic disorders in this species (EMA, 2004).

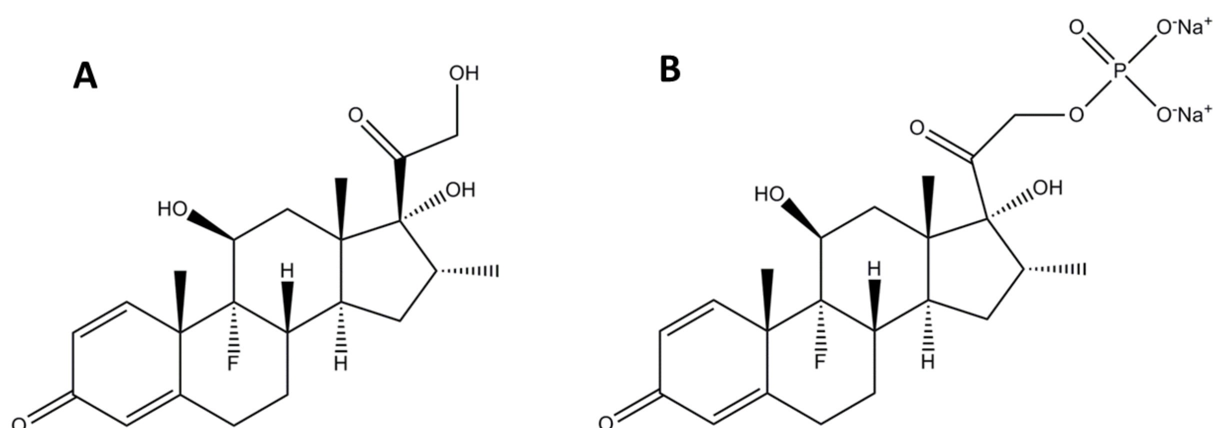


Figure 1. Chemical structures of dexamethasone (DEX; A) and its ester, DEX-sodium phosphate (B)

Therefore, the aim of this study was to determine the PK properties of DEX in pigs. These characteristics will be applied in ongoing research to study the immunomodulatory properties of DEX, either alone or in combination with several antibiotics in a porcine lipopolysaccharide (LPS) inflammation model (Wyns et al., 2013).

2. Materials and methods

2.1. Experimental design

Six clinically healthy male pigs (Landrace) with a mean body weight (BW) of 28.3 ± 3.01 kg were randomly divided in two groups. The animals had free access to feed and drinking water.

The experiment was performed as a two-way cross-over design with a wash-out period of 5 days. A bolus of 0.3 mg/kg BW DEX sodium phosphate (DEXA 0.2%®, Kela Laboratoria) was injected intravenously (IV) in the ear vein or intramuscularly (IM) in the *gluteus* muscle via a sterile winged infusion needle (25G; Micro-flo^{TM}, Novolab). Blood was collected by venipuncture of the jugular vein into EDTA-coated tubes (Vacutest Kima) before administration (time 0 h) and at 0.08, 0.25, 0.5, 0.75, 1, 2, 4, 6, 8, 10, 12 and 24 h post-administration (p.a.). Blood samples were centrifuged at 2800 *x g* for 10 min at 4 °C and plasma was stored at ≤ -15 °C until analysis within 2 months.

The animal experiment was approved by the Ethical Committee of the Faculty of Veterinary Medicine of Ghent University (EC 2011/156).

2.2. Chemicals and solutions

High-performance liquid chromatography (HPLC) grade water, HPLC methanol (MEOH) and pro-analysis grade glacial acetic acid and diethylether were purchased from VWR. Trichloroacetic (TCA) was obtained from Sigma-Aldrich. A 20 % solution of TCA was prepared in HPLC water. Ultra-performance liquid chromatography (UPLC) grade water and acetonitrile (ACN) were obtained from Biosolve.

The analytical standards of DEX and desoximetasone (internal standard, IS) were obtained from Sigma-Aldrich. Stock solutions of 1 mg/mL of DEX and IS were prepared in HPLC methanol and stored at 2 – 8 °C. These stock solutions were found to be stable for at least three months. Working solutions of 100 and 1 µg/mL (DEX) and 10 µg/mL (IS) were obtained by diluting the stock solutions with HPLC water and were stored at 2 – 8 °C for at least one month.

2.3. Analysis of dexamethasone

Quantification of DEX in porcine plasma was performed using a validated liquid chromatography-tandem mass spectrometry (LC-MS/MS) method.

Prior to LC-MS/MS analysis, the plasma sample was subjected to protein precipitation. More specifically, sample preparation was performed by adding 25 µL of the IS working solution to 250 µL of porcine plasma. After a vortex mixing (15 s), 25 µL of a 20 % TCA solution were added, followed by a vortex mixing (15 s) and a centrifugation step (10 min, 7800 *x g*). The supernatant was transferred to a screw-capped extraction tube (15 mL, polyethylene, SPL Life Science, Novolab) and 3 mL diethylether were added. A liquid–liquid extraction was performed by gentle rolling (20 min). After centrifugation (10 min, 1500 *x g*), the upper ether layer was transferred into a new tube and evaporated to dryness using a nitrogen stream (35 °C). The residue was re-dissolved into 200 µL of UPLC water by vortex mixing (15 s) and transferred to an autosampler vial. A 10 µL aliquot was injected onto the LC-MS/MS system.

The LC system consisted of a quaternary, low-pressure mixing pump with vacuum degassing, type Surveyor MSpump Plus and an autosampler with temperature controlled tray and column oven, type Autosampler Plus, from Thermo Scientific. Chromatographic separation was achieved on a Hypersil Gold (internal diameter, 50 x 2.1 mm; particle size, dp, 1.9 µm) column in combination with a Hypersil Gold Drop-in guard cartridge (internal diameter, 10 x 2.1 mm), both from Thermo Scientific. Two mobile phases were used, the mobile phase A consisted of 0.01 % acetic acid in UPLC grade water, while mobile phase B was UPLC ACN. A gradient elution was performed (0-1 min: 60 % A, 1-2 min: linear gradient to 30 % A, 2-3 min: 30 % A, 3-3.2 min: linear gradient to 60 % A, 3.2-6 min: 60 % A) at a flow-rate of 300 µL/min. The temperatures of the column oven and autosampler tray were set at 45 °C and 5 °C, respectively.

The LC column effluent was interfaced to a TSQ Quantum Ultra triple quadrupole mass spectrometer, equipped with a heated electrospray ionisation (h-ESI) probe operating in the positive ionisation mode (all from Thermo Scientific). Instrument parameters were optimised for both analytes (DEX and IS). For each compound, the two most intense product ions were selected and monitored in the selected reaction monitoring (SRM)

mode. For quantification, the most intense product ion was used (i.e. DEX: *m/z* 393.0 > 355.3; IS: *m/z* 377.1 > 339.3).

The method was validated by a set of parameters that were in compliance with the recommendations defined by EU standards (Commission Decision 2002/657/EC; Heitzman, 1994; VICH GL 49) and by Knecht and Stork (1974). The following parameters were evaluated: linearity, within-run and between-run accuracy and precision, limit of quantification (LOQ), limit of detection (LOD), extraction recovery, matrix effect and specificity. Linear calibration curves were constructed using matrix-matched calibrator samples (concentration range: 2–10000 ng/mL) and the correlation coefficients (r = 0.9967 ± 0.001, n = 3) and goodness-of-fit coefficients (g = 7.57 ± 0.001 %, n = 3) fell within the accepted ranges, i.e. $r \geq 0.99$ and $g \leq 20$ %, respectively.

Within-run and between-run precision and accuracy were evaluated by analysing six independently spiked blank plasma samples at three concentration levels, i.e. 10, 100 and 1000 ng/mL, respectively. The following mean results were obtained: within-run accuracy and precision (n = 6) 11.0 ± 2.1 ng/mL, 108.0 ± 14.59 ng/mL and 1072.2 ± 20.524 ng/mL; between-run accuracy and precision (n = 6) 10.2 ± 0.78 ng/mL, 96.5 ± 10.9 ng/mL and 965.5 ± 139.7 ng/mL. The results fell within the accepted ranges for accuracy (−20 % to +10 % of the theoretical concentration) and precision (within-run precision: relative standard deviation (RSD) ≤ RSD_{max} with $RSD_{max} = 2^{(1-0.5\log Conc)}$ x 2/3, i.e. 21.3 %, 15.1 % and 10.7 % at 10 ng/mL, 100 ng/mL and 1000 ng/mL, respectively; between-run precision: RSD ≤ RSD_{max} with $RSD_{max} = 2^{(1-0.5\log Conc)}$, i.e. 32.0 %, 22.6 % and 16.0 % at 10 ng/mL, 100 ng/mL and 1000 ng/mL, respectively).

The LOQ was defined as the lowest concentration for which the method was validated with a within-run accuracy and precision that fell within the specified ranges. The LOQ was also the lowest point of the calibration curve and was set at 2 ng/mL (n = 6, mean result: 2.0 ± 0.24 ng/mL). The LOD was defined as the concentration corresponding with a signal-to-noise ratio of 3 and was found to be 1.17 ng/mL. The specificity of the method was shown, since no peaks of endogenous interferences could be determined in blank plasma samples. Correction for extraction recovery was not necessary since the method of internal standardisation was applied for quantification.

2.4. Pharmacokinetic and statistical analysis

The PK properties were determined by means of WinNonlin®, version 6.2.0 software program (Pharsight Corporation) using a one-compartmental model. Values below the LOQ of 2 ng/mL were not included in the analysis.

The mean area under the plasma concentration-time curve ($AUC_{0\rightarrow\infty}$) was calculated using the linear trapezoidal method with extrapolation to infinity. The absorption and elimination rate constant (k_{abs} and k_{el}, respectively), half-life of absorption and elimination ($t_{1/2abs}$ and $t_{1/2el}$, respectively) expressed as the harmonic mean, volume of distribution (V_d), clearance (Cl), maximum plasma concentration (C_{max}), and time to maximum plasma concentration (t_{max}) were also determined. The absolute bioavailability (F) was calculated from the following equation:

$$F(\%) = \frac{AUC_{0\rightarrow\infty IM}}{AUC_{0\rightarrow\infty IV}} \text{ x } 100$$

Data are presented as mean ± standard deviation (SD) and were statistically analysed by single-factor analysis of variance (ANOVA), using SPSS Version 20.0 software for Windows. The level of significance was $\alpha = 0.05$.

3. Results

No adverse effects following IV or IM administration were observed during the course of the study. Quantifiable concentrations of DEX were measured in plasma until 4 h after both IV and IM administration. The PK properties of DEX (mean ± SD) are summarised in Table 1. Semi-logarithmic plots of the mean plasma concentration-time curves following IV and IM administration are presented in Figure 2.

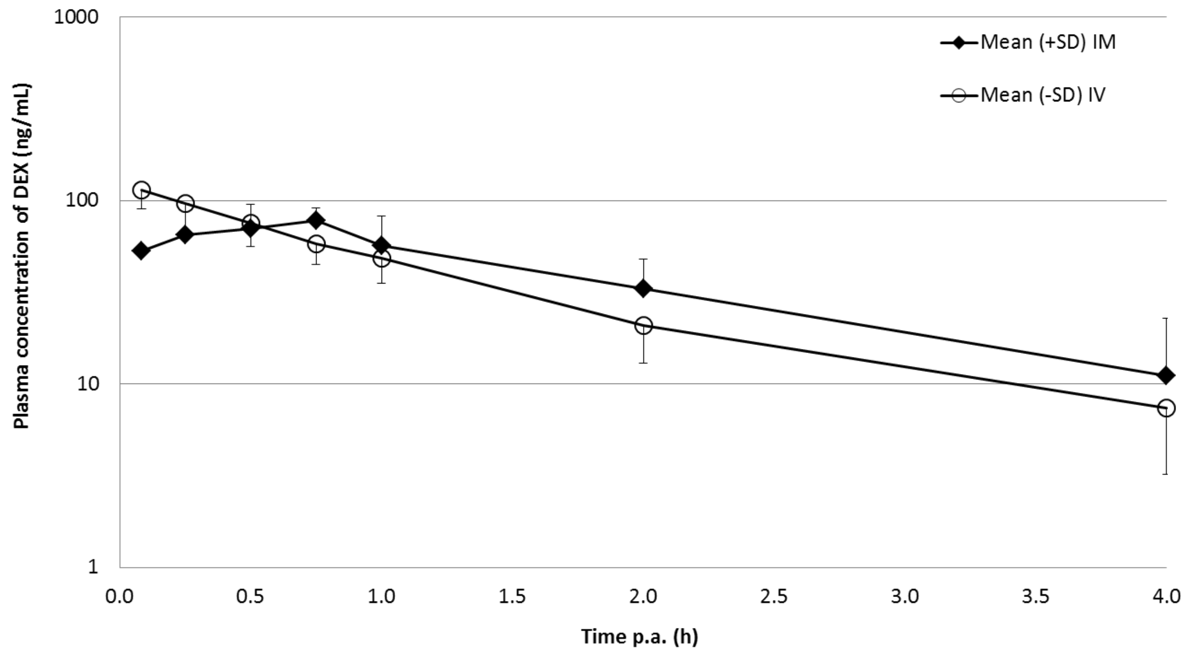


Figure 2. Mean (± SD) plasma concentration-time profiles of dexamethasone (DEX) after IV and IM bolus injection of 0.3 mg/kg BW DEX sodium phosphate in pigs (*n* = 6). Quantifiable concentrations of DEX were measured in plasma for 4 h after IV and IM administration (LOQ = 2 ng/mL).

Following an IV bolus injection of 0.3 mg/kg BW DEX sodium phosphate, the $AUC_{0\rightarrow\infty}$ was 133.07 ± 39.59 ng.h/mL. The $t_{1/2el}$ was 0.77 h and the V_d and Cl were 2.78 ± 0.88 L/kg and 2.39 ± 0.57 L/h.kg, respectively.

Following an IM bolus injection of 0.3 mg/kg BW DEX sodium phosphate, the $AUC_{0\rightarrow\infty}$ was 173.24 ± 53.59 ng.h/mL. A C_{max} of 80.94 ± 21.29 ng/mL was reached at 0.35 ± 0.21 h (t_{max}). The $t_{1/2el}$ was 1.06 h and the absolute bioavailability of DEX after IM injection was 131.06 ± 26.05%.

None of the PK properties significantly differed between both administration routes.

Table 1. Pharmacokinetic parameters (mean ± SD) of dexamethasone in pigs after IV and IM bolus administration of 0.3 mg/kg BW DEX-sodium phosphate (*n* = 6)

Parameter	Units	IV	IM
$AUC_{0\to\infty}$	h.ng/mL	133.07 ± 39.59	173.24 ± 53.59
k_{abs}	/h	-	12.90 ± 10.57
k_{el}	/h	0.90 ± 0.28	0.65 ± 0.30
$t_{1/2abs}$	h	-	0.05^A
$t_{1/2el}$	h	0.77^A	1.06^A
V_d	L/kg	2.78 ± 0.88	-
Cl	L/h.kg	2.39 ± 0.57	-
t_{max}	h	-	0.35 ± 0.21
C_0	ng/mL	114.89 ± 26.56	-
C_{max}	ng/mL	-	80.94 ± 21.29
F	%	-	131.06 ± 26.05

AUC, area under the curve; k_{abs}, absorption rate constant; k_{el}, elimination rate constant; $t_{1/2abs}$, half-life of absorption; $t_{1/2el}$, half-life of elimination; V_d, volume of distribution; Cl, clearance; t_{max}, time to maximum plasma concentration; C_0, maximum plasma concentration at 0 h; C_{max}, maximum plasma concentration; F, absolute bioavailability.

^{A}: harmonic mean

4. Discussion

The present study is the first to describe the pharmacokinetics and bioavailability of DEX after IV and IM administration in pigs.

Sibila et al. (2008) demonstrated that the concomitant administration of a glucocorticoid with an antibiotic possesses beneficial effects in an experimental model of severe pneumonia in pigs. Particularly, in bronchial tissue a reduced local inflammatory response, and in the bronchoalveolar lavage and lung a reduced bacterial load could be observed.

In this study, DEX was supplied as a water-soluble and stable sodium phosphate ester to pigs. As previously established by Rohdewald et al. (1987), DEX sodium phosphate is rapidly converted to the free and active alcohol form of DEX. Therefore, DEX alcohol, and not the esterified prodrug, was currently measured in plasma using the validated LC-MS/MS method.

An IM bolus injection of 0.3 mg/kg BW DEX sodium phosphate in pigs resulted in a relatively fast absorption and a C_{max} of DEX at 0.35 h (t_{max}). However, in horses a faster t_{max} of 0.16 h was recently observed (Soma et al., 2013). After an IM injection, DEX sodium phosphate is fully bioavailable in pigs (F = 131 %), horses (F = 100 %; Soma et al., 2013) and broiler chickens (F = 123%; Watteyn et al., 2013).

In dogs, an IM administration of DEX alcohol resulted in a relatively early C_{max} at 0.63 h and a complete absorption, while in cattle a delayed C_{max} at 4.27 h and an absolute bioavailability of only 67 % was observed. Likewise, after an IM bolus injection of DEX 21-isonicotinate, an early C_{max} at 0.50 h and a delayed C_{max} at 3.57 h was observed in dogs and cattle, respectively. However, the absolute bioavailability was barely 40 % in dogs compared to 72 % in cattle (Toutain et al. 1982; 1983).

In comparison with other mammalian species, a remarkably high clearance of DEX was observed in pigs (2.39 L/h.kg). Conversely, in dogs, cattle, horses and humans, a clearance of < 0.6 L/h.kg was reported (Toutain et al., 1982, 1983; Rohdewald et al., 1987; Greco et al., 1993; Hochhaus et al., 2001; Soma et al., 2013). In most species, the V_d of DEX is less than 2 L/kg (Toutain et al., 1982, 1983; Hochhaus et al., 2001; Soma et al., 2013). Interestingly, the V_d of DEX in pigs was 2.78 L/kg which suggests a good tissue penetration of this glucocorticoïd drug. However, a relatively short $t_{1/2el}$ (0.77 h) was observed in pigs. In other species, DEX is considered to be a long-acting (> 48 h) glucocorticoid in comparison to hydrocortisone and prednisolone. Notwithstanding the PK results observed in this study, DEX could have a longer action in pigs from a pharmacodynamical point of view.

In conclusion, an IM bolus injection of DEX sodium phosphate in pigs resulted in a fast and complete absorption, followed by a fast elimination of the active DEX alcohol. The V_d and particularly the clearance of DEX are much higher in pigs in comparison with other mammalian species.

Acknowledgements

The authors would like to thank G. Antonissen, M. Devreese, J. Goossens, J. Lambrecht and A. Van den Bussche for their technical assistance during the animal experiment.

CHAPTER 2.

Immunomodulatory properties of veterinary drugs

2.1. *In vitro* effect of gamithromycin, ketoprofen and dexamethasone on pro-inflammatory cytokine and prostaglandin E_2 production in LPS-stimulated porcine peripheral blood mononuclear cells

Adapted from

Wyns, H., Meyer, E., Plessers, E., Watteyn, A., van Bergen, T., Schauvliege, S., De Baere, S., Devreese, M., De Backer, P., Croubels, S. (2014) Modulation of gamithromycin and ketoprofen on *in vitro* and *in vivo* porcine lipopolysaccharide-induced inflammation *(Submitted to PLoS ONE).*

Abstract

Immunomodulation is predominantly studied at the level of cytokine production, inflammatory mediators and cell functions. In this respect, isolated peripheral blood mononuclear cells (PBMCs) have been frequently used as an *in vitro* model to study the lipopolysaccharide (LPS)-induced immune response in different animal species. The aim of the present study was to investigate the effects of gamithromycin (GAM), ketoprofen (KETO), dexamethasone (DEX) and their combinations on the production of prostaglandin (PG) E_2, tumor necrosis factor (TNF)-α, interleukin (IL)-1β and IL-6 in LPS-stimulated porcine PBMCs.

Following an 18 h-incubation period with 1.25 µg/mL LPS, significantly increased levels of TNF-α, IL-1β and IL-6 ($p < 0.05$) were measured in the PBMC cell supernatants. Whereas DEX was successfully included as a positive control for cytokine inhibition ($p < 0.05$), KETO had no inhibiting effect on cytokine production. Remarkably, KETO showed a trend to enhance TNF-α levels. Combinations of GAM and KETO, on the other hand, significantly reduced levels of LPS-induced IL-1β ($p < 0.05$) and IL-6 ($p < 0.01$). KETO, DEX and their respective combinations with GAM, significantly reduced PGE_2 production ($p < 0.001$).

1. Introduction

Modulation of the immune response by drugs is of major interest. Research on this subject is predominantly performed at the level of cytokine production, inflammatory mediators and cell functions (Blecha, 1988; Kanoh and Rubin, 2010). *In vitro* research can offer a first indication of the expected *in vivo* outcome. Since blood monocytes and tissue macrophages are responsible for the onset of inflammation, isolated peripheral blood mononuclear cells (PBMCs) have been frequently used as a model of *in vitro* immune response stimulation (Bailly et al., 1991a; Baumann and Gauldie, 1994; Morikawa et al., 1996, Ianaro et al., 2000; Cao et al., 2006). As a major part of the Gram-negative bacterial outer membrane, lipopolysaccharide (LPS) is a key molecule in several porcine diseases. Binding of LPS to Toll-like receptor 4 (TLR4) ultimately leads to the activation of nuclear factor-κB (NF-κB), subsequent gene transcription and production of pro-inflammatory cytokines, including tumor necrosis factor (TNF)-α, interleukin (IL)-1β and IL-6, and other mediators (Pålsson-McDermott and O'Neill, 2004). Additionally, NF-κB-mediated cyclooxygenase (COX)-2 expression is induced, resulting in prostaglandin (PG) E_2 and thromboxane (TX) A_2 production (Yamamoto et al., 1995). On the other hand, COX-1 is constitutively expressed in most cells and is mainly involved in the maintenance of physiological processes (Vane, 1971; Tegeder et al., 2001). The d*e novo* formation of PGE_2 comprises a three-step cascade reaction catalyzed by phospholipase A_2 (PLA_2), which liberates arachidonic acid from membrane phospholipids; cyclooxygenase (COX), which converts arachidonic acid to PGH_2; and PGE synthase, which finally isomerizes PGH_2 to PGE_2. Subsequently, PGE_2 is rapidly converted to its major inactive metabolite 13,14-dihydro-15-keto PGE_2, and can be subject to further metabolism to 13,14-dihydro-15-keto PGA_2 and finally bicyclic PGE_2 (Fitzpatrick et al., 1980; Granström et al., 1980; Ivanov and Romanovsky, 2004).

Non-steroidal anti-inflammatory drugs (NSAIDs) exert both their therapeutic and side effects by binding and blocking COX. However, it is hypothesized that some NSAIDs might also possess COX-independent properties, involving inhibition of NF-κB-related gene transcription (Tegeder et al., 2001). Ketoprofen (KETO) is an NSAID which has been commonly used in veterinary medicine because of its anti-inflammatory, antipyretic and analgesic properties (Mustonen et al., 2012a; Salichs et al., 2012). It was previously

suggested that KETO has not the ability to inhibit NF-κB activation (Matasić et al., 2000), yet Donalisio et al. (2013) recently described an inhibiting effect of KETO on the *ex vivo* LPS-induced TNF-α production in dairy cows.

Glucocorticoids induce lipocortin which inhibits PLA_2. Dexamethasone (DEX) is a potent synthetic glucocorticoid and inhibition of LPS-induced cytokine production by DEX has been well-documented (Morikawa et al., 1996; Bessler et al., 1999).

Numerous review articles have been dedicated to the diversity of clinically important *in vitro* and *in vivo* immunomodulatory effects of macrolide antibiotics (Labro, 1998; Čulić et al., 2001; Tamaoki et al., 2004; Kanoh and Rubin, 2010). In this respect, these drugs are reported to influence monocyte-mediated cytokine release and PG production, as well as neutrophil adhesion, migration and accumulation (Čulić et al., 2001). In addition, it is suggested that macrolides prevent NF-κB activation and the following protein expression and production (Ianaro et al., 2000; Healy, 2007). Gamithromycin (GAM) is a macrolide antibiotic recently approved for the treatment of bovine respiratory disease. Furthermore, the influence of GAM on the porcine immune response has not yet been studied. Recently, Salichs et al. (2013) demonstrated that the combination of an NSAID and an antibiotic was advantageous in the treatment of swine respiratory disease compared to treatment with the antibiotic alone.

The aim of the present study was therefore to investigate the effects of GAM, KETO and their combinations on the production of pro-inflammatory cytokines and PGE_2 in LPS-stimulated porcine PBMCs. DEX was included as a positive control inhibiting pro-inflammatory cytokines.

2. Materials and methods

2.1. Standards and reagents

Analytical standards of ketoprofen (KETO), dexamethasone (DEX) and indomethacin (IND) were purchased from Sigma-Aldrich. The standard of gamithromycin (GAM) was kindly donated by Merial. High-performance liquid chromatography (HPLC) grade methanol (MeOH) and ethanol absolute for analysis (EtOH) were obtained by VWR. Stock solutions of 1 mg/mL of the analytical standards of GAM, KETO and DEX were prepared in HPLC MeOH

and were stored at 2-8°C (DEX) or at ≤ -15°C (GAM and KETO). Working solutions were prepared in RPMI 1640 medium (Invitrogen) supplemented with 10 % heat-inactivated fetal bovine serum (FBS), 1 % L-glutamine, 1 % sodium pyruvate and 1 % non-essential amino acids (complete RPMI). Ultrapure LPS from *E. coli* serotype O111:B4 was obtained by InvivoGen. A stock solution of 100 µg/mL of IND was prepared in EtOH. Subsequently, 100 µL of the stock solution was dispensed in microcentrifuge tubes and evaporated to dryness. Tubes were kept closed and stored at 2-8°C until the start of the experiment.

Standards of PGE_2 and 13,14-dihydro-15-keto PGE_2 (PGE_2-*met*); as well as the internal standards (IS), deuterated PGE_2 (PGE_2-d_4) and PGB_2 (PGB_2-d_4) were all obtained by Cayman Chemical. Ultra-performance liquid chromatography (UPLC) grade MeOH, acetonitrile (ACN) and water were obtained by Biosolve. Stock solutions of 1 mg/mL of PGE_2, PGE_2-*met*, PGE_2-d_4 and PGB_2-d_4 were prepared in UPLC MeOH and stored at < -70°C. Mixed working solutions of PGE_2 and PGE_2-*met* of 0.1, 1.0 and 10 ng/mL; and a mixed IS working solution of PGE_2-d_4 and PGB_2-d_4 of 10 ng/mL were prepared in UPLC MeOH and stored at < -70°C.

2.2. Isolation and stimulation of porcine peripheral blood mononuclear cells

Jugular venipuncture was performed in six clinically healthy donor pigs, weighing on average 30 kg (Biocentrum Agri-Vet, Ghent University), and 10 mL blood was collected in lithium heparinized tubes and diluted 1:1 with 0.9% NaCl. Peripheral blood mononuclear cells (PBMCs) were subsequently isolated by density gradient centrifugation (800 *x g* for 25 min at 18 °C) using Lymphoprep™ (Lucron). Remaining erythrocytes were lysed in an ice-cold isotonic ammonium chloride (NH_4Cl) solution (138 mM NH_4Cl and 21 mM Tris, pH 7.4). PBMCs were washed twice with RPMI 1640 containing 10 % heat-inactivated FBS and pelleted at 350 *x g* for 10 min at 4°C. Cells were counted using BD TruCOUNT™ tubes by means of a FACSArray Bioanalyzer™, following the manufacturer's instructions (BD Biosciences). PBMCs were finally resuspended at a concentration of 2.5×10^6 cells/mL in complete RPMI.

The cells were cultured in 15 mL polypropylene tubes at a density of 2×10^6 cells/tube (800 µL) in the presence of LPS (1.25 µg/mL) with or without GAM, KETO and DEX or a combination of drugs (a total volume of 1 mL in each tube; Table 1) for 18 h on a

horizontal shaker (IKA® HS 260, Sigma-Aldrich) in a 37 °C humidified incubator containing 5 % CO_2. Subsequently, the cells were pelleted at 350 *x g* for 10 min at 4 °C and the supernatant was harvested in uncoated or IND-coated (10 µg/mL) microcentrifuge tubes for analysis of TNF-α, IL-1β and IL-6; and PGE_2, respectively, and immediately stored at ≤ -70 °C until analysis.

Blood sampling was approved by the Ethical Committee of the Faculty of Veterinary Medicine of Ghent University (EC2013/23).

Table 1. Overview of the drugs and their combination tested

Groups	GAM (µg/mL)	KETO (µg/mL)	DEX (µg/mL)	% MeOH
CONTR	-	-	-	0
LPS	-	-	-	0
GAM 10	10	-	-	1
GAM 20	20	-	-	2
KETO 10	-	10	-	1
KETO 20	-	20	-	2
DEX 1	-	-	1	0.1
DEX 10	-	-	10	1
GAM 10 – KETO 10	10	10	-	2
GAM 20 – KETO 20	20	20	-	4
GAM 10 – KETO 20	10	20	-	3
GAM 20 – KETO 10	20	10	-	3
GAM 10 – DEX 1	10	-	1	1.1
GAM 20 – DEX 1	20	-	1	2.1

GAM: gamithromycin; KETO: ketoprofen; DEX: dexamethasone; MeOH: methanol

2.3. Cell viability

Prior to LPS stimulation, cell viability was assessed after incubation of 2.5 x 10^5 cells for 10 min with 1 µg/mL propidium iodide (PI; Sigma-Aldrich). Five thousand events were evaluated by means of a FACSArray Bioanalyzer™ and analysed using FACSDiva software (BD Biosciences). The influence of MeOH, GAM, KETO, DEX and the combination of drugs on cell viability was investigated in a similar manner after incubation of PBMCs for 18 h at 37 °C with 5 % CO_2.

To exclude bacterial contamination of the cell culture after the 18 h-incubation period, the cell suspension was plated onto a trypticase soy agar (TSA) plate and incubated for 48 h at 37 °C. The TSA plate was visually inspected for the presence of bacterial growth.

2.4. Sample analysis

The concentration of PGE_2 and its inactive metabolite PGE_2-*met* in cell supernatant was measured using an in-house validated UPLC-tandem mass spectrometry method (UPLC-MS/MS), which was initially developed for plasma samples (De Baere et al., unpublished data). In brief, sample preparation was performed by adding 25 µL of the IS working solution to 100 µL of cell supernatant. After a vortex mixing step (10s), 525 µL of UPLC ACN were added, followed by a vortex mixing (20s) and a centrifugation step (7800 *x g* for 10 min at 4 °C). The supernatant was transferred to a glass extraction tube and evaporated to dryness using a nitrogen stream (35 °C). The residue was re-dissolved in 200 µL of UPLC water/ACN (80/20, v/v), filtered through a Millex®-GV PVDF filter unit (0.22 µm - Millipore) and transferred to an autosampler vial. A 10 µL-aliquot was injected onto the UPLC-MS/MS instrument.

The LC system consisted of an Acquity UPLC H-Class Quaternary Solvent Manager and Flow-Through-Needle Sample Manager with temperature controlled tray and column oven from Waters. Chromatographic separation was achieved on an Acquity UPLC BEH C18 column (50 mm x 2.1 mm i.d., dp: 1.7 µm) in combination with an Acquity BEH C18 1.7 µm Vanguard pre-column (Waters). The mobile phase A consisted of 0.01 % formic acid in water, while the mobile phase B was 0.01 % formic acid in ACN.

The UPLC column effluent was interfaced to a XEVO TQ-S® MS/MS system, equipped with an electrospray ionization (ESI) probe operating in the negative mode (all from Waters). A divert valve was used and the UPLC effluent was directed to the mass spectrometer from 2.0 to 4.9 min.

MS/MS acquisition was performed in the multiple reaction monitoring (MRM) mode. The MRM transitions monitored as quantification and identification ion, respectively, were m/z = 351.22 > 271.26 and 351.22 > 315.21 for PGE_2; m/z = 355.16 > 319.28 and 355.16 > 275.27 for PGE_2-d4 (IS); m/z = 333.20 > 175.00 and 333.20 > 235.00 for PGE_2-*met*; m/z = 337.20 > 179.00 and 337.20 > 319.20 for PGB_2-d4 (IS).

The method was validated by a set of parameters that were in compliance with the recommendations defined by EU standards (Commission Decision 2002/657/EC, VICH GL 49, Heitzman, 1994) and by Knecht and Stork (1974). In this respect, linearity, within- and between-run accuracy and precision, limit of quantification (LOQ), limit of detection (LOD)

and specificity were evaluated. Linear calibration curves (r > 0.99) were constructed in a concentration range of 50 and 5000 pg/mL. Within- and between-run accuracy and precision were evaluated by analysing independently spiked samples (*n* = 6) at four concentration levels i.e. 50, 125, 500 and 5000 pg/mL, respectively. The results fell within the accepted ranges for accuracy and precision (2002/657/EC; VICH GL49). The LOQ was defined as the lowest concentration for which the method was validated with a within-run accuracy and precision that fell within the specified ranges and was established at 50 pg/mL for both PGE_2 and PGE_2-*met*. The LOD was defined as the concentration corresponding with a signal-to-noise ratio of 3 and was found to be 14.0 and 10.0 pg/mL for PGE_2 and PGE_2-*met*, respectively. The specificity of the method was proven, since no peaks of endogenous interferences could be determined in blank samples.

All samples were analysed for detection of TNF-α, IL-1β and IL-6 using commercially available porcine DuoSet® ELISA Development Systems according to the manufacturer's instructions (R&D Systems). Optical densities were determined using a plate reader (Multiskan MS Labsystems, Thermo Scientific). Data were analysed by means of DeltaSoft JV (BioMetallics Incorporated).

2.5. Statistical analysis

Data were statistically analyzed by single factor analysis of variance (ANOVA), using SPSS Statistics 22.0 software for Windows. Pairwise comparisons of means of the different groups were performed using the Tukey test. For those parameters that were not normally distributed, the Kruskal-Wallis ANOVA on ranks was used. A p-value of < 0.05 was considered statistically significant.

3. Results

3.1. Cell viability

Before incubation with LPS and/or drugs, the mean viability of the cells was 99.7 ± 0.06%. Neither MeOH (up to a concentration of 4%, Table 1), nor GAM, KETO, DEX or their

combination displayed harmful effects for porcine PBMCs, since the viability of the cells of all groups was still ≥ 96.3% after the 18 h-incubation period.

No bacterial contamination was observed on the TSA plate after a 48 h-incubation at 37°C.

3.2. Prostaglandin E_2 production

Incubation of porcine PBMCs with LPS significantly increased PGE_2 in the supernatant of the LPS, GAM 10 and GAM 20 groups in comparison with unstimulated PBMCs (CONTR) after 18 h ($p < 0.001$). In the supernatant of the other groups, PGE_2 could not be quantified (< LOQ). Incubation of the cells in the presence of KETO, DEX and their respective combinations with GAM, significantly reduced PGE_2 production compared to the LPS group ($p < 0.001$) (Figure 1).

No concentrations of PGE_2-*met* could be quantified (< LOQ) (data not shown).

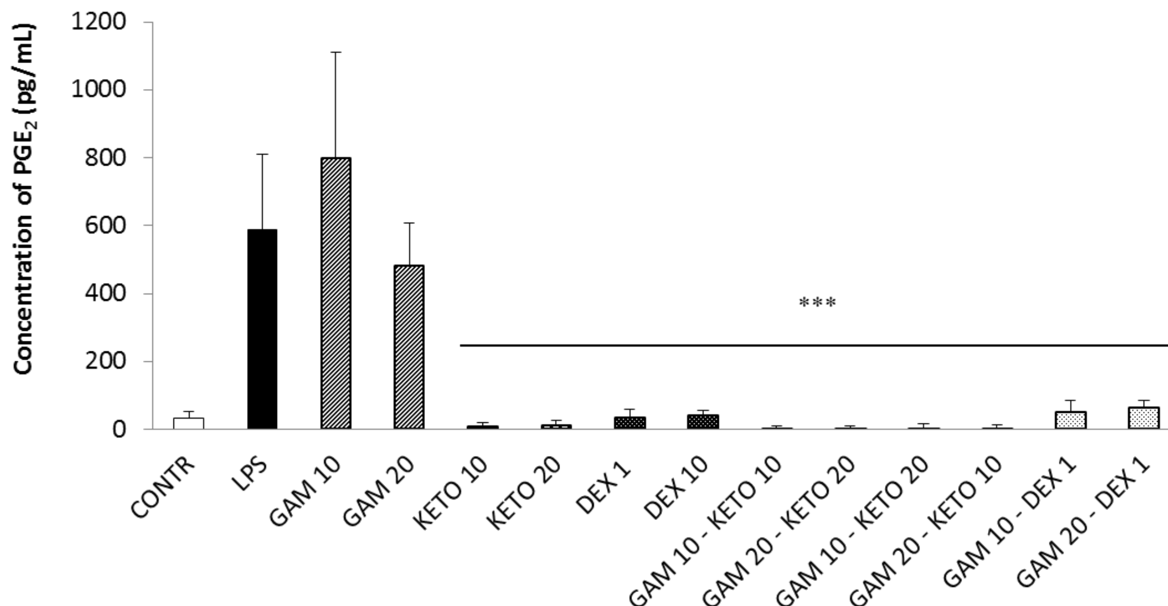


Figure 1. Effect of gamithromycin (GAM), ketoprofen (KETO), dexamethasone (DEX) and their combination on lipopolysaccharide (LPS)-induced (1.25 µg/mL) PGE_2 production by PBMCs. Data are presented as means + SD. Statistically significant differences compared to the LPS group are visualized as *** $p < 0.001$.

3.3. Cytokine production

Following incubation with LPS, the LPS, GAM 10, GAM 20, KETO 10, KETO 20, GAM 10 – KETO 10, GAM 10 – KETO 20 and GAM 20 – KETO 10 groups showed a marked increase in supernatant levels of TNF-α ($p < 0.001$) and IL-1β ($p < 0.01$) in comparison with unstimulated PBMCs (CONTR) after 18 h (Figure 2A-B). As unstimulated PBMCs (CONTR) also produced considerable amounts of IL-6 (Figure 2C), only the LPS group showed a significant higher IL-6 level compared to the CONTR group ($p < 0.05$). Combinations of GAM and KETO significantly reduced levels of LPS-induced IL-1β (GAM 20 – KETO 20; $p < 0.05$) and IL-6 (GAM 20 – KETO 20, GAM 10 – KETO 20 and GAM 20 – KETO 10; $p < 0.01$) in comparison with the LPS group.

As expected, the presence of the positive control DEX significantly suppressed the production of TNF-α ($p < 0.05$), IL-1β ($p < 0.01$) and IL-6 ($p < 0.01$) compared to the LPS group.

