# Habilitation à diriger des recherches Ignacio Caballero

Ignacio Caballero-Posadas

# HABILITATION À DIRIGER DES RECHERCHES

**Sciences de la Vie et de la Santé**

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présentée et soutenue publiquement par :

Ignacio CABALLERO

le 14 Octobre 2020

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**JURY :**
**(Par ordre alphabétique)**

| | | | | |
|---|---|---|---|---|
| -M. | Pascal | BARBRY | Directeur de Recherche, CNRS | Université Côte d'Azur |
| -M. | Nicolas | BERTHO | Chargé de Recherche,INRA, HDR | INRAe, ONIRIS Nantes |
| -M. | Pablo | CHAMERO | Chargé de Recherche INRA, HDR | INRAe, Université Tours |
| -Mme | Isabelle | DIMIER-POISSON | Professeur des Universités | Université de Tours |
| -Mme | Isabelle | OSWALD | Directeur de Recherche, INRA | INRAe Occitanie Toulouse |
| -Mme | Nathalie | WINTER | Directeur de Recherche, INRA | INRAe, Université Tours |

# INDEX

# Section 1: Curriculum Vitae

# SECTION 1.1 CURRICULUM VITAE

## PERSONAL INFORMATION

**CABALLERO, Ignacio**

Chargé de recherche classe normale

Date of birth: 22 September 1978

Place of birth: Murcia, Spain

Nationality: Spain

Address: Infection and innate immunity in monogastric livestock (3IMo) team
UMR1282 Infectiologie et Santé Publique
INRA – Centre Val de Loire
Route de Crotelles, ISP311
37380 Nouzilly, France

Tél : +33 (0)2 47 42 78 65

Email: ignacio.caballero-posadas@inrae.fr

URL for web site: https://www6.val-de-loire.inra.fr/infectiologie-santepublique_eng/

## EDUCATION

2007 European Doctorate in Veterinary Medicine (PhD)
University of Murcia (Spain)

2003 Veterinary Medicine Degree (DVM)
University of Murcia (Spain)

## CURRENT POSITION

2013- Chargé de recherché classe normale
UMR1282 Infectiologie et Santé Publique, Centre Val de Loire INRA

## PREVIOUS POSITIONS

2011-2013 Postdoctoral Marie Curie fellow (IIF).
Academic Unit of Reproductive and Developmental Medicine, Human Metabolism Department, University of Sheffield, UK

2010-2011 Postdoctoral fellow

Animal Reproduction research group. University of Murcia, Spain

2008-2010 Postdoctoral fellow
Molecular Biomedical Science department, North Carolina State University, Raleigh, USA

2003-2007 PhD student (Supervisors Prof. J.M. Vazquez Rojas and Prof. I. Parrilla Riera)
Animal Reproduction research group. University of Murcia, Spain

2001-2002 Undergraduate research assistant.
Animal Reproduction research group. University of Murcia, Spain

## COMMISSIONS OF TRUST

Scientific Evaluator: Spanish National Agency of Research (2015 – present)

Reviewer activities: Journal of Andrology; International Journal of Andrology; Theriogenology; Reproduction and Reproduction in domestic animals, PLoS ONE

Editorial activities: Review Editor for Frontiers in Veterinary Science

## MAJOR COLLABORATORS

- Sascha Trapp, INRA Centre Val de Loire
- Rodrigo Guabiraba, INRA Centre Val de Loire
- Pablo Chamero, INRA Centre Val de Loire
- Mustapha Si-Tahar, INSERM U1100, Centre d'étude des pathologies respiratoires, Tours
- Pascal Barbry, CNRS, Institut de Pharmacologie Moléculaire et Cellulaire, F06560 Sophia Antipolis
- Catherine Robbe-Masselot, CNRS/USTL, UMR 8576, Unité de Glycobiologie Structurale et Fonctionnelle, Université Lille1
- Nikolai Klymiuk, CIMM-Gene Center and Center for Innovative Medical Models, LMU Munich, Germany
- Jean-Claude Sirard, CNRS, INSERM, CHU Lille, Institut Pasteur de Lille, U1019 – UMR9017 - CIIL - Center for Infection and Immunity of Lille, F-59000 Lille, France
- Laura Soler-Vasco, Toxalim (Research Centre in Food Toxicology), Université de Toulouse, INRA, ENVT, INP-Purpan, UPS, Toulouse, France
- Michael White, Michael Smith Building, Faculty of Life Sciences, University of Manchester, Manchester, United Kingdom
- Christopher Sanderson, Department of Cellular and Molecular Physiology, University of Liverpool, Liverpool, United Kingdom

- Alireza Fazeli, Academic Unit of Reproductive and Developmental Medicine, The Medical School, University of Sheffield, Sheffield, United Kingdom

## AWARDS

- Phd with distiction: “Premio Extraordinario de Doctorado” by University of Murcia, Spain 2008.
- First prize i-patent for the transfer of research results in the Region of Murcia, Spain. 2006.
- Pfizer Animal Health Award for Research Excellence as member of Dr Piedrahita’s lab. 2009.
- Best presentation at EYIM conference 2017.

# SECTION 1.2 ACADEMIC ACTIVITIES

## TEACHING ACTIVITIES

2014- Lecturer for the Master programme "Infectiologie Cellulaire Et Moleculaire Vaccinologie, Anticorps Therapeutiques", University Francois Rabelois, Tours, France

2011-2013 Advisor for rotation students and Ph.D. candidates; Academic Unit of Reproductive and Developmental Medicine, Human Metabolism Department, University of Sheffield, UK

2008-2010 Advisor for rotation students and Ph.D. candidates; Molecular Biomedical Science department, North Carolina State University, Raleigh, USA

2003-2007 Lecturer for animal reproduction course – Veterinary Masters programme; Department of Animal Medicine and Surgery, University of Murcia, Murcia, Spain

## SUPERVISION OF STUDENTS AND POSTDOCTORAL FELLOWS

Postdoctoral fellows : Dr. Raquel López-Gálvez (INRA Centre-Val de Loire; 2019-present)

Graduate students : Javier Arturo Sánchez-López (Medical School, University of Sheffield, United Kingdom, 2011-2013)

Mehrnaz Montazeri (Medical School, University of Sheffield, United Kingdom, 2011-2013)

Master students : Robin Sanchez-Avila (M2 in Infectiologie, Vaccinologie et Biomédicaments, Université de Tours; 2019)

Melissa Thevanne (M1 in Bio agro prc durabilité qualité des filières de production animal, Université de Tours, 2019)

Sumiah Al Ghareeb (MsC in Molecular Medicine, Medical School, University of Sheffield, United Kingdom, 2011)

Gloria Marcela Mayor (MsC of Pig Reproduction, University of Murcia, Spain, 2007)

## SUPERVISED MASTER THESES

| | |
|---|---|
| 2019 | Robin Sanchez-Avila (M2 Thesis; Université de Tours): " Evaluation of differences in virulence between Salmonella strains from super-shedders and low-shedders individuals" |
| 2011 | Sumiah Al Ghareeb (MsC Thesis, University of Sheffield, United Kingdom): "Understanding innate immunity in maternal-gamete interaction" |
| 2007 | Gloria Marcela Mayor (MsC Thesis, University of Murcia, Spain): "Effect of the PSP-I/PSP-II heterodimer on sperm capacitation" |

**MEMBER OF PHD JURY**

| | |
|---|---|
| 2018 | Alicia Nohalez Ruiz, University of Murcia, Spain (Directors: Profs. Emilio Martinez Garcia and Cristina Cuello Medina) |
| 2017 | Isabel Barranco Cascales, University of Murcia, Spain (Directors: Profs. Jordi Roca Aleu and Heriberto Rodriguez-Martinez) |
| 2016 | Luis Anel Lopez, University of Castilla la Mancha, Spain (Directors: Profs. José Julián Garde López-Brea) |
| 2016 | Enrique del Olmo Medina, University of Castilla la Mancha, Spain (Directors: Profs. José Julián Garde López-Brea and María del Rocío Fernández Santos) |
| 2015 | Vicente Seco-Rovira, University of Murcia, Spain (Directors: Prof. Luis Miguel Pastor Garcia) |

# SECTION 1.3 GRANT PROJECTS AND FUNDING

## ON-GOING GRANTS

1. **Projet Recherche d'Initiative Académique 2019 ; Period :** 2019-2021 ; **Title :** Etude des facteurs virologiques associés à l'évolution chronique de l'hépatite E chez les patients immunodéprimés avec modélisation de l'infection in vivo chez le porc. **Finacial entity** : Region Centre ; **Amount :** 200k€ ; **Role :** Partner

2. **Santé Animale INRA 2019 ; Period :** 2020-2021 ; **Title :** Exploration of molecular mechanisms leading to endothelial dysfunction during ASF infection. **Finacial entity** : SA INRA ; **Amount :** 20k€ ; **Role :** Coordinator

3. **ANR JCJC 2017; Period:** 2019-2022; **Title :** PIGIMMUNITY: a systems biology approach to boost innate immunity in the pig. **Finacial entity** : French National Research Agency (ANR) ; **Amount :** 312k€ ; **Role :** Coordinator

## PAST GRANTS

1. **Recherche Fondamentale 2018 ; Period :** 2019-2020 ; **Title :** Explorer l'atlas d'expression des cellules des voies aériennes chez le cochon CFTR-/-. **Finacial entity** : Vaincre la Mucoviscidose; **Amount :** 161k€ ; **Role :** Partner (Workpackage leader).

2. **Recherche Fondamentale 2016 ; Period :** 2016-2018 ; **Title :** Traitements innovants par voie respiratoire combinant immunostimulation de TLR5 et antibiothérapies pour augmenter l'efficacité traitements contre les infections à Pseudomonas aeruginosa: preuves de concept et optimisation. **Finacial entity** : Vaincre la Mucoviscidose ; **Amount :** 233k€ ; **Role :** Partner (Workpackage leader)

3. **Recherche Fondamentale 2015 ; Period :** 2016-2018 ; **Title :** The European Cystic Fibrosis pig: a unique model to decipher glycosylation alterations in lung mucins. **Finacial entity** : Vaincre la Mucoviscidose ; **Amount :** 188k€ ; **Role :** Partner (Workpackage leader)

4. **ANR Projet Générique 2014 ; Period :** 2015-2018 ; **Title :** PIGLETBIOTA: an integrative biology-based study of the influence of intestinal microbiota composition on piglet robustness at weaning in a perspective of a limited use of antibiotics in livestock production ; **Amount :** 794k€ ; **Role :** Partner

5. **Santé Animale Crédit Jeune Chercheur 2014**. **Period :** 2014-2015 ; **Title :** The development of a novel PMN-iRFP transgenic pig model ; **Amount :** 10k€ ; **Role :**Coordinator

6. **Projet Recherche d'Initiative Académique 2011 ; Period :** 2011-2013 ; **Title :** Modèle d'Infections Respiratoires par Pseudomonas aeruginosa chez le porc. **Finacial entity** : Region Centre ; **Amount :** 100k€ ; **Role :** Partner

7. **Recherche Fondamentale 2013 ; Period :** 2014-2015 ; **Title :** Développement d'un modèle de colonisation pulmonaire par Pseudomonas aeruginosa chez le porc CFTR-/. **Finacial entity** : Vaincre la Mucoviscidose ; **Amount :** 200€ ; **Role :** Partner

8. **Projet Recherche d'Initiative Académique 2012 ; Period :** 2013-2015 ; **Title :** Modèle de colonisation pulmonaire chronique à Pseudomonas aeruginosa chez le porc CFTR-/-. **Finacial entity** : Region Centre ; **Amount :** 200k€ ; **Role :** Partner

9. **Medical School, University of Sheffield funding ; Period :** 2011 ; **Title :** Understanding innate immunity in maternal gamete interaction. **Finacial entity** : Medical School, University of Sheffield, UK ; **Amount :** 4k€ ; **Role :** Principal Investigator

10. **Marie Curie IIF Funding 2010; Period:** 2011-2013; **Title:** Regulation of innate immunity in the female reproductive tract. **Financial entity:** 7th Framework programme. European Commission; **Amount:** 173k€; **Role:** Applicant

# Section 2: Publications

# SECTION 2.1 PUBLICATIONS

## PUBLICATIONS IN INTERNATIONAL PEER-REVIEWED JOURNALS

1. **Ignacio Caballero**, Bélinda Ringot-Destrez, Mustapha Si-Tahar, Pascal Barbry, Antoine Guillon, Isabelle Lantier, Mustapha Berri, Claire Chevaleyre, Isabelle Fleurot, Céline Barc, Reuben Ramphal, Nicolas Pons, Agnès Paquet, Kévin Lebrigand, Carole Baron, Andrea Bähr, Nikolai Klymiuk, Renaud Léonard, Catherine Robbe-Masselot. Evidence of early increased sialylation of airway mucins and defective mucociliary clearance in CFTR-deficient piglets. J Cyst Fibros 2020 (Submitted).

2. Fleurot I, López-Gálvez, Melo S, Olivier M, Chevaleyre C, Barc C, Riou M, Si-Tahar M, Barbry P, Bähr A, Klymiuk N, Sirad JC, **Caballero I**. Flagellin administration modulates defective TLR5 signalling in cystic fibrosis airways. European Respiratory Journal 2020 (Submitted).

3. Bréa D, Soler L, Fleurot I, Melo S, Chevaleyre C, Berri M, Labas V, Teixeira-Gomes AP, Pujo J, Cenac N, Bähr A, Klymiuk N, Guillon A, Si-Tahar M, **Caballero I**. Intrinsic alterations in peripheral neutrophils from cystic fibrosis newborn piglets. J Cyst Fibros 2020 (accepted). Times cited:

4. Ruiz Garcia S, Deprez M, Lebrigand K, Paquet A, Cavard A, Arguel MJ, Magnone V, Truchi M, **Caballero I,** Leroy S, Marquette CH, Marcet B, Barbry P, Zaragosi LE. Novel dynamics of human mucociliary differentiation revealed by single-cell RNA sequencing of nasal epithelial cultures. Development 2019. Oct 23;146(20). Times cited: 2

5. Braux J, Jourdain ML, Guillaume C, Untereiner V, Piot O, Baehr A, Klymiuk N, Winter N, Berri M, Buzoni-Gatel D, **Caballero I**, Si-Tahar M, Jacquot J, Velard F. Lack of cystic fibrosis transmembrane conductance regulator causes low cortical bone thickness and high cortical porosity in newborn pigs. J Cyst Fibros 2019 (In press). Times cited:

6. **Caballero I**, Riou M, Hacquin O, Chevaleyre C, Barc C, Pezant J, Pinard A, Rezzonico R, Mari B, Heuzé-Vourc'h N, Pitard B, Vassaux G. Tetrafunctional block copolymers promote lung gene transfer in newborn piglets. Mol Ther Nucleic Acids 2019 Jun 7;16:186-193. Times cited: 1

7. Sper RB, Koh S, Zhang X, Simpson S, Collins B, Sommer J, et al. Generation of a Stable Transgenic Swine Model Expressing a Porcine Histone 2B-eGFP Fusion Protein for Cell Tracking and Chromosome Dynamics Studies. PloS one. 2017;12(1):e0169242. Times cited: 4

8. **Caballero I**, Boyd J, Alminana C, Sanchez-Lopez JA, Basatvat S, Montazeri M, et al. Understanding the dynamics of Toll-like Receptor 5 response to flagellin and its regulation by estradiol. Sci Rep. 2017;7:40981. Times cited: 3

9. Montazeri M, Sanchez-Lopez JA, **Caballero I**, Maslehat Lay N, Elliott S, Fazeli A. Interleukin-1 receptor antagonist mediates toll-like receptor 3-induced inhibition of

trophoblast adhesion to endometrial cells in vitro. Hum Reprod. 2016;31(9):2098-107. Times cited: 5

10. Chevaleyre C, Riou M, Brea D, Vandebrouck C, Barc C, Pezant J, Melo S, Olivier M, Delaunay R, Boulesteix O, Berthon P, Rossignol C, Burlaud Gaillard J, Becq F, Gauthier F, Si-Tahar M, Meurens F, Berri M, **Caballero-Posadas I**, Attucci S. The Pig: A Relevant Model for Evaluating the Neutrophil Serine Protease Activities during Acute Pseudomonas aeruginosa Lung Infection. PloS one. 2016;11(12):e0168577. Times cited: 4

11. Montazeri M, Sanchez-Lopez JA, **Caballero I**, Maslehat Lay N, Elliott S, Lopez-Martin S, et al. Activation of Toll-like receptor 3 reduces actin polymerization and adhesion molecule expression in endometrial cells, a potential mechanism for viral-induced implantation failure. Hum Reprod. 2015;30(4):893-905. Times cited: 11

12. Guillon A, Chevaleyre C, Barc C, Berri M, Adriaensen H, Lecompte F, Villemagne T, Pezant J, Delaunay R, Moënne-Loccoz J, Berthon P, Bähr A, Wolf E, Klymiuk N, Attucci S, Ramphal R, Sarradin P, Buzoni-Gatel D, Si-Tahar M, **Caballero I**. Computed Tomography (CT) Scanning Facilitates Early Identification of Neonatal Cystic Fibrosis Piglets. PloS one. 2015;10(11):e0143459. Times cited: 2

13. Sanchez-Lopez JA, **Caballero I**, Montazeri M, Maslehat N, Elliott S, Fernandez-Gonzalez R, et al. Local activation of uterine Toll-like receptor 2 and 2/6 decreases embryo implantation and affects uterine receptivity in mice. Biol Reprod. 2014;90(4):87. Times cited: 9

14. Alminana C, **Caballero I**, Heath PR, Maleki-Dizaji S, Parrilla I, Cuello C, et al. The battle of the sexes starts in the oviduct: modulation of oviductal transcriptome by X and Y-bearing spermatozoa. BMC Genomics. 2014;15:293. Times cited: 69

15. **Caballero I,** Al Ghareeb S, Basatvat S, Sanchez-Lopez JA, Montazeri M, Maslehat N, et al. Human trophoblast cells modulate endometrial cells nuclear factor kappaB response to flagellin in vitro. PloS one. 2013;8(1):e39441. Times cited: 6

16. Parrilla I, del Olmo D, **Caballero I**, Tarantini T, Cuello C, Gil MA, et al. The effect of glycerol concentrations on the post-thaw in vitro characteristics of cryopreserved sex-sorted boar spermatozoa. Reprod Domest Anim. 2012;47(6):965-74. Times cited: 7

17. **Caballero I**, Parrilla I, Alminana C, del Olmo D, Roca J, Martinez EA, et al. Seminal plasma proteins as modulators of the sperm function and their application in sperm biotechnologies. Reprod Domest Anim. 2012;47 Suppl 3:12-21. Times cited: 66

18. Sanchez-Osorio J, Cuello C, Gil MA, Parrilla I, Alminana C, **Caballero I**, et al. In vitro postwarming viability of vitrified porcine embryos: effect of cryostorage length. Theriogenology. 2010;74(3):486-90. Times cited: 15

19. Alminana C, Gil MA, Cuello C, Parrilla I, **Caballero I**, Sanchez-Osorio J, et al. Capability of frozen-thawed boar spermatozoa to sustain pre-implantational embryo development. Anim Reprod Sci. 2010;121(1-2):145-51. Times cited: 13

20. Parrilla I, Vazquez JM, **Caballero I,** Gil MA, Hernandez M, Roca J, et al. Optimal characteristics of spermatozoa for semen technologies in pigs. Soc Reprod Fertil Suppl. 2009;66:37-50. Times cited:

21. **Caballero I,** Vazquez JM, Mayor GM, Alminana C, Calvete JJ, Sanz L, et al. PSP-I/PSP-II spermadhesin exert a decapacitation effect on highly extended boar spermatozoa. Int J Androl. 2009;32(5):505-13. Times cited: 42

22. **Caballero I,** Piedrahita JA. Evaluation of the Serratia marcescens nuclease (NucA) as a transgenic cell ablation system in porcine. Anim Biotechnol. 2009;20(4):177-85. Times cited: 1

23. Vazquez JM, Roca J, Gil MA, Cuello C, Parrilla I, **Caballero I**, et al. Low-dose insemination in pigs: problems and possibilities. Reprod Domest Anim. 2008;43 Suppl 2:347-54. Times cited: 13

24. Vazquez JM, Parrilla I, Gil MA, Cuello C, **Caballero I**, Vazquez JL, et al. Improving the efficiency of insemination with sex-sorted spermatozoa. Reprod Domest Anim. 2008;43 Suppl 4:1-8. Times cited: 9

25. Sanchez-Osorio J, Cuello C, Gil MA, Alminana C, Parrilla I, **Caballero I**, et al. Factors affecting the success rate of porcine embryo vitrification by the Open Pulled Straw method. Anim Reprod Sci. 2008;108(3-4):334-44. Times cited: 34

26. **Caballero I**, Vazquez JM, Garcia EM, Parrilla I, Roca J, Calvete JJ, et al. Major proteins of boar seminal plasma as a tool for biotechnological preservation of spermatozoa. Theriogenology. 2008;70(8):1352-5. Times cited: 42

27. Alminana C, Gil MA, Cuello C, **Caballero I,** Roca J, Vazquez JM, et al. In vitro fertilization (IVF) in straws and a short gamete coincubation time improves the efficiency of porcine IVF. Reprod Domest Anim. 2008;43(6):747-52. Times cited: 8

28. Alminana C, Gil MA, Cuello C, **Caballero I,** Roca J, Vazquez JM, et al. In vitro maturation of porcine oocytes with retinoids improves embryonic development. Reprod Fertil Dev. 2008;20(4):483-9. Times cited: 21

29. Spjuth L, Gil MA, **Caballero I,** Cuello C, Alminana C, Martinez EA, et al. Pre-pubertal di(2-ethylhexyl) phthalate (DEHP) exposure of young boars did not affect sperm in vitro penetration capacity of homologous oocytes post-puberty. Arch Androl. 2007;53(3):141-7. Times cited: 5

30. Maxwell WM, Parrilla I, **Caballero I,** Garcia E, Roca J, Martinez EA, et al. Retained functional integrity of bull spermatozoa after double freezing and thawing using PureSperm density gradient centrifugation. Reprod Domest Anim. 2007;42(5):489-94. Times cited: 38

31. Garcia EM, Vazquez JM, Parrilla I, Calvete JJ, Sanz L, **Caballero I,** et al. Improving the fertilizing ability of sex sorted boar spermatozoa. Theriogenology. 2007;68(5):771-8. Times cited: 32

32. Cuello C, Gil MA, Alminana C, Sanchez-Osorio J, Parrilla I, **Caballero I**, et al. Vitrification of in vitro cultured porcine two-to-four cell embryos. Theriogenology. 2007;68(2):258-64. Times cited: 15

33. Wongtawan T, Saravia F, Wallgren M, **Caballero I**, Rodriguez-Martinez H. Fertility after deep intra-uterine artificial insemination of concentrated low-volume boar semen doses. Theriogenology. 2006;65(4):773-87. Times cited: 51

34. Garcia EM, Vazquez JM, Calvete JJ, Sanz L, **Caballero I,** Parrilla I, et al. Dissecting the protective effect of the seminal plasma spermadhesin PSP-I/PSP-II on boar sperm functionality. J Androl. 2006;27(3):434-43. Times cited: 36

35. De Ambrogi M, Ballester J, Saravia F, **Caballero I**, Johannisson A, Wallgren M, et al. Effect of storage in short--and long-term commercial semen extenders on the motility, plasma membrane and chromatin integrity of boar spermatozoa. Int J Androl. 2006;29(5):543-52. Times cited: 52

36. **Caballero I**, Vazquez JM, Garcia EM, Roca J, Martinez EA, Calvete JJ, et al. Immunolocalization and possible functional role of PSP-I/PSP-II heterodimer in highly extended boar spermatozoa. J Androl. 2006;27(6):766-73. Times cited: 40

37. Parrilla I, Vazquez JM, Gil MA, **Caballero I,** Alminana C, Roca J, et al. Influence of storage time on functional capacity of flow cytometrically sex-sorted boar spermatozoa. Theriogenology. 2005;64(1):86-98. Times cited: 25

38. **Caballero I**, Vazquez JM, Rodriguez-Martinez H, Gill MA, Calvete JJ, Sanz L, et al. Influence of seminal plasma PSP-I/PSP-II spermadhesin on pig gamete interaction. Zygote. 2005;13(1):11-6. Times cited: 24

39. **Caballero I**, Vazquez JM, Gil MA, Calvete JJ, Roca J, Sanz L, et al. Does seminal plasma PSP-I/PSP-II spermadhesin modulate the ability of boar spermatozoa to penetrate homologous oocytes in vitro? J Androl. 2004;25(6):1004-12. Times cited: 26

40. **Caballero I**, Vazquez JM, Centurion F, Rodriguez-Martinez H, Parrilla I, Roca J, et al. Comparative effects of autologous and homologous seminal plasma on the viability of largely extended boar spermatozoa. Reprod Domest Anim. 2004;39(5):370-5. Times cited: 49

## BOOKS AND BOOK CHAPTERS

**1.** Hernández M, Bolarín A, Gil MA, Almiñana C, Cuello C, Parrilla I, García E, **Caballero I**, Sánchez-Osorio J, Lucas X, Vázquez JM, Martínez EA, Roca J. Criopreservación espermática. Manual de técnicas de reproducción asistida en porcino (spanish). Eds Universidad de Girona. Red temática nacional de porcino. ISBN:84-8458-241-8. pages: 185-200.

**2.** Parrilla I, **Caballero I**, García E, Gil MA, Almiñana C, Cuello C, Sánchez-Osorio J, Hernández M, Lucas X, Roca J, Martínez EA, Vázquez JM. 2006. Determinación del sexo de la descendencia: sexaje de espermatozoides. Manual de técnicas de reproducción asistida en porcino (spanish). ISBN:84-8458-241-8. pages: 215-236.

**3.** Cuello C, Gil MA, Almiñana C, Sánchez-Osorio J, Parrilla I, Hernández M, García E, **Caballero I**, Lucas X, Vázquez JM, Roca J, Martínez EA. 2006. Obtención y cultivo in vitro de embriones. Manual de técnicas de reproducción asistida en porcino (spanish). Eds Universidad de Girona. Red temática nacional de porcino. ISBN:84-8458-241-8. pages: 259-266.

**4.** Gil MA, Almiñana C, Cuello C, Parrilla I, Hernández M, García E, **Caballero I**, Sánchez-Osorio J, Lucas X, Vázquez JM, Roca J, Martínez EA. 2006. Producción in vitro de embriones porcinos. Manual de técnicas de reproducción asistida en porcino (spanish). Eds Universidad de Girona. Red temática nacional de porcino. ISBN:84-8458-241-8. pages: 267-278.

**5.** Cuello C, Gil MA, Almiñana C, Sánchez-Osorio J, Parrilla I, Hernández M, García E, **Caballero I**, Lucas X, Vázquez JM, Roca J, Martínez EA. 2006. Técnicas de transferencia de embriones. Manual de técnicas de reproducción asistida en porcino (spanish). Eds Universidad de Girona. Red temática nacional de porcino. ISBN:84-8458-241-8. pages: 279-284.

**6.** Cuello C, Gil MA, Almiñana C, Sánchez-Osorio J, Parrilla I, Hernández M, García E, **Caballero I**, Lucas X, Vázquez JM, Roca J, Martínez EA. 2006. Técnicas de vitrificación de embriones. Manual de técnicas de reproducción asistida en porcino (spanish). Eds Universidad de Girona. Red temática nacional de porcino. ISBN:84-8458-241-8. pages: 285-292.

**7.** Parrilla I, **Caballero I**, García EM, Gil MA, Cuello C, Roca J, Martínez EA, Vázquez JM. 2006. Preselección del sexo de la descendencia en el ganado porcino. Biotecnología de la reproducción porcina. Porci (monograph) (spanish). ISSN:1130-8451. pages: 39-50.

**8.** Parrilla I, **Caballero I**, García EM, Gil MA, Cuello C, Roca J, Martínez EA, Vázquez JM. 2006. Preselección del sexo de la descendencia en el ganado porcino. Producir carne de cerdo en el siglo XXI, generando un nuevo orden zootécnico (spanish). Eds. Acalanthis Comunicación Estrategia, S.L.U. ISBN:84-935067-2-9. pages: 263-276.

**9.** Spjuth L; Gil MA, **Caballero I**, Cuello C, Almiñana C, Martínez EA, Lundeheim N, Rodríguez-Martínez H. Pre-pubertal Di(2-ethylhexyl) Phthalate (DEHP) Exposure of Young Boars Did Not Affect Sperm In vitro Penetration Capacity of Homologous Oocytes Post-puberty. Di(2-ethylhexyl) Phthalate and Semen Quality in Boars (English). Eds. Acta Universitatis Agriculturae Sueciae. ISBN 91-576-7253-9. pages:1-11 (chapter 4).

**10. Caballero I.** Estudio del plasma seminal y la espermadhesina PSP-I/PSP-II sobre la funcionalidad de los espermatozoides de verraco (spanish). ISBN:978-84-690-6075-9. (245 pages). Ed. Servicio de publicaciones de la Universidad de Murcia. Murcia.

## INVITED PRESENTATIONS TO INTERNATIONAL CONFERENCES

1. Novel approaches boosting innate immunity against *Pseudomonas aeruginosa. 15 Congrès national de la SFM. Paris, France. 30 september-2 October, 2019.*
2. The European CF pig model: ups, downs and perspectives. International Conference on Cystic fibrosis: contribution of animal models to therapeutic development. Tours, France. November 2015.
3. Maintenance and phenotyping of a pig model for the study of early pathogenesis of cystic fibrosis. First Conference COST action SALAAM. Munich, Germany, December 2014.
4. Applications of seminal plasma in sperm biotechnologies. 17$^{th}$ Conference of the European Society for Domestic Animal Reproduction (ESDAR). Bologna, Italy, September 2013.
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# SECTION 2.2 SUMMARY OF EARLY ACHIEVEMENTS

I completed my PhD in 2007 at the University of Murcia in 2003. The main goal of the PhD project was to improve the outcome of sperm cryopreservation and flow cytometric sex-sorting (that allows choosing the sex of the offspring) in porcine using seminal plasma and its proteins. I performed a first post-doctoral research state at Dr Piedrahita's lab in North Carolina State University (2008-2010). Here, I was trained in the development of transgenic pig models for human medicine research. During this project, I generated a stable transgenic pig model expressing H2B-eGFP for cell tracking in xenograft studies and transgenic pigs with targeted ablation of Natural Killer cells. After this research stage, I was awarded with a Marie Curie International Incoming Fellowship at the University of Sheffield, United Kingdom (2011-2013). There, I studied the role of innate immunity in in pregnancy failure. Specifically, I focused on the role of Toll-like receptors (TLRs) signalling on embryo implantation and how this signalling is modulated by the oestrus cycle and the presence of the embryo. In 2013, I was appointed for a permanent researcher position at the INRA-Centre-Val de Loire in France as the leader of the "Porcine Mucosal Immunology" team. In 2019 this team merged with the "Avian Immune Response and Pathogenesis" to form the "Infection and innate immunity in monogastric livestock" team. My major research interest lies in understanding the mechanisms that modulate pig innate immune response in order to develop novel alternative therapies and immune-stimulation strategies. The pig is an important research model, both for his great economic impact as livestock and its growing importance as a biomedical model. In this regard, I have established in my lab a transgenic pig model of cystic fibrosis, a disease characterized for an excessive lung inflammatory response, to determine the mechanisms leading to this excessive inflammation and the use of flagellin as an immune-modulatory molecule.

I have been awarded with several research grants, both as PI (4) and partner (9). It can be highlighted an ANR jeune chercheur grant as PI. I have built an international network of collaborators, from France and the United Kingdom. I have published a total of 38 manuscript in peer-reviewed international journals (2 more submitted) with a total of 791 citations and an h-index of 16. I participated actively at international and national conferences with 49 poster presentations and oral communications and I have also been an invited speaker in 7 international conferences. As part of my academic activities I have directed 4 Master students, I have been the day-to-day supervisor of 2 PhD students and I am currently directing a postdoctoral student. I participate actively as a lecturer in the Master program ICMV from the University of Tours.

# Section 3: Scientific activities

# SECTION 3.1 INTRODUCTION

By 2050, the world population is expected to increase to more than 9 billion people. Providing animal food resources while respecting the societal demands for food safety and animal welfare is a priority for the UN Sustainable Development Agenda [1]. Pork accounts for more than one-third of meat produced worldwide. It is a major component of global food security, agricultural economies, and trade (22.1 million tonnes of pig meat production in the European Union in 2014, highest of all livestock species). The demand for pork has led to intensification of production, with farms often housing thousands of animals in densities conducive to rapid pathogen transmission. Infectious diseases result in direct losses to livestock production through mortality, loss of productivity, trade restrictions, reduced market value, and often food insecurity [2]. Among those diseases, the porcine respiratory disease complex (PRDC) is a cause massive economic loss to swine industries worldwide. PRDC results from a combination of environmental stress and respiratory tract infection, with swine influenza A virus (SIAV) being the leading viral pathogen. Although mono-infections with SIAV generally cause low mortality (<1%), they predispose the host to secondary infections with pathogenic bacteria, ie. *Actinobacillus pleuropneumoniae* (App) and *Mycoplasma hyopneumoniae* (Mhp) [3]. These viral-bacterial co-infections lead to a profuse and deleterious secretion of pro-inflammatory cytokines, severely aggravating the clinical picture in the herd and resulting in important economic losses (up to 6.4€ reduced return/pig) [4]. In addition, SIAV is an epizootic/zoonotic pathogen causing respiratory infections and extensive morbidity and mortality in livestock and man [5-7].

In addition to its value as livestock, the last years has seen a tremendous increase in the interest on the pig as a biomedical model. Pigs have become an attractive research model in the field of surgical and procedure training, disease progression and pathology, translational research, and regenerative medicine/stem cell therapy, due to their anatomic, genetic, and physiological similarities with humans. In addition, the availability of the pig genome sequence, the development of somatic cells nuclear transfer (SCNT) and high efficiency genome editing tools such as Transcription activator-like effector nucleases (TALENs) and Clustered regularly interspaced short palindromic repeats (CRISPR-Cas9), have allowed precise and efficient genetic engineering in the pig [8]. A clear example of the pig as a translational biomedical model is cystic fibrosis (CF). CF, the most common lethal genetic disease in the Caucasian population, is a recessive genetic disorder caused by mutations in the cystic fibrosis transmembrane conductance regulator (*CFTR*) gene, rendering the protein non-functional [9]. CFTR has been shown to function as a regulated anionic channel at the apical cell surface. CF mutations affect the rheology of secretions, which become thick and difficult to clear from respiratory airways [10]. These conditions lead to chronic bacterial infection, often dominated by *Pseudomonas aeruginosa* (*P. aeruginosa*), persistent inflammation with exacerbated recruitment of polymorphonuclear neutrophils (PMNs) into the lungs, excessive release of proteases and ultimately lung tissue destruction [9, 11, 12]. The latter is the main cause of death in CF patients. Understanding the processes leading to this altered inflammatory response is crucial to improve CF therapies. Since the *CFTR* gene was first identified and isolated in 1989 [13], several transgenic mouse strains have been generated in order to study the pathophysiology of CF. However, their usefulness have been limited by the lack of a phenotype in the respiratory tract that mimics the complications observed in human CF [14, 15]. More recently, this drawback has been tackled by the development of a mutated $CFTR^{\Delta F508/\Delta F508}$ pig [16] and a $CFTR^{-/-}$ knockout pig [17, 18]. Studies in the $CFTR^{\Delta F508/\Delta F508}$ pig [21] and the *CFTR*-

$^{/-}$ knockout pig [18, 21] suggest that airways inflammation in naive, non-infected lungs [22], is related to an altered immune response that facilitate lung colonization by the pathogen. This would lead to a chronic infection and consequently a pro-inflammatory lung environment [23], similar to the alterations described in CF patients [19, 20]. These features make the CF pig a very valuable model to study the pathogenesis of CF and to evaluate new therapies.

Modulation of innate immunity has been identified as a promising prophylactic and/or therapeutic strategy to combat infectious diseases due to their ability to activate host immune mechanisms with a broad spectrum of protection. A deep understanding of the mechanisms that control the nature and intensity of the innate immune response would be crucial to improve CF therapies, as well as to fight viral and bacterial infections in the pig. During the last years, **my research have been dedicated to the study of innate immunity and host-pathogen interactions in the pig**, with special focus on the CF pig model and Toll-like receptor (TLR) signalling.

The specific objectives of my research have been:

1. Development of an experimental model of *P. aeruginosa* infection in the pig.
2. Development of a cystic fibrosis pig model at the INRA, Val de Loire centre.
3. Determine whether there are intrinsic alterations in the CF pig model innate immune response that are responsible for the development of CF lung disease.
4. Development of novel therapies targeting innate immunity.

## STATE OF THE ART

### Toll-like receptors, a major family of pattern recognition receptors of innate immunity

The immune response is generally divided in two main arms, innate and adaptive immune response. Although, traditionally, innate immunitiy has garnered less attention than adaptive immunity, the last 20 years has seen an important growth in this field, with key discoveries shedding light in the mechanisms of innate immunity. Among those are the mechanisms that control the detection of the pathogens and the downstream cascade signalling that will lead to an effective inflammatory response.

The innate immune system detects the presence of microbes and initiates mechanisms to eliminate potentially infectious threats. Microbial detection is achieved through germline-encoded pattern-recognition receptors (PRRs) that survey both the extracellular and intracellular space for conserved microbial determinants that serve as indicators of infection [24]. Most PRRs can be classified into one of five families based on protein domain homology. These five families consist of C-type lectin receptors (CLRs), nucleotide-binding domain, leucine-rich repeat (LRR)-containing (or NOD-like) receptors (NLRs), RIG-I-like receptors (RLRs), and the AIM2-like receptors (ALRs) and the Toll-like receptors (TLRs) [25].

Toll-like receptors (TLRs) are the main family of pattern recognition receptors (PRRs) of the innate immune system [24]. They are characterized by an extracellular leucine-rich repeat (LRRs) domain, which mediates recognition of Pathogen-associated molecular pattern molecules (PAMPs), a transmembrane domain along with its cytosolic intracellular Toll/IL-1R-like (TIR) domains required for downstream signalling pathways. TLRs were first identified in the fruit fly in 1988 [26] and then its homolog, called TLR4, was discovered in humans in 1997 [27]. Since then, this family of receptors have been found in all animals and even in plants,

illustrating the ancient origin of this gene family, with most mammalian species presenting 10 TLR genes (12 in mice) [28].

TLRs are expressed in most cell types, including airway epithelial and hematopoietic cells [24]. They are synthesized in the endoplasmic reticulum and transported to their ultimate destination in the cell, which are the plasma membrane or endosomal membranes [29]. Cell surface TLRs include TLR1, TLR2, TLR4, TLR5, TLR6, and TLR10, whereas intracellular TLRs are localized in the endosome and include TLR3, TLR7, TLR8, TLR9 [30]. Cell surface TLRs mainly recognize microbial membrane components such as lipids, lipoproteins, and proteins (e.g. TLR4 recognizes bacterial lipopolysaccharide (LPS), TLR2 along with TLR1 or TLR6 recognizes lipoproteins and TLR5 recognizes bacterial flagellin [31]). On the other hand, intracellular TLRs recognize nucleic acids from bacterial and viral origin, but also from the host damaged cells [32] (Figure 1). The importance of spatially restricting TLR recognition of nucleic acids to endosomes was revealed by studies that artificially positioned TLR9 at the cell surface [33]. Under these conditions, inflammatory responses are induced upon cellular exposure to extracellular DNA, which can lead to lethal autoinflammatory responses in mice [34]. Yet recognition of nucleic acid is also problematic because there is little to distinguish self-RNA and -DNA from nonself. One solution to this apparent conundrum is to recognize particular chemical motifs that are not present on self-nucleic acids. For example, TLR9 predominantly recognizes unmethylated CpG DNA motifs, which are uncommon in the mammalian genome [35]. Upon activation, TLRs anchor different adaptor molecules, the myeloid differentiation primary response 88 (MyD88) pathway and/or the TIR domain-containing adaptor inducing IFN-β (TRIF), depending on the TLR involved.

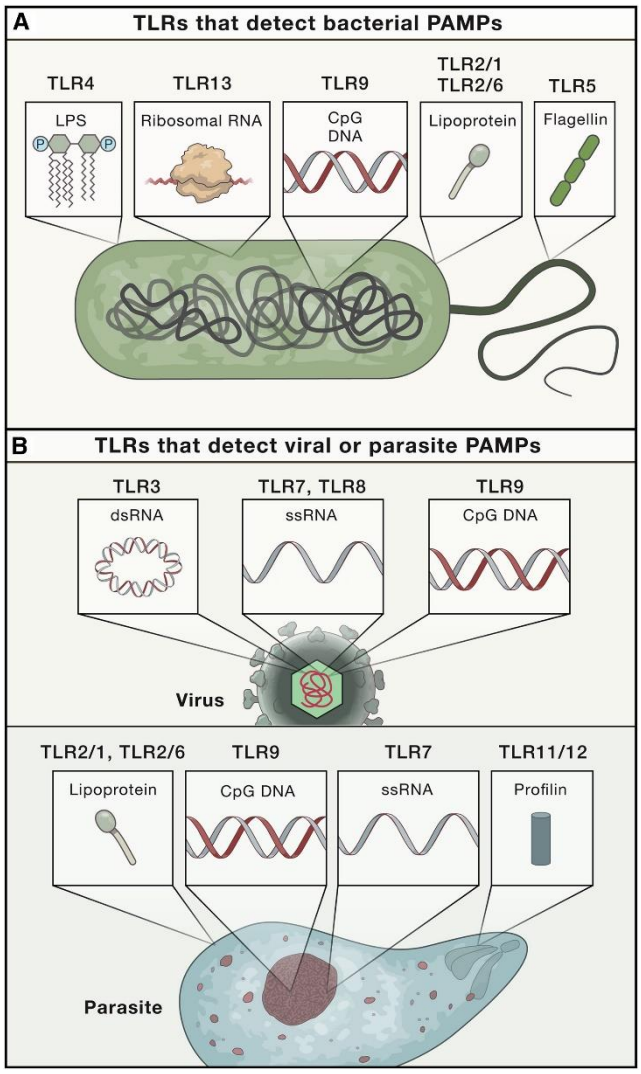


*Figure 1. Multiple TLR Family Members Can Detect PAM Ps on Individual Microorganisms. (adapted from Fitzgerald and Kagan [31])*

## Toll-like receptor signalling

Upon ligand recognition, a fundamental feature of the signalling process is the formation of TLR ectodomains dimers that result in the coordinate dimeration of the cytosolic TIR domains and the assembly of a large oligomeric scaffold of cytosolic proteins known as a supramolecular organizing center (SMOCs). The SMOCs that govern TLRs signalling are the Myddosome (based on the MyD88 protein) and the putative triffosome (based on the TRIF protein) [31]. The signalling cascade triggered will lead to transcriptional regulation of distinct genes, depending of the TLR and cell types involved. With the exception of TLR3, all TLRs use MyD88 for signal transduction. MyD88 recruits the IL-1 receptor associated kinases IRAK1 and IRAK4. The tight packing of the IRAKs within the myddosome activates their latent kinase activity, driving autophosphorylation and the subsequent recruitment of the E3 ubiquitin ligase TRAF6 [36].

TRAF6 activation is essential in the activation of mitogen-activated protein kinases (MAPK) and the nuclear factor κB transcription factor (NF-κB). NF-κB critically regulates innate

immunity and inflammation and has a key role in cell division and cell death. This transcription factor family is formed by 5 members, RelA, RelB, c-Rel, p50/p105 (NF-κB1), and p52/p100 (NF-κB2). In the absence of stimulus NF-κB proteins are bound to IκBα and IκBε in the cytoplasm, preventing its translocation to the nucleus. Upon stimulation, this inhibitory proteins will be phosphorylated and degraded, allowing the NF-κB dimers to move into the nucleus and bind to the DNA, which will trigger the expression of genes involved in a great array of inflammatory processes [37] (Figure 2).

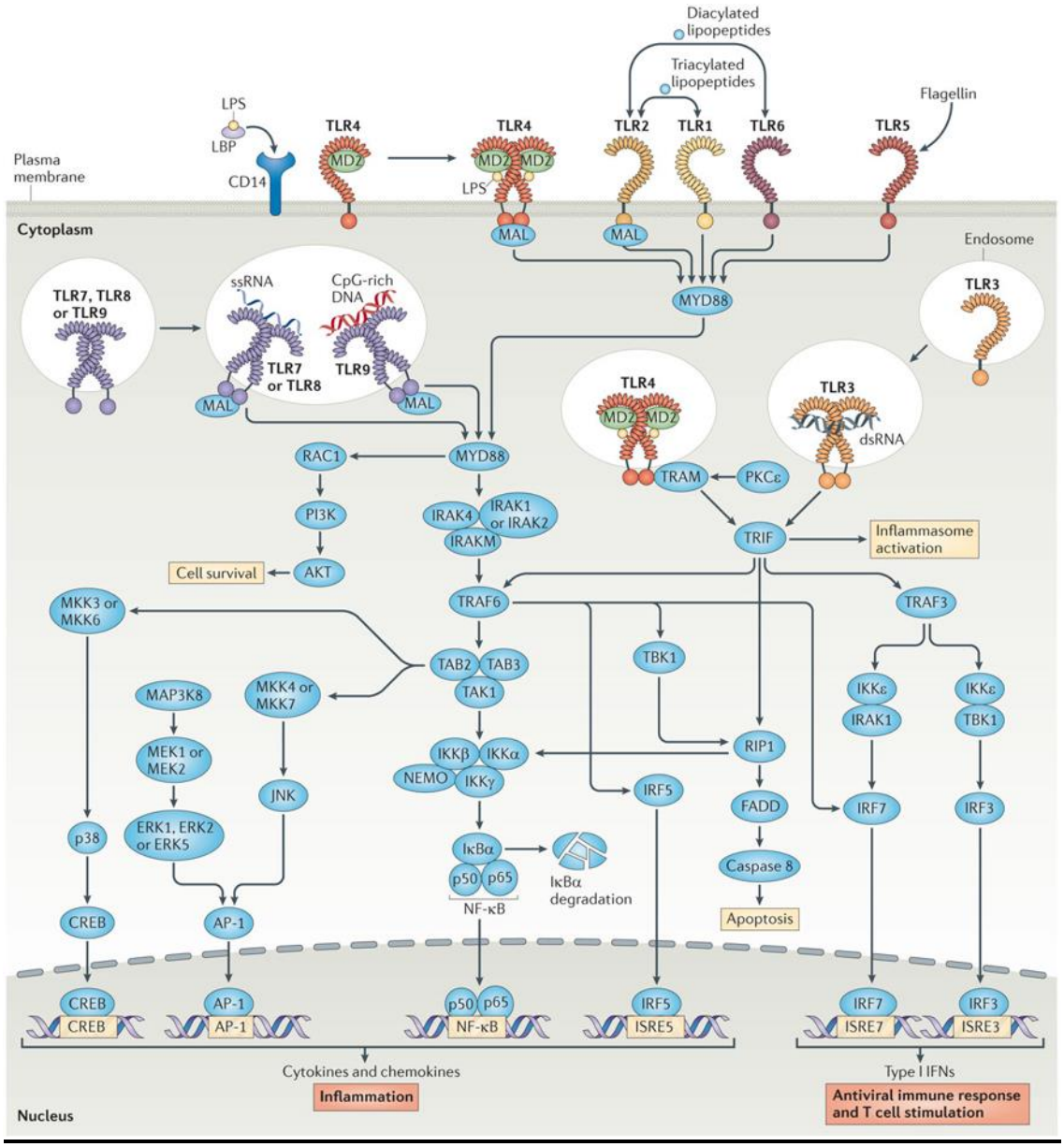


*Figure 2 Schematic representation of TLR signaling (adapted from Gay et al. [38])*

The means by which TRAF6 coordynates myddosome activities, activation of MAPK and/or NF-κB pathways and regulates transcriptional activity are still unclear.

On the other hand, studies using MyD88 deficient cell lines have shown that, in addition to TLR3, TLR4 can also use TRIF signalling. Interestingly, unlike the MyD88 pathway, downstream of TLR4 and TLR3, MyD88-independent cellular responses have been shown to upregulate the expression of genes encoding interferons (IFNs) and IFN-stimulated genes (ISGs).

Molecular mechanisms modulating cell response upon an inflammatory stimulus

During inflammation, TLRs from epithelial and immune cells will encounter a myriad of signals from bacterial and endogenous origin. Although TLRs share the main signaling hubs (NF-κB, MAPK), different TLRs will transform the outside information into different cell responses. Understanding how cells can efficiently process this information in a rapidly changing and noisy environment is a fundamental question in TLR signalling. A primary mechanism to control the cell response would be the location of the SMOC. A clear example of this is TLR4 signalling. TLR4 is unique in that it utilizes both MyD88 and TRIF adaptors. However, TLR4-induced MyD88-dependent signalling seems to originate from the cell surface, whereas TRF-signalling comes from the endosomes [25]. Other mechanisms that serve to drive a precise and controlled immune response involve differential activation of NF-κB and MAPK signalling, as well as the dynamics of their activation. It is noteworthy that not all the cells in a population respond to an inflammatory stimulus in the same manner, with some cells that do not respond at all and variations in timing, intensity and type of response in those that react to the stimulus.

Cells must discriminate between “dangerous” and “non-dangerous” signals and take the decision to whether activate or not the inflammatory response and the nature of this response. Different mechanisms regulate these processes and cells can be activated in a digital (on/off) or analogous (gradual) manner. The MAPK pathway consists of several levels (usually three), where the activated kinase at each level phosphorylates the kinase at the next level down the cascade. MAPKs are the kinases of the terminal level of the cascades. They are activated by the MAPK kinases (MKKs) that phosphorylate MAPK at two sites (Figure 3).

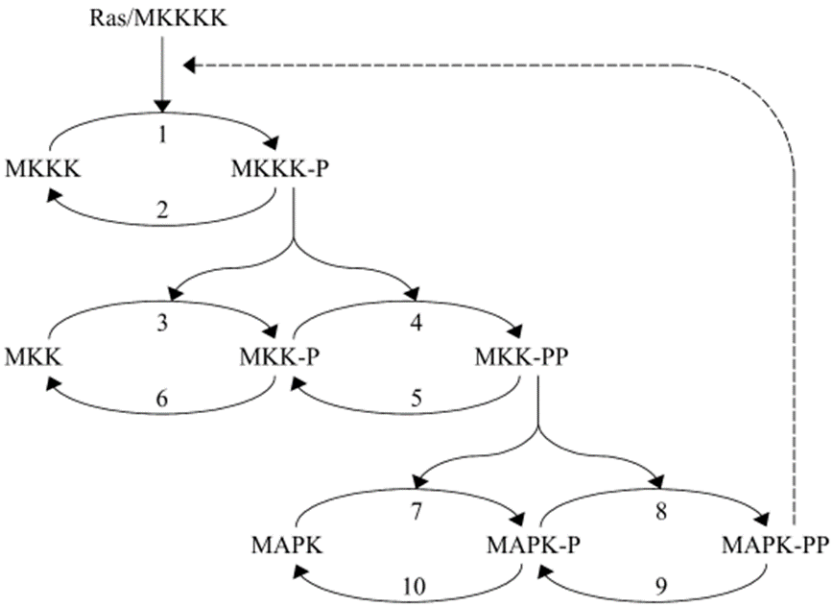


*Figure 3. Kinetic scheme of the MAPK cascade adapted from Kholodenko [39].*

Mathematical modelling have shown that the three layers architecture of the MAPK pathway may serve as an amplification signal and lead to a remarkable increase in sensitivity. This would mean that a graded stimulus would be converted into a sigmoidal 'ultrasensitive' switch-like response with a negative feedback that will produce oscillations in the system [39]. In this regard, studies on macrophages at the population level have shown different activation thresholds on MAPK and NF-κB pathway upon LPS stimulation. Above a threshold of ligand, MAPK is activated in an ultrasensitive switch-like manner, facilitating production of inflammatory mediators. At ligand concentrations below this threshold, NF-κB signalling occurs, promoting expression of a restricted set of genes and macrophage priming. This system is used to filter out "noisy" signals that should not trigger a potent response and would allow a dual control of the inflammatory response, yielding sequential barriers to inflammation that could serve as a "danger discrimination" system [40].

Further insights into the mechanisms that drive the inflammatory response have been obtained with the implementation of single cell analysis and mathematical modelling. They have become an important methodology to understand cell heterogeneity, adding a new level of complexity to the study of the immune response. Single cell confocal live imaging have been used to track transcription factor oscillations (mostly P65, a subunit of the NF-κB complex). It is known that the "intensity" and nature of NF-κB signalling is under different layers of regulation that involve the expression of positive and negative feedback loops. Upon stimulation by an inflammatory signal, IκB proteins will be phosphorylated and degraded, allowing NF-κB translocation into the nucleus to activate the expression of several genes. These includes the expression of IκB inhibitors, which will work as a first negative feedback by binding to NF-κB in the nucleus and relocating it to the cytoplasm. A second negative feedback loop involves the expression the A20 protein, which inhibits IKKs upstream of the signalling pathway [37] (Figure 4A). Different timescales in the expression of these feedback loops serve to fine-tune the system [41]. Therefore, constant stimulation will lead to dampened oscillations in the nuclear concentration of NF-κB at the single cell level with heterogeneous dynamics according to each cell susceptibility [37]. Due to such cell asynchrony, oscillatory response at the population level would appear as almost non-oscillating (Figure 4B).

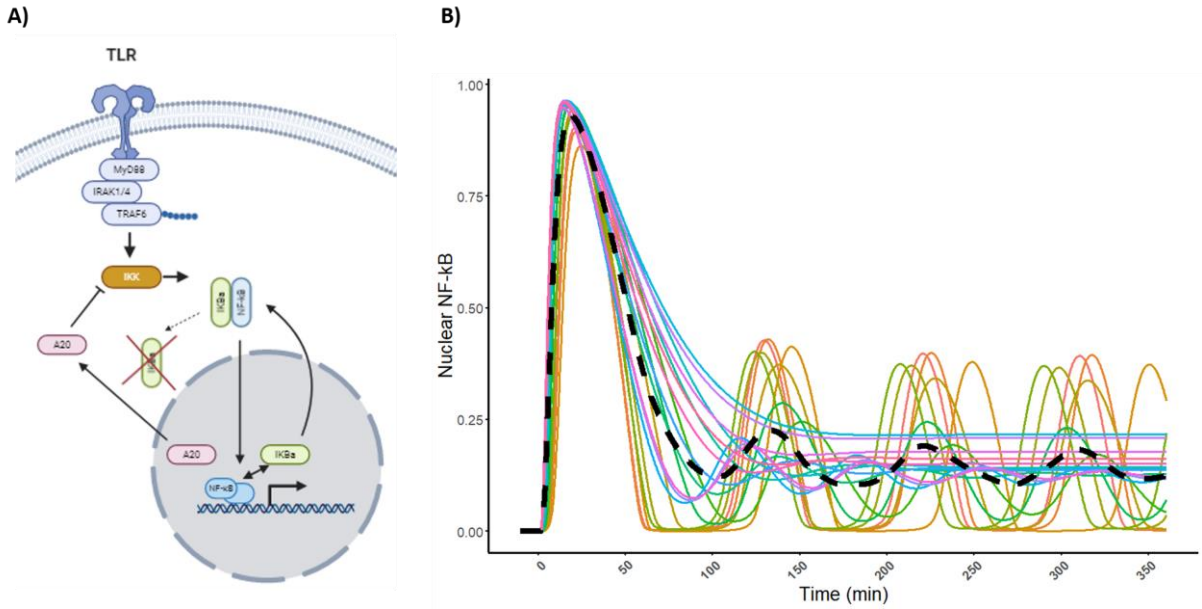


*Figure 4. (A) Schematic representation of NF-κB signalling and negative feedback loops. (B) Mathematical simulations showing oscillatory behavior NF-κB signalling in single cells after a continuous 6h LPS stimulation (200 ng/ml). Each color represent a single cell (n=20) were the amounts of TLR4, TRAF6 and NF-κB have been randomized to simulate extrinsic noise. The dashed black line represents the average nuclear NF-κB for all cells. Simulations are based on [42].*

The oscillatory pattern of the NF-κB transcription factor plays a main role in the determination of the cell response to an inflammatory stimulus. Single cell experiments have shown that modulation of these oscillations using repeated short pulses of the input signal at different intervals could synchronize NF-κB translocation cycles into the nucleus and give repeated full amplitud translocations. Alterations of NF-κB oscillations will give result to different patterns of gene expression [37, 43]. These studies support the idea that transcription factor oscillation frequency has an important functional role in the cell response to an inflammatory cue. There are different mechanisms through which the cell can modulate NF-κB signalling. Single cell live imaging of 3T3 fibroblasts stimulated with LPS and TNFα showed that in a population of cells, the fraction that will activate NF-κB signalling depends on the integration of the stimulus dose and duration of that stimulus (dose concentration x signal duration=area). This "area" of the input stimulus will be important not only to determine the fraction of activated cells but it will have an impact in NF-κB oscillatory patterns in individual cells [42]. Another important aspect to take into account is to understand how cells can process different types of inflammatory inputs. Cells receive a multitude of signals from the environment, and during infection, several PAMPs will be present at the same time, resulting in the potential stimulation of different TLRs. Kellogg et al. [44] showed that activation of different TLRs lead to different NF-κB dynamics. More interesting, when cells where under co-stimulation with both TLR2 and TLR4 ligands, cells did not integrate this information into a "mixed" signalling pattern. They would rather continue to show ligand-specific dynamics (either TLR2 or TLR4) and the percentage of cells in the population displaying any of the two profiles would depend on the concentration of each stimulus. This heterogeneous response at the single cell level could play an important collaborative role in the innate immune response by synergistically activating the production of cytokines at the tissue level.

Cross-talk between signalling pathways play also an important role in the modulation of the innate immune response. New evidences point towards calcium ($Ca^{2+}$) signalling as an intriguing new mechanism for the regulation of TLR response. Extracellular $Ca^{2+}$may elicit TLR3, TLR4 and TLR9 feedforward regulation and control the intensity of the response

through regulation of MAPK signalling [45]. Moreover, solid evidence emphasizes the role of mitochondria $Ca^{2+}$ homeostasis as key decoding stations of cellular signals. In this model, mitochondrial $Ca^{2+}$ seems to play a role in cell response to *Pseudomonas aeruginosa* infections in cystic fibrosis [46]. It has also been described as a potential modulator of the immune response by activating a TLR9 alternative pathway with anti-inflammatory activity [47].

**The use of flagellin to boost innate immunity**

Several studies are targeting TLRs as a promising prophylactic and/or therapeutic strategy to combat infectious diseases since modulation of their function and responsiveness to pathogens could improve innate immunity.[48, 49]. Stimulation of innate immunity through TLR activation can mobilize multiple host defence mechanisms that accelerate clearance of microorganisms. A protective effect against bacterial and viral infections has been observed when using specific agonists targeting TLRs [48].

Flagellin is the structural protein of the bacterial flagellum. Its amino acid composition ranges between 250 and 1250 residues, including a conserved region flanking a central hypervariable region (Figure 5). Flagellin from *Salmonella* Typhimurium (FLiC) is 495 amino acids long and composed of 4 domains (D0, D1, D2, D3). Mutagenesis studies showed that flagellin D1 domain, especially the conserved residues 89-96 (QRIRELAV), as well as the D0 domain are essential for TLR5 recognition [50]. Flagellin-based interventions have demonstrated protective activity in many infectious diseases, from viral and bacterial origin. Mucosal or systemic administrations of flagellin had a protective effect against Gram-negative bacteria (*Salmonella sp., Pseudomonas aeruginosa, Burkholderia cepacia, Yersinia pseudotuberculosis*) in mice. Flagellin has also demonstrated its protective and adjuvant effect in the context of influenza and post-influenza pneumococcal infection [49]. This protection seems to be dependent on the secretion of pro-inflammatory cytokines, which are essential for the recruitment of immune cells, the secretion of antimicrobial molecules and mucins, and maturation of the dendritic cell (DC)/ type 3 innate lymphoid cells [49]. The nature of signals that contribute to DC maturation remains to be defined. Interestingly, protection does not seem to be limited to prophylaxis since the combination of flagellin with antibiotics boosts antibiotic therapeutic efficiency during acute respiratory infections [51].

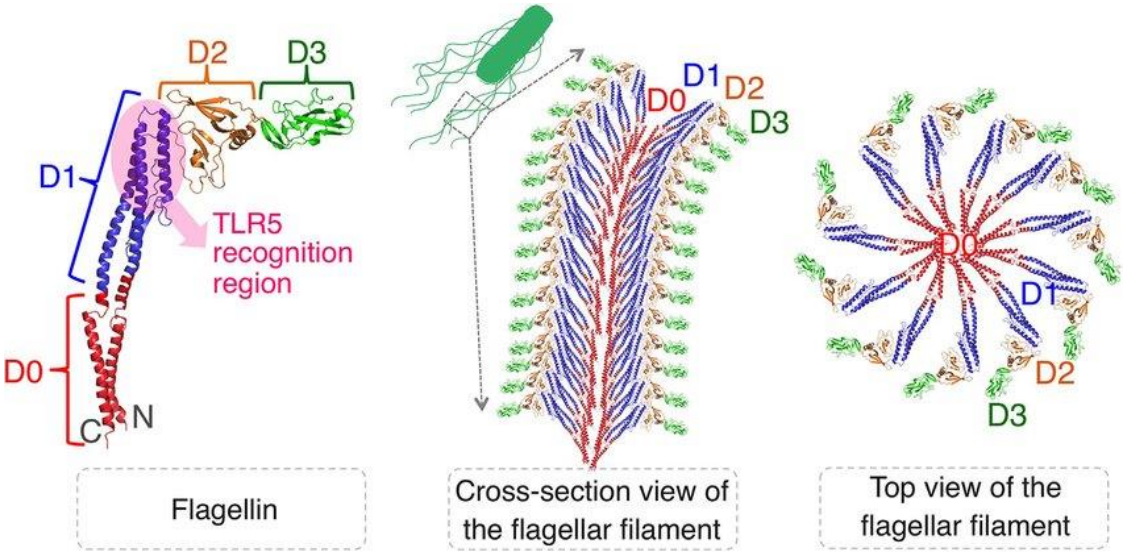


*Figure 5. The structure of flagellin and the cross-sectional and top views of the flagellar filament. Adapted from [52]*

There could be some risks associated to this type of strategies, since they may lead to exacerbated inflammation, tissue destruction, and development of autoimmunity, as it has been observed when employing agonists for TLR3 and TLR9 [48]. However, this does not seem to be the case for flagellin and TLR5-mediated immune stimulation. On the contrary, flagellin seems to improve the general health status, promoting a more rapid and efficient resolution of inflammation, leading to full restoration of lung architecture in experimental models of acute lung infection [53]. The mechanisms underlying the observed improvements of flagellin-based treatments, whether enhanced bacterial clearance, induction of Th2 responses or switching on resolution of the inflammation and tissue repair processes, are not completely understood, especially in livestock. Further studies, both *in vitro* and *in vivo* are needed, to shed light on the processes triggered by flagellin responsible for its observed protective effect.

# SECTION 3.2 SCIENTIFIC ACTIVITIES

## Development of an experimental model of *Pseudomonas aeruginosa* infection in the pig

*Pseudomonas aeruginosa (P. aeruginosa),* a bacterium that rarely infects human lungs unless the host immune system has been impaired [54], is one of the main pathogens found in CF, COPD and VAP [55-57]. A hallmark of *P. aeruginosa* infection in CF is a massive recruitment of neutrophils to the lungs [58-60]. During this process neutrophils secrete large amounts of neutrophil serine proteases (NSPs: neutrophil elastase (NE), protease 3 (Pr3) and cathepsin G (cat G)) that overwhelm the capacity of endogenous antiproteases to control their activity, ultimately leading to the destruction of lung tissue [61-63]. A wide array of transgenic mice models has been developed to study chronic lung inflammatory diseases, such as CF [64]. Mice models are also used to evaluate the role of neutrophils in the progress of acute and chronic neutrophil-associated lung diseases that frequently involve *P. aeruginosa* infection [65-69]. Despite the important advances made with these models, several research groups have acknowledged that they have inherent limitations [18-21]. There are important differences in the lifespans of mice and humans as well as in their airway architecture that make it impossible to study the chronicity of lung diseases. Thus, rodent models cannot reproduce the complex features of human diseases like CF [22, 70, 71]. Differences in the substrate specificities of mouse and human NSPs [72] also seriously complicate testing and validating anti-inflammatory therapies that target NSPs. Thus, anti-infectious and/or anti-inflammatory treatments must be tested on a suitable animal model.

Porcine lungs share many anatomical, histological, biochemical, and physiological features with those of humans [73]. Porcine and human neutrophils behave similarly *in vitro,* both releasing NSPs and neutrophil extracellular traps (NETs) in response to *P. aeruginosa* infection [74]. Pig NSPs also have the same substrate specificities and similar immunochemical properties as their human homologues. Thus, they can be inhibited by the human natural inhibitors α1-proteinase inhibitor (α1-Pi) and α1-antichymotrypsin (ACT), which makes the pig a relevant model for developing drugs that target human NSPs. Therefore, we aimed to develop an experimental model of *P. aeruginosa* lung infection and acute inflammation in wild-type (WT) pigs in which to evaluate the neutrophilic response to infection. For that, pigs were infected with *P. aeruginosa* and neutrophil recruitment to the lungs, the production of proinflammatory mediators and the proteolytic activities of secreted NSPs and neutrophil extracellular trap formation were evaluated.

Our data showed that inoculating the lungs of wild-type pigs with *P. aeruginosa* induces an acute predominantly local inflammatory response, similar to what is observed in humans. This response is characterised by a transient increase in the bronchoalveolar lavage (BAL) pro-inflammatory cytokines (IL-8, IL-6 and TNF-α) with a peak at 6h post-infection that returned to normal at 24h (Figure 6A-C).

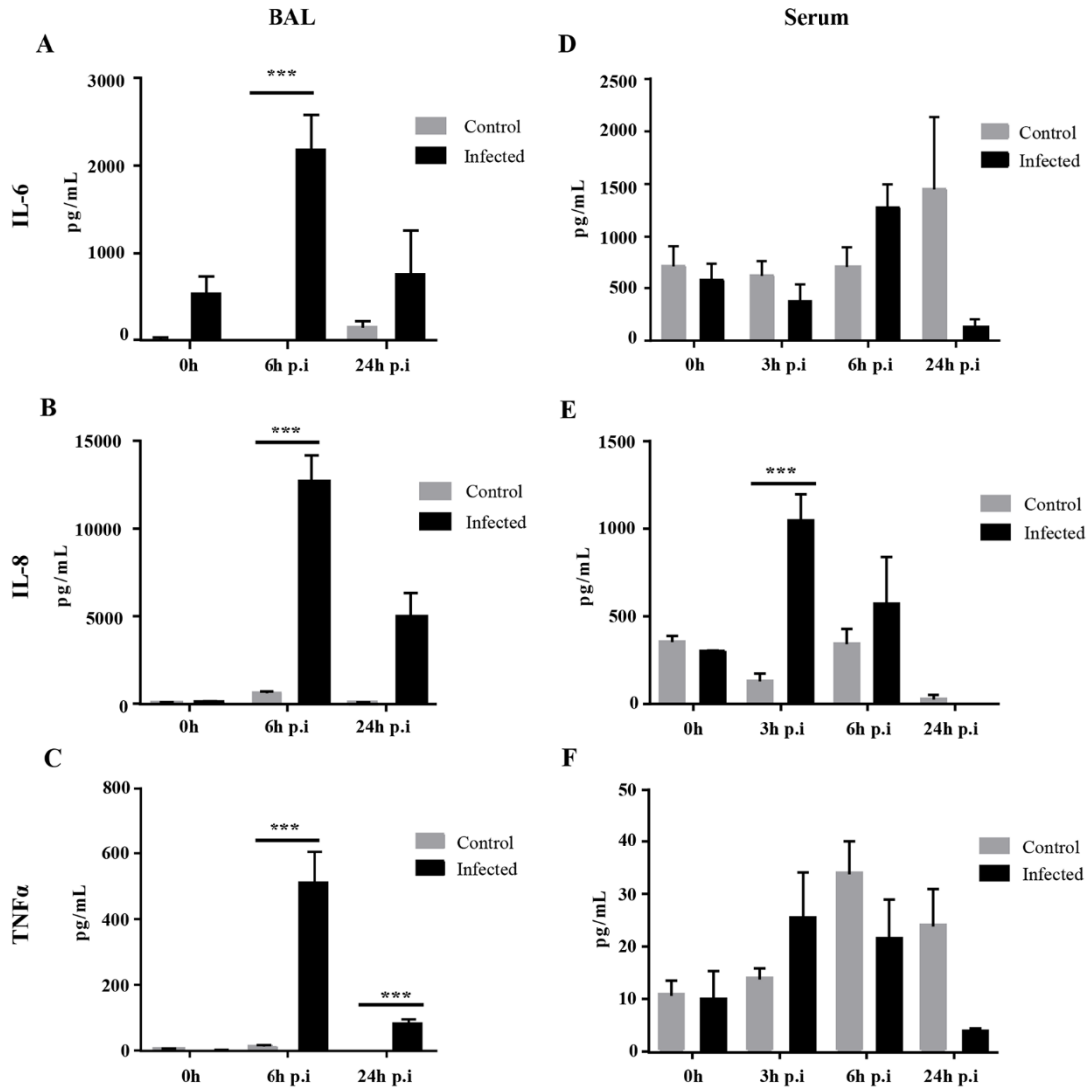


*Figure 6. Cytokine concentrations in the bronchoalveolar lavage (BAL) and serum of pigs after P. aeruginosa infection. A. IL-6, IL-8 and TNF-α in BAL fluid. B. IL-6, IL-8 and TNF-α in serum. Data were analysed by two-way ANOVA followed by Bonferroni's post hoc test. Data are means ± S.E.M. * indicates p<0.05. *** indicates p<0.001.*

These pro-inflammatory molecules play an important role in the response to a *P. aeruginosa* infection by regulating neutrophil trafficking from the blood to the inflamed tissues [75-77]. The numbers of neutrophils in the differential WBC increased transiently (from 20% at 0 h to 60% at 6 h) and there was a massive influx of neutrophils into the lungs (Figure 7A-D), despite the fact that the blood of pigs contains a smaller fraction of neutrophils (20-45%) than does human blood (40-80%). This event was associated with transient increases in all the neutrophil serine proteases and the secretion of NETs, even though they were barely detectable using BAL fluids, but quite readily detected in purified activated pig neutrophils (Figure 8) [74], probably for mechanical reasons. Such an intense neutrophilic response is a hallmark of *Pseudomonas* lung infections in both CF and VAP [78, 79]. The acute immune response led to an important decrease in the lungs bacterial load at 24 h p.i. (Figure 9). In contrast, most of the biochemical parameters analysed as well as the inflammatory cytokines and bacteraemia remained low. These findings indicate the importance of a local immune response in the lungs rather than a systemic one, much like the data reported for the porcine models of VAP infected with *P. aeruginosa* [80-82]. Previous reports found that the inflammatory response was sustained for

up to 96 h [80, 81], but we observed a very acute inflammatory response in the lungs that peaked 6 h after infection and decreased by 24 h.

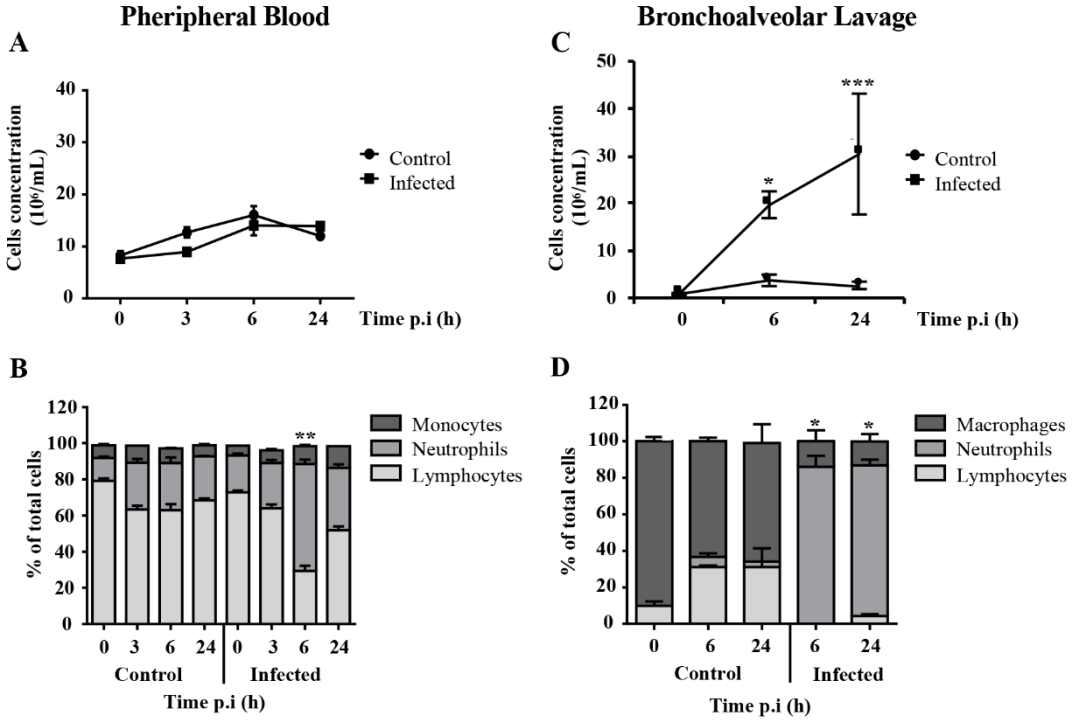


*Figure 7. Total white blood cell counts and cell profiles in the peripheral blood and BAL fluid of pigs at different times after P. aeruginosa infection. A. Total white blood cells (WBC) in peripheral blood. B. Profile of the WBC populations in peripheral blood. C. Total WBC in BAL fluid. D. Profile of the WBC populations in BAL fluid. Total WBC and cell profiles of the control and infected groups were compared using the Mann-Whitney U test for each time point. Data are means ± S.E.M. * indicates p<0.05. ** indicates p<0.01. *** indicates p<0.001.*

The efficacy of drugs targeting NSPs has presently been tested only in mice. Their results are difficult to interpret due to differences in the physicochemical properties and substrate specificities of mouse enzymes and those of human neutrophils [83, 84]. We have recently shown that human and porcine blood neutrophils and their proteases behave very similarly *in vitro* [74]. However, the neutrophil phenotype and responsiveness change once they are activated during migration from the peripheral blood to the airways [76, 85, 86] and lung secretions may alter the function and proteolytic potential of NSPs due to the presence of inhibitors. We have now shown that the *in vivo* physicochemical properties and substrate specificities of pig lung NSPs are similar to those of humans. The experiments with the anti-peptide antibodies raised against human proteases confirmed the presence of all three NSPs at the surface of neutrophils and on the NETs of pigs. Immunoblotting analysis using anti-human protease antibodies indicated that the endogenous NSP inhibitors were overwhelmed: we detected both free proteases and complexed forms, as in human BAL fluids (Figure 10) [87]. These data are especially important in animal models of lung diseases where neutrophils play a prominent role. For example, the pig model of CF develops lung abnormalities similar to those observed in humans, unlike the mouse model [20]. Hence, therapeutic inhibitors that target the active sites of human NSPs can be tested in the pig.

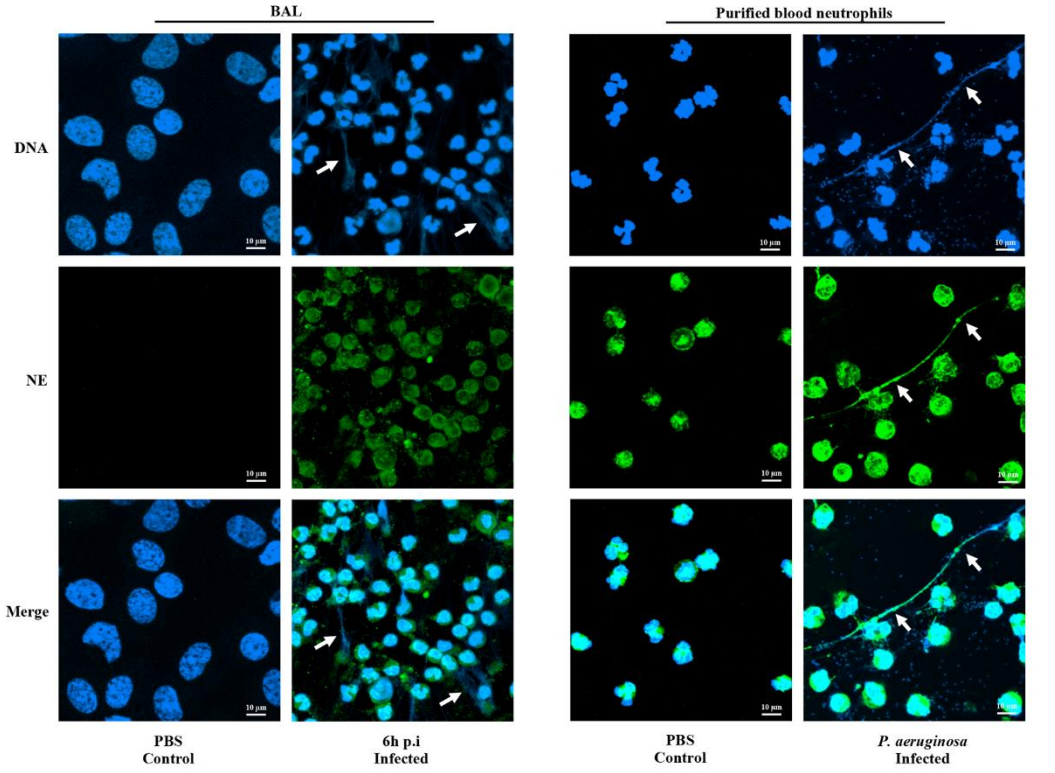


*Figure 8. Neutrophil serine proteases in BAL fluids of pigs infected with P. aeruginosa and purified blood neutrophils. Confocal microscopy showing DNA (blue) and elastase (NE; green). Arrows show NET filaments.*

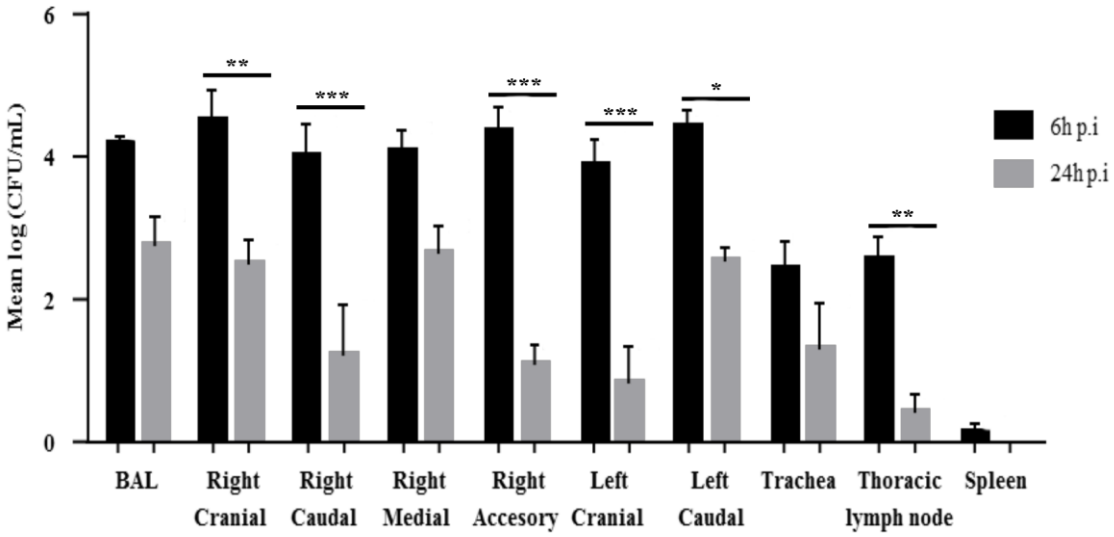


*Figure 9. Bacterial loads in the BAL fluid, lung lobes, trachea, thoracic lymph nodes and spleen of pigs infected with P. aeruginosa. Pigs were inoculated with 70 mL of an 8 x $10^6$ cfu/mL suspension of P. aeruginosa PAK strain. Controls were obtained by inoculating 70 mL of 1X sterile PBS. Data were analysed by two-way ANOVA followed by Bonferroni's post hoc test. Data are means ± S.E.M. * indicates p<0.05. ** indicates p<0.01. *** indicates p<0.001.*

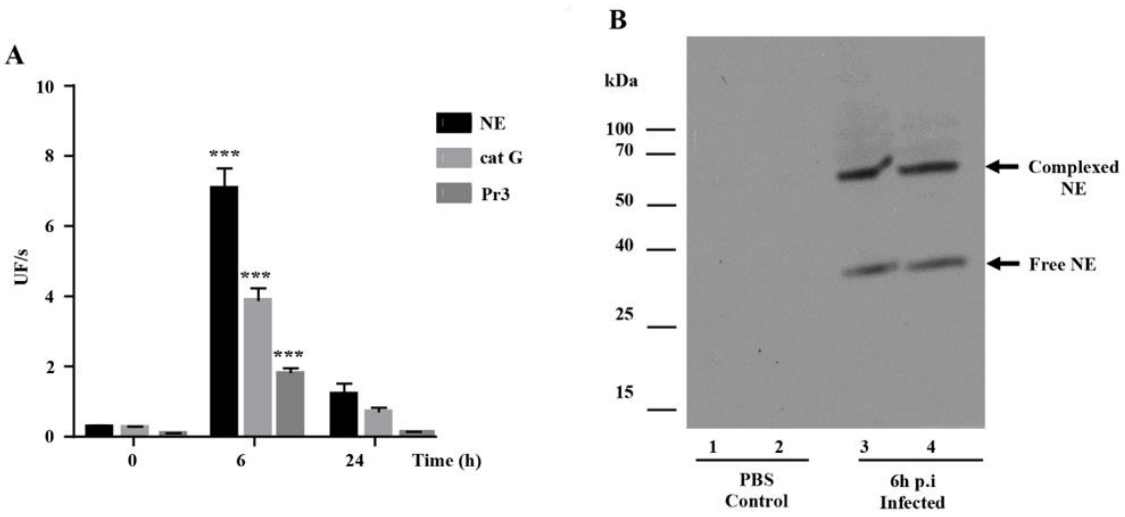


*Figure 10. Peptidase activity of neutrophil serine proteases from the BAL fluid of pigs infected with P. aeruginosa. A. Peptidase activities of NE, Pr3 and cat G (means ± SEM; n=6).*

We conclude that inoculating pigs with *P. aeruginosa* produces a neutrophilic response similar to that seen in humans. The resemblance between the functions of pig and human neutrophils in their response to inhibitors, and the *in vivo* data presented here indicate that pigs are suitable candidates to model neutrophil-dependent lung inflammatory diseases such as CF. Further studies will be performed in the CF pig model to determine if there are any intrinsic differences in the innate immune response to *P. aeruginosa.*

**Development of a pig model of cystic fibrosis**

In order to understand the pathogenesis of CF, we established a pig model of CF in collaboration with the University of Munich (LMU). *CFTR*$^{+/-}$ pigs were produced by replacing the exon 1 of the *CFTR* gene by a STOP box and a neo cassette using homologous recombination by BAC vectors [88]. Single male and female *CFTR*$^{+/-}$ transgenic pigs were moved to INRA, Nouzilly (France) and mated to generate *CFTR*$^{+/+}$, *CFTR*$^{+/-}$ and *CFTR*$^{-/-}$ piglets. Genotype was confirmed by multiplex PCR. The CF pig model is hampered by a major drawback, which is the high occurrence of a severe intestinal obstruction by MI, which leads to early death [17]. Histopathological analysis of the ileum and colon of newborn *CFTR*$^{-/-}$ piglets showed a pathological phenotype in agreement with previous studies [18, 89]. Briefly, *CFTR*$^{-/-}$ piglets showed an atrophy of the intestinal mucosa with a hypertrophy of the mucus cells and accumulation of mucus in the lumen compared to WT animals. The muscular wall of the ileum and colon was thickened and more fragile in the *CFTR*$^{-/-}$ piglets with the presence of diverticulosis. The pathogenesis of diverticulosis is likely the result of pressure increase by the meconium that leads to herniation of the mucosa [89] (Figure 11). This situation could be exacerbated in the first hours after birth due to the food ingestion by the piglets leading to intestine perforation and peritonitis. Performing an ileostomy in these piglets is thus an emergency to decrease the occurrence of fatal intestine perforation.

We attempted to reduce the birth-to-surgery time of the newborn *CFTR*$^{-/-}$ piglets by using CT scan imaging as diagnostic tool. CT scan imaging is a very sensitive and specific tool to detect the presence of MI in the intestine. When the results obtained by CT scan were compared to those obtained by PCR genotyping, we observed that 84 piglets were accurately diagnosed as CF and non-CF piglets, with only 1 false negative and 8 false positive results out of 93 piglets analysed. Indeed, the prediction of CT scan to diagnose *CFTR*$^{-/-}$ newborn piglets had a sensitivity of 94 (73 – 99) %, a specificity of 89 (80 - 95) %, a positive predictive value of 68 (47 - 85) % and a negative predictive value of 99 (92 - 100) % (Table 1). CT scan images of MI showed a very distinct dense and homogenous mass that filled enlarged bowel loops in accordance with the descriptions found in the literature [90, 91] (Figure 12). This tool allowed us to quickly sort those pigs carrying the homozygous mutation and perform a surgical correction in the intestinal obstruction. During our study, we have managed to keep alive newborn piglets diagnosed by CT scan for 13 days, an improvement when compared to a maximum of 2.5 days before early diagnosis of the CF piglets (Figure 13). Our results suggest that a better survival rate may be obtained in those piglets that were quickly diagnosed and subjected to an ileostomy. Despite early treatment of the *CFTR*$^{-/-}$ piglets, the survival rate was less than that obtained by other laboratories using piglets with different genetic background. Previous studies have reported a survival of 2 months or longer in piglets that underwent ileostomy [22]. These differences in the survival rate and the intestinal disease severity may be related to the genetic background of the piglets. In fact, different genetic predisposition to diverticulosis has been proposed for different pig breeds [92].

**Table 1. CT scan imaging sensitivity, specificity, positive predictive value (PPV) and negative predictive value (NPV) to diagnose CFTR$^{-/-}$ newborn piglets.**

| | Number of animals | Number of *CFTR*$^{-/-}$ | Sensitivity | Specificity | PPV | NPV |
|---|---|---|---|---|---|---|
| **CT scan** | 93 | 18 | 94.4% (72.63-99.07) | 89.33% (80.05-95.27) | 68% (46.50-85.01) | 98.53% (92.05-99.75) |

95% confidence interval is indicated between brackets.