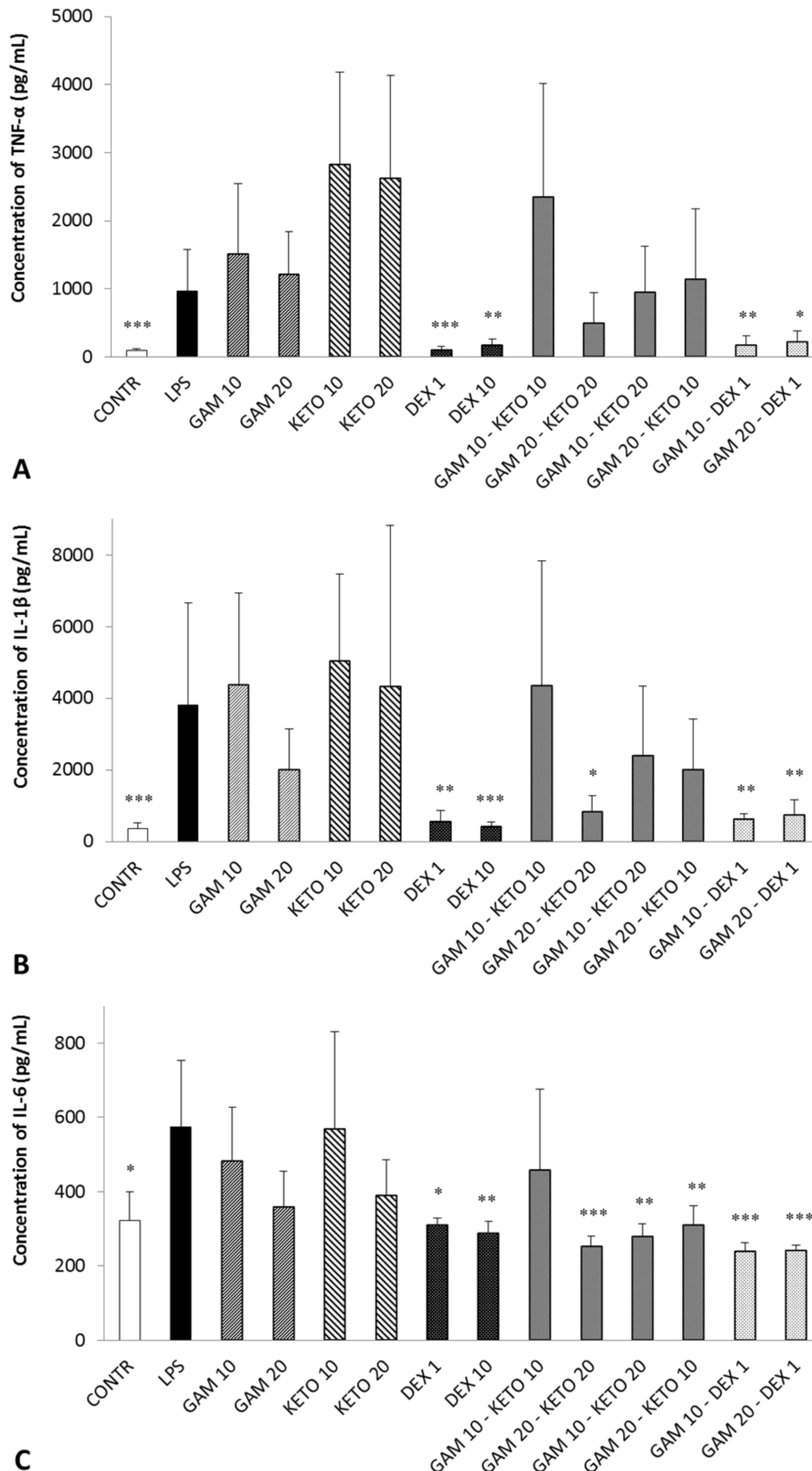


Figure 2. Effect of gamithromycin (GAM), ketoprofen (KETO), dexamethasone (DEX) and their combination on lipopolysaccharide (LPS)-induced (1.25 µg/mL) TNF-α (A), IL-1β (B) and IL-6 production by PBMCs (C). Data are presented as means + SD. Statistically significant differences compared to the LPS group are visualized as * $p < 0.05$; ** $p < 0.01$ and *** $p < 0.001$.

4. Discussion

The present study reports the influence of GAM, KETO and DEX on the production of TNF-α, IL-1β and IL-6; and PGE_2 using an *in vitro* model of LPS-induced (1.25 µg/mL) inflammation in pigs. The experimental design concerning LPS dose, incubation period and doses of GAM tested was largely based on similar research (Ianaro et al., 2000; Cao et al., 2006). Moreover, an in-house pilot study demonstrated that an incubation period of 18 h was more suitable in comparison with 6 h and 24 h (data not shown). In a previous pharmacokinetic study by our group, maximal plasma concentrations of 0.27-0.49 µg/mL were established for GAM in pigs after a single subcutaneous injection of 6 mg/kg (Wyns et al., 2014). Taking into account the total blood volume and estimated number of monocytes in the peripheral circulation of pigs, ± 6% of the total leukocyte count (Friendship et al., 1984; Clark and Coffer, 2008; Williams et al., 2009), GAM doses of 10 and 20 µg/mL were considered appropriate for evaluating the possible immunomodulatory capacities of this macrolide antibiotic after incubation with 2 x 10^6 PBMCs.

Due to instability and following rapid metabolism of PGs, the simultaneous detection of multiple PG metabolites is desirable for a more comprehensive understanding of the PG biosynthesis. Therefore, a highly specific LC-MS/MS method was in-house developed for the simultaneous determination of PGE_2 and its inactive metabolite, PGE_2-*met* (De Baere et al., unpublished data). The presence of albumin and alkaline conditions (pH ≥ 9) promotes further degradation of 13,14-dihydro-15-keto PGE_2 to PGE_2-*met* and other inactive metabolites (Fitzpatrick et al., 1980; Granström et al., 1980; Kelly et al., 1992). In this study, however, none of the groups showed detectable concentrations of PGE_2-*met* (data not shown). This might be explained by the fact that the complete RPMI medium used was protein-free and showed a physiological pH. Furthermore, the *in vitro* incubation set-up might lack essential physiological circumstances, such as enzymes, as compared to the *in vivo*-situation.

Both SAIDs and NSAIDs exert their main anti-inflammatory effects by inhibition of the PG biosynthesis. As expected, the administration of DEX as well as KETO to LPS-stimulated PBMCs efficiently inhibited the production of PGE_2. Conversely, high levels of PGE_2 were detected in the supernatant of the LPS, GAM 10 and GAM 20 groups (Figure 1).

Macrolide antibiotics have been previously reported to reduce the COX-mediated eicosanoid production in LPS-stimulated murine mononuclear cells. In contrast to NSAIDs, it is suggested that macrolides do not interact directly with LPS-induced COX-2, but rather suppress its expression through inhibition of NF-κB (Ianaro et al., 2000; Cao et al., 2006; Leiva et al., 2008). Nevertheless, GAM did not show any (indirect) effect on the production of PGE_2 by porcine PBMCs compared to the LPS group.

In vivo, the cytokine-mediated activation of the hypothalamic-pituitary-adrenal axis and following endogenous glucocorticoid synthesis suppress further cytokine production, in order to protect the host from an excessive and possibly harmful immune response (Fantuzzi and Ghezzi, 1993; Coelho et al., 1995; Tuchscherer et al., 2010). DEX has been repeatedly reported to inhibit LPS-induced TNF-α, IL-1β and IL-6 levels both *in vitro* and *in vivo* in different animal species (Morikawa et al., 1996, Bessler et al., 1999; Teeling et al., 2010; Myers et al., 2003). As expected, DEX also significantly suppressed the LPS-induced production of TNF-α, IL-1β and IL-6 in the current study.

Administration of KETO neither inhibited LPS-induced TNF-α, IL-1β nor IL-6 production by porcine PBMCs. In contrast, PBMCs of the KETO 10, KETO 20 as well as the combination group GAM 10 – KETO 10, showed a trend, yet not significant, to produce even higher levels of TNF-α compared to the LPS group. In this respect, our results are in agreement with previous studies, reporting that NSAIDs increase rather than inhibit TNF-α production both *in vitro* and *in vivo* (Pettipher and Wimberly, 1994; Ghezzi et al., 1998; Roth et al., 2002). Since PGE_2 has been established as a feedback inhibitor of TNF-α production, increased TNF-α levels can be expected following COX-inhibition (Kunkel et al., 1988; Sironi et al., 1992). Indeed, no PGE_2 was detected in the groups receiving KETO.

In contrast to many previous reports concerning inhibition of cytokine production by macrolide antibiotics, GAM had no influence on LPS-induced cytokine levels in the present study. Macrolides, including azithromycin, clarithromycin, erythromycin, roxithromycin, telithromycin, tilmicosin and tylosin are all reported to decrease the *in vitro* and/or *in vivo* production of TNF-α, IL-1β and IL-6 in humans and rodent species (Morikawa et al., 1996; Ianaro et al., 2000; Cao et al., 2006; Sugiyama et al., 2007; Leiva et al., 2008). In this respect, GAM seems to differ from other macrolide antibiotics. On the other hand, Bailly et al. (1991a) also observed no effect of roxithromycin on cytokine production, and even a stimulating effect of spiramycin, and to a lesser extent erythromycin, on IL-6

production in LPS-stimulated human monocytes. An alternative explanation is that the concentrations of GAM used in the present study (i.e. 10 and 20 μg/mL) were not sufficient to influence the cytokine as well as PGE_2 production as these effects are generally considered to be concentration-dependent (Morikawa et al., 1996; Ianaro et al., 2000; Cao et al., 2006). Nevertheless, Cao et al. (2006) reported significant effects with similar concentrations of tilmicosin and tylosin. Being weak basic (and lipophilic) molecules, macrolides can easily penetrate and accumulate in cells and tissues reaching substantial intracellular and therapeutic concentrations, despite inadequate plasma concentrations (Anadón and Reeve-Johnson, 1999). Lung concentrations of GAM in cattle were established 247-410 times higher compared to plasma (Huang et al., 2010). However, experimental conditions, such as the incubation temperature and the presence of extracellular Ca^{2+}, can also influence their uptake and thus their immunomodulatory capacities (Vazifeh, 1997; Čulić et al., 2001). Furthermore, it has been described that some macrolides become inactive in acidic intracellular conditions, i.e. in phagosomes (Van Bambeke and Tulkens, 2001). These results indicate that the effects of macrolides on cytokine production are equivocal and require a case-specific approach. Additional research using a broader range of GAM concentrations can further elucidate this hiatus.

Interestingly, the combination of a high concentration of GAM as well as KETO (GAM 20 – KETO 20) reduced TNF-α, yet not significantly, IL-1β and IL-6 levels. This effect is not due to enhanced toxicity of higher drug concentrations since more than 96% of the cells of the GAM 20 – KETO 20 group was still viable after the 18 h-incubation period. High concentrations of both GAM and KETO might act synergistically and possibly lower the threshold to observe immunomodulatory effects. While these *in vitro* results provide a first, promising indication of the possible advantageous effects of the combination of GAM and KETO, the efficacy of this combination will be further evaluated in LPS-challenged pigs.

Acknowledgements

The authors would like to thank Merial for providing the analytical standard of GAM.

2.2. *In vivo* influence of gamithromycin and ketoprofen on the acute phase response in lipopolysaccharide-challenged pigs

Adapted from

Wyns, H., Meyer, E., Plessers, E., Watteyn, A., van Bergen, T., Schauvliege, S., De Baere, S., Devreese, M., De Backer, P., Croubels, S. (2014) Modulation of gamithromycin and ketoprofen on *in vitro* and *in vivo* porcine lipopolysaccharide-induced inflammation *(Submitted to PLoS ONE).*

Abstract

The immunomodulatory properties of gamithromycin (GAM), ketoprofen (KETO) and their combination (GAM-KETO) were studied on the production of prostaglandin (PG) E_2 and pro-inflammatory cytokines tumor necrosis factor (TNF)-α, interleukin (IL)-1β and IL-6 in lipopolysaccharide (LPS)-challenged pigs. Additionally, the effects on the production of the acute phase proteins (APPs) pig-major acute phase protein (pig-MAP) and C-reactive protein (CRP), as well as on the development of fever, pulmonary symptoms and sickness behaviour were investigated.

Maximal plasma concentrations of TNF-α and IL-6 were observed at 1 h and 2.5 h following intravenous LPS challenge in pigs, respectively. Neither GAM, KETO nor the combination GAM-KETO was able to inhibit the *in vivo* LPS-induced cytokine production. Furthermore, none of the drugs influenced the APPs production.

Pretreatment of the pigs with KETO significantly reduced PGE_2 production ($p < 0.001$) and prevented the development of fever and severe symptoms following LPS challenge, including dyspnea, anorexia, vomiting and lateral decubitus. A crucial role for cyclooxygenase (COX)-1 mediated products, including PGE_2 and thromboxane A_2 is suggested in the development of early LPS-associated symptoms in pigs.

1. Introduction

Lipopolysaccharide (LPS), a structural part of the outer membrane of Gram-negative bacteria, is one of the most effective stimulators of the immune system and has been widely applied in pigs as an experimental model for bacterial infection and septic shock (Dinarello, 1997; Schrauwen et al., 1988; Wyns et al., manuscript submitted). Both neutrophils and monocytes are important cells in the innate immune response (Baumann and Gauldie, 1994; Cavaillon and Adib-Conquy, 2005; Sanz-Santos et al., 2011). Bacterial LPS is recognized by Toll-like receptor 4 (TLR4) which is constitutively expressed on the surface of innate immune cells, including monocytes and tissue macrophages. Extracellular binding of LPS to TLR4 and following intracellular signaling ultimately leads to the activation of nuclear factor-κB (NF-κB) and subsequent gene transcription of pro-inflammatory cytokines and mediators (Baumann and Gauldie, 1994; Peri et al., 2010). This results in the further recruitment and migration of immune cells to target tissues and the induction of the hepatic acute phase proteins (APPs) production, including C-reactive protein (CRP), haptoglobin (Hp) and pig major acute phase protein (pig-MAP) (Baumann and Gauldie, 1994; Cavaillon and Adib-Conquy, 2005).

This acute phase response (APR) can be modulated by drugs. Immunomodulation is defined as a reorganization of the inflammatory response by modifying or regulating functions of the hosts' immune system. The immunomodulatory capacities of (non)-steroidal anti-inflammatory drugs (N)SAIDs and antibiotics have been studied after both *in vitro* and *in vivo* LPS challenges in various animal species. In contrast to the rather restricted *in vitro* research, *in vivo* research includes the APPs and febrile response and thus offers a more complete evaluation of the APR.

The *Escherichia (E.) coli* LPS-induced fever model has been accepted for the evaluation of the antipyretic effect of NSAIDs (EMA, 2001). These drugs exert both their therapeutic and their side effects by (non-)selective central and peripheral cyclooxygenase (COX)-inhibition, consequently blocking prostaglandin (PG) biosynthesis (Curry et al., 2005; Ferguson et al., 2009). Ketoprofen (KETO) is an NSAID which has been commonly used in veterinary medicine because of its anti-inflammatory, antipyretic and analgesic properties. It has been repeatedly established as a major antipyretic drug in pigs (Swinkels et al., 1994; Mustonen et al., 2012a; Salichs et al., 2012).

Compared to NSAIDs, antimicrobial drugs are even more frequently used in veterinary medicine. In pigs, bacterial respiratory infections are responsible for considerable economic losses and reduced animal welfare. Bacteria, including *Actinobacillus pleuropneumoniae*, *Pasteurella multocida*, *Haemophilus parasuis* and *Mycoplasma hyopneumoniae* are major pathogens involved in swine respiratory disease (SRD). In addition to their anti-infectious properties, especially macrolide antibiotics are recurrently reported to possess clinically important immunomodulatory effects by influencing a variety of inflammatory processes varying from the release of cytokines and mediators, neutrophil function, migration, infiltration and accumulation to mucus (hyper)secretion (Čulić et al., 2001; Tamaoki et al., 2004; Kanoh and Rubin, 2010). Macrolides, including azithromycin, clarithromycin, erythromycin, roxithromycin, telithromycin, tilmicosin and tylosin are reported to decrease the *in vitro* and/or *in vivo* LPS-induced production of tumor necrosis factor (TNF)-α, interleukin (IL)-1β and IL-6 as well as PGE_2 (Morikawa et al., 1996; Ianaro et al., 2000; Cao et al., 2006; Sugiyama et al., 2007; Leiva et al., 2008). To date, tulathromycin (Draxxin®) and tildipirosin (Zuprevo®) have been approved for treatment of SRD (EMA, 2008a; 2011). Gamithromycin (GAM), on the other hand, is a 15-membered semi-synthetic macrolide antibiotic of the azalide subclass, which has been recently developed for the treatment and prevention of bovine RD (Huang et al., 2010). However, GAM has not yet been applied in porcine research, which makes it interesting to evaluate its therapeutic applicability in pigs. Recently, Salichs et al. (2013) demonstrated a significant improvement of the clinical condition of pigs with symptoms of SRD, including dyspnea, coughing, depression and fever when a combination of the antibiotic doxycycline and KETO was administered compared to the treatment with doxycycline solely.

The aim of the present study was therefore to investigate the immunomodulatory activities of KETO, GAM and the combination of both drugs in LPS-challenged pigs. Immunomodulation was studied at the level of cytokine production (TNF-α, IL-1β and IL-6), APPs (pig-MAP and CRP), PGE_2; and of the behavioural and febrile responses. It is hypothesized that administration of the combination of KETO and GAM, would be more beneficial compared to the exclusive administration of KETO or GAM due to possible synergistic and/or additive effects.

2. Materials and methods

2.1. Animals

Twenty-six clinically healthy 10-week-old male pigs of a stress resistant breed (Seghers Hybrid) with a mean (± SD) body weight (BW) of 28.5 ± 3.04 kg were housed in group. The animals had free access to feed and drinking water. After a one-week acclimatization period, a double-lumen jugular catheter was inserted for accurate intravenous (IV) LPS administration and blood sampling facilitation.

In brief, after intramuscular (IM) premedication with midazolam, morphine and ketamine, anaesthesia was IV induced with propofol. Following endotracheal intubation, anaesthesia was maintained with isoflurane in a mixture of oxygen and air. Subsequently, the pigs were randomly assigned to receive either a constant rate infusion (CRI) of alfaxalone (*n* = 13) or a CRI of saline (*n* = 13) (Cerasoli et al., 2014). A central venous catheter (two-lumen central venous catheterization set, 7 Fr, 60 cm; Arrow® International) was surgically implanted in the left external jugular vein using a slightly adapted technique from Gasthuys et al. (1999). Briefly, the external jugular vein was surgically exposed after which the catheter was introduced with the Seldinger technique (Seldinger, 1953; 1984) and tunnelled subcutaneously to its dorsal exit point on the dorsal part of the neck. Finally, the catheter was fixed with a suture on the caudal part of the neck and a suture in the midline of the back. The surgical incision at the level of the jugular groove was closed in a two-layer continuous pattern.

In order to reduce the risk of bacterial infections after anaesthesia and surgery, 24 h before and immediately after anaesthesia, the pigs received an IM injection of 3 mg/kg BW sodium ceftiofur (Excenel®, Zoetis).

After surgery, the pigs were housed individually, the catheters were flushed at least once daily with heparinized NaCl 0.9 % (100 IU/mL) and the bandages were changed daily.

2.2. Experimental design

After a three-day recovery period, the pigs were randomly divided into four groups of six animals and an additional control group of two animals, considering that 50% of the

pigs of each group had been assigned to the alfaxalone anaesthesia protocol. All groups received either a single bolus of respectively 12 mg/kg BW GAM (Zactran®; Merial) subcutaneously (SC) in the flank region (*n* = 6), 6 mg/kg BW KETO (Ketofen® 10%; Merial) intramuscularly in the gluteus muscle (*n* = 6), the combination of both drugs (GAM-KETO at again 12 mg/kg and 6 mg/kg, respectively; *n* = 6) or no drugs (LPS, *n* = 6). Following a 1-hour-interval, all these 24 pigs were intravenously challenged with 15 µg/kg BW (15 x 10^3 EU/kg BW) ultrapure LPS from *E. coli* serotype O111:B4 through the proximal lumen of the catheter. The two additional control animals only received an equivalent volume of 0.9% NaCl IV (CONTR, *n* = 2).

Since the SC administration of Zactran® to pigs turned out to be particularly painful, a local anaesthetic, lidocaine hydrochloride (Xylocaine® 2 %; AstraZeneca), was applied simultaneously to GAM injection in both the GAM and GAM-KETO group.

The animals' clinical condition was scored continuously by a qualified veterinarian observing the clinical symptoms and behaviour during the first 8 h after LPS challenge. More specifically, the occurrence of anorexia, nausea, vomiting, tachypnea, dyspnea, shivering and lateral decubitus were registered.

Rectal body temperature (RT) was measured and blood samples were collected from the distal lumen of the catheter into lithium heparinized tubes before (0 h) and at 0.5, 1, 1.5, 2, 2.5, 3, 3.5, 4, 5, 6, 8, 12, 18, 24, 30, 36, 48 and 72 h post administration (p.a.) of LPS. Additionally, 1 mL of blood was collected in a heparin- and indomethacin-coated (10 µg/mL) microcentrifuge tube for PGE_2 analysis, as previously described. Prior to the *in vivo* blood collection, heparin (10 IU; Leo Pharma) was added to the tubes. Indomethacin was added in order to prevent *ex vivo* artefactual eicosanoid generation (Pelligand et al., 2012). Samples were immediately placed on ice and centrifuged (at 2800 *x g* and 1000 *x g*, respectively, for 10 min at 4 °C) within 2 h. Plasma was isolated, aliquoted and stored at ≤ -70 °C until analysis.

All procedures were approved by the Ethical Committee of the Faculty of Veterinary Medicine of Ghent University (EC2013/128).

2.3. Analysis of cytokines, APPs and PGE_2 in porcine plasma

All plasma samples were analysed for detection of the cytokines TNF-α, IL-1β and IL-6; and the APPs CRP and pig-MAP using commercially available enzyme-linked immunosorbent assays (ELISAs). The concentrations of TNF-α, IL-1β and IL-6 as well as CRP, were determined using porcine DuoSet® ELISA Development Systems (R&D Systems) according to the manufacturer's instructions. The suitability of these assays for porcine plasma samples was approved by a preceding in-house validation procedure. In this respect, imprecision (mean intra- and inter-assay coefficients of variation (CV)) and linearity were calculated for each parameter (Maddens et al., 2010). Intra- and inter-assay CV values of < 10 % and < 15 %, respectively, were considered to be acceptable. For the evaluation of linearity, a recovery range of 80 – 120 % for the linear dilutions was postulated. With the exception of IL-1β, all ELISAs were successfully validated and considered suitable for use on porcine plasma samples. For IL-1β, in which an inter-assay CV of 33 % was established, a higher dilution factor was required to obtain more reliable and reproducible results. Yet, the plasma concentration of this cytokine appeared to be relatively low, allowing no further dilution. Plasma levels of pig-MAP were evaluated using a ready-to-use ELISA kit according to the manufacturer's instructions (PigCHAMP Pro Europa S.L.). Optical densities were determined using a plate reader (Multiskan MS Labsystems, Thermo Scientific). Data were analysed by means of DeltaSoft JV (BioMetallics Incorporated).

The plasma concentrations of PGE_2 and its inactive metabolite 13,14-dihydro-15-keto PGA_2 (PGE_2-*met*) were measured using an in-house validated ultra-performance liquid chromatography-tandem mass spectrometry method (UPLC-MS/MS), as previously described. The limit of quantification (LOQ) in plasma was established at 50 pg/mL for both PGE_2 and PGE_2-*met*. The limit of detection (LOD) in plasma was found to be 28.2 and 9.0 pg/mL for PGE_2 and PGE_2-*met*, respectively.

2.4. Statistical analysis

Data were statistically analyzed by single factor analysis of variance (ANOVA), using SPSS Statistics 22.0 software for Windows. Pairwise comparisons of means of the different

groups were performed using the Tukey test. For those parameters that were not normally distributed, the Kruskal-Wallis ANOVA on ranks was used. A *p*-value of <0.05 was considered statistically significant.

3. Results

3.1. Rectal body temperature

At the start of the experiment (time 0 h), the mean RT of all pigs was 39.1 ± 0.55 °C. Following LPS challenge, the mean RT of the LPS and GAM group gradually increased to reach a maximum of 41.1 ± 0.56 °C and 41.0 ± 0.24 °C, respectively, as early as 2.5 h after LPS administration ($p < 0.001$) (Figure 1). The mean RT then steadily decreased to regain a normal RT by 8 h after LPS administration. Conversely, the pigs receiving KETO (both the KETO and GAM-KETO groups) showed no increase in RT. The CONTR group, which received neither LPS nor drugs, also showed no significant changes in RT (Figure 1).

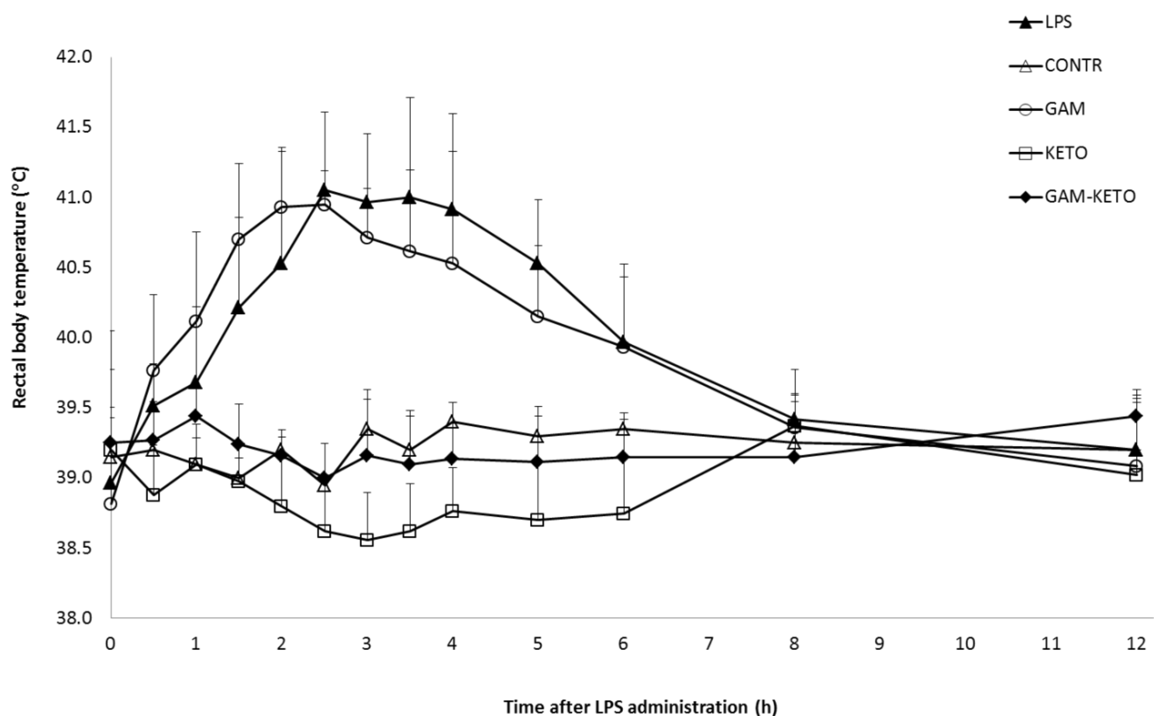


Figure 1. Rectal body temperature after intravenous administration of saline (CONTR; *n* = 2) or LPS (15 µg/kg BW) and pretreatment with gamithromycin (GAM; *n* = 6), ketoprofen (KETO; *n* = 6), the combination of both drugs (GAM-KETO; *n* = 6) or no drugs (LPS; *n* = 6). Data are presented as means + SD.

3.2. Clinical scoring

Table 1 provides an overview of the clinical symptoms occurring after LPS challenge. The severity of these symptoms was comparable between the animals.

At the start of the experiment, all pigs were in good health and displayed no adverse signs. Within 15 min after LPS administration, all pigs of the LPS and GAM groups demonstrated a marked tachypnea (at 11.6 ± 3.51 min and 11.1 ± 3.12 min p.a., respectively) which was followed by a severe dyspnea in most animals of both these groups (at 21.8 ± 1.25 min and 25.8 ± 9.08 min p.a., respectively). All pigs of the LPS and GAM group showed anorexia, as demonstrated by a complete loss of interest in feed and drinking water. Vomiting was preceded by clear signs of nausea, including salivation, chewing movements and retching. With the exception of one pig, vomiting was observed in all animals of the LPS group at 20.9 ± 7.54 min after LPS administration, while in the GAM group, only two pigs vomited. Shivering was observed in all pigs of both groups from 42.0 ± 10.1 min and 29.7 ± 8.82 min, respectively, p.a. of LPS.

Following this first phase of general sickness and the onset of respiratory symptoms, all pigs of the LPS and GAM group experienced a depression phase, which was manifested by lateral decubitus with persistent respiratory distress. However, the start of this depression varied among both groups. Within 1h p.a. of LPS, the majority of the pigs of the GAM group already showed lateral decubitus (at 26.2 ± 19.4 min), while all the pigs of the LPS group showed this depression remarkably later (135 ± 42.3 min) (Table 1).

The recovery phase, which is recognized by regaining alertness, vitality and appetite, already occurred at 335 ± 30.8 min after LPS administration in the LPS group, while the pigs of the GAM group recovered noticeably later (at 416 ± 23.3 min).

After the SC injection of GAM (Zactran®), the pigs of the GAM (*n* = 6) and the GAM-KETO groups (*n* = 6), all showed a local inflammation reaction, which was characterized by an immediate marked and painful swelling at the site of injection. During the first hours of the experiment, this swelling was experienced as extremely uncomfortable.

Apart from two pigs of both the KETO and GAM-KETO group showing tachypnea (Table 1), and the inconvenience related to the Zactran® injection by the GAM-KETO group, the animals of the KETO as well as the GAM-KETO group displayed no adverse effects following LPS challenge. These pigs showed normal behaviour throughout the experiment, as demonstrated by exploring the environment, social interaction, playing, sleeping and eating.

As expected, neither clinical symptoms nor behavioural changes were observed by the two pigs of the CONTR group during the experiment.

Table 1. Overview of the onset of clinical symptoms p.a. of LPS (LPS, GAM, KETO and GAM-KETO groups) or saline (CONTR group)

Clinical symptoms	**LPS (*n* = 6)**		**GAM (*n* = 6)**		**KETO (*n* = 6)**		**GAM-KETO (*n* = 6)**		**CONTR (*n* = 2)**	
	Pigs (%)	**Time p.a. (min.)**	**Pigs (%)**	**Time p.a. (min.)**	**Pigs (%)**	**Time p.a. (min.)**	**Pigs (%)**	**Time p.a. (min.)**	**Pigs (%)**	**Time p.a. (min.)**
Tachypnea	100	11.6 ± 3.51	100	11.1 ± 3.12	33	75.5 ± 6.36	33	27.3 ± 25.1	0	-
Dyspnea	83	21.8 ± 1.25	100	25.8 ± 9.08	0	-	0	-	0	-
Nausea	100	20.5 ± 6.84	67	14.8 ± 5.74	0	-	0	-	0	-
Vomiting	83	20.9 ± 7.54	33	16.5 ± 3.54	0	-	0	-	0	-
Shivering	100	42.0 ± 10.1	100	29.7 ± 8.82	0	-	0	-	0	-
Lateral decubitus ≤ 1h p.a.	0	-	83	26.2 ± 19.4	0	-	0	-	0	-
Lateral decubitus > 1h p.a.	100	135 ± 42.3	17	247	0	-	0	-	0	-
Recovery ≤ 6h p.a.	83	335 ± 30.8	17	310	N/A	-	N/A	-	N/A	-
Recovery > 6h p.a.	17	440	83	416 ± 23.3	N/A	-	N/A	-	N/A	-

Data are presented as mean ± SD; p.a.: post administration; min.: minutes; N/A: not applicable

3.3. Cytokine plasma concentrations

A time-dependent increase of TNF-α and IL-6 was observed in all pigs of the LPS, GAM, KETO and GAM-KETO groups following LPS challenge, whereas the CONTR group presented no detectable or basal levels of both cytokines (Figure 2A and 2B). Significant higher TNF-α levels were observed in the former groups compared to the CONTR group from 0.5 h to 5 h p.a. of LPS ($p < 0.05$). Levels of TNF-α dramatically increased in all groups reaching major mean peak concentrations as soon as 1 h p.a. of LPS (Figure 2A).

While significant higher IL-6 levels were observed in all groups at 2 h p.a. of LPS ($p \leq 0.01$), the KETO group showed significantly increased levels of IL-6 compared to the CONTR group from 1.5 h to 3 h p.a. of LPS ($p < 0.05$). Maximal mean concentrations of IL-6 were recorded after a more gradual increase in these groups at 2.5 h p.a. of LPS (Figure 2B).

Both TNF-α and IL-6 returned to pre-challenge concentrations within 6 h p.a. of LPS. No significant differences were observed between the GAM, KETO and GAM-KETO groups compared to the LPS group for both cytokines.

Although increased, overall low IL-1β plasma concentrations were measured in most pigs of the LPS, GAM, KETO and GAM-KETO groups (data not shown).

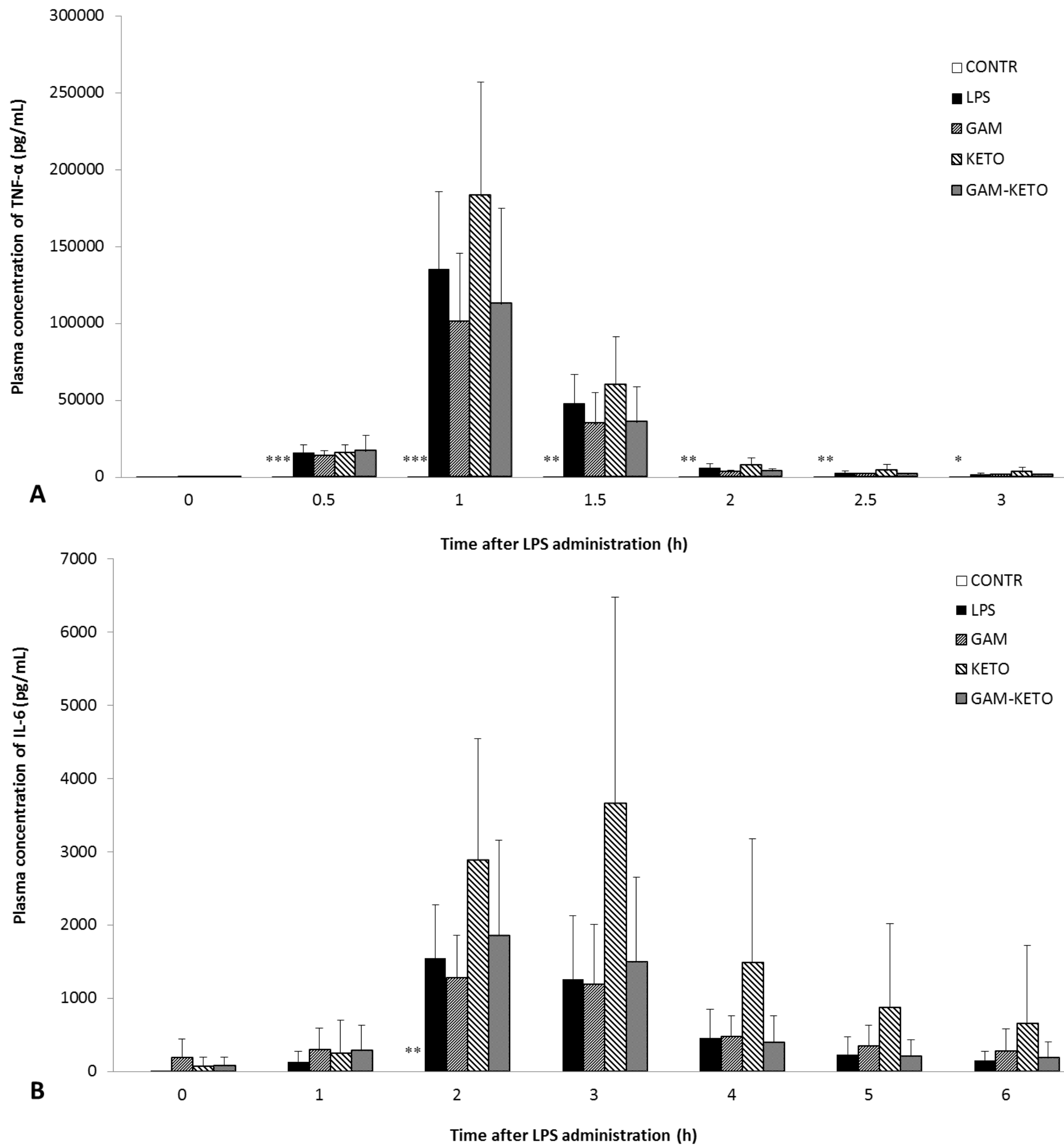


Figure 2. Plasma concentrations of TNF-α (A) and IL-6 (B) after intravenous administration of saline (CONTR; n = 2) or LPS (15 µg/kg BW) and pretreatment with gamithromycin (GAM; n = 6), ketoprofen (KETO; n = 6), the combination of both drugs (GAM-KETO; n = 6) or no drugs (LPS; n = 6). Data are presented as means + SD. Statistically significant differences compared to the LPS group are visualized as * $p < 0.05$; ** $p < 0.01$ and *** $p < 0.001$.

3.4. Acute phase protein plasma concentrations

At the start of the experiment (time 0 h), all pigs showed basal levels of both pig-MAP and CRP. Increased plasma concentrations of both APPs could be observed in all groups following LPS challenge, whereas the CONTR group presented basal levels during the complete course of the experiment (Figure 3A and 3B).

From 18 h until 30 h p.a. of LPS, significant increased pig-MAP levels were observed in all groups receiving LPS compared to the CONTR group ($p \leq 0.01$). Maximal concentrations of pig-MAP were attained around 24 h p.a. of LPS (Figure 3A). At 36 h and 48 h p.a., plasma concentrations of CRP were significantly increased in all groups receiving LPS compared to the CONTR group ($p < 0.01$). For CRP, a marked biphasic profile could be recognized in all groups (Figure 3B). With the exception of the GAM and GAM-KETO groups, showing significant higher concentrations of CRP at 36 h p.a. of LPS ($p < 0.05$), neither GAM nor KETO nor their combination had a significant influence on the production of pig-MAP and CRP compared to the LPS group.