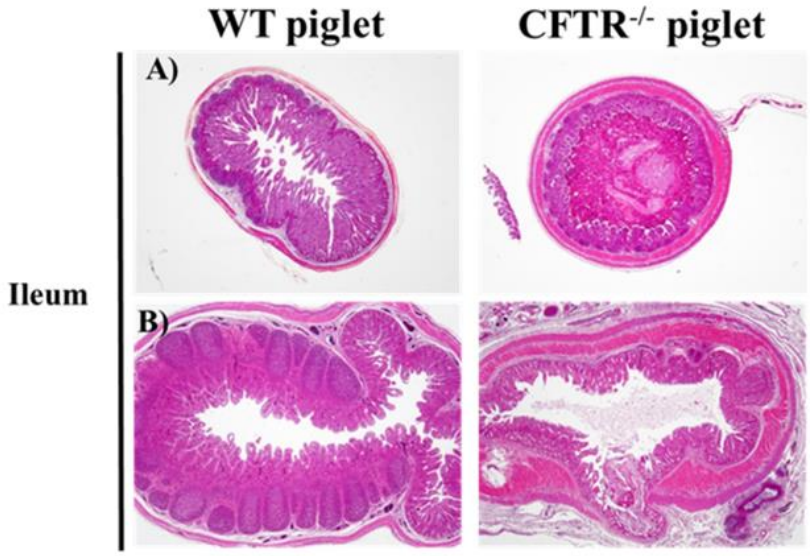


*Figure 11. H&E staining of WT and CFTR$^{-/-}$ ileum and colon. Intestinal phenotype of newborn and 13 days old CFTR$^{-/-}$ piglets. H&E staining of WT and CFTR$^{-/-}$ ileum at day 1 [A (x 20)] and day 13 [B (x 20)] after birth. CFTR$^{-/-}$ piglets showed a hypertrophy of mucus cells with mucus accumulation, thickening of the muscular wall and presence of diverticuli. A significant atrophy of the lymphoid follicles in the mucosal lamina propria of the ileum can be observed at day 13 after birth.*

It is important to highlight the importance of animal models with different genetic backgrounds in order to better understand CF pathogenesis. In this regard, CF is a complex disease characterized by substantial clinical heterogeneity [93]. This variability may be related with the expression of different "modifier" genes expressed by different genetic backgrounds, which can affect the severity of the observed phenotype [64]. These differences between genetic backgrounds should be regarded as an advantage of the model, since they may provide the basis for later studies on "modifier" genes that may play a role in the severity of the disease.

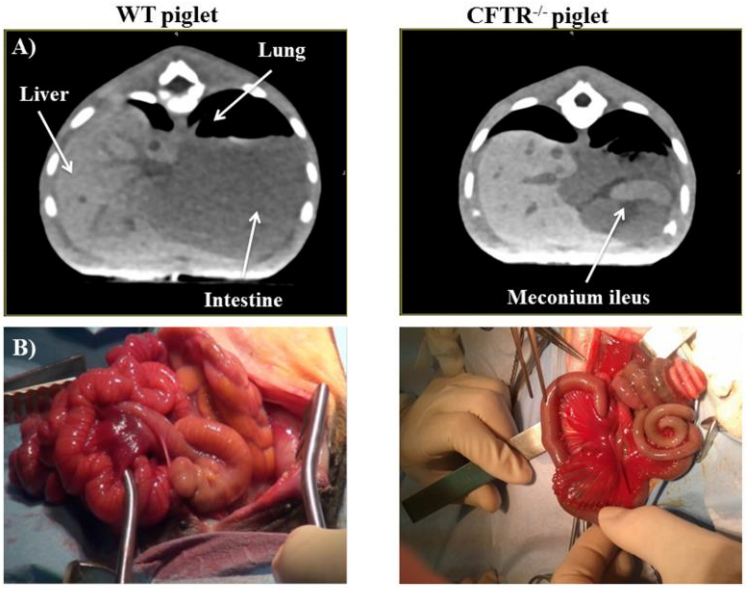


*Figure 12. Meconium ileus in the CFTR$^{-/-}$ newborn piglet. A) CT scan images of WT and CFTR$^{-/-}$ abdomen. The presence of MI is observed in CFTR$^{-/-}$ as a homogenous dense material filling the intestinal loops of the piglets. B) Macroscopic image of WT and CFTR$^{-/-}$ intestines. The presence of MI and microcolon can be observed in the CFTR$^{-/-}$ piglet.*

In summary, the CF pig model produced in our laboratory develops a very severe form of the intestinal phenotype. Despite the advances using the CT scan for early diagnosis and care of CF pigs, the life expectancy remains too low to allow the development of chronic studies of life infection and inflammation. However, this model represents a highly valuable tool to study the early pathogenesis of CF, including alterations in the lung environment that may favor bacterial colonization, and intrinsic defects in the innate immune system, including TLR response and neutrophil alterations.

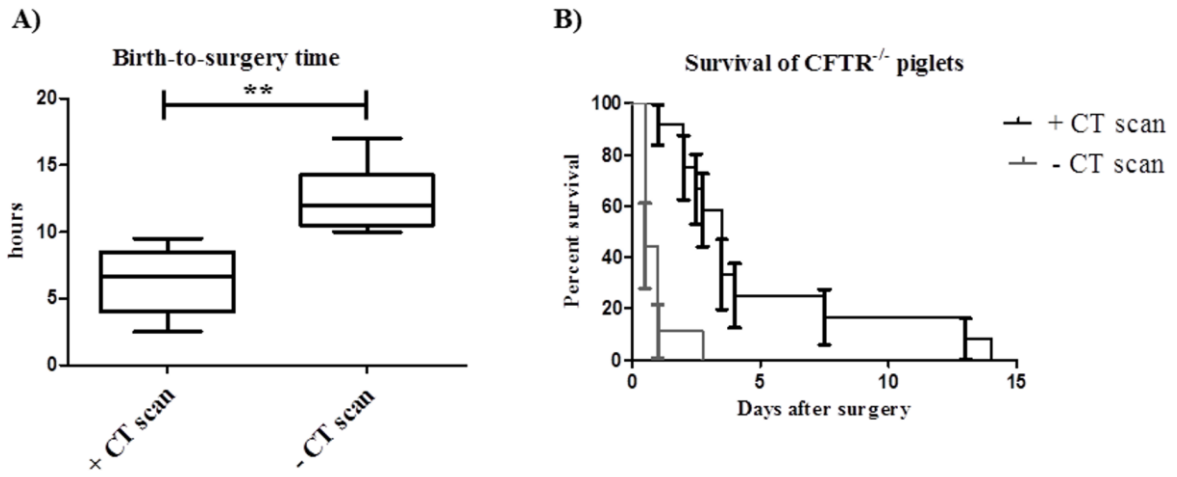


*Figure 13. The use of CT scan decreases the birth-to-surgery time and increases the survival rate of CFTR$^{-/-}$ piglets. A) Box and whiskers plot of the birth-to-surgery time of CFTR$^{-/-}$ piglets diagnosed or not by CT scan (** indicates p<0.001). B) Survival curve of CFTR$^{-/-}$ piglets diagnosed or not by CT scan.*

**Evaluation of intrinsic alterations in the innate immune response of the cystic fibrosis pig**

The difficulties encountered to maintain the CF pig in good health for a long period of time made impossible developing studies of chronic infection with *P. aeruginosa* in these animals. Despite this important drawback, the CF pigs represent an important tool to understand the origins of the CF lung disease. Understanding the initial host defense defects in cystic fibrosis airways could suggest novel preventions and treatments and the means to assess disease status and efficacy of therapeutics. In order to determine whether there are important alterations in CF innate immune response to pathogens, we focused our studies in: 1) peripheral neutrophils at birth; 2) alterations of the lung environment at birth and its response to a pathogenic challenge.

Intrinsic alterations in peripheral neutrophils from cystic fibrosis newborn piglets

A hallmark of the CF lung disease is a neutrophil dominated lung environment. The paradox is that despite the large number of neutrophils presents in the lungs, they fail to kill colonizing bacteria. At the same time, neutrophils are central orchestrators of the inflammatory process as a major source of regulatory lipid mediators, playing an important role in the resolution of the inflammation [94]. Whether neutrophil defective response is due to an innate effect of CFTR alteration or to the lung inflammatory environment is unclear. For instance, chronic infection and inflammation in the lungs of CF patients could lead to altered neutrophil maturation and function [95-98]. This is supported by the fact that chronic bacterial colonization in CF is mostly restricted to the lungs. On the other hand, the presence of CFTR in neutrophils and the phagolysosome suggests a direct role in several neutrophil functions, including degranulation bacteria phagocytosis and killing [99, 100]. Studies aiming to clarify the role of CFTR in neutrophils are hampered by the inherent variability in the degree of inflammation when sampling human patients, due to age and course of disease. The use of the CF pig model can tackle these issues. Porcine and human neutrophils present also similar biochemical properties, and the pig is a suitable model for testing new therapies targeting neutrophil inflammation [74, 101]. In addition, no signs of lung or systemic inflammation are observed at birth in the CF pig model that could mask or confound the innate differences between CF and non-CF neutrophils.

Thus, we aimed to search for potential differences in bioactive inflammatory and pro-resolving lipids as well as peptide/protein content between CF neutrophils and their wild-type counterparts before the appearance of an inflammatory environment that could mask their innate defects. For that, we isolated peripheral blood neutrophils from newborn $CFTR^{+/+}$ and $CFTR^{-/-}$ piglets. Neutrophils immunophenotype was evaluated by flow cytometry. Lipidomic and proteomic profile were characterized by liquid chromatography/tandem mass spectrometry (LC-MS/MS), intact cell matrix-assisted laser desorption/ionization mass spectrometry (ICM-MS) followed by top-down high-resolution mass spectrometry (HRMS), respectively.

We did not observe any differences in terms of total leukocyte population in peripheral blood differences between $CFTR^{+/+}$, and $CFTR^{-/-}$ newborn piglets (Figure 14A). Differential cell counts of leukocyte subsets did not show significant differences between genotypes, either (Figure 14B). Phenotypical characterization of purified peripheral neutrophils by flow cytometry showed no difference between genotypes. Furthermore, no difference was observed in the expression of CD11b and SWC3 (CD172a), two cell surface markers involved in neutrophil migration [102, 103] (Figure 14C). In a similar fashion, no differences were observed in CF and WT neutrophils ability to kill *Pseudomonas aeruginosa* in vitro nor the concentrations of bioactive lipids between each genotype. However, $CFTR^{+/+}$ and $CFTR^{-/-}$ neutrophils showed significant global phenotypic differences as evidenced after supervised machine-learning analysis with spectra from neutrophils of both genotypes. More specifically,

the prediction model based in the use of the genetic algorithm was able to distinguish between the ICM-MS spectra generated from *CFTR*$^{+/+}$ and *CFTR*$^{-/-}$ neutrophils with a recognition capability of 100% independently of the variability among spectra (indicated by a cross-validation value of 92.73%). The analysis of ICM-MS spectra from *CFTR*$^{+/+}$ and *CFTR*$^{-/-}$ neutrophils indicated that 89 out of the 214 m/z masses detected in the range of 2-20 kDa showed significant signal intensity differences (PWKW $p<0.01$), and from these, 49 showed very significant signal intensity differences (PWKW $p<0.001$). Eleven of these differentially abundant masses showed a good diagnostic performance, indicated by values of area under receiver operating characteristic curves higher than 0.75. Two-dimensional peak distribution of peaks 6 at *m/z* 2136 and peak 36 at *m/z* 3247 allowed for a good discrimination of *CFTR*$^{+/+}$ and *CFTR*$^{-/-}$ neutrophils (Figure 15).

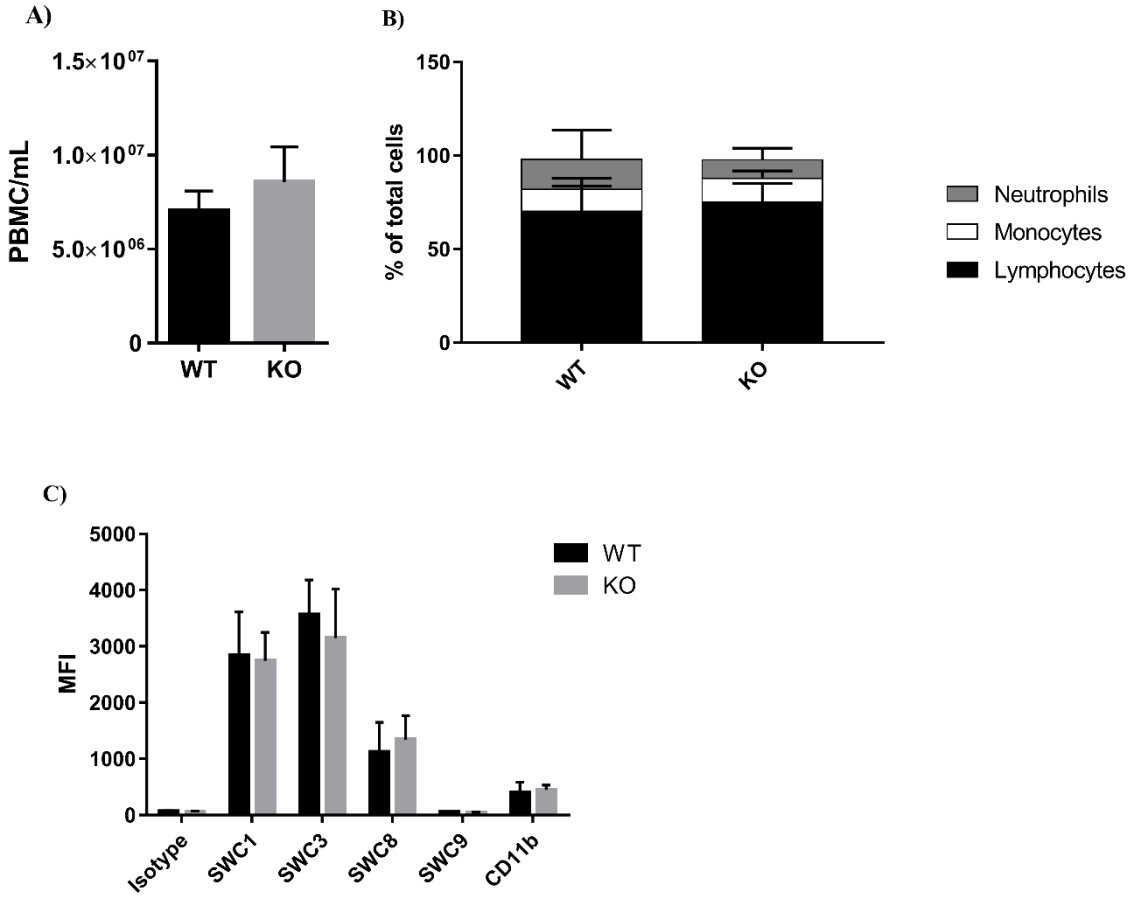


*Figure 14. Total white blood cell counts and cell profiles in the peripheral blood and phenotypic characterization of peripheral neutrophils from CFTR+/+ (WT), and CFTR-/- (KO) newborn piglets. (A)Total white blood cells (WBC) in peripheral blood. (B) Profile of the WBC populations in peripheral blood. (C) Flow cytometry analysis of purified neutrophils using antibodies raised against the surface marker of leucocytes (SWC1), myeloid cells (SWC3), neutrophils (SC8), macrophages (SWC9) and CD11b with IgM isotype as control.*

Our data showed for the first time that CF pig peripheral blood neutrophils are intrinsically different to their WT counterparts at birth. We identified for the first time differences in the phenotype of peripheral blood neutrophils from CF newborn pigs (6-12h after birth). ICM-MS spectra was used to build mathematical models that accurately differentiated neutrophils from either *CFTR*$^{+/+}$ or *CFTR*$^{-/-}$ origin. These results strongly pointed to intrinsic differences in the peptide/protein composition of *CFTR*$^{-/-}$ neutrophils already at birth, in the absence of an

inflammatory environment, which could have a potential impact in their functionality. Similarly, a recent report showed an increase neutrophil survival as well as neutrophil extracellular trap formation in CF neutrophils that was associated to a primary defect of CFTR rather than a secondary effect of inflammation [104].

Once determined that *CFTR*$^{+/+}$ and *CFTR*$^{-/-}$ peripheral neutrophils are intrinsically different, we aimed to identify those proteins that are differentially abundant and whether they are related with defects in neutrophil function. The top-down proteomic analysis was able to identify 57 *m/z* masses from the total peak list retrieved from the *CFTR*$^{+/+}$ and *CFTR*$^{-/-}$ neutrophils spectra.

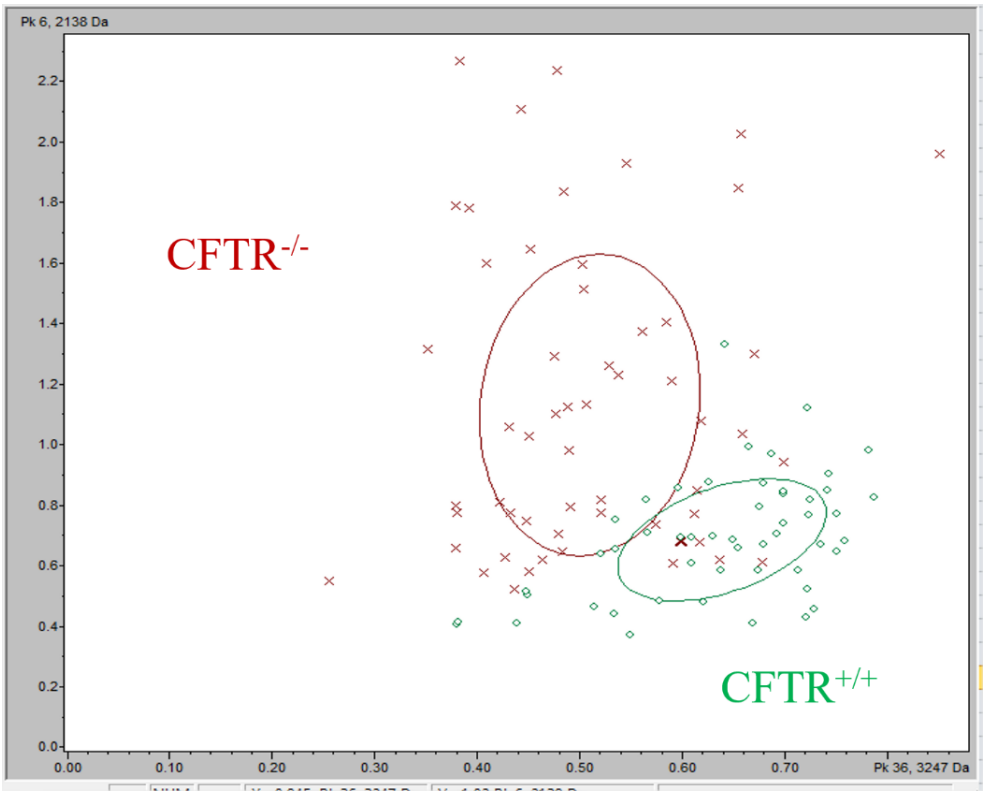


*Figure 15. Two-dimensional peak distribution diagrams of the two m/z peaks with the highest ability to discriminate CFTR*$^{+/+}$ *and CFTR-/- genotypes. The peak area and the m/z values are indicated on the x- and y-axes. The ellipses represent the standard deviation of the class average of the peak areas/intensities. Red represents CF (CFTR-/-) piglets and green represents WT (CFTR*$^{+/+}$*) piglets.*

Among these, 19 corresponded to masses showing significant changes (PWKW p<0.01), and corresponded mainly with proteins related with the antimicrobial response. Antimicrobial peptides, such as PR-39 [105] and Prophenin-2 [106], as well as SRGN, PTPRN2 and CANX, which are implicated in phagosome and granule maturation and degranulation processes were downregulated in *CFTR*$^{-/-}$ neutrophils. In addition, we identified several proteins that play an important role in reactive oxygen species (ROS) generation to be differentially abundant in the *CFTR*$^{-/-}$ neutrophils. Four of the masses showing good diagnostic performance (AUC> 0.75) were identified (Table 2). Western blotting analysis confirmed the differential expression of Annexin A1, Histone H2A and Pr3 observed by the ICM-MS and top-down analysis.

Despite the observed differences, in the abundance of antimicrobial proteins, no alteration was observed in neutrophil killing ability from *CFTR*$^{-/-}$ pigs *in vitro*. In this regard, the effect of a CFTR deficiency in neutrophils ability to kill bacteria has not been clarified [107]. Upon bacterial phagocytosis, NADPH oxidase subunits assemble at the phagolysosome and the cell membrane to direct the production of hypoclorous acid (HOCl), an oxygen reactant with high

antimicrobial activity. This procedure seems to be defective in CF neutrophils phagolysosome, but not extracellularly, and lead to a decrease ability of peripheral blood CF neutrophils to kill opsonized *P. aeruginosa* [99]. Other reports have not found differences in the ability to kill *P. aeruginosa*, which could vary depending on the bacterial isolate [108] or the proteolytic environment [107]. In addition to their ability to kill bacteria, other important features of neutrophil functionality remain to be evaluated. Generation of ROS, apoptosis rates and chemotaxis can greatly influence neutrophil response to a pathogen. Further studies would be needed to determine the specific role of the observed alterations in $CFTR^{-/-}$ neutrophils in their different functional aspects and how they can impact their ability to kill bacteria.

**Table 2. Characteristics of the intact cell MALDI-TOF Mass Spectrometry m/z masses found to be differential between *CFTR*+/+ and *CFTR*-/- pig neutrophils (p<0.05) that were confidently identified after top-down High Resolution Mass Spectrometry. AUC: area under the receiving-operating curve.**

| Mass | Intensity Average Difference | p-value | Fold-change CFTR+/CFTR- | AUC | Symbol | Protein name | Main function |
|---|---|---|---|---|---|---|---|
| 8634.95 | 0.51 | 0.0002 | 1.9 | 0.74 | NPG2 | Prophenin-2 | Antibacterial protein |
| 8678.73 | 0.24 | 0.0053 | 1.5 | 0.75 | HIST2H2AA4 | Histone H2A type 2-A | Nucleosome; Immunostimulation |
| 2100.11 | 0.63 | 0.0097 | 1.3 | 0.68 | ANXA1 | Annexin A1 | Regulator of the inflammatory process |
| 8338.1 | 0.12 | 0.0001 | 1.3 | 0.74 | PMAP37 | Antibacterial peptide PMAMP-37 | Antibacterial protein |
| 3247.48 | 0.14 | 0.0000 | 1.2 | 0.82 | CANX | Calnexin | Chaperone; Maturation of phagosomes |
| 2980.76 | 0.13 | 0.0000 | 1.2 | 0.84 | PR39 | Antibacterial protein PR-39 | Antibacterial protein |
| 9368.06 | 0.09 | 0.0016 | 1.2 | 0.68 | MLLT10 | Protein AF-10 | Transcriptional regulation |
| 2300.84 | 0.11 | 0.0034 | 1.2 | 0.77 | LOC100736951 | Cathelicidin | Antibacterial protein |
| 8396.36 | 0.09 | 0.0008 | 1.2 | 0.68 | SUPT5H | Transcription elongation factor SPT5 | Regulates mRNA processing and transcription elongation |
| 3377.72 | 0.09 | 0.0071 | 1.2 | 0.65 | HNRNP | Heterogeneous nuclear ribonucleoprotein | RNA binding protein |
| 9313.07 | 0.07 | 0.0080 | 1.1 | 0.65 | HIST2H2BE | Histone H2B type 2-E | Nucleosome; Immunostimulation |
| 6382.27 | 0.08 | 0.0067 | 1.1 | 0.63 | CAP2 | Adenylyl cyclase-associated protein | Regulates filament dynamics |
| 4599.95 | 0.08 | 0.0013 | 1.1 | 0.66 | PR39 | Antibacterial protein PR-39 | Antibacterial protein |
| 6787.34 | 0.07 | 0.0032 | 1.1 | 0.64 | LOC100154508 | Histone H2A type 1-F like | Nucleosome; Immunostimulation |
| 5146.05 | 0.08 | 0.0067 | 1.1 | 0.61 | LOC100512420 | Histone H2B type 1-K | Nucleosome; Immunostimulation |
| 7388.33 | 0.06 | 0.0093 | 1.1 | 0.62 | NPG2 | Prophenin-2 | Antibacterial protein |
| 6715.07 | 0.16 | 0.0087 | 0.8 | 0.68 | H2AFX | Histone H2A | Nucleosome; Immunostimulation |
| 4090,36 | 1.96 | 0.0048 | 0.7 | 0.68 | SERPINB1 | Leukocyte elastase inhibitor | Regulates the activity of the neutrophil proteases |
| 7260.62 | 4.2 | 0.0001 | 0.5 | 0.75 | ATP6AP2 | Renin receptor | Neutrophil degranulation |

Determination of intrinsic alterations in the CF lung environment that favour bacterial infection

There is a long-standing debate regarding the links between CFTR mutations and the development of persistent infection and inflammation [9]. While some suggest that CFTR dysfunction leads to intrinsic inflammation, others hypothesize that excessive inflammation is rather the result of a lung environment that favours bacterial colonization, being indirectly linked to specificities of the CF mucus [109]. Contradictory reports have shown increased or decreased sialylation and sulfation of O-glycan content in CF patients [110-112]. These discrepancies are probably explained by altered expression of sialyltransferases under inflammatory conditions, such as those observed in CF patients [110, 113]. We used newborn CF pigs to determine whether there are alterations in the biochemical composition of airway mucins, mucociliary transport (MCT), as well as the lung inflammatory status before and after *P. aeruginosa* infections, a major pathogen in CF patients.

Periodic acid-Schiff (PAS) and alcian blue staining of the airways of $CFTR^{+/+}$ pigs showed a mix neutral of sialylated mucins in the goblet cells (Fig. 16A), while, $CFTR^{-/-}$ pigs displayed a high density of sialylated mucins (Fig. 16B). These results were confirmed by mass spectrometry analysis of bronchoalveolar lavages (BAL) purified mucins which showed a significant increase in mucin sialylation in $CFTR^{-/-}$ pigs (Figure 16C). These results were confirmed using *ex vivo* tracheal explants from WT piglets that had been treated for 24h with the CFTR inhibitor GlyH-101, followed by separation and purification of mucosal mucins.

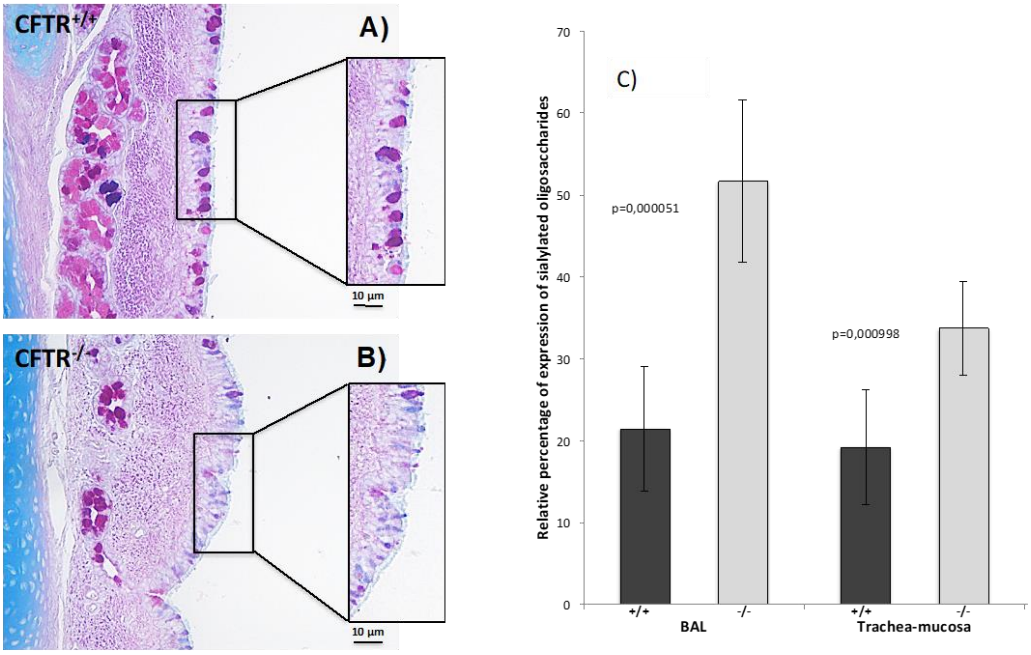


*Figure 16. $CFTR^{-/-}$ pig mucins show altered glycosylation at birth. A-B). PAS/Alcian blue histochemical staining of $CFTR^{-/-}$ and $CFTR^{+/+}$ pig tracheas. A) Section (200x) of a $CFTR^{+/+}$ trachea showing neutral/acidic mucins in the surface goblet cells (stained in purple). The right figure is a zoom of the surface epithelium goblet cells of $CFTR^{+/+}$ pig trachea. B) Section (200x) of a $CFTR^{-/-}$ trachea showing acidic mucins in the surface goblet cells (stained in blue). The right figure is a zoom of the surface epithelium goblet cells of $CFTR^{-/-}$ pig trachea. Images are representative of 20 $CFTR^{-/-}$ and 22 $CFTR^{+/+}$ pig tracheas. C) Relative percentage of whole sialylated O-glycans in mucins. Mucin O-glycans were released from BAL (n=20 $CFTR^{-/-}$ and n=22 $CFTR^{+/+}$ pigs) and from epithelial surface of the tracheas (n=4 $CFTR^{-/-}$ and n=7 $CFTR^{+/+}$ pigs). O-glycans were permethylated before analysis by MALDI-TOF mass spectrometry in the positive ion mode $[M+Na]^+$.*

Contrasted differences in the degree of mucin sulfation and sialylation of CF mucins have been reported in the literature [110, 112, 114-116]. These differences have been explained by: (i) differences in the lung inflammatory status of the CF patients, (ii) use of non-CF pulmonary

diseases as control patients, who often have difficulties in expectorating mucus [115, 117-119], (iii) regional differences along the vertical axis, with higher levels of sialylation in the distal than in the proximal lung. Our studies on newborn CF pigs were designed in order to control these different parameters. Samples were collected in the absence of metacholine stimulation, so that mucins purified from the BAL were representative of the airway surface, including in *CFTR*$^{-/-}$ piglets, where mucus is more difficult to detach [120]. All mucus samples were systematically purified after BAL collection or scrapping of the mucosal surface, so that our measurements were specific to mucins. Pre-existing inflammation in *CFTR*$^{-/-}$ airways was ruled out since RNA-seq analyses showed no differences in the expression of inflammatory genes between the two genotypes under basal conditions (Figure 17). In addition, no differences were observed in the expression of the 12 sialyltransferases that were identified in our pig transcriptomes. These data, which support the absence of inflammation in non-infected newborn piglet airways, were confirmed: (i) by real-time quantitative PCR on a selection of 7 markers; (ii) by the absence of neutrophils in the lumen of *CFTR*$^{-/-}$ and *CFTR*$^{+/+}$ piglets; (iii) by low levels of IL-8 cytokine in BAL.

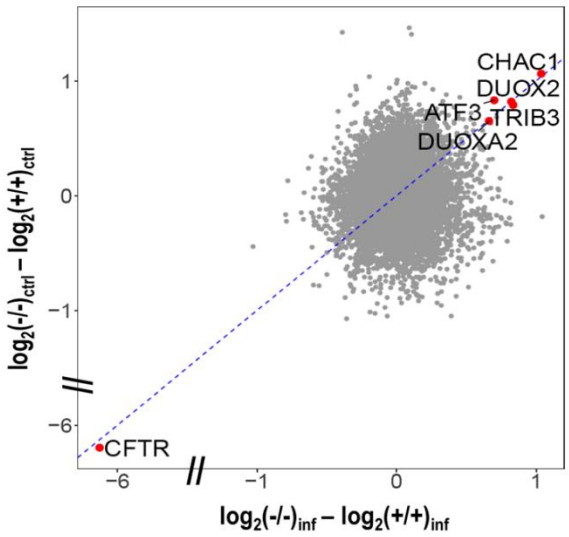


*Figure 17. Gene expression modifications in airway mucosa from newborn CFTR*$^{-/-}$ *piglets. Relationship between log2(CF/WT) in infected (horizontal) and not infected (vertical) piglets.*

We also noticed that mucins from CF pigs had an increased binding capacity to *P. aeruginosa* (Figure 18A), which is probably linked to the ability of *P. aeruginosa* to bind to the sialic acid moiety of mucins [112, 121-123], *via* the bacterial flagellar cap FliD protein [124]. Increased binding should help bacterial clearance from the lungs. In a previous report, Hoegger *et al.* suggested that a defective mucociliary transport occurs in lung from CF pigs due to mucus tethering to gland ducts, which impairs detachment [120]. That study was performed after a cholinergic stimulation by methacholine, using inert tantalum particles and fluorescent beads. This set-up did not address the possible impact of bacteria on MCT and secretion of ASL [125, 126]. In order to test any impairment of anti-bacterial host defence in *CFTR*$^{-/-}$ pigs, live *P. aeruginosa* were inoculated at the apical surface of a 3D-reconstituted bronchial epithelium, obtained after differentiation under air liquid interface conditions. We noticed a significant increase of bacterial growth when inoculation was performed on the *CFTR*$^{-/-}$ bronchial epithelium (Figure 18B). *In vivo* evaluation of mucociliary clearance of *P. aeruginosa* showed significant differences between *CFTR*$^{+/+}$ and *CFTR*$^{-/-}$ piglets. Six hours after bacterial inoculation into the tracheal carina, *P. aeruginosa* was mainly found in the larynx, pharynx and

nose of *CFTR*$^{+/+}$ piglets. On the contrary, *P. aeruginosa* remained in the tracheal carina of *CFTR*$^{-/-}$ piglets, near the site of inoculation, suggesting a defective MCT (Figure 18C).

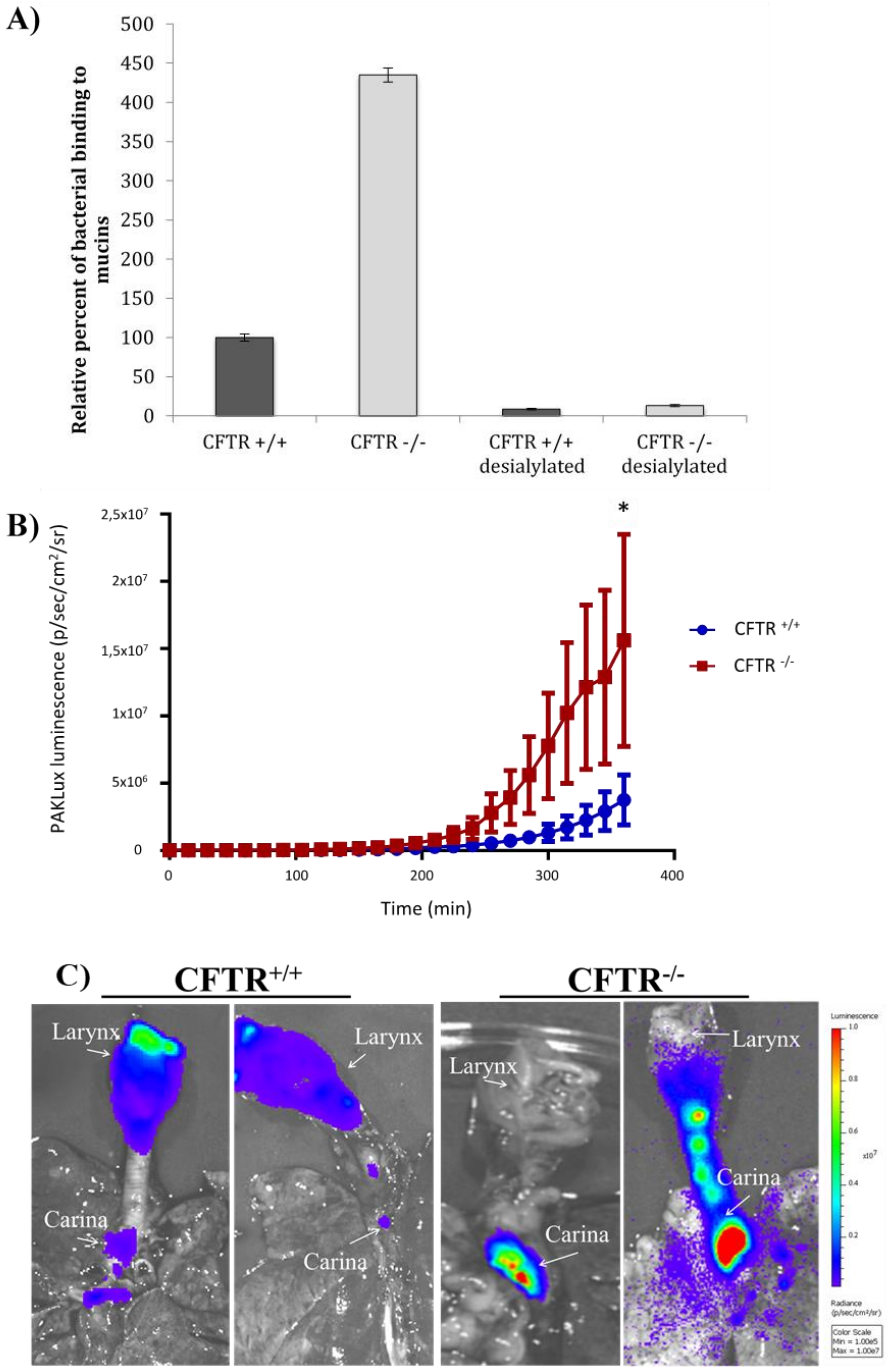


*Figure 18. CFTR*$^{-/-}$ *pigs show impaired bacterial clearance from the lungs. A) Binding of DAPI-labeled P. aeruginosa bacteria to purified airway mucins from CFTR*$^{-/-}$ *and CFTR*$^{+/+}$ *pigs was quantified by slot-blot overlay assays. P. aeruginosa binds to CFTR*$^{-/-}$ *airway mucins. After chemical desialylation of mucins, no binding of the bacteria was observed. Data shown is a representative experiment +/- S.D of 3 replicates, representative of at least 20 experiments. B) Time series of luminescent P. aeruginosa imaged under the IVIS spectrum system. Bacteria was inoculated to a CFTR*$^{+/+}$ *or a CFTR*$^{-/-}$ *bronchial epithelium (MOI=0.1) and cultured for 6h. Data are mean±S.E.M. Curves were fitted using a non-linear exponential growth model and best-fits compared using the extra-sum-of-squares F test. C) Defect in mucociliary clearance in CFTR*$^{-/-}$ *pigs. CFTR*$^{+/+}$ *and CFTR*$^{-/-}$ *pigs were inoculated with luminescent P. aeruginosa into the tracheal carina for 6 h and imaged under the IVIS Spectrum system.*

Our data fully confirmed the severe impairment of MCT in CF airways at an early stage of development observed by these authors. Incidentally, our observation that CF airway epithelium favoured *P. aeruginosa* growth, is probably in line with the reduced pH of the ASL that can inactivate host antimicrobials [127]. The inflammatory response 6 h after *P. aeruginosa* infection was also evaluated by RNA-seq. Although there was a tendency towards an increased inflammation in the CFTR$^{-/-}$ airways, those differences did not reach statistical significance (Figure 19).

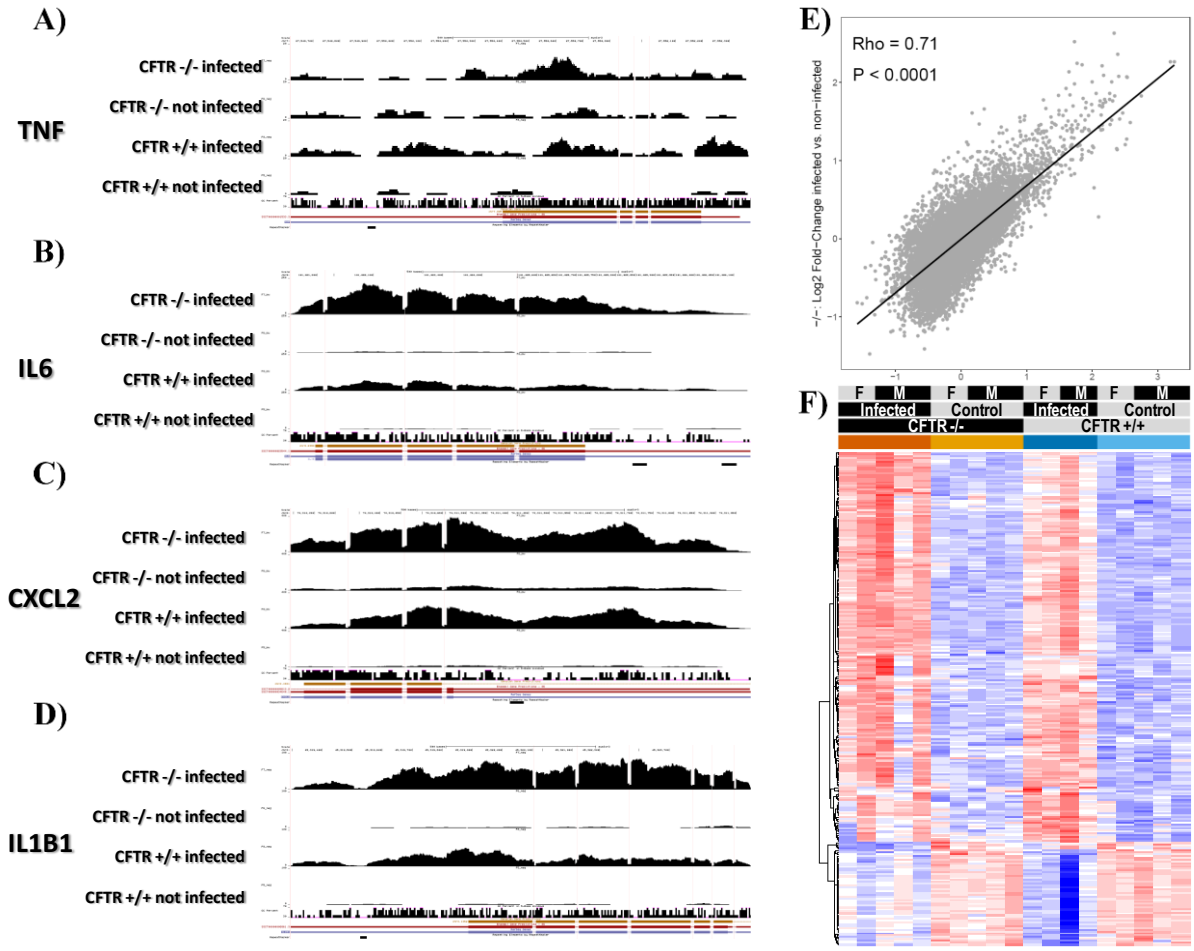


*Figure 19. CFTR$^{-/-}$ piglet transcriptional response following infection by Pseudomonas aeruginosa. A-D) RNA-seq read densities (vertical scale of reads) in the TNF (A), IL-6 (B), CXCL2 (C) and IL1B1 (D) genomic locus in CFTR$^{-/-}$ and CFTR$^{+/+}$, with or without infection. E) Log2(infected/control) relationship between CFTR$^{+/+}$ (horizontal) and CFTR$^{-/-}$ (vertical). F) Heatmap of the most differentially expressed transcripts during infection.*

Altogether, the CF lung environment promoted bacterial colonization and increased bacterial residency time in the lungs. This can indeed represent a likely explanation for the slight increase in the host defence response observed in RNA-seq experiments. A blunted immune response to heat-killed *Staphylococcus aureus* has been previously reported by Bartlett *et al.* [23]. This difference could be explained by the use of killed bacteria, unable to colonize the lungs. Overall, the most notable differences that we measured in gene expression were related to an increased expression of a few transcripts associated with the reticulum stress response. This observation is consistent with the known relationship between reticulum stress and increased activity of the inflammatory pathways [128]. It has however not been reported before in other gene expression studies performed on other CF models. Understanding the underlying mechanisms will thus require future investigations.

In conclusion, we report here for the first time an early increase of respiratory mucin sialylation in the lungs of CF pigs that results in an increased adherence of *P. aeruginosa* to the CF lung, in the absence of any previous inflammation. This increased sialylation, which can also occur as a result of inflammation in non-CF individuals, is potentially beneficial for host defence as it may allow better mucociliary clearance of inhaled pathogens. However, in the CF lung these altered mucin properties may be detrimental as a result of a concomitant decrease in mucociliary clearance and impaired bacterial killing at the epithelial surface. This may result in an increased time of residency of live bacteria, allowing them to escape from the mucus layer and survive long enough in the periciliary liquid to infect neighbouring tissue. We propose that these three properties cooperatively contribute to bacterial colonization and development of CF lung inflammation.

Development of novel therapies targeting innate immunity in cystic fibrosis

Our previous observations showed that the lung environment in CF favours bacterial colonization already at birth. This could be one of the mechanisms that trigger the chronic bacterial colonization in the lungs and inflammation that is a classical hallmark of the disease. Understanding the processes leading to excessive lung inflammatory environment would be crucial to improve CF therapies. TLRs are key to drive a balanced immune response [24]. Several studies are targeting TLRs as a promising prophylactic and/or therapeutic strategy to combat infectious diseases since modulation of their function and responsiveness to pathogens could improve innate immunity.[48, 49]. In the CF context, TLR4 and TLR5, which interact with LPS and flagellin, respectively, are known to be major factors in the immune response to *P. aeruginosa* [129]. In particular, TLR5 response to flagellin has been suggested to play a role in the excessive inflammation observed in CF airway epithelial cells and, therefore, it is a candidate target for anti-inflammatory therapies [130, 131]. Apical localization of TLR5 in the airway mucosa allows airway epithelial cells (AECs) to be amongst the first responders to flagellated bacteria [132], and a cell of interest for immune-stimulation therapies. In this regard, several studies have shown that the bacterial protein flagellin (TLR5 ligand) can provide protection against different bacterial species at the respiratory level. This protection seems to be linked to a pro-inflammatory activity of flagellin that promotes the recruitment and maturation of immune cells [53, 133], therefore preparing the mucosal environment for the arriving bacteria. At the same time, mucosal administration of flagellin seems also to reduce tissue damage due to the exacerbated inflammation [53], promoting full resolution of the process. Recent findings in the CF pig model show that newborn animals do no present intrinsic inflammation in the lungs, which spontaneously develops later in life [22]. This feature makes the newborn CF pig model especially well-suited to evaluate early defects in innate immune signalling. At that time, a lack of previous infections and/or chronic inflammation avoid confounding effects that could modify the airway epithelium response to an inflammatory stimulus. Here, we aimed to determine whether TLR5 signalling is defective in the CF airway epithelium and the effect of flagellin pre-stimulation on the innate immune response against *P. aeruginosa* in the CF pig model.

To assess whether alterations in CFTR function may lead to a defective innate immune response, primary airway epithelial cells (AECs) from WT piglets (cultured under ALI conditions) were treated with GlyH-101 (a CFTR inhibitor) and then stimulated apically with the recombinant flagellin $FliC_{\Delta 174\text{-}400}$. We observed that flagellin administration significantly increased the expression of genes coding for inflammatory cytokines (*CXCL8*, *CCL2*, *CXCL2*, *IL1A*) and regulation of the immune response (*TNFAIP3*, *NFKBIA*). Interestingly, the response was exacerbated in the samples pre-treated with GlyH-101 (Figure 20). These data suggest that TLR5 signalling could be altered after pharmacological inhibition of the CFTR channel. These observations were confirmed in AECs from newborn $CFTR^{+/+}$ or $CFTR^{-/-}$. Stimulation of $CFTR^{-/-}$ AECs with $FliC_{\Delta 174\text{-}400}$ resulted in a significantly increased expression of *CXCL2* and *CXCL8* (Figure 21), coding for chemokines involved in neutrophil recruitment [134, 135]. In contrast, LPS did not induce any significant changes in $CFTR^{-/-}$ cells compared to $CFTR^{+/+}$ cells. Altogether, these data indicate that CFTR plays an important role in the regulation of TLR5 signalling.

Previous studies in the CF pig model hypothesized that bacterial colonization of the lungs could be related to acidification of the airway-surface liquid, making anti-microbial molecules ineffective [136, 137]. Another, and not exclusive, hypothesis was the existence of a blunted immune response at birth [23]. The latter would favour initial bacterial colonization of the lungs, which would ultimately lead to increased inflammation. Our data, however, pointed to

an exacerbated inflammatory response driven by TLR5 signalling dysregulation, with an increased IL8 and CXCL2 gene expression.

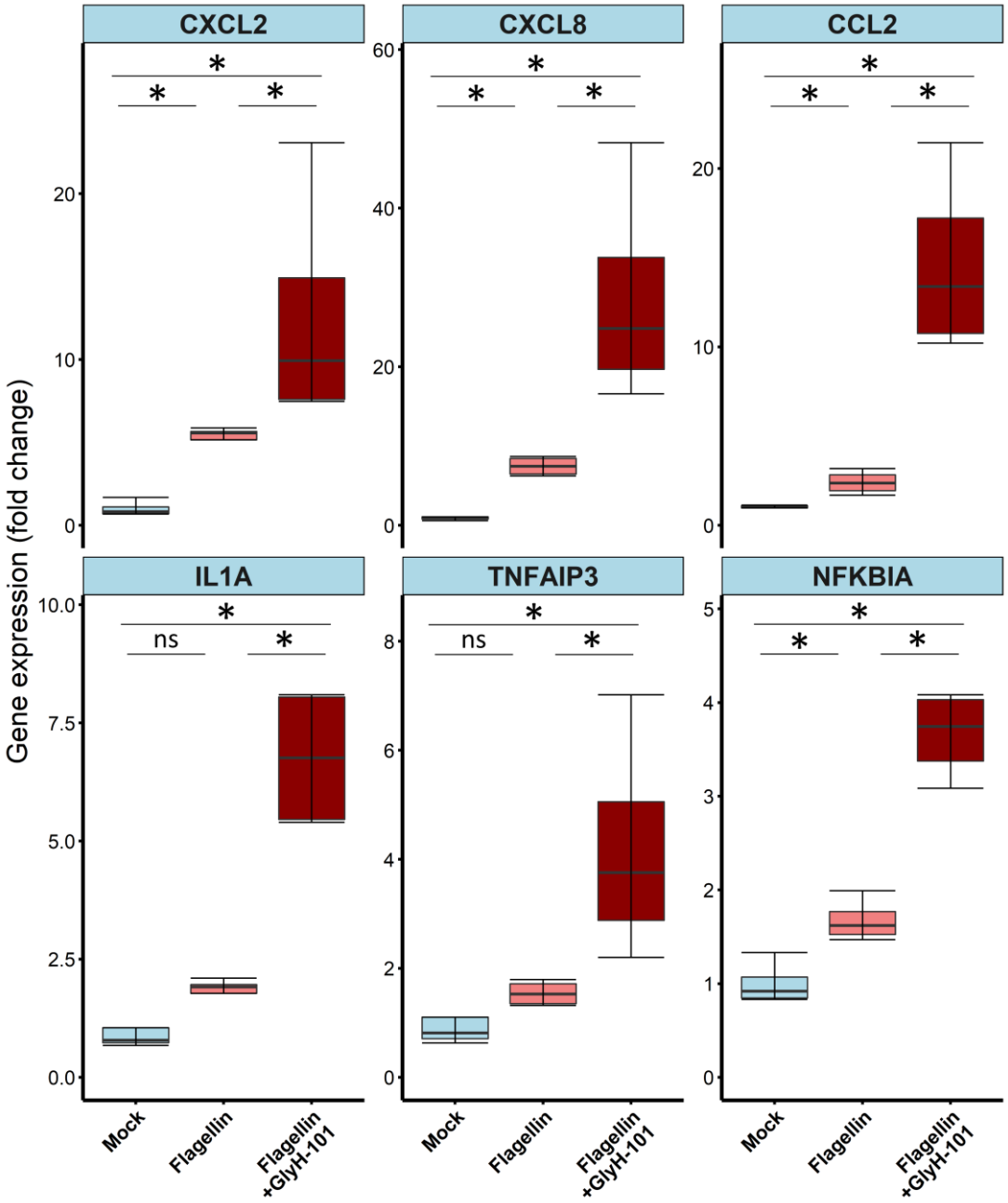


*Figure 20. Inhibition of CFTR channel activity dysregulates TLR5 signalling. Gene transcription analysis in airway epithelial cells from wild-type pigs treated with 50 µM of GlyH-101 or left untreated for 24 h and stimulated for 5 h with 200 µl of either 100 ng/ml of apical $FliC_{\Delta 174\text{-}400}$ or the vehicle. Gene expression levels are shown relative to the mock group (treated with the vehicle). Intergroup differences were analysed using the Kruskal-Wallis test followed by a pairwise Mann-Whitney test with Benjamini-Hochberg false discovery rate correction. Data are representative of four experiments. * indicates $p<0.05$. ns indicates not significant.*

This is in line with earlier studies that describe an overproduction of pro-inflammatory cytokines in the CF cell lines, and airway epithelia from CF patients [138-140]. This fits also with the known major role played by TLR5 signalling in the exaggerated cytokine production [130]. Our results differ somehow from a study by Bartlett et al [23] who analysed the impact on airway epithelium of heat-killed *Staphylococcus aureus* [23], which does not express flagellin [126], and which is therefore unable to activate TLR5 signalling, unlike *P. aeruginosa*

and purified flagellin. Very consistently, a recent study showed opposite effect in the induction of IL-8 *by S. aureus* and *P. aeruginosa* in BEAS-2B airway epithelial cells, with *P. aeruginosa* strongly inducing IL-8 secretion, and *S. aureus* having a minimal effect [141].

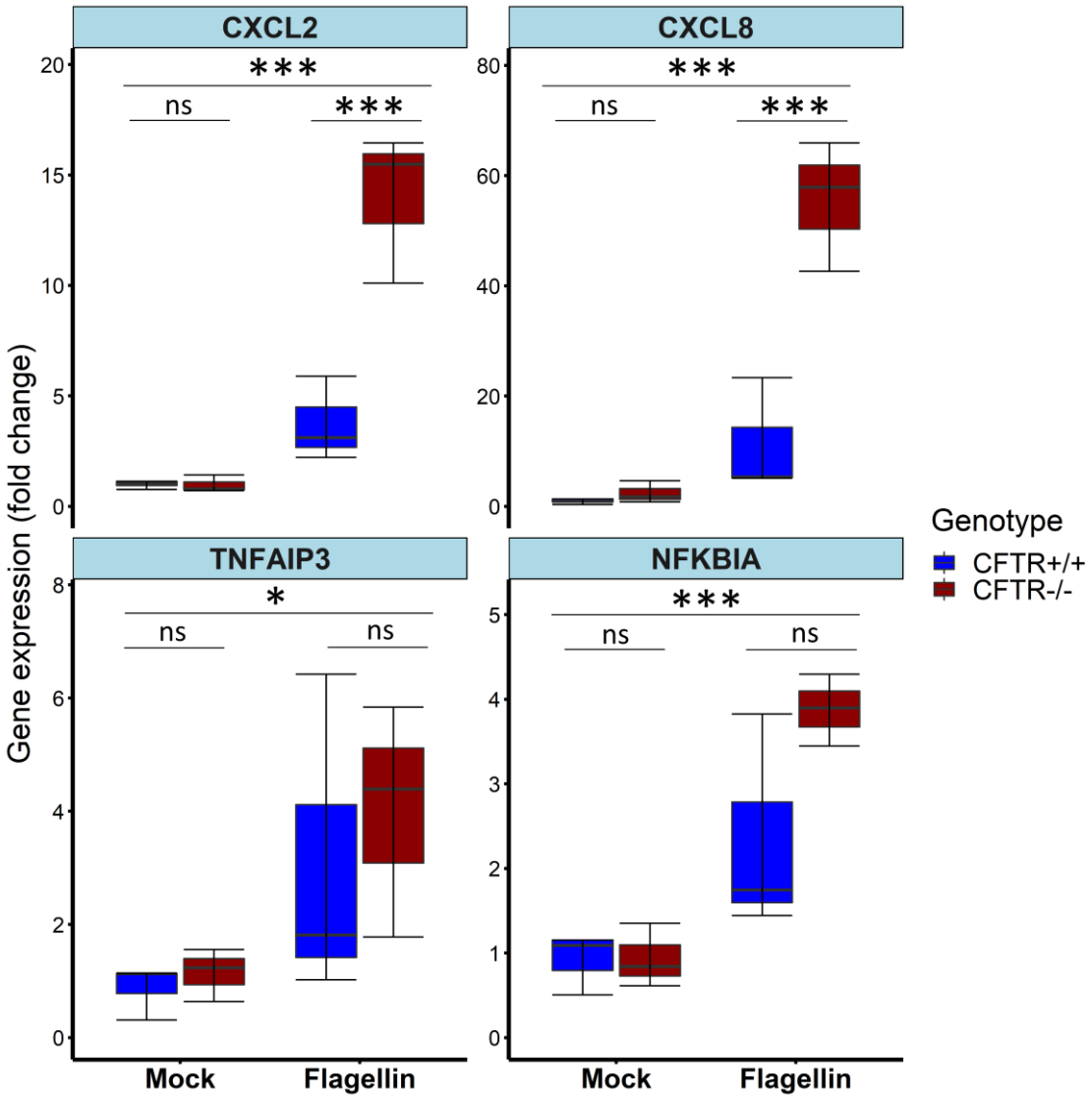


*Figure 21. Newborn CFTR-/- piglets present an exacerbated TLR5 response. Real-time RT-qPCR analysis of airway epithelial cells from newborn CFTR$^{+/+}$ and CFTR$^{-/-}$ piglets stimulated for 5 h with either 200 µl of 100 ng/ml of apical FliC$_{\Delta 174\text{-}400}$ or the vehicle. Gene expression was normalized against the unstimulated (Mock) CFTR$^{+/+}$ group. Data were analysed by two-way ANOVA, using pig genotype and flagellin stimulation as factors, followed by Tukey's HSD post hoc test. Data are representative of three experiments. * indicates p<0.05. ** indicates p<0.01. *** indicates p<0.001. ns indicates not significant.*

Our observations suggest that TLR5 signalling may represent an important mediator of the immune response in CF, which could contribute to the excessive inflammation observed in CF lungs. As such, it may represent an interesting target to develop novel immune-modulatory therapies in CF. Modulation of TLR5 signalling has been previously achieved through flagellin stimulation in several disease backgrounds (*Salmonella* sp., *Burkholderia cepacia*, *Yersinia pseudotuberculosis*) [49]. This protection seems to be dependent on the secretion of pro-inflammatory cytokines, which are essential for the recruitment of immune cells, the secretion of antimicrobial molecules and mucins, and maturation of dendritic cells and type 3 innate lymphoid cells [49]. Thus, we aimed to determine the effect of flagellin treatment on the response to an inflammatory stimulus. First, we observed that a first flagellin treatment

produced a significant desensitization of TLR5 signalling to a second $FliC_{\Delta 174\text{-}400}$ stimulus (Figure 22) in WT AECs.

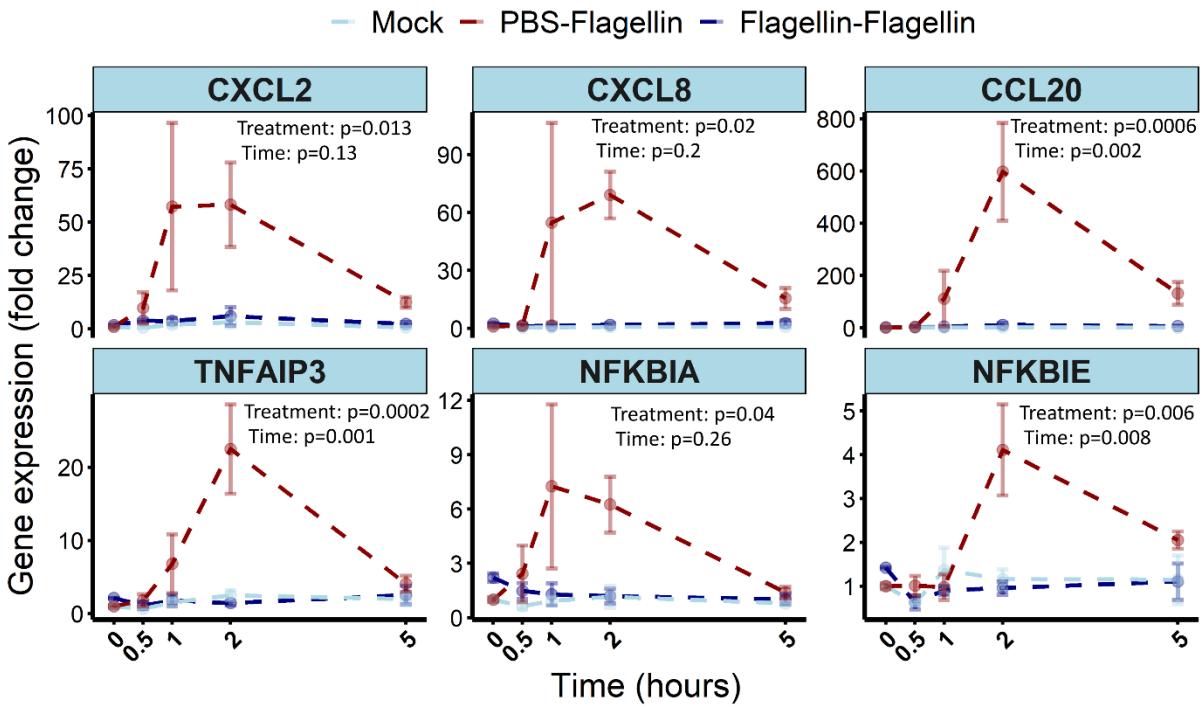


*Figure 22. Flagellin treatment decreases TLR5 response to a second flagellin input in airway epithelial cells. Real-time RT-qPCR analysis of differentiated airway epithelial cells from wild-type pigs pre-treated or not with 100 ng/ml of apical $FliC_{\Delta 174\text{-}400}$ for 24 h and later stimulated or not with a second flagellin input (100 ng/ml) for 0, 0.5, 1, 2 and 5 h. Mock indicates PBS-treated an non-stimulated airway epithelial cells. Gene expression levels are shown relative to the mock group. Intergroup differences were analysed by two-way ANOVA using time and treatment as factors, followed by Tukey's HSD post hoc test. Data are representative of three experiments.*

We further investigated whether flagellin would play a role on the immune response to a pathogenic challenge. Infection of WT AECs with *P. aeruginosa* PAK strain triggered an important inflammatory response with the upregulation of *CXCL2*, *CXCL8*, *CCL20*, *TNFAIP3*, *NFKBIA* and *NFKBIE* genes. The inflammatory response was significantly decreased when WT AECs were before exposed to $FliC_{\Delta 174\text{-}400}$ (Figure 23). We next analysed the time course of the flagellin-mediated effect on the $CFTR^{-/-}$ airways in response to *P. aeruginosa*. For that, we employed a PCLS culture system that allows obtaining a large number of lung slices where the architecture of the tissue is preserved. As expected, *P. aeruginosa* infection upregulated the transcription of genes involved in the inflammatory response. Interestingly, flagellin treatment before *P. aeruginosa* infection was able to reduce significantly the expression of *CXCL2* and *A20* genes (Figure 24). Taken as a whole, these data suggest that flagellin treatment can modulate the immune response to a pathogen, even in the CF airways context.

Then, we evaluated the effect of *in vivo* bronchial administration of $FliC_{\Delta 174\text{-}400}$ in WT pigs to validate the *in vitro* and *ex-vivo* data. Bronchial administration of $FliC_{\Delta 174\text{-}400}$ in WT pigs produced within three hours a mild inflammatory response in the lungs, the increase in lung *CXCL8* expression (Figure 25A), and the secretion of IL-8 into the conducting airways. We later evaluated whether pre-exposure to flagellin also modulated the inflammatory response to *P. aeruginosa in vivo*. We observed a trend for decreased *CXCL8* gene expression when pigs were treated with $FliC_{\Delta 174\text{-}400}$ for 24 h before *P. aeruginosa* infection (Figure 25B). No difference was observed in the level of secreted IL-8 in the BAL fluid, between flagellin treated and mock-treated animals.

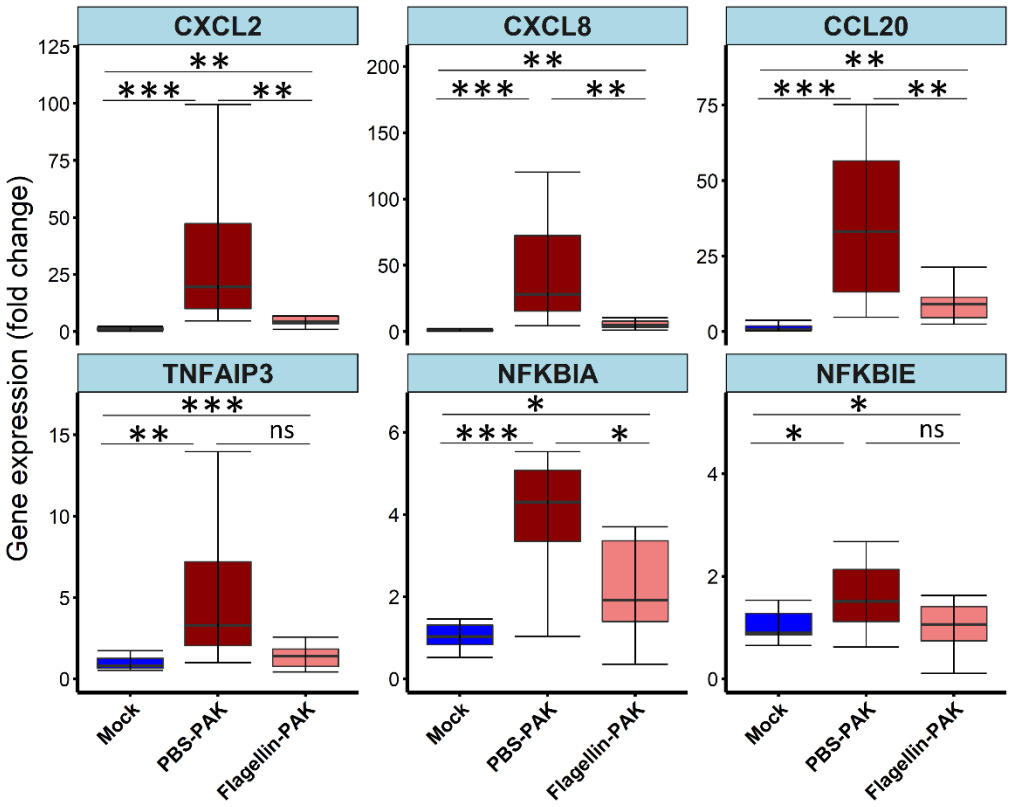


*Figure 23. Flagellin treatment decreases the immune response to Pseudomonas aeruginosa in wild-type airway epithelial cells. Real-time RT-qPCR analysis of wild-type airway epithelial cells pre-treated or not with 200 µl of 100 ng/ml of apical $FliC_{\Delta 174-400}$ or left untreated for 24 h and later infected with P. aeruginosa strain PAK (MOI= 0.2) for 5 h. Gene expression levels are shown relative to the mock group (treated with the vehicle). Intergroup differences were analysed using the Kruskal-Wallis test followed by a pairwise Mann-Whitney test with Benjamini-Hochberg false discovery rate correction. Data are representative of at least three experiments. * indicates p<0.05. ** indicates p<0.01. *** indicates p<0.001.*

Histological analysis of the lungs showed dilated interlobular septa and cellular infiltration into the alveolar spaces after *P. aeruginosa* infection. This inflammatory response was significantly reduced in those animals that had been treated with flagellin before the infection (Figure 26 and Table 3). These results indicate that flagellin is able to induce a transient inflammatory response in the airways that has an impact in the lung immune response to *P. aeruginosa*.

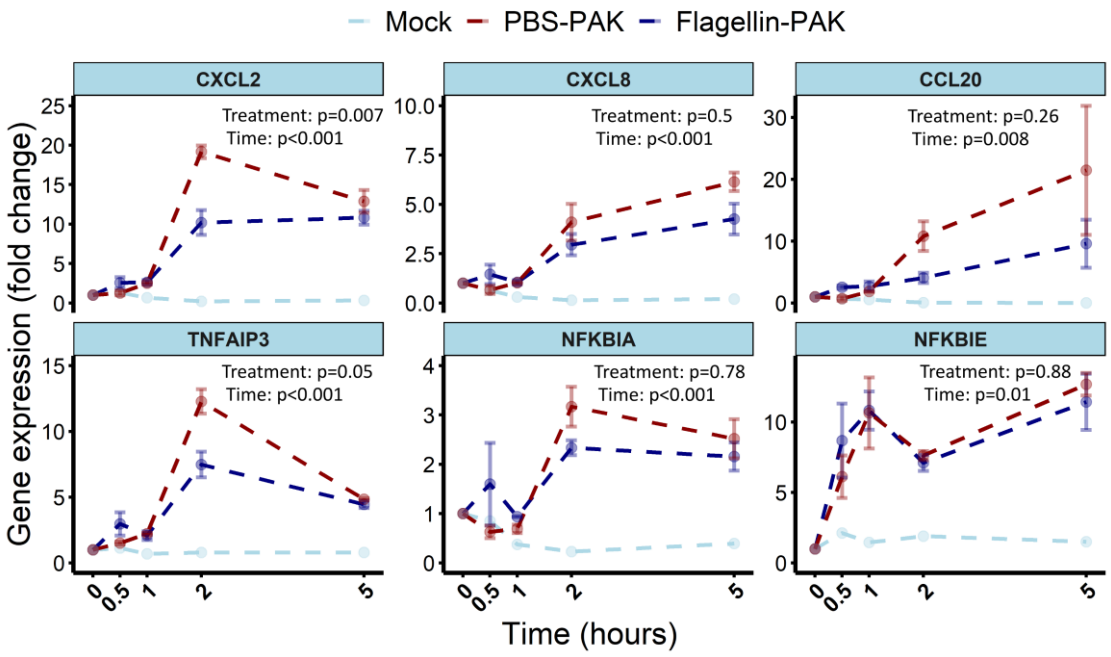


*Figure 24. Flagellin treatment reduces the inflammatory response to Pseudomonas aeruginosa in newborn CFTR-/- piglets precision-cut lung slices. Real-time qPCR analysis of precision-cut lung slices from newborn CFTR-/- piglets treated or not with 200µl of 100 ng/ml of FliC$_{\Delta 174\text{-}400}$ for 24 h and later infected with P. aeruginosa strain PAK (MOI= 0.2) for 0, 0.5, 1, 2 and 5 h. Gene expression levels are shown relative to the mock group (non-treated and non-infected samples). Intergroup differences were analysed by two-way ANOVA using time and treatment as factors, followed by Tukey's HSD post hoc test. Data are representative of three experiments.*

Flagellin activates TLR5 signalling by triggering the NF-κB pathway, but at the same time, the response is short-lived due to strong feedback regulatory mechanisms [37, 142]. *P. aeruginosa* lacking the expression of flagellin, fail to elicit an immune response [143], suggesting that TLR5 signalling is key to regulate the response to *P. aeruginosa*. The observed decreased inflammatory response could be related to TLR5 signalling desensitization, due to a decrease in receptor availability. However, flagellin is rapidly degraded in the airways (less than 12 h) [132], and our observations on the lungs inflammatory response to *P. aeruginosa* were performed 48 h after flagellin administration. It is also possible that flagellin pre-treatment also interfere with feedback loops that regulate NF-κB signalling. Although further studies are clearly needed to understand the mechanisms triggered by flagellin treatment that lead to a less severe inflammatory response, it is noteworthy that flagellin pre-treatment improved the lung status after infection with *P. aeruginosa*. These results suggests a better resolution of the inflammation after flagellin therapy as already indicated by Muñoz et al [53].

In conclusion, our results suggest that innate immune response is dysregulated in CF airways. We point to TLR5 signalling as a key player in this exacerbated inflammatory response. Moreover, we show that modulation of TLR5 signalling through flagellin administration can improve innate immune response to *P. aeruginosa*, thus decreasing inflammation in the lungs. Modulation of the inflammatory response is crucial for CF patients during pulmonary exacerbations, which are characterised by excessive neutrophilic inflammation. Our data open new leads toward the design of novel immune-modulatory therapies targeting TLR5 that could improve the management of lung inflammation in CF.

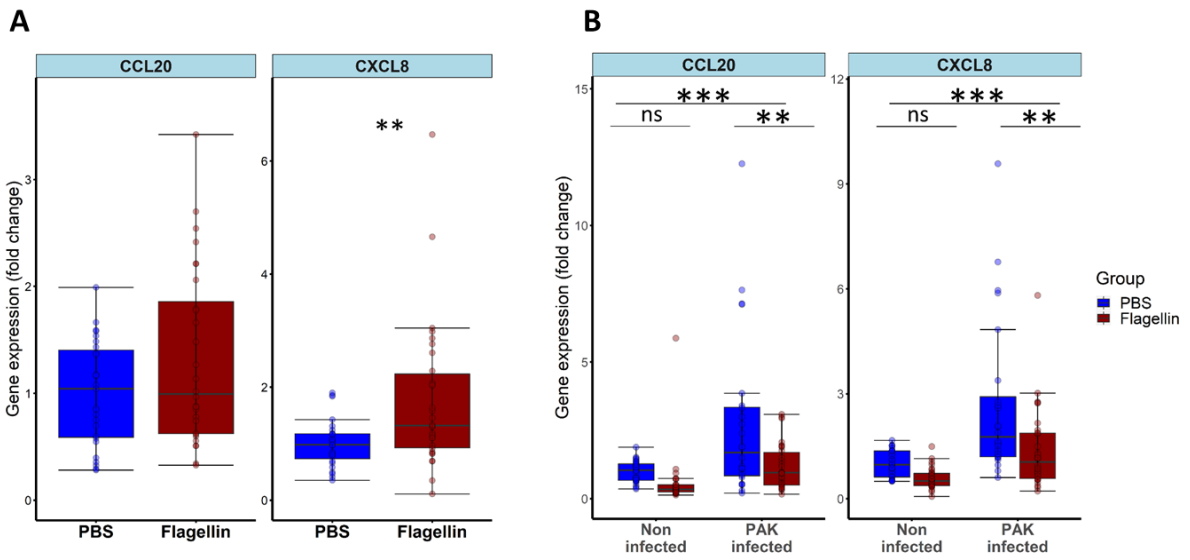


*Figure 25. Flagellin produces a mild transient inflammatory response in the lungs and decreases immune response to Pseudomonas aeruginosa* infection. (A) Pigs (n=5) received a bronchial administration of 0.012 mg/kg of FliCΔ174-400 or PBS and the inflammatory response evaluated after 3 h. (B) Pigs (n=5) received a bronchial administration of 0.012 mg/kg of FliCΔ174-400 or PBS and 24 h later infected or not with 10 ml of a 107 cfu/ml suspension of P. aeruginosa strain PAK. The inflammatory response was evaluated 24 h after infection. CCL20 and CXCL8 gene expression were evaluated by real-time RT-qPCR. Gene expression levels are shown relative to the PBS group. Intergroup differences were compared using the Mann-Whitney test. * indicates p<0.05. ** indicates p<0.01. *** indicates p<0.001. ns indicates not significant.

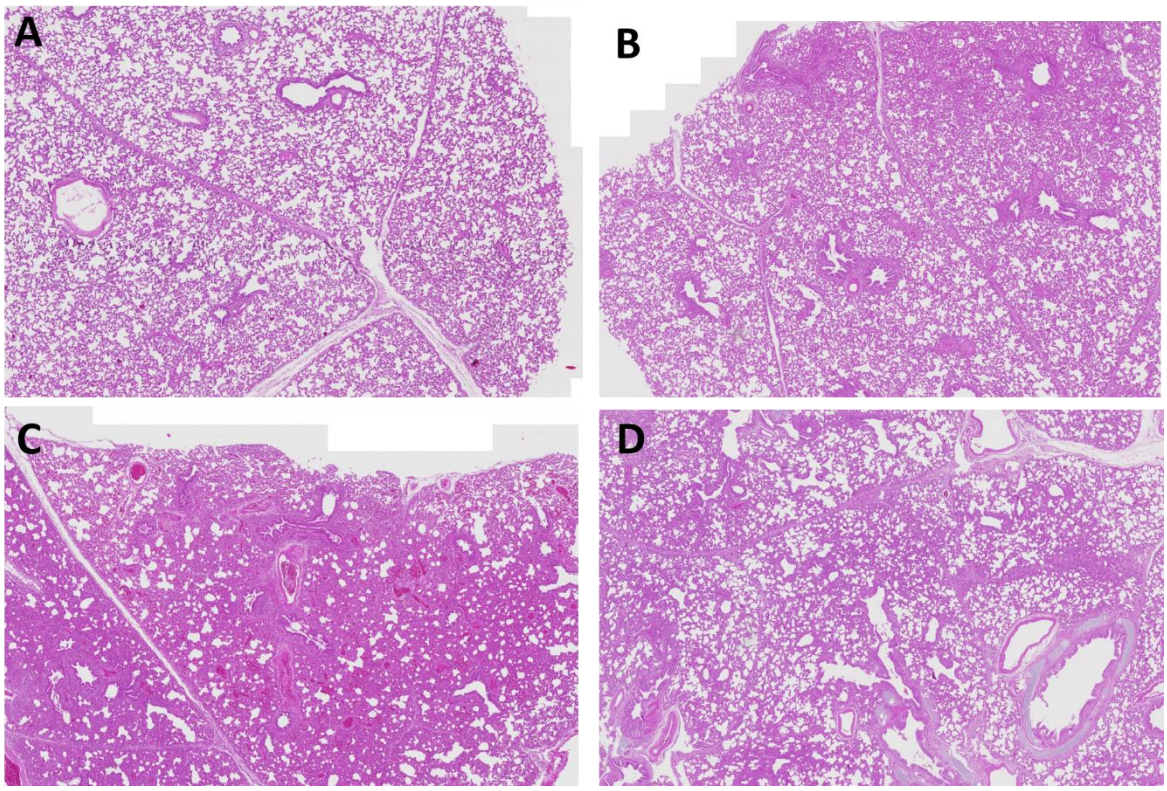


*Figure 26. Flagellin treatment prior to P. aeruginosa infection dampens lung inflammation in pigs. Flagellin was administered in the respiratory tract of pigs 24 hours prior to P. aeruginosa infection. Lung sections were stained 24 h after infection with hematoxylin & eosin. Images are shown of (A) PBS-treated and non-infected pig (PBS-PBS), (B) flagellin-treated and non-infected pig (flagellin-PBS), (C) PBS-treated and infected pig (PBS-PAK), and (D) flagellin-treated and infected pig (flagellin-PAK).*

**Table 3. Histological evaluation of pig lungs treated or not with flagellin before *Pseudomonas aeruginosa* infection.**

| | None or mild (%) | Medium (%) | Severe (%) | *p* value |
|---|---|---|---|---|
| **PBS-PAK** | 30.43 | 43.47 | 26.8 | <0.021 |
| **Flagellin-PAK** | 71.42 | 21.42 | 7.14 | |

Flagellin was administered in the respiratory tract of pigs 24 hours prior to *P. aeruginosa* infection. Lung sections from accessory, left caudal, left cranial, middle, right caudal and right cranial lobes were stained 24 h after infection with hematoxylin & eosin. This table shows the percentages of lung lobes classified according to lesion severity (n= 5 pigs). None or mild: histological score lower than 2; Medium: histological scores 2 and 3; Severe: histological score higher than 3. *p* values were calculated using the contingency chi-squared test.

# SECTION 3.3 PERSPECTIVES

The research work presented focused mainly in the study of the innate immune response in a biomedical model such as the CF pig. There has been some difficulties to build and continue the European Consortium on the CF pig model. In addition, this topic partially isolated my team from rest of the ISP unit. For that, it was decided that my team would merge with Dr Trapp's team, where I would continue my research on the modulation of innate immunity but targeting diseases of importance for both humans and livestock.

In this regard, we have previously mentioned PRDC. This is a multifactorial and complex disease caused by a combination of infectious pathogens causing important economic losses in the pig industry. [4]. SIAV, an epizootic/zoonotic pathogen causing respiratory infections and extensive morbidity and mortality in livestock and man, is one of the leading viral pathogens in PRDC. In addition, its relevance as a zoonotic pathogen is best illustrated by the central role of swine in the emergence of the 4 influenza pandemics of the $20^{th}/21^{st}$ century. Addressing the threat posed by swine influenza and/or SIAV-bacterial co-infections has been proven difficult, because currently available SIAV vaccines fail to provide protective immunity against all present virus clades. Confronted with this challenge, new immune stimulation strategies in the pig are gaining more and more importance [144]. Stimulation of innate immunity mobilizes multiple host defence mechanisms that accelerate clearance of microorganisms. Mucosal pre-treatment with the TLR5 ligand flagellin has demonstrated protective activity in bacterial and viral disease models in mice, which could be attributed to its pro-inflammatory activity and a positive effect on resident immune cells maturation. Although this effect could be a double-edge sword, since it could provoke excessive inflammation and tissue damage [48], flagellin pre-treatment seems also to reduce tissue damage due to the exacerbated inflammation [53], promoting full resolution of the process. A similar result have been observed using our pig model of CF, where flagellin pre-treatment was able to dampen inflammation and improve the lung status after *P. aeruginosa* infection. Our own preliminary experiments revealed that flagellin pre-stimulation increases cytokine secretion in vivo in pig lungs while it decreases the response to a subsequent inflammatory stimulus in airway epithelial cells by modulating NF-κB activation. Moreover, intranasal flagellin inoculation triggered the expression of antiviral genes in mice.

Studies defining the mechanisms used by the cells to process an inflammatory/infections stimulus are mostly based on bulk cell population analyses, failing to capture the complexity of the airways immune response [145]. This is clear in the case of SIAV infections. Upon infection, SIAV is sensed by infected cells via PRRs. Signaling by these receptors leads to the activation of the NF-κB and IRF3 transcription factors and the production of type I interferons and pro-inflammatory cytokines to combat and resolve infection. However, when unregulated, profuse and/or aberrant antiviral cytokine responses can also lead to severe immunopathologies [146]. The role of NF-κB in the cellular response to influenza virus infection is controversial, as anti- and pro-viral activities of this transcription factor have been described. **NF-κB activation is crucial in the onset of an antiviral innate immune response, but at the same time, its functions are hijacked by influenza viruses to promote viral replication**, which may increase the host susceptibility to concomitant bacterial infections (e.g. App) [5-7].

Despite the use of NF-κB inhibitors as anti-influenza drugs in pre-clinical trials [147], the mechanisms that dictate the anti- or proviral roles of NF-κB during influenza virus infection are still poorly understood. The airway epithelium, the principal target of SIAV, is a complex mixture of cell subtypes [148], and the inflammatory response to SIAV is heterogeneous. Thus,

an infected epithelium will present "infected" as well as "bystander" (exposed but uninfected) cells [145, 149], and population-level averages will likely miss critical information on the inflammatory response. In addition, recent data clearly indicates a crucial role of NF-κB oscillatory dynamics [37, 44] and the crosstalk with other signalling mechanisms, such as mitochondrial calcium [45, 150] as major determinants of the inflammatory response. Single cell approaches are a powerful mean to decode the mechanisms that modulate cell response during inflammation and can overcome the limits imposed by bulk population studies. It will lead to novel insights into the mechanisms used by the cell to determine the response to an infection. It will help to better design novel therapies, like flagellin administration, developing better and safer strategies to combat swine influenza and virus-mediated PRDC and provide a high level of protection in the absence of exacerbated inflammation.

Here, we **aim to use a multidisciplinary approach to bridge the knowledge gap between flagellin-induced changes in signal processing and the cellular response to SIAV and secondary bacterial infections**.

Our **hypothesis** predicts that flagellin stimulation preconditions airway epithelial and resident immune cells and helps to preserve mucosal homeostasis by modulating the cellular response to pathogenic stimulation. The **specific aims** of the project are:

1. To identify SIAV-mediated alterations in porcine epithelial cells that predispose the host to secondary bacterial infections.

2. To determine the signals triggered by flagellin pre-stimulation that play a role in the modulation of the immune response to pathogens.

3. To determine NF-κB and IRF3 signalling dynamics and oscillatory pattern alterations in influenza-infected pig cells and build a predictive mathematical model to understand the effect of flagellin pre-treatment on the cell response to pathogens.

The proposed project is based on the unique environment accessible at INRA Nouzilly that allows the development of single cell studies in large animal models of infection. It proposes a systems biology approach combining the use of a pig model of SIAV infection with single cell RNA-seq and NF-κB (master regulator of inflammation) single cell live imaging. This is a competitive interdisciplinary project aiming to decipher the mechanisms triggered by flagellin to modulate the immune response to SIAV. It integrates complementary "state of the art" approaches to perform research related to various scientific disciplines, spanning cell biology, virology, immunology, veterinary sciences and mathematics. The cross-talk between $Ca^{2+}$ signalling, TLR-signalling and the antimicrobial response is gaining relevance in recent years, but still remains largely unexplored. In addition, systems biology studies in livestock species are scarce. Most studies have been done in mice and human cell lines and focused on TNFα, and, more recently, on TLR4 signal transduction. However, TLR5 signalling dynamics at the single cell level have only been studied by the laboratory of the present project coordinator [142] (Annex 2). Better understanding TLR5 signalling dynamics and its regulation is of particular relevance due to the protective effect shown by flagellin against pathogen infections. We will use "state of the art" *in vivo*, *in vitro* and *ex vivo* models, as well as single cell technologies, which highlights the **novelty and originality** of the project.

**Work programme**

*WP 1: Determination of the single cell RNA "signature" of infected and bystander cells in the pig lungs.*

Pigs will be infected with SIAV (H3N2/Bissendorf03) and lungs collected at 2 and 5 days post-infection. Single cell RNA-seq will be performed using a procedure that was already validated in our lab in collaboration with Dr Barbry [148] (Annex 2). We will determine the signatures of infected cells [148], with the aim to determine the different RNA fingerprint between infected and bystander cells in the same animal.

*WP 2: Determination of cell signals triggered by flagellin that shape the immune response to SIAV.*

Recombinant flagellin will be produced and quality/control of purification and activity will be assessed *in vitro* using a Caco-CCL20-Luc reporter cell line. Different concentrations of recombinant flagellin will be used to determine the threshold of activation for the different TLR5 signalling pathways (MAPK, NF-κB) in differentiated primary airway epithelial cells in an air-liquid interface (AECs/ALI). Activation thresholds of gene expression and their dynamics will be determined using high throughput Fluidigm qRT-PCR. Changes in mitochondrial $Ca^{2+}$ will be measured in AECs/ALI as recently described [150]. Once the activation thresholds are determined, different flagellin inputs followed by SIAV infection will be used to determine the effect of flagellin pre-treatment on influenza virus infection. A mutant flagellin that does not activate TLR5 signalling will be used to assess the specificity of the response. Single-cell RNA-seq from AECs/ALI samples will be used to dissect the impact of the treatments on the multiple populations of resident cells. The effect of flagellin on viral replication and pro-apoptotic caspase activity will be assessed in AECs/ALI. Data from WP 1-2 will integrate a modelling and biomarker discovery pipeline as described in task 3.

*WP 3: Determination NF-κB and IRF3 signalling dynamics in SIAV infected cells.*

Traditional biochemical approaches can often misinterpret cellular signalling events that occur in dynamic and heterogeneous processes, such as the immune response [151]. In order to better understand the dynamic behaviour of the response to SIAV and the effect of flagellin, live single-cell confocal imaging will be used to determine oscillatory patterns of NF-κB and IRF3 [151] transcription factors, in AECs/ALI and the new-born porcine tracheal epithelial (NPTr) cell line. The latter is frequently used for studies on the interaction of mammalian/avian influenza A viruses with porcine host cells. Lentiviral transfection of AECs/ALI and/or chemical transfection of immortalized bronchial epithelial cells will be used to track NF-κB and IRF3 tagged with fluorescent markers. The role of flagellin pre-treatment and SIAV infection on the modulation of the cell response will be studied to determine alterations in the onset/reset of activation, the number of refractory cells and putative pathway cross-talks. Modelling would be done alongside experimental work with a view towards aiding experimental design. A stochastic mathematical model that captures the cell-to-cell variability in response will be built based on the previously published TLR3/RIG-I model [152]. Dose response data collected in WPs 1-3 will be used to constrain model parameters and to elucidate network topology and points of cross-talk. We will test *in silico* the activity of the molecules and genes involved in TLR3/RIG-I signalling in response to influenza to produce new insights into the alterations driven by flagellin pre-treatment. This data will serve to determine new molecular targets to test in WP 4. Sensitivity analyses will lead to insights on how levels and duration of stimuli determine lead to sustained inflammatory signalling responses and oscillatory dynamics. Together, this will enable identification of new potential molecular targets to improve immunity.

*WP 4: Determination of the mechanisms through which flagellin modulates the response to SIAV.*

Specific signalling pathways (e.g. p38, RelA, mitochondrial calcium uniporter) as wells as candidate molecules selected in WPs 1-3 will be pharmacologically inhibited or silenced in AECs/ALI or knocked-out in the NPTr cell line, to determine the mechanisms through which flagellin modulate the immune response to SIAV. Gene expression profiles, viral replication, caspase activity, calcium signalling will be assessed as described in WPs 1 and 2.