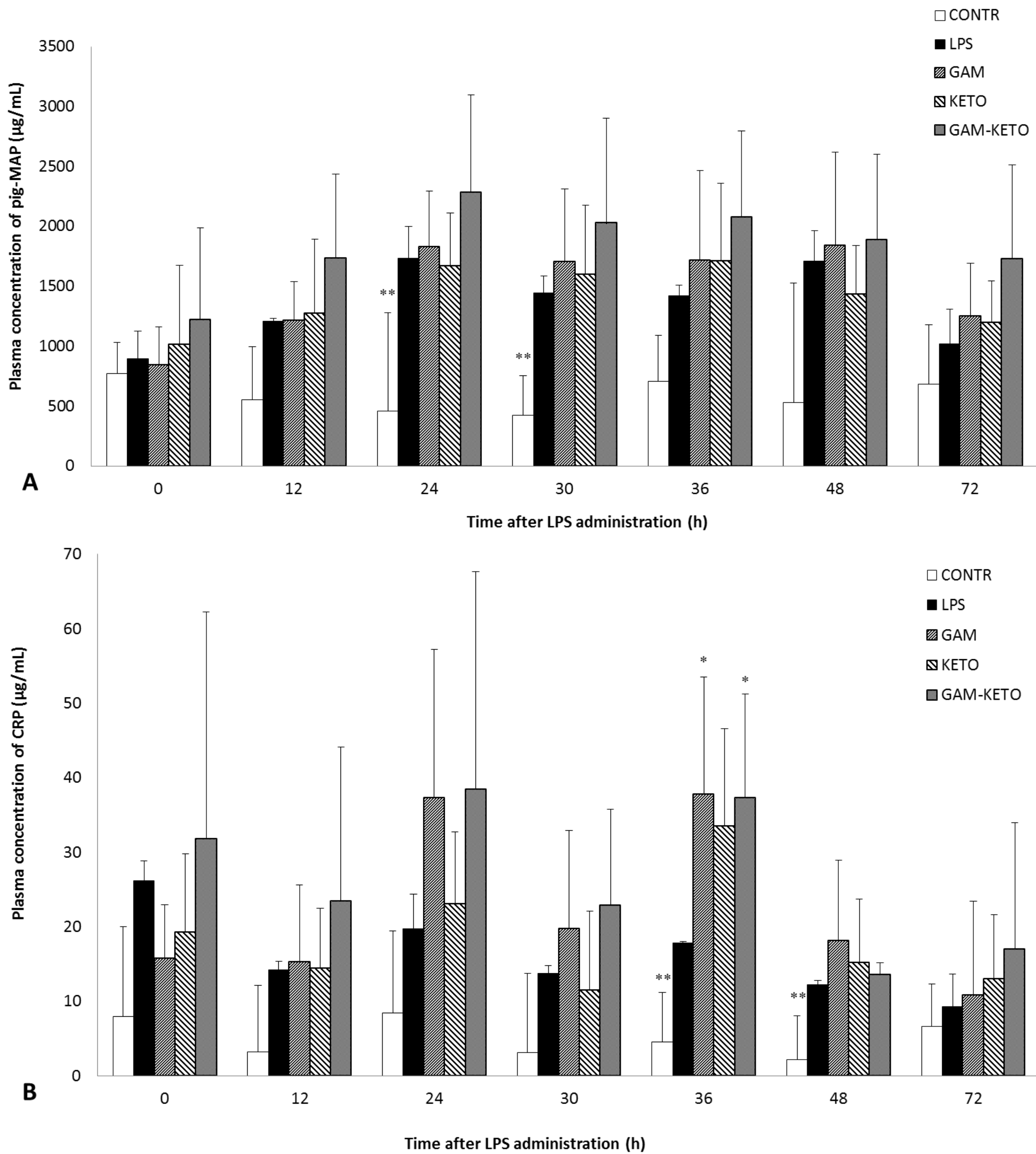


Figure 3. Plasma concentrations of pig-MAP (A) and CRP (B) after intravenous administration of saline (CONTR; *n* = 2) or LPS (15 µg/kg BW) and pretreatment with gamithromycin (GAM; *n* = 6), ketoprofen (KETO; *n* = 6), the combination of both drugs (GAM-KETO; *n* = 6) or no drugs (LPS; *n* = 6). Data are presented as means + SD. Statistically significant differences compared to the LPS group are visualized as * $p < 0.05$ and ** $p < 0.01$.

3.5. Prostaglandin E_2 (-*met*) plasma concentrations

In the LPS and GAM groups, both PGE_2 (data not shown) and PGE_2-*met* increased to reach a peak concentration already at 1 h p.a. of LPS, whereas the CONTR group presented no detectable levels of PGE_2 (data not shown) or PGE_2-*met* (Figure 4).

Administration of KETO significantly reduced PGE_2 production compared to the LPS groups at 1, 1.5 and 2 h p.a. of LPS ($p < 0.05$). Pigs of the KETO and the GAM-KETO groups showed no quantifiable levels of both PGE_2 (data not shown) and PGE_2-*met* (Figure 4).

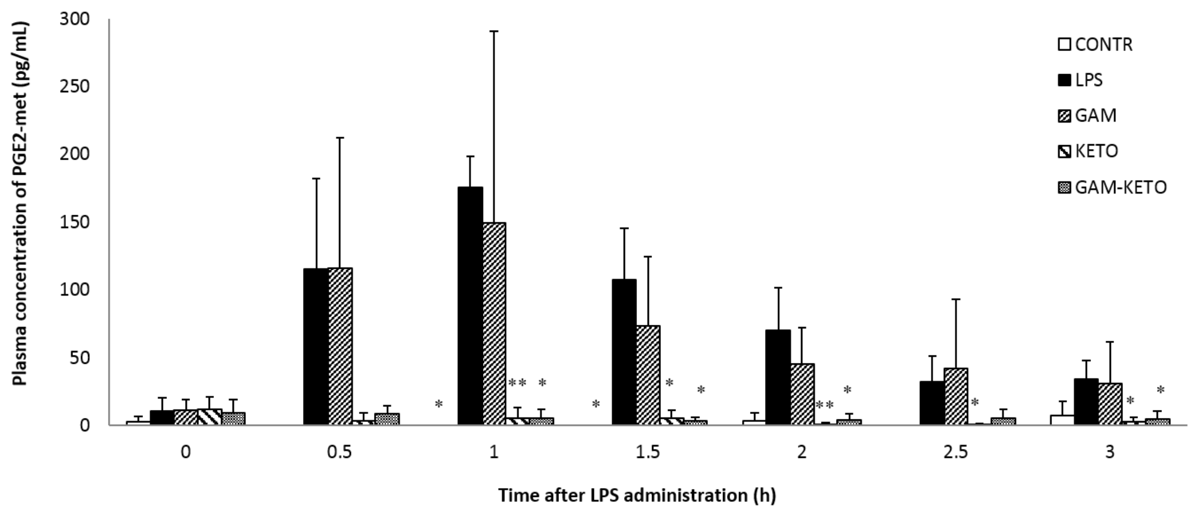


Figure 4. Plasma concentrations of PGE_2-*met* after intravenous administration of saline (CONTR; *n* = 2) or LPS (15 µg/kg BW) and pretreatment with gamithromycin (GAM; *n* = 6), ketoprofen (KETO; *n* = 6), the combination of both drugs (GAM-KETO; *n* = 6) or no drugs (LPS; *n* = 6). Data are presented as means + SD. Statistically significant differences compared to the LPS group are visualized as * $p < 0.05$ and ** $p < 0.01$.

4. Discussion

The present study is the first to report the possible immunomodulatory properties of GAM and KETO as well as their combination in LPS-challenged pigs. Therefore the influence of both drugs on the production of pro-inflammatory cytokines, APP and PGE_2, as well as the development of fever, pulmonary symptoms and sickness behaviour was investigated.

All 26 pigs experienced an acclimatization period in which the animals were habituated to human presence, contact and experimental manipulations, including measurement of the RT. The pigs of the CONTR group were included in the experiment to exclude possible interfering influences related to handling stress (Straw et al., 1999; Clark and Coffer, 2008). These pigs showed neither clinical symptoms nor an increase in RT, systemic cytokines or APP during the experiment.

E. coli LPS has been widely applied in porcine research to study the APR (Frank et al., 2003; Llamas Moya et al., 2006; Williams et al., 2009; Wyns et al., manuscript submitted). Pigs are very sensitive to the administration of LPS since relatively low doses induce pronounced cardiovascular and pulmonary effects. In this respect, the LD_{100} for *E. coli* LPS has been established to be 500 µg/kg BW for pigs (Schrauwen and Houvenaghel, 1985; Olson et al., 1995). The dose of 15 µg/kg BW (15 x 10^3 EU/kg BW) LPS used in this study, which can be considered as moderate, was selected to provoke an apparent inflammation reaction, without causing mortality. *E. coli* serotype O111:B4 is the most frequently used one in porcine research (Williams et al., 2009; Wyns et al., manuscript submitted). In contrast to most commercial LPS preparations, the ultrapure LPS variant used in this study selectively activates TLR4 signaling, providing a more univocal immune response.

LPS was supplied 1 h after IM and/or SC administration of KETO and/or GAM, respectively, when nearby maximal concentrations of both drugs were expected to be present in plasma. Indeed, the maximal plasma concentration (C_{max}) of KETO has been reported to occur between 0.68 and 1.27 h after a single IM administration in pigs (Raekallio et al., 2008, Fosse et al., 2011; Mustonen et al., 2012b), while the C_{max} of GAM was established at 0.63 h following a single SC administration in pigs (Wyns et al., 2014).

Clear symptoms of acute lung failure were induced in the LPS and GAM groups. As fast as within 15 min after LPS administration, all pigs of both the LPS and GAM group displayed a marked tachypnea followed by a severe dyspnea. The occurrence of these early symptoms and the increased sensitivity of pigs to LPS in general, might be explained by the abundant presence and activation of mononuclear phagocytic cells within the porcine and bovine pulmonary capillaries. These pulmonary intravascular macrophages (PIMs) comprise a major resident population of differentiated lung macrophages junctionally anchored to the lung endothelium (Winkler and Cheville, 1985). In humans and laboratory animal

species, such as rats and mice, clearance of particles from the circulation predominantly occurs by hepatic Kupffer cells and splenic macrophages. In pigs and ruminants, on the other hand, PIMs are responsible for the rapid and efficient removal of blood-borne particles, including LPS, even before their hepatic passage (Winkler, 1988; Brain et al., 1999). However, besides these excellent clearance capacities for blood LPS, PIMs also considerably contribute to the inflammatory response and the development of acute lung failure by the excessive release of inflammatory mediators, including vasoactive eicosanoids, after pathogen phagocytosis (Bertram, 1986; Winkler, 1988). It is therefore hypothesized that PIMs respond to LPS with a rapid and massive release of thromboxane A_2 (TXA_2), resulting in a severe pulmonary hypertension and possibly right heart dysfunction in pigs and calves (Ball et al., 1986; Klosterhalfen et al., 1992; Plessers et al., manuscript submitted). In pigs, peak plasma concentrations of TXB_2, a stable metabolite of TXA_2, are reported to appear as soon as 30 to 45 min after LPS challenge (Klosterhalfen et al., 1992; Mustonen et al., 2012a). Furthermore, the cytoplasmic presence of LPS in PIMs was confirmed as soon as 8-10 min after the IV administration of *E. coli* LPS in sheep, simultaneously with the development of pulmonary interstitial oedema (Singh and Atwal, 1997). Also in pigs, LPS was found in lung macrophages (Nakajima et al., 1995). In the present study, pigs of the LPS and GAM groups showed marked respiratory symptoms before 15 min after LPS challenge, which is largely in accordance with the above-mentioned findings. Administration of the COX-inhibitor KETO successfully prevented the development of severe pulmonary symptoms after LPS challenge in pigs of both the KETO and GAM-KETO groups, suggesting the involvement of eicosanoids. Only two pigs of both the KETO and GAM-KETO group showed a moderate and delayed tachypnea, while their clinical condition was not compromised. This might be attributed to the presence of high circulating TNF-α levels in both animals, since the latter cytokine has been reported to be, along with TXA_2, involved in the pathogenesis of hypoxia, pulmonary oedema and hypertension (Li et al., 1995; Petrache et al., 2003).

It is generally accepted that the prostaglandin PGE_2 is responsible for the development of fever in mammals. It exerts its pyrogenic action by elevating the body temperature set point at the thermoregulatory center in the preoptic area of the anterior hypothalamus. Subsequently, fever is developed by an increased heat production and decreased heat loss (Baumann and Gauldie, 1994; Netea et al., 2000; Blatteis et al., 2005).

The half-life of PGE_2 is estimated to be only a few seconds in plasma (Hamberg and Samuelsson, 1971; Bygdeman, 2003; Ivanov and Romanovsky, 2004). Due to this very rapid metabolism and the instability of its main metabolite 13,14-dihydro-15-keto PGE_2, it is often preferred to quantify a more stable metabolite which in turn reflects the PGE_2 concentration (Granström et al., 1980). In the current study, the concentrations of both PGE_2 and its inactive dehydration product PGE_2-*met* were measured. Maximal plasma concentrations of PGE_2 (data not shown) and PGE_2-*met* were observed already at 1 h after LPS administration in the LPS and GAM groups. Accordingly, the RT of the pigs of both groups started to increase as soon as 0.5 h after LPS administration, reaching a maximum already at 2.5 h p.a. and normalizing by 8 h p.a. Earlier reports in pigs demonstrated a maximal febrile response at 4 h following LPS challenge (Johnson and von Borell, 1994; Warren et al., 1997; Leininger et al., 2000; Wright et al., 2000; Mustonen et al., 2012a). The occurrence of shivering in all pigs of the LPS and GAM groups was an external reflection of this enhanced heat production. In the present study GAM showed no inhibiting effect on the production of PGE_2, which is inconsistent with some previous *in vitro* and *in vivo* studies on macrolide antibiotics (Ianaro et al., 2000; Cao et al., 2006). Since pigs of the KETO and GAM-KETO groups, on the other hand, showed no increase in either RT or PGE_2 levels, a crucial role of PGE_2 in the porcine febrile response was suggested. These *in vivo* results also confirmed our earlier *in vitro* data, as no PGE_2 levels could be measured in the supernatant of LPS-stimulated porcine peripheral blood mononuclear cells (PBMCs) in both the KETO and GAM-KETO group. While PGE_2-*met*, on the other hand, could not be measured *in vitro* in a buffer with similar pH and temperature, *in vivo* plasma levels of PGE_2-*met* were formed considerably faster (Granström et al., 1980).

As expected, increased levels of pro-inflammatory cytokines were observed following IV LPS administration. Plasma concentrations of TNF-α reached a major and sharp peak at 1 h p.a. of LPS in all groups, while IL-6 showed a more gradual increase and attained maximal levels at 2.5 h after LPS challenge in all groups. These findings in relation to these pro-inflammatory cytokines were largely in accordance to previous reports in pigs (Frank et al., 2003; Carroll et al., 2005; Williams et al., 2009). In contrast to TNF-α and IL-6, plasma concentrations of IL-1β could not be measured reproducibly. The latter results were in accordance with previous studies in which IL-1β has been reported either not detectable or not increasing in plasma after LPS challenge in pigs (Myers et al., 2003; Tuchscherer et

al., 2004; Llamas Moya et al., 2006; Ruud et al., 2007). In contrast, a significant increase of IL-1β following LPS administration has yet been formerly described in pigs (Frank et al., 2003; John et al., 2008; Williams et al., 2009). In previous research from our group, a ready-to-use ELISA (Quantikine®, R&D Systems), approved for use in porcine plasma, was applied and yielded a significant, albeit low maximal concentration of IL-1β at 3 h after LPS administration (Wyns et al., 2013). Since the less expensive ELISA (DuoSet®, R&D Systems) used in the present study was considered not suitable for measurement of IL-1β in porcine plasma and the IL-1β concentrations are again indicated to be relatively low, multiplex analysis of TNF-α, IL-1β and IL-6 might be a valuable and more sensitive alternative, as previously demonstrated (Wyns et al., 2013). The administration of neither GAM, KETO nor the combination of both drugs before LPS challenge, had a significant influence on the amplitude of the TNF-α and IL-6 concentrations after LPS challenge compared to the untreated LPS group. Although Donalisio et al. (2013) recently described an inhibiting effect of KETO on the *ex vivo* LPS-induced TNF-α production in dairy cows, it has previously been put forward that KETO has not the ability to inhibit NF-κB activation (Matasić et al., 2000). In our experiments, on the contrary, we have noticed that pigs of the KETO group even tended to show slightly enhanced plasma concentrations, yet not significant, of TNF-α and IL-6 after LPS challenge. Similar results were obtained *in vitro*, since LPS-stimulated porcine PBMCs of the KETO groups showed a trend to produce higher levels of TNF-α. Therefore, the current results *in vivo* confirm that NSAIDs, including KETO rather increase than decrease TNF-α production which has been associated with a significant increased mortality in animal models of endotoxic shock (Pettipher and Wimberly, 1994; Ghezzi et al., 1998; Roth et al., 2002). It has been suggested that PGE_2 is a feedback inhibitor of TNF-α production (Kunkel et al., 1988; Sironi et al., 1992). As mentioned before, no PGE_2 levels could be quantified in the KETO group. Thus, and in marked contrast to most macrolide antibiotics studied so far, including azithromycin, clarithromycin, erythromycin, roxithromycin, telithromycin, tilmicosin and tylosin, GAM had no inhibiting effect on the LPS-induced production of cytokines in pigs. However, some macrolides have been occasionally reported to have no significant effect on cytokine production (Bailly et al., 1991b; Er and Yazar, 2012).

General sickness behaviour, including loss of appetite, nausea, vomiting and depression, is, along with the febrile response, regarded as a strategy of the host to

counter infection. These physiological changes are centrally mediated and pro-inflammatory cytokines are assumed to have a major role in this process. Indeed, intracerebroventricular injection of TNF-α to pigs induces anorexia and depression (Warren et al., 1997). Two major pathways have been postulated to explain how peripherally released, hydrophilic cytokines can transfer immune signals to the central nerve system as they cannot passively pass the blood-brain barrier. These include a fast neural transmission via afferent vagal nerves from the site of inflammation and a slower humoral transmission to induce local cytokine production in the choroid plexus and circumventricular organs, where the blood-brain barrier is deficient. Additionally, local production of mediators, including PGE_2 occurs in endothelial cells of brain vessels in animal and man (Konsman et al., 1999; Conti et al., 2004; Dantzer, 2009). IL-1β has been put forward to be a key cytokine for induction of behavioural changes following LPS challenge (Bluthé et al., 1991; 2000; Dantzer, 2009). Neuronal IL-1 receptors have been established in the circumventricular organ of the brainstem, where the *area postrema* is located. This could possibly explain the anorexia, nausea and vomiting observed following LPS challenge (Ericsson et al., 1995). Interestingly, the early clinical symptoms observed following LPS administration in pigs of the LPS and GAM group preceded the increasing levels of peripheral pro-inflammatory cytokines. Already at 15 and 20 min after LPS challenge, clear signs of nausea and subsequently vomiting were observed, respectively, whereas increasing plasma levels of TNF-α and IL-6 were recorded not earlier than 0.5 and 1.5h after LPS administration, respectively. Additionally, similar to the pigs of the LPS and GAM groups, pigs of the KETO and GAM-KETO groups displayed high cytokine levels in plasma, while showing no sickness behaviour at all. It has already been demonstrated that LPS-induced behavioural changes in pigs can be reversed by pretreatment with a COX-inhibitor (Johnson and von Borell, 1994; Friton et al., 2006; Mustonen et al., 2012a; Peters et al., 2012). The results of the present study therefore question the fundamental role for pro-inflammatory cytokines in the LPS-mediated early sickness behaviour and rather suggest a pivotal role for arachidonic acid metabolites, more specifically PGE_2. While COX-1 is mainly constitutively expressed in most cell types and is responsible for the production of PGs involved in a number of physiological functions, COX-2 is induced by diverse inflammatory stimuli and is responsible for the production of PGs in several inflammatory processes (Vane, 1971; Curry et al., 2005). Indeed, in contrast to the pigs of the LPS and GAM groups, those of the KETO and GAM-

KETO groups, which were all pretreated with the COX-inhibitor KETO, demonstrated no quantifiable levels of PGE_2 (data not shown) or PGE_2-*met*, nor sickness behaviour. Paradoxically, the NF-κB-regulated transcription and *de novo* synthesis of cytokines and subsequently COX-2 requires considerable time, about 90 min, and hence the role of COX-2 in generating early clinical symptoms or fever is controversial (Conti et al., 2004; Teeling et al., 2010). Of relevance, it has been ultimately hypothesized that COX-1-mediated PGE_2 is induced by the immediate complement cascade activation by LPS and binding of the anaphylatoxic complement component C5a to hepatic Kupffer cells as soon as minutes after LPS administration (Blatteis et al., 2005). The PGE_2 immune signal is then transferred to the brain via hepatic afferent nerves and possibly by passing the blood-brain barrier or diffusion in the brain parenchyma (Blatteis et al., 2005; Blatteis, 2007). Since it has been demonstrated that PIMs and not the Kupffer cells are the principal LPS-clearing mononuclear phagocytes in pigs, it is likely that the latter cells considerably contribute to the instant peripheral PGE_2 synthesis after LPS challenge. Indeed, KETO is a non-selective COX-inhibitor, but is repeatedly reported to be relatively COX-1 selective in different animal species (Streppa et al., 2002; Curry et al., 2005; Donalisio et al., 2013). This might to some extent explain the often superior anti-inflammatory and antipyretic capacities of KETO compared to other, recently developed mainly COX-2 selective NSAIDs, including firocoxib (Jackman et al., 1994; Swinkels et al., 1994; Mustonen et al., 2012a; Salichs et al., 2012). Interestingly, pigs of the GAM group showed a remarkable early and long-lasting depression phase following LPS challenge. Within one half h p.a. of LPS, most of the animals were already in lateral decubitus, which persisted until 7 h p.a. of LPS. It is generally accepted that macrolides accumulate within inflammatory cells, including monocytes and neutrophils, and thus can be accurately delivered in high concentrations to the bacterial infection site (Gladue et al., 1989; Čulić et al., 2001). Azalides, such as azythromcin and GAM have been reported to concentrate preferably within lung macrophages (Mattoes and Nightingale, 2002; Huang et al., 2010). Since the lung is a major target for both circulating LPS and GAM, it is not unthinkable that porcine PIMs and PIM-related inflammatory mediators, including TXA_2, contribute to the more severe symptoms of pigs of the GAM group in comparison to those of the LPS group. TXA_2 has indeed been associated with more severe clinical symptoms (Ball et al., 1986; Mustonen et al., 2012a). Additionally, excipients included in the Zactran® formulation might also contribute to those

symptoms. Remarkably, only one pig of the GAM group showed lateral decubitus at a far later stage p.a. of LPS. However, this particular pig experienced a notable long-lasting irritation after its SC Zactran® injection, which appeared to prevent lateral decubitus despite severe LPS-related symptoms.

Low basal concentrations of both APPs were detectable prior to LPS administration in all pigs and were in accordance with previous reports (Heegaard et al., 1998; Llamas Moya et al., 2006; Williams et al., 2009). Consequently, a major, negative impact of the previous surgery on APP levels could be excluded. Following LPS challenge, increased levels of pig-MAP and CRP were observed in all groups. The administration of GAM, KETO or the combination of both drugs (GAM-KETO) before LPS challenge revealed no major influences on APP production after LPS challenge. Similarly, it has been reported that meloxicam had no effect on the production of CRP and haptoglobin in LPS-challenged pigs (Friton et al., 2006). Er and Yazar (2012), on the other hand, reported a decrease in LPS-induced CRP levels in bronchoalveolar lavage fluid of rats by tylosin, tulathromycin, and to a lesser extent by tilmicosin. However, at 36 h p.a. of LPS, the GAM and GAM-KETO groups demonstrated significantly higher concentrations of CRP compared to the LPS group. This might be explained and looks to be linked to the marked local inflammation reaction observed in pigs of the GAM and GAM-KETO group following the SC injection of the used GAM formulation. Local irritation has also been quoted after administration of macrolides (Zhanel et al., 2001). In some reports, local inflammation induced by a SC turpentine injection has been reported to elicit also considerable systemic CRP concentrations in pigs (Lampreave et al., 1994; Eckersall et al., 1996).

In conclusion, KETO was demonstrated to have a clear inhibiting effect on PGE_2 production and the development of fever, pulmonary symptoms and general sickness behaviour. Therefore, it can be hypothesized that the combination of GAM and KETO, would be beneficial compared to the administration of exclusively GAM. Preliminary *in vitro* research indeed confirmed the inhibition of TNF-α, yet not significant, IL-1β and IL-6 production by LPS-stimulated PBMC when adding both GAM and KETO to the cells. Apart from the inconvenience and local inflammation reaction related to the Zactran® injection, no differences were observed between KETO and GAM-KETO concerning clinical symptoms, RT, cytokines and APP in this study. However, beneficial effects of GAM could be demonstrated in an appropriate bacterial infection model.

Acknowledgements

The help of G. Antonissen, N. Broekaert, T. De Mil, S. Fraeyman, J. De Loor, J. Lambrecht, V. De Rop, I. Cerasoli and the surgery staff during the animal experiments was gratefully appreciated. Special thanks to Prof. dr. F. Gasthuys for his excellent guidance during the surgical catheterizations of the pigs.

CHAPTER 3.

Comparative study of immuno-assays for cytokine detection:

ELISA vs flow cytometry

3.1. Development of a cytometric bead array screening tool for the simultaneous detection of pro-inflammatory cytokines in porcine plasma

Adapted from

Wyns, H., Croubels, S., Demeyere, K., Watteyn, A., De Backer, P., Meyer, E. (2013) Development of a cytometric bead array screening tool for the simultaneous detection of pro-inflammatory cytokines in porcine plasma. *Veterinary Immunology and Immunopathology* 151, 28-36.

Abstract

Lipopolysaccharide (LPS) has been widely used as a model of immune challenge in pigs as it induces the immediate synthesis of pro-inflammatory cytokines such as tumor necrosis factor (TNF)- α, interleukin (IL)-1β and IL-6, which trigger the production of the acute phase proteins (APPs) C-reactive protein (CRP), haptoglobin (Hp) and pig-major acute phase protein (pig-MAP). To measure secreted proteins in porcine plasma, specific and sensitive enzyme-linked immuno sorbent assays (ELISAs) are well-suited to perform single parameter analysis, yet this approach is time-consuming and expensive for multi-parameter analyses. During the last decade, multiplex bead-based flow cytometry has been increasingly applied as it offers the opportunity to estimate protein ratios in a small sample volume.

Cytometric bead array (CBA) is a flow cytometric application using a diversity of beads with unique fluorescence intensities, covalently coupled to a capture antibody for each protein of interest. Detection antibodies, either directly or indirectly conjugated to a fluorochrome, are added to accomplish the desired sandwich format. The aim of the present study was to develop a CBA 3-plex assay for the major pro-inflammatory cytokines TNF-α, IL-1β and IL-6, and an additional CBA 2-plex assay for the major APPs, CRP and pig-MAP, in porcine plasma. Results were compared to commercial ELISA kits. For the CBA 3-plex assay, the limits of detection (LODs) varied between 0.005 and 0.363 ng/mL, the intra- and inter-assay coefficients of variation were < 10 % and < 16 %, respectively. For TNF-α, IL-1β, IL-6 and pig-MAP, CBA time-concentration profiles similar to those obtained with commercial ELISAs were observed. In conclusion, the novel validated CBA 3-plex assay provides a fast and economical screening tool for determination of pro-inflammatory cytokine profiles in limited porcine plasma volumes. This tool will be applied to study the immunomodulatory properties of drugs in a porcine LPS inflammation model. This study also demonstrated the applicability of CBA for measurement of APPs in pigs, although a different combination than pig-MAP with CRP is recommended.

1. Introduction

Lipopolysaccharide (LPS), an intrinsic component of the outer membrane of Gram-negative bacteria such as the ubiquitous *Escherichia coli (E. coli)*, is a potent stimulator of the immune system. When released from bacteria during cellular division or lysis, it elicits the synthesis and release of pro-inflammatory cytokines such as tumor necrosis factor (TNF)-α, interleukin (IL)-1β and IL-6, which orchestrate the acute phase response by inducing fever and stimulating hepatocyte production of acute phase proteins (APPs) (Baumann and Gauldie, 1994; Feghali and Wright, 1997). The immune response following challenge with a diversity of LPS doses and serotypes has been widely studied in pigs (Webel et al., 1997; Frank et al., 2003; Myers et al., 2003; Carroll et al., 2005; Llamas Moya et al., 2006; Williams et al., 2009). Important APPs in this species are C-reactive protein (CRP) and haptoglobin (Hp) (Lampreave et al., 1994; Eckersall et al., 1996; Alava et al., 1997). Less studied, is a 120-kDa glycoprotein, named pig-major acute phase protein (pig-MAP), though the latter has been described as particularly relevant in pigs (Lampreave et al., 1994) and as an excellent biomarker of different porcine pathologies (Alava et al., 1997).

To measure secreted proteins in porcine plasma or serum, researchers commonly rely on enzyme-linked immuno sorbent assays (ELISAs) (Frank et al., 2003; Llamas Moya et al., 2006; Williams et al., 2009). Indeed, ELISAs are highly specific and sensitive and are still considered as the gold standard (Elshal and McCoy, 2006). However, they are restricted to the quantitation of only one parameter at a time and are consequently time-consuming and expensive for multi-parameter screening analyses. Moreover, since the procedure has to be repeated for every analyte of interest it requires multiple sample aliquots. Additionally, dilution steps are often indispensable to fit the generally narrow calibration range. Therefore, porcine research would benefit from a more elegant immuno-assay strategy allowing the fast simultaneous profiling of a number of selected parameters in a limited sample volume. In line with this need, an increasing number of multiplex bead-based flow cytometric protocols were reported which capture the secreted analytes of interest on target particles (Carson and Vignali, 1999; Jiménez et al., 2005; Heijmans-Antonissen et al., 2006). Both the Luminex xMAP and the cytometric bead array (CBA) technologies use a diversity of beads with unique fluorescence intensities, covalently

coupled to a capture antibody for each protein of interest. Subsequently, detection antibodies, either directly or indirectly conjugated to a fluorochrome, are added to accomplish the desired sandwich format. Compared to ELISA, multiplex bead-based assays minimize the sample volume and analysis time while maximizing the number of proteins that can be simultaneously analyzed (Morgan et al., 2004). The latter approach also provides a wider dynamic range limiting the required number of dilutions. Furthermore, particles in suspension are assumed to be more efficient in capturing soluble antigens than the immobilized antibodies on the surface of ELISA wells (Tarnók et al., 2003). While ELISAs typically use enzyme amplification of a colorimetric substrate, multiplex bead-based assays use a fluorescence reporter system. In addition, multiplexing allows to estimate the protein ratio in a sample, which might be even more biologically relevant than absolute concentrations since interacting proteins can significantly alter each other's functions (Kelso, 1998). Another advantage of multiplex bead-based assays is their flexibility, enabling addition and withdrawal of proteins (Tarnók et al., 2003). Nevertheless, a powerful CBA tool is currently lacking in porcine research.

Generally, multiplex bead-based assays for human and murine cytokines are well-documented in marked contrast to the scarce reports for their porcine homologues. In 2006, Johannisson *et al.* multiplexed 3 porcine pro-inflammatory mediators, i.e. TNF-α, IL-1β and IL-8, in pig serum and plasma by means of the Luminex xMAP technology. More recently, Bjerre et al. (2009) developed both a 4-plex Luminex assay for TNF-α, IL-6, IL-8 and IL-10, and a 2-plex assay for IL-1β and heat shock protein (Hsp) 32 in pig plasma. In 2010, Lawson et al. created an 8-plex Luminex assay to simultaneously detect the swine cytokines TNF-α, IL-1β, IL-4, IL-8, IL-10, IL-12, IFN-α and IFN-γ. These Luminex assays require a specialized Luminex instrument. CBA, on the other hand, can be performed on a common dual-laser flow cytometer, which is an attractive feature of CBA (Elshal and McCoy, 2006).

The aim of this study was to develop a CBA 3-plex assay combining for the first time the major pro-inflammatory cytokines TNF-α, IL-1β and IL-6, and an additional CBA 2-plex assay for the major APPs CRP and pig-MAP in porcine plasma after LPS-challenge. Using commercial ELISAs for each of these mediators, a critical comparison between both immuno-assays was made.

2. Materials and methods

2.1. Experimental design

Ultrapure LPS from *E. coli* serotype O111:B4 was purchased from Cayla-InvivoGen. Four male pigs (Seghers Hybrid), with a mean (± SD) body weight (BW) of 24.9 ± 3.17 kg were intravenously challenged with 15 µg LPS/kg BW through the proximal lumen of a double-lumen jugular catheter (13 G, 60 cm, Arrow) (Gasthuys et al., 2009). Rectal body temperature was measured and blood samples were collected from the distal lumen of the catheter at 0, 0.5, 1, 1.5, 2, 2.5, 3, 3.5, 4, 6, 12, 24, 36 and 48 h after LPS administration. The samples were collected in EDTA-coated tubes, immediately placed on ice and centrifuged (500 *x g* for 10 min at 4 °C) within 2 h. Subsequently, plasma was isolated, aliquoted into polypropylene microcentrifuge tubes and stored at ≤ -70 °C until analysis.

All procedures were approved by the Ethical Committee of the Faculty of Veterinary Medicine of Ghent University (EC 2009/099 and 2010/072).

2.2. Covalent coupling of capture antibodies to functional beads

A CBA Functional Bead Conjugation Buffer Set was purchased from Becton Dickinson Biosciences (BD). Capture antibodies were covalently coupled to the surface of different color-coded 7.5 µm polystyrene functional beads according to the manufacturer's instructions (Table 1). All functional beads have distinct fluorescence intensities and an alphanumeric label (A4 to E9) is assigned to each bead population, indicating its position relative to the other beads. As a result, beads with different positions can be combined to create a multiplex assay. DL-Dithiothreitol (DTT) and N-ethylmaleimide (NEM) (Sigma-Aldrich), sulfosuccinimidyl-4-(N-maleimidomethyl)cyclohexane-1-carboxylate (sulfo-SMCC) (Thermo Scientific) and Bio-Spin® 30 Tris Columns (Bio-Rad Laboratories) were used. The integral procedure was carried out at room temperature efficiently protecting functional beads and antibodies from light. Washing step centrifugations were performed at 900 x g for 3 min and incubation was performed on a shaker (BioSan Mini-Rocker).

Briefly, the selected beads (at positions A7, B4, B8, D5 and D9) were vortexed thoroughly for 30 s after which 75 µL of bead suspension was transferred into a

polypropylene microcentrifuge tube and sonicated during 1 min. After addition of 1.9 µL 1M DTT the tube was vortexed for 5 s and incubated for 1 h. Subsequently, the beads were washed 4 times with 1 mL of coupling buffer and the bead pellet was finally resuspended in 20 µL of coupling buffer. Next, 2 µL of sulfo-SMCC was added to 90 µg of capture antibody at a stock concentration of 1 mg/mL in a polypropylene microcentrifuge tube to accomplish the protein modification. The tube was again vortexed for 5 s and incubated for 1 h. A Bio-Rad Spin Column was inverted vigorously several times, placed into a 12 x 75 mm polystyrene test tube (BD) and centrifuged at 1000 x g for 2 min to remove the packing-buffer. The Spin Column was then completely filled 3 times with coupling buffer, placed into a clean test tube and centrifuged at 1000 x g for 1 min. In order to remove unreacted components, the antibody/sulfo-SMCC solution was completely transferred to the Spin Column and centrifuged at 1000 x g for 2 min and 15 s. The modified antibody was then transferred to the tube with beads, again vortexed for 5 s and incubated for 1 h. After addition of 2 µL of NEM, the tube was vortexed for 5 s and incubated for 15 min. Subsequently, the beads were washed 4 times with 1 mL of storage buffer. Finally, the bead pellet was resuspended in 500 µL of storage buffer at a final concentration of approximately 6×10^6 beads/mL and stored in the dark at 2 - 8°C.

Table 1. Antibodies, beads and standards used in the CBA 3-plex assay

Analyte	Reagent	Catalog #	Description / Beadposition	Source
TNF-α	capture Ab	MP390	mAb Mouse IgG_1	Thermo Scientific
	detection Ab	MP391	mAb Mouse IgG_1	Thermo Scientific
	standard	RPTNFAI	recombinant protein	Thermo Scientific
	functional bead	558657	D9	BD
IL-1β	capture Ab	MAB6811	mAb Mouse IgG_1	R&D Systems
	detection Ab	BAF681	Biotinylated pAb Goat IgG	R&D Systems
	standard	681-PI	recombinant protein	R&D Systems
	functional bead	560035	B4	BD
IL-6	capture Ab	AF686	pAb Goat IgG	R&D Systems
	detection Ab	BAF686	Biotinylated pAb Goat IgG	R&D Systems
	standard	686-PI-025	recombinant protein	R&D Systems
	functional bead	560033	A7	BD
Pig-MAP	capture Ab	AbM1*	mAb Mouse IgG_{2A}	PigCHAMP Pro Europa S.A.
	detection Ab	AbM3*	mAb Mouse IgG_1	PigCHAMP Pro Europa S.A.
	standard	ELISA**	diluted serum fraction	PigCHAMP Pro Europa S.A.
	functional bead	558583	D5	BD
CRP	capture Ab	18-374-130062	pAb Chicken IgY	GenWay Biotech
	detection Ab	MAB1707	mAb Mouse IgG_{2A}	R&D Systems
	standard	ELISA**	-	ALPCO Diagnostics
	functional bead	558581	B8	BD

*The pig-MAP antibodies and standard were kindly donated by Dr. M. Piñeiro (PigCHAMP Pro Europe S.A.); **The standards were used from the respective ELISA kits

Coupling of antibodies to the beads was confirmed by appropriate fluorochrome-conjugated secondary antibodies. Depending on the used fluorochrome, analyses were performed either on a BD FACSCanto™ flow cytometer, equipped with a 488 and 633 nm laser or on a BD FACSArray™ Bioanalyzer, equipped with a 532 and 635 nm laser. When the signal of the test sample was at least a median fluorescence intensity (MFI) of 500 higher than the MFI signal of the negative control sample, the coupling was considered successful.

2.3. Conjugation of detection antibodies to R-Phycoerythrin

A Lightning-Link™ (LL) R-Phycoerythrin (R-PE) conjugation kit was used for the R-PE conjugation of the TNF-α, CRP and pig-MAP detection antibodies according to the manufacturer's instructions (Innova Biosciences).