**Expected results**

This project will generate comprehensive knowledge on the mechanisms that regulate pig innate immunity, more specifically cell response to SIAV. In addition, the evaluation of TLR5 signalling is expected to allow us to identify new pathways and molecular targets that enhances pig immunity. Previous data suggest that flagellin can be used as a broad-spectrum therapy for multiple diseases, improving production yields and pig industry competitiveness, as well as reducing the use of antibiotics. The main expected results include:

- Identification of the specific "signature" of cells infected by SIAV.
- Determine TLR5-mediated MAPK and NF-κB activation thresholds, time to activation as well as its oscillatory dynamics in the airway epithelium. We will also determine TLR5-mediated MAPK and NF-κB signalling weight on the modulation of the response to SIAV, as well as the role of calcium on TLR5 signalling.
- Define the set of genes regulated flagellin that can influence the immune response to a pathogen.
- Generation of a mathematical model for TLR3 signalling. This should lead to a deeper understanding of the molecules regulating cell response to SIAV and develop new hypothesis for the identification of the most sensitive molecules that can be targeted by novel drugs.

**Project consortium**

**Ignacio Caballero** (coordinator, H-index=16), completed his PhD in February 2007. Leader of the Porcine Mucosal Immunology (PMI) team at the ISP unit of Centre **INRA Val de Loire** from September 2013. His research focuses in the evaluation of host-pathogen interactions and modulation of innate immunity in the pig, especially TLR, producing the first data available on TLR5 signalling on single cells[142]. The team has extensive experience in pig models, including transgenic pigs, CRISPR-Cas9, as well as all the molecular biology and cell culture techniques required. All the facilities and equipment required are present at INRA Val de Loire, including confocal imaging facilities, flow cytometry analyser, Biomark HD Fluidigm, pig facilities and local slaughterhouse.

**Rodrigo Guabiraba** (H-index=20), biologist, is a researcher at the UMR ISP unit, **INRA Val de Loire**. He has been developing strategies to unveil the participation of leukocytes and chemical mediators in the onset and resolution phases of inflammation for > 10 years. He will contribute to the understanding of cell signalling processes and cytokine regulation in the ex vivo experimental systems.

**Sascha Trapp** is head of the "Pathologie et Immunologie Aviaire" team at the UMR ISP (**INRA, Centre VdL**) and has >10 years' experience with viral epizootic diseases incl. avian influenza. He is co-coordinator of the EU H2020 infrastructure project VetBioNet (assembling most of Europe's leading research organisations in the field of livestock infectious diseases) and workpackage leader/partner in a number of European/national research projects related to avian influenza. To date, his team has authored > 15 articles on avian, human or swine influenza in internal peer-reviewed journals.

**Pablo Chamero** (H-index=20) is a researcher at the NICS team at **INRA, Val de Loire**, specialized in high-resolution, live-cell $Ca^{2+}$ imaging with publications in Nature, Cell, PNAS and Current Biology. They have established new imaging methods for many cell types in combination with virus-based expression systems in the context of $Ca^{2+}$ measure in organelles. They are currently using a novel $Ca^{2+}$ ratiometric sensor, with a broad $Ca^{2+}$ affinity range to target specific organelles [153].

**Pascal Barbry** (180 publications, 4 patents, 9200 citations, H-index = 53) is the creator (1999) and head of the group "Physiological Genomics of the Eukaryotes", of the UCAGenomiX platform, and one of the creators of the national infrastructure France Génomique. Director of the Institut de Pharmacologie Moléculaire et Cellulaire in Sophia Antipolis (19 research groups, 220 people) from 2004-2017. His group has developed an expertise in single cell RNA seq and bioinformatics, being the first (and so far only) French recipient of a Chan Zuckerberg Foundation grant, in the context of the Human Cell Atlas. He already validated with Dr Caballero the protocols to perform single cell RNAseq on pig lung tissue.

**Jean-Claude Sirard** (76 publications, 5 patents, 3800 citations, H-index= 30), principal investigator in Team "Lung Infection and Innate Immunity", **Institut Pasteur de Lille** (http://www.ciil.fr/recherche/l3i/). He is an expert on innate immunity and particularly flagellint-TLR5 interaction and the development of immune-interventions to induce protection against infection. He will provide flagellin and its mutant variants.

**Christopher Sanderson** (H-index=24**),** Professor at the **University of Liverpool**. He is expert in mammalian protein-protein interaction networks and quantitative microscopy approaches. He leads an interdisciplinary team, including researcher James Boyd who has collaborated with the coordinator, and will provide additional imaging analysis support and will build the mathematical models.

**Experimental infectious disease platform (PFIE):** The PFIE experimental unit, part of INRA Val de Loire, is the largest European experimental facility for infectious diseases in livestock animals. It provides BSL-2 and BSL-3 containment installations and present a large experience in pig models of influenza infection[154, 155]. This resource is open to other teams as part of a European Network of experimental facilities.

**Means available to achieve the objectives**

All the facilities and equipment required for the project are present in the consortium, including: **Pig facilities and local slaughterhouse** to obtain the biological material required. L2/L3 cell culture rooms. **Confocal microscopy** Leica SP8 with controlled temperature and incubation chamber in L2 conditions. **Flow cytometry** facilities including FACs analyser and MoFlo Astrios sorter (Beckman Coulter), equipped with 4 lasers and installed in laminar flow hood. Production-scale throughput gene expression analyser (**Biomark HD Fluidigm**). An imaging room dedicated to fluorescent microscopy and imaging is available. In this room, a **Ca-imaging**

**set-up** (Olympus IX-71) inverted microscope-imaging workstation, consisting on an inverted microscope allowing fluorescence videomicroscopy and auxiliary equipment for cell picking such as electrode puller and microforge, tissue preparation area and a small electronics bench. *In vivo* experimentations will be performed at the **PFIE that provides level A2 and A3 containment** installations for livestock animals including equipment for large animals anaesthesia and drug delivery into the lungs. **Single cell RNA-seq** will be performed at Pascal Barbry's team at the IPMC, Sophia-Antipolis functional genomics platform. This platform has developed an expertise in single cell RNA-seq since 2016[148, 156-158]. It provides the following equipment: 10xGenomics Chromium, Fluidigm C1, Fluidigm Biomark and Illumina NextSeq500 for sequencing. IPMC contributes to the Human Lung Atlas project (CZI-funded Human Lung Seed Network) and the H2020-funded Discovair project. A dedicated single-cell equipment will be intstalled in a BSL3 laboratory to allow the analysis of influenza-infected samples. Samples collected in Nouzilly will be carried at 4°C in Hypothermasol, as proposed by Meyer et al[159], using a established procedure between partners.

**Impact**

By 2050, the world population will increase to more than nine billion people. Despite the technological advances and increase in agricultural productivity, extreme hunger and malnutrition remain a huge barrier to development in many countries. The UN Sustainable Development Agenda aims to ensure access to safe and sufficient food in order to end all forms of hunger and malnutrition as part of the "Zero Hunger" goal. Ensuring food availability in a perspective of a safe and sustainable bioeconomy is also a major objective in the H2020 programme "Societal Challenge- Food security, sustainable agriculture and forestry" and the challenge 5 "Securité Alimentaire et Défi Démographique" from the ANR 2018 work programme (WP). This project addresses these issues by identifying new alternative strategies that increases pig resistance to disease. The project will identify new pathways and molecular targets that enhances pig immunity and resistance to disease, improving production yields and pig industry competitiveness.

The project proposed will have an important impact at the scientific level, delivering new and important knowledge in the molecular mechanisms that control pig immunity that can translate to humans. Moreover, it will deepen our knowledge of a new mechanisms of pathway cross-talk between TLR and $Ca^{2+}$ signalling, which have not been much studied. The mathematical model developed as well as RNA-seq the data will be public in specialized databases and come become an important research tool for the scientific community. Altogether, the results produced by this project will produce novel pharmacological targets that can become of interest for the development of new research lines. Although we focus in a specific pig disease, it is noteworthy that boosting innate immunity can provide general protection against diverse pathogens.

In addition, this multidisciplinary project, funded by the ANR, will help to develop my research career. It will consolidate a research network for the development of systems biology in livestock, aiming to tackle important biological questions using original approaches that are not common in animal production. We will publish the results obtained in the highest ranked journals. As a young researcher, high quality publications delivered by this project, the leadership obtained and the network established will increase the coordinator's chance to succeed in highly competitive calls such as those from the European Research Council. It is expected that this project will provide the perfect opportunity and appropriate framework to supervise and train new PhD and postdoctoral students.

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# Section 4: Annex

# SECTION 4.1 SYNTHÈSE EN FRANÇAIS

Il est prévu que la population mondiale atteigne plus de 9 milliards de personnes d'ici 2050. Fournir des ressources alimentaires animales tout en respectant les exigences sociétales en matière de sécurité alimentaire et de bien-être des animaux est une priorité du programme de développement durable des Nations Unies. La viande de porc représente plus d'un tiers de la viande produite dans le monde. Elle est une composante majeure de la sécurité alimentaire, des économies agricoles et du commerce mondial (22,1 millions de tonnes de viande porcine produites dans l'Union Européenne en 2014, soit la production la plus élevée de toutes les espèces de bétail). La demande de viande porcine a entraîné une intensification de la production, les exploitations agricoles abritant souvent des milliers d'animaux dans des densités propices à une transmission rapide des agents pathogènes. Les maladies infectieuses entraînent des pertes directes pour la production animale par la mortalité, la perte de productivité, les restrictions commerciales, la réduction de la valeur marchande et souvent l'insécurité alimentaire. Parmi ces maladies, le complexe des maladies respiratoires porcines (PRDC) est une cause de perte économique massive pour les industries porcines du monde entier. Le PRDC résulte d'une combinaison de stress environnemental et d'infection des voies respiratoires, le virus de la grippe porcine A (SIAV) est le principal agent pathogène viral.

Outre sa valeur en tant qu'animal d'élevage, le porc a connu ces dernières années une augmentation considérable de l'intérêt qu'il suscite en tant que modèle biomédical. Les porcs sont devenus un modèle de recherche attrayant dans le domaine de la formation aux procédures chirurgicales, de la progression et de la pathologie des maladies, de la recherche translationnelle et de la médecine régénératrice / thérapie par cellules souches, en raison de leurs similitudes anatomiques, génétiques et physiologiques avec les humains. En outre, la disponibilité de la séquence du génome du porc, le développement du transfert de noyaux de cellules somatiques et d'outils d'édition du génome à haute efficacité tels TALENs et CRISPR-Cas9, ont permis une ingénierie génétique précise et efficace chez le porc. La mucoviscidose est un exemple clair du porc comme modèle biomédical. La mucoviscidose, la maladie génétique mortelle la plus courante dans la population caucasienne, est un trouble génétique récessif causé par des mutations du gène *CFTR*, rendant la protéine non fonctionnelle. Il a été démontré que CFTR fonctionne comme un canal anionique régulé à la surface de la cellule apicale. Les mutations de CFTR affectent la rhéologie des sécrétions, qui deviennent épaisses et difficiles à éliminer des voies respiratoires. Ces conditions conduisent à une infection bactérienne chronique, souvent dominée par *Pseudomonas aeruginosa* (*P. aeruginosa*), à une inflammation persistante avec un recrutement exacerbé de neutrophiles polymorphonucléaires (PMN) dans les poumons, à une libération excessive de protéases et finalement à la destruction des tissus pulmonaires. La compréhension des processus conduisant à cette réponse inflammatoire altérée est cruciale pour améliorer les thérapies de la mucoviscidose.

Depuis que le gène CFTR a été identifié et isolé pour la première fois en 1989, plusieurs souches de souris transgéniques ont été générées afin d'étudier la physiopathologie de la mucoviscidose. Toutefois, leur utilité a été limitée par l'absence d'un phénotype dans les voies respiratoires qui imite les complications observées chez l'homme. Plus récemment, cet inconvénient a été résolu par le développement d'un porc $CFTR^{\Delta F508/\Delta F508}$ muté et d'un porc $CFTR^{-/-}$ knockout. Ce dernier modèle de porc atteint de mucoviscidose développe une pathologie intestinale (iléus méconial, microcolon) et pulmonaire similaire aux altérations décrites chez les patients

mucoviscidosiques. Ces caractéristiques font du porc atteint de mucoviscidose un modèle très précieux pour étudier la pathogénie de la mucoviscidose et évaluer de nouvelles thérapies.

La modulation de l'immunité innée a été identifiée comme une stratégie prophylactique et/ou thérapeutique prometteuse pour combattre les maladies infectieuses en raison de leur capacité à activer les mécanismes immunitaires de l'hôte avec un large spectre de protection. Une compréhension approfondie des mécanismes qui contrôlent la nature et l'intensité de la réponse immunitaire innée serait cruciale pour améliorer les thérapies contre la mucoviscidose, ainsi que pour lutter contre les infections virales et bactériennes chez le porc. Au cours des dernières années, mes recherches ont été consacrées à l'étude de l'immunité innée et des interactions hôte-pathogène chez le porc, avec un accent particulier sur le modèle du porc atteint de mucoviscidose et la signalisation du récepteur type Toll (TLR).

Les objectifs spécifiques de mes recherches ont été les suivants :

1. Développement d'un modèle expérimental d'infection à *P. aeruginosa* chez le porc.

2. Développement d'un modèle de porc atteint de mucoviscidose à l'INRAe, centre du Val de Loire.

3. Déterminer s'il existe des altérations intrinsèques dans la réponse immunitaire innée du modèle porcin de la mucoviscidose qui sont responsables du développement de la maladie pulmonaire de la mucoviscidose.

4. Développement de nouvelles thérapies ciblant l'immunité innée.

## SYNTHÈSE DE L'ACTIVITÉ DE RECHERCHE

### Développement d'un modèle expérimental d'infection à Pseudomonas aeruginosa chez le porc

*Pseudomonas aeruginosa*, une bactérie qui infecte rarement les poumons humains à moins que le système immunitaire de l'hôte ne soit affaibli, est l'un des principaux agents pathogènes que l'on trouve dans la mucoviscidose, la COPD et la VAP. L'une des caractéristiques de l'infection à *P. aeruginosa* dans la mucoviscidose est le recrutement massif de neutrophiles dans les poumons. Au cours de ce processus, les neutrophiles sécrètent de grandes quantités de sérine-protéases neutrophiles (PSN : élastase neutrophile (NE), protéase 3 (Pr3) et cathepsine G (cat G)) qui dépassent la capacité des antiprotéases endogènes à contrôler leur activité, entraînant finalement la destruction du tissu pulmonaire. Les traitements anti-infectieux et/ou anti-inflammatoires doivent être testés sur un modèle animal approprié. Les modèles de souris ne reproduisent pas plusieurs aspects des maladies pulmonaires humaines. Cela est particulièrement vrai pour la mucoviscidose, qui a conduit la communauté scientifique à rechercher de nouveaux modèles animaux. Nous avons montré que les souris ne sont pas appropriées pour la caractérisation des médicaments ciblant l'inflammation dépendante des neutrophiles et que les neutrophiles porcins et leurs PSN sont similaires à leurs homologues humains. Nous avons induit des réponses inflammatoires neutrophiles aiguës dans les poumons de porcs en utilisant *P. aeruginosa*, un pathogène respiratoire opportuniste. Des échantillons de sang, des écouvillons nasaux et des liquides de lavage bronchoalvéolaires (LBA) ont été prélevés à 0, 3, 6 et 24 heures après l'infection (p.i.) et les paramètres biochimiques, les cytokines sériques et les LBA, les cultures bactériennes et l'activité des neutrophiles ont été

évalués. La libération de médiateurs pro-inflammatoires, les paramètres biochimiques et hématologiques du sang, le recrutement cellulaire et la réactivité bronchique ont atteint un pic 6 heures après l'injection. Nous avons également utilisé des substrats synthétiques spécifiques pour les protéases des neutrophiles humains afin de montrer que l'activité des PSN porcins dans les BALF a augmenté. Ces protéases ont également été détectées à la surface des neutrophiles pulmonaires à l'aide d'anticorps anti-NSP humains. L'infection pulmonaire induite par *P. aeruginosa* chez les porcs entraîne une réponse neutrophile similaire à celle décrite pour la mucoviscidose et la pneumonie sous ventilation chez l'homme. Dans l'ensemble, cela indique que le porc est un modèle approprié pour tester des médicaments anti-infectieux et/ou anti-inflammatoires pour combattre les effets protéolytiques néfastes des neutrophiles dans les maladies pulmonaires humaines.

**Développement d'un modèle de porc atteint de mucoviscidose**

Une fois que nous avons développé un modèle de l'infection à *P. aeruginosa*, nous avons dû établir le modèle du porc atteint de mucoviscidose à l'INRAe, Centre du Val de Loire. Des porcs transgéniques $CFTR^{+/-}$ mâles et femelles ont été déplacés de la LMU de Munich (Allemagne) à l'INRAe de Nouzilly (France) et accouplés pour générer des porcelets $CFTR^{+/+}$, $CFTR^{+/-}$ et $CFTR^{-/-}$. Cependant, les porcs $CFTR^{-/-}$ présentent une prévalence de 100 % d'iléus méconial qui entraîne la mort dans les premières heures suivant la naissance, ce qui nécessite un diagnostic rapide et une intervention chirurgicale pour soulager l'obstruction intestinale. L'identification des porcelets $CFTR^{-/-}$ est généralement réalisée par génotypage PCR, une procédure qui dure entre 4 et 6 h. Nous avons donc cherché à développer une procédure d'identification rapide des porcelets CFTR-/- qui permettra de les placer en soins intensifs peu après la naissance et de procéder immédiatement à la correction chirurgicale. Nous avons tenté de réduire le temps de passage de la naissance à la chirurgie des porcelets $CFTR^{-/-}$ nouveau-nés en utilisant l'imagerie par tomodensitométrie comme outil de diagnostic. L'imagerie par tomodensitométrie est un outil très sensible et spécifique pour détecter la présence d'un infarctus intestinal. La tomodensitométrie présente une sensibilité de 94,4 % pour diagnostiquer les porcelets $CFTR^{-/-}$. Le diagnostic par tomodensitométrie a permis de réduire le temps de passage de la naissance à la chirurgie d'un minimum de 10 h à un minimum de 2,5 h et d'augmenter la survie des porcelets CFTR-/- à un maximum de 13 jours après la chirurgie, contre seulement 66 h après une chirurgie ultérieure. Malgré les progrès réalisés dans l'utilisation de la tomodensitométrie pour le diagnostic et les soins précoces des porcs atteints de mucoviscidose, l'espérance de vie reste trop faible pour permettre le développement d'études chroniques sur l'infection et l'inflammation au cours de la vie. Toutefois, ce modèle représente un outil très précieux pour étudier la pathogénèse précoce de la mucoviscidose, notamment les altérations de l'environnement pulmonaire qui peuvent favoriser la colonisation bactérienne, et les défauts intrinsèques du système immunitaire inné, y compris la réponse TLR et les altérations des neutrophiles.

**Évaluation des altérations intrinsèques de la réponse immunitaire innée du porc atteint de mucoviscidose**

Les difficultés rencontrées pour maintenir le porc atteint de mucoviscidose en bonne santé pendant une longue période ont rendu impossible le développement d'études sur l'infection chronique par *P. aeruginosa* chez ces animaux. Malgré cet inconvénient majeur, les porcs atteints de mucoviscidose représentent un outil important pour comprendre les origines de la maladie pulmonaire de la mucoviscidose. La compréhension des défauts de défense initiaux de l'hôte dans les voies respiratoires de la mucoviscidose pourrait suggérer de nouvelles préventions et de nouveaux traitements, ainsi que les moyens d'évaluer le statut de la maladie

et l'efficacité des thérapeutiques. Afin de déterminer s'il existe des altérations importantes de la réponse immunitaire innée aux agents pathogènes de la mucoviscidose, nous avons concentré nos études sur 1) les neutrophiles périphériques à la naissance ; 2) les altérations de l'environnement pulmonaire à la naissance et sa réponse à un défi pathogène.

Altérations intrinsèques des neutrophiles périphériques chez les porcelets nouveau-nés atteints de mucoviscidose

La caractéristique de la maladie pulmonaire liée à la mucoviscidose est un environnement pulmonaire dominé par les neutrophiles qui est associé à la destruction chronique du tissu pulmonaire et, en fin de compte, à la mort du patient. Il n'est pas clair si l'exacerbation de la réponse des neutrophiles est principalement liée à un CFTR défectueux ou plutôt secondaire à une colonisation bactérienne chronique et à une inflammation. Ici, nous avons émis l'hypothèse que les neutrophiles du sang périphérique de la mucoviscidose présentent une altération intrinsèque à la naissance avant le début d'un processus inflammatoire. Les neutrophiles du sang périphérique ont été isolés chez des porcelets $CFTR^{+/+}$ et $CFTR^{-/-}$ nouveau-nés et caractérisés respectivement par chromatographie liquide/spectrométrie de masse en tandem (LC-MS/MS), désorption laser/spectrométrie de masse par ionisation assistée par matrice de cellules intactes (ICM-MS) suivie d'une spectrométrie de masse à haute résolution (HRMS) de haut en bas. La capacité des neutrophiles de porc atteint de mucoviscidose à tuer *P. aeruginosa* a également été évaluée.

L'analyse des métabolites d'acides gras polyinsaturés n'a montré aucune différence entre les neutrophiles $CFTR^{+/+}$ et $CFTR^{-/-}$. En revanche, un modèle mathématique prédictif basé sur le profil protéomique ICM-MS a permis de distinguer les deux génotypes. Une analyse protéomique descendante a permis d'identifier des masses différentiellement abondantes de 19 m/z qui correspondaient principalement à des protéines liées à la réponse antimicrobienne et à la génération d'espèces réactives de l'oxygène (ROS). Cependant, aucune altération de la capacité des neutrophiles $CFTR^{-/-}$ à tuer *P. aeruginosa* in vitro n'a été observée. En conclusion, l'ICM-MS a démontré que les neutrophiles $CFTR^{-/-}$ présentent des altérations intrinsèques dès la naissance, avant la présence de toute infection ou inflammation.

Détermination des altérations intrinsèques dans l'environnement pulmonaire des porcs atteint de la mucoviscidose qui favorisent l'infection bactérienne

Malgré les progrès réalisés dans la prise en charge de la maladie respiratoire de la mucoviscidose, son origine est encore largement débattue. Un lien entre le dysfonctionnement de CFTR, une fonction mucociliaire défectueuse et le déclin de la fonction respiratoire a constamment été émise. La colonisation bactérienne dans les poumons de patients atteint de la mucoviscidose a été directement associée à la perte de la fonction CFTR, et/ou secondairement liée à des cycles répétitifs d'inflammation/infection chronique. Nous avons émis l'hypothèse que l'altération des propriétés moléculaires des mucines pourrait contribuer à ce processus. Les $CFTR^{+/+}$ et $CFTR^{-/-}$ du nouveau-né ont été sacrifiés avant et 6h après l'inoculation de *P. aeruginosa* luminescent dans la carène trachéale. La muqueuse trachéale et le liquide de lavage bronchoalvéolaire (LBA) ont été prélevés pour déterminer le niveau d'O-glycosylation de la mucine, les bactéries se liant aux mucines et le transcriptome des voies respiratoires. Les perturbations du transport mucociliaire ont été déterminées par l'imagerie *ex-vivo* de *P. aeruginosa* luminescent.

Nous fournissons des preuves d'une sialylation accrue des mucines des voies respiratoires des porcs atteint de mucoviscidose et d'une perturbation du transport mucociliaire qui se produisent

avant le début de l'inflammation. L'hypersialylation des mucines a été reproduite sur des explants trachéaux provenant d'animaux non atteints de mucoviscidose traités avec GlyH101, un inhibiteur de l'activité du canal CFTR, indiquant une relation de cause à effet entre l'absence d'expression de CFTR et la sialylation des mucines. Cette sialylation accrue a été corrélée à une adhérence accrue de *P. aeruginosa* aux mucines. L'infection *in vivo* de porcelets atteint de mucoviscidose nouveau-nés par *P. aeruginosa* vivant et luminescent a démontré une altération du transport mucociliaire de cette bactérie, sans aucune preuve d'inflammation préexistante. Nos résultats documentent pour la première fois dans un modèle animal de mucoviscidose bien défini les modifications qui affectent les chaînes de mucines O-glycanes. Ces modifications précèdent l'infection et l'inflammation des tissus des voies respiratoires, et fournissent un contexte favorable au développement microbien dans le poumon atteint de mucoviscidose qui caractérise cette maladie.

Développement de nouvelles thérapies ciblant l'immunité innée chez la mucoviscidose

Nos observations précédentes ont montré que l'environnement pulmonaire de la mucoviscidose favorise la colonisation bactérienne dès la naissance. Cela pourrait être l'un des mécanismes qui déclenchent la colonisation bactérienne chronique des poumons et l'inflammation qui est une caractéristique classique de la maladie. Il serait essentiel de comprendre les processus qui conduisent à un environnement inflammatoire pulmonaire excessif pour améliorer les traitements de la mucoviscidose. Les TLR sont la clé d'une réponse immunitaire équilibrée. Plusieurs études ciblent les TLR comme stratégie prophylactique et/ou thérapeutique prometteuse pour lutter contre les maladies infectieuses, car la modulation de leur fonction et de leur réactivité aux agents pathogènes pourrait améliorer l'immunité innée. Dans le contexte de la mucoviscidose, les TLR4 et TLR5, qui interagissent respectivement avec le LPS et la flagelline, sont connues pour être des facteurs majeurs de la réponse immunitaire à *P. aeruginosa*. En particulier, la réponse de TLR5 à la flagelline a été suggérée comme jouant un rôle dans l'inflammation excessive observée dans les cellules épithéliales des voies aériennes atteint de mucoviscidose et, par conséquent, elle est une cible candidate pour les thérapies anti-inflammatoires.

Nous avons utilisé un modèle de porc atteint de mucoviscidose nouveau-né pour étudier les altérations intrinsèques de la réponse immunitaire innée de l'épithélium des voies respiratoires. Les cellules épithéliales des voies aériennes (AEC) et les tranches de poumon coupées avec précision (PCLS) ont été stimulées avec de la flagelline ou un lipopolysaccharide (LPS) comme témoin pour déterminer les réponses spécifiques pour TLR5 et TLR4, respectivement. Ensuite, nous avons évalué les propriétés immunomodulatrices de la flagelline *in vitro* et *in vivo* dans un modèle porcin d'infection par *P. aeruginosa*. Nous avons observé une augmentation significative de la sécrétion de cytokines lorsque les AEC de porcs mucoviscidosiques étaient stimulées par la flagelline par rapport aux AEC de type sauvage (WT). Ces résultats ont été répétés lorsque les AEC ont été traitées avec un inhibiteur de l'activité du canal CFTR. Le prétraitement à la flagelline a permis de réduire la réponse immunitaire à *P. aeruginosa* à la fois dans les AEC des porcs sauvage et dans les PCLS de porcs atteint de mucoviscidose. En outre, l'inoculation *in vivo* de flagelline dans les poumons de porc a diminué la réponse inflammatoire à *P. aeruginosa*, diminuant la gravité de la pneumonie pulmonaire causée par l'infection. En conclusion, nos données montrent que le TLR5 est intrinsèquement défectueux dans la mucoviscidose à la naissance. La modulation de la signalisation TLR5 pourrait contribuer à mieux contrôler la réponse inflammatoire excessive observée dans les poumons mucoviscidosiques.

**PERSPECTIVES**

Au cours de mon activité de recherche, je me suis concentré sur l'étude de l'immunité innée dans un modèle de porc transgénique et sur l'utilisation de nouvelles stratégies pour moduler la réponse immunitaire innée. Ces stratégies ne sont pas limitées au modèle de porc atteint de mucoviscidose, puisque la stimulation de la réponse immunitaire innée pourrait servir de thérapie à large spectre dans différentes maladies infectieuses. Le virus de la grippe porcine A (SIAV) est une maladie difficile à combattre. Le SIAV prédispose aux maladies respiratoires porcines complexes, ce qui entraîne d'énormes pertes économiques. Il s'est avéré très difficile de faire face à cette menace, car les vaccins contre le SIAV ne procurent pas d'immunité stérilisante. En cas d'infection, le SIAV peut détourner la machinerie cellulaire de l'hôte (c'est-à-dire la signalisation NF-κB) à son propre profit, modifiant la réponse inflammatoire et augmentant le risque d'infections secondaires. Cependant, les mécanismes par lesquels le SIAV modifie l'homéostasie des cellules ne sont pas totalement compris.

La stimulation par la flagelline, un agoniste spécifique du système immunitaire inné, est apparue comme une stratégie alternative possible pour augmenter la résistance aux maladies. Nous proposons une approche de biologie systémique pour déterminer les facteurs qui contrôlent la modulation du système immunitaire induite par la flagelline et la résistance aux maladies. La plupart des études sont basées sur des analyses de population en masse qui font la moyenne des réponses cellulaires des cellules infectées et non infectées, ce qui entraîne la perte d'informations essentielles. Les approches « single cell » et la modélisation mathématique sont un moyen puissant de décoder les mécanismes qui modulent la réponse cellulaire pendant l'inflammation et peuvent surmonter les limites imposées par les études de population en masse. Notre projet s'appuie sur l'environnement unique accessible à l'INRAe de Nouzilly qui permet le développement d'études « single cell »chez les grands modèles animaux d'infection. Il propose une approche de biologie systémique combinant l'utilisation d'un modèle porcin d'infection par le SIAV avec le séquençage d'ARN à cellule unique et l'imagerie de NF-κB (régulateur majeur de l'inflammation). Le rôle de la flagelline en tant que modulateur de l'immunité innée sera également étudié. Cette combinaison unique d'un modèle infectieux très pertinent et d'approches "de pointe" nous permettra d'étudier les altérations induites par le SIAV qui conduisent à une réponse inflammatoire défectueuse. Elle permettra d'acquérir des connaissances approfondies sur les mécanismes qui régulent la réponse inflammatoire à la grippe et d'identifier de nouvelles voies cellulaires et cibles moléculaires qui pourraient servir à mettre au point de nouvelles thérapies.

# SECTION 4.2 PUBLICATION ABSTRACTS

**Submitted to European Respiratory Journal (ERJ-00477-2020)**

**Title (90 characters)**

**Flagellin administration modulates defective TLR5 signalling in cystic fibrosis airways**

Isabelle Fleurot[1#], Raquel López-Gálvez[1#], Sandrine Melo[1], Michel Olivier[1], Claire Chevaleyre[1], Céline Barc[2], Mickael Riou[2], Mustapha Si-Tahar[3,4], Pascal Barbry[5], Andrea Bähr[6], Nikolai Klymiuk[6], Jean-Claude Sirad[7], Ignacio Caballero[1*]

[1]INRAE, Université de Tours, UMR-1282 Infectiologie et Santé Publique (ISP), Centre de Recherche Val de Loire, 37380 Nouzilly, France;

[2]INRAE, UE-1277 Plateforme d'infectiologie expérimentale (PFIE), Centre de Recherche Val de Loire, 37380 Nouzilly, France;

[3]Université de Tours, 37000 Tours, France;

[4]INSERM U1100, Centre d'étude des pathologies respiratoires (CEPR), 37000 Tours, France;

[5]Université Côte d'Azur, CNRS, Institut de Pharmacologie Moléculaire et Cellulaire, F06560 Sophia Antipolis, France;

[6]CIMM-Gene Center and Center for Innovative Medical Models, LMU Munich, Germany

[7]Univ. Lille, CNRS, INSERM, CHU Lille, Institut Pasteur de Lille, U1019 – UMR9017 - CIIL - Center for Infection and Immunity of Lille, F-59000 Lille, France

#These authors contributed equally to the work.

*Correspondence and requests for reprints should be addressed to Ignacio Caballero, UMR1282 Infectiologie et Santé Publique, INRA – Centre Val de Loire, Route de Crotelles, ISP311, 37380, Nouzilly, France; Tel: +33 247427865; Email: ignacio.caballero-posadas@inrae.fr

**Take home message (256 characters):**

TLR5 signalling is defective in CF, contributing to the development of the CF lung hyper-inflammatory environment. Modulation of TLR5 signalling by flagellin decreases excessive inflammation after *pseudomonas aeruginosa* infection, restoring lung function.

**Submitted to Journal of Cystic Fibrosis (JCF-D-20-00032)**

# Evidence of early increased sialylation of airway mucins and defective mucociliary clearance in CFTR-deficient piglets

Ignacio Caballero[a#], Bélinda Ringot-Destrez[b#], Mustapha Si-Tahar[c,d], Pascal Barbry[e], Antoine Guillon[c,d,f], Isabelle Lantier[a], Mustapha Berri[a], Claire Chevaleyre[a], Isabelle Fleurot[a], Céline Barc[g], Reuben Ramphal[c,d], Nicolas Pons[e], Agnès Paquet[e], Kévin Lebrigand[e], Carole Baron[e], Andrea Bähr[h], Nikolai Klymiuk[h], Renaud Léonard[b], Catherine Robbe-Masselot[b*]

[a]INRAE, Université de Tours, ISP, F-37380, Nouzilly, France;

[b]Univ.lille, CNRS, UMR8576-UGSF-Unité de Glycobiologie Structurale et Fonctionnelle, F59000 Lille, France;

[c]Université de Tours, 37000 Tours, France;

[d]INSERM U1100, Centre d'étude des pathologies respiratoires (CEPR), 37000 Tours, France;

[e]Université Côte d'Azur, CNRS, Institut de Pharmacologie Moléculaire et Cellulaire, F06560 Sophia Antipolis, France;

[f]CHU Tours, service de Médecine Intensive-Réanimation, Tours, France

[g]Plateforme d'Infectiologie expérimentale (UE-1277 PFIE), INRA, Nouzilly, France;

[h]CIMM-Gene Center and Center for Innovative Medical Models, LMU Munich, Germany

#These authors contributed equally to the work.

*Correspondence and requests for reprints should be addressed to Catherine Robbe-Masselot, Unité de Glycobiologie Structurale et Fonctionnelle, Campus CNRS de la Haute Borne, 50 avenue de Halley, 59658 Villeneuve d'Ascq, France; Tel: +33 362531719; Fax: +33 320436555; Email: catherine.robbe-masselot@univ-lille.fr

## Highlights:

- CFTR-deficient pigs present increased lung mucin sialylation at birth in the absence of inflammation
- Increased mucin sialylation is linked to increased *Pseudomonas aeruginosa* adhesion to mucins
- CF pigs also present a mucociliary clearance defect, in the absence of inflammation, which may contribute bacterial colonization and development of early lung disease. These defects seems to be linked to the absence of CFTR functionality since they are independent of an inflammatory environment in the lungs

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Original Article

# CFTR-deficient pigs display alterations of bone microarchitecture and composition at birth

Julien Braux[a], Marie-Laure Jourdain[a], Christine Guillaume[a], Valérie Untereiner[b], Olivier Piot[c], Andrea Baehr[d], Nikolai Klymiuk[d], Nathalie Winter[e], Mustapha Berri[e], Dominique Buzoni-Gatel[e], Ignaccio Caballero[e], Antoine Guillon[f], Mustapha Si-Tahar[f], Jacky Jacquot[a,*], Frédéric Velard[a,*]

[a] Université de Reims Champagne Ardenne, BIOS EA 4691, Biomatériaux et Inflammation en site osseux, SFR CAP-Santé (FED 4231), 1, Avenue du Maréchal Juin, 51097 Reims, France
[b] Université de Reims Champagne Ardenne (URCA), PICT Platform, Reims, 1, Avenue du Maréchal Juin, 51097 Reims, France
[c] Université de Reims Champagne-Ardenne, BioSpecT (Translational BioSpectroscopy) EA 7506, 1, Avenue du Maréchal Juin, 51097 Reims, France
[d] Institute of Molecular Animal Breeding and Biotechnology, Gene Center, Ludwig-Maximilians-Universitat Munchen, Hackerstrasse 27, 85764, Oberschleissheim, Germany
[e] INRA, UMR1282 ISP, Centre de recherches INRA Val de Loire, 37380, Nouzilly, France
[f] Inserm, Centre d'Etude des Pathologies Respiratoires, UMR1100/EA6305, 10 Boulevard Tonnellé, 37032, Tours, France

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ABSTRACT

*Background:* The lack of cystic fibrosis transmembrane conductance regulator (CFTR) function causes cystic fibrosis (CF), predisposing to severe lung disease, reduced growth and osteopenia. Both reduced bone content and strength are increasingly recognized in infants with CF before the onset of significant lung disease, suggesting a developmental origin and a possible role in bone disease pathogenesis. The role of CFTR in bone metabolism is unclear and studies on humans are not feasible. Deletion of CFTR in pigs (*CFTR* $^{-/-}$ pigs) displays at birth severe malformations similar to humans in the intestine, respiratory tract, pancreas, liver, and male reproductive tract.

*Methods:* We compared bone parameters of *CFTR* $^{-/-}$ male and female pigs with those of their wild-type (WT) littermates at birth. Morphological and microstructural properties of femoral cortical and trabecular bone were evaluated using micro-computed tomography ($\mu$CT), and their chemical compositions were examined using Raman microspectroscopy.

*Results:* The integrity of the *CFTR* $^{-/-}$ bone was altered due to changes in its microstructure and chemical composition in both sexes. Low cortical thickness and high cortical porosity were found in *CFTR* $^{-/-}$ pigs compared to sex-matched WT littermates. Moreover, an increased chemical composition heterogeneity associated with higher carbonate/phosphate ratio and higher mineral crystallinity was found in *CFTR* $^{-/-}$ trabecular bone, but not in *CFTR* $^{-/-}$ cortical bone.

*Conclusions:* The loss of *CFTR* directly alters the bone composition and metabolism of newborn pigs. Based on these findings, we speculate that bone defects in patients with CF could be a primary, rather than a secondary consequence of inflammation and infection.



## 1. Introduction

Cystic fibrosis (CF) disease, which is caused by mutations of the CF transmembrane conductance regulator (*CFTR*) gene, is characterized by multiorgan deficiencies that begin early in life [1,2]. With the increasing life expectancy due to improvements in the treatment of the disease's pulmonary and gastrointestinal disorders, other CF complications and comorbidities have become more prevalent, such as diabetes and CF-related bone disease (CFBD) with 55–65% of affected patients being older than 45 [3–5]. Individuals with CF have low bone mineral density (BMD) [6–8] and increased fracture rate as early as adolescence, [9] which leads to

* Corresponding authors.
*E-mail addresses:* jacky.jacquot@inserm.fr (J. Jacquot), frederic.velard@univ-reims.fr (F. Velard).

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Original Article

# Intrinsic alterations in peripheral neutrophils from cystic fibrosis newborn piglets

Déborah Bréa[a,b,1], Laura Soler[c,1], Isabelle Fleurot[d], Sandrine Melo[d], Claire Chevaleyre[d], Mustapha Berri[d], Valérie Labas[e,f], Ana-Paula Teixeira-Gomes[d,f], Julien Pujo[g], Nicolas Cenac[g], Andrea Bähr[h], Nikolai Klymiuk[h], Antoine Guillon[a,b,i], Mustapha Si-Tahar[a,b], Ignacio Caballero[d,*]

[a] INSERM, Centre d'Etude des Pathologies Respiratoires, UMR 1100, Tours cedex, France
[b] Université de Tours, Faculté de Médecine de Tours, Tours, France
[c] Toxalim (Research Centre in Food Toxicology), Université de Toulouse, INRA, ENVT, INP-Purpan, UPS, Toulouse, France
[d] ISP, INRA, Université de Tours, UMR 1282, 37380, Nouzilly, France
[e] INRA UMR85-CNRS UMR7247 Physiologie de la Reproduction et des Comportements, Nouzilly, France
[f] INRA, Plate-forme de Chirurgie et d'Imagerie pour la Recherche et l'Enseignement (CIRE), Pôle d'Analyse et d'Imagerie des Biomolécules (PAIB), Nouzilly, France
[g] IRSD, Université de Toulouse, INSERM, INRA, ENVT, UPS, Toulouse, France
[h] Gene Center and Center for Innovative Medical Models (CiMM), LMU Munich, Germany
[i] CHRU Tours, service de médecine intensive réanimation, Tours, France

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ABSTRACT

*Background:* The hallmark of the cystic fibrosis (CF) lung disease is a neutrophil dominated lung environment that is associated to chronic lung tissue destruction and ultimately the patient's death. It is unclear whether the exacerbated neutrophil response is primary related to a defective CFTR or rather secondary to chronic bacterial colonization and inflammation. Here, we hypothesized that CF peripheral blood neutrophils present intrinsic alteration at birth before the start of an inflammatory process.

*Methods:* Peripheral blood neutrophils were isolated from newborn $CFTR^{+/+}$ and $CFTR^{-/-}$ piglets. Neutrophils immunophenotype was evaluated by flow cytometry. Lipidomic and proteomic profile were characterized by liquid chromatography/tandem mass spectrometry (LC-MS/MS), intact cell matrix-assisted laser desorption/ionization mass spectrometry (ICM-MS) followed by top-down high-resolution mass spectrometry (HRMS), respectively. The ability of CF neutrophils to kill *pseudomonas aeruginosa* was also evaluated.

*Results:* Polyunsaturated fatty acid metabolites analysis did not show any difference between $CFTR^{+/+}$ and $CFTR^{-/-}$ neutrophils. On the other hand, a predictive mathematical model based on the ICM-MS proteomic profile was able to discriminate between both genotypes. Top-down proteomic analysis identified 19 m/z differentially abundant masses that corresponded mainly to proteins related to the antimicrobial response and the generation of reactive oxygen species (ROS). However, no alteration in the ability of $CFTR^{-/-}$ neutrophils to kill *pseudomonas aeruginosa in vitro* was observed.

*Conclusions:* ICM-MS demonstrated that $CFTR^{-/-}$ neutrophils present intrinsic alterations already at birth, before the presence of any infection or inflammation.



## 1. Introduction

Cystic fibrosis (CF) is a recessive genetic disease caused by mutations in the cystic fibrosis transmembrane regulator (CFTR) gene, a cAMP-regulated chloride channel [1]. CFTR loss of function leads to aberrant ion regulation and disturbed homeostasis in the epithelial surface. Although a multi-systemic disease, lung alterations

*Abbreviations:* ICM-MS, intact cell matrix-assisted laser desorption/ionization mass spectrometry; PWKW, P-value from combined Wilcoxon rank-sum test and Kruskal-Wallis test.

* Corresponding author: Ignacio Caballero.
*E-mail address:* ignacio.caballero-posadas@inra.fr (I. Caballero).

[1] These authors contributed equally to the work

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TECHNIQUES AND RESOURCES HUMAN DEVELOPMENT ARTICLE

# Novel dynamics of human mucociliary differentiation revealed by single-cell RNA sequencing of nasal epithelial cultures

**Sandra Ruiz García[1,*], Marie Deprez[1,*], Kevin Lebrigand[1], Amélie Cavard[1], Agnès Paquet[1], Marie-Jeanne Arguel[1], Virginie Magnone[1], Marin Truchi[1], Ignacio Caballero[2], Sylvie Leroy[1,3], Charles-Hugo Marquette[3], Brice Marcet[1], Pascal Barbry[1,‡] and Laure-Emmanuelle Zaragosi[1,‡]**

## ABSTRACT

The upper airway epithelium, which is mainly composed of multiciliated, goblet, club and basal cells, ensures proper mucociliary function and can regenerate in response to assaults. In chronic airway diseases, defective repair leads to tissue remodeling. Delineating key drivers of differentiation dynamics can help understand how normal or pathological regeneration occurs. Using single-cell transcriptomics and lineage inference, we have unraveled trajectories from basal to luminal cells, providing novel markers for specific populations. We report that: (1) a precursor subgroup of multiciliated cells, which we have entitled deuterosomal cells, is defined by specific markers, such as DEUP1, FOXN4, YPEL1, HES6 and CDC20B; (2) goblet cells can be precursors of multiciliated cells, thus explaining the presence of hybrid cells that co-express markers of goblet and multiciliated cells; and (3) a repertoire of molecules involved in the regeneration process, such as keratins or components of the Notch, Wnt or BMP/TGFβ pathways, can be identified. Confirmation of our results on fresh human and pig airway samples, and on mouse tracheal cells, extend and confirm our conclusions regarding the molecular and cellular choreography at work during mucociliary epithelial differentiation.

**KEY WORDS: Airway epithelium, Single-cell RNA-seq, Differentiation, Multiciliated cells, Club cells, Goblet cells, Basal cells, Deuterosome, Keratins, Pathways**

## INTRODUCTION

The airway epithelium makes an efficient line of defense against inhaled substances. It is mainly composed of multiciliated cells (MCCs), goblet cells (GCs), club cells (CCs) and basal cells (BCs) (Gras et al., 2013; Kotton and Morrisey, 2014). Decreased numbers of MCCs and increased number of GCs hallmark many chronic respiratory diseases, during which frequent injuries, repair defects, tissue remodeling and altered mucociliary clearance occur (Cohn, 2006; Curran and Cohn, 2010; Merigo et al., 2002). Characteristics contributing to efficient airway regeneration after injuries have been extensively investigated in mouse, establishing mouse BCs as the main airway stem cells, with self-renewal capacities and the ability to differentiate into MCCs, CCs and GCs (Cole et al., 2010; Kotton and Morrisey, 2014; Rock et al., 2009). BCs are abundant in upper mouse airways but absent from lower airways (Hogan et al., 2014). Human BCs populate the whole airways, and their abundance also decreases in smaller airways (Boers et al., 1998). A direct differentiation of BCs into MCCs has been reported after injury (Pardo-Saganta et al., 2015a), but the current consensus is that BCs can differentiate first into CCs (Watson et al., 2015), i.e. club/secretory or Clara cells. CCs are widespread in the whole mouse airways. They are less abundant in human, being nearly absent from upper airways but enriched in terminal and respiratory bronchioles (Boers et al., 1999). CCs are luminally located, show a characteristic columnar shape and contribute to xenobiotic metabolism through the production of anti-microbial and anti-inflammatory peptides (Wang et al., 2003; Jones et al., 1983), such as the secretoglobin SCGB1A1. CCs can give rise to MCCs, as detected by the expression of transcription factor FOXJ1 (Rawlins et al., 2009; Watson et al., 2015) and to GCs, as detected by the expression of mucin MUC5AC (Chen et al., 2009; Kotton and Morrisey, 2014).

Distinct molecular mechanisms regulate cell fate decisions in airway epithelium lineages. Notch signaling plays a pivotal role during commitment of BCs: activation leads to CC/GC lineages, while inhibition leads to MCC lineages (Morimoto et al., 2010; Pardo-Saganta et al., 2015b; Rock et al., 2011; Tsao et al., 2009). We have shown that Notch pathway inhibition by the *miR-34/449* families of microRNAs is required for MCC differentiation (Marcet et al., 2011a,b; Mercey et al., 2017). *In vivo* lineage-tracing studies have some limitations: observations in animal models do not necessarily transfer to human; use of drastic forms of injuries may not completely reveal physiological tissue turnover; and strategies of specific genetic cell labeling (usually *Krt5* for BCs and *Scgb1a1* for CCs) are not necessarily comprehensive and do not necessarily provide a full picture of the airway epithelial cell hierarchies. In human, in which lineage tracing is impossible, cell lineage hierarchies in homeostatic bronchi have been indirectly inferred by assessing somatic mitochondrial mutations (Teixeira et al., 2013); however, *in vitro* approaches are still necessary to study cell lineage during epithelial regeneration.

Single-cell RNA-sequencing has emerged as a powerful approach to measure cell lineage hierarchies (Fletcher et al., 2017; Karamitros et al., 2018; Pal et al., 2017), by capturing cells at different levels of differentiation (Plass et al., 2018). After a first study that delineated lineage hierarchies of mouse alveolar cells (Treutlein et al., 2014), several atlases of the airways have recently been released in mouse (Montoro et al., 2018) and human (Ordovas-

[1]Université Côte d'Azur, CNRS, IPMC, Sophia-Antipolis 06560, France. [2]ISP, INRA, Université Tours, Nouzilly 37380, France. [3]Université Côte d'Azur, CHU de Nice, Pulmonology Department, Nice 06000, France.
*These authors contributed equally to this work

‡Author for correspondence (barbry@ipmc.cnrs.fr; zaragosi@ipmc.cnrs.fr)

P.B., 0000-0001-9632-6483; L.-E.Z., 0000-0001-6747-7928



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Original Article

# Tetrafunctional Block Copolymers Promote Lung Gene Transfer in Newborn Piglets

Ignacio Caballero,[1] Mickaël Riou,[2] Océane Hacquin,[3,4] Claire Chevaleyre,[1] Céline Barc,[2] Jérémy Pezant,[2] Anne Pinard,[2] Julien Fassy,[3,4] Roger Rezzonico,[3,4] Bernard Mari,[3,4] Nathalie Heuzé-Vourc'h,[5] Bruno Pitard,[6] and Georges Vassaux[3,4]

[1]INRA Centre Val de Loire – Université de Tours, UMR-1282 Infectiologie et Santé Publique (ISP), 37380 Nouzilly, France; [2]INRA Centre Val de Loire, UE-1277 Plateforme d'Infectiologie expérimentale (PFIE), 37380 Nouzilly, France; [3]Université Côte d'Azur, INSERM, CNRS, IPMC, Valbonne, France; [4]FHU-OncoAge, Nice, France; [5]Centre d'Etude des Pathologies Respiratoires, U1100, INSERM, Tours, France; [6]CRCINA, INSERM, Université d'Angers, Université de Nantes, Nantes, France

**Tetrafunctional block copolymers are molecules capable of complexing DNA. Although ineffective *in vitro*, studies in mice have shown that the tetrafunctional block copolymer 704 is a more efficient lung gene transfer agent than the cationic liposome GL67A, previously used in a phase II clinical trial in cystic fibrosis patients. In the present study, we compared the gene transfer capacity of the 704-DNA formulation and a cationic liposome-DNA formulation equivalent to GL67A in a larger-animal model, the newborn piglet. Our results indicate an efficacy of the 704-DNA formulation well above one order of magnitude higher than that of the cationic liposome-DNA formulation, with no elevated levels of interleukin-6 (IL-6), taken as a marker of inflammation. Transgene expression was heterogeneous within lung lobes, with expression levels that were below the detection threshold in some samples, while high in other samples. This heterogeneity is likely to be due to the bolus injection procedure as well as to the small volume of injection. The present study highlights the potential of tetrafunctional block copolymers as non-viral vectors for lung gene therapy.**

## INTRODUCTION

Lung gene transfer has potential application in a large range of pathologies. These include inherited diseases such as cystic fibrosis (CF)[1] and pathologies with mixed origins, such as asthma and chronic obstructive pulmonary disease.[1] Several acquired diseases that lack satisfactory treatments, such as primary lung cancers or metastases of distant cancers and idiopathic pulmonary fibrosis, could also be considered as indications for gene therapy.[1] More surprisingly, gene transfer to the lungs has been proposed as a method to produce proteins for release into the circulation.[2–4]

To achieve sufficient gene delivery, the development of vectors relevant to the pathology and to the expected therapeutic schedule is central. Viral vectors are highly efficient but their utilization has been hampered by their immunogenic-proinflammatory properties,[5] which render them inappropriate for applications in pathologies where the lungs are severely inflamed and/or that require repeated administrations. Recent developments have led to the emergence of integrating pseudotyped lentiviruses.[6] These can promote long-lasting transgene expression in rodent models,[7–11] and their relevance to the clinical situation is currently under investigation.[10,12]

Non-viral gene delivery vectors represent an attractive alternative. These are synthetic, chemically defined organic molecules complexed with DNA. They are less likely to induce a strong inflammatory response and are particularly relevant in indications that require transient gene expression and/or repeated administrations. Within this field, the most studied reagent is the cationic lipid formulation, GL67A. This formulation has been shown to transfect mouse and ovine lungs *in vivo*[13–15] and to be suitable for repeated administration.[14,15] GL67A has been administered successfully to healthy volunteers and to CF patients.[16,17] Repeated administration of GL67A to CF patients resulted in stabilization of the disease in a randomized, double-blind, placebo-controlled, phase 2b trial.[18]

Formulations composed of DNA and non-ionic amphiphilic block copolymer have been reported to successfully transfect skeletal and cardiac muscles.[19–22] Intratracheal delivery of a similar formulation led to a gene transfer level equivalent to that promoted by a polyethylenimine-based formulation, but with reduced inflammation.[23] Tetrafunctional block copolymers have a tetrafunctional structure consisting of four poly(ethylene oxide) -poly(propylene oxide) blocks centered on an ethylenediamine moiety. They form small complexes with DNA,[24] and their potential as vectors for cardiac and muscle gene transfer has been described.[20,24–26] The ability of tetrafunctional block copolymers to deliver DNA in muscles for expression of genes of therapeutic interest has been demonstrated in mouse models of hepatocellular carcinoma,[27] allergic asthma,[28,29] and colorectal cancer,[30] against the CF *Mycobacterium abscessus*,[31,32] against transposase-derived proteins encoded by human neogenes,[33] and against Zika virus.[34] In an attempt to identify more efficient non-viral formulations

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**Correspondence:** Georges Vassaux, Université Côte d'Azur, INSERM, CNRS UMR 7275, IPMC, 660 Route des Lucioles, 06560 Valbonne, France
**E-mail:** vassaux@ipmc.cnrs.fr

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# Understanding the dynamics of Toll-like Receptor 5 response to flagellin and its regulation by estradiol

Ignacio Caballero[1,2], James Boyd[3], Carmen Almiñana[1], Javier A. Sánchez-López[1], Shaghayegh Basatvat[1], Mehrnaz Montazeri[1], Nasim Maslehat Lay[1], Sarah Elliott[1], David G. Spiller[3], Michael R. H. White[3] & Alireza Fazeli[1,4]

**Toll-like receptors (TLRs) are major players of the innate immune system. Once activated, they trigger a signalling cascade that leads to NF-κB translocation from the cytoplasm to the nucleus. Single cell analysis shows that NF-κB signalling dynamics are a critical determinant of transcriptional regulation. Moreover, the outcome of innate immune response is also affected by the cross-talk between TLRs and estrogen signalling. Here, we characterized the dynamics of TLR5 signalling, responsible for the recognition of flagellated bacteria, and those changes induced by estradiol in its signalling at the single cell level. TLR5 activation in MCF7 cells induced a single and sustained NF-κB translocation into the nucleus that resulted in high NF-κB transcription activity. The overall magnitude of NF-κB transcription activity was not influenced by the duration of the stimulus. No significant changes are observed in the dynamics of NF-κB translocation to the nucleus when MCF7 cells are incubated with estradiol. However, estradiol significantly decreased NF-κB transcriptional activity while increasing TLR5-mediated AP-1 transcription. The effect of estradiol on transcriptional activity was dependent on the estrogen receptor activated. This fine tuning seems to occur mainly in the nucleus at the transcription level rather than affecting the translocation of the NF-κB transcription factor.**

Toll-like receptors (TLRs) are a family of evolutionary conserved pattern recognition receptors (PRRs) from the innate immune system. They are membrane bound-receptors, with up to 10 members in humans and 12 in mice. Traditionally, they are considered to be a part of the first line of defence against pathogens, their major role being to detect specific microbial motifs, known as pathogen-associated molecular patterns (PAMPs) and to trigger an inflammatory response[1]. Abnormal TLR signalling has been related to a variety of pathologies, including acute and chronic infections, autoimmune diseases, immunodeficiencies and cancer[2–4].

TLRs downstream signalling involves the activation of mitogen-activated protein kinases (MAPK) and the nuclear factor κB transcription factor (NF-κB)[1]. This transcription factor family is formed by 5 members, RelA, RelB, c-Rel, p50/p105 (NF-κB1), and p52/p100 (NF-κB2). In the absence of stimuli, NF-κB proteins are bound in the cytoplasm to proteins from the inhibitor of nuclear factor κB (IκB) protein family, preventing NF-κB translocation to the nucleus. Upon stimulation, IκBα is phosphorylated by the IκB kinase (IKK) and degraded, allowing the NF-κB dimers to move into the nucleus and bind to the DNA, triggering the expression of target genes[5,6]. After translocation into the nucleus, NF-κB target genes including cytokines and negative feedback regulators of the system, such as IκB and A20, that have the potential to produce oscillations[7]. NF-κB is a major network hub processing many different inflammatory signals that lead to different transcriptional response. However, understanding how this genetic circuit can provide the adequate transcriptional response to each stimulus is unclear. Single cell studies and mathematical modeling are clarifying the biological significance of the dynamics of several signalling pathways (especially NF-κB) and their oscillatory behavior[8,9]. They show that the type (TNF-α, LPS)

[1]Academic Unit of Reproductive and Developmental Medicine, Department of Oncology and Metabolism, The Medical School, University of Sheffield, Sheffield, United Kingdom. [2]ISP, INRA, Université Tours, 37380, Nouzilly, France. [3]Michael Smith Building, Faculty of Life Sciences, University of Manchester, Manchester, United Kingdom. [4]Institute of Biomedicine and Translational Medicine, Department of Pathophysiology, University of Tartu, Tartu, Estonia. Correspondence and requests for materials should be addressed to A.F. (email: Fazeli@ut.ee)

PLOS ONE

RESEARCH ARTICLE

# Generation of a Stable Transgenic Swine Model Expressing a Porcine Histone 2B-eGFP Fusion Protein for Cell Tracking and Chromosome Dynamics Studies

**Renan B. Sper[1,2☯], Sehwon Koh[3☯¤a], Xia Zhang[1,2☯], Sean Simpson[1,2], Bruce Collins[1,4], Jeff Sommer[4], Robert M. Petters[1,4], Ignacio Caballero[1¤b], Jeff L. Platt[3], Jorge A. Piedrahita[1,2]***

1 Comparative Medicine Institute, North Carolina State University, Raleigh, North Carolina, United States of America, 2 Department of Molecular Biomedical Sciences, College of Veterinary Medicine, North Carolina State University, Raleigh, North Carolina, United States of America, 3 Department of Surgery and Microbiology and Immunology, University of Michigan Health System, Ann Arbor, Michigan, United States of America, 4 Department of Animal Science, College of Agriculture and Life Sciences, North Carolina State University, Raleigh, North Carolina, United States of America

☯ These authors contributed equally to this work.
¤a Current address: Department of Cell Biology, Duke University Medical Center, Durham, North Carolina, United States of America.
¤b Current address: French National Institute for Agricultural Research (INRA), Nouzilly, France
* japiedra@ncsu.edu



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**Competing Interests:** The authors have declared that no competing interests exist.

## Abstract

Transgenic pigs have become an attractive research model in the field of translational research, regenerative medicine, and stem cell therapy due to their anatomic, genetic and physiological similarities with humans. The development of fluorescent proteins as molecular tags has allowed investigators to track cell migration and engraftment levels after transplantation. Here we describe the development of two transgenic pig models via SCNT expressing a fusion protein composed of eGFP and porcine Histone 2B (pH2B). This fusion protein is targeted to the nucleosomes resulting a nuclear/chromatin eGFP signal. The first model (I) was generated via random insertion of pH2B-eGFP driven by the CAG promoter (chicken beta actin promoter and rabbit Globin poly A; pCAG-pH2B-eGFP) and protected by human interferon-β matrix attachment regions (MARs). Despite the consistent, high, and ubiquitous expression of the fusion protein pH2B-eGFP in all tissues analyzed, two independently generated Model I transgenic lines developed neurodegenerative symptoms including Wallerian degeneration between 3–5 months of age, requiring euthanasia. A second transgenic model (II) was developed via CRISPR-Cas9 mediated homology-directed repair (HDR) of IRES-pH2B-eGFP into the endogenous β-actin (ACTB) locus. Model II transgenic animals showed ubiquitous expression of pH2B-eGFP on all tissues analyzed. Unlike the pCAG-pH2B-eGFP/MAR line, all Model II animals were healthy and multiple pregnancies have been established with progeny showing the expected Mendelian ratio for the transmission of the pH2B-eGFP. Expression of pH2B-eGFP was used to examine the timing of the maternal to zygotic transition after IVF, and to examine chromosome segregation of SCNT embryos. To our knowledge this is the first viable transgenic pig model with chromatin-associated

PLOS ONE

RESEARCH ARTICLE

# The Pig: A Relevant Model for Evaluating the Neutrophil Serine Protease Activities during Acute *Pseudomonas aeruginosa* Lung Infection

**Claire Chevaleyre[1☯], Mickaël Riou[2☯], Déborah Bréa[3], Clarisse Vandebrouck[4], Céline Barc[2], Jérémy Pezant[2], Sandrine Melo[1], Michel Olivier[1], Rémy Delaunay[2], Olivier Boulesteix[2], Patricia Berthon[1], Christelle Rossignol[1], Julien Burlaud Gaillard[5], Frédéric Becq[4], Francis Gauthier[3], Mustapha Si-Tahar[3], François Meurens[6], Mustapha Berri[1], Ignacio Caballero-Posadas[1], Sylvie Attucci[3]***

**1** Infectiologie et Santé Publique (UMR 1282 ISP), INRA, Université Tours, Nouzilly, France, **2** Plateforme d'Infectiologie expérimentale (UE-1277 PFIE), INRA, Nouzilly, France, **3** INSERM, Centre d'Etude des Pathologies Respiratoires, UMR 1100, Tours cedex, France, **4** Laboratoire Signalisation et Transports Ioniques Membranaires, Université de Poitiers, Centre National de la Recherche Scientifique, Poitiers cedex, France, **5** Département des Microscopies (Plateau technologique Analyse des systèmes Biologiques), Université François-Rabelais, Tours cedex, France, **6** BioEpAR, Oniris, Nantes Atlantic National College of Veterinary Medicine, Food Science and Engineering La Chantrerie, Nantes Cedex 3, France

☯ These authors contributed equally to this work.
* attucci@univ-tours.fr



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## Abstract

The main features of lung infection and inflammation are a massive recruitment of neutrophils and the subsequent release of neutrophil serine proteases (NSPs). Anti-infectious and/or anti-inflammatory treatments must be tested on a suitable animal model. Mice models do not replicate several aspects of human lung disease. This is particularly true for cystic fibrosis (CF), which has led the scientific community to a search for new animal models. We have shown that mice are not appropriate for characterizing drugs targeting neutrophil-dependent inflammation and that pig neutrophils and their NSPs are similar to their human homologues. We induced acute neutrophilic inflammatory responses in pig lungs using *Pseudomonas aeruginosa*, an opportunistic respiratory pathogen. Blood samples, nasal swabs and bronchoalveolar lavage fluids (BALFs) were collected at 0, 3, 6 and 24 h *post*-insfection (p.i.) and biochemical parameters, serum and BAL cytokines, bacterial cultures and neutrophil activity were evaluated. The release of proinflammatory mediators, biochemical and hematological blood parameters, cell recruitment and bronchial reactivity, peaked at 6h p.i.. We also used synthetic substrates specific for human neutrophil proteases to show that the activity of pig NSPs in BALFs increased. These proteases were also detected at the surface of lung neutrophils using anti-human NSP antibodies. *Pseudomonas aeruginosa*-induced lung infection in pigs results in a neutrophilic response similar to that described for cystic fibrosis and ventilator-associated pneumonia in humans. Altogether, this indicates that the pig is an appropriate model for testing anti-infectious and/or anti-inflammatory drugs to combat adverse proteolytic effects of neutrophil in human lung diseases.

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human reproduction **ORIGINAL ARTICLE** ***Reproductive biology***

# Interleukin-1 receptor antagonist mediates toll-like receptor 3-induced inhibition of trophoblast adhesion to endometrial cells *in vitro*

**M. Montazeri[1], J.A. Sanchez-Lopez[1], I. Caballero[1,2], N. Maslehat Lay[1], S. Elliott[1], and A. Fazeli[1,*]**

[1]Academic Unit of Reproductive and Developmental Medicine, The University of Sheffield, Level 4, Jessop Wing, Tree Root Walk, Sheffield S10 2SF, UK [2]UMR1282 ISP, INRA, Nouzilly, France

*Correspondence address. E-mail: a.fazeli@sheffield.ac.uk

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**STUDY QUESTION:** Is interleukin-1 receptor antagonist (IL-1RA) involved in the toll-like receptor 3 (TLR 3)-induced inhibition of trophoblast cells' adhesion to endometrial cells *in vitro*?

**SUMMARY ANSWER:** IL-1RA mediates the TLR 3-induced inhibition of trophoblast cells' adhesion to endometrial cells *in vitro*.

**WHAT IS KNOWN ALREADY:** It is well documented that endometrial TLR 3 activation leads to impairment of trophoblast binding to endometrial cells *in vitro*. IL-1RA is known as an anti-implantation factor, as its injection significantly reduced implantation rates in mice by an effect on endometrial receptivity.

**STUDY DESIGN, SIZE, DURATION:** Poly I:C was used as a TLR3 specific ligand and endometrial cells were either treated or not with Poly I:C (treated versus control) *in vitro*. IL-1RA was applied to block IL-1 signal transduction. IL-1RA was knocked down by Accell Human IL1RN siRNA. Flagellin was used to stimulate TLR 5. SP600125 (JNK) was applied to inhibit the mitogen-activated protein kinases (MAPK) pathway. BAY11 -7082 was used to inhibit the nuclear factor-κB (NF-κB) pathway. The experiments were performed in three replicates on three separate days.

**PARTICIPANTS/MATERIALS, SETTING, METHODS:** An *in vitro* assay was developed using RL95-2 (an endometrial cell line) and JAr (a trophoblast cell line) cells. Initially, the production of IL-1RA in RL95-2 cells in response to TLR 3 activation was measured. To determine whether the TLR 3-induced inhibition of trophoblast binding was mediated through IL-1RA: (i) we evaluated the effect of IL-1RA on the attachment of trophoblast cells to endometrial cells; (ii) we knocked down TLR3-induced IL-1RA gene expression by IL-1RA Small interfering RNA (siRNA) and evaluated trophoblast attachment to endometrial cells. Finally, to clarify through which pathway TLR 3-induced inhibition of trophoblast binding occurs: (i) activation of NF-κB and MAPK was detected by transfecting the cells with secreted placental alkaline phosphatase reporter plasmids bearing promoter sequences for each transcription factor; (ii) the inhibitors for NF-κB and MAPK were used to block signaling; (iii) it was then investigated whether addition of these inhibitors could restore the TLR 3-induced impairment of trophoblast attachment to the endometrial cells.

**MAIN RESULTS AND THE ROLE OF CHANCE:** Our results showed that addition of polyinosinic:polycytidylic acid (Poly I:C) to RL95-2 cells significantly increased the production of IL-1RA ($P < 0.05$). Addition of human recombinant IL-1RA to RL95-2 cells remarkably decreased the adhesion rate of trophoblast cells to endometrial cells ($P < 0.05$). In addition, suppression of TLR3-induced IL-1RA gene expression in RL95-2 cells significantly restored trophoblast cells attachment to endometrial cells in the presence of Poly I:C ($P < 0.05$). Only TLR3 and not TLR5 induced MAPK activation ($P < 0.05$). TLR3 ligation did not affect NF-κB activation. Of NF-kB and MAPK inhibitors, only MAPK's inhibitor could achieve restoration of spheroid adhesion to endometrial cells ($P < 0.05$).

**LIMITATIONS, REASONS FOR CAUTION:** This study has been only done *in vitro*. Future *in vivo* studies will confirm our data.

**WIDER IMPLICATIONS OF THE FINDINGS:** The findings of this study have a potential clinical application in introducing IL-1RA as one of the diagnostic infertility markers in the endometrium, which can affect the process of embryo adhesion at the time of implantation. Moreover,

PLOS ONE

RESEARCH ARTICLE

# Computed Tomography (CT) Scanning Facilitates Early Identification of Neonatal Cystic Fibrosis Piglets

**Antoine Guillon[1,2], Claire Chevaleyre[3], Celine Barc[4], Mustapha Berri[3], Hans Adriaensen[5], François Lecompte[5], Thierry Villemagne[6], Jérémy Pezant[4], Rémi Delaunay[4], Joseph Moënne-Loccoz[7], Patricia Berthon[3], Andrea Bähr[8], Eckhard Wolf[8], Nikolai Klymiuk[8], Sylvie Attucci[1], Reuben Ramphal[1], Pierre Sarradin[4], Dominique Buzoni-Gatel[3], Mustapha Si-Tahar[1], Ignacio Caballero[3]***

**1** INSERM, Centre d'Etude des Pathologies Respiratoires, UMR 1100/EA6305, Tours, France, **2** CHU Tours, service de réanimation polyvalente, Tours, France, **3** INRA, UMR1282 ISP, Nouzilly, France, **4** INRA, UMR1277 PFIE, Nouzilly, France, **5** INRA, CIRE, Nouzilly, France, **6** CHU Clocheville, Tours, France, **7** CHU Tours, service anesthésie-réanimation, Tours, France, **8** Gene Center and Center for Innovative Medical Models (CiMM), LMU Munich, Germany

* icaballerop@tours.inra.fr



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**Competing Interests:** The authors have declared that no competing interests exist.

## Abstract

### Background

Cystic Fibrosis (CF) is the most prevalent autosomal recessive disease in the Caucasian population. A cystic fibrosis transmembrane conductance regulator knockout (CFTR-/-) pig that displays most of the features of the human CF disease has been recently developed. However, *CFTR*$^{-/-}$ pigs presents a 100% prevalence of meconium ileus that leads to death in the first hours after birth, requiring a rapid diagnosis and surgical intervention to relieve intestinal obstruction. Identification of *CFTR*$^{-/-}$ piglets is usually performed by PCR genotyping, a procedure that lasts between 4 to 6 h. Here, we aimed to develop a procedure for rapid identification of *CFTR*$^{-/-}$ piglets that will allow placing them under intensive care soon after birth and immediately proceeding with the surgical correction.

### Methods and Principal Findings

Male and female *CFTR*$^{+/-}$ pigs were crossed and the progeny was examined by computed tomography (CT) scan to detect the presence of meconium ileus and facilitate a rapid post-natal surgical intervention. Genotype was confirmed by PCR. CT scan presented a 94.4% sensitivity to diagnose *CFTR*$^{-/-}$ piglets. Diagnosis by CT scan reduced the birth-to-surgery time from a minimum of 10 h down to a minimum of 2.5 h and increased the survival of *CFTR*$^{-/-}$ piglets to a maximum of 13 days post-surgery as opposed to just 66 h after later surgery.

### Conclusion

CT scan imaging of meconium ileus is an accurate method for rapid identification of *CFTR*$^{-/-}$ piglets. Early CT detection of meconium ileus may help to extend the lifespan of *CFTR*$^{-/-}$ piglets and, thus, improve experimental research on CF, still an incurable disease.

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human reproduction **ORIGINAL ARTICLE** ***Reproductive biology***

# Activation of Toll-like receptor 3 reduces actin polymerization and adhesion molecule expression in endometrial cells, a potential mechanism for viral-induced implantation failure

**M. Montazeri[1], J.A. Sanchez-Lopez[1], I. Caballero[1,2], N. Maslehat Lay[1], S. Elliott[1], S. López-Martín[3], M. Yáñez-Mó[3], and A. Fazeli[1,*]**

[1]Academic Unit of Reproductive and Developmental Medicine, The University of Sheffield, Level 4, Jessop Wing, Tree Root Walk, Sheffield S10 2SF, UK [2]UMR1282 ISP, INRA, Nouzilly, France [3]Unidad de Investigación, Hospital Santa Cristina, Instituto de Investigación Sanitaria Princesa, C/Maestro Amadeo Vives 2, 28009 Madrid, Spain

*Correspondence address. E-mail: a.fazeli@sheffield.ac.uk

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**STUDY QUESTION:** Does activation of endometrial Toll-like receptor 3 (TLR 3) affect cell receptivity to trophoblast adhesion?

**SUMMARY ANSWER:** TLR 3 activation *in vitro* reduces the attachment of trophoblast cells to endometrial cells by altering the cell cytoskeleton and reducing the expression of adhesion molecules in human endometrial cells.

**WHAT IS KNOWN ALREADY:** It is well documented that the presence of an infection at the time of implantation can lead to implantation failure. The female reproductive tract recognizes invading micro-organisms through the innate pathogen recognition receptors such as the TLRs.

**STUDY DESIGN, SIZE, DURATION:** Poly I:C was used as a TLR 3-specific ligand and endometrial cells were either treated or not with Poly I:C (treated versus control) *in vitro*. The experiments were performed in three replicates on three separate days.

**PARTICIPANTS/MATERIALS, SETTING, METHODS:** An *in vitro* assay was developed using RL95-2 (a human endometrial cell line) and JAr (a human trophoblast cell line) cells. Initially, the percentage of attached JAr spheroids to RL95-2 was measured in response to TLR 3 activation. Next, actin polymerization in RL95-2 cells was assessed in response to TLR 2/6, 3 and 5 activation. Phalloidin was used to assess the mean fluorescence intensity of F-actin by flow cytometry or confocal microscopy. Secondly, the influence of TLR 2/6, 3 and 5 activation on the expression of cluster of differentiation 98 (CD98) and β3 integrin was determined. To further understand through which pathways the TLR 3-induced alterations occur, inhibitors were applied for Toll/interleukin-1 receptor domain-containing adaptor inducing interferon-beta (TRIF), myeloid differentiation primary response 88 (MYD88), mitogen-activated protein kinases (MAPK) and nuclear factor pathways.

**MAIN RESULTS AND THE ROLE OF CHANCE:** We observed that stimulation of TLR 3 in endometrial cells with different concentrations of Poly I:C led to a reduction in the percentage of trophoblasts attached to the endometrial cells in a dose-dependent manner ($P < 0.05$). This decrease was consistent in the Poly I:C treated group regardless of the co-incubation time ($P < 0.05$). In addition, our results demonstrated that actin polymerization and CD98 expression significantly decreased only in response to TLR 3 activation ($P < 0.05$). Activation of endometrial cells with TLR 2/6, 3 and 5 significantly reduced β3 integrin expression ($P < 0.05$). These alterations were shown to work via MYD88-MAPK pathways ($P < 0.05$).

**LIMITATIONS, REASONS FOR CAUTION:** This study has been performed *in vitro*. Future *in vivo* studies will be required in order to confirm our data.

**WIDER IMPLICATIONS OF THE FINDINGS:** This is a novel discovery which extends our current knowledge concerning diagnosis and treatment of viral-induced infertility cases.

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http://www.biomedcentral.com/1471-2164/15/293

BMC Genomics

RESEARCH ARTICLE Open Access

# The battle of the sexes starts in the oviduct: modulation of oviductal transcriptome by X and Y-bearing spermatozoa

Carmen Almiñana[1,2], Ignacio Caballero[1], Paul Roy Heath[3], Saeedeh Maleki-Dizaji[4], Inmaculada Parrilla[2], Cristina Cuello[2], Maria Antonia Gil[2], Jose Luis Vazquez[2], Juan Maria Vazquez[2], Jordi Roca[2], Emilio Arsenio Martinez[2], William Vincent Holt[1] and Alireza Fazeli[1*]

**Abstract**

**Background:** Sex allocation of offspring in mammals is usually considered as a matter of chance, being dependent on whether an X- or a Y-chromosome-bearing spermatozoon reaches the oocyte first. Here we investigated the alternative possibility, namely that the oviducts can recognise X- and Y- spermatozoa, and may thus be able to bias the offspring sex ratio.

**Results:** By introducing X- or Y-sperm populations into the two separate oviducts of single female pigs using bilateral laparoscopic insemination we found that the spermatozoa did indeed elicit sex-specific transcriptomic responses. Microarray analysis revealed that 501 were consistently altered (P-value < 0.05) in the oviduct in the presence of Y-chromosome-bearing spermatozoa compared to the presence of X-chromosome-bearing spermatozoa. From these 501 transcripts, 271 transcripts (54.1%) were down-regulated and 230 transcripts (45.9%) were up-regulated when the Y- chromosome-bearing spermatozoa was present in the oviduct. Our data showed that local immune responses specific to each sperm type were elicited within the oviduct. In addition, either type of spermatozoa elicits sex-specific signal transduction signalling by oviductal cells.

**Conclusions:** Our data suggest that the oviduct functions as a biological sensor that screens the spermatozoon, and then responds by modifying the oviductal environment. We hypothesize that there might exist a gender biasing mechanism controlled by the female.

**Keywords:** X and Y-chromosome bearing spermatozoa, Oviduct, Transcriptome, Sex selection

## Background

For many years gender allocation of offspring in mammals, including humans, has been regarded as a matter of chance, depending on whether an X- or a Y- chromosome-bearing spermatozoon reaches the oocyte first. Since an equal number of X- and Y- spermatozoa are produced during spermatogenesis [1], and fertilization is a random event, it stands to reason that in each generation equal numbers of males and females should be born. Evidence from the field and laboratory challenges this classic dogma and suggests that some kind of adaptive control of offspring gender may exist in mammals [2,3]. Evidence for this ability exists in many invertebrates and some avian species are able to adjust their progeny sex ratio predictably in response to environmental conditions [4]. Numerous factors such as population density, resource availability (famine), season, mother's age, levels of hormones, time of insemination and stress are known to influence the sex ratio in mammals [5-8]. However, the biological mechanism(s) through which mammals can bias the offspring ratio is still unknown.

Several hypothetical mechanisms have been proposed to explain sex ratio skewing in mammals. On the male side, any shift from the expected 1:1 sex ratio among offspring has been related to intrinsic differences in sperm motility, viability and fertilization ability of the two

* Correspondence: A.Fazeli@Sheffield.AC.UK
[1]Academic Unit of Reproductive and Developmental Medicine, Department of Human metabolism, The University of Sheffield, Level 4, The Jessop Wing, Tree Root Walk, Sheffield S10 2SF, UK
Full list of author information is available at the end of the article

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# Local Activation of Uterine Toll-Like Receptor 2 and 2/6 Decreases Embryo Implantation and Affects Uterine Receptivity in Mice[1]

**Javier Arturo Sanchez-Lopez,[3] Ignacio Caballero,[3] Mehrnaz Montazeri,[3] Nasim Maslehat,[3] Sarah Elliott,[3] Raul Fernandez-Gonzalez,[4] Alexandra Calle,[4] Alfonso Gutierrez-Adan,[4] and Alireza Fazeli[2,3]**

[3]*Academic Unit of Reproductive and Developmental Medicine, The University of Sheffield, Sheffield, United Kingdom*
[4]*Departamento de Reproduccion Animal, Instituto Nacional de Investigacion y Tecnología Agraria y Alimentaria (INIA), Madrid, Spain*

## ABSTRACT

**Embryo implantation is a complex interaction between maternal endometrium and embryonic structures. Failure to implant is highly recurrent and impossible to diagnose. Inflammation and infections in the female reproductive tract are common causes of infertility, embryo loss, and preterm labor. The current work describes how the activation of endometrial Toll-like receptor (TLR) 2 and 2/6 reduces embryo implantation chances. We developed a morphometric index to evaluate the effects of the TLR 2/6 activation along the uterine horn (UH). TLR 2/6 ligation reduced the endometrial myometrial and glandular indexes and increased the luminal index. Furthermore, TLR 2/6 activation increased the proinflammatory cytokines such as interleukin (IL)-1beta and monocyte chemotactic protein (MCP)-1 in UH lavages in the preimplantation day and IL-1 receptor antagonist in the implantation day. The engagement of TLR 2/6 with its ligand in the UH during embryo transfer severely affected the rate of embryonic implantation (45.00% ± 6.49% vs. 16.69% ± 5.01%, $P < 0.05$, control vs. test, respectively). Furthermore, this interference with the embryo implantation process was verified using an in vitro model of human embryo implantation where trophoblast spheroids failed to adhere to a monolayer of TLR 2- and TLR 2/6-activated endometrial cells. The inhibition of TLR receptors 2 and 6 in the presence of their specific ligands restored the ability of the spheroids to bind to the endometrial cells. In conclusion, the activation of the innate immune system in the uterus at the time of implantation interfered with the endometrial receptivity and reduced the chances of implantation success.**

*endometrium, female infertility, implantation, toll-like receptors (TLR), trophoblast spheroids*

## INTRODUCTION

Embryonic implantation is a critical event leading to a successful pregnancy [1]. The implantation process requires a complex orchestration of cellular and molecular events that include expression of adhesion molecules, remodeling of the uterine extracellular matrix, and an intricate cross-talk of hormones, cytokines, and growth factors between the embryo and the endometrium [2–6]. The immune system plays an important role in the modulation of the mechanisms involved in implantation. Pregnancy represents an immunological contradiction in which a semiallogeneic foreign entity, the embryo, is not rejected by the maternal immune system but accepted and nourished. This is achieved by several mechanisms, including the modulation of the maternal immune system by the preimplantation embryo [7]. Consequently, any interference that may imbalance the immune system responses could result in embryo loss and infertility. In fact, many infertility problems in women are associated with infections of the upper female reproductive tract (FRT) compartments: endocervix, fallopian tubes, and uterus. The pathogens responsible for sexually transmitted diseases (STD) such as *Mycoplasma genitalium*, *Neisseria gonorrhoeae*, and *Chlamydia trachomatis* have been associated with infertility in women [1, 8].

The FRT is able to respond against these pathogenic entities and initiate an immune response. The initial recognition of pathogens in the FRT is mediated by the innate immune system of epithelial, resident dendritic, and natural killer cells [3, 7, 9]. This innate response will later prime and instruct the adaptive immune system to initiate cellular and humoral responses against the pathogens [7, 9, 10]. In order to detect the potential pathogens, endometrial epithelial cells express pattern-recognition receptors to common pathogen-associated molecular patterns (PAMPs). One of the main families of pattern-recognition receptors is the TLR family [9, 11]. They can recognize a great variety of PAMPs from bacterial, fungal, parasitical, and viral origin. The TLR family is formed by 10 members where each responds to a specific ligand that promotes the secretion of a different subset of cytokines and chemokines [12–15]. TLR 2 and its heterodimers with TLR 1 and TLR 6 can sense a great variety of PAMPs from pathogens that might be present in the FRT and can be activated by peptidoglycans (PGNs) from bacteria (*C. trachomatis*, *Staphylococcus aureus*, *N. gonorrhoeae*, and *M. genitalium* lipoproteins) [16], yeast (*Candida albicans* phospholipomannan) [17], and parasites (*Trichomonas vaginalis* lipophosphoglycan) [18]. The heterodimer of TLR 2 and TLR 6 has been found to recognize the macrophage-activated lipoprotein derived from *Mycoplasma fermentans* (MALP-2) [19]. FSL-1 is a specific and potent ligand for the heterodimer TLR 2/6 and was synthesized based on the MALP-2 structure by changing its amino acid sequence [20]. The ligation of TLRs activates signaling pathways that end up activating the transcription factors nuclear factor (NF)-κB or AP-1 that promote the activation of proinflammatory genes [21].

The expression of TLRs 1–10 has been observed in mammalian uterus and in different human endometrial epithelial cell lines [13]. This expression follows the menstrual

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[2]Correspondence: E-mail: A.Fazeli@sheffield.ac.uk

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# Human Trophoblast Cells Modulate Endometrial Cells Nuclear Factor κB Response to Flagellin *In Vitro*

**Ignacio Caballero, Sumiah Al Ghareeb, Shaghayegh Basatvat, Javier A. Sánchez-López, Mehrnaz Montazeri, Nasim Maslehat, Sarah Elliott, Neil R. Chapman, Alireza Fazeli***

Academic Unit of Reproductive and Developmental Medicine, Department of Human Metabolism, The Medical School, University of Sheffield, Sheffield, United Kingdom

## Abstract

*Background:* Implantation is a complex process that requires a delicate cooperation between the immune and reproductive system. Any interference in the fine balance could result in embryo loss and infertility. We have recently shown that Toll-like receptor 5 activation results in a decrease of trophoblast cells binding to endometrial cells in an *in vitro* model of human implantation. However, little is known about the downstream signalling leading to the observed failure in implantation and the factors that modulate this immune response.

*Methods and Principal Findings:* An *in vitro* model of embryo implantation was used to evaluate the effect of trophoblasts and flagellin on the activation of NF-κB in endometrial cells and whether TLR5-related *in vitro* implantation failure is signalled through NF-κB. We generated two different NF-κB reporting cell lines by transfecting either an immortalized endometrial epithelial cell line (hTERT-EECs) or a human endometrial carcinoma cell line (Ishikawa 3-H-12) with a plasmid containing the secreted alkaline phosphatase (SEAP) under the control of five NF-κB sites. The presence of trophoblast cells as well as flagellin increased NF-κB activity when compared to controls. The NF-κB activation induced by flagellin was further increased by the addition of trophoblast cells. Moreover, blocking NF-κB signalling with a specific inhibitor (BAY11-7082) was able to restore the binding ability of our trophoblast cell line to the endometrial monolayer.

*Conclusions:* These are the first results showing a local effect of the trophoblasts on the innate immune response of the endometrial epithelium. Moreover, we show that implantation failure caused by intrauterine infections could be associated with abnormal levels of NF-κB activation. Further studies are needed to evaluate the target genes through which NF-κB activation after TLR5 stimulation lead to failure in implantation and the effect of the embryo on those genes. Understanding these pathways could help in the diagnosis and treatment of implantation failure cases.

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* E-mail: A.Fazeli@Sheffield.ac.uk

## Introduction

Implantation of the embryo in the uterus is considered to be one of the most critical steps during pregnancy. This complex biological process represents a paradoxical immune status where a semi-allogenic body (embryo), which under normal circumstances would be rejected by the recipient immune system, is nourished and nurtured [1]. In this regard, different microarray studies have shown that a tight control of the maternal immune system is necessary to promote immune tolerance to the conceptus whilst protecting against infection during the implantation period [1,2]. However, the mechanisms through which all these processes are regulated are unclear. A successful implantation is dependent on a two-way crosstalk between the embryo and maternal signals [3]. This embryo-maternal dialogue should provide endometrial receptivity in synchrony with an optimal embryo development [4].

Providing appropriate endometrial receptivity is crucial for implantation since approximately two-thirds of implantation failures are imputable to inadequate uterine receptivity [5]. Uterine receptivity to the embryo is clearly influenced by the hormones, growth factors and cytokines present in the uterine environment during the window of implantation. This cytokine network is extremely sensitive to systemic and local changes and needs to be kept in balance for a successful implantation [6,7]. One of the main regulators of the immune response is the Toll-like receptor family (TLR). TLRs are the main family of pattern recognition receptors (PRRs) of the innate immune system [8,9]. This family of receptors have been seen to be expressed in human endometrial tissue and trophoblasts [10,11] and are known to have a key role in the modulation of immune and inflammatory responses in mammals [12]. Although their principal role has been generally assumed to be the defence against infection, TLRs are able to modulate the cytokine environment in response to endogenous factors called "danger-associated molecular patterns" (DAMPs) [13,14].

TLR signalling involves activation of nuclear factor κB transcription factor (NF-κB). There are two best-described pathways, the canonical and non-canonical, leading to NF-κB

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# The Effect of Glycerol Concentrations on the Post-thaw *In Vitro* Characteristics of Cryopreserved Sex-sorted Boar Spermatozoa

**I Parrilla, D del Olmo, I Caballero, T Tarantini, C Cuello, MA Gil, J Roca, EA Martinez and JM Vazquez**

*Department of Animal Medicine and Surgery, University of Murcia, Murcia, Spain*

**Contents**

The objective of this study was to optimize protocols for the cryopreservation of sex-sorted boar spermatozoa. In the experiment 1, we evaluated the effects of a standard boar sperm cryopreservation procedure (3% final glycerol concentration) on the *in vitro* characteristics of sex-sorted sperm frozen at low sperm concentrations ($20 \times 10^6$ sperm/ml; S20 group). Non-sorted spermatozoa frozen at $1000 \times 10^6$ (C1000 group) and $20 \times 10^6$ (C20 group) sperm/ml were used as the freezing control groups. In experiment 2, the effects of different final glycerol concentrations (0.16%, 0.5%, 1.0%, 2.0% and 3.0%) on post-thaw quality of the S20 and C20 groups were evaluated. In both experiments, the samples were evaluated prior to freezing (5°C) and at 30, 90 and 150 min after thawing. Experiment 1 indicated that freezing sperm at low concentrations decreased ($p < 0.05$) the total motility (TM) and progressive motility (PM) at 90 and 150 min after thawing regardless of whether the sperm were sorted or not. However, the sperm membrane integrity was not affected at any evaluation step. Inexperiment 2, significant effects on the TM and PM because of increased glycerol concentrations in the S20 and C20 groups were observed only at 90 and 150 min after thawing. The samples frozen in 3% glycerol showed lower ($p < 0.05$) TM and PM values when compared to those frozen in the presence of 0.5% and 1% glycerol. In both experiments, non-sorted control samples displayed higher percentages of spermatozoa with damaged DNA than sorted spermatozoa. In conclusion, the optimization of cryopreservation conditions by decreasing the glycerol concentrations can improve post-thaw motility of sex-sorted spermatozoa frozen at low concentrations.