2.4. CBA multiplex assays

2.4.1. CBA 3-plex assay for TNF-α, IL-1β and IL-6

Antibodies, beads and standards used in the CBA 3-plex assay are listed in Table 1. The CBA Human Enhanced Sensitivity Master Buffer Kit was also obtained from BD. Indeed, because of the inclusion of the more sensitive commercial biotinylated detection antibodies in this porcine CBA, a two-step detection system was required. Bead-coupled capture antibodies (A7, B4 and D9) were sonicated and vortexed. For the assay, 1 µL/test of each capture bead was mixed and diluted in capture bead diluent to a total volume of 50 µL/test. Recombinant porcine TNF-α, IL-1β and IL-6 were carefully mixed and diluted in assay diluent to generate appropriate standard curves (0.0, 0.5, 1.0, 2.5, 5.0, 7.5, 10, 20, 50 ng/mL). EDTA-plasma samples were vortexed thoroughly and diluted 1/4 with assay diluent. A new aliquot of recombinant cytokines and plasma samples was used for each assay to avoid repeated freeze-thaw cycles. A mixture of detection antibodies (200, 100 and 50 ng/test for TNF-α, IL-1β and IL-6, respectively) was prepared and diluted in detection reagent diluent. The assay was carried out in a polypropylene 96-well plate with V-bottom (BD). First, 100 µL of wash buffer was added to the wells and completely

removed, then 50 µL of each standard concentration or diluted plasma samples were applied to the plate. Subsequently, 50 µL of the bead mixture was added to each well. The plate was covered with a plate sealer, gently vortexed and incubated for 30 min in the dark at room temperature. Next, 50 µL of the detection antibody mixture was added to each well and the plate was covered, vortexed and incubated for 2 h as mentioned above. After incubation, the plate was centrifuged at 200 *x g* for 5 min and the supernatant was gently discarded. Next, 150 µL of wash buffer was added to the wells, the plate was centrifuged once more at 200 *x g* for 5 min and the supernatant was gently discarded. Enhanced sensitivity detection reagent was prepared following the manufacturer's instructions and 100 µL was added to each well. The plate was covered, vortexed and incubated for 1 h. After incubation, the plate was centrifuged at 200 *x g* for 5 min and the supernatant was gently discarded. Next, 150 µL of wash buffer was added to the wells, the plate was vortexed and immediately analyzed on a validated BD FACSArray™ Bioanalyzer. The flow cytometer was calibrated with set-up beads and 1500 events were acquired for each well. After forward versus side scatter gating of the 7.5 µm beads, the different bead populations were displayed in a 2-color dot plot (Near-infrared vs. Red). For each bead, the PE-signal was measured as a parameter for the analyte concentration. Subsequently, data were analyzed by means of the FCAPArray™ software (BD). A 4-parameter logistic regression model was used to fit the curve.

2.4.2. CBA 2-plex assay for pig-MAP and CRP

Antibodies, beads and standards used in the CBA 2-plex assay are listed in Table 1. For this purpose, a CBA Human Soluble Protein Master Buffer Kit was used (BD). With the exception of the following variations, the 2-plex assay was performed similar to the 3-plex assay. The capture beads for standard samples were mixed and diluted in regular capture bead diluent, yet, the capture beads for plasma samples were mixed and diluted in capture bead diluent for serum/plasma. Standard curves for CRP (0.0, 15.6, 31.3, 62.5, 125, 250, 500, 750, 1000 ng/mL) and pig-MAP (0.0, 156, 312, 625, 1250, 2500, 5000, 7500, 10000 ng/mL) were prepared in assay diluent. EDTA-plasma samples were vortexed thoroughly and diluted 1/1000 with assay diluent. Unlike the 3-plex assay, 50 µL of the bead mixture was first added to the wells. After adding the detection antibody mixture to the wells (200

ng/test for both CRP and pig-MAP), the plate was incubated for 2.5 h before flow cytometric analysis.

2.5. Validation of the CBA 3-plex assay for TNF-α, IL-1β and IL-6

The limit of detection (LOD) was determined for each cytokine and defined as the lowest detectable cytokine concentration within the linear range of the individual cytokine standard curve in the multiplex assay. The LOD was calculated by evaluation of 7 standard curves for each of the three cytokines.

Intra-assay variation was evaluated for each cytokine by analyzing multiple replicates (n = 6) of a sample with a known concentration of recombinant protein (5000 pg/mL) spiked in a 1/4 diluted plasma pool prepared from 5 healthy pigs.

Inter-assay variation was determined for each cytokine by evaluating the MFI-values of 2 different plasma samples of an LPS-challenged pig in duplicate, which were each analysed on 3 different days. Intra- and inter-assay coefficients of variation (CVs) were subsequently calculated for each cytokine.

2.6. ELISAs

Porcine cytokines and APPs were also analyzed using 6 ready-to-use porcine ELISA kits. TNF-α, IL-1β and IL-6 kits were all purchased from R&D Systems (Quantikine® ELISA PTA00, PLB00B and P6000B, respectively). The dynamic ranges of these assays were 0.023 to 1.500 ng/mL, 0.039 to 2.500 ng/mL and 0.010 to 1.200 ng/mL, respectively. Table 2 summarizes the LODs, intra- and inter-assay variations of the ELISAs as provided by the manufacturer. The pig-MAP ELISA was obtained from PigCHAMP Pro Europe S.A., while concentrations of CRP were determined using porcine-specific immuno-assays from both Tridelta Development Ltd and ALPCO Diagnostics. The dynamic ranges of these assays were 440 to 3500 ng/mL, 46.9 to 1500 ng/mL and 6.25 to 200 ng/mL, respectively. All ELISAs contained precoated 96-well plates and were performed as described by the manufacturers. Optical densities were determined using a plate reader (Multiskan MS Labsystems, Thermo Scientific). Data were analyzed by means of DeltaSoft JV (BioMetallics Incorporated).

Table 2. Limits of detection (LOD), mean intra- and inter-assay variations (CV %) and dynamic ranges of each cytokine in the in-house validated CBA 3-plex assay and commercially available ELISAs (R&D Systems)

Cytokine	LOD (ng/mL)		Intra-assay (CV %)		Inter-assay (CV %)		Dynamic range (ng/mL)	
	CBA	ELISA	CBA	ELISA	CBA	ELISA	CBA	ELISA
TNF-α	0.363	0.004	8.45	4.86	15.71	8.90	0.50 - 50	0.023 - 1.50
IL-1β	0.109	0.007	2.26	5.77	15.48	6.27	0.50 - 50	0.039 - 2.50
IL-6	0.005	0.002	2.33	4.00	11.41	5.97	0.50 - 50	0.010 - 1.20

LOD: limit of detection; CV: coefficient of variation

2.7. Statistical analysis

A linear mixed model with pig as random effect was fitted for the 3 different cytokines. The fixed categorical effect was time after administration. Comparisons to time 0 were adjusted according to the Bonferroni technique and the corresponding adjusted *p*-values are reported for CBA.

A Pearson's correlation coefficient (r) was determined for the single plex vs. multiplex comparisons of TNF-α, IL-1β and IL-6. The level of significance was α = 0.05. All values represent mean + standard deviation (SD). Statistical analysis was performed using SPSS Version 20.0 for Windows.

3. Results

3.1. Selection of the capture-detection antibody pairs

To establish appropriate antibody pairs for the 3-plex assay, diverse combinations of commercially available antibodies were tested. The selected compatible antibody pairs are shown in Table 1. For IL-1β and IL-6, respectively 5 and 3 combinations of antibodies evaluated as capture and/or as detection antibody were considered unsuitable and therefore excluded for use in our CBA assay for porcine plasma samples (Table 3).

For CRP, the 18-374-130062 (GenWay Biotech) / PP169 (Tridelta Development Ltd) antibody pair was considered unsuitable for use in our CBA assay for porcine plasma samples.

Table 3. Incompatible pairs of antibodies

Analyte	Capture Ab	Source	Detection Ab	Source
IL-1β	PP425	Thermo Scientific	PP425	Thermo Scientific
	MAB6811	R&D Systems	MAB681	R&D Systems
	MAB6811	R&D Systems	PP425	Thermo Scientific
	MAB681	R&D Systems	MAB6811	R&D Systems
	PP425	Thermo Scientific	MAB681	R&D Systems
IL-6	PP690	Thermo Scientific	PP690	Thermo Scientific
	MAB6861	R&D Systems	PP690	Thermo Scientific
	MAB6861	R&D Systems	MAB686	R&D Systems
CRP	18-374-130062	GenWay Biotech	PP169*	Tridelta

*The CRP detection antibody was kindly donated by M. Gallagher (Tridelta Development Ltd)

3.2. Confirmation of adequate bead conjugation for selected capture antibodies

As a first step in the development of the 3-plex and 2-plex assays, the successful coupling of the 5 selected capture antibodies to their beads had to be confirmed. For this purpose, the MFI of each test sample was compared to the MFI of a negative control sample. The following MFI values of test vs. control samples were obtained for the 5 parameters included in our CBA set-up: TNF-α (235325 vs. 93), IL-1β (199834 vs. 109), IL-6 (261294 vs. 171), pig-MAP (229180 vs. 61) and CRP (262143 vs. 192). These data clearly indicated that all 5 bead conjugations were adequately performed, as the manufacturer considers a capture antibody-bead coupling step successful when the MFI signal of the test sample exceeds that of the negative control sample at least 500 times.

3.3. Validation of the CBA 3-plex assay for TNF-α, IL-1β and IL-6

Representative standard curves of the CBA 3-plex cytokine assay are shown in Figure 1, while Table 2 reports its corresponding LODs, intra- and inter-assay CVs and dynamic ranges. The LODs were found to be acceptable, varying between 0.005 and 0.363 ng/mL. The intra-assay CVs were also satisfactory and ranged from 2.26 to 8.45%. As expected, the inter-assay CVs were found to be higher than those obtained within-day and ranged from 11.41 to 15.71%. The dynamic ranges of the standard curves for TNF-α, IL-1β and IL-6 ranged from 0.50 to 50 ng/mL. The comparison between both immuno-assays is described in the next paragraph.

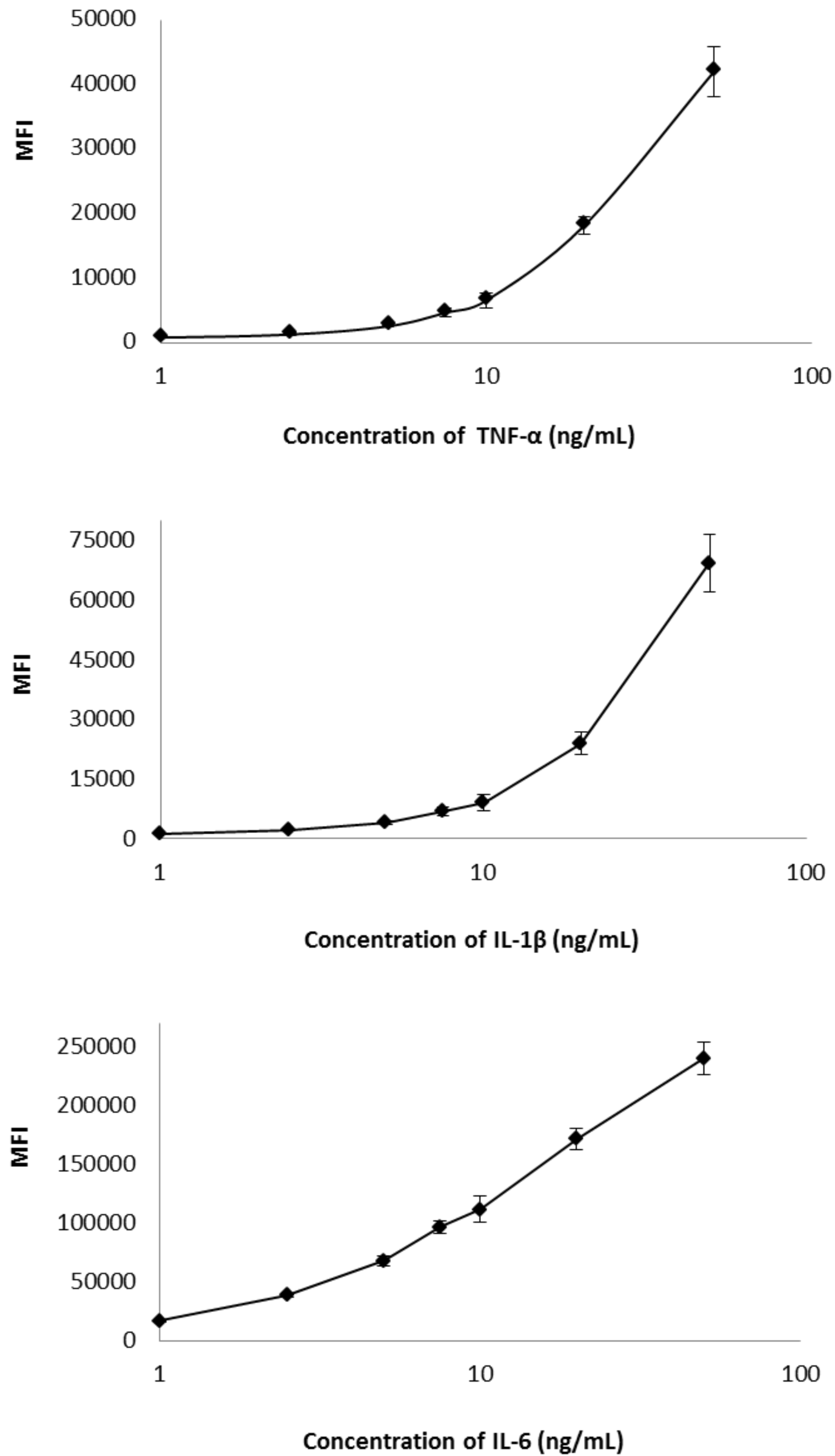


Figure 1. Representative standard curves of TNF-α, IL-1β and IL-6 from the CBA 3-plex assay. Each standard curve shows median fluorescence intensities (MFIs) presented as mean ± SD obtained from 5 independent assays.

3.4. Analysis of porcine plasma samples after LPS-challenge

3.4.1. CBA 3-plex assay for TNF-α, IL-1β and IL-6

Plasma samples from LPS-challenged pigs were analysed using the developed CBA 3-plex cytokine assay and levels were compared to the results obtained by commercially available ELISAs. A single 1/4 dilution was found to be sufficient for all time points to fit the dynamic range of the standard curves, while for the ELISAs dilutions ranging from 1/2 to 1/100 were required. As expected, the intravenous administration of 15 µg/kg BW LPS induced a time-dependent increase of cytokines in all pigs ($n = 4$). For the three cytokines measured, a similar trend was observed with both immuno-assay techniques as shown in Figure 2. Prior to LPS administration, systemic concentrations of TNF-α were very low. However, plasma levels of TNF-α rapidly increased after LPS administration, reaching peak concentrations at 1 h post administration (p.a.) ($p < 0.0005$ for CBA and ELISA). Plasma TNF-α already resumed levels comparable to basal levels at 6 h p.a. Similar to TNF-α, IL-6 concentrations prior to LPS administration were negligible. Following LPS administration, IL-6 increased to maximal concentrations at 2.5 h ($p = 0.019$ for CBA), whereas basal concentrations were not yet resumed at 6 h p.a. Remarkably, CBA, and to a lesser degree also ELISA, demonstrated elevated IL-1β plasma levels already prior to LPS administration. After a gradual increase, maximal concentrations of IL-1β were attained at 3 h p.a. ($p = 0.005$ for CBA) and similar to IL-6, these systemic levels remained elevated at 6 h p.a. In contrast to CBA, the results obtained for IL-1β and IL-6 by ELISA were not significantly different compared to time 0.

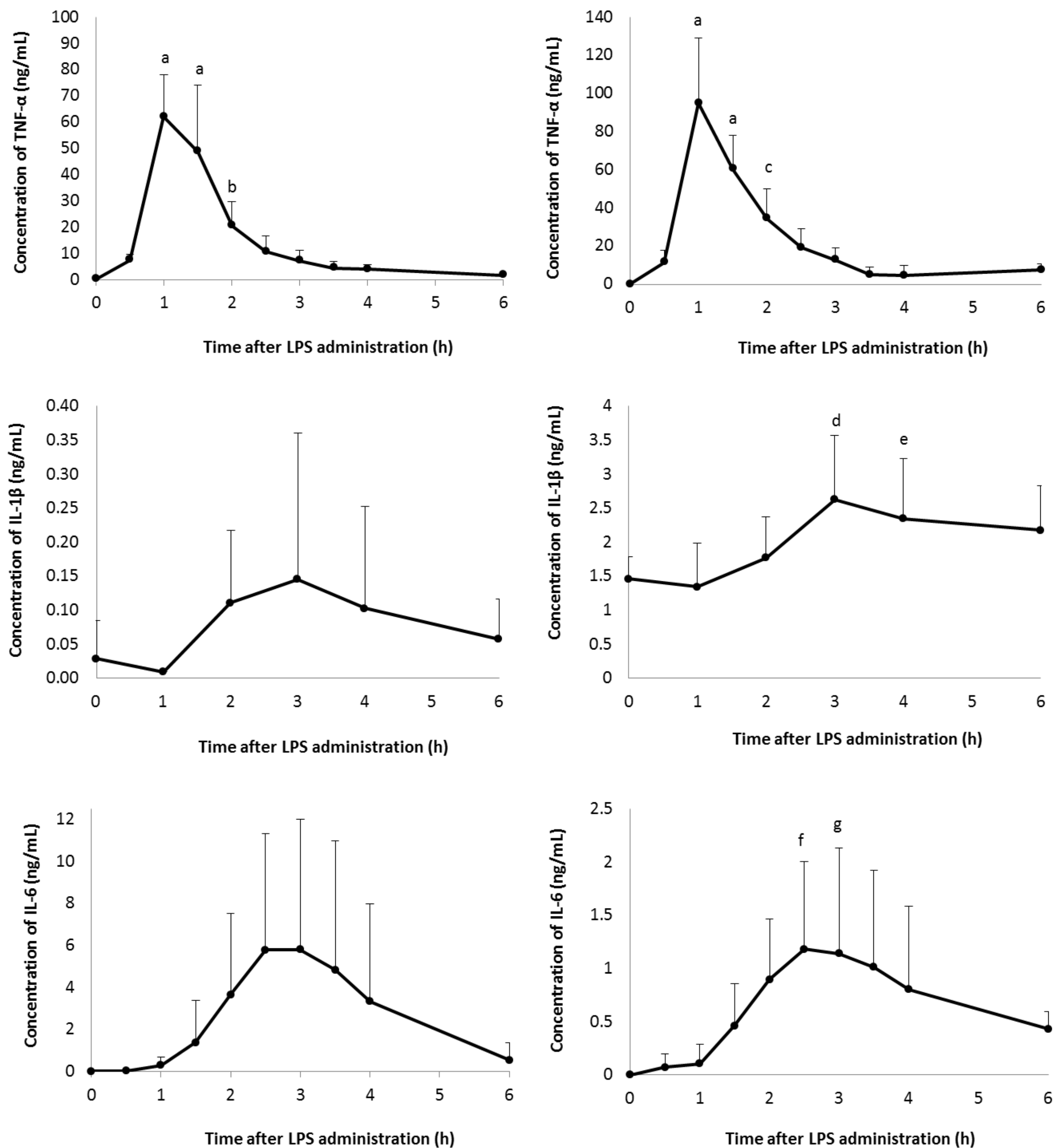
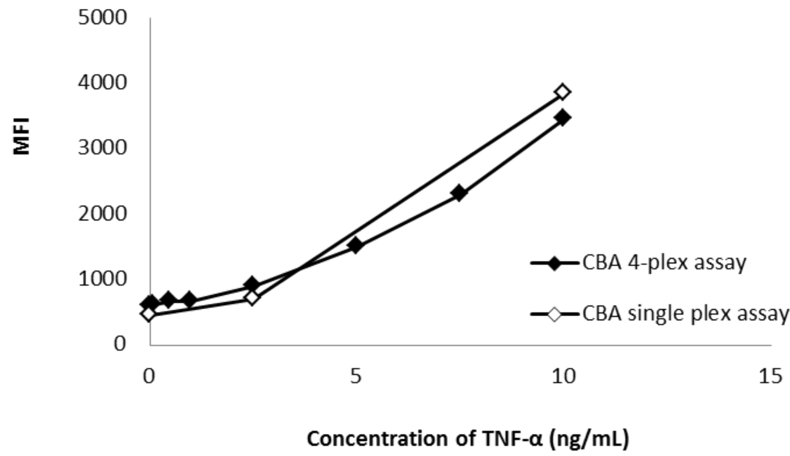
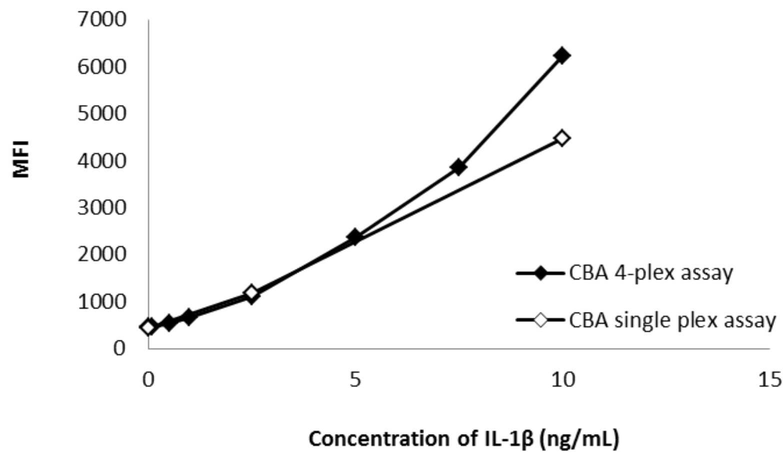


Figure 2. Mean (+ SD) plasma concentration-time curves of TNF-α, IL-1β and IL-6 obtained from LPS-challenged pigs (*n* = 4). Adjusted *p*-values are reported as a ($p < 0.0005$), b ($p = 0.033$), c ($p = 0.004$), d ($p = 0.005$), e ($p = 0.039$), f ($p = 0.019$) and g ($p = 0.026$). Concentrations were measured using both ELISA (left panel) and CBA (right panel). Note that the detection sensitivities, mentioned in Table 2 allowed the reliable quantification of all 3 parameters for both ELISA and CBA. Remarkable differences between both techniques were however observed for IL-1β and IL-6.

Before the CBA 3-plex assay, it was attempted to develop a CBA 4-plex assay for the simultaneous detection of TNF-α, IL-1β, IL-6 and pig-MAP. This 4-plex analysis of LPS-challenged porcine plasma generated comparable results as with the CBA 3-plex assay (data not shown). Furthermore, the CBA 4-plex assay was successfully compared to the single plex assays for all three cytokines as illustrated in Figure 3. Nevertheless, finally a CBA 3-plex and an additional CBA 2-plex format was preferred since the peak concentrations of cytokines and APPs severely differ in order of magnitude and time points after LPS administration.





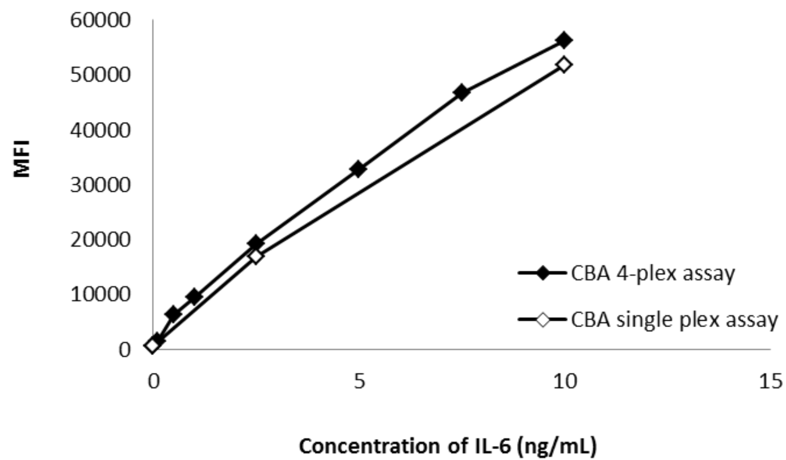


Figure 3. Comparison of median fluorescence intensities (MFIs) between the CBA 4-plex assay (solid rhomb) and the single plex assays (open rhomb) of TNF-α, IL-1β and IL-6. Pearson's correlation coefficients were 0.999, 0.995 and 0.999, respectively ($p < 0.05$). Note different scales on the y-axes for TNF-α, IL-1β and IL-6.

3.4.2. CBA 2-plex assay for pig-MAP and CRP

Representative standard curves of the CBA 2-plex APP assay are shown in Figure 4. Considerable plasma levels of pig-MAP were detected prior to LPS administration, which gradually increased to maximal concentrations at 24 h p.a. Systemic pig-MAP levels remained elevated at 36 p.a. A similar trend was observed with ELISA as shown in Figure 5.

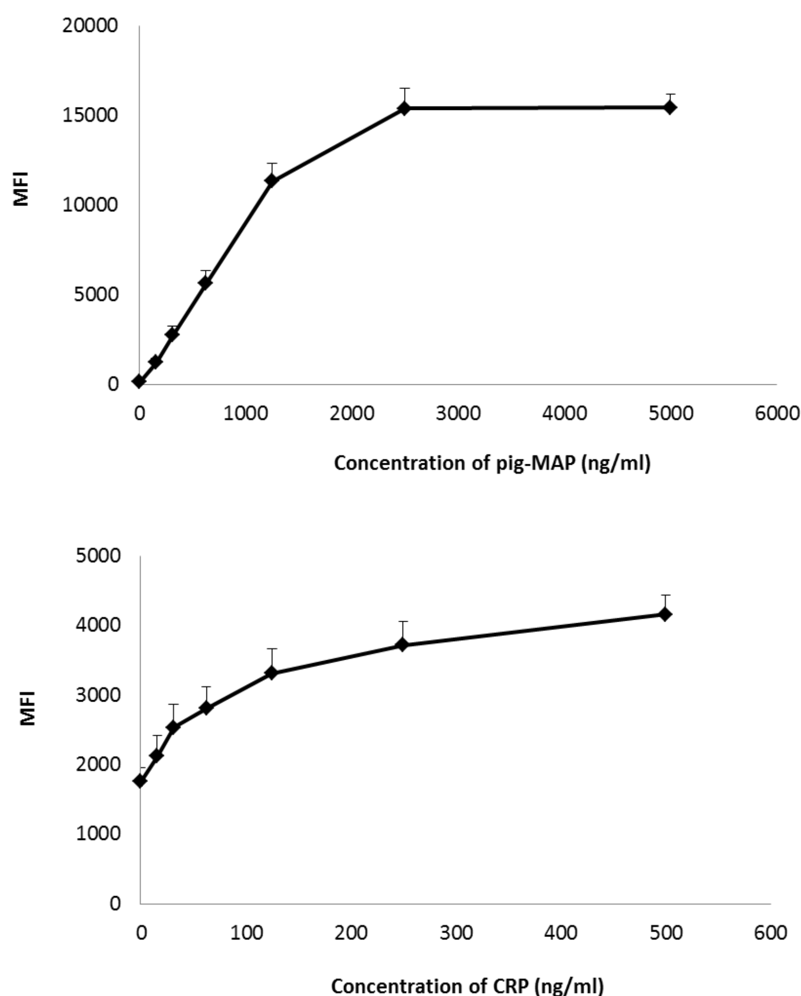


Figure 4. Representative standard curves of pig-MAP and CRP from the CBA 2-plex assay. Each standard curve shows median fluorescence intensities (MFIs) presented as means and standard deviations obtained from 3 independent assays.

It should be remarked that inclusion of CRP in a CBA 2-plex format with pig-MAP severely decreased the measured plasma concentrations of pig-MAP in comparison to the CBA single plex and 4-plex analysis (Figure 5). Moreover, for CRP, no plasma levels were

detectable with this CBA 2-plex assay. Results for CRP obtained using the Tridelta ELISA were too dubious and consequently could not be taken into account (data not shown). It should be remarked that in line with our observations, Slagman *et al.* (2011) also considered the results generated by the latter immuno-assay as controversial. Conversely, the ALPCO ELISA demonstrated low plasma concentrations of CRP prior to LPS-challenge, reaching maximal concentrations at 24 h p.a. Systemic CRP levels also remained elevated at 36 h p.a. (data not shown).

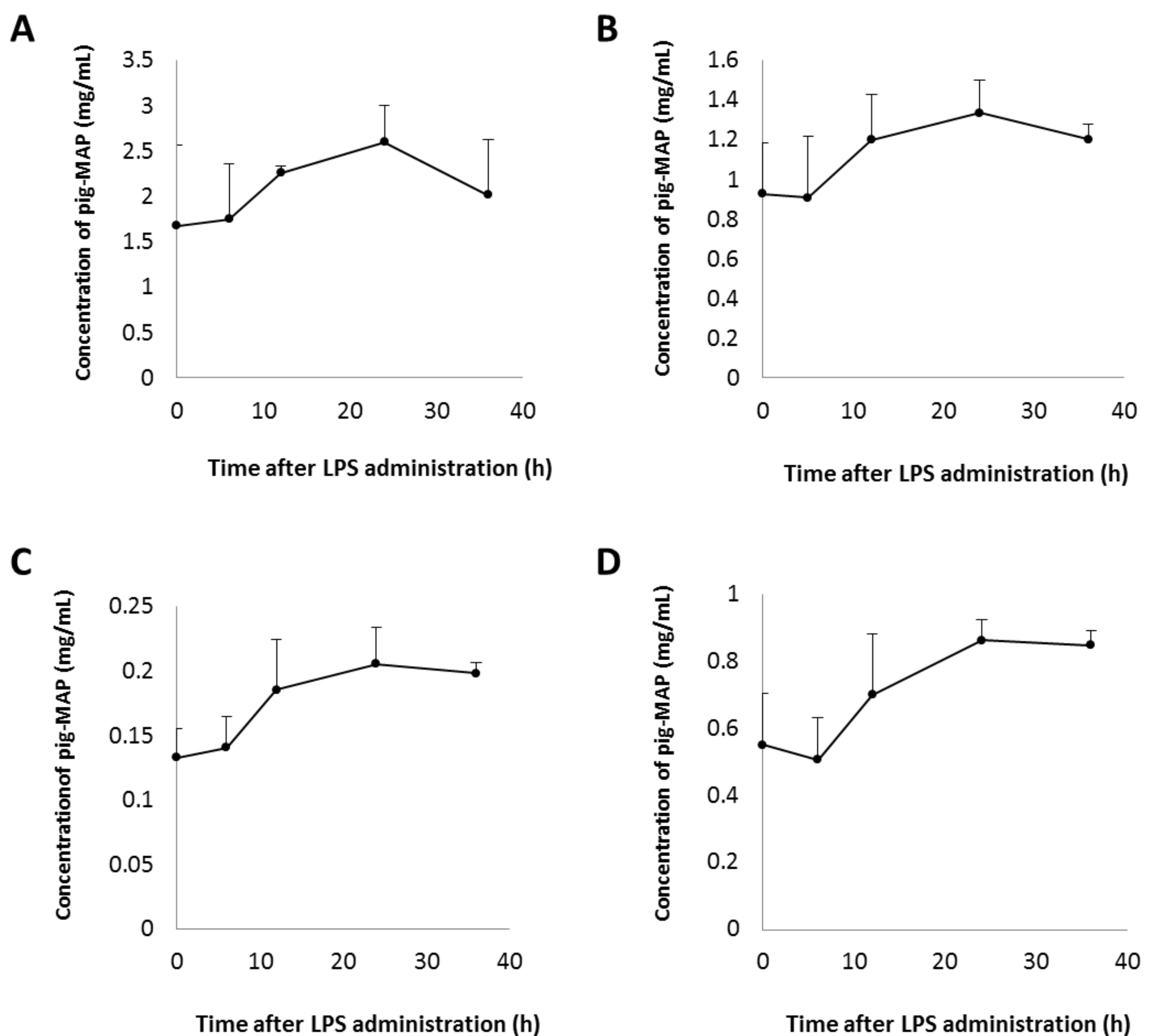


Figure 5. Mean (+ SD) plasma concentration-time curves of pig-MAP obtained from LPS-challenged pigs (*n* = 4). Concentrations were measured using ELISA (A), CBA single plex (B), CBA 2-plex (C) and CBA 4-plex (D) assays. Note different scales on the y-axes.

4. Discussion

This is the first CBA study describing a validated multiplex protocol for the simultaneous measurement of the major porcine pro-inflammatory cytokines TNF-α, IL-1β and IL-6. Additionally, a concept for porcine APPs is provided. This technology was used to analyze porcine plasma samples obtained in an LPS inflammation model and the results were compared to those obtained with commercial ELISA kits.

Similar concentration-time profiles were observed for all three cytokines and for pig-MAP with CBA and ELISA in plasma from LPS-challenged pigs. However, the absolute concentrations of the systemic cytokines and APP were found to differ considerably.

The acceptance by the scientific community of multiplex bead-based assays largely depends on achieving similar results as with ELISA, which is still generally considered as the gold standard. However, comparisons between multiplex assays and ELISA kits are only straightforward when the same antibodies and cytokine standards are used in both immuno-assay formats (Chen et al., 1999; Khan et al., 2004). Several divergent absolute concentrations between both types of immuno-assays have been reported and attributed to the use of reagents from different manufacturers (Prabhakar et al., 2002; Ray et al., 2005; Maier et al., 2006). To develop our CBA 3-plex assay, we also used different antibodies and standards than those used in the commercial porcine ELISA kits. Furthermore, the CBA concept with capture beads in suspension versus surface-coated in ELISA, as well as the execution of both procedures and the final analysis method, imply major dissimilarities between the two immuno-assays.

As the primary focus of our developed methodology lays on the time- and cost-efficient study of relative changes in the systemic levels of TNF-α, IL-1β and IL-6 after LPS-challenge in pigs, the results currently obtained with the novel CBA 3-plex assay serve our purpose. Moreover, multiplex flow cytometry additionally allows the estimation of the cytokine ratio in a biological sample, which might be more relevant than absolute concentrations. Since cytokines interact in a complex immunological network, multiplexing carried out on analytes in suspension may even provide a more accurate view of the immune response than the single parameter and surface-immobilized ELISA strategy. Furthermore, results obtained with multiplex bead-based assays are generally more accurate since these are calculated from the mean of at least 300 beads, each functioning

as an individual replicate. In contrast, ELISA data are derived from a single reading of an individual well (Carson and Vignali, 1999). Consequently, CBA provides a suitable screening tool for large sample numbers. Our CBA 3-plex data corroborate those from Williams et al. (2009) who reported similar cytokine profiles with ELISA after an intravenous LPS-challenge in pigs except for a second peak of IL-6 at 3.5 h p.a. Although the presence of plasma IL-1β prior to LPS-challenge in the current study was rather unexpected, Myers et al. (2003) also detected constitutive plasma levels of this cytokine with ELISA. According to Dernfalk et al. (2007), comparisons of the (absolute) concentrations obtained with multiplex bead-based assays and ELISA kits are rather redundant, since a standard curve of recombinant protein is included in each immuno-assay providing an adequate internal control.

Notwithstanding the fact that generally ELISA kits are specific, sensitive and well-suited to perform single parameter analysis, some limitations have to be taken into account. For our porcine cytokine ELISAs, 50 µL of plasma and a total incubation time of 4.5 h were required per cytokine, while the CBA 3-plex assay for simultaneous quantitation of TNF-α, IL-1β and IL-6 required 50 µL of plasma and a total incubation time of 3.5 h. In our CBA 3-plex assay, LODs were higher than in the respective ELISAs (Table 2). On the other hand, the dynamic range of ELISAs only covered 2 to 3 logs, as mentioned above, whereas CBA substantially expanded this range to 4 logs, which is particularly attractive for the measurement of samples from an LPS inflammation model as few sample dilutions will suffice.

Although this implies that the CBA 3-plex assay presented here does not allows the detection of equally low concentrations, it is certainly a valuable tool to screen a panel of porcine biomarkers of which high systemic levels are typically expected during severe inflammation. When we consider the reported LODs either in comparable porcine or in bovine 3-plex Luminex assays, i.e. those developed by Johannisson et al. (2006) (range 0.92 - 12 ng/mL) and Dernfalk et al. (2007) (range 2.0 - 6.5 ng/mL), respectively, our LODs certainly are acceptable. On the other hand, for TNF-α and IL-1β, Bjerre et al. (2009) achieved lower LODs in their multiplex assays, respectively 0.010 and 0.020 ng/mL, but for optimal sensitivity the assays had to be performed as a 4-plex and 2-plex assay.

The intra-assay CVs of the CBA 3-plex assay were < 10 %, which is considered acceptable. As a result, the use of duplicate samples becomes redundant, again saving both time and sample volume. Although we recognize that the inter-assay CVs were higher, a low inter-

assay variation is only important to compare analysis results in studies where repeated analyses are performed over a longer period of time. We therefore recommend to perform analyses preferably within one day which should reduce analytical variation to a minimum.

Both the CBA and Luminex xMAP techniques, are increasingly applied in human (clinical) and mouse (preclinical) research. Indeed, to date commercial CBA kits are offered for a wide variety of analytes for these species. In marked contrast, only a limited number of studies report the simultaneous detection of biomarkers by multiplex flow cytometry in veterinary science. For veterinary species, the lack of specific antibody pairs suitable for detecting secreted proteins has been defined as a major limitation (Wagner and Freer, 2009). Moreover, it is hard to obtain the commercial antibodies as single reagents from either the ELISA companies or their suppliers. On the other hand, commercial antibody pairs that were found to be optimal for ELISA format, may not be suitable for multiplex bead-based immuno-assays. Therefore, antibodies should be carefully screened to select the suited antibody pairs (Kellar and Iannone, 2002). In the current study, several combinations of commercial antibodies were preliminary evaluated, which was a very labor- and cost-intensive process that often did not provide satisfactory results (Table 3). It should be remarked that we were able to generate accurate standard curves for IL-6 with the monoclonal antibody pair MAB6861/MAB686 (Table 3) as capture and detection antibody, respectively (data not shown). Unfortunately, no IL-6 signal was observed even not with a CBA single plex in porcine plasma samples obtained after LPS-challenge and analyzed as positive for this cytokine with ELISA. Likewise, Lawson *et al.* (2010) also experienced major problems finding a suitable antibody pair for the determination of porcine IL-6 in their Luminex assay.

As described, pig-MAP was originally included in a CBA 4-plex assay together with TNF-α, IL-1β and IL-6. This initial 4-plex analysis of LPS plasma samples yielded satisfactory results for all 4 parameters (data not shown). Nevertheless, the simultaneous measurement of pro-inflammatory cytokines and APPs proved to be rather redundant within an acute LPS inflammation model as their peak concentrations differ in order of magnitude and time points after LPS administration. Consequently, pig-MAP was withdrawn from this original 4-plex format and we decided to develop a separate 3-plex cytokine and a separate 2-plex APP assay. Regardless the presence of pig-MAP, comparable

absolute concentrations were obtained with the original CBA 4-plex and final CBA 3-plex assay, demonstrating the flexibility of the CBA technique.