## Introduction

Preselection of offspring sex is a potentially important tool for improving reproductive management in pig production, particularly in nucleus herds. The implementation of sex pre-selection would allow producers to optimize male or female lines according to the farm's needs. Although success has been achieved in other species, such as cattle [with millions of commercial inseminations with frozen-thawed sex-sorted spermatozoa every year (Seidel 2009)], the limitations of incorporating sex-sorted sperm into pig production have not been completely overcome (Vazquez et al. 2009). One of these impediments is the low number of spermatozoa that can be sex-sorted relative to the high number of spermatozoa required for successful standard intracervical insemination. However, efficient techniques for insemination using low numbers of spermatozoa are now available in pigs. Currently, laparoscopic insemination is a powerful new tool in which the number of spermatozoa needed per inseminated sow is approximately $3–5 \times 10^6$ (Roca et al. 2011). Another limitation of using sex-sorting technology in pigs is the poor freezability of sex-sorted spermatozoa, especially when compared with other species (Rath and Johnson 2008; Vazquez et al. 2009). The development of a successful procedure for freezing sex-sorted boar spermatozoa is essential for the practical application of sorting technology because it would provide farm managers greater flexibility in pig production. Although several attempts to freeze sex-sorted boar sperm have been reported, fertility results obtained in these studies have been extremely poor (Bathgate et al. 2007, 2008). Moreover, results from our laboratory indicate that the *in vitro* fertilizing ability of cryopreserved sex-sorted sperm is compromised by freezing.

The sex-sorting process has been shown to produce a population of weak, highly diluted spermatozoa with a reduced lifespan (Maxwell and Johnson 1997; Caballero et al. 2008; Spinaci et al. 2010). For the successful cryopreservation of sex-sorted boar spermatozoa and to increase their functionality after thawing, it is necessary to adjust the standard spermatozoa cryopreservation procedures according to the specific requirements of this type of spermatozoa. The concentration of glycerol used during freezing could be one of these critical adjustments. Final glycerol concentrations of 2–4% have been recommended for freezing boar spermatozoa when the final sperm concentration is $1000 \times 10^6$ sperm/ml (Hernández et al. 2007). However, despite its importance as a cryoprotectant, glycerol has toxic chemical and osmotic effects on sperm (Woods et al. 2004) that can cause decreases in post-thaw sperm motility and viability with a subsequent decline in the fertilizing ability. Moreover, the negative effects of glycerol could be related to the concentration and the number of spermatozoa present in dilute samples prior to freezing, as previously reported for other species (Evans and Maxwell 1987; Beilby et al. 2010). Because of the low number of available sex-sorted spermatozoa per time unit with the currently available flow cytometry sorters and to optimize the use of laparoscopic insemination with frozen sex-sorted sperm, it would be necessary to adapt the number of spermatozoa per insemination straw based on the number of spermatozoa required per insemination. Currently, we are developing a freezing procedure using 0.25-ml straws with a final sex-sorted sperm concentration of $20 \times 10^6$ sperm/ml ($5 \times 10^6$ sperm/straw). This concentration is 50 times lower than that used in current boar semen cryopreservation protocols. Therefore, adjustments of the current cryopreservation protocols with respect to the concentration of glycerol in the freezing extender are

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# Seminal Plasma Proteins as Modulators of the Sperm Function and Their Application in Sperm Biotechnologies

**I Caballero[1], I Parrilla[2], C Almiñana[1], D del Olmo[2], J Roca[2], EA Martínez[2] and JM Vázquez[2]**

[1]*Academic Unit of Reproductive and Developmental Medicine, Department of Human Metabolism, The Medical School, University of Sheffield, Sheffield, UK;* [2]*Department of Medicine and Animal Surgery, Veterinary Faculty, University of Murcia, Murcia, Spain*

**Contents**

Seminal plasma (SP) is known to play an important role in mammalian fertilization. However, the variability found in its composition among species, males and even fractions of the same ejaculate has made difficult to completely understand its effect in sperm function. Proteins are one of the major SP components that modulate sperm functionality. During the last years, intensive work has been performed to characterize the role of these proteins. They have been found to influence sperm capacitation, formation of the oviductal sperm reservoir and sperm–oocyte interaction. Sperm biotechnologies, such as sperm cryopreservation and flow cytometric sex-sorting, that involve a substantial dilution of the SP are detrimental to sperm quality. Attempts to improve the outcome of these biotechnologies include the restoration of SP, which has produced contradictory results. To overcome this variability, different research groups have proposed the application of isolated SP proteins. Herein, we will review the current knowledge in the role of the major SP proteins as modulators of sperm functionality. Furthermore, we will discuss the possible applications of the SP proteins in sperm cryopreservation and flow cytometric sex-sorting.

## Introduction

Since the early years of the 20th century, researchers realized that the seminal plasma (SP) played more roles in sperm biology other than serving as a mere vehicle for the spermatozoa (Goldblatt 1935). Growing evidence point to this 'acellular' part of the semen, the SP, as a fundamental player in the modulation of sperm function. Seminal plasma provides metabolic support, as an energy source for the sperm cells, and influences sperm functionality in a very complex and not completely understood manner (Mann 1978; Mann and Lutwak-Mann 1981; Rodríguez-Martínez et al. 1984; Rodríguez-Martínez 1991; Caballero et al. 2004a). This fluid is a complex mix of secretions from the epididymis and the accessory sexual glands. Variations in the presence as well as size of these glands among species of mammals generate obvious differences in the composition of the SP. Moreover, these differences in SP composition have also been found amongst males, ejaculates from males and fractions of the same ejaculate (Killian et al. 1993; Ashworth et al. 1994; Zhu et al. 2000; Rodríguez-Martínez et al. 2011).

Several difficulties to understand the role of SP in the modulation of the sperm functionality have arisen because of the complexity and variability in SP composition. In this regard, in most domestic species, semen is ejaculated sequentially in the so-called spurts or jets. During natural mating, the sperm-rich fraction is deposited in the female genital tract and does not come into contact with posterior fractions of the ejaculate. However, when semen is collected, in most artificial insemination (AI) centres and research laboratories, all the different fractions of the SP (with the exception of the gel fraction that is usually removed) are blended together in the same vial. During this period, the spermatozoa come into contact with both positive and negative factors of the SP, a situation that does not happen under physiological conditions (Rodríguez-Martínez et al. 2011). A common practice in the AI industry includes the partial removal or dilution of the SP and replacement by extenders for handling and storage of sperm samples (Rodríguez-Martínez and Barth 2007). The rationale behind this action is that certain SP fractions, the prostatic one (England and Allen, 1992), the gel fraction in stallion, boar or camelids (Mann and Lutwak-Mann 1981), as well as the secretion of the seminal vesicles (ram, Ashworth et al. 1994; bull, Way et al. 2000; buck, Azerêdo et al. 2000), are considered by some authors as detrimental for sperm survival *in vitro*. Despite the aforementioned practice, the application of SP in biotechnologies, such as flow cytometric sex-sorting and sperm cryopreservation, has been a topic of a great number of studies. These biotechnologies involve large dilutions of the spermatozoa and subsequently partial or complete removal of SP, which leads to motility loss, and decrease in metabolic activity and fertilizing capacity (Dott et al. 1979; Ashworth et al. 1994; Maxwell and Johnson 1999). This detrimental effect in sperm functionality, described by Mann (1954) as the 'dilution effect', has been associated with the dilution of SP factors that protects the sperm from membrane damage and premature capacitation (Maxwell et al. 1997, 1998). It would be reasonable to think that a certain proportion of SP in the semen sample is necessary to decrease the detrimental effects associated with this 'dilution effect'. Unfortunately, several studies involving the addition of SP to sperm subjected to these biotechnologies have shown variable results. Initial research in sperm cryopreservation showed that the addition of SP protected boar spermatozoa from cold shock and improved sperm motility (Pursel et al. 1973; Pursel and Johnson 1975). In the same way, SP has proven beneficial when added to cryopreserved bull (Garner et al. 2001), stallion (Aurich et al. 1996) and boar (Hernández et al. 2007; Vadnais and Roberts 2007) spermatozoa. On the other hand, different studies have shown no improvement or even detrimental effects in quality parameters evaluated when SP was added after thawing (Dott et al. 1979; Moore et al. 2005; Muiño-Blanco et al. 2008). The

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# Capability of frozen–thawed boar spermatozoa to sustain pre-implantational embryo development

C. Almiñana, M.A. Gil, C. Cuello, I. Parrilla, I. Caballero, J. Sanchez-Osorio, J.M. Vazquez, J. Roca, E.A. Martinez*

*Department of Animal Medicine and Surgery, Faculty of Veterinary Medicine, University of Murcia, Murcia, Spain*

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ABSTRACT

The present study was designed to evaluate the competence of frozen–thawed (FT) boar spermatozoa on the developmental ability of early porcine embryos under *in vitro* and *in vivo* conditions. Repeat deep uterine insemination was applied to sows ($n = 12$) at 30 h and 36 h after estrus detection, using either $750 \times 10^6$ of liquid or FT motile spermatozoa in a volume of 5 mL. Semen was pooled from mature Pietrain boars ($n = 3$) of proven fertility and classified as "good sperm freezers" in previous experiments. Only sows with preovulatory follicles identified during the first insemination, and those that had ovulated 6 h after the second insemination were used in the experiment. Sows were subjected to laparotomy on Day 2 of the estrous cycle (the onset of estrus classified as Day 0), and only one oviduct of each animal was flushed. The collected embryos (zygotes and two to four cell embryos) were cultured in vitro for 96 h. Embryos from the contralateral oviduct were permitted to develop *in vivo* for the same period of time. Fertilization rates were 94.4% and 90.9% for liquid ($n = 90$) and FT ($n = 77$) insemination groups, respectively, and did not differ significantly between groups. The use of FT semen for insemination did not affect embryo development and embryo quality in terms of total cells number per embryo. In contrast, these parameters were affected by the culture system ($P < 0.001$). These data indicate that when an optimal protocol for insemination with FT semen is used, normal fertilization rates, embryonic development, and embryo quality are obtained, and consequently acceptable farrowing rates and prolificacy can be expected.



## 1. Introduction

There has been an increased development and improvement of emerging reproductive technologies in the pig industry in recent years. Sperm cryopreservation, which contributes to the conservation of porcine genetic resources and to the introduction of new genetics into the porcine breeding population is one of these technologies. Frozen–thawed (FT) semen is a useful alternative to the transportation of live boars and allows large numbers of females to be inseminated over extended periods. Despite the growing interest, the commercial application of FT semen at the beginning of the new millennium was restricted to 1% of the porcine artificial insemination worldwide (Wagner and Thibier, 2000). Reduced fertility and litter size achieved when FT semen is used under field conditions, as compared with those obtained with liquid semen, discouraged its use (Johnson et al., 2000; Martinez et al., 2005; Roca et al., 2006a,b). Increased fertility rates have been reported using improvements in cryopreservation protocols (Bussiere et al., 2000; Eriksson et al., 2002) and new procedures for artificial insemination (AI), such

* Corresponding author at: Departamento Medicina y Cirugía Animal (Reproducción y Obstetricia), Hospital Clínico Veterinario, Universidad de Murcia, 300071 Murcia, Spain. Tel.: +34 968364734; fax: +34 968367069.
*E-mail address:* emilio@um.es (E.A. Martinez).

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Technical note

# *In vitro* postwarming viability of vitrified porcine embryos: Effect of cryostorage length

J. Sanchez-Osorio[a], C. Cuello[a], M.A. Gil[a], I. Parrilla[a], C. Almiñana[a], I. Caballero[a], J. Roca[a], J.M. Vazquez[a], H. Rodriguez-Martinez[b], E.A. Martinez[a,*]

[a] *Department of Animal Medicine and Surgery, Faculty of Veterinary Medicine, University of Murcia, E-30071 Murcia, Spain*
[b] *Division of Reproduction, Faculty of Veterinary Medicine & Animal Science (FVMAS), Swedish University of Agricultural Sciences (SLU), POB 7054, SE-75007 Uppsala, Sweden*

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**Abstract**

Porcine embryos, which had been vitrified and stored in liquid nitrogen for up to three yr, were retrospectively analyzed to evaluate the influence of duration of storage on their *in vitro* viability post-warming. All embryos were vitrified (OPS or SOPS) and warmed (three-step or direct warming) using procedures that resulted in the same *in vitro* survival, hatching rates, and numbers of cells. Therefore, embryo data obtained using the different procedures were pooled according to their developmental stage as morulae (n = 571) or blastocysts (n = 797) and to the length of their storage in liquid nitrogen: a) 1–9 d; b) 10–30 d; c) 31–90 d; d) 1–3 yr. Non-vitrified embryos of corresponding developmental stages were used as a fresh control group (n = 280). Survival and hatching rates were evaluated after *in vitro* culture to assess embryo viability. The total number of cells was counted in the resulting viable blastocysts as an indicator of quality. A total of 1,648 fresh and vitrified embryos were analyzed. *In vitro* survival and hatching rates, but not the number of cells, differed significantly between vitrified morulae and their fresh counterparts irrespective of the duration of cryostorage. Length of storage in liquid nitrogen ($LN_2$) did not influence *in vitro* viability among different groups of vitrified/warmed morulae nor embryos at the blastocyst stage. In conclusion, duration of storage in $LN_2$ has no effect on the post-warming viability of porcine embryos vitrified at morula or blastocyst stage.


*Keywords:* Porcine; Embryo; Vitrification; Storage; Cryopreservation

## 1. Introduction

For years, intensive breeding programmes have allowed the improvement of genetic progress in the pig industry. However, increased selection pressure is causing a reduction in genetic diversity and losses of relevant breeding lines. Conservation of genetic resources is crucial to allow the maintenance of diversity and make a diverse range of genotypes available for future use. Cryostorage of embryos enables preservation of individual genetic information and also provides an efficient system to transmit genetic potential [1]. Furthermore, associating cryopreservation of embryos with new procedures for non-surgical embryo transfer [2] allows the movement of genetic resources without compromising animal welfare, minimizes the risk of disease transmission, and reduces transportation costs.

Open Pulled Straw (OPS) vitrification [3] is the most employed method for cryopreservation of pig

* Corresponding Author. Tel.: 34868884812; fax: 34868887069.
*E-mail address:* emilio@um.es (E.A. Martinez).

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ORIGINAL ARTICLE

# PSP-I/PSP-II spermadhesin exert a decapacitation effect on highly extended boar spermatozoa

Ignacio Caballero,* Juan M. Vazquez,* Gloria M. Mayor,* Carmen Almiñana,* Juan J. Calvete,† Libia Sanz,† Jordi Roca* and Emilio A. Martinez*

*Department of Medicine and Surgery, Faculty of Veterinary Medicine, University of Murcia, Murcia, and †Institute of Biomedicine, C.S.I.C., Valencia, Spain

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**Correspondence:**
Ignacio Caballero, Departamento de Medicina y Cirugía Animal, Facultad de Veterinaria, Universidad de Murcia, 30071 Murcia, Spain.
E-mail: nachof@um.es

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## Summary

PSP-I/PSP-II heterodimer is a major protein of boar seminal plasma that is able to preserve, in vitro, the viability, motility and mitochondrial activity of highly-extended boar spermatozoa. However, a relationship between the protective effects of the heterodimer and sperm capacitation is still unclear. The present study investigated the effect of the PSP-I/PSP-II (1.5 mg/mL) on membrane stability, intracellular calcium concentration ($[Ca^{2+}]_I$) and plasma membrane and acrosome integrity of highly extended boar spermatozoa. Boar spermatozoa were diluted to $1 \times 10^6$ spermatozoa/mL and incubated at 38 °C in Phosphate-buffered saline (PBS) for 10, 30, 60, 120 and 300 min or in modified Tris-buffered medium (mTBM) for 10, 20, 30, 60 and 120 min. After each incubation time, the membrane stability (using Merocyanine-540/Yo-Pro-1), elevation of $[Ca^{2+}]_I$ (using Fluo-3-AM/PI) and the sperm plasma membrane and acrosome integrity (using SYBR-14/PI/PE-PNA) were evaluated by flow cytometry. As expected, exposure of the spermatozoa to the PSP-I/PSP-II preserved the plasma membrane and acrosome integrity compared to non-exposed spermatozoa in both media PBS and mTBM ($p < .01$). The evaluation of membrane stability showed no differences in the percentages of viable sperm with instable plasma membrane in the presence of the PSP-I/PSP-II compared to controls irrespective of the dilution media. The evaluation of the $[Ca^{2+}]_I$ levels showed that while spermatozoa incubated in mTBM and exposed to PSP-I/PSP-II had lower $[Ca^{2+}]_I$ than controls (39.08% vs. 47.97%, respectively; $p < .05$), no differences were observed in those samples incubated in PBS. However, a temporal evaluation of the samples showed that a similar proportion of live spermatozoa were able to achieve high levels of $[Ca^{2+}]_I$ and membrane instability independent of the presence of PSP-I/PSP-II. In conclusion, PSP-I/PSP-II exert a non-permanent decapacitation effect on highly extended boar spermatozoa that is related with a delay in the increase of $[Ca^{2+}]_I$ levels.

## Introduction

After ejaculation, spermatozoa are bathed in seminal plasma (SP), deposited in the female genital tract and transported to the oviduct where they will undergo capacitation, a variety of cellular modifications prior to fertilization (Yanagimachi, 1994; Rodríguez-Martínez *et al.*, 2005). These modifications involve destabilization of the sperm plasma membrane, elevation of the intracellular calcium concentration ($[Ca^{2+}]_I$) and the cAMP level and stimulation of protein tyrosine phosphorylation (Yanagimachi, 1994; Flesch & Gadella, 2000; Vadnais *et al.*, 2007; Witte & Schäfer-Somi, 2007). At present, to achieve a detailed understanding in this process is a topic of considerable interest to improve the application of sperm biotechnologies, such as cryopreservation and flow cytometric sex-sorting. These processes require sperm manipulations, including dilution in different extenders, which trigger a series of phenomena that resemble those occurring during sperm capacitation (Maxwell *et al.*,

 

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# EVALUATION OF THE *SERRATIA MARCESCENS* NUCLEASE (NucA) AS A TRANSGENIC CELL ABLATION SYSTEM IN PORCINE

**I. Caballero and J. A. Piedrahita**
*Department of Molecular Biomedical Sciences and Center for Comparative Medicine and Translational Research, North Carolina State University, Raleigh, North Carolina*

***The efficiency of the Serratia marcescens nuclease encoded by the NucA gene, with or without a nuclear localization signal (NLS), and the commonly used diphtheria toxin A (DTA) were compared for their ability to ablate cells in culture. Constructs containing the test genes driven by the β-actin promoter coupled with enhancer elements from the cytomegalovirus promoter and rabbit β-globin gene (pCAG) and the blasticidin resistance gene driven by the phosphoglycerate kinase (PGK) promoter were generated and electroporated into porcine fetal fibroblasts. Three independent replicates were completed. Following blasticidin selection, the number of surviving colonies was counted to assess the efficiency of the toxic gene. Both NucA and DTA proved to be effective in killing porcine fibroblasts compared to controls. However, the efficiency of cell ablation was significantly higher with DTA than with NucA or NucANLS ($p < 0.05$). Gene expression analysis of surviving colonies indicated that survival is related to low or absent expression of the toxic genes. These results indicate that the NucA gene, while capable of mammalian cell ablation, is less efficient than DTA.***

*Keywords*: Ablation; DTA; Fibroblasts; NucA; Porcine

## INTRODUCTION

The study of specific cell lineages, tissues, and tissue interactions or the development of animal models for human diseases may require the ablation of a specific cell lineage (1, 2). Several strategies have been developed to delete the target cells including microdissection, laser ablation, and the use of antibodies or chemicals that target specific cell types. An alternate approach is the use of transgenic cell ablation whereby a toxic "suicide" gene, driven by a cell specific promoter, is introduced into the cell or animal (3, 4). This approach can be modified to achieve total ablation of a cell lineage (target and all descendents) or be strictly confined to a targeted differentiated

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Address correspondence to Jorge A. Piedrahita, Department of Molecular Biomedical Sciences and Center for Comparative Medicine and Translational Research, North Carolina State University, Raleigh, North Carolina. E-mail: jorge_piedrahita@ncsu.edu

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# *In Vitro* Fertilization (IVF) in Straws and a Short Gamete Coincubation Time Improves the Efficiency of Porcine IVF

**C Almiñana, MA Gil, C Cuello, I Caballero, J Roca, JM Vazquez and EA Martinez**

*Department of Animal Medicine and Surgery, Faculty of Veterinary Medicine, University of Murcia, Murcia, Spain*

**Contents**

The present study was designed to evaluate three different *in vitro* fertilization (IVF) systems: a straw-IVF system with 10 min of coincubation, a straw-IVF system with 6-h coincubation and the microdrop-IVF system with 6-h coincubation (the traditional IVF system used routinely in most of IVF laboratories) in an attempt to reduce polyspermic penetration (Experiment 1). When the straw-IVF system was tested in combination with two coincubation times, the use of 10 min of coincubation significantly increased ($p < 0.001$) the penetration rate and the efficiency of fertilization (67.7 ± 6.4% vs 31.9 ± 6.5% and 41.5 ± 2.5% vs 17.6 ± 2.5% for 10 min and 6 h, respectively), while there were no significant differences in the incidence of monospermy between both systems (64.3 ± 5.1% and 67.7 ± 3.4%, for 10 min and 6 h, respectively). The penetration rate in the 6-h microdrop-IVF system was higher (93.8 ± 3.6%; $p < 0.001$) compared with the 10-min straw-IVF system (67.7 ± 6.4%), however, monospermy was severely reduced (25.0 ± 4.3% vs 67.7 ± 3.4%, for the 6-h microdrop-IVF system and 10-min straw-IVF system, respectively). The efficiency of the IVF showed similar values between microdrop and 6-h straw-IVF systems, but efficiency was significantly improved ($p < 0.05$) when the 10-min straw-IVF system was used. Experiment 2 was designed to compare porcine *in vitro* embryo production in two IVF systems, the 6-h microdrop-IVF system (1000 sperm per oocyte) and 10-min straw-IVF system (30 000 sperm per oocyte). The blastocyst formation rates tended ($p = 0.06$) to be higher when the 10-min straw-IVF system was used compared with the 6-h microdrop-IVF system. In addition, the number of total cells per blastocyst increased significantly ($p < 0.05$) in the 10-min straw-IVF system. These results showed that the 10-min straw-IVF system is an effective way to decrease polyspermic penetration, and improve the efficiency of fertilization and the quality of blastocysts in terms of cell number per embryo.

## Introduction

*In vitro* production of mammalian embryos is no longer an exclusive technique for research laboratories. It is considered as an initial step to obtain a large source of embryos from abattoir-derived ovaries for the application of new biotechnologies. In pigs, the efficiency of embryos produced *in vitro* is remarkably low because of the unacceptable high incidence of polyspermy obtained during *in vitro* fertilization (IVF) (Niwa 1993; Matas et al. 1996; Abeydeera and Day 1997; Funahashi and Day 1997; Wang et al. 1997; Abeydeera 2002), and the poor quality of blastocysts derived following *in vitro* culture of putative zygotes (Han et al. 1999a,b; McCauley et al. 2003). For this reason, the improvement of IVF protocols is critical to overcome the low rates of porcine blastocysts currently produced *in vitro*.

The conditions under which IVF is performed differ among laboratories. However, a common factor in current IVF systems is the exposition of oocytes to an excessive and non-physiological number of sperm during gamete coincubation. This high number of spermatozoa per oocyte causes simultaneous sperm penetration and results in polyspermic fertilization (Wang et al. 2003). In addition, the environment conditions during *in vitro* gamete coincubation are far from the oviductal environment in the female genital tract. These IVF conditions appear to override the *in vivo* strategies for sperm capacitation and natural selection of spermatozoa after insemination (Hunter and Rodriguez-Martinez 2004). Thus, a reduction in spermatozoa number during IVF has been studied in order to decrease polyspermic penetration, however, it also reduces overall sperm penetration rates (Abeydeera and Day 1997) resulting in low efficiency of fertilization. New strategies such as the straw-IVF system (Li et al. 2003), the climbing-over-wall method (Funahashi and Nagai 2000), or microfluidic technology (Beebe et al. 2002; Clark et al. 2002, 2003, 2005) have been reported in an attempt to mimic the process of *in vivo* fertilization in the oviductal tract, where spermatozoa are gradually capacitated during transport to the site of fertilization, (Rodriguez-Martinez et al. 2005), reducing the incidence of polyspermic fertilization (Funahashi and Nagai 2000).

On the other hand, several studies suggest that longer gamete coincubation times (Coy et al. 1993a; Martinez et al. 1996; Abeydeera and Day 1997; Marchal et al. 2002; Gil et al. 2007) parallel higher penetration rates and higher incidence of polyspermic penetration. In addition, it has been reported that there are sufficient spermatozoa bound to zona pellucida within the first 2–10 min of gamete coincubation to penetrate a high number of oocytes (Gil et al. 2004a, 2007; Almiñana et al. 2008) and, therefore, a short gamete coincubation time (10 min) has been suggested as an alternative method to increase the efficiency of IVF of porcine oocytes (Grupen and Nottle 2000; Funahashi and Romar 2004; Gil et al. 2004a; Almiñana et al. 2005).

Based on these findings, the present study was designed to evaluate whether the combination of a straw-IVF system and a short coincubation time (10 min) is a suitable strategy to decrease polyspermic fertilization and improve *in vitro* production of porcine blastocysts.

## Materials and Methods

### Culture media

The medium used for the collection and washing of cumulus–oocyte complexes (COCs) was Dulbecco's phosphate-buffered saline (DPBS) medium composed of 136.89 mM NaCl, 2.68 mM KCl, 8.1 mM $Na_2HPO_4$ and 1.46 mM $CaCl_2.2H_2O$ supplemented with 4 mg/ml

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# Major proteins of boar seminal plasma as a tool for biotechnological preservation of spermatozoa

I. Caballero [a,*], J.M. Vazquez [a], E.M. García [a], I. Parrilla [a,c], J. Roca [a], J.J. Calvete [b], L. Sanz [b], E.A. Martínez [a]

[a] Department of Animal Medicine and Surgery, University of Murcia, Murcia Spain
[b] Institute of Biomedicine, C.S.I.C., Valencia, Spain
[c] Institute for Animal Breeding, Mariensee (FAL), Germany

**Abstract**

Boar seminal plasma is a complex mixture of secretions from the testes, epididymides, and the male accessory reproductive organs which bathe the spermatozoa at ejaculation. The seminal plasma contains factors, mostly proteins, which influence the spermatozoa, the female genital tract, and the ovum. In boars, most of the proteins belong to the spermadhesin family and bind to the sperm surface. Spermadhesins are multifunctional proteins with a wide range of ligand-binding abilities to heparin, phospholipids, protease inhibitors and carbohydrates; the family can be roughly divided into heparin-binding (AQN-1, AQN-3, AWN) and non-heparin-binding spermadhesins (PSP-I/PSP-II heterodimer). These proteins have various effects promoting or inhibiting sperm functions including motility, oviduct binding, zona binding/penetration, and ultimately fertilization. The complexity of the environmental signals that influence these actions have implications for the uses of these proteins *in vivo* and *in vitro*, and may lead to uses in improving sperm storage.


*Keywords:* Seminal plasma protein; Spermadhesin; Sperm capacitation; Oviductal sperm reservoir; Boar semen

## 1. Introduction

Boar seminal plasma (SP) is a complex mixture of secretions originating from the testes, epididymides, and the male accessory reproductive organs in which the spermatozoa are bathed at ejaculation. It is well established that SP contains factors that influence both the spermatozoa and the female genital tract during sperm transport [1–4]; most of these factors are SP proteins. In boars, the bulk of SP proteins (>90%) belong to the spermadhesin family, a group of 12–16 kDa glycoproteins that bind to the sperm surface. These proteins, containing a single CUB domain architecture [5,6], comprise five members: AQN-1, AQN-3, AWN, PSP-I and PSP-II. The latter two proteins form a glycosylated PSP-I/PSP-II heterodimer and represent over 50% of the total protein content in boar SP. Spermadhesins are multifunctional proteins exhibiting a wide range of ligand-binding abilities to heparin, phospholipids, protease inhibitors and carbohydrates that change with glycosylation and aggregation states. Depending on their binding capability, spermadhesins can be classified into heparin-binding (AQN-1, AQN-3, AWN) and non-heparin-binding spermadhesins (PSP-I/PSP-II heterodimer) [6–12].

* Corresponding author at: Departamento de Medicina y Cirugía Animal, Facultad de Veterinaria, Universidad de Murcia, 30071 Murcia, Spain. Tel.: +34 968 364812; fax: +34 968 367069.
*E-mail address:* nachof@um.es (I. Caballero).

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# Factors affecting the success rate of porcine embryo vitrification by the Open Pulled Straw method

J. Sanchez-Osorio, C. Cuello, M.A. Gil, C. Almiñana, I. Parrilla, I. Caballero, E.M. Garcia, J.M. Vazquez, J. Roca, E.A. Martinez*

*Department of Animal Medicine and Surgery, Veterinary Science, University of Murcia, E-30071 Murcia, Spain*

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**Abstract**

The objectives of this study were: (1) to evaluate the influence of porcine embryo developmental stage on *in vitro* embryo development after vitrification, (2) to study the efficiency of the one-step dilution procedure, compared with conventional warming, for vitrified embryos at different stages of development, and (3) to determine the influence of the embryo donor on the *in vitro* survival of vitrified embryos at morulae and blastocyst stages. Two to four cell embryos, morulae and blastocysts were collected by laparotomy from weaned crossbred sows ($n = 55$). Vitrification and conventional warming were performed using the OPS procedure with Superfine Open Pulled Straws (SOPS). For one-step dilution, embryos were placed in 800 μl TCM199-HEPES containing 20% of new born calf serum and 0.13 M sucrose for 5 min. To evaluate development, two to four cell embryos, morulae and blastocysts were cultured *in vitro* for 120, 48 and 24 h, respectively. Some fresh embryos from each developmental stage were not vitrified and cultured as controls. Embryos were morphologically evaluated for their developmental capacity during the *in vitro* culture by stereomicroscopy. The total cell number of embryos was assessed by Hoechst-33342 staining and fluorescence microscope observation. There was a significant effect of the stage of development on the *in vitro* survival, perihatching rate and the number of cells of embryos after vitrification and warming (Experiment 1; $p < 0.001$). The survival and perihatching rates of two to four cell embryos were lower than those obtained for morulae and blastocysts ($p < 0.001$). No differences ($p > 0.05$) in survival rates were found between vitrified and fresh blastocysts. The warming procedure did not affect the development and total cell number of vitrified two to four cell embryos, morulae or blastocysts (Experiment 2). However, donor had a significant effect ($p < 0.001$) on the *in vitro* development and the number of cells of morulae and blastocysts after vitrification and warming (Experiment 3). In conclusion, the embryo developmental stage and the embryo donor were important factors that affected the development of porcine embryos after

* Corresponding author at: Departamento Medicina y Cirugía Animal (Reproducción y Obstetricia), Hospital Clínico Veterinario, Universidad de Murcia, 30071 Murcia, Spain. Tel.: +34 968364812; fax: +34 968367069.
*E-mail address:* emilio@um.es (E.A. Martinez).


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## Review Article

# Improving the Efficiency of Insemination with Sex-sorted Spermatozoa

**JM Vazquez, I Parrilla, MA Gil, C Cuello, I Caballero, JL Vazquez, J Roca and EA Martínez**

*Department of Animal Medicine and Surgery, Faculty of Veterinary Medicine, University of Murcia, Murcia, Spain*

**Contents**

The sorting of X- and Y-chromosome-bearing spermatozoa by flow cytometry is nowadays one of the most apt assisted-reproduction technologies in livestock production. Potential economic and biological benefits, as well as those related to easier management of herds, have been reported arising out of the application of this technique, especially in cattle. Yet, the sex-sorting procedure induces damage to spermatozoa, affecting their function and fertilizing ability. Different species present varying degrees of susceptibility to damage from the sorting process and each has its own requirements for sex-sorted insemination procedures. Thus, several new protocols and strategies have been designed for the handling of sorted spermatozoa, with the main objective of optimizing their fertilizing ability and the consequent application of flow-cytometric sex-sorting technology. This article reviews current advances in this technology, pointing out the components to be improved before this technology may be widely applied in different domestic species.

## Introduction

Pre-selection of the sex of offspring by sexing of spermatozoa is widely accepted as one of the most important advances in assisted reproductive technology (ART). The only effective sex pre-selection methodology, validated in several laboratories, is based on measuring the differential amount of DNA present in X- and Y-chromosome-bearing spermatozoa (Johnson and Pinkel 1986), and using a modified flow cytometer–cell sorter, which is able to identify and sort spermatozoa into X- or Y-populations with a high percentage of purity (Johnson and Welch 1999; Parrilla et al. 2003). This sperm-sexing technology is being applied commercially to produce offspring of the desired sex mainly in bovine livestock (Garner 2006).

Offspring of the pre-determined sex have been obtained, using this technology, in species of domestic mammals such as pigs, rabbits, horses, sheep and cats and also in non-domestic mammals including the dolphin, elk and buffalo. Even in humans, sperm-sexing by flow cytometry has been successfully applied to obtain offspring of a pre-determined sex (Cran 2007).

The benefits derived from the application of this biotechnology are numerous and change according to the species of application. In humans, it provides the possibility of avoiding critical hereditary sex-linked diseases. In wildlife species, sex-sorted spermatozoa can help to recover critically small populations in captive breeding programmes (Hermes et al. 2007). Yet, the application of this technology has the greatest advantage when applied to species used in animal production because of the derived economic impact, enhancement of genetic management and optimization of reproductive efficiency.

At present, the commercialization of sexed spermatozoa is a reality in cattle. Producers are able to buy sexed-frozen spermatozoa from selected bulls and use it for optimizing the outputs of their farms. Bull spermatozoa are easier to sort because the difference in DNA content between X- and Y-chromosome-bearing cells is higher in bulls than in stallions or boars (Garner 2006). In addition, bull spermatozoa have a better response to the freezing and also to the sorting procedure than spermatozoa from the latter species. Recently, the ability of bull spermatozoa to retain their functional integrity after double freezing and thawing has been reported (Maxwell et al. 2007a). For these reasons, together with the ease of performing artificial insemination (AI) into the uterus using a small number of spermatozoa, sex-sorting technology has been successfully applied for cattle production (Hamano 2007).

Unlike the spermatozoa of most other livestock species, ram spermatozoa appear to be highly resistant to sex-sorting-induced damage, exhibiting improved motility, viability, acrosomal integrity and mitochondrial function, but reduced straight-line velocity and progression through artificial cervical mucus, after cryopreservation when compared with non-sorted controls (De Graaf et al. 2006). Recently, Beilby et al. (2008) showed that these advantages, presumably because of the selection of spermatozoa resistant to damage by the flow-cytometric sorting process, were reflected in better fertility of ewes inseminated with sex-sorted frozen–thawed spermatozoa than the unsorted frozen–thawed ones.

The current situation of sperm-sexing technology is the result of many years of intense work from several research groups and companies, trusting in the great possibilities of the technique in the animal production field. Efforts have been directed to improve sorting efficiency, in terms of yield and quality of spermatozoa after sorting, and to optimize the results derived from the application of this biotechnology. Recently, several reviews about the procedure and its application have been published. These reviews mainly explain the status of the technology in cattle and the impact of its application in bovine livestock production (De Vries et al. 2008; Garner and Seidel 2008). Our review is focussed on the two critical steps that need to be improved before the commercial application of this technology in domestic species: (i) the development of

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# Low-Dose Insemination in Pigs: Problems and Possibilities

**JM Vazquez, J Roca, MA Gil, C Cuello, I Parrilla, I Caballero, JL Vazquez and EA Martínez**

*Department of Animal Medicine and Surgery, Faculty of Veterinary Medicine, Campus de Espinardo, University of Murcia, Murcia, Spain*

**Contents**

Low-dose AI procedures are required by the pig industry to efficiently utilize emerging sperm technologies, such as cryopreservation and sex-sorting. Currently, several different procedures for inseminating with a low or very low number of spermatozoa have been described. Deep intrauterine insemination allows the deposition of the spermatozoa in the depth of the uterine horn, allowing a significant reduction in the number of spermatozoa inseminated with maintenance of optimal reproductive performance. Intra-oviductal laparoscopic insemination has been recently applied in pigs. This technique has proved to be applicable with diluted and sex-sorted spermatozoa. This review discusses several problems encountered during the development of deep intrauterine insemination and intra-oviductal laparoscopic insemination of pigs and provides potential solutions for the practical application of both the technologies.

## Introduction

Low-dose insemination techniques are recommended in pigs when the available number of spermatozoa is limited, since standard pig artificial insemination (AI) protocols employ $3 \times 10^9$ spermatozoa per dose deposited intracervically two or three times during oestrus. Under these standard conditions, one ejaculate can be used to inseminate only a limited number of sows, thus constraining the efficient use of the boars. The standard AI technique is either unsuitable or inefficient when applied to the emerging sperm technologies, such as frozen-thawed spermatozoa and sperm sexing. For these reasons, a new procedure has been developed for depositing spermatozoa deep into the uterine horn (DUI), which allows a reduction of the number of spermatozoa per dose (Martinez et al. 2001a,b, 2002). When fresh semen is used under field conditions, the sperm dose can be reduced to $150 \times 10^6$ spermatozoa with acceptable fertility results (Martinez et al. 2002, 2006). This practical procedure should offer a great benefit for the optimization of the use of fresh semen from superior boars or in sanitary contingencies when the number of doses to be used is decreased. Currently, the DUI technique has the potential to achieve high-fertility results using as few as $1–2 \times 10^9$ total frozen-thawed spermatozoa (Roca et al. 2003, 2006). The DUI technology has the potential to counteract factors limiting routine application of frozen-thawed spermatozoa, such as the normally high number of spermatozoa required per dose and the low fertility achieved.

The most sought after reproductive technology is pre-conception sex pre-selection. The only accurate and potentially cost-effective approach for achieving sex pre-selection involves separating the X- from the Y-chromosome bearing spermatozoa using flow cytometry and sperm sorting (Johnson et al. 2005). However, the number of available flow-sorted spermatozoa is too low for an extended use of the technology in pig production, even using DUI methodology (Vazquez et al. 2003, 2005). Laparoscopic insemination into the oviduct (ILI) might be an alternative method, at least when applied under specialized production situations (Vazquez et al. 2006).

The following review identifies objections and problems, which appeared during the development of the DUI and ILI procedures, and describes potential solutions for practical application of both technologies.

## Low Dose: Deep Intrauterine Insemination

Two main objections have been raised to the procedure of DUI in sows: (1) putative damage to the cervix and the uterine wall by the insemination device during its advancement along the lumen of the cervical canal and the uterus and (2) the incidence of the unilateral fertilization when $150 \times 10^6$ fresh spermatozoa are used.

### Putative damage of the cervix and uterine wall by the DUI catheter

It has been suggested that the DUI catheter may cause damage to the cervix and the uterus mucosa during its insertion in the sow reproductive tract, potentially compromising subsequent fertility. Using a fibre optic endoscope procedure developed in our laboratories for non-surgical DUI in non-sedated sows (Martinez et al. 2001a), slight bleeding of the cervical canal was observed in 3 out of 33 sows (9.1%) during the insertion of the endoscope. In addition, during removal of the endoscope from the genital tract, a visible mark on the endometrium of the first uterine curvature was found in seven animals (21.2%). The endometrial damage was not accompanied by internal or external bleeding in any sow and did not have a detrimental effect on fertility of hormonally treated oestrous sows used in that study.

Recently, results from Australia indicated that a high proportion of sows (27%) bled during or after DUI and that 22% of the inseminations resulted in some form of bleeding from the reproductive tract (Bathgate et al. 2007). In that paper, the type of bleeding was divided subjectively into three categories: cervical (blood was seen on the catheter or at anytime during insemination), uterine (blood not noticed during insemination, but became evident 12 or more hours after insemination) and both (both types of blood were observed). The authors did not find significant differences in non-return rates, farrowing rates and litter sizes between sows with or without bleeding. The high proportion of sows with

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# *In vitro* maturation of porcine oocytes with retinoids improves embryonic development

*C. Almiñana*[A], *M. A. Gil*[A], *C. Cuello*[A], *I. Caballero*[A], *J. Roca*[A], *J. M. Vazquez*[A], *E. Gomez*[B] *and E. A. Martinez*[A,C]

[A]Department of Animal Medicine and Surgery, Faculty of Veterinary Medicine, University of Murcia, Campus de Espinardo, 30100 Murcia, Spain.
[B]Department of Genetic and Animal Reproduction, SERIDA, Camino de los Claveles, 604, Somió 33203 Gijón, Spain.
[C]Corresponding author. Email: emilio@um.es

**Abstract.** In the present study, the effects of retinoid metabolite administration during *in vitro* maturation (IVM) on oocyte maturation, parameters of *in vitro* fertilisation (IVF) and embryo development were examined. Varying concentrations of 9-*cis* retinoic acid (RA; 0, 5, 50 and 500 nM; Experiment 1) and all-*trans* retinol (ROH; 0, 125, 1250 and 12 500 nM; Experiment 2) were included in the maturation medium. Cumulus–oocyte complexes were matured *in vitro* and inseminated with frozen–thawed spermatozoa. Presumptive zygotes were cultured for 16 h to assess IVF parameters or for 7 days to assess embryo development and quality. In Experiment 1, the oocyte maturation rate to metaphase II was significantly decreased ($P < 0.001$), with values below 5%, in the presence of the highest concentration of RA (500 nM). However, 5 and 50 nM RA had no effect compared with control. Treatment with 5 nM RA improved the blastocyst development rate ($P < 0.001$). In Experiment 2, the oocyte maturation rate did not differ between 125 and 1250 nM ROH treatment groups and control. However, treatment with 12 500 nM ROH was deleterious because no matured oocytes were observed following the treatment. The penetration rate was lower in the group treated with 1250 nM ROH compared with the 125 nM ROH-treated and control groups, but the blastocyst formation rate did not differ among the three groups. In conclusion, 5 nM RA in the IVM medium significantly increased the blastocyst formation rate, suggesting that RA may play an important role during IVM.

**Additional keywords:** all-*trans* retinol, blastocyst formation, 9-*cis* retinoic acid, *in vitro* fertilisation, oocyte maturation.

## Introduction

With growing interest in the generation of embryonic stem cells for the production of transgenic animals and the study of developmental gene regulation, there is an increasing reliance on the *in vitro* fertilisation (IVF) laboratory to maximise embryo viability and quality. However, the *in vitro* production of porcine embryos has been limited by low rates of development to the blastocyst stage (Abeydeera and Day 1997; Kikuchi *et al*. 2002) and their poor quality compared with blastocysts produced *in vivo* (Wang *et al*. 1999). The low quality of oocytes after *in vitro* maturation (IVM; Funahashi *et al*. 1997; Nagai 2001; Kikuchi *et al*. 2002), increased polyspermy after IVF (Niwa 1993; Abeydeera and Day 1997; Wang *et al*. 1997*a*; Abeydeera 2002; Gil *et al*. 2004, 2007; Almiñana *et al*. 2005, 2007*a*, 2007*b*) and poor developmental ability of embryos produced by IVM–IVF (Abeydeera 2001) are the main reasons for this limited performance, together with the unsuitability of *in vitro* culture systems (Kikuchi *et al*. 2002).