As demonstrated, for CRP, adequate standard curves were created with the 18-374-130062/MAB1707 antibody pair (Table 1). Unfortunately, in porcine plasma, no reproducible nor sensitive signals were obtained with neither the CBA single plex nor the CBA 2-plex assay for this APP. Furthermore, the inclusion of CRP negatively influenced concentration levels of pig-MAP compared to the CBA single plex and the original CBA 4-plex assay format (data not shown).

To the best of our knowledge, this is the first study attempting to detect porcine APPs by means of (multiplex) bead-based flow cytometry. As the first antibody pair failed on the plasma level, the use of an alternative second antibody pair is indicated. However, currently no alternative porcine CRP antibodies are available to further optimize our CBA 2-plex APP assay. An alternative strategy might be the use of bead-coupled phosphocholine as a catching agent, since CRP possesses a Ca^{2+}-dependent binding affinity for phosphocholine (Deegan et al. 2003). The substitution of CRP by the third important APP in pigs, haptoglobin, is also conceived as an option.

Finally, it can be remarked that CBA assays should not be restricted to the detection of cytokines or APPs, since other biomarkers of infection and inflammation can be included. The major restriction of the applicability of this technique probably remains the accessibility of suitable porcine-specific antibody pairs. Another challenge remains the requirement to balance the individual optimal dilution factors for the single analytes with an acceptable universal one for all analytes.

5. Conclusion

Our optimized and validated CBA 3-plex cytokine protocol provides a fast, flexible and cost-effective screening tool for simultaneous determination of pro-inflammatory cytokine profiles in a limited porcine plasma volume. Similar cytokine profiles were obtained for TNF-α, IL-1β, IL-6 and pig-MAP compared to conventional ELISAs, which are specific, sensitive and well-suited to perform single parameter analysis. Hence, this technique will be applied in future research to study the immunomodulating properties of drugs in a porcine LPS inflammation model. This study also demonstrated the applicability of CBA for measurement of APPs, although further optimization is required.

Acknowledgements

The authors would like to thank BD for the helpful hints and Prof. dr. S. Sys for his advice on the statistical analyses. The help of E. Plessers and A. Van den Bussche during the animal experiment was gratefully appreciated.

3.2. Multiplex analysis of pro-inflammatory cytokines in serum of *Actinobacillus pleuropneumoniae*-infected pigs

Adapted from

Wyns, H., Croubels, S., Vandekerckhove, M., Demeyere, K., De Backer, P., Goddeeris, B.M., Meyer, E. (2014) Multiplex analysis of pro-inflammatory cytokines in serum of *Actinobacillus pleuropneumoniae*-infected pigs *(manuscript in preparation).*

Abstract

Porcine pleuropneumonia is a severe respiratory disease caused by the Gram-negative pathogen *Actinobacillus (A.) pleuropneumoniae*. Reports on systemic cytokine production following experimental infections with this pathogen in pigs are not straightforward. Nevertheless, IL-6 has been consistently detected in the majority of these infected pigs and has consequently been put forward as a valuable biomarker for monitoring bacterial infections.

As multiplex bead-based flow cytometry is gaining popularity for the simultaneous detection of diverse inflammatory markers in veterinary research, the aim of the present study was to analyze serum samples of *A. pleuropneumoniae*-infected pigs at the onset of infection for TNF-α, IL-1β and IL-6 using our previously developed CBA 3-plex assay for porcine plasma (Chapter 3.1). Additionally, these samples were also analyzed for IL-6 by means of a commercial porcine-specific ELISA to compare the results of both immuno-assays.

The CBA 3-plex assay was successfully validated for its use in serum samples. The limits of detection (LODs) varied between 0.012 and 0.333 ng/mL, and the inter- and inter-assay coefficients of variation were < 5 % and < 10%, respectively.

Using the CBA 3-plex assay, increased levels were observed for all 3 cytokines following experimental infection with *A. pleuropneumoniae*. Mean serum peak concentrations of TNF-α and IL-6 were recorded at 12 h p.i. and at 10 h p.i., respectively. Serum concentrations of IL-1β also increased, albeit without reaching a maximum in the 0-18 h period of analysis. A similar concentration-time profile as with CBA was observed for IL-6 when analyzing these serum samples in parallel with ELISA.

In conclusion, the feasibility of the CBA 3-plex assay for pro-inflammatory cytokine detection in porcine serum beside plasma samples was confirmed. Moreover, we propose that this elegant immuno-assay can be applied for the screening of immunomodulatory properties of drugs and vaccine adjuvants in infection, inflammation and vaccination.

1. Introduction

Multiplex bead-based flow cytometry is gaining popularity for the simultaneous detection of diverse inflammatory markers in veterinary research. Compared to other commonly used methods, including the gold standard i.e. enzyme-linked immunosorbent assay (ELISA), multiplex bead-based assays significantly minimize the sample volume and analysis time required, while maximizing the number of parameters that can be simultaneously analyzed. Christopher-Hennings et al. (2013) provided a comprehensive overview of current multiplex technologies with attention to veterinary diagnostic purposes. This highlighted that in contrast to humans and rodent species, multiplex assays for porcine research are scarce. During the past decade, several multiplex bead-based assays have been in-house developed to characterize the immune response in pigs (Johannisson et al., 2006; Bjerre et al., 2009; Lawson et al., 2010; Bongoni et al., 2013; Wyns et al., 2013).

Porcine pleuropneumonia is a severe respiratory disease caused by the Gram-negative pathogen *Actinobacillus (A.) pleuropneumoniae*, characterized by a fibrinohemorrhagic necrotizing bronchopneumonia and a fibrinous pleuritic. It is responsible for major economic losses and reduced animal welfare. *A. pleuropneumoniae* has a multifactorial pathogenicity, involving different secreted and non-secreted virulence factors, including lipopolysaccharide (LPS), cytotoxins (Apx toxins), hemolysin, proteases, host and environmental factors (Haesebrouck, 1997; Huang et al., 1998; Chiers et al., 2010). Reports on systemic cytokine production following experimental infections with *A. pleuropneumoniae* in pigs are not equivocal (Fossum et al., 1998; Huang et al., 1999; Balaji et al., 2002; Myers et al., 2004). Only IL-6 has been consistently detected in the majority of infected pigs and has consequently been put forward as a valuable marker for bacterial infections (Fossum et al., 1998; Tambuyzer et al., 2014).

The present study was designed to test out serum samples of *A. pleuropneumoniae*-infected pigs at the onset of infection for pro-inflammatory cytokines using a novel developed cytometric bead array (CBA) 3-plex assay for a fast and simultaneous profiling of TNF-α, IL-1β and IL-6, as previously described for porcine plasma in an LPS-induced inflammation model by Wyns et al. (2013), with special attention to the IL-6 response.

2. Materials and methods

2.1. Serum samples

Serum samples were obtained from early weaned outbred pigs (n = 9) of approximately 20 kg body weight (BW) (Rattlerow Seghers Holding N.V.) in an experimental design with *A. pleuropneumoniae* as described by Tambuyzer et al. (2014). Five pigs were endobronchially infected with 1 x 10^7 CFU (biotype 1-serotype 9, strain no. 13261; Van Overbeke et al., 2001) *A. pleuropneumoniae,* whereas four other pigs received endobronchially sterile medium and served as negative controls. The pigs were catheterized three days before infection for repetitive blood sampling. Serum samples were collected immediately before (baseline value; 0 h) and at 2, 4, 6, 8, 10, 12, 14, 16 and 18 h post infection (p.i.) and serum separated. Samples were stored aliquoted at -70 °C until analysis for presence of TNF-α, IL-1β and IL-6.

2.2. CBA 3-plex assay for TNF-α, IL-1β and IL-6

With the exception of the selected beads (A4, C7 and D9 for IL-6, IL-1β and TNF-α, respectively), antibodies, beads and standards were applied as previously described (Wyns et al., 2013). Standard curves of 0.0, 0.125, 0.25, 0.5, 1.0, 2.5, 5.0, 7.5, 10.0, 20.0 and 50.0 ng/mL were generated. A single universal 4-fold dilution was used for all samples. Analyses were performed on a validated FACSCanto™ dual-laser (488 and 633 nm) flow cytometer (BD Biosciences). After forward vs side scatter gating of the single bead population, the distinct bead populations were displayed in a 2-color dot plot (FL-5 and FL-6).

The applicability of the CBA 3-plex assay for serum samples was evaluated prior to use. The limit of detection (LOD) as well as intra- and inter-assay coefficients of variation (CVs) were assessed and compared to the values previously obtained in porcinplasma (Wyns et al., 2013).

2.3. ELISA for IL-6

Serum samples were additionally analyzed for IL-6 by means of a commercial porcine ELISA (DuoSet® ELISA Development Systems; R&D Systems) to confirm the results obtained by CBA. The lowest quantifiable IL-6 concentration was approximately 0.125 ng/mL (i.e. the lowest calibration point, the LOQ was not in-house determined for serum).

2.4. Statistical analysis

Data were statistically analyzed by a Student's t-test comparing the mean area under the curve (AUC)-values of the negative control pigs (CONTR) and the *A. pleuropneumoniae*-infected pigs using SPSS Statistics 22.0 software for Windows. For those parameters that were not normally distributed, the Mann-Whitney U test was used. A *p*-value of < 0.05 was considered statistically significant.

3. Results

3.1. Validation of the CBA 3-plex assay for porcine serum

The LOD, defined as the lowest detectable cytokine concentration within the linear range of the standard curve, was established at 0.333, 0.054 and 0.012 ng/mL for serum TNF-α, IL-1β and IL-6, respectively. Intra- and inter-assay CVs of each cytokine were calculated by evaluating MFI-values of two different serum samples from infected pigs in duplicate within a day or at different analysis days, respectively. The intra- and inter-assay CV % were, 3.83 and 4.72 for TNF-α, 2.67 and 3.47 for IL-1β; and 2.83 and 6.31 for IL-6, respectively.

Validation parameters for serum samples are summarized in Table 1 and compared to those previously obtained in porcine plasma by our group (Wyns et al., 2013).

Table 1. Validation parameters of the CBA 3-plex assay of TNF-α, IL-1β and IL-6 for both serum and plasma samples

	LOD (ng/mL)		**Intra-assay (CV %)**		**Inter-assay (CV %)**	
	serum	**plasma**	**serum**	**plasma**	**serum**	**plasma**
TNF-α	0.333	0.363	3.83	8.45	4.72	15.71
IL-1β	0.054	0.109	2.67	2.26	3.47	15.48
IL-6	0.012	0.005	2.83	2.33	6.31	11.41

LOD: limit of detection; CV: coefficient of variation

3.2. Analysis of TNF-α, IL-1β and IL-6 with CBA 3-plex assay

Following experimental infection with *A. pleuropneumoniae*, increased levels were present for all 3 cytokines. In the CONTR group, levels of TNF-α and IL-6 remained undetectable, whereas basal levels were present for IL-1β (Figure 1A-C). As the inter-animal (biological) variation of these cytokine responses was high.

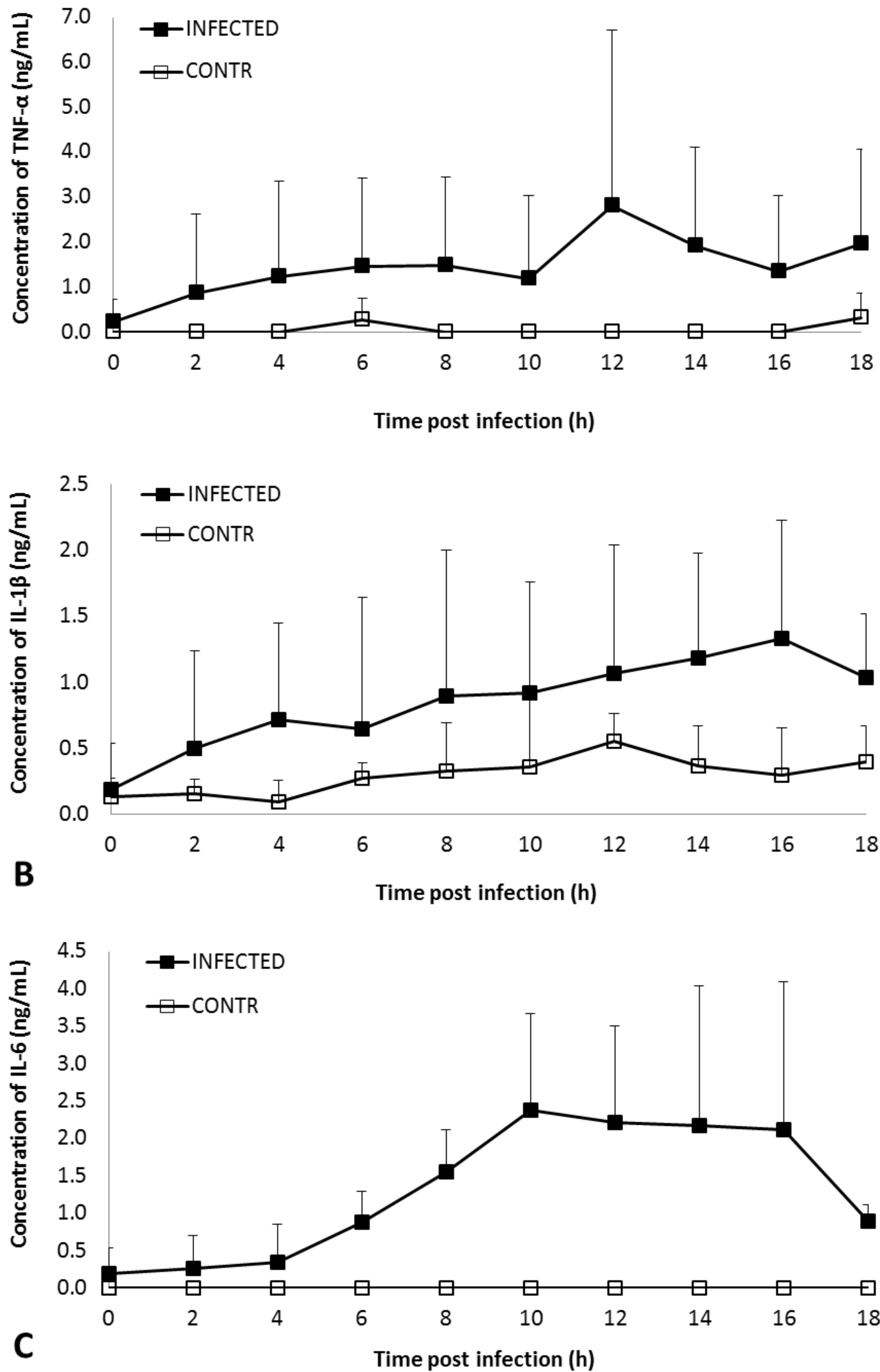


Figure 1. Mean (+ SD) serum concentration-time profiles of TNF-α (A), IL-1β (B) and IL-6 (C) obtained from *A. pleuropneumoniae*-infected (*n* = 5) and negative control pigs (*n* = 4), using the CBA 3-plex porcine cytokine assay.

A mean maximal serum concentration of TNF-α was recorded at 12 h p.i. (range 0 - 10.4 ng/mL), which transiently declined and increased again at 16 h p.i. (Figure 1A). In one infected pig, TNF-α was undetectable, whereas two other infected pigs demonstrated a delayed and only moderate TNF-α response (data not shown), which explains the gradual mean increase of this cytokine (Figure 1A). The mean serum concentration of IL-1β increased without reaching a maximum in the 0-18 h period of analysis (Figure 1B). The levels of IL-1β substantially increased in all infected pigs, yet again one pig showed both a slower and lower response compared to the other pigs of this group (range 0.40 - 2.99 ng/mL). Due to the large biological variations, no significant differences were observed for TNF-α and IL-1β between the negative control group and the *A. pleuropneumoniae*-infected pigs.

For IL-6, on the other hand, a clear mean maximal serum concentration ($p < 0.05$) was observed at 10 h p.i. (range 1.35 - 6.04 ng/mL), which started to decline consistently at 16 h p.i. (Figure 1C). Remarkably, one pig already had substantial IL-6 serum levels at the baseline (0 h) and showed no noticeable IL-6 response following infection. In one other pig, IL-6 increased considerably, without reaching a maximal concentration within the 18 h-analysis-period (data not shown).

For all three cytokines, basal levels were not reached within 18 h p.i. in the infected animals.

3.3. Analysis of IL-6 with ELISA

Compared to the results obtained by the CBA 3-plex assay, a similar concentration-time profile was observed for IL-6 when analyzing the serum samples with ELISA. Again after a gradual increase, a mean maximal serum concentration was reached at 14 h p.i. and a marked decrease started at 16 h p.i. (Figure 2). The IL-6 levels in the pigs of the CONTR group remained below the concentration of the lowest standard (Figure 2). No significant differences were observed between CBA and ELISA for IL-6 analysis.

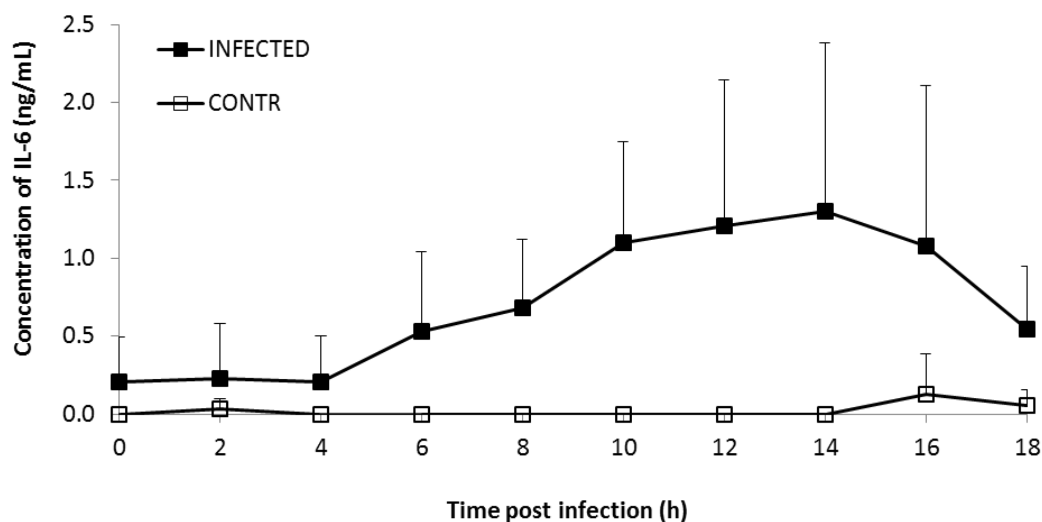


Figure 2. Mean (+ SD) serum concentration-time profiles of IL-6 obtained from *A. pleuropneumoniae*-infected (*n* = 5) and negative control pigs (*n* = 4), using a porcine ELISA.

4. Discussion

This study validated and subsequently demonstrated the applicability of the in-house developed CBA 3-plex assay in porcine serum samples. The LOD and intra-assay parameters obtained for serum were comparable with those previously obtained for plasma and are considered to be again acceptable (Wyns et al., 2013). The inter-assay CVs for serum were even a bit lower, indicating an even better reproducibility of this immuno-assay in serum compared to plasma samples. Its wide dynamic range and the single 4-fold dilution offer the opportunity of measuring both high (infected pigs) and low (negative control pigs) TNF-α, IL-1β and IL-6 levels in a standardized and reproducible manner.

In the present study, increased levels of TNF-α, IL-1β and IL-6 were demonstrated in the serum of pigs infected with *A. pleuropneumoniae*, while the control animals did not show an increased cytokine production. The first 18 h p.i. were selected for analysis using the CBA 3-plex cytokine assay, as the induction of pro-inflammatory cytokines TNF-α, IL-1β and IL-6 can be expected immediately after infection (Baarsch et al., 1995; Fossum et al., 1998; Huang et al., 1999; Myers et al., 2004). Indeed, TNF-α reached a maximal concentration at 12 h p.i., while IL-1β levels increased steadily p.i. of *A. pleuropneumoniae*. These results are inconsistent with some previous reports, in which no systemic changes in TNF-α and IL-1β could be detected in *A. pleuropneumoniae*-infected pigs (Fossum et al., 1998; Balaji et al., 2002; Myers et al., 2004). Yet, Huang et al. (1999) described an increase

in TNF-α as well as IL-1β after both an *in vitro* and *in vivo* stimulation and infection with *A. pleuropneumoniae*, respectively. In contrast to the rather unpredictable TNF-α and IL-1β responses, IL-6 has been postulated as a valuable biomarker for monitoring (porcine) bacterial infections (Fossum et al., 1998; Tambuyzer et al., 2014) as well as for the evaluation of antibiotic treatment (Lauritzen et al., 2003). In the current study, 80 % of the pigs showed a marked IL-6 response following experimental infection with *A. pleuropneumoniae* and a maximal mean IL-6 concentration was recorded at 10 h p.i. Of relevance, these findings are largely in agreement with other reports including a very recent one from the Tambuyzer et al. (2014) (Fossum et al., 1998; Lauritzen et al., 2003; Myers et al., 2004).

Still, the discrepancies between some of these studies can be partially explained by the fact that the outcome of an *in vivo* experimental bacterial infection model is far less predictable compared to a more standardized and reproducible LPS model (Olson et al., 1995; Myers et al., 2003). The sequence of cytokine release following LPS administration is quite straightforward (Gerros et al., 1993). In contrast, the substantial biological variations observed between outbred animals in a bacterial infection model, which was also obvious in our experiments, remain of major concern (Fossum et al., 1998; Huang et al., 1999; Myers et al., 2004). Moreover, the mode of infection, bacterial dosage, strain and associated virulence factors might also contribute to a different outcome (Huang et al., 1999; Balaji et al., 2002).

Although the concentration-time profiles of IL-6 were similar between CBA and ELISA, different absolute mean concentrations were clearly registered between both immuno-assay. These dissimilarities can be ascribed to the use of different antibodies, standards and reagents in both immuno-assay formats, as also previously mentioned by our group and other authors (Prabhakar et al., 2002; Khan et al., 2004; Wyns et al., 2013). In addition, the simultaneous measurement of three parameters is more complex compared to the single-parameter-approach of the gold standard ELISA. Nevertheless, multiplexing offers the opportunity to characterize the relative changes in the systemic cytokine levels more completely in a time- and cost-efficient manner, avoiding freeze-thaw cycles and aliquot variations.

Additionally, our developed and validated CBA 3-plex assay was applied to re-analyze the porcine plasma samples from LPS-challenged pigs as described in Chapter 2.2. As shown for plasma (Chapter 3.1) and here for serum of infected pigs, similar trend was demonstrated between ELISA and CBA, with peak concentrations of TNF-α and IL-6 observed at 1 h and 2.5 h in these plasma samples from LPS-induced inflammation. Concentrations of IL-1β were confirmed to be low and inconsistent (data not shown).

In summary, this acute infection model study has demonstrated at first the feasibility of the CBA 3-plex assay for pro-inflammatory cytokine simultaneous detection in porcine serum samples. Moreover, we suggest that this immuno-assay can now be applied for the evaluation of immunomodulatory properties of drugs and vaccine adjuvants in infection and vaccination.

Modulation of the host immune response is of growing interest in human and veterinary medicine. Innate immunity guarantees the first, non-specific line of defense against invading micro-organisms. It mainly comprises of peripheral blood monocytes (PBMCs) and resident tissue macrophages which are concentrated at locations exposed to pathogen infiltration (Ulevitch, 2004; Fairnbairn et al., 2011). Additionally, neutrophils, dendritic, endothelial and epithelial cells participate in this complex process. Pattern recognition comprises the basic concept of innate immunity, with lipopolysaccharide (LPS) as a major pathogen-associated molecular pattern (PAMP), that triggers innate immunity through stimulation of toll-like receptor (TLR) 4 (Heumann and Roger, 2002; Sanz-Santos et al., 2011).

Administration of LPS has been widely applied to study the immunomodulatory properties of drugs in various animal species, including the pig. These studies were predominantly carried out at the levels of leukocyte function and cytokine production. Cytokines coordinate a wide variety of intercellular interactions after their secretion (Healy, 2007). The ongoing quest for a multi-efficient drug has focused especially on antibiotics, as these may also possess marked beneficial immunomodulatory properties besides their main antimicrobial activities. The past decade, rising evidence concerning the immunomodulatory abilities of macrolide antibiotics has emerged.

The main accomplishments of this thesis were summarized in Figure 1.

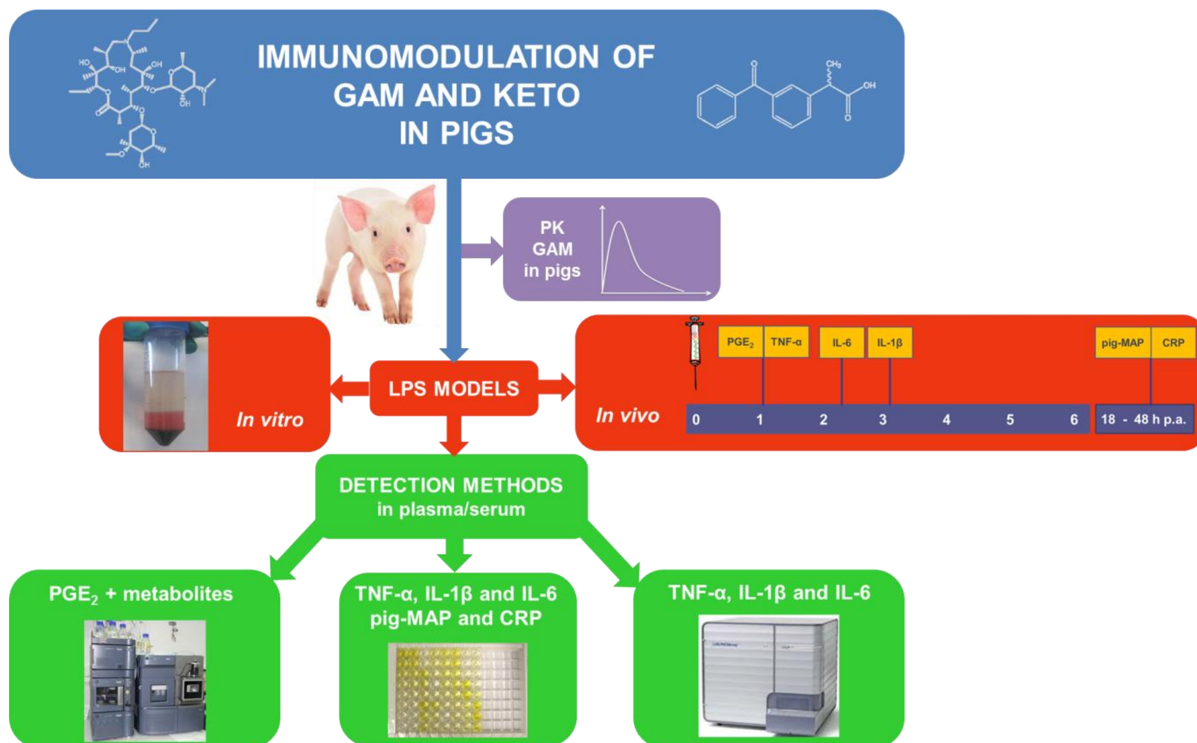


Figure 1. Overview of the main realizations achieved in this work.

Pharmacokinetic studies on gamithromycin, ketoprofen and dexamethasone

Pharmacokinetic (PK) studies provide essential information concerning maximal concentrations (C_{max}) that can be expected in the pig's plasma. Additionally, the time to this C_{max} (T_{max}) and thus the time at which a high efficacy is expected, can be estimated.

Gamithromycin (GAM) is a 2nd generation macrolide antibiotic of the azalide subclass, which is registered for use as a single subcutaneous (SC) administration at 6 mg/kg body weight (BW) in cattle (Zactran®; EMA, 2008b). Nevertheless, the PK properties of GAM have been previously described not only in cattle, but also in foals and recently by our group in broiler chickens (Huang et al., 2010; Berghaus et al., 2011; Giguère et al., 2011; Watteyn et al., 2013). Our PK study in pigs at first demonstrates that GAM is rapidly absorbed and fully bioavailable (F = 117%) after a SC injection of 6 mg/kg BW. A mean C_{max} of 0.41 µg/mL was attained at 0.63 h, which was highly comparable to the C_{max} in calves (0.43 µg/mL; Giguère et al., 2011), yet plasma concentrations generally remained far below the estimated minimum inhibitory concentration (MIC) for *Mycoplasma (M.) hyopneumoniae* (0.25 µg/mL) and *Actinobacillus (A.) pleuropneumoniae* (2 µg/mL) in pigs (unpublished data). As GAM was eliminated 3 to 4 times faster in pigs compared to cattle,

it likely requires more frequent dosage in pigs (Huang et al., 2010; Giguère et al., 2011). However, plasma concentrations of macrolides are usually considerably lower than the MIC of the pathogens against which these drugs have been proven to be effective. Of more clinical relevance is the evaluation of the drug concentration at the site of bacterial infection (Van Bambeke and Tulkens, 2001). Indeed, the high volume of distribution (V_d) of 31 L/kg indicates that much higher concentrations of GAM can be expected within porcine pulmonary epithelial lining fluid (PELF), bronchoalveolar lavage (BAL) fluid and cells and lung tissue, as is the case in cattle and foals. In addition, therapeutic concentrations persisted in these compartments for 7 to > 15 days after a single SC or intramuscular (IM) administration of 6 mg/kg BW in both the latter species (Huang et al., 2010; Berghaus et al., 2011; Giguère et al., 2011). Overall, the plasma PK properties of GAM in pigs are comparable to those obtained in other animal species. In future research, the disposition and efficacy of GAM can be evaluated in *A. pleuropneumoniae* or *M. hyopneumoniae*-infected pigs, as these are major pathogens involved in swine respiratory disease (SRD).

As clearly shown by the data obtained in this thesis, the current formulation of GAM for use in cattle might be of concern in its therapeutic applicability in pigs. Indeed, the SC administration of Zactran® to pigs was experienced as painful and was accompanied by a marked local inflammation reaction. An IM injection, which is a more appropriate route of drug administration in pig practice, was therefore not considered. On the other hand, after 3-fold dilution, as applied in our PK study, no local or systemic adverse effects were noticed after SC injection. It can thus be stated that the Zactran® formulation as such is not yet pig-proof, but can be adapted to become so.

The NSAID ketoprofen (KETO) was selected in this research for its excellent antipyretic properties and promising effects regarding combination therapy for the treatment of SRD. KETO or 2-(phenyl 3-benzoyl) propionic acid is a chiral compound and consequently exists in two enantiomeric forms. All veterinary formulations, including Ketofen 10%®, are racemic mixtures, containing both enantiomers in equal amounts. The S(+) enantiomer has been generally accepted to be pharmacologically active, through inhibition of the cyclooxygenase (COX) pathway (Cabré et al., 1998). The PK characteristics of KETO have been well-established in pigs. In this respect, the maximal plasma concentration (C_{max}) of KETO has been reported to occur between 0.68 and 1.27 h after a

single IM administration in pigs (Raekallio et al., 2008, Fosse et al., 2011; Mustonen et al., 2012b).

Glucocorticoids are among the most widely (mis)used drugs in veterinary medicine. Regardless their potent anti-inflammatory effect, this class of drugs is mainly immunosuppressive and therefore potentially harmful (Ferguson et al., 2009). Additionally, the use of glucocorticoids in both cattle and pigs remains highly controversial as they can be illegally used as growth promoters (Courtheyn et al., 2002; Vincenti et al., 2009). Dexamethasone (DEX) is expected to be a long-acting (> 48 h) synthetic glucocorticoid. Yet in our research its PK characteristics in pigs revealed a remarkably higher clearance (Cl; 2.39 L/h) and a rather short half-life of elimination ($t_{1/2el}$; 0.77 h) compared to those reported for dogs, cattle and horses (Toutain et al., 1982; Greco et al., 1993; Soma et al., 2013). However, the V_d (2.78 L/kg) suggests a good tissue penetration in pigs. Although DEX is a well-known inhibitor of cytokine production both *in vitro* and *in vivo*, its influence on LPS-induced sickness behaviour was quite disappointing in mice and, as recently evaluated by our group, in cattle too (Morikawa et al., 1996; Myers et al., 2003; Teeling et al., 2010; Plessers et al., manuscript in preparation). Therefore, DEX was only included as a positive control in our *in vitro* research.

Appropriate *in vitro* and *in vivo* porcine LPS inflammation models

To evaluate the influence of GAM and KETO on the acute phase response (APR), as well as the animals' clinical condition, we opted for *in vitro* and *in vivo* porcine LPS-induced inflammation models. *In vitro* research is expected to offer a first indication of the expected *in vivo* outcome (Ianaro et al., 2000; Leiva et al., 2008). Peripheral blood mononuclear cells have been frequently used as a model of *in vitro* immune response stimulation. In this study, incubation of porcine PBMCs with LPS induced a marked increase in the pro-inflammatory mediators PGE_2, TNF-α, IL-1β and IL-6.

The *Escherichia coli* LPS-induced fever model has been accepted and applied for the evaluation of the antipyretic effect of NSAIDs (EMA, 2001; Salichs et al., 2012). Compared to infection with live bacteria, this rather straightforward LPS model provides major advantages as the sequence of mediator release following LPS administration is well-documented and quite standardized (Gerros et al., 1993).

The administration of LPS to pigs immediately initiates a cascade of events which can be approached from both an immunological and a clinical point of view. On the one hand, binding of LPS to its TLR4 leads to the activation of transcription factors initiating the expression, production and/or release of COX, prostaglandins (PGs), thromboxane (TX), cytokines and at a later stage also of acute phase proteins (APPs). On the other hand, severe clinical symptoms including anorexia, nausea, tachypnea, dyspnea, fever and a prolonged depression phase are simultaneously induced which considerably restrict the animals' clinical condition and well-being.

Nevertheless, it should be emphasized that the development of a suitable *in vivo* model in pigs is quite challenging and needs to be subjected to some critical considerations, as illustrated below.

A first step in applying such an LPS model in pigs is the selection of an appropriate LPS serotype and of pig-related variables, including age, weight and breed.

Different LPS serotypes and preparations are commercially available of which the O111:B4 preparation provided by Sigma is indisputably the most frequently used in porcine research (Frank et al., 2003; Carroll et al., 2005; Williams et al., 2009; Wyns et al., manuscript submitted). However, protein impurities in those LPS preparations may influence the transcriptional profile as both TLR2- and TLR4-mediated signaling can occur (Lorne et al., 2010; Rutledge et al., 2012). To date, our research group is the only one applying the ultrapure O111:B4 variant of InvivoGen, which is recognized to selectively activate TLR4 but not TLR2.

The response to LPS significantly differs between animal species, with pigs and ruminants being very sensitive since relative low LPS doses induce marked effects on the cardiovascular system. This has been ascribed to the presence of pulmonary intravascular macrophages (PIMs) in the pulmonary circulation of both species. Cardiovascular effects of endotoxin in cats, rabbits, ponies and horses are less pronounced (Olson et al., 1995). In dogs, rodents and broiler chickens, extremely high endotoxin dosages (> 1 mg/kg) are administered (Olson et al., 1995; De Boever et al., 2010).

In this respect, we had to find a compromise between eliciting a marked clinical and immunological inflammatory response with systemically measurable PGE_2, TNF-α, IL-1β, IL-6, pig-MAP and CRP concentrations, but without causing shock or mortality. Based on previous reports, a dose of 15 µg/kg BW (or 15 x 10^3 EU/kg BW) was selected and can be

considered as moderate for use in pigs. As pigs with a BW > 20 kg are considered immunologically mature animals with respect to their cellular functionality and cytokine production (Myers et al., 1999), in our LPS experiments they all had a BW of 20-30 kg. We also opted to work with a central venous catheter for accurate IV LPS administration and atraumatic repetitive blood sampling in sick animals.

To perform this catheterization, a team of surgeons and anesthetists had to be mobilized and subsequently a careful individual follow-up of the catheterized animals was required. For the latter purpose, the surgical suture at the jugular groove was visually evaluated, the catheters were flushed at least once daily with heparinized saline, and the bandages protecting the catheter from trauma and contamination, were changed daily.

In our study, the IV administration of 15 µg/kg BW ultrapure LPS induced systemic peak concentrations of PGE_2 and TNF-α at 1 h p.a., and maximal concentrations of IL-6 at 2.5 h p.a. As expected, levels of pig-MAP and CRP started to increase not earlier than 18 h after LPS administration. In contrast to previous reports, the mean maximal rectal body temperatures occurred as early as 2.5 h p.a. of LPS. Severe clinical symptoms of acute lung failure and general sickness, including tachypnea, dyspnea, anorexia, nausea, vomiting and depression were developed in all LPS-induced pigs.

Compared to the standardized and reproducible LPS model, the APR of an *in vivo* experimental bacterial infection model, on the other hand, is far less predictable (Olson et al., 1995; Myers et al., 2003). In contrast to TNF-α and IL-1β, IL-6 has been consistently detected in the majority, if not all, of the infected pigs (Fossum et al., 1998; Myers et al., 2004). Consequently, IL-6 has been postulated as a valuable biomarker for early disease detection in bacterial infections (Fossum et al., 1998; Tambuyzer et al., 2014), as well as for the evaluation of antibiotic treatment (Lauritzen et al., 2003). The appearance of TNF-α, IL-1β and IL-6 in the serum of *A. pleuropneumoniae*-infected pigs occurred remarkably later compared to our LPS model, with mean maximal serum concentrations of TNF-α and IL-6 at 12 h and 10 h after infection, respectively.

In summary, we can conclude that both our *in vitro* and *in vivo* LPS models were appropriate for testing the immunomodulatory effect of GAM, KETO and their combination on the animal's clinical condition and the inflammatory mediator release, using DEX as a positive control.

As a counterpart for IL-6 in porcine bacterial infections, PGE_2 can be put forward as a candidate valuable early-disease-monitoring biomarker for LPS-induced fever and inflammation in pigs.

Immunology orchestrates symptomatology

"Can the deteriorating clinical condition following LPS administration be ascribed to the systemic appearance of PGE2 and/or of TNF-α, IL-1β, IL-6, pig-MAP, CRP?"

Cytokines have long been regarded as responsible for the various and often severe clinical symptoms that occur after LPS administration, including the induction of fever and the species-specific expression of sickness behaviour (Bluthé et al., 1991; Konsman et al., 2002; Dantzer, 2009). Indeed, the peripheral injection of TNF-α, IL-1 and IL-6 all mimic LPS-induced symptoms, whereas the administration of anti-TNF antibodies, IL-1 receptor antagonists or the use of IL-1 receptor or IL-6-deficient animals to a larger or lesser extent reverse these symptoms (Fong et al., 1989; Bluthé et al., 1991; 2000a; 2000b; Teeling et al.; 2007; Dantzer, 2009). Since cytokines act in a complex interacting network after their release, it is relatively difficult to address specific actions to a single cytokine (Dantzer, 2009). On the one hand, similar to LPS, the intra-cerebroventricular injection of recombinant porcine TNF-α induced anorexia and depression in pigs (Warren et al., 1997). On the other hand, Johnson and von Borell (1994) suggested a crucial role for COX and PGs, as LPS-induced symptoms could be completely inhibited by pretreatment of pigs with the selective COX-1-inhibitor indomethacin. This COX-pathway of thinking was also suggested by other authors (Teeling et al., 2007 and 2010) and found to be plausible to explain our results as discussed below.

Although pigs of the KETO and GAM-KETO groups demonstrated high systemic cytokine levels, which were sometimes even higher than those measured in pigs of the LPS group, these animals surprisingly showed no sickness behaviour and no febrile response following LPS administration. Furthermore, these pigs had no quantifiable levels of PGE_2.

On the other hand, DEX which is a known repressor of NF-κB-regulated gene transcription, including cytokines and COX-2, was reported to efficiently block TNF-α, IL-1β and IL-6, but had no effect on the LPS-induced behavioural changes in mice (Adcock et al., 1999; Bezugla et al., 2006; Teeling et al., 2010). Similarly, it has been shown by our group

that DEX only attenuated the depression phase of the APR in calves (Plessers et al., manuscript in preparation). In our *in vitro* study, DEX successfully and significantly suppressed the production of PGE_2, TNF-α, IL-1β and IL-6. Additionally, and somehow surprisingly, selective COX-1 inhibitors, including indomethacin, were more potent inhibitors of LPS-induced behavioural changes (Botting, 2006; Teeling et al., 2010).

These findings offer different perspectives on the functions of both COX enzymes and suggest that COX-1, besides its function as a housekeeping enzyme, plays a significant role in inflammation-related sickness behaviour and the development of fever. COX-1 is also considered responsible for the early and massive production of TXA_2 in platelets following LPS administration (Botting, 2006). These findings might also partially explain why KETO, which is a COX-1-selective NSAID, has been proven to be a superior antipyretic drug in pigs (Swinkels et al., 1994; Salichs et al., 2012).

Pharmacology encounters immunology

"Can modulation of the innate immune response contribute to an improved pharmacodynamic activity?"

It has become nearly generally accepted that macrolide antibiotics reduce the production of pro-inflammatory mediators. However, unlike the reports on azithromycin, erythromycin, clarithromycin, roxithromycin, telithromycin, tylosin and tilmicosin (Kanoh and Rubin, 2010), GAM did not affect the production of PGE_2, TNF-α, IL-1β and IL-6 either *in vitro* or *in vivo*. Moreover, pretreatment with GAM did not improve the pigs' welfare. In contrast, these pigs even showed a worse clinical condition as demonstrated by a remarkably early and longer-lasting depression phase compared to the pigs receiving only LPS.

Azalides, including GAM, have been reported to concentrate preferably within lung macrophages (Mattoes and Nightingale, 2002; Huang et al., 2010). In this context, it is not unlikely that GAM stimulates porcine pulmonary intravascular macrophages (PIMs) to release more TXA_2 which at least in part can explain the observed discrepancy between the LPS and GAM groups. However, it has to be taken into account that a likely cause would be the GAM formulation and related marked local inflammation reaction.

Pretreatment of the pigs with KETO nearly completely counteracted the deleterious effects of LPS. This was both surprising and expected. Indeed, the efficacy of KETO, and its superiority to other NSAIDs, has already been demonstrated after LPS administration, in *A. pleuropneumoniae*-infected pigs as well as in pigs with clear symptoms of SRD (Swinkels et al., 1994; Mustonen et al., 2012a; Salichs et al., 2012; 2013). Still, it was surprising that those pigs experienced no adverse clinical effects of LPS administration at all. Being a potent COX-inhibitor, KETO significantly reduced the concentration of PGE_2, both *in vitro* and *in vivo,* whereas no influence on the production of TNF-α, IL-1β and IL-6 was observed. In this respect, KETO has certainly to be considered as a pharmacological agent to improve animal welfare.

The concomitant use of an antibiotic and an (N)SAID has been recommended by some authors to decrease the severity of clinical symptoms, including anorexia and inflammation-induced lung failure. This therapeutic strategy can limit the impact of bacterial respiratory diseases on weight gain and animal welfare (Francoz et al., 2012). In this respect, it was hypothesized that the combination of GAM and KETO would be more beneficial in decreasing LPS-associated symptoms compared to the exclusive administration of each of the respective drugs. It should be nevertheless be stressed that in this study, we demonstrated that the administration of both GAM and KETO was not advantageous compared to administration of KETO alone. Although this was a disappointing observation, a positive finding of this research was that the combination KETO and GAM did not evoke any adverse effects in marked contrast to GAM alone. Overall, we can suggest that its combination with KETO might be beneficial in experimental porcine bacterial infection models, including *A. pleuropneumoniae* or *M. hyopneumoniae* and should subsequently be investigated under field conditions.

Last but not least, macrolides indirectly facilitate their direct antimicrobial activity by stimulating innate immunity through enhanced leukocyte phagocytosis, degranulation, oxidative burst and chemotaxis (Parnham, 2005). As these drugs are potent weak bases they become ion-trapped within acidic intracellular compartments such as lysosomes and phagosomes. This mechanism potentially increases their antimicrobial activity against intracellular pathogens too (Labro, 1996). It was recently demonstrated that tulathromycin inhibits cytosolic phospholipase A_2 and the production of PGE_2 in bovine neutrophils and zymosan-challenged calves, respectively (Fischer et al., 2014). In *A. pleuropneumoniae*-

infected pigs, tulathromycin significantly improved the clinical condition of the animals (Prof. dr. Buret; personal communication).

In brief, while KETO can guarantee appetite, weight gain and animal welfare, GAM can efficiently battle the bacterial infection and limit persisting lung damage, supporting the combined use of both drugs.

Both *in vitro* and *in vivo*, KETO showed a trend to enhance TNF-α levels, which is in agreement with previous reports (Pettipher and Wimberly, 1994; Ghezzi et al., 1998; Roth et al., 2002). Additionally, KETO and the combination of GAM and KETO significantly reduced the production of PGE_2. In this respect, the results of our *in vitro* study were largely confirmed by our *in vivo* study. Remarkably, the combination of GAM and KETO significantly reduced the *in vitro* production of IL-1β and IL-6, and showed a trend to reduce TNF-α production. This latter effect was not confirmed *in vivo*.

Analytical methodology profiles immunology

"Towards an improved multi-screening method for cytokine profiling"

From the three major pro-inflammatory cytokines, IL-1β remains a major source of concern from an analytical point of view. Indeed, several authors have vainly attempted to include this cytokine in the characterization of the porcine innate immune response, yet IL-1β was reported recurrently as neither detectable, nor increasing following LPS administration (Myers et al., 2003; Tuchscherer et al., 2004; Llamas Moya et al., 2006; Ruud et al., 2007). Nevertheless, it is still widely assumed that IL-1β plays a pivotal role in the LPS-induced sickness behaviour in several species (Bluthé et al., 1991; 2000a; Dantzer, 2009). During our research, we used two different porcine-specific commercial ELISA kits, both provided by R&D Systems: a less expensive one (DuoSet®), which needed in-house validation before its use for plasma samples and a more expensive ready-to-use kit (Quantikine®). Using the latter kit, a maximal yet low plasma concentration of IL-1β was demonstrated at 3 h after LPS administration. However the large standard deviations (SDs) measured indicated major biological variability between the individual animals. The economical more attractive ELISA was even considered not suitable for measurement of IL-1β in porcine plasma, as a higher dilution factor was required to reduce matrix interferences and the IL-1β concentrations in these diluted samples were too low to remain

quantifiable. Overall, concentrations of porcine IL-1β are low and inconsistent, which is in marked contrast to the well-characterised sky-rocketing levels of TNF-α and substantial increase of IL-6 following LPS administration in our model.

From this point of view, we intended to develop a cytometric bead array (CBA) screening tool for the simultaneous detection (multiplexing) of TNF-α, IL-1β and IL-6, and compared these data with our gold standard ELISA results. Our CBA screening tool has been proven to be a valuable time-saving multi-parameter screening alternative for the time-consuming single-parameter approach offered by ELISA. Similar to ELISA, a maximal plasma concentration of IL-1β was demonstrated at 3 h after LPS administration. CBA offers the opportunity to evaluate the cytokine profiles relative to each other in one sample analysis. Within the scope of the above-mentioned cytokine network, this strategy has been regarded to be potentially more biologically relevant than the measurement of absolute concentrations (Kelso, 1999).

"Towards an improved efficient multi-method for eicosanoid profiling"

PGE_2 is generally measured by means of immuno-based assays such as ELISA, which is considered as the gold standard. As PGE_2 is rapidly metabolized both *in vitro* and *in vivo*, we succeeded in developing and validating a specific and accurate ultra-performance liquid chromatography (UPLC)-tandem mass spectrometry (MS/MS) method for the simultaneous detection of PGE_2 and its major inactive metabolite 13,14-dihydro-15-keto PGE_2 in plasma. In order to determine the *in vivo* PG concentration, it is of major importance to block the *ex vivo* PG generation immediately after blood sampling, by adding a COX-inhibitor to the samples (Pelligand et al., 2012). This LC-MS/MS methodology offers the advantage of a very high specificity and sensitivity, especially needed when dealing with the analysis of hundreds of eicosanoid metabolites with very similar chemical structures and physico-chemical properties, and present in small quantities of biological samples. Our data suggest that it may be useful e.g. to monitor eicosanoid biomarkers as readouts reflecting disease, or as tool in (veterinary) drug research for efficacy testing.

In conclusion, we succeeded in developing an appropriate LPS inflammation model with efficient multi-parameter methods for both pro-inflammatory cytokines and eicosanoids, allowing for the state-of-the-art evaluation of immunomodulatory properties of drugs in pigs.

Future perspectives

Our current research has provided an ideal basis to:

- Evaluate the *in vivo* influence of glucocorticoids on the LPS-induced production of PGE_2, TNF-α, IL-1β and IL-6 in pigs, and thus gain a more profound insight and confirmation of the additional rather than essential role of cytokines in the development of severe symptoms following LPS administration.
- Evaluate the *in vivo* immunomodulatory properties of other antibiotics in porcine LPS and/or experimental bacterial infection models such as *A. pleuropneumoniae* or *M. hyopneumoniae*, either with or without the combined administration of KETO.
- Measure the concentration of TXA_2 in our developed porcine LPS inflammation model, as this mediator is considered to be responsible for the LPS-associated symptoms of acute lung failure and can be used as an early detection parameter of bacterial infections. It might be interesting to include this mediator in a multi-eicosanoid ultra-performance LC-MS/MS method for the qualitative and quantitative detection of eicosanoids.

Adams, H.R. (2001) Prostaglandins, related factors, and cytokines. In: *Veterinary Pharmacology and Therapeutics*. 8th edition. Ames, Iowa, USA, pp.

Adcock, I.M., Nasuhara, Y., Stevens, D.A., Barnes, P.J. (1999) Ligand-induced differentiation of glucocorticoid receptor (GR) trans-repression and transactivation: preferential targetting of NF-κB and lack of I-κB involvement. *British Journal of Pharmacology* 127, 1003-1011.

Alava, M.A., González-Ramón, N., Heegaard, P., Guzylack, S., Toussaint, M.J.M., Lipperheide, C., Madec, F., Gruys, E., Eckersall, P.D., Lampreave, F., Piñeiro, A. (1997) Pig-MAP, porcine acute phase proteins and standardisation of assays in Europe. *Comparative Haematology International* 7, 208-213.

Algarra, M., Gomes, D., Esteves da Silva, J.C. (2013) Current analytical strategies for C-reactive protein quantification in blood. *Clinica Chimica Acta* 415, 1-9.

Anadon, A., Reeve-Johnson, L. (1999) Macrolide antibiotics, drug interactions and microsomal enzymes: implications for veterinary medicine. *Research in Veterinary Science* 66, 197-203.

Araujo, P., Mengesha, Z., Lucena, E., Grung, B. (2013) Development and validation of an extraction method for the determination of pro-inflammatory eicosanoids in human plasma using liquid chromatography-tandem mass spectrometry. *Journal of Chromatography A*.

Baarsch, M.J., Scamurra, R.W., Burger, K., Foss, D.L., Maheswaran, S.K., Murtaugh, M.P. (1995) Inflammatory cytokine expression in swine experimentally infected with *Actinobacillus pleuropneumoniae*. *Infection and Immunity* 63, 3587-3594.

Bailly, S., Fay, M., Roche, Y., Gougerot-Pocidalo, M.A. (1990) Effects of quinolones on tumor necrosis factor production by human monocytes. *International Journal of Immunopharmacology* 12, 31-36.

Bailly, S., Fay, M., Ferrua, B., Gougerot-Pocidalo, M.A. (1991a) Ciprofloxacin treatment *in vivo* increases the *ex vivo* capacity of lipopolysaccharide-stimulated human monocytes to produce IL-1, IL-6 and tumour necrosis factor-alpha. *Clinical and Experimental Immunology* 85, 331-334.

Bailly, S., Pocidalo, J.J., Fay, M., Gougerot-Pocidalo, M.A. (1991b). Differential modulation of cytokine production by macrolides: interleukin-6 production is increased by spiramycin and erythromycin. *Antimicrobial Agents and Chemotherapy* 35, 2016-2019.

Balaji, R., Wright, K.J., Turner, J.L., Hill, C.M., Dritz, S.S., Fenwick, B., Carroll, J.A., Zannelli, M.E., Beausang, L.A., Minton, J.E. (2002) Circulating cortisol, tumor necrosis factor-alpha interleukin-1beta, and interferon-gamma in pigs infected with *Actinobacillus pleuropneumoniae*. *Journal of Animal Science* 80, 202-207.

Ball, H.A., Cook, J.A., Wise, W.C., Halushka, P.V. (1986) Role of thromboxane, prostaglandins and leukotrienes in endotoxic and septic shock. *Intensive Care Medicine* 12, 116-126.

Baumann, H., Gauldie, J. (1994) The acute phase response. *Immunology Today* 15, 74-80.

Beinke, S., Ley, S.C. (2004) Functions of NF-kappaB1 and NF-kappaB2 in immune cell biology. *Biochemical Journal* 382, 393-409.

Benchaoui, H.A., Nowakowski, M., Sherington, J., Rowan, T.G., Sunderland, S.J. (2004) Pharmacokinetics and lung tissue concentrations of tulathromycin in swine. *Journal of Veterinary Pharmacology and Therapeutics* 27, 203-210.

Berghaus, L.J., Giguère, S., Sturgill, T.L., Bade, D., Malinski, T.J., Huang, R. (2012) Plasma pharmacokinetics, pulmonary distribution, and in vitro activity of gamithromycin in foals. *Journal of Veterinary Pharmacology and Therapeutics* 35, 59-66.

Bertram, T.A. (1986) Intravascular macrophages in lungs of pigs infected with *Haemophilus pleuropneumoniae. Veterinary Pathology* 23, 681-691.

Bessler, H., Mendel, C., Straussberg, R., Gurary, N., Aloni, D., Sirota, L. (1999) Effects of dexamethasone on IL-1beta, IL-6, and TNF-alpha production by mononuclear cells of newborns and adults. *Biology of the Neonate* 75, 225-233.

Bezugla, Y., Kolada, A., Kamionka, S., Bernard, B., Scheibe, R., Dieter, P. (2006) COX-1 and COX-2 contribute differntially to the LPS-induced release of PGE_2 and TxA_2 in liver macrophages. *Prostaglandins & other Lipid Mediators* 79, 93-100.

Bi, J., Song, S., Fang, L., Wang, D., Jing, H., Goa, L., Cai, Y., Luo, R., Chen, H., Xiao, S. (2014) Porcine reproductive and respiratory syndrome virus induces IL-1β production depending on TLR4/MyD88 pathway and NLRP3 inflammasome in primary porcine alveolar macrophages. *Mediators of Inflammation* (http://dx.doi.org/10.1155/2014/403515).

Biswas, S.K., Lopez-Collazo, E. (2009) Endotoxin tolerance: new mechanisms, molecules and clinical significance. *Trends in Immunology* 30, 475-487.

Bjerre, M., Hansen, T.K., Flyvbjerg, A., Tønnesen, E., (2009) Simultaneous detection of porcine cytokines by multiplex analysis: Development of magnetic bioplex assay. *Veterinary Immunology Immunopathology* 130, 53-58.

Blatteis, C.M. (2007) The onset of fever: new insights into its mechanism. *Progress in Brain Research* 162, 3-14.

Blatteis, C.M., Li, S.X., Li, Z.H., Feleder, C., Perlik, V. (2005) Cytokines, PGE(2) and endotoxic fever: a re-assessment. *Prostaglandins & other Lipid Mediators* 76, 1-18.

Blecha, F. (1988) Immunomodulation: a means of disease prevention in stressed livestock. *Journal of Animal Science* 66, 2084-2090.

Bluthé, R.M., Dantzer, R., Kelley, K.W. (1991) Interleukin-1 mediates behavioural but not metabolic effects of tumor necrosis factor alpha in mice. *European Journal of Pharmacology* 209, 281-283.

Bluthé, R.M., Laye, S., Michaud, B., Combe, C., Dantzer, R., Parnet, P. (2000a) Role of interleukin-1beta and tumour necrosis factor-alpha in lipopolysaccharide-induced sickness behaviour: a study with interleukin-1 type I receptor-deficient mice. *The European Journal of Neuroscience* 12, 4447-4456.

Bluthé, R.M., Michaud, B., Poli, V., Dantzer, R. (2000b) Role of IL-6 in cytokine-induced sickness behavior: a study with IL-6 deficient mice. *Physiology & Behavior* 70, 367-373.

Bongoni, A.K., Lanz, J., Rieben, R., Banz, Y. (2013) Development of a bead-based multiplex assay for the simultaneous detection of porcine inflammation markers using xMAP technology. *Cytometry A* 83, 636-647.

Botting, R.M. (2006) Inhibitors of cyclooxygenases: mechanisms, selectivity and uses. Journal of Physiology and Pharmacology 57, 113-124.

Brain, J.D., Molina, R.M., DeCamp, M.M., Warner, A.E. (1999) Pulmonary intravascular macrophages: their contribution to the mononuclear phagocyte system in 13 species. *American Journal of Physiology-Lung Cellular and Molecular Physiology* 276, L146-L154.

Brown, J., Wang, H., Hajishengallis, G.N., Martin, M. (2011) TLR-signaling networks: an integration of adaptor molecules, kinases, and cross-talk. *Journal of Dental Research* 90, 417-427.

Buras, J.A., Holzmann, B., Sitkovsky, M. (2005) Animal models of sepsis: setting the stage. *Nature Reviews Drug Discovery* 4, 854-865.

Buret, A.G. (2010) Immuno-modulation and anti-inflammatory benefits of antibiotics: the example of tilmicosin. *Canadian Journal of Veterinary Research* 74, 1-10.

Bygdeman, M. (2003) Pharmacokinetics of prostaglandins. *Best Practice & Research Clinical Obstetrics* 17, 707-+.

Cabré, F., Fernandez, M.F., Calvo, L., Ferrer, X., Garcia, M.L., Mauleon, D. (1998) Analgesic, antiinflammatory, and antipyretic effects of S(+)-ketoprofen *in vivo*. *Journal of Clinical Pharmacology* 38, 3S-10S.

Cao, X.Y., Dong, M., Shen, J.Z., Wu, B.B., Wu, C.M., Du, X.D., Wang, Z., Qi, Y.T., Li, B.Y. (2006) Tilmicosin and tylosin have anti-inflammatory properties via modulation of COX-2 and iNOS gene expression and production of cytokines in LPS-induced macrophages and monocytes. *International Journal of Antimicrobial Agents* 27, 431-438.

Carroll, J.A., Carter, D.B., Korte, S.W., Prather, R.S. (2005) Evaluation of the acute phase response in cloned pigs following a lipopolysaccharide challenge. *Domestic Animal Endocrinology* 29, 564-572.

Carson, R.T., Vignali, D.A.A. (1999) Simultaneous quantitation of 15 cytokines using a multiplexed flow cytometric assay. *Journal of Immunological Methods* 227, 41-52.

Cavaillon, J.M., Adib-Conquy, M. (2005) Monocytes/macrophages and sepsis. *Critical Care Medicine* 33(12 Suppl), S506-509.

Cerasoli, I., Marcilla, M.G., Wyns, H., van Bergen, T., Gasthuys, F., Duchateau, L., Schauvliege, S. (2014) Alfaxalone constant rate infusion during isoflurane anaesthesia in midazolam, morphine and ketamine premedicated pigs. *Veterinary Anaesthesia and Analgesia. Association of Veterinary Anaesthesists meeting*, Vienna, Austria.

Chen, R., Lowe, L., Wilson, J.D., Crowther, E., Tzeggai, K., Bishop, J.E., Varro, R. (1999) Simultaneous quantification of six human cytokines in a single sample using microparticle-based flow cytometric technology. *Clinical Chemistry* 45, 1693-1694.

Chiers, K., De Waele, T., Pasmans, F., Ducatelle, R., Haesebrouck, F. (2010) Virulence factors of Actinobacillus pleuropneumoniae involved in colonization, persistence and induction of lesions in its porcine host. *Veterinary Research* 41, 65.

Christopher-Hennings, J., Araujo, K.P., Souza, C.J., Fang, Y., Lawson, S., Nelson, E.A., Clement, T., Dunn, M., Lunney, J.K. (2013) Opportunities for bead-based multiplex assays in veterinary diagnostic laboratories. *Journal of Veterinary Diagnostic Investigation* 25, 671-691.

Clark, S.G., Coffer, N. (2008) Normal hematology and hematologic disorders in potbellied pigs. *The Veterinary Clinics of North America. Exotic animal practice* 11, 569-582, vii.

Coceani, F., Bishai, I., Lees, J., Sirko, S. (1986) Prostaglandin-E2 and Fever - a Continuing Debate. *Yale Journal of Biology and Medicine* 59, 169-174.

Coelho, M.M., Luheshi, G., Hopkins, S.J., Pela, I.R., Rothwell, N.J. (1995) Multiple mechanisms mediate antipyretic action of glucocorticoids. *The American journal of physiology* 269, R527-535.

Commission Decision 2002/657/EC. Implementing Council Directive 96/23/EC Concerning the Performances of Analytical Methods and the Interpretation of Results. Official Journal of the European Communities, No. L221, 17/08/2002, Decision of 12 August 2002, *European Commission, Directorate General for Public Health and Consumers Protection.*

Conti, B., Tabarean, I., Andrei, C., Bartfai, T., 2004. Cytokines and fever. *Frontiers in Bioscience* 9, 1433-1449.

Courtheyn, D., Le Bizec, B., Brambilla, G., De Brabander, H.F., Cobbaert, E., Van de Wiele, M., Vercammen, J., De Wasch, K. (2002) Recent developments in the use and abuse of growth promoters. *Analytica Chimica Acta* 473, 71-82.

Čulić, O., Erakovic, V., Parnham, M.J. (2001) Anti-inflammatory effects of macrolide antibiotics. *European Journal of Pharmacology* 429, 209-229.

Curry, S.L., Cogar, S.M., Cook, J.L. (2005) Nonsteroidal antiinflammatory drugs: a review. *Journal of American Animal Hospital Association* 41, 298-309.

Dantzer, R. (2009) Cytokine, sickness behavior, and depression. *Immunology and Allergy Clinics of North America* 29, 247-264.

De Boever, S., Neirinckx, E.A., Meyer, E., de Baere, S., Beyaert, R., de Backer, P., Croubels, S. (2010) Pharmacodynamics of tepoxalin, sodium-salicylate and ketoprofen in an intravenous lipopolysaccharide inflammation model in broiler chickens. *Journal of Veterinary Pharmacology and Therapeutics* 33, 564-572.

Deegan, O., Walshe, K., Kavanagh, K., Doyle, S. (2003) Quantitative detection of C-reactive protein using phosphocholine-labelled enzyme or microspheres. *Analytical Biochemistry* 312, 175-181.

Dernfalk, J., Persson Waller, K., Johannisson, A. (2007) The xMAP^{TM} technique can be used for detection of the inflammatory cytokines IL-1β, IL-6 and TNF-α in bovine samples. *Veterinary Immunology Immunopathology* 118, 40-49.

Dickneite, G., Leithäuser, B. (1999) Influence of antithrombin III on coagulation and inflammation in porcine septic shock. *Arteriosclerosis, Thrombosis, and Vascular Biology* 19, 1566-1572.

Dinarello, C.A. (1997) Proinflammatory and anti-inflammatory cytokines as mediators in the pathogenesis of septic shock. *Chest*, 112(6 Suppl), 321S-329S.

Donalisio, C., Barbero, R., Cuniberti, B., Vercelli, C., Casalone, M., Re, G. (2013) Effects of flunixin meglumine and ketoprofen on mediator production in *ex vivo* and *in vitro* models of inflammation in healthy dairy cows. *Journal of Veterinary Pharmacology and Therapeutics* 36, 130-139.

Drieghe, S.A., Alsaadi, H., Tugirimana, P.L., Delanghe, J.R. (2014) A new high-sensitive nephelometric method for assaying serum C-reactive protein based on phosphocholine interaction. *Clinical Chemistry and Laboratory Medicine* 52, 861-867.

Ebdrup, L., Krog, J., Granfeldt, A., Larsen, P.O., Vestergaard, C., Hokland, M., Tonnesen, E. (2008) Leukocyte, plasma, and organ-associated cytokine profiles in an animal model of acute inflammation. *Acta Pathologica, Microbiologica, et Immunologica Scandinavica* 116, 352-360.

Eckersall, P.D., Saini, P.K., McComb, C. (1996) The acute phase response of acid soluble glycoprotein, alpha(1)-acid glycoprotein, ceruloplasmin, haptoglobin and C-reactive protein, in the pig. *Veterinary Immunology and Immunopathology* 51, 377-385.

Elshal, M.F., McCoy, J.P. (2006) Multiplex bead array assays: Performance evaluation and comparison of sensitivity to ELISA. *Methods* 38, 317-323.

EMA, European Medicines Agency (2001) Guideline for the conduct of efficacy studies for non-steroidal anti-inflammatory drugs. EMEA/CVMP/237/01-FINAL. Date latest accessed: 9 February 2014.

EMA, European Medicines Agency (2004) The European Agency for the Evaluation of Medicinal Products. *Dexamethasone: Summary Report (3).* Date latest accessed: 8 July 2013

EMA, European Medicines Agency (2008a) The European Agency for the Evaluation of Medicinal Products. *Draxxin: EPAR – Product Information.* Date latest accessed: 8 July 2013.

EMA, European Medicines Agency (2008b) The European Agency for the Evaluation of Medicinal Products. *Zactran: EPAR - Scientific Discussion.* Date latest accessed: 28 September 2014.

EMA, European Medicines Agency (2011) The European Agency for the Evaluation of Medicinal Products. *Zuprevo: EPAR - Product information.* Date latest accessed: 28 September 2014.

Er, A., Yazar, E. (2012) Effects of tylosin, tilmicosin and tulathromycin on inflammatory mediators in bronchoalveolar lavage fluid of lipopolysaccharide-induced lung injury. *Acta Veterinaria Hungarica* 60, 465-476.

Ericsson, A., Liu, C., Hart, R.P., Sawchenko, P.E. (1995) Type 1 interleukin-1 receptor in the rat brain: distribution, regulation, and relationship to sites of IL-1-induced cellular activation. *The Journal of Comparative Neurology* 361, 681-698.

Escribano, D., Campos, P.H., Gutierrez, A.M., Le Floc'h, N., Ceron, J.J., Merlot, E. (2014) Effect of repeated administration of lipopolysaccharide on inflammatory and stress markers in saliva of growing pigs. *The Veterinary Journal*, 200(3), 393-397.

Fairnbairn, L., Kapetanovic, R., Sester, D.P., Hume, D.A. (2011) The mononuclear phagocyte system of the pig as a model for understanding human innate immunity and disease. Journal of Leukocyte Biology 89, 855-871.

Fantuzzi, G., Ghezzi, P. (1993) Glucocorticoids as cytokine inhibitors: role in neuroendocrine control and therapy of inflammatory diseases. *Mediators of Inflammation* 2, 263-270.

Feghali, C.A., Wright, T.M. (1997) Cytokines in acute and chronic inflammation. *Frontiers in Bioscience* 2, 12-26.

Ferguson, D.C., Dirikolu, L., Hoenig, M. (2009) Glucocorticoids, mineralocorticoids, and adrenolytic drugs. In: Veterinary Pharmacology and Therapeutics, 9th edition, Wiley-Blackwell, Iowa, USA, pp. 771-802.

Fischer, C.D., Duquette, S.C., Renaux, B.S., Feener, T.D., Morck, D.W., Hollenberg, M.D., Lucas, M.J., Buret, A.G. (2014) Tulathromycin exerts preresolving effects in bovine neutrophils by inhibiting phospholipases and altering leukotriene B_4, prostaglandin E_2, and lipoxin A_4 production. *Antimicrobial Agents and Chemotherapy* 58, 4298-4307.

Fink, M.P., Heard, S.O. (1990) Laboratory models of sepsis and septic shock. *Journal of Surgical Research* 49, 186-196.

Fitzpatrick, F.A., Aguirre, R., Pike, J.E., Lincoln, F.H. (1980) The stability of 13,14-dihydro-15 keto-PGE2. *Prostaglandins* 19, 917-931.

Fong, Y., Tracey, K.J., Moldawer, L.L., Hesse, D.G., Manogue, K.B., Kenney, J.S., Lee, A.T., Kuo, G.C., Allison, A.C., Lowry, S.F., Cerami, A. (1989) Antibodies to cachectin/tumor necrosis factor reduce interleukin 1β and interleukin 6 appearance during lethal bacteremia. *Journal of Experimental Medicine* 170, 1627-1633.

Fosse, T.K., Toutain, P.L., Spadavecchia, C., Haga, H.A., Horsberg, T.E., Ranheim, B. (2011) Ketoprofen in piglets: enantioselective pharmacokinetics, pharmacodynamics and PK/PD modelling. *Journal of Veterinary Pharmacology and Therapeutics* 34, 338-349.

Fossum, C., Wattrang, E., Fuxler, L., Jensen, K.T., Wallgren, P. (1998) Evaluation of various cytokines (IL-6, IFN-alpha, IFN-gamma, TNF-alpha) as markers for acute bacterial infection in swine--a possible role for serum interleukin-6. *Veterinary Immunology and Immunopathology* 64, 161-172.

Francoz, D., Buczinski, S., Apley, M. (2012) Evidence related to the use of ancillary drugs in bovine respiratory disease (anti-inflammatory and others): are they justified or not? *Veterinary Clinics of North America: Food Animal Practice* 28, 23-38.

Frank, J.W., Carroll, J.A., Allee, G.L., Zannelli, M.E. (2003) The effects of thermal environment and spray-dried plasma on the acute-phase response of pigs challenged with lipopolysaccharide. *Journal of Animal Science* 81, 1166-1176.

Frank, J.W., Mellencamp, M.A., Carroll, J.A., Boyd, R.D., Allee, G.L. (2005) Acute feed intake and acute-phase protein responses following a lipopolysaccharide challenge in pigs from two dam lines. *Veterinary Immunology and Immunopathology* 107, 179-187.

Friendship, R.M., Lumsden, J.H., Mcmillan, I., Wilson, M.R. (1984) Hematology and Biochemistry Reference Values for Ontario Swine. *The Canadian Journal of Comparative Medicine* 48, 390-393.

Friton, G.M., Schmidt, H., Schrodl, W. (2006) Clinical and anti-inflammatory effects of treating endotoxin-challenged pigs with meloxicam. *Veterinary Record* 159, 552-557.

Gasthuys, F., Baert, K., De Backer, P. (1999) Repetitive intravenous catheterisation for pharmacokinetic studies in pigs. *Vlaams Diergeneeskundig Tijdschrift* 68, 85-90.

Gasthuys, F., De Boever, S., Schauvliege, S., Reyns, T., Levet, T., Cornillie, P., Casteleyn, C., De Backer, P., Croubels, S. (2009) Transsplenic portal catheterization combined with a jugular double-lumen catheter for pharmacokinetic and presystemic metabolization studies in pigs. *Journal of Veterinary Pharmacology and Therapeutics* 32, 137-145.

Gerros, T.C., Semrad, S.D., Proctor, R.A., LaBorde, A. (1993) Effect of dose and method of administration of endotoxin on cell mediator release in neonatal calves. *American Journal of Veterinary Research* 54, 2121-2127.

Ghezzi, P., Melillo, G., Meazza, C., Sacco, S., Pellegrini, L., Asti, C., Porzio, S., Marullo, A., Sabbatini, V., Caselli, G., Bertini, R. (1998) Differential contribution of R and S isomers in ketoprofen anti-inflammatory activity: role of cytokine modulation. *The Journal of Pharmacology and Experimental Therapeutics* 287, 969-974.

Giguère, S., Huang, R., Malinski, T. J., Dorr, P.M., Tessman, R.K., Somerville, B.A. (2011) Disposition of gamithromycin in plasma, pulmonary epithelial lining fluid, bronchoalveolar cells, and lung tissue in cattle. American Journal of Veterinary Research 72, 326-330.

Gladue, R.P., Bright, G.M., Isaacson, R.E., Newborg, M.F. (1989) *In vitro* and *in vivo* uptake of azithromycin (CP-62,993) by phagocytic cells: possible mechanism of delivery and release at sites of infection. *Antimicrobial Agents and Chemotherapy* 33, 277-282.

Godinho, K.S. (2008) Susceptibility testing of tulathromycin: interpretative breakpoints and susceptibility of field isolates. *Veterinary Microbiology* 129, 426-432.

González-Ramón, N., Alava, M.A., Sarsa, J.A., Piñeiro, M., Escartin, A., Garcia-Gil, A., Lampreave, F., Piñeiro, A. (1995) The major acute phase serum protein in pigs is homologous to human plasma kallikrein sensitive PK-120. *FEBS Letters* 371, 227-230.

Goscinski, G., Lipcsey, M., Eriksson, M., Larsson, A., Tano, E., Sjolin, J. (2004) Endotoxin neutralization and anti-inflammatory effects of tobramycin and ceftazidime in porcine endotoxin shock. *Critical Care* 8, R35-41.

Granström, E., Hamberg, M., Hansson, G., Kindahl, H. (1980) Chemical instability of 15-keto-13,14-dihydro-PGE2: the reason for low assay reliability. *Prostaglandins* 19, 933-957.

Greco, D.S., Brown, S.A., Gauze, J.J., Weise, D.W., Buck, J.M. (1993) Dexamethasone pharmacokinetics in clinically normal dogs during low- and high-dose dexamethasone suppression testing. *American Journal of Veterinary Research* 54, 580-585.

Gruys, E., Toussaint, M.J., Niewold, T.A., Koopmans, S.J. (2005) Acute phase reaction and acute phase proteins. *Journal of Zhejiang University Science B* 6, 1045-1056.

Gutsmann, T., Muller, M., Carroll, S.F., MacKenzie, R.C., Wiese, A., Seydel, U. (2001) Dual role of lipopolysaccharide (LPS)-binding protein in neutralization of LPS and enhancement of LPS-induced activation of mononuclear cells. *Infection and Immunity* 69, 6942-6950.

Haesebrouck, F., Chiers, K., Van Overbeke, I., Ducatelle, R. (1997) *Actinobacillus pleuropneumoniae* infections in pigs: the role of virulence factors in pathogenesis and protection. *Veterinary Microbiology* 58, 239-249.

Hamberg, M., Samuelsson, B. (1971) On the metabolism of prostaglandins E_1 and E_2 in man. *The Journal of Biological Chemistry* 246, 6713-6721.

Healy, D.P. (2007) Macrolide immunomodulation of chronic respiratory diseases. *Current Infectious Disease Reports* 9, 7-13.

Heegaard, P.M., Klausen, J., Nielsen, J.P., González-Ramón, N., Piñeiro, M., Lampreave, F., Alava, M.A. (1998) The porcine acute phase response to infection with Actinobacillus pleuropneumoniae. Haptoglobin, C-reactive protein, major acute phase protein and serum amyloid A protein are sensitive indicators of infection. *Comparative Biochemistry and Physiology Part B: Biochemistry and Molecular Biology* 119, 365-373.

Heegaard, P.M.H., Stockmarr, A., Piñeiro, M., Carpintero, R., Lampreave, F., Campbell, F.M., Eckersall, P.D., Toussaint, M.J.M., Gruys, E., Sorensen, N.S. (2011) Optimal combinations of acute phase proteins for detecting infectious disease in pigs. *Veterinary Research* 42.

Heinrich, P.C., Castell, J.V., Andus, T. (1990) Interleukin-6 and the acute phase response. *Biochemical Journal* 265, 621-636.

Heitzman, R.J. (Ed.) (1994) *Veterinary Drug Residues*, Report Eur. 14126-EN, Commission of the EC. Brussels-Luxembourg.

Heijmans-Antonissen, C., Wesseldijk, F., Munnikes, R.J.M., Huygen, F.J.P.M., van der Meijden, P., Hop, W.C.J., Hooijkaas, H., Zijlstra, F.J. (2006) Multiplex bead array assay for detection of 25 soluble cytokines in blister fluid of patients with complex regional pain syndrome type 1. *Mediators of Inflammation* 2006, 1-8.

Hersh, E.V., Moore, P.A., Ross, G.L. (2000) Over-the-counter analgesics and antipyretics: a critical assessment. *Clinical Therapeutics* 22, 500-548.

Heumann, D., Roger, T. (2002) Initial responses to endotoxins and Gram-negative bacteria. *Clinica Chimica Acta* 323, 59-72.

Hochhaus, G., Barth, J., Al-Fayoumi, S., Suarez, S., Derendorf, H., Hochhaus, R., Möllmann, H. (2001) Pharmacokinetics and pharmacodynamics of dexamethasone sodium-m-sulfobenzoate (DS) after intravenous and intramuscular administration: a comparison with dexamethasone phosphate (DP). *Journal of Clinical Pharmacology* 41, 425-434.

Holger, J.S., Dries, D.J., Barringer, K.W., Peake, B.J., Flottemesch, T.J., Marini, J.J. (2010) Cardiovascular and metabolic effects of high-dose insulin in a porcine septic shock model. *Academic Emergency Medicine* 17, 429-435.

Holst, O. (2007) The structures of core regions from enterobacterial lipopolysaccharides - an update. *FEMS Microbiology Letters* 271, 3-11.

Horvath, G.L., Schrum, J.E., De Nardo, C.M., Latz, E. (2011) Intracellular sensing of microbes and danger signals by the inflammasomes. *Immunological Reviews* 243, 119-135.

Huang, R.A., Letendre, L.T., Banav, N., Fischer, J., Somerville, B. (2010) Pharmacokinetics of gamithromycin in cattle with comparison of plasma and lung tissue concentrations and plasma antibacterial activity. *Journal of Veterinary Pharmacology and Therapeutics* 33, 227-237.

Huang, H., Potter, A.A., Campos, M., Leighton, F.A., Willson, P.J., Haines, D.M., Yates, W.D. (1999) Pathogenesis of porcine Actinobacillus pleuropneumonia, part II: roles of proinflammatory cytokines. *Canadian Journal of Veterinary Research* 63, 69-78.

Huang, H., Potter, A.A., Campos, M., Leighton, F.A., Willson, P.J., Yates, W.D. (1998) Pathogenesis of porcine *Actinobacillus pleuropneumonia*: Part I. Effects of surface components of *Actinobacillus pleuropneumoniae in vitro* and *in vivo*. *Canadian Journal of Veterinary Research* 62, 93-101.

Ianaro, A., Ialenti, A., Maffia, P., Sautebin, L., Rombola, L., Carnuccio, R., Iuvone, T., D'Acquisto, F., Di Rosa, M. (2000) Anti-inflammatory activity of macrolide antibiotics. *The Journal of Pharmacology and Experimental Therapeutics* 292, 156-163.

Ivanov, A.I., Romanovsky, A.A. (2004) Prostaglandin E2 as a mediator of fever: synthesis and catabolism. *Frontiers in Bioscience* 9, 1977-1993.

Jackman, B.R., Moore, J.N., Barton, M.H., Morris, D.D. (1994) Comparison of the effects of ketoprofen and flunixin meglumine on the *in vitro* response of equine peripheral blood monocytes to bacterial endotoxin. *Canadian Journal of Veterinary Research* 58, 138-143.

Jain, R., Danziger, L.H. (2004) The macrolide antibiotics: a pharmacokinetic and pharmacodynamic overview. *Current Pharmaceutical Design* 10, 3045-3053.

Jerala, R. (2007) Structural biology of the LPS recognition. *Int J Med Microbiol*, 297(5), 353-363.

Jiménez, R., Ramírez, R., Carracedo, J., Agüera, M., Navarro, D., Santamaría, R., Pérez, R., Del Castillo, D., Aljama, P. (2005) Cytometric bead array (CBA) for the measurement of cytokines in urine and plasma of patients undergoing renal rejection. *Cytokine* 32, 45-50.

Johannisson, A., Jonasson, R., Dernfalk, J., Jensen-Waern, M. (2006) Simultaneous detection of porcine proinflammatory cytokines using multiplex flow cytometry by the xMAP^{TM} technology. *Cytometry Part A* 69A, 391-395.

John, E., Pais, P., Furtado, N., Chin, A., Radhakrishnan, J., Fornell, L., Lumpaopong, A. & Beier, U.H. (2008) Early effects of lipopolysaccharide on cytokine release, hemodynamic and renal function in newborn piglets. *Neonatology*, 93, 106-112.

Johnson, R.W., von Borell, E. (1994) Lipopolysaccharide-induced sickness behavior in pigs is inhibited by pretreatment with indomethacin. *Journal of Animal Science* 72, 309-314.

Kanitz, E., Tuchscherer, M., Tuchscherer, A., Stabenow, B., Manteuffel, G. (2002) Neuroendocrine and immune responses to acute endotoxemia in suckling and weaned piglets. *Biology of the Neonate* 81, 203-209.

Kanoh, S., Rubin, B.K. (2010) Mechanisms of action and clinical application of macrolides as immunomodulatory medications. *Clinical Microbiology Reviews* 23, 590-615.

Kayagaki, N., Wong, M.T., Stowe, I.B., Ranjani Ramani, S., Gonzalez, L.C., Akashi-Takamura, S., Miyake, K., Zhang, J., Lee, W.P., Muszyński, A., Forsberg, L.S., Carlson, R.W., Dixit, V.M. (2013) Noncanonical inflammasome activation by intracellular LPS independent of TLR4. *Science* 341, 1246-1249.

Kellar, K.L., Iannone, M.A. (2002) Multiplexed microsphere-based flow cytometric assays. *Experimental Hematology* 30, 1227-1237.

Kelly, R.W., Gallacher, A.F., Johnston, T.A., Harrison, N.W. (1992) The measurement of the main PGE metabolite, 13,14-dihydro-15-keto prostaglandin E by radioimmunoassay using methyl oxime stabilization. *Prostaglandins, Leukotrienes and Essential Fatty Acids* 45, 185-189.

Kelso, A. (1998) Cytokines: Principles and prospects. *Immunology & Cell Biology* 76, 300-317.

Khan, S.S., Smith, M.S., Reda, D., Suffredini, A.F., McCoy, J.P. (2004) Multiplex bead array assays for detection of soluble cytokines: Comparisons of sensitivity and quantitative values among kits from multiple manufacturers. *Cytometry Part B-Clinical Cytometry* 61B, 35-39.

Kim, J., Ahn, H., Woo, H.-M., Lee, E., Lee, G.-S. (2014) Characterization of porcine NLRP3 inflammasome activation and its upstream mechanism. *Veterinary Research Communication* 38, 193-200.

Klosterhalfen, B., Horstmann-Jungemann, K., Vogel, P., Flohe, S., Offner, F., Kirkpatrick, C.J., Heinrich, P.C. (1992) Time course of various inflammatory mediators during recurrent endotoxemia. *Biochemical Pharmacology* 43, 2103-2109.

Knecht, J., Stork, G. (1974). Percentage and logarithmic procedures for calculation of calibration curves. *Fresenius Zeitschrift für Analytische Chemie* 270, 97–99.

Konsman, J.P., Kelley, K., Dantzer, R. (1999) Temporal and spatial relationships between lipopolysaccharide-induced expression of Fos, interleukin-1beta and inducible nitric oxide synthase in rat brain. *Neuroscience* 89, 535-548.

Kunkel, S.L., Spengler, M., May, M.A., Spengler, R., Larrick, J., Remick, D. (1988) Prostaglandin E2 regulates macrophage-derived tumor necrosis factor gene expression. *The Journal of Biological Chemistry* 263, 5380-5384.

Labro, M.T. (1996) Intracellular bioactivity of macrolides. *Clinical Microbiology Infection* 1 (Suppl. 1), S24–S30.

Labro, M.T. (1998) Anti-inflammatory activity of macrolides: a new therapeutic potential? *The Journal of Antimicrobial Chemotherapy* 41 Suppl B, 37-46.

Lamkanfi, M., Dixit, V.M. (2014) Mechanisms and functions of inflammasomes. *Cell* 157, 1013-1022.

Lampreave, F., González-Ramón, N., Martinezayensa, S., Hernandez, M.A., Lorenzo, H.K., Garciagil, A., Piñeiro, A. (1994) Characterization of the acute phase serum protein response in pigs. *Electrophoresis* 15, 672-676.

Lauritzen, B., Lykkesfeldt, J., Skaanild, M.T., Angen, O., Nielsen, J.P., Friis, C. (2003) Putative biomarkers for evaluating antibiotic treatment: an experimental model of porcine *Actinobacillus pleuropneumoniae* infection. *Research in Veterinary Science* 74, 261-270.

Lawson, S., Lunney, J., Zuckermann, F., Osorio, F., Nelson, E., Welbon, C., Clement, T., Fang, Y., Wong, S., Kulas, K., Christopher-Hennings, J. (2010) Development of an 8-plex Luminex assay to detect swine cytokines for vaccine development: Assessment of immunity after porcine reproductive and respiratory syndrome virus (PRRSV) vaccination. *Vaccine* 28, 5356-5364.

Leininger, M.T., Portocarrero, C.P., Schinckel, A.P., Spurlock, M.E., Bidwell, C.A., Nielsen, J.N., Houseknecht, K.L. (2000) Physiological response to acute endotoxemia in swine: effect of genotype on energy metabolites and leptin. *Domestic Animal Endocrinology* 18, 71-82.

Leiva, M., Ruiz-Bravo, A., Jimenez-Valera, M. (2008) Effects of telithromycin in *in vitro* and *in vivo* models of lipopolysaccharide-induced airway inflammation. *Chest* 134, 20-29.

Lepper, P.M., Held, T.K., Schneider, E.M., Bolke, E., Gerlach, H., Trautmann, M. (2002) Clinical implications of antibiotic-induced endotoxin release in septic shock. *Intensive Care Medicine* 28, 824-833.

Levenbrown, Y., Penfil, S., Rodriguez, E., Zhu, Y., Hossain, J., Bhat, A.M., Hesek, A., O'Neil, K.B., Tobin, K., Shaffer, T.H. (2013) Use of Insulin to Decrease Septic Shock-Induced Myocardial Depression in a Porcine Model. *Inflammation* 36, 1494-1502.

Li, J.X., Oliver, J.R., Lu, C.Y., Philips, J.B. (1995) Delayed Thromboxane or Tumor-Necrosis-Factor-Alpha, but Not Leukotriene Inhibition, Attenuates Prolonged Pulmonary-Hypertension in Endotoxemia. *The American Journal of Medical Sciences* 310, 103-110.

Llamas Moya, S., Boyle, L., Lynch, P.B., Arkins, S. (2006) Pro-inflammatory cytokine and acute phase protein responses to low-dose lipopolysaccharide (LPS) challenge in pigs. *Animal Science* 82, 527-534.

Lorne, E., Dupont, H., Abraham, E. (2010) Toll-like receptors 2 and 4: initiators of non-septic inflammation in critical care medicine? *Intensive Care Medicine* 36, 1826-1835.

Maddens, B.E., Daminet, S., Demeyere, K., Demon, D., Smets, P., Meyer, E. (2010) Validation of immunoassays for the candidate renal markers C-reactive protein, immunoglobulin G, thromboxane B2 and retinol binding protein in canine urine. *Veterinary Immunology and Immunopathology* 134, 259-264.

Maier, R., Weger, M., Haller-Schober, E.M., El-Shabrawi, Y., Theisl, A., Barth, A., Aigner, R., Haas, A. (2006) Application of multiplex cytometric bead array technology for the measurement of angiogenic factors in the vitreous. *Molecular Vision* 12, 1143-1147.

Martich, G.D., Boujoukos, A.J., Suffredini, A.F. (1993) Response of man to endotoxin. *Immunobiology* 187, 403-416.

Martin, T.R., Mathison, J.C., Tobias, P.S., Leturcq, D.J., Moriarty, A.M., Maunder, R.J., Ulevitch, R.J. (1992) Lipopolysaccharide binding protein enhances the responsiveness of alveolar macrophages to bacterial lipopolysaccharide. Implications for cytokine production in normal and injured lungs. *Journal of Clinical Investigation* 90, 2209-2219.

Matasic, R., Dietz, A.B., Vuk-Pavlovic, S. (2000) Cyclooxygenase-independent inhibition of dendritic cell maturation by aspirin. *Immunology* 101, 53-60.

Mattoes, H.M., Nightingale, C.H. (2002) Pharmacokinetics ⁄ pharmcodynamics of macrolides. In: *Macrolide Antibiotics.* Eds Schonfeld, W., Kirst, H.A., Birkhauser Verlag, Basel, Boston and Berlin. pp. 25–36.

Miyake, K. (2004) Innate recognition of lipopolysaccharide by Toll-like receptor 4-MD-2. *Trends in Microbiology* 12, 186-192.

Morgan, E., Varro, R., Sepulveda, H., Ember, J.A., Apgar, J., Wilson, J., Lowe, L., Chen, R., Shivraj, L., Agadir, A., Campos, R., Ernst, D., Gaur, A. (2004) Cytometric bead array: a multiplexed assay platform with applications in various areas of biology. *Clinical Immunology* 110, 252-266.

Morikawa, K., Watabe, H., Araake, M., Morikawa, S. (1996) Modulatory effect of antibiotics on cytokine production by human monocytes *in vitro*. *Antimicrobial Agents and Chemotherapy* 40, 1366-1370.

Murtaugh, M.P., Baarsch, M.J., Zhou, Y., Scamurra, R.W., Lin, G. (1996) Inflammatory cytokines in animal health and disease. *Veterinary Immunology and Immunopathology* 54, 45-55.

Mustonen, K., Banting, A., Raekallio, M., Heinonen, M., Peltoniemi, O.A.T., Vainio, O. (2012a) Dose-response investigation of oral ketoprofen in pigs challenged with *Escherichia coli* endotoxin. *Veterinary Record* 171, 70.

Mustonen, K., Niemi, A., Raekallio, M., Heinonen, M., Peltoniemi, O.A.T., Palviainen, M., Siven, M., Peltoniemi, M., Vainio, O. (2012b) Enantiospecific ketoprofen concentrations in plasma after oral and intramuscular administration in growing pigs. *Acta Veterinaria Scandinavia* 54:55.

Mutak, S. (2007) Azalides from azithromycin to new azalide derivatives. *The Journal of Antibiotics* 60, 85-122.

Myers, M.J., Farrell, D.E., Baker, J.D., Cope, C.V., Evock-Clover, C.M., Steele, N.C. (1999) Challenge differentially affects cytokine production and metabolic status of growing and finishing swine. *Domestic Animal Endocrinology* 17, 345-360.

Myers, M.J., Farrell, D.E., Palmer, D.C., Post, L.O. (2003) Inflammatory mediator production in swine following endotoxin challenge with or without co-administration of dexamethasone. *International Immunopharmacology* 3, 571-579.

Myers, M.J., Farrell, D.E., Snider, T.G., 3rd, Post, L.O. (2004) Inflammatory cytokines, pleuropneumonia infection and the effect of dexamethasone. *Pathobiology* 71, 35-42.

Nakajima, Y., Momotani, E., Takahashi, H., Ishikawa, Y., Ito, T., Kanesaki, M., Madarame, H. (1995) Endogenous tumor necrosis factor (TNF) production and modification of pathological lesions in experimental Escherichia coli endotoxemia of piglets. *Veterinary Immunology and Immunopathology* 45, 45-54.

Netea, M.G., Kullberg, B.J., Van der Meer, J.W.M. (2000) Circulating cytokines as mediators of fever. *Clinical Infectious Diseases* 31, S178-S184.

Nielsen, J.S., Larsson, A., Rix, T., Nyboe, R., Gjedsted, J., Krog, J., Ledet, T., Tonnesen, E. (2007) The effect of activated protein C on plasma cytokine levels in a porcine model of acute endotoxemia. *Intensive Care Medicine*, 33(6), 1085-1093.

Nightingale, C.H. (1997) Pharmacokinetics and pharmacodynamics of newer macrolides. *The Pediatrics Infectious Disease Journal* 16, 438-443.

Olson, N.C., Brown, T.T., Jr., Anderson, D.L. (1985) Dexamethasone and indomethacin modify endotoxin-induced respiratory failure in pigs. *J Appl Physiol (1985)*, 58(1), 274-284.

Olson, N.C., Hellyer, P.W., Dodam, J.R. (1995) Mediators and vascular effects in response to endotoxin. *The British Veterinary Journal* 151, 489-522.

Opal, S.M., DePalo, V.A. (2000) Anti-inflammatory cytokines. *Chest* 117, 1162-1172.

Pålsson-McDermott, E.M., O'Neill, L.A. (2004) Signal transduction by the lipopolysaccharide receptor, Toll-like receptor-4. *Immunology* 113, 153-162.

Papich, M.G., Riviere, J.E. (2009) Chloramphenicol and derivatives, macrolides, lincosamides, and miscellaneous antimicrobials. In: *Veterinary Pharmacology and Therapeutics.* 9th edition. Ames, Iowa, USA pp. 945-982.

Parnham, M.J. (2005) Immunomodulatory effects of antimicrobials in the therapy of respiratory tract infections. *Current Opinion in Infectious Diseases* 18, 125-131.

Parrott, R.F., Vellucci, S.V., Goode, J.A., Lloyd, D.M., Forsling, M.L. (1995) Cyclooxygenase Mediation of Endotoxin-Induced Fever, Anterior and Posterior Pituitary-Hormone Release, and Hypothalamic C-Fos Expression in the Prepubertal Pig. *Experimental Physiology* 80, 663-674.

Parrott, R.F., Vellucci, S.V., Goode, J.A., Lloyd, D.M., Forsling, M.L. (1997) Interrelated adrenocortical and neurohypophysial responses associated with fever in endotoxin-treated pigs. *American Journal of Physiology* 273, R1046-1052.

Pelligand, L., House, A.K., Summers, B.A., Hatzis, A., Tivers, M., Elliott, J., Lees, P. (2012) Development and validation of a tissue cage model of acute inflammation in the cat. *Journal of Veterinary Pharmacology and Therapeutics* 35, 239-248.

Peri, F., Piazza, M., Calabrese, V., Damore, G., Cighetti, R. (2010) Exploring the LPS/TLR4 signal pathway with small molecules. *Biochemical Society Transactions* 38, 1390-1395.

Peters, S.M., Yancy, H., Deaver, C., Jones, Y.L., Kenyon, E., Chiesa, O.A., Esparza, J., Screven, R., Lancaster, V., Stubbs, J.T., 3rd, Yang, M., Wiesenfeld, P.L., Myers, M.J. (2012) *In vivo* characterization of inflammatory biomarkers in swine and the impact of flunixin meglumine administration. *Veterinary Immunology and Immunopathology* 148, 236-242.

Petersen, H.H., Nielsen, J.P., Heegaard, P.M. (2004) Application of acute phase protein measurements in veterinary clinical chemistry. *Veterinary Research* 35, 163-187.

Petrache, I., Birukova, A., Ramirez, S.I., Garcia, J.G.N., Verin, A.D. (2003) The role of the microtubules in tumor necrosis factor-alpha-induced endothelial cell permeability. *American Journal of Respiratory Cell and Molecular Biology* 28, 574-581.

Pettipher, E.R., Wimberly, D.J. (1994) Cyclooxygenase inhibitors enhance tumour necrosis factor production and mortality in murine endotoxic shock. *Cytokine* 6, 500-503.

Philippart, F., Cavaillon, J.M. (2007) Sepsis mediators. *Current Infectious Disease Reports* 9, 358-365.

Piñeiro, M., Piñeiro, C., Carpintero, R., Morales, J., Campbell, F.M., Eckersall, P.D., Toussaint, M.J.M., Lampreave, F. (2007) Characterisation of the pig acute phase protein response to road transport. *The Veterinary Journal* 173, 669-674.

Plessers, E., Wyns, H., Watteyn, A., Pardon, B., De Backer, P., Croubels, S. (2014) Characterization of an intravenous lipopolysaccharide inflammation model in calves with respect to the acute phase response. *Veterinary Immunology and Immunopathology (http://dx.doi.org/10.1016/j.vetimm.2014.11.005).*

Poli-de-Figueiredo, L.F., Garrido, A.G., Nakagawa, N., Sannomiya, P. (2008) Experimental models of sepsis and their clinical relevance. *Shock* 30 Suppl 1, 53-59.

Prabhakar, U., Eirikis, E., Davis, H.M. (2002) Simultaneous quantification of proinflammatory cytokines in human plasma using the LabMAP™ assay. *Journal of Immunological Methods* 260, 207-218.

Prats, C., El Korchi, G., Francesch, R., Arboix, M., Perez, B. (2002) Disposition kinetics of tylosin administered intravenously and intramuscularly to pigs. *Research in Veterinary Science* 73, 141-144.

Purswani, M.U., Eckert, S.J., Arora, H.K., Noel, G.J. (2002) Effect of ciprofloxacin on lethal and sublethal challenge with endotoxin and on early cytokine responses in a murine *in vivo* model. *Journal of Antimicrobial Chemotherapy* 50, 51-58.

Raekallio, M.R., Mustonen, K.M., Heinonen, M.L., Peltoniemi, O.A., Sakkinen, M.S., Peltoniemi, S.M., Honkavaara, J.M., Vainio, O.M. (2008) Evaluation of bioequivalence after oral, intramuscular, and intravenous administration of racemic ketoprofen in pigs. *American Journal of Veterinary Research* 69, 108-113.

Ray, C.A., Bowsher, R.R., Smith, W.C., Devanarayan, V., Willey, M.B., Brandt, J.T., Dean, R.A. (2005) Development, validation, and implementation of a multiplex immunoassay for the simultaneous determination of five cytokines in human serum. *Journal of Pharmaceutical and Biomedical Analysis* 36, 1037-1044.

Rietschel, E.T., Kirikae, T., Schade, F.U., Mamat, U., Schmidt, G., Loppnow, H., Ulmer, A.J., Zähringer, U., Seydel, U., Di Padova, F., Schreier, M., Brade, H. (1994) Bacterial endotoxin: molecular relationships of structure to activity and function. *The FASEB Journal* 8, 217-225.

Rietschel, E.T., Kirikae, T., Schade, F.U., Ulmer, A.J., Holst, O., Brade, H., Schmidt, G., Mamat, U., Grimmecke, H.-D., Kusumoto, S., Zähringer, U. (1993) The chemical structure of bacterial endotoxin in relation to bioactivity. *Immunobiology* 187, 169-190.

Rohdewald, P., Möllmann, H., Barth, J., Rehder, J., Derendorf, H. (1987) Pharmacokinetics of dexamethasone and its phosphate ester. *Biopharmaceutics & drug disposition* 8, 205-212.

Røkke, O., Revhaug, A., Osterud, B., Giercksky, K.E. (1988) Increased plasma levels of endotoxin and corresponding changes in circulatory performance in a porcine sepsis model: the effect of antibiotic administration. *Progress in Clinical and Biological Research* 272, 247-262.

Rose, M., Menge, M., Bohland, C., Zschiesche, E., Wilhelm, C., Kilp, S., Metz, W., Allan, M., Ropke, R., Nurnberger, M. (2013) Pharmacokinetics of tildipirosin in porcine plasma, lung tissue, and bronchial fluid and effects of test conditions on in vitro activity against reference strains and field isolates of *Actinobacillus pleuropneumoniae*. *Journal of Veterinary Pharmacology and Therapeutics* 36, 140-153.

Roth, J., Hubschle, T., Pehl, U., Ross, G., Gerstberger, R. (2002) Influence of systemic treatment with cyclooxygenase inhibitors on lipopolysaccharide-induced fever and circulating levels of cytokines and cortisol in guinea-pigs. *Pflügers Archiv: European journal of physiology* 443, 411-417.

Rutledge, H.R., Jiang, W., Yang, J., Warg, L.A., Schwartz, D.A., Pisetsky, D.S., Yang, I.V. (2012) Gene expression profiles of RAW264.7 macrophages stimulated with preparations of LPS differing in isolation and purity. Innate Immunity 18, 80-88.

Ruud, T.E., Gundersen, Y., Wang, J.E., Foster, S.J., Thiemermann, C., Aasen, A.O. (2007) Activation of cytokine synthesis by systemic infusions of lipopolysaccharide and peptidoglycan in a porcine model *in vivo* and *in vitro*. *Surgical Infections (Larchmt)* 8, 495-503.

Salichs, M., Sabate, D., Ciervo, O., Homedes, J. (2012) Comparison of the antipyretic efficacy of ketoprofen, acetylsalicylic acid, and paracetamol, orally administered to swine. *Journal of Veterinary Pharmacology and Therapeutics* 35, 198-201.

Salichs, M., Sabate, D., Homedes, J. (2013) Efficacy of ketoprofen administered in drinking water at a low dose for the treatment of porcine respiratory disease complex. *Journal of Animal Science* 91, 4469-4475.

Sanz-Santos, G., Jimenez-Marin, A., Bautista, R., Fernandez, N., Claros, G.M., Garrido, J.J. (2011) Gene expression pattern in swine neutrophils after lipopolysaccharide exposure: a time course comparison. *BMC Proceedings* 5 Suppl 4, S11.

Schmidhammer, R., Wassermann, E., Germann, P., Redl, H., Ullrich, R. (2006) Infusion of increasing doses of endotoxin induces progressive acute lung injury but prevents early pulmonary hypertension in pigs. *Shock* 25, 389-394.

Schrauwen, E., Cox, E., Houvenaghel, A. (1988) Escherichia coli sepsis and endotoxemia in conscious young pigs. *Veterinary Research Communications* 12, 295-303.

Schrauwen, E.M., Houvenaghel, A.M. (1984) Endotoxin shock in the pig: beneficial effects of pretreatment with prednisolone sodium succinate. *American Journal of Veterinary Research* 45, 1650-1653.

Schrauwen, E.M., Houvenaghel, A.M. (1985) Endotoxic shock in the awake young pig: absence of beneficial effect of prednisolone sodium succinate treatment. *American Journal of Veterinary Research* 46, 1770-1774.

Schrauwen, E., Thoonen, H., Hoorens, J., Houvenaghel, A. (1986) Pathophysiological effects of endotoxin infusion in young pigs. *British Veterinary Journal* 142, 364-370.

Schrauwen, E., Vandeplassche, G., Laekeman, G., Houvenaghel, A. (1984) Endotoxic shock in the piglet: beneficial effects of prostaglandin synthesis inhibition. *Prostaglandins, Leukotrienes, and Medicine* 13, 271-278.

Seldinger, S.I. (1953) Catheter replacement of the needle in percutaneous arteriography; a new technique. *Acta Radiologica* 39, 368-376.

Seldinger, S.I. (1984) The Seldinger Technique - Catheter Replacement of the Needle in Percutaneous Arteriography - a New Technique (Reprinted). *American Journal of Roentgenology* 142, 5-7.

Seydel, U., Hawkins, L., Schromm, A.B., Heine, H., Scheel, O., Koch, M.H., Brandenburg, K. (2003) The generalized endotoxic principle. *European Journal of Immunology* 33, 1586-1592.

Shen, J., Li, C., Jiang, H., Zhang, S., Guo, P., Ding, S., Li, X. (2005) Pharmacokinetics of tilmicosin after oral administration in swine. *American Journal of Veterinary Research* 66, 1071-1074.

Shi, J., Zhao, Y., Wang, Y., Gao, W., Ding, J., Li, P., Hu, L., Shao, F. (2014) Inflammatory caspases are innate immune receptors for intracellular LPS. *Nature* 514, 187-194.

Sibila, O., Luna, C.M., Agusti, C., Baquero, S., Gando, S., Patrón, J.R., Morato, J.G., Absi, R., Bassi, N., Torres, A. (2008) Effects of glucocorticoids in ventilated piglets with severe pneumonia. *European Respiratory Journal* 32, 1037-1046.

Singh, B., Atwal, O.S. (1997) Ultrastructural and immunocytochemical study of the pulmonary intravascular macrophages of Escherichia coli lipopolysaccharide-treated sheep. *The Anatomical Record* 247, 214-224.

Sironi, M., Gadina, M., Kankova, M., Riganti, F., Mantovani, A., Zandalasini, M., Ghezzi, P. (1992) Differential sensitivity of *in vivo* TNF and IL-6 production to modulation by anti-inflammatory drugs in mice. *International Journal of Immunopharmacology* 14, 1045-1050.

Slagman, A.C., Bock, C., Abdel-Aty, H., Vogt, B., Gebauer, F., Janelt, G., Wohlgemuth, F., Morgenstern, R., Yapici, G., Puppe, A., Modersohn, D., Mans, D., Jerichow, T., Ott, S., Kunze, R., Schrödl, W., Janko, C., Hermann, M., Kalden, J.R., Kern, P., Parsch, H., Kirschfink, M., Schulz-Menger, J., Röttgen, R., Unger, J.K., Frei, U., Schindler, R., Möckel, M., Sheriff, A. (2011) Specific removal of C-reactive protein by apheresis in a porcine cardiac infarction model. *Blood Purification* 31, 9-17.

Soma, L.R., Uboh, C.E., Liu, Y., Li, X., Robinson, M.A., Boston, R.C., Colahan, P.T. (2013) Pharmacokinetics of dexamethasone following intra-articular, intravenous, intramuscular, and oral administration in horses and its effects on endogenous hydrocortisone. Journal of *Veterinary Pharmacology and Therapeutics* 36, 181-191.

Sorensen, N.S., Tegtmeier, C., Andresen, L.O., Piñeiro, M., Toussaint, M.J., Campbell, F.M., Lampreave, F., Heegaard, P.M. (2006) The porcine acute phase protein response to acute clinical and subclinical experimental infection with *Streptococcus suis*. *Veterinary Immunology and Immunopathology* 113, 157-168.

Straw B.E., Meuten D.J., Thacker B.J. (1999) Physical examination and diagnosis. In: *Diseases of Swine*, 8th edition. Blackwell Science Ltd., Ames, Iowa, USA, pp. 3–18.

Streppa, H.K., Jones, C.J., Budsberg, S.C. (2002) Cyclooxygenase selectivity of nonsteroidal anti-inflammatory drugs in canine blood. *American Journal of Veterinary Research* 63, 91-94.

Sugiyama, K., Shirai, R., Mukae, H., Ishimoto, H., Nagata, T., Sakamoto, N., Ishii, H., Nakayama, S., Yanagihara, K., Mizuta, Y., Kohno, S. (2007) Differing effects of clarithromycin and azithromycin on cytokine production by murine dendritic cells. *Clinical & Experimental Immunology* 147, 540-546.

Swinkels, J.M., Pijpers, A., Vernooy, J.C., Van Nes, A., Verheijden, J.H. (1994) Effects of ketoprofen and flunixin in pigs experimentally infected with *Actinobacillus pleuropneumoniae*. *Journal of Veterinary Pharmacology and Therapeutics* 17, 299-303.

Swinkels, J.M., Pijpers, A., Vernooy, J.C., Van Nes, A., Verheijden, J.H. (1994) Effects of ketoprofen and flunixin in pigs experimentally infected with *Actinobacillus pleuropneumoniae. Journal of Veterinary Pharmacology and Therapeutics* 17, 299-303.

Tamaoki, J., Kadota, J., Takizawa, H., 2004. Clinical implications of the immunomodulatory effects of macrolides. *The American Journal of Medicine* 117 Suppl 9A, 5S-11S.

Tambuyzer, T., De Waele, T., Chiers, K., Berckmans, D., Goddeeris, B.M., Aerts, J-M. (2014) Interleukin-6 dynamics as a basis for an early-warning monitor for sepsis and inflammation in individual pigs. *Research in Veterinary Science* 96, 460-463.

Tárnok, A., Hambsch, J., Chen, R., Varro, R. (2003) Cytometric bead array to measure six cytokines in twenty-five microliters of serum. *Clinical Chemistry* 49, 1000-1002.

Tauber, S.C., Nau, R. (2008) Immunomodulatory properties of antibiotics. *Current Molecular Pharmacology* 1, 68-79.

Teeling, J.L., Cunningham, C., Newman, T.A., Perry, V.H. (2010) The effect of non-steroidal anti-inflammatory agents on behavioural changes and cytokine production following systemic inflammation: Implications for a role of COX-1. *Brain Behavior and Immunity* 24, 409-419.

Teeling, J.L., Felton, L.M., Deacon, R.M.J., Cunningham, C., Rawlins, J.N.P., Perry, V.H. (2007) Sub-pyrogenic systemic inflammation impacts on brain and behavior, independent of cytokines. *Brain, Behavior, and Immunity* 21, 836-850.

Tegeder, I., Pfeilschifter, J., Geisslinger, G. (2001) Cyclooxygenase-independent actions of cyclooxygenase inhibitors. *The Federation of American Societies for Experimental Biology Journal* 15, 2057-2072.

Tobias, P.S., Tapping, R.I., Gegner, J.A. (1999) Endotoxin interactions with lipopolysaccharide-responsive cells. *Clinical Infectious Diseases* 28, 476-481.

Toutain, P.L., Alvinerie, M., Ruckebusch, Y. (1983) Pharmacokinetics of dexamethasone and its effect on adrenal gland function in the dog. *American Journal of Veterinary Research* 44, 212-217.

Toutain, P.L., Brandon, R.A., Alvinerie, M., Garcia-Villar, R., Ruckebusch, Y. (1982) Dexamethasone in cattle: pharmacokinetics and action on the adrenal gland. *Journal of Veterinary Pharmacology and Therapeutics* 5, 33-43.

Triantafilou, M., Triantafilou, K. (2002) Lipopolysaccharide recognition: CD14, TLRs and the LPS-activation cluster. *Trends in Immunology* 23, 301-304.

Tuchscherer, M., Kanitz, E., Puppe, B., Tuchscherer, A. (2010) Altered immunomodulation by glucocorticoids in neonatal pigs exposed to a psychosocial stressor. *Pediatric research* 68, 473-478.

Tuchscherer, M., Kanitz, E., Puppe, B., Tuchscherer, A., Stabenow, B. (2004) Effects of postnatal social isolation on hormonal and immune responses of pigs to an acute endotoxin challenge. *Physiology & Behavior* 82, 503-511.

Tugirimana, P.L., De Clercq, D., Holderbeke, A.L., Kint, J.A., De Cooman, L., Deprez, P., Delanghe, J.R. (2011) A functional turbidimetric method to determine C-reactive protein in horses. *Journal of Veterinary Diagnostic Investigation* 23, 308-311.

Ulevitch, R.J. (2004) Therapeutics targeting the innate immune system. *Nature Reviews Immunology* 4, 512-520.

Underhill, D.M., Goodridge, H.S. (2012) Information processing during phagocytosis. *Nature Reviews Immunology* 12, 492-502.

Van Bambeke, F., Tulkens, P.M. (2001) Macrolides: pharmacokinetics and pharmacodynamics. *International Journal of Antimicrobial Agents* 18 Suppl 1, S17-23.

Vane, J.R. (1971) Inhibition of prostaglandin synthesis as a mechanism of action for aspirin-like drugs. *Nature: New Biology* 231, 232-235.

Van Overbeke, I., Chiers, K., Ducatelle, R., Haesebrouck, F. (2001). Effect of endobronchial challenge with *Actinobacillus pleuropneumoniae* serotype 9 of pigs vaccinated with a vaccine containing Apx toxins and transferrin-binding proteins. *Journal of Veterinary Medicine Series B-Infectious Diseases and Veterinary Public Health* 48, 15-20.

Vazifeh, D., Abdelghaffar, H., Labro, M.T. (1997) Cellular accumulation of the new ketolide RU 64004 by human neutrophils: comparison with that of azithromycin and roxithromycin. *Antimicrobial agents and chemotherapy* 41, 2099-2107.

Vellucci, S.V., Parrott, R.F. (1994) Hyperthermia-Associated Changes in Fos Protein in the Median Preoptic and Other Hypothalamic Nuclei of the Pig Following Intravenous Administration of Prostaglandin E(2). *Brain Research* 646, 165-169.

Verstrepen, L., Bekaert, T., Chau, T.L., Tavernier, J., Chariot, A., Beyaert, R. (2008) TLR-4, IL-1R and TNF-R signaling to NF-kappaB: variations on a common theme. *Cellular and Molecular Life Sciences* 65, 2964-2978.

VICH GL 49 (MRK) draft 1. *Guideline for the validation of analytical methods used in residue depletion studies*, November 2009, Brussels, Belgium.

Villarino, N., Garcia-Tapia, D., Lesman, S., Lucas, M., Robinson, J., Brown, S.A., Martin-Jimenez, T. (2013) An acute reversible experimental model of pneumonia in pigs: time-related histological and clinical signs changes. *Journal of Veterinary Pharmacology and Therapeutics* 36, 241-247.

Vincenti, M., Girolami, F., Capra, P., Pazzi, M., Carletti, M., Gardini, G., Nebbia, C. (2009) Study of dexamethasone urinary excretion profile in cattle by LC-MS/MS: comparison between therapeutic and growth-promoting administration. *Journal of Agricultural and Food Chemistry* 57, 1299-1306.

Wagner, B., Freer, H. (2009) Development of a bead-based multiplex assay for simultaneous quantification of cytokines in horses. *Veterinary Immunology Immunopathology* 127, 242-248.

Wang, X., Tao, Y.F., Huang, L.L., Chen, D.M., Yin, S.Z., Ihsan, A., Zhou, W., Su, S.J., Liu, Z.L., Pan, Y.H., Yuan, Z.H. (2012) Pharmacokinetics of tulathromycin and its metabolite in swine administered with an intravenous bolus injection and a single gavage. *Journal of Veterinary Pharmacology and Therapeutics* 35, 282-289.

Wang, J.F., Wang, M., Ma, J.L., Jiao, L.G., Zhou, X.Y., Lindberg, J.E. (2006) The influence of intramammary lipopolysaccharide infusion on serum Ca, P, vitamin D, cytokines and cortisol concentrations in lactating sows. *Journal of Veterinary Medicine Series A* 53, 113-118.

Warren, E.J., Finck, B.N., Arkins, S., Kelley, K.W., Scamurra, R.W., Murtaugh, M.P., Johnson, R.W. (1997) Coincidental changes in behavior and plasma cortisol in unrestrained pigs after intracerebroventricular injection of tumor necrosis factor-alpha. *Endocrinology* 138, 2365-2371.

Watteyn, A., Plessers, E., Wyns, H., De Baere, S., De Backer, P., Croubels, S. (2013) Pharmacokinetics of gamithromycin after intravenous and subcutaneous administration in broiler chickens. *Poultry Science* 92, 1516-1522.

Watteyn, A., Wyns, H., Plessers, E., Russo, E., De Baere, S., De Backer, P., Croubels, S. (2013) Pharmacokinetics of dexamethasone after intravenous and intramuscular administration in broiler chickens. *The Veterinary Journal* 195, 216-220.

Webel, D.M., Finck, B.N., Baker, D.H., Johnson, R.W. (1997) Time course of increased plasma cytokines, cortisol, and urea nitrogen in pigs following intraperitoneal injection of lipopolysaccharide. *Journal of Animal Science* 75, 1514-1520.

Williams, P.N., Collier, C.T., Carroll, J.A., Welsh, T.H., Jr., Laurenz, J.C. (2009) Temporal pattern and effect of sex on lipopolysaccharide-induced stress hormone and cytokine response in pigs. *Domestic Animal Endocrinology* 37, 139-147.

Winkler, G.C. (1988) Pulmonary Intravascular Macrophages in Domestic-Animal Species - Review of Structural and Functional-Properties. *American Journal of Anatomy* 181, 217-234.

Winkler, G.C., Cheville, N.F. (1985) Monocytic origin and postnatal mitosis of intravascular macrophages in the porcine lung. *Journal of Leukocyte Biology* 38, 471-480.

Wittebole, X., Castanares-Zapatero, D., Laterre, P.F. (2010) Toll-like receptor 4 modulation as a strategy to treat sepsis. *Mediators of Inflammation* 2010, 568396.

Wright, A., Kanegasaki, S. (1971) Molecular aspects of lipopolysaccharides. *Physiological Reviews* 51, 748-784.

Wright, K.J., Balaji, R., Hill, C.M., Dritz, S.S., Knoppel, E.L., Minton, J.E. (2000) Integrated adrenal, somatotropic, and immune responses of growing pigs to treatment with lipopolysaccharide. *Journal of Animal Science* 78, 1892-1899.

Wyns, H., Croubels, S., Demeyere, K., Watteyn, A., De Backer, P., Meyer, E. (2013) Development of a cytometric bead array screening tool for the simultaneous detection of pro-inflammatory cytokines in porcine plasma. *Veterinary Immunology and Immunopathology* 151, 28-36.

Wyns, H., Meyer, E., Plessers, E., Watteyn, A., De Baere, S., De Backer, P., Croubels, S. (2014) Pharmacokinetics of gamithromycin after intravenous and subcutaneous administration in pigs. *Research in Veterinary Science* 96, 160-163.

Wyns, H., Plessers, E., De Backer, P., Meyer E., Croubels, S. (2014) *In vivo* porcine lipopolysaccharide inflammation models to study immunomodulation of drugs. *Veterinary Immunology and Immunopathology (under revision).*

Yamamoto, K., Arakawa, T., Ueda, N., Yamamoto, S. (1995) Transcriptional roles of nuclear factor kappa B and nuclear factor-interleukin-6 in the tumor necrosis factor alpha-dependent induction of cyclooxygenase-2 in MC3T3-E1 cells. *The Journal of Biological Chemistry* 270, 31315-31320.

Zhanel, G.G., Dueck, M., Hoban, D.J., Vercaigne, L.M., Embil, J.M., Gin, A.S., Karlowsky, J.A. (2001) Review of macrolides and ketolides: focus on respiratory tract infections. *Drugs* 61, 443-498.

Zhang, X., Song, Y., Ci, X., An, N., Fan, J., Cui, J., Deng, X. (2008) Effects of florfenicol on early cytokine responses and survival in murine endotoxemia. *International Immunopharmacology* 8, 982-988.

Zuckerman, J.M., Qamar, F., Bono, B.R. (2011) Review of macrolides (azithromycin, clarithromycin), ketolids (telithromycin) and glycylcyclines (tigecycline). *Medical Clinics of North America* 95, 761-791, viii.

Lipopolysaccharide (LPS), a major part of the Gram-negative bacterial outer membrane, has been widely applied to study the acute phase response (APR). This APR is well-established in pigs. Modulation of the host immune response is of growing interest in both human and veterinary medicine, yet is beyond the frontiers of knowledge in porcine research. This immune response can be influenced by either (non)steroidal anti-inflammatory drugs ((N)SAIDs) or antibiotics such as macrolides. It largely remains to be determined whether a combination therapy of antibiotics and (N)SAIDs would be beneficial in the treatment of pigs compared to single drug administration.

Therefore, the **General Aim** of this thesis was to investigate immunomodulation by the macrolide antibiotic gamithromycin (GAM), the NSAID ketoprofen (KETO) and their combination in appropriate *in vitro* and *in vivo* porcine LPS inflammation models. Dexamethasone (DEX), a glucocorticoid and a well-known cytokine inhibitor, was included as a positive control.

In the **General Introduction**, selected aspects of the molecular structure, extracellular recognition and intracellular signaling pathways of LPS are highlighted. The LPS-induced porcine APR with regard to the systemic release of (pro-)inflammatory mediators and clinical symptoms, including the febrile response is extensively described. Additionally, a comprehensive overview is provided about the use of LPS-induced inflammation models in pigs to characterize the host immune response and to study immunomodulation by antibiotics and (N)SAIDs.

In **Chapter 1**, the pharmacokinetics of GAM and DEX were evaluated in pig to determine their disposition and to estimate the time to maximal plasma concentration (T_{max}). Both GAM and DEX are rapidly absorbed and fully bioavailable after subcutaneous (SC) and intramuscular (IM) injection, respectively. Mean maximal plasma concentration of GAM was observed at 38 min, whereas the T_{max} of DEX was established at 21 min. For both drugs, a remarkably higher clearance was observed in pigs in comparison with other animal

species. Additionally, GAM showed a similar volume of distribution compared to cattle and was therefore eliminated 3 to 4 times faster in pigs. In cattle, a withdrawal period of 64 days is taken into account for meat.

In **Chapter 2**, the immunomodulatory properties of GAM, KETO, DEX and their combinations were investigated in appropriate *in vitro* and *in vivo* porcine LPS-induced inflammation models. Peripheral blood mononuclear cells (PBMCs) were used as a model of *in vitro* immune response stimulation. Incubation of porcine PBMCs with LPS induced a marked increase in the pro-inflammatory mediators prostaglandin (PG) E_2, tumor necrosis factor (TNF)-α, interleukin (IL)-1β and IL-6. A highly specific and sensitive ultra-performance liquid chromatography-tandem mass spectrometry (UPLC-MS/MS) method was developed and validated for the quantification of PGE_2 and its metabolite in cell supernatant and plasma.

In vivo, 15 µg/kg BW ultrapure LPS was intravenously administered to pigs 1 h after SC and/or IM administration of GAM and/or KETO, respectively, when nearby maximal concentrations of both drugs (T_{max}) were expected in plasma. Lipopolysaccharide induced systemic plasma peak concentrations of PGE_2 and TNF-α at 1 h post administration (p.a.), and maximal concentrations of IL-6 at 2.5 h p.a. A maximal febrile response was recorded at 2.5 h p.a. of LPS. Concomitantly, severe symptoms of acute lung failure and general sickness, including tachypnea, dyspnea, anorexia, nausea, vomiting and depression developed in the pigs. Levels of pig-major acute phase protein (pig-MAP) and C-reactive protein (CRP) increased not earlier than 18 h p.a. of LPS.

The macrolide GAM had no significant effect on the production of PGE_2, TNF-α, IL-1β and IL-6 either *in vitro* or *in vivo,* which was in contrast to previous reports on other macrolides. The NSAID KETO had no significant effect on cytokine production, yet showed a trend to enhance TNF-α levels both *in vitro* and *in vivo*. On the other hand, KETO and its combination with GAM, significantly reduced PGE_2 production both *in vitro* and *in vivo*. Additionally, pigs of both groups showed no increase in rectal body temperature and developed no severe symptoms following LPS challenge. In general, none of these drugs or their combination had a major influence on the production of acute phase proteins pig-MAP and CRP. DEX, which was used as a positive control in the *in vitro* research,

significantly suppressed the production of PGE_2, TNF-α, IL-1β and IL-6, also in combination with GAM.

These results suggest a major role for both COX-1- and COX-2-mediated PGs in the APR, and a more additional role for pro-inflammatory cytokines.

In **Chapter 3**, a comparative study of immuno-assays was performed for the detection of porcine cytokines. In porcine research, detection and quantification of IL-1β remains a major source of concern from an analytical point of view. In this respect, a cytometric bead array (CBA) screening tool was developed and validated for the simultaneous detection of TNF-α, IL-1β and IL-6 in porcine plasma and critically compared with the gold standard enzyme-linked immuno sorbent assay (ELISA). A similar trend was observed with both immuno-assays for the three cytokines. Overall, LPS-induced concentrations of IL-1β were low and inconsistent, yet a maximal plasma concentration of IL-1β was demonstrated at 3 h after LPS administration with both immuno-assays. This CBA screening tool has therefore been proven a valuable time-saving multi-parameter screening alternative for the time-consuming single-parameter approach offered by ELISA methods.

Additionally, our CBA 3-plex assay was successfully tested and validated for use in serum samples of *Actinobacillus (A.) pleuropneumoniae*-infected pigs. The appearance of TNF-α, IL-1β and IL-6 in the serum of the *A. pleuropneumoniae*-infected pigs occurred remarkably later compared to our LPS model, with mean maximal serum concentrations of TNF-α and IL-6 observed only at 12 h and 10 h after infection, respectively.

In **Conclusion**, we succeeded in developing appropriate *in vitro* and *in vivo* LPS inflammation models for the evaluation of immunomodulation of drugs in pigs. The NSAID KETO as well as its combination with the macrolide GAM was clearly demonstrated to have an inhibiting effect on PGE_2 production as well as the development of fever, pulmonary symptoms and general sickness following LPS administration, while GAM alone did not.

Additionally, a cost-effective CBA screening tool for pro-inflammatory cytokines TNF-α, IL-1β and IL-6, as well as a specific UPLC-MS/MS method for PGs quantification were developed and validated for use in cell media and porcine samples.

We developed appropriate porcine *in vitro* and *in vivo* LPS inflammation models for testing of different classes of drugs, as well as efficient multi-parameter methods for both pro-inflammatory cytokines and eicosanoids.

Lipopolysaccharide (LPS) is een belangrijke component van de buitenste membraan van Gram-negatieve bacteriën. Deze substantie werd reeds uitvoerig gebruikt voor het bestuderen van de acute fase reactie (AFR) bij verschillende diersoorten. Bij het varken werd de AFR reeds in detail beschreven. Zowel vanuit de humane- als de diergeneeskunde is er een groeiende belangstelling voor het moduleren van de immuunrespons, maar dit aspect is nog relatief weinig bestudeerd bij het varken. De immuunrespons zou vooral kunnen beïnvloed worden door zowel (niet-)steroïdale anti-inflammatoire geneesmiddelen ((N)SAIDs), als door antibiotica waaronder de macroliden. Hierbij is ook belangrijk in welke mate het gecombineerd gebruik van een antibioticum en een (N)SAID voordeel kan hebben voor de behandeling van varkens in vergelijking met de toediening van beide klassen van geneesmiddelen afzonderlijk.

De **Algemene Doelstelling** van dit onderzoek was daarom het bestuderen van de immunomodulerende eigenschappen van het macrolide antibioticum gamithromycine (GAM), het NSAID ketoprofen (KETO) en de combinatie van beide geneesmiddelen in reproduceerbare *in vitro* en *in vivo* LPS inflammatie modellen bij het varken. Dexamethasone (DEX), een glucocorticoïde en een gekende inhibitor van cytokines, werd hierbij gebruikt als positieve controle.

In de **Algemene Inleiding** werden eerst specifieke aspecten van de moleculaire structuur, de extracellulaire herkenning en de intracellulaire signaaltransductie van LPS toegelicht. Vervolgens werd de LPS-geïnduceerde AFR bij het varken beschreven met bijzondere aandacht voor de systemische vrijstelling van (pro-)inflammatoire mediatoren en klinische symptomen, waaronder de koortsreactie. Aansluitend werd een uitgebreid overzicht gegeven over het gebruik van LPS-geïnduceerde inflammatie modellen voor het bestuderen van de immuunrespons bij het varken.

In **Hoofdstuk 1** werden de farmacokinetische eigenschappen van GAM en DEX bestudeerd bij het varken om zowel de dispositie als de tijd nodig om maximale plasma

concentraties (T_{max}) te bereiken, te bepalen. Zowel GAM als DEX worden snel geabsorbeerd en zijn volledig biologisch beschikbaar na subcutane (SC) en intramusculaire (IM) injectie, respectievelijk. De gemiddelde maximale plasma concentratie van GAM werd vastgesteld op 38 minuten (min), de T_{max} van DEX werd gemeten op 21 min. Voor beide geneesmiddelen werd een opvallend hogere klaring genoteerd bij het varken in vergelijking met andere diersoorten. Aangezien GAM een vergelijkbaar distributievolume vertoonde met het rund, kunnen we besluiten dat GAM bij het varken 3 tot 4 keer sneller geëlimineerd wordt. Bij het rund wordt voor GAM een wachttijd van 64 dagen voor vlees in acht genomen.

In **Hoofdstuk 2** worden de immunomodulerende eigenschappen van GAM, KETO, DEX en hun respectievelijke combinaties bestudeerd in reproduceerbare *in vitro* en *in vivo* LPS-geïnduceerde inflammatie modellen bij het varken. Perifere bloed mononucleaire cellen (PBMCs) van varkens werden gebruikt in een *in vitro* model voor de studie van de immuunrespons. Incubatie van deze PBMCs met LPS induceerde een duidelijke stijging van pro-inflammatoire mediatoren zoals tumor necrosis factor (TNF)-α, interleukine (IL)-1β, IL-6 en prostaglandine (PG) E_2. Een uiterst specifieke en gevoelige ultra-performantie vloeistofchromatografie-tandem massaspectrometrische (UPLC-MS/MS) methode werd ontwikkeld en gevalideerd voor de kwantitatieve bepaling van PGE_2 en zijn metaboliet in celcultuur supernatans en plasma.

Voor het *in vivo* onderzoek werd 15 µg/kg lichaamsgewicht (LG) (15 x 10^3 EU/kg LG) ultra-zuiver LPS intraveneus toegediend aan varkens op 1 u na de SC en/of IM toediening van GAM en/of KETO, respectievelijk. Op dit tijdstip worden namelijk hoge plasma concentraties van beide geneesmiddelen (T_{max}) verwacht. LPS induceerde inderdaad systemische piekconcentraties van PGE_2 en TNF-α in het plasma op het tijdstip 1 u, en maximale concentraties van IL-6 op 2,5 u na LPS toediening. De koortspiek werd reeds vastgesteld 2,5 u na LPS toediening. De varkens ontwikkelden simultaan ernstige symptomen van acuut longfalen zoals een versnelde en bemoeilijkte ademhaling en ook een algemeen ziektebeeld gekarakteriseerd door anorexie, misselijkheid, braken en algemene depressie. De concentraties van de acute fase eiwitten "pig major” acute fase proteïne (pig-MAP) en C-reactief proteïne (CRP) namen pas toe vanaf 18 u na LPS toediening.

Het macrolide antibioticum GAM had geen significant effect op de productie van PGE_2, TNF-α, IL-1β en IL-6 *in vitro* noch *in vivo*. Het NSAID KETO had eveneens geen significant effect op de productie van cytokines, maar vertoonde wel een trend tot het verhogen van TNF-α (plasma)concentraties, zowel *in vitro* als *in vivo*. Zowel KETO als zijn combinatie met GAM, reduceerden significant de concentratie van PGE_2 *in vitro* en *in vivo*. De varkens van beide groepen vertoonden geen stijging van de rectale lichaamstemperatuur en ontwikkelden geen ernstige symptomen na LPS toediening. Over het algemeen hadden de individueel geteste geneesmiddelen, noch hun combinatie een belangrijke invloed op de productie van pig-MAP en CRP. DEX daarentegen, dat werd ingesloten als een positieve controle in het *in vitro* onderzoek, onderdrukte wel significant de productie van PGE_2, TNF-α, IL-1β en IL-6 en dit ook in combinatie met GAM.

De resultaten van dit onderzoek suggereren een belangrijke rol voor zowel COX-1- als COX-2-gemedieerde PGs in de AFR en een minder ingrijpende rol voor pro-inflammatoire cytokines.

In **Hoofdstuk 3** werd een vergelijkende studie uitgevoerd van twee immunologische methoden voor de bepaling van porciene cytokines. Vooral het bepalen en kwantificeren van IL-1β is een uitdaging. Daarom werd eerst een cytometric bead array (CBA) screeningsmethode ontwikkeld en gevalideerd voor de gelijktijdige bepaling van TNF-α, IL-1β en IL-6 in varkensplasma. Vervolgens werd een kritische vergelijking gemaakt met de algemeen aanvaarde enzyme-linked immuno sorbent assay (ELISA). Eenzelfde trend was duidelijk waarneembaar tussen beide immunologische methoden voor alle cytokines. Ondanks het feit dat de LPS-geïnduceerde concentraties van IL-1β doorgaans laag en variabel waren tussen de varkens, kon met beide technieken toch een maximale concentratie 3 u na LPS toediening worden genoteerd. Deze multiplex CBA screeningsmethode kan daarom worden beschouwd als een waardevol en tijdbesparend alternatief voor de enkelvoudige-parameter benadering van ELISA.

De CBA screeningsmethode werd vervolgens ook succesvol getest en gevalideerd voor gebruik in serumstalen van varkens geïnfecteerd met *Actinobacillus pleuropneumoniae*. De systemische vrijstelling van TNF-α, IL-1β en IL-6 in het serum van de varkens trad opmerkelijk later op in vergelijking met deze in het LPS-geïnduceerd

inflammatie model. Zo werden maximale serumconcentraties van TNF-α en IL-6 pas bereikt op 12 u en 10 u na infectie, respectievelijk.

Samengevat kunnen we stellen dat we erin geslaagd om reproduceerbare *in vitro* en *in vivo* LPS inflammatie modellen te ontwikkelen bij het varken voor de studie van de immunomodulerende eigenschappen van geneesmiddelen. Zowel het NSAID KETO, als de combinatie met GAM, vertoonde een duidelijk inhiberend effect op de PGE_2 productie, de koortsrespons, longsymptomen en het algemene ziektebeeld na LPS toediening. De toediening van GAM alleen vertoonde deze gunstige werking echter niet.

Tijdens ons onderzoek werd tevens een CBA screeningsmethode voor de pro-inflammatoire cytokines TNF-α, IL-1β en IL-6 en een specifieke UPLC-MS/MS methode voor PG kwantificatie ontwikkeld en gevalideerd voor gebruik in celcultuur media en biologische stalen van varkens.

Er werden reproduceerbare porciene *in vitro* en *in vivo* LPS inflammatie modellen ontwikkeld voor het bestuderen van verschillende klassen van geneesmiddelen, alsook werden efficiënte multi-parameter methodes ontwikkeld voor het bepalen van zowel pro-inflammatoire cytokines, als eicosanoiden.

CURRICULUM VITAE

Heidi Wyns werd geboren op 21 september 1984 te Dendermonde. Na het behalen van het diploma hoger secundair onderwijs aan het Sint-Vincentiusinstituut te Dendermonde (Moderne Talen-Wetenschappen), begon zij in 2002 met de studie Diergeneeskunde aan de Universiteit Gent en behaalde in 2008 het diploma van dierenarts (Optie Gezelschapsdieren) met onderscheiding.

In oktober 2008 startte zij als assistent bij de vakgroep Farmacologie, Toxicologie en Biochemie van de Faculteit Diergeneeskunde. Onder begeleiding van prof. dr. P. De Backer, prof. dr. S. Croubels en prof. dr. E. Meyer, verrichtte zij er onderzoek naar de immunomodulerende eigenschappen van geneesmiddelen in een lipopolysaccharide inflammatie model bij varkens. Verder begeleidde zij als assistent de practica biochemie en farmacologie en was zij promotor van meerdere studenten in het kader van hun Masterproef. In 2014 vervolledigde zij het trainingsprogramma van de Doctoral School of Life Sciences and Medicine van de Universiteit Gent.

Heidi Wyns is auteur en mede-auteur van meerdere wetenschappelijke publicaties in internationale tijdschriften. Tevens nam zij actief deel aan verschillende nationale en internationale congressen.

Scientific papers

- **Wyns, H.**, Croubels, S., Vandekerckhove, M., Demeyere, K., De Backer, P., Goddeeris, B.M., Meyer, E. (2014) Multiplex analysis of pro-inflammatory cytokines in serum of *Actinobacillus pleuropneumoniae*-infected pigs *(manuscript in preparation).*

- **Wyns, H.**, Meyer, E., Plessers, E., Watteyn, A., van Bergen, T., Schauvliege, S., De Baere, S., Devreese, M., De Backer, P., Croubels, S. (2014) Modulation of gamithromycin and ketoprofen on *in vitro* and *in vivo* porcine lipopolysaccharide-induced inflammation *(Submitted to PLoS ONE).*

- **Wyns, H.**, Plessers, E., De Backer, P., Meyer E., Croubels, S. (2014) *In vivo* porcine lipopolysaccharide inflammation models to study immunomodulation of drugs. *Veterinary Immunology and Immunopathology (under revision)*.

- Plessers E., **Wyns, H.**, Watteyn, A., Pardon, B., De Backer, P., Croubels, S. (2014) Characterization of an intravenous lipopolysaccharide inflammation model in calves with respect to the acute phase response. *Veterinary Immunology and Immunopathology (http://dx.doi.org/10.1016/j.vetimm.2014.11.005)*

- Plessers, E., Watteyn, A., **Wyns, H.**, Pardon, B., De Baere, S., De Backer, P., Croubels, S. (2014) Enantioselective pharmacokinetics of ketoprofen in calves after intramuscular administration of a racemic mixture. *Journal of Veterinary Pharmacology and Therapeutics (doi:10.1111/jvp.12186).*

- **Wyns, H.**, Meyer, E., Plessers, E., Watteyn, A., De Baere, S., De Backer, P., Croubels, S. (2014) Pharmacokinetics of gamithromycin after intravenous and subcutaneous administration in pigs. *Research in Veterinary Science* 96, 160 – 163.

- Watteyn, A., Plessers, E., **Wyns, H.**, De Baere, S., De Backer, P., Croubels, S. (2013) Pharmacokinetics of gamithromycin after intravenous and subcutaneous administration in broiler chickens. *Poulty Science* 92, 1516 – 1522.

- **Wyns, H.**, Meyer, E., Watteyn, A., Plessers, E., De Baere, S., De Backer, P., Croubels, S. (2013) Pharmacokinetics of dexamethasone after intravenous and intramuscular administration in pigs. *The Veterinary Journal* 198, 286 – 288.

- **Wyns, H**., Croubels, S., Demeyere, K., Watteyn, A., De Backer, P., Meyer, E. (2013) Development of a cytometric bead array screening tool for the simultaneous detection of pro-inflammatory cytokines in porcine plasma. *Veterinary Immunology and Immunopathology* 151, 28 – 36.

- Watteyn, A., **Wyns, H.**, Plessers, E., Russo, E., De Baere, S., De Backer, P. and Croubels, S. (2013) Pharmacokinetics of dexamethasone after intravenous and intramuscular administration in broiler chickens. *The Veterinary Journal*, 195, 216 – 220.

Scientific abstracts

- Watteyn, A., Devreese, M., De Baere, S., Plessers, E., **Wyns, H.**, De Backer, P., Croubels, S. (2014) Pharmacokinetics of gamithromycin in turkey plasma and lung tissue. *Antimicrobial Agents in Veterinary Medicine, 7th International Conference,* Berlin, Germany.

- Plessers, E., **Wyns, H.**, Watteyn, A., Pardon, B., De Baere, S., De Backer, P., Croubels, S. (2014) Comparative efficacy of dexamethasone, ketoprofen and gamithromycin in a lipopolysaccharide inflammation model in calves. *XIV Middle European Buiatrics Congress*, Warsaw, Poland.

- Watteyn, A., Plessers E., **Wyns, H.**, Reddy, V., Pasmans, F., Martel, A., Haesebrouck, F. De Backer, P., Croubels, S. (2014) Efficacy of a single bolus administration of gamithromycin against *Ornithobacterium rhinotracheale* infection in turkeys. *14th European Poultry Conference*, Stavanger, Norway.

- Cerasoli, I., Gozalo Marcilla, M., **Wyns, H.**, van Bergen, T., Gasthuys, F., Duchateau, L., Schauvliege, S. (2014) Alfaxalone constant rate infusion in midazolam, morphine and ketamine premedicated pigs. *Veterinary Anaesthesia and Analgesia. Association of Veterinary Anaesthesists meeting*, Vienna, Austria.

- **Wyns, H.**, Meyer, E., Plessers, E., Watteyn, A., De Baere, S., van Bergen, T., Schauvliege, S., De Backer, Croubels, S. (2014) Influence of gamithromycin and ketoprofen on the acute phase response in LPS-challenged pigs. *6th European Symposium on Porcine Health Management*, Sorrento, Italy.

- Plessers, E., Watteyn, A., **Wyns, H.**, Pardon, B., De Backer, P., Croubels, S. (2012). Influence of dexamethasone and gamithromycin on the acute phase response in LPS-challenged calves. *Journal of Veterinary Pharmacology and Therapeutics* 35, suppl. 1 - *12th European Association for Veterinary Pharmacology and Toxicology Congress*, Noordwijkerhout, The Netherlands.

- **Wyns, H.**, Meyer, E., **Watteyn, A.**, Plessers, E., De Baere, S., De Backer, P., Croubels, S. (2012). Pharmacokinetics of dexamethasone in pigs. *Journal of Veterinary Pharmacology and Therapeutics* 35, suppl. 1 - *12th European Association for Veterinary Pharmacology and Toxicology Congress*, Noordwijkerhout, The Netherlands.

- Watteyn, A., **Wyns, H.**, Plessers, E., De Baere, S., De Backer, P., Croubels, S. (2012) Pharmacokinetics of dexamethasone in broiler chickens. *Journal of Veterinary Pharmacology and Therapeutics* 35, suppl. 1 - *12th European Association for Veterinary Pharmacology and Toxicology Congress*, Noordwijkerhout, The Netherlands.

- **Wyns, H.**, Croubels, S., Demeyere, K., Watteyn, A., De Backer, P., Meyer, E. (2012) Development of a cytometric bead array screening tool for the simultaneous detection of pro-inflammatory cytokines and acute phase proteins in porcine plasma. *Journal of Veterinary Pharmacology and Therapeutics* 35, suppl. 1 - *12th European Association for Veterinary Pharmacology and Toxicology Congress*, Noordwijkerhout, The Netherlands.

- Watteyn, A., Plessers, E., **Wyns, H.**, De Baere, S., De Backer, P., Croubels, S. (2012) Pharmacokinetics of gamithromycin in broiler chickens. *6th International Conference on Antimicrobial Agents in Veterinary Medicine*, Washington, USA.

- **Wyns, H.**, Meyer, E., Plessers, E., Watteyn, A., De Baere, S., De Backer, P., Croubels, S. (2012) Pharmacokinetics of gamithromycin in pigs. *6th International Conference on Antimicrobial Agents in Veterinary Medicine*, Washington, USA.

- **Wyns, H.**, Croubels, S., Demeyere, K., Watteyn, A., De Backer, P., Meyer, E. (2012) Development of a cytometric bead array screening tool for the simultaneous detection of pro-inflammatory cytokines in plasma of lipopolysaccharide-challenged pigs. *IPVS Belgian Branch Studienamiddag,* Merelbeke, Belgium.

- Plessers, E., **Wyns, H.**, Watteyn, A., Pardon, B., De Backer, P., Croubels, S. (2011) Study of the immunomodulating properties of drugs in a lipopolysaccharide-inflammation model in calves. *6th European Congress of Bovine Health Management,* Liège, Belgium.

- **Wyns, H.**, Croubels, S., Demeyere, K., De Backer, P., Meyer, E. (2010) Multiplex analysis of pro-inflammatory cytokines and pig-major acute phase protein in plasma of lipopolysaccharide-challenged pigs. *IPVS Belgian Branch Studiedag,* Ghent, Belgium.

Beste lezer, na het doorworstelen van de lijvige brok wetenschap, bent u nu aanbeland bij het dankwoord. U mag het gerust beschouwen als een laatste, persoonlijke aim van dit proefschrift. Heel eenvoudig, maar tegelijkertijd zeer moeilijk...

Eerst en vooral wil ik mijn drie promotoren bedanken voor de aangeboden kans mijn doctoraatsonderzoek op deze vakgroep aan te vatten.

Prof. dr. Patrick De Backer, voor mij beantwoordt u perfect aan het beeld van de wijze (en een beetje grijze) professor. Op elke vraag, wetenschappelijk of niet, heeft u steeds snel een antwoord klaar - vaak nog vergezeld van een komische kwinkslag. Bedankt voor de kritische interpretaties, constructieve inbreng en wijze raad bij het schrijven van deze doctoraatsthesis!

Prof. dr. Croubels, Siska, u verslindt manuscripten van allerhande pluimage - abstracts, artikels, posters, verslagen - bij de vleet. Toch maakt u steeds met een glimlach de nodige tijd vrij om uw standpunten toe te lichten. Het is dan ook bewonderenswaardig hoe u met uw legendarisch oog voor detail overbodige punten, spaties en komma's geen schijn van kans gunt. Bedankt voor alles!

Prof. dr. Meyer, Evelyne, hoe hopeloos en uitzichtloos een resultaat er op het eerste zicht ook mag uitzien, u probeert er steeds iets positief in terug te vinden. Dit weerspiegelt eveneens hoe u - ondanks enkele tegenslagen - in het leven staat. Bedankt voor alle suggesties en het aanbrengen van alternatieve denkpistes!

Bijzondere dank gaat uit naar de leden van de lees- en examencommissie, **prof. dr. Claerebout**, **prof. dr. Maes**, **prof. dr. Goddeeris**, **prof. dr. Pasmans** en **dr. Meyns**, voor het kritisch nalezen van dit proefschrif, de rake opmerkingen en het aanreiken van waardevolle suggesties. Special thanks to **prof. dr. Werling** for the valuable suggestions and for attending my PhD defense.

Mijn oprechte dank aan **prof. dr. Gasthuys**, en het hele heelkunde-team, voor de chirurgische katheterisaties van de varkens.

En dan de collega's...
Graag wil ik starten met het bedanken van twee personen die al lang geen gewone collega's meer zijn, maar echte vriendinnen zijn geworden, Anneleen en Elke. Er is geen onderwerp dat ik niet met jullie kon bespreken. Jullie waren mijn twee rechterhanden wanneer nodig, een luisterend oor, partners-in-crime! We hebben heel wat afgezeverd en gelachen. En we zetten, samen met Eva, uiteraard onze hippe DDD-traditie verder!

Anneleen, samen met jou heb ik meermaals de nacht doorgebracht op onze bureau voor de nachtelijke bloednames bij onze beestjes, en hebben we onze kinderen tijdens de weekends vaak 'meegelokt' voor het verzorgen van de kippen, kalkoenen en/of varkens. Ik wens je heel veel geluk met je - (hopelijk ondertussen) uitgebreide - gezinnetje, de verdere afwerking van jullie gezellige 'nest' en uiteraard het finaliseren van je doctoraat!

Elke - tegenwoordig a.k.a. Elke P. - mijn koffiezuster. We waren een geoliede tandem voor het nemen van bloed en de toediening van geneesmiddelen bij de varkens. Ook voor jou is het einde van je doctoraat in zicht - je hebt de resultaten letterlijk en figuurlijk uit je ontelbare ELISA's geklopt - maar eerst heb je nog enkele andere noten te kraken! Ik wens je veel sterkte, het beste en succes ;-) met de afwerking van je doctoraat, de bevalling en je ongetwijfeld prachtig gezinnetje!

Jorien, ook jij bent ondertussen een echte vriendin geworden. Jij staat altijd klaar om te helpen waar en wanneer nodig. Ik kijk al uit naar onze toekomstige stapjes in de wereld, ook samen met Nele. Hopelijk bezorgt sloeber Yari je niet al te veel kopzorgen meer. Ik wens je nog heel veel succes met je verdere onderzoek!

Sophie, het koffieverslaafde Mytox koekoeksjong in onze LPS-nest! ☺ Met je droge humor weet je overal een komische noot aan toe te voegen. Ik wens je heel veel succes met je verdere onderzoek en het afwerken van jullie gezellige huisje, tuintje en wie weet ook kindje ;-)!

Elke G., je werkt nog niet zo heel lang bij ons, maar door jouw enthousiaste gezelligheid lijkt het alsof we elkaar al jaren kennen! Ik wens je nog heel veel succes met die kleine, schattige - en na enkele weken iets minder schattige - varkentjes!

Kristel, de leading lady van het labo biochemie. Voor elk probleem bedenk jij een creatieve oplossing. Jij begeleidde mij in de mysterieuze en fascinerende wereld van de flow cytometrie. Bedankt voor alle hulp met de ELISA's, CBA-analyses én de vele fijne babbels!

Koen, jou zal ik mij herinneren als die toffe, gemotiveerde collega met een licht ochtendhumeur. Ik wil je alvast heel veel succes toewensen met de laatste loodjes van je doctoraat en het verder 'uitdoctoren' van ingewikkelde inflammasoom-aangelegenheden in je postdoc-onderzoek!

Donna, jouw eeuwige optimisme en bijhorende aanstekelijke lach zinderen nog na in mijn hoofd. Bij jou kon ik steeds terecht voor een luchtige babbel over onvermoeibare en slechtslapende kindjes. Bedankt voor alle PhD tips & tricks!

En dan ben ik aanbeland bij de 'mannen van het mycotoxine-team'. Eerst en vooral wil ik een collectief woordje van dank richten voor de hulp bij de dierproeven!

Mathias, jou wens ik heel veel succes met je postdoc en je PROFessionele carrière ;-), alsook in je persoonlijke leven samen met Joke.

Nathan, de nonchalante bon vivant van het gezelschap. Jouw gezangen weerklonken aandoenlijk vals door de gangen. Veel succes met het afronden van je doctoraat!

Thomas, jou zal ik me steeds herinneren als de gemotiveerde doctoraatsstudent die in witte labojas door de gangen raast, maar stipt om 11u30 onze bureau binnenspringt om te vragen of er iemand mee gaat eten ☺.

Gunther, je zou een toevallige passant in de gang kunnen geweest zijn, maar we kwamen elkaar meestal ook nog tegen in de stallen. Veel succes met je verdere onderzoek!

Jelle, één van de weinige vaste waarden tijdens de middagpauze in de refter. Bedankt voor de hulp bij het oneindige aliquoteren tijdens de varkensproeven en de vele gezellige babbels. Veel geluk met de verbouwingen en de beestenboel.

Siegrid, er bestaat geen HPLC-UV noch LC-MS/MS analysemethode die niet door jou ontwikkeld en gevalideerd kan worden! Bedankt voor alle goede raad en hulp met de analyses en validaties!

Ann S., wij hebben nooit rechtstreeks samengewerkt - dit dankwoord is bijgevolg (nog) niet GLP-approved - maar toch zal ik me jou herinneren als die altijd goedlachse collega!

An, meermaals heb je mij - terecht - op de vingers getikt, wanneer ik weer eens iets niet volgens het laboboekje had gedaan. Dankzij jou verloopt alles op rolletjes in het labo. Bedankt voor de fijne babbels - oa. over de fratsen van Nacho.

Kris, jarenlang heb ik je gestalkt met de meest uiteenlopende en onmogelijke computerproblemen - aangezien ik verkeerdelijk dacht dat dit een ware hobby en pure ontspanning was voor jou - maar toch was je steeds bereid om deze met een glimlach meteen op te lossen.

Marc, jouw vrolijke fluitdeuntjes en lachsalvo's weerklinken dagdagelijks door het labo. Jij bent steeds je opgewekte zelve en altijd bereid raad te geven wanneer nodig.

Veerle, aan jou wil ik nog een speciaal woordje van dank richten voor de hulp met de PGE_2-analyses.

Nathalie, jij staat steeds met de glimlach klaar om te helpen met eender welke administratieve rompslomp. Bedankt hiervoor!

Toen ik hier 6 jaar geleden aan de vakgroep begon, behoorde ik bij de jongste doctoraatsstudenten en waren er nog met vitamientjes overgoten fruitpauzes. Intussen ben ik helaas langzaam, maar zeker geëvolueerd tot een 'ancien' en deden ook steeds decadenter wordende en uitdijende koekjespauzes hun intrede. Graag wil ik van de gelegenheid gebruik maken om enkele andere anciens te bedanken en even terug te denken aan 'die tijd van toen':

Eva, toen ik als groentje bij jou in de bureau werd gedropt, heb je mij meteen moederlijk onder jouw vleugels genomen. Ik ben blij dat we elkaar na al die jaren nog steeds niet uit het oog verloren zijn en kijk alvast reikhalzend uit naar onze volgende decadente bijeenkomst! Ik wens je nog heel veel geluk samen met je grote en je kleine ventje!

Joline en **Anja**, samen met jullie heb ik mijn eerste stapjes op deze vakgroep gezet. Ik herinner me nog levendig onze fotoshoots, wie-is-het? en pictionary spelletjes op dode momenten in de varkensstal. Een feestje was met jullie nooit ver af. Bedankt voor alle hulp met de dierproeven en de vele leuke momenten!

Ann O., jij hebt ondertussen ook je (kippen)vleugels elders uitgeslaan. Ik wens je veel geluk in je verdere leven, zowel op persoonlijk als professioneel gebied.

Jella, jij bent slechts twee verdiepingen naar beneden gemigreerd en we komen elkaar nog regelmatig tegen op de AAP-vergaderingen. Heel veel succes met je verdere carrière, je gezinnetje en al je beestjes thuis!

Bert en **Dieter**, veel succes met jullie verdere professionele carrière.

Sandra en **Virginie**, jullie waren een vaste waarde toen ik hier begon. Sandra, jij leerde me de LPS-kneepjes van het vak. Virginie, jij hebt mij op de meet geklopt door één dag voor mij te bevallen van Andreas. Ik wens jullie nog veel succes in jullie persoonlijke en professionele leven!

Ons aller **Maggy**, ik kijk steeds uit naar doctoraatsverdedigingen om jou nog eens terug te zien. Helaas kan je er vandaag niet bij zijn. Bedankt voor het prachtige kaartje en de mooie woorden!

En uiteraard mag ik de jonge garde niet vergeten:

Mijn huidige bureaugenootjes, **Jonas** en **Nermin**, ik duim alvast mee voor (super)goed nieuws van het IWT-front!
Joske en **Joren**, de vluchtige schim die af en toe mijn bureau inpalmde, heel veel succes met jullie onderzoek!

En, last but not least, een welgemeende dankjewel en tegelijkertijd oprechte sorry aan alle varkentjes die hebben bijgedragen aan dit proefschrift.

En ja,... **Nemo**, ook al haal je met je kattenkwaad regelmatig het bloed vanonder mijn nagels, bedankt voor je rustgevend gespin en je attente en aanmoedigende schouderkopjes.

Graag wens ik ook nog enkele mensen te bedanken die iets verder van mijn onderzoek staan, maar veel dichter bij mezelf.

Elke S., **Stephanie** en **Sieglinde**, we moeten dringend nog eens werk maken van een reünieke!
P.S. Stephanie, mocht je hier aanwezig zijn, gelieve plaats te nemen op een onopvallend plaatsje ;-).

Tineke en **Jelle**, bedankt voor de steun tijdens de voorbije maanden. Ik wens jullie nog heel veel geluk, samen met de mooie Annabel.

Meters, jullie zijn er vandaag helaas niet bij, maar ik weet dat jullie fier op mij zijn.

Mijn ‘kleine’ broertje, **Steven**, en **Xandrine**, ik wens jullie nog heel veel geluk met jullie twee prachtige kindjes, **Lentl** en **Milo.** Ik ben dan ook nog steeds zeer fier en blij dat ik meter mocht zijn van de kleine Milo!

Vake en **moeke**, bedankt voor de onvoorwaardelijke steun de voorbije jaren, maar vooral de voorbije maanden. Jullie staan altijd paraat om de kindjes op te vangen, wanneer nodig. Zonder jullie had ik hier nu niet gestaan! Voor duizend en één dingen. BEDANKT!

Noémi en **Senne**, het is met geen pen te beschrijven hoeveel jullie voor mij betekenen. Jullie zijn mijn twee zonnetjes - met af en toe een beetje bewolking - in huis. Ik besef dat ik de voorbije maanden niet zoveel tijd heb kunnen vrijmaken voor jullie, maar ik beloof dat ik dit zeker en vast zal goedmaken! Binnenkort verhuizen we naar ons ‘nieuwe’ huisje, waar jullie zo naar uitkijken. Met ons 3-tjes slaan we ons overal wel door!

En om af te sluiten: een doctoraat maak je niet alleen...
...maar vele handen maken licht(er) werk!