Successful nuclear maturation of oocytes *in vitro* is obtained with a high level of repeatability in most current porcine IVF systems. However, although a large proportion of oocytes reaches metaphase II (MII) after IVM, the IVM environment may not support adequate cytoplasmic and molecular maturation (Sirard *et al*. 2006), which is required to prepare the oocyte for post-fertilisation events, allowing the oocyte to reach the blastocyst stage. Compared with *in vivo* maturation, IVM conditions are simple and materially limited, which can profoundly affect the maturation status of oocyte. For this reason, improved culture conditions are essential to obtain consistently successful and reliable oocyte maturation (both cytoplasmic and nuclear), leading to marked improvements in the efficiency of *in vitro* embryo production.

There is growing evidence for the essential role of retinol and its metabolites, namely all-*trans* retinol (ROH) and 9-*cis* retinoic acid (RA), in cell growth, differentiation and embryonic development under *in vivo* and *in vitro* conditions (Shaw *et al*. 1995; Hidalgo *et al*. 2003). Both ROH and RA are natural cellular metabolites of retinol. The ROH metabolite is converted to its isomer RA and other isomers in a reversible way. These metabolites enter the cell nucleus and are able to activate retinoic acid receptors (RAR), whereas retinoid X receptors (RXR) are activated only by RA (Mangelsdorf *et al*. 1992; Chambon 1996).

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# Retained Functional Integrity of Bull Spermatozoa after Double Freezing and Thawing Using PureSperm® Density Gradient Centrifugation

**WMC Maxwell[1], I Parrilla[2], I Caballero[2], E Garcia[2], J Roca[2], EA Martinez[2], JM Vazquez[2] and D Rath[3]**

[1]*Faculty of Veterinary Science, University of Sydney, Sydney, Australia;* [2]*Department of Medicine and Animal Surgery, Faculty of Veterinary Medicine, Campus de Espinardo, University of Murcia, Murcia, Spain;* [3]*Institute for Animal Breeding, Mariensee (FAL), Neustadt, Germany*

**Contents**

The main aim of this study was to compare the motility and functional integrity of bull spermatozoa after single and double freezing and thawing. The viability and morphological integrity of spermatozoa selected by PureSperm® density gradient centrifugation after cryopreservation of bovine semen in two commercial extenders (Experiment 1) and the function of bull spermatozoa before and after a second freezing and thawing assisted by PureSperm® selection (Experiment 2) were examined. On average, 35.8 ± 12.1% of sperm loaded onto the PureSperm® density gradient were recovered after centrifugation. In Experiment 1, post-thaw motility and acrosome integrity were higher for spermatozoa frozen in Tris-egg yolk extender than in AndroMed®, whether the assessments were made immediately after thawing [80.4 ± 12.7 vs 47.6 ± 19.0% motile and 78.8 ± 8.3 vs 50.1 ± 19.5% normal apical ridge (NAR), $p < 0.05$] or after preparation on the gradient (83.3 ± 8.6 vs 69.4 ± 15.9% motile and 89.5 ± 7.2 vs 69.1 ± 11.4% NAR, $p < 0.05$). For semen frozen in Tris-egg yolk extender, selection on the PureSperm® gradient did not influence total motility but significantly improved the proportion of acrosome-intact spermatozoa. After the gradient, both the total motility and percentage of normal acrosomes increased for spermatozoa frozen in AndroMed® (Minitüb Tiefenbach, Germany). In Experiment 2, there was no difference in sperm motility after the first and second freeze-thawing (82.9 ± 12.7 vs 68.8 ± 18.7%). However, the proportion of acrosome-intact spermatozoa was significantly improved by selection through the PureSperm® gradient, whether measured by phase contrast microscopy (78.9 ± 9.7 vs 90.4 ± 4.0% NAR, $p < 0.05$) or flow cytometry (53.4 ± 11.7 vs 76.3 ± 6.0% viable acrosome-intact spermatozoa, $p < 0.001$). The improvement in the percentage of spermatozoa with normal acrosomes was maintained after resuspension in the cooling extender and cooling to 4°C (88.2 ± 6.2) and after re-freezing and thawing (83.6 ± 6.56% NAR). However, flow cytometric assessment of the sperm membranes revealed a decline in the percentage of viable spermatozoa with intact membranes after the second freezing and thawing compared with after gradient centrifugation (76.3 ± 6.0% vs 46.6 ± 6.6%, $p < 0.001$) to levels equivalent to those obtained after the first round of freeze-thawing (53.4 ± 11.7% viable acrosome-intact spermatozoa). Sperm movement characteristics assessed by computer-assisted analysis were unaffected in the population selected on the PureSperm® gradients but declined after cooling of the selected and extended spermatozoa to 4°C. There was no further change in these kinematic measurements after the cooled spermatozoa had undergone the second round of freeze-thawing. These results demonstrate that bull semen can be frozen and thawed, followed by a second freeze-thawing cycle of a population of spermatozoa selected by PureSperm®, with retained motility and functional integrity. This points to the possibility of using double frozen spermatozoa in bovine artificial insemination programmes and to the potential benefits of PureSperm® density gradient centrifugation for the application of cryopreserved bull spermatozoa to other biotechnological procedures such as flow cytometric sex sorting followed by re-freezing and thawing.

## Introduction

Gene preservation programmes for endangered species and livestock are often based on long-term cryopreservation of semen. To comply with changing hygiene regulations when the semen is finally thawed, it may be necessary to purify the spermatozoa and treat them with specific antibiotics prior to re-freezing for shipment to the market and to allow exchange of genetic material between countries. Another application of re-freezing of spermatozoa is for flow cytometric sex sorting, where it may be necessary to use semen from proven bulls that have already been frozen-stored or imported, or where the sex-sorting facility is a long distance from the semen collection centre.

The PureSperm® (Nidacon, Gothenburg, Sweden) density gradient centrifugation technique is designed to select viable and morphologically intact human spermatozoa and to purify them for assisted reproductive technologies (ART), such as artificial insemination (AI) and *in vitro* fertilization (Sonderland and Lundin 2000). PureSperm® is a sterile colloidal silica suspension in an isotonic salt solution, claimed to separate normal sperm from lymphocytes, epithelial cells, abnormal or immature sperm, cell debris, bacteria and seminal plasma. Rodriguez-Martinez et al. (1997) reported the first application of glycerolpropylsilane (GS)-silica density gradient centrifugation for the isolation of spermatozoa with improved motility and proportions of intact membranes from frozen-thawed bull spermatozoa. Also there have been a number of reports on the advantages of PureSperm®, with improved viability, membrane integrity and even DNA integrity of primate and human spermatozoa, compared with other sperm selection gradients such as Percoll (Claassens et al. 1998; Sakkas et al. 2000; Morrell et al. 2004; Mousset-Simeon et al. 2004; Hernandez-Lopez et al. 2005).

O'Brien et al. (2003) first applied PureSperm® gradients for the preparation of frozen-thawed ram and primate semen for flow cytometric sex sorting, as a means to remove the egg yolk component of the freezing extender. PureSperm® separated frozen-thawed ram (Hollinshead et al. 2004a) and bull spermatozoa (Hollinshead et al. 2004b) were subsequently sex-sorted, re-frozen and thawed, and the former were used *in vitro* to produce sheep embryos of pre-selected sex that resulted in the birth of lambs after transfer to recipient ewes

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# Improving the fertilizing ability of sex sorted boar spermatozoa

E.M. García [a], J.M. Vázquez [a,*], I. Parrilla [a], J.J. Calvete [b], L. Sanz [b], I. Caballero [a], J. Roca [a], J.L. Vazquez [a], E.A. Martínez [a]

[a] *Department of Animal Medicine and Surgery, Faculty of Veterinary, University of Murcia, 30071 Murcia, Spain*
[b] *Institute of Biomedicine, C.S.I.C, Valencia, Spain*

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**Abstract**

The sex sorting of spermatozoa by flow cytometry induces damage, since sperm cells are highly diluted, affecting their functionality and fertilizing ability. In this work it was investigated whether the concentration of sex sorted spermatozoa by the sedimentation method, rather than centrifugation, in combination with the presence of the seminal plasma protein PSP-I/PSP-II heterodimer may improve their fertilizing ability.

Spermatozoa were sorted by flow cytometry and collected in BTS with 10% of seminal plasma (group C: control) or with 1.5 mg/mL of PSP-I/PSP-II heterodimer (group H). Collected spermatozoa from each medium were split into two aliquots. One aliquot of each group was centrifuged (800 × *g*/5 min) just after sorting and stored 16–18 h at 17 °C (groups Cc and Hc) at $6 \times 10^6$ sperm/mL. The second aliquot was directly stored at 17 °C for 16–18 °C (group Cs and Hs). After storage the supernatant was discarded and the sedimented pellet adjusted to $6 \times 10^6$ sperm/mL. Membrane integrity, acrosome status and motility characteristics of spermatozoa from all groups were assessed. Post-weaning pre-ovulatory sows were inseminated by laparoscopy into the oviduct with $0.3 \times 10^6$ sex sorted spermatozoa to assess their ability to penetrate oocytes in vivo. Putative zygotes were collected 18 h after insemination by washing the oviduct. Penetration and monospermic rates were evaluated.

After 16–18 h of storage, centrifuged spermatozoa collected with 10% seminal plasma or 1.5 mg/mL PSP-I/PSP-II heterodimer after sex sorting showed lower ($p < 0.05$) percentages of membrane integrity, motility and fertilization than sedimented spermatozoa. Overall, the presence of 10% seminal plasma or PSP-I/PSP-II heterodimer did not affect the results. However, a positive effect of PSP-I/PSP-II heterodimer ($p < 0.05$) was observed in sedimented spermatozoa. Hence, our results indicate that the sedimentation method in the presence of PSP-I/PSP-II heterodimer improves the in vivo fertilizing ability of sex sorted boar spermatozoa.


*Keywords:* Sex sorted boar spermatozoa; PSP-I/PSP-II heterodimer; Intraoviductal laparoscopic insemination; Sedimentation

## 1. Introduction

The development of semen sexing represents a major advance in reproductive technology. The preselection of the sex in swine improves the efficiency in the production of male or female crossbred lines. Moreover, animal welfare laws in some countries could forbid the use of castration and alternatives for producing higher numbers of females will need to be found. However, the success in the implementation and wide spread of this technology depends on economics, efficiency, safety and ease of use.

The major problem with the preselection of the sex by flow cytometric sorting, the only procedure available to produce sex sorted spermatozoa, is related

* Corresponding author. Tel.: +34 968 364736; fax: +34 968 367069.
*E-mail address:* vazquez@um.es (J.M. Vázquez).

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# Vitrification of *in vitro* cultured porcine two-to-four cell embryos

C. Cuello [a,b,*], M.A. Gil [a], C. Almiñana [a], J. Sanchez-Osorio [a], I. Parrilla [a], I. Caballero [a], J.M. Vazquez [a], J. Roca [a], H. Rodriguez-Martinez [b], E.A. Martinez [a]

[a] *Department of Animal Medicine and Surgery, Veterinary Science, University of Murcia, E-30071 Murica, Spain*
[b] *Division of Reproduction, Faculty of Veterinary Medicine and Animal Science, SLU, SE-750 07 Uppsala, Sweden*

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**Abstract**

The objective of this experiment was to evaluate the effect of a 5-day period of *in vitro* culture of two-to-four cell porcine embryos up to the blastocyst stage on their ability to survive vitrification and warming. In order to increase the cooling rate, superfine open pulled straws and Vit-Master® technology were used for vitrification. Two-to-four cell embryos were collected from weaned sows ($n = 11$) on day 2 (D0 = onset of estrus). Some embryos ($N = 63$) were vitrified within 3 h after collection, warmed and cultured for 120 h (Group V2). Additionally, 81 two-to-four cell embryos were cultured for 96 h in order to obtain blastocysts; these were then vitrified, warmed and cultured for 24 h (Group VB; $N = 65$). The remaining two-to-four cell embryos were used as controls and thus not vitrified (control embryos; $N = 70$) but were cultured *in vitro* for 120 h. The V2, VB and control embryos were evaluated for their developmental progression and morphology during culture. All embryos (V2, VB and controls) were fixed on the same day of development in order to assess the total number of blastomeres. The survival and blastocyst formation rates obtained from V2 embryos were very poor ($9.6 \pm 0.7\%$ and $3.2 \pm 0.5\%$, respectively). The survival and hatching rates of VB embryos ($75.0 \pm 0.69\%$ and $33.6 \pm 0.13\%$) were lower ($p < 0.001$) than those obtained with control embryos ($89.1 \pm 0.8\%$ and $47.5 \pm 0.12\%$). Hatched VB embryos had a lower ($p < 0.01$) total cell number than hatched control embryos ($70.3 \pm 4.5$ versus $90.6 \pm 3.2$, respectively). There was no difference between expanded VB and control blastocysts. In conclusion, blastocysts derived from *in vitro* culture of two-to-four cell pig embryos could be successfully vitrified using SOPS straws and Vit-Master®.


*Keywords:* Porcine; Embryo; Vitrification; Two-to-four cell

## 1. Introduction

In the past few years, important advances in porcine embryo cryopreservation have been made, particularly in vitrification, with protocols becoming simpler and better adapted for routine use [1–5]. Among vitrification methods, open pulled straw (OPS) technology has become the most widely employed to date [6]. *In vitro* survival rates obtained with porcine *in vivo* derived morulae and blastocysts after OPS vitrification are remarkable [7–10], and high pregnancy rates have been achieved after surgical transfer of OPS-vitrified blastocysts and morulae [11–18]. Recently, piglets have been obtained with vitrified blastocysts [19] using a non-surgical deep intrauterine embryo transfer system [20], and after surgical transfer of vitrified–warmed *in vitro* produced transgenic blastocysts [21]. However, successful vitrification of porcine embryos has, to date, been limited to the morula and blastocysts stages. Pig embryos are very sensitive to temperatures below 15 °C

* Corresponding author at: Departamento Medicina y Cirugía Animal (Reproducción y Obstetricia), Hospital Clínico Veterinario, Universidad de Murcia, 30071 Murcia, Spain. Tel.: +34 968364812; fax: +34 968367069.
*E-mail address:* ccuello@um.es (C. Cuello).

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**Communication**

# Pre-pubertal Di(2-ethylhexyl) Phthalate (DEHP) Exposure of Young Boars Did Not Affect Sperm *In vitro* Penetration Capacity of Homologous Oocytes Post-puberty

**Linda Spjuth**
Department of Clinical Sciences, Division of Reproduction, Faculty of Veterinary Medicine and Animal Science, Swedish University of Agricultural Sciences (SLU), Uppsala, Sweden

**M. A. Gil, I. Caballero, C. Cuello, C. Almiñana, and E. A. Martínez**
Department of Animal Medicine & Surgery, Division of Animal Reproduction & Obstetrics, Faculty of Veterinary Medicine, University of Murcia, Murcia, Spain

**N. Lundeheim**
Department of Animal Breeding and Genetics, Faculty of Veterinary Medicine and Animal Science, Swedish University of Agricultural Sciences (SLU), Uppsala, Sweden

**H. Rodríguez-Martínez**
Department of Clinical Sciences, Division of Reproduction, Faculty of Veterinary Medicine and Animal Science, Swedish University of Agricultural Sciences (SLU), Uppsala, Sweden

Di(2-ethylhexyl) phthalate (DEHP), a plastic softener used in polyvinylchloride (PVC) products (e.g., plastic bags and medical equipment), has been reported to have toxic effects on animal reproduction and is considered an environmental hazard based, mostly, on rodent studies. However, the doses used in these studies are often considerably higher than that presumed in human exposure. In the present study we used young boars as model animals to assess the effects of pre-pubertal DEHP exposure on the ability of spermatozoa to penetrate homologous oocytes *in vitro*. Eight pairs of cross-bred male boar siblings were used. One brother in each pair became, at random, the test animal exposed to DEHP *per os*, three times a week, from 3 to 7 weeks of age while the other acted as the control, i.e., placebo-exposed. Semen was collected and frozen between 8 and 9 months of age and stored until spermatozoa were evaluated for their ability to *in vitro* penetrate *in vitro*-matured homologous oocytes post-thaw. Both the penetration rate and the number of spermatozoa per oocyte were considered within expected ranges for frozen boar semen of good quality. Penetration rate did not significantly differ ($p > 0.05$) between the groups with DEHP-exposed: 50%; control: 59%, which could be owing to a large variation between boars, and between replicates. The number of spermatozoa in the ooplasm was low and similar ($p > 0.05$) between the groups with DEHP-exposed: 1.5 and the control: 1.7. Under the conditions of the present experiment, pre-pubertal exposure to DEHP does not seem to cause a deleterious effect on the *in vitro* fertilizing ability of frozen spermatozoa post-puberty.

**KEYWORDS** boar, DEHP, *in vitro* penetration, IVF, phthalate, pre-pubertal

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# Immunolocalization and Possible Functional Role of PSP-I/PSP-II Heterodimer in Highly Extended Boar Spermatozoa

IGNACIO CABALLERO,* JUAN M. VÁZQUEZ,* EVA M. GARCÍA,* JORDI ROCA,* EMILIO A. MARTÍNEZ,* JUAN J. CALVETE,† LIBIA SANZ,† HANS EKWALL,‡ AND HERIBERTO RODRÍGUEZ-MARTÍNEZ‡

*From the *Department of Medicine and Surgery, Faculty of Veterinary Medicine, University of Murcia, Murcia, Spain; the †Institute of Biomedicine, C.S.I.C., Valencia, Spain; and the ‡Division of Comparative Reproduction, Faculty of Veterinary Medicine and Animal Science, Swedish University of Agricultural Sciences (SLU), Uppsala, Sweden*

**ABSTRACT:** PSP-I/PSP-II heterodimer is a major protein of boar seminal plasma which is able to preserve, in vitro, the viability, motility, and mitochondrial activity of highly extended boar spermatozoa for at least 5 hours. However, little is known about the binding pattern of the heterodimer to the sperm plasma membrane and its eventual relation with the maintenance of the sperm functionality. The present study investigated the effect of exposing highly extended boar spermatozoa (1 million/mL) to 1.5 mg/mL of PSP-I/PSP-II for 0.5, 5, and 10 hours at 38°C on sperm characteristics and the changes in PSP-I/PSP-II localization as a result of both the addition of PSP-I/PSP-II to the extender and the incubation time. Exposure of the spermatozoa to PSP-I/PSP-II preserved sperm viability, motility, and mitochondrial activity when compared to nonexposed spermatozoa. This protective effect lasted for 10 hours ($P < .05$). After immunolabeling of highly extended semen with rabbit monospecific polyclonal antibody against PSP-I/PSP-II, the percentage of immunopositive spermatozoa declines over time from 71% (0.5 hours) to 49% (10 hours). However, more than 80% of spermatozoa remained labeled during the 10-hour incubation period if PSP-I/PSP-II was added. Scanning electron microscopy revealed 4 different binding patterns. The heterodimer was mainly localized to the acrosomal area, being redistributed to the postacrosomal area or lost during in vitro incubation. In conclusion, the protective effect of the heterodimer appears to be related to its adhesion to the acrosomal area, and the loss of this protective effect coincides with a stepwise redistribution of PSP-I/PSP-II during incubation.

Key words: Reproductive tract, semen, sperm, seminal plasma, sperm capacitation.

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In the boar, the majority of seminal plasma (SP) proteins belong to the spermadhesin family, a group of (glyco)proteins built by a single CUB domain architecture (Romero et al, 1997) and thought to play important roles in individual steps of the fertilization process, such as capacitation and zona pellucida binding (Töpfer-Petersen et al, 1998). The spermadhesin family comprises 5 members: AQN-1, AQN-3, AWN, PSP-I, and PSP-II. The last 2 form a glycosylated PSP-I/PSP-II heterodimer under physiological conditions (Calvete et al, 1995). In vitro, low doses of PSP-I/PSP-II heterodimer (1.5 mg/mL) appear to preserve membrane integrity, motility, and mitochondrial activity of highly extended spermatozoa (Centurión et al, 2003). Processes linked to the removal of factors present in the SP that coat the sperm surface and maintain the stability of the plasma membrane, such as high extension of the spermatozoa (Maxwell and Johnson, 1999), trigger a series of phenomena that resemble those occurring during sperm capacitation, culminating in premature acrosome exocytosis, thus decreasing the life span of the spermatozoa (Maxwell and Johnson, 1999). Although the addition of homologous seminal plasma (from 1% to 10% v/v) is a possible counter-measure to alleviate the consequences of such an "extension effect" (Asworth et al, 1994; Maxwell et al, 1997), differences in SP-protein profiles have been found between males of different fertility. These differences may be related to the variability between different sources of SP (Fournier-Delpech and Thibault, 1993; Killian et al, 1993; Caballero et al, 2004a) which leads to both beneficial and detrimental effects on the spermatozoa.

Either whole SP or specific SP components of low molecular weight affect survivability of boar spermatozoa, depending on how long they are exposed to the PSP-I/PSP-II (Centurión et al, 2003). However, there is an advantage to using an isolated protein instead of

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Correspondence to: Juan M Vazquez, Departamento de Medicina y Cirugía Animal, Facultad de Veterinaria, Universidad de Murcia, 30071 Murcia, Spain (e-mail: vazquez@um.es).

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ORIGINAL ARTICLE

# Effect of storage in short- and long-term commercial semen extenders on the motility, plasma membrane and chromatin integrity of boar spermatozoa

Marco De Ambrogi,*†‡ Juan Ballester,*† Fernando Saravia,* Ignacio Caballero,*§ Anders Johannisson,† Margareta Wallgren,*¶ Magnus Andersson** and Heriberto Rodriguez-Martinez*

*Division of Comparative Reproduction, Obstetrics, and Udder Health, Department of Clinical Sciences, Faculty of Veterinary Medicine and Animal Science, Swedish University of Agricultural Sciences, Uppsala, †Unit of Cell Analysis, Department of Anatomy and Physiology, Faculty of Veterinary Medicine and Animal Science, Swedish University of Agricultural Sciences, Uppsala, Sweden, ‡Department of Veterinary Morphophysiology and Animal Production (DIMORFIPA), Faculty of Veterinary Medicine, University of Bologna, Bologna, Italy, §Department of Medicine and Surgery, Faculty of Veterinary Medicine, University of Murcia, Murcia, Spain, ¶Quality Genetics, Kävlinge, Sweden, and **Saari Unit, Department of Clinical Veterinary Sciences, Faculty of Veterinary Medicine, University of Helsinki, Mäntsälä, Finland

**Keywords:**
boar, chromatin stability, liquid semen, sperm viability, storage

**Correspondence:**
Heriberto Rodriguez-Martinez, Division of Comparative Reproduction, PO Box 7054, SLU, SE-750 07 Uppsala, Sweden. E-mail: heriberto.rodriguez@kv.slu.se

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## Summary

For artificial insemination (AI) in pigs, preservation of liquid boar semen at 16–20 °C is still common practice as sperm cryopreservation remains suboptimal in this species. To meet the different needs of the swine industry, several extenders have been developed to preserve semen in liquid form for short- and long-term storage. In the present study, three different commercial extenders devised for short-term (BTS+) or long-term preservation (MR-A and X-Cell), were used to test whether storage of semen from four mature, fertile boars at 17 °C for 96 h would affect sperm characteristics relevant for fertility, such as motility, membrane integrity and chromatin stability. Computer-assisted sperm analysis, and stainings with the acylated membrane dye SYBR-14/propidium iodide, and acridine orange in connection with flow cytometry were used to evaluate these variables. Percentages of total motile spermatozoa decreased slightly, but significantly, after 72–96 h. While membrane integrity values varied during the period of study, no significant changes in either membrane integrity or chromatin stability were, however, registered. This suggests a customary 96-day storage at 17 °C in these extenders was too short an interval to cause losses of integrity in nuclear DNA in the boar population studied.

## Introduction

In the swine industry, more than 99% of artificial inseminations (AIs) performed around the world today are carried out with extended liquid semen (Wagner & Thibier, 2000) whether on the same day or stored at 15–20 °C for 1–5 days before AI (Johnson *et al.*, 2000). A major advantage of using extended, non-frozen semen is that fertility is maintained even with low numbers of spermatozoa in the AI dose. The lower the number of spermatozoa included per dose and the shorter the storage prior to AI, the higher the number of usable AI doses produced from one ejaculate. Consequently, preservation of the fertilizing capacity of boar semen for several days remains a major target for the industry.

However, a concern is that use of AI doses older than 12–24 h following extension of the semen may lead to fertility losses, particularly in terms of litter size, as discussed in Waberski *et al.* (1994) and Christensen *et al.* (2004), among others. For example, in a field fertility study of 41 327 AIs using semen extended with MR-A (KUBUS, S.A., Las Rozas, Spain), with $2.5 \times 10^9$ spermatozoa (spz)/dose, the conception rate (%) and litter size (based on 6924 litters) decreased with storage of semen, progressively from day 1 (day of collection) to day 5. The conception rate decreased from 85.6% to 80.4%, and litter size decreased from 11.9 to 10.8 piglets (Ratto & Jokinen, 1991). Using extension with Beltsville Thawing Solution (BTS), and semen doses of $2.5 \times 10^9$ total spz/dose for a total of 76 822 first AIs on 2859 herds, and

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# Dissecting the Protective Effect of the Seminal Plasma Spermadhesin PSP-I/PSP-II on Boar Sperm Functionality

EVA M. GARCIA,*JUAN M. VÁZQUEZ,* JUAN J. CALVETE,† LIBIA SANZ,† IGNACIO CABALLERO,* INMACULADA PARRILLA,* MARIA A. GIL,* JORDI ROCA,* AND EMILIO A. MARTINEZ*

*From the *Departamento de Medicina y Cirugía Animal, Facultad de Veterinaria, Universidad de Murcia, Murcia, Spain; and the †Instituto de Biomedicina de Valencia, CSIC, Valencia, Spain.*

**ABSTRACT:** To dissect the protective activity of PSP-I/PSP-II, the effect of the isolated subunits PSP-I and PSP-II and their affinity-purified tryptic peptide and glycan fractions on the viability, mitochondrial activity, and motility of highly diluted boar spermatozoa was investigated. High dilution exerted a negative effect on control spermatozoa. Incubation of spermatozoa with PSP-I/PSP-II or with its PSP-II subunit had a protective effect on sperm functionality, high mitochondrial membrane potential, and sperm motility. These effects were less pronounced when spermatozoa were incubated with the PSP-I subunit. It was noteworthy that motility was abolished by incubation of spermatozoa with isolated PSP-I. Trypsin-degraded PSP-I/PSP-II, PSP-I, and PSP-II reproduced the effects of the native proteins. Incubating spermatozoa with the glycan-depleted tryptic-peptide fraction of PSP-I/PSP-II for 5 hours preserved a higher percentage of viable spermatozoa than when sperm was incubated for the same time with the native heterodimer, trypsin-digested PSP-I/PSP-II, the glycan fraction or without added proteins. However, sperm motility decreased as the concentration of added peptide fraction increased. On the other hand, spermatozoa incubated with the glycan fraction showed lower values than spermatozoa incubated with the peptide fraction. We concluded that the subunits of the PSP-I/PSP-II heterodimeric spermadhesin exert different activities on sperm functions. The finding that the beneficial effect of the native PSP-I/PSP-II on the functionality of highly diluted boar spermatozoa is largely preserved in its isolated PSP-II subunit and does not appear to require the glycan moiety points to a peptide moiety as a potential sperm function-preserving additive of highly diluted boar spermatozoa.

Key words: Boar seminal plasma, spermadhesin PSP-I/PSP-II, protective effect on sperm function, sperm survival, highly diluted spermatozoa.

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The seminal plasma, consisting of secretions from the testes, epididymis, and the accessory sex glands, contains a variety of factors (amino acids, lipids, fatty acids, osmolytes, peptides, and proteins) that influence the viability and fertilizing capacity of ejaculated spermatozoa (Mann and Lutwak-Mann, 1981; Shivaji et al, 1990; Yanagimachi, 1994). Thus, the seminal plasmas of a variety of mammalian species contain both factors that prevent inappropriate acrosome reactions and proteins that upon binding to the sperm surface enhance the fertilizing potential of spermatozoa (Killian et al, 1993; Thérien et al, 1997; Rodríguez-Martínez et al, 2005). The concerted action of these regulatory seminal plasma factors modulates the capacitation state of spermatozoa. However, the precise role of most of the seminal plasma proteins on sperm physiology remains obscure. In addition, the effect of seminal plasma on spermatozoa is variable among species, males of the same species, and ejaculates from a single male. The distinct effects exerted by different seminal plasmas on sperm functionality have been in part ascribed to variability of the composition and concentration of some proteins (Maxwell and Johnson, 1999; Centurión et al, 2003).

Spermadhesins are male secretory proteins detected so far in ungulates (pig, cattle, and horse) (Haase et al, 2005). In the pig, this family of proteins consists of 5 members—AQN-1, AQN-3, AWN, PSP-I, and PSP-II (Töpfer-Petersen et al, 1998)—and together they represent over 90% of the total boar seminal plasma proteins (Dostàlovà et al, 1994). The porcine spermadhesin genes are clustered on SCC 14q28–q29 (Haase et al, 2005). Porcine spermadhesins, 110- to 133-residue polypeptides built by a single CUB domain architecture (Romero et al, 1997), are synthesized by the epididymis and accessory glands (Ekhlasi-Hundrieser et al, 2002) and exhibit distinct sperm-coating and ligand-binding capabilities. Sequence variation, glycosylation, and their aggregation state of spermadhesins contribute to their specific pattern of biological activities (Calvete et al,

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Correspondence to: Juan M. Vázquez, Departamento de Medicina y Cirugía Animal, Facultad de Veterinaria, Universidad de Murcia, Campus de Espinardo, 30071 Murcia, Spain (e-mail: vazquez@um.es).

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# Fertility after deep intra-uterine artificial insemination of concentrated low-volume boar semen doses

Tuempong Wongtawan [a], Fernando Saravia [a], Margareta Wallgren [a,b,*], Ignacio Caballero [a,c], Heriberto Rodríguez-Martínez [a]

[a] *Division of Comparative Reproduction, Obstetrics and Udder Health, Department of Clinical Sciences, Faculty of Veterinary Medicine and Animal Science, Swedish University of Agricultural Sciences (SLU), Ullsvägen 14 C, Clinical Centre, P.O. Box 7054, Ultuna, SE-75007 Uppsala, Sweden*
[b] *Quality Genetics, Kävlinge, Sweden*
[c] *Department of Medicine and Surgery, Faculty of Veterinary Medicine, University of Murcia, Murcia, Spain*

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**Abstract**

Boar semen can be successfully frozen – highly packed – in small containers (medium-straw, MS or MiniFlatPack, MFP). The use of deep intra-uterine artificial insemination (DIU-AI) can make possible the deposition of small volumes of this thawed, non re-extended semen deeply intra-uterine, close to the sperm reservoir. The present experiments studied the fertility achieved after single or double DIU-AI per oestrus, with special attention to the interval between AI and spontaneous ovulation. Semen from two boars of proven fertility was frozen in MS or MFP holding $1 \times 10^9$ total spermatozoa. Multiparous (2–5 parity, $n = 42$) crossbred sows were checked for oestrous behaviour after weaning and the occurrence of spontaneous ovulation was checked with transrectal ultrasonography (TUS) to establish the mean interval between onset of oestrus (OO) and ovulation which was found to be when approximately 2/3 of the oestrus period has passed. The sows were, in the following standing oestrus, subjected to DIU-AI using thawed semen from either MS ($n = 20$) or MFP ($n = 22$), inseminated without further re-extension. The sows were randomly allotted to one of three groups: (1) single DIU-AI 8 h before expected ovulation (control group, $n = 19$); (2) single DIU-AI

* Corresponding author.
*E-mail address:* margareta.wallgren@kv.slu.se (M. Wallgren).

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# Influence of storage time on functional capacity of flow cytometrically sex-sorted boar spermatozoa

Inmaculada Parrilla, Juan M. Vazquez*, Maria A. Gil, Ignacio Caballero, Carmen Almiñana, Jordi Roca, Emilio A. Martinez

*Department of Animal Medicine and Surgery, Faculty of Veterinary, University of Murcia, 30071 Murcia, Spain*

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**Abstract**

Sex-sorting of boar spermatozoa is an emerging biotechnology, still considered suboptimal owing to the slowness of the process, which requires long sorting periods to obtain an adequate number of spermatozoa to perform a non-surgical insemination. This period involves storage of sorted cells that could impair their functional capacity. Here, we have studied how the storage of sex-sorted boar spermatozoa affects their functional capacity. Sorted spermatozoa were assessed at various times (0, 2, 5 h or 10 h) during storage after sorting and compared with diluted and unsorted spermatozoa for sperm motility patterns, plasma membrane and acrosomal integrity and their ability to penetrate homologous IVM oocytes. Sex-sorted sperm motility and membrane integrity only decreased significantly ($p < 0.05$) by the end of the storage period (10 h) compared to unsorted spermatozoa. Sperm velocity, ALH and Dance increased significantly ($p < 0.05$), immediately post-sorting, returning to unsorted sperm values during storage. Acrosome integrity was not seriously affected by the sorting process, but decreased ($p < 0.05$) during storage after sorting. Sorted spermatozoa stored 2 h after sorting did not differ from unsorted in penetration rates and numbers of spermatozoa per oocyte, reaching the highest ($p < 0.05$) penetration rates and sperm numbers per oocyte, when co-cultured for 6 or more hours. Non-storage or storage for 5 h or 10 h negatively ($p < 0.05$) affected sperm penetration ability. In conclusion, although flow cytometrically sex-sorted spermatozoa are able to maintain motility, viability and acrosomal integrity at optimal levels until 10 h of storage after sorting, fertilizing ability is maintained only over shorter storage times (<5 h).


*Keywords:* Flow cytometry; Sex-sorting; Seminal plasma; Sperm quality; Oocyte penetration; In vitro; Pig

* Corresponding author. Tel.: +34 968 364736; fax: +34 968 367069.
*E-mail address:* vazquez@um.es (J.M. Vazquez).

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# Influence of seminal plasma PSP-I/PSP-II spermadhesin on pig gamete interaction

*Ignacio Caballero*[1], *Juan M. Vázquez*[1], *Heriberto Rodríguez-Martínez*[1,2], *Maria A. Gil*[1], *Juan J. Calvete*[3], *Libia Sanz*[3], *Eva M. Garcia*[1], *Jordi Roca*[1] *and Emilio A. Martínez*[1]

Faculty of Veterinary Medicine, University of Murcia, Murcia, Spain; Faculty of Veterinary Medicine and Animal Science, Swedish University of Agricultural Sciences (SLU), Uppsala, Sweden; and Institute of Biomedicine, CSIC, Valencia, Spain

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## Summary

The seminal plasma PSP-I/PSP-II spermadhesin is able to preserve, *in vitro*, the viability of highly extended boar spermatozoa, suggesting it might be used as a suitable ameliorator for the damaging effects of sperm handling, including *in vitro* fertilization. However, little is known about the ligand capability of PSP-I/PSP-II as regards the zona pellucida (ZP) or its possible role in gamete interaction. The present study evaluated the effect of the presence of PSP-I/PSP-II (1.5 mg/ml) during *in vitro* oocyte maturation and also during co-incubation of frozen-thawed boar spermatozoa with either immature (IM) or *in vitro* matured (IVM) oocytes, either enclosed by cumulus cells or denuded. Exposure of the gametes to the heterodimer during *in vitro* gamete co-incubation showed a significant blocking effect of sperm penetration rates and a decreased number of spermatozoa per oocyte in both IM and IVM denuded oocytes. Such an effect was not present in cumulus-enclosed oocytes, suggesting the effect could be mediated by exposed ZP receptors. In addition, when PSP-I/PSP-II was added to the IVM medium, oocyte maturation rates were significantly reduced. In conclusion, the results suggest that PSP-I/PSP-II, when present *in vitro*, blocks sperm–ZP binding.

Keywords: Gametes, IVF, Pig, PSP-I/PSP-II, Seminal plasma

## Introduction

Gamete interaction is an early critical step in mammalian fertilization, involving at least three morphologically disparate cell types: the capacitated acrosome-intact spermatozoon, the mature oocyte and the surrounding cumulus cells (Yanagimachi, 1994). The events of gamete recognition, binding and fusion are highly regulated processes that imply a number of biochemical reactions until a new zygote is formed. In domestic species, this mechanism of cell-to-cell adhesion seems to be mediated by protein–carbohydrate interactions between sperm-associated lectins and glycan structures of the oocyte zona pellucida (ZP), the latter synthesized by the concerted action of the oocyte and the granulosa cells during oocyte maturation (Sinowatz *et al.*, 2001).

One of these sperm lectins constitutes the spermadhesin family (Sinowatz *et al.*, 1997). Boar spermadhesins are a group of (glyco)proteins built by a single CUB domain architecture (Romero *et al.*, 1997), coating the sperm surface (Dostálová *et al.*, 1994). They play a role in several biological functions, including sperm capacitation and, as already mentioned, gamete recognition and binding (Calvete *et al.*, 1994). According to their binding properties, spermadhesins can be divided into two groups, depending on their ability to either bind heparin (AQN-1, AQN-3, AWN) or not (PSP-I/PSP-II heterodimer).

The PSP-I/PSP-II heterodimer appears to preserve, *in vitro*, the membrane integrity, motility and mitochondrial activity of highly extended spermatozoa for as long these are exposed to the heterodimer (Centurión

Correspondence to: J.M. Vázquez, Departamento de Medicina y Cirugía Animal, Facultad de Veterinaria, Universidad de Murcia, 30071 Murcia, Spain. Tel: +34 968 364736. Fax: +34 968 367069. e-mail: vazquez@um.es
[1] Department of Medicine and Surgery, Faculty of Veterinary Medicine, University of Murcia, Murcia, Spain.
[2] Department of Obstetrics & Gynaecology, Faculty of Veterinary Medicine and Animal Science, Swedish University of Agricultural Sciences (SLU), Uppsala, Sweden.
[3] Institute of Biomedicine, CSIC, Valencia, Spain.

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# Does Seminal Plasma PSP-I/PSP-II Spermadhesin Modulate the Ability of Boar Spermatozoa to Penetrate Homologous Oocytes In Vitro?

IGNACIO CABALLERO,* JUAN M. VAZQUEZ,* MARIA A. GIL,* JUAN J. CALVETE,† JORDI ROCA,* LIBIA SANZ,† INMACULADA PARRILLA,* EVA M. GARCIA,* HERIBERTO RODRIGUEZ-MARTINEZ,*‡ AND EMILIO A. MARTINEZ*

*From the *Department of Medicine and Surgery, Faculty of Veterinary Medicine, University of Murcia, Murcia, Spain; †Institute of Biomedicine, C.S.I.C., Valencia, Spain; and ‡Department of Obstetrics and Gynaecology, Faculty of Veterinary Medicine and Animal Science, Swedish University of Agricultural Sciences (SLU), Uppsala, Sweden.*

**ABSTRACT:** Low concentration (0.15 mg per million of spermatozoa) of seminal plasma–derived PSP-I/PSP-II spermadhesin heterodimer is able to preserve the viability of highly extended boar spermatozoa. Whether spermatozoa also keep their fertilizing capacity is not yet known. The present study evaluated the effect of exposing freshly extended and frozen-thawed boar spermatozoa (10 million/mL) to PSP-I/PSP-II (1.5 mg/mL) for 30 or 120 minutes on sperm characteristics and the outcome of in vitro penetration of immature (IM) and in vitro matured (IVM) homologous oocytes, aiming to identify this spermadhesin as a suitable modulator for sperm-handling protocols. Although exposure to the heterodimer improved sperm viability and motility without increasing the levels of sperm acrosome exocytosis in both freshly extended and frozen-thawed spermatozoa, this pretreatment did not affect sperm penetration rates or sperm numbers per oocyte when pretreated fresh spermatozoa were coincubated with IM or IVM oocytes compared with controls. When cryopreserved spermatozoa were tested, however, on IVM oocytes, already a 30-minute preincubation exposure to PSP-I/PSP-II showed a significant blocking effect on penetration rate (from 90% to 32%, $P < .05$) and on mean sperm numbers per oocyte (2.9 to 1.6, $P < .05$). To disclose the nature of this paradox, frozen-thawed spermatozoa were cleansed (by centrifugation in saline bovine serum albumin or through Percoll density gradient separation) and the procedure repeated. Oocyte penetration (but not number of spermatozoa per oocyte) increased ($P < .05$) when spermatozoa were cleansed with Percoll compared with either washed or unwashed controls (53% vs 13% vs 31%, respectively). In addition, the percentages of polyspermic oocytes remained lower than control (38.5% vs 68.7%, respectively; $P < .05$). In conclusion, the results confirm that exposure of fresh or frozen-thawed boar spermatozoa to a low dose of seminal PSP-I/PSP-II spermadhesin preserves sperm viability and motility in vitro. Although there was no obvious influence of the heterodimer on the capability of freshly extended boar spermatozoa to penetrate homologous oocytes (either IM or IVM), PSP-I/PSP-II exerted a deleterious effect when frozen-thawed spermatozoa were used to penetrate IVM oocytes. Such an effect of cryopreservation seems to a certain extent reversible, since cleansing of the sperm surface decreased, at least partially, this blocking effect, increasing both penetration and the monospermic rates.

Key words: Seminal plasma, preservation, sperm viability, IVF, pig.

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Seminal plasma (SP), the fluid in which mammalian spermatozoa are suspended in semen, is a complex mixture of secretions that originate from the testes, epididymides, and male accessory sexual glands. The SP contains factors that influence both spermatozoa and the female genital tract during sperm transport (Shivaji et al, 1990; Yanagimachi, 1994; Waberski et al, 1995). In particular, SP proteins play a role in the modulation of sperm function before they reach the oocyte(s) at the tubal site of fertilization, during gamete recognition, and when spermatozoa and oocytes bind at fertilization (Calvete et al, 1995a). In boars, the major protein component of the SP is the spermadhesin family (Calvete et al, 1995a; Töpfer-Petersen et al, 1998), a group of (glyco)proteins built by a single CUB domain architecture (Romero et al, 1997), coating the sperm surface (Dostálová et al, 1994). According to their binding properties, spermadhesins can be divided into 2 groups, depending on their ability to either bind heparin (AQN-1, AQN-3, AWN) or not (PSP-I/PSP-II heterodimer). The PSP-I/PSP-II heterodimer appears to preserve in vitro membrane integrity, motility, and mitochondrial activity of highly extended spermatozoa for as long as these are exposed to the heterodimer

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Correspondence to: Dr Vazquez, Departamento de Medicina y Cirugía Animal, Facultad de Veterinaria, Universidad de Murcia, 30071 Murcia, Spain (e-mail: vazquez@um.es).

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# Comparative Effects of Autologous and Homologous Seminal Plasma on the Viability of Largely Extended Boar Spermatozoa

**I Caballero[1], JM Vazquez[1], F Centurión[1,2], H Rodríguez-Martinez[1,3], I Parrilla[1], J Roca[1], C Cuello[1] and EA Martinez[1]**

[1]*Department of Medicine and Surgery (Animal Reproduction), Faculty of Veterinary Medicine, University of Murcia, Murcia, Spain;* [2]*Department of Animal Reproduction, Faculty of Veterinary, University of Yucatan, Merida, Mexico;* [3]*Department of Obstetrics & Gynaecology, Faculty of Veterinary Medicine and Animal Science, Swedish University of Agricultural Sciences (SLU), Uppsala, Sweden*

**Contents**

Sperm handling, associated to artificial reproduction technologies (ART) such as *in vitro* fertilization (IVF) or the use of flow cytometry for cell analysis or sorting imposes volumetric extension of the sperm suspension and decreases sperm viability, presumably because of the removal of seminal plasma (SP) components. This study evaluated whether a 10% v/v of autologous SP (retrieved from the same donor boar) or homologous SP (e.g. from any of the four fertile boars included, other than the one providing the spermatozoa) would differently affect the viability of boar spermatozoa subjected to large extension in a simple saline medium [phosphate-buffered saline and 0.1% ethylenediaminetetraacetic acid (EDTA), PBSm] to a concentration of $0.3 \times 10^6$ spermatozoa/ml and incubated for 2 h at 30°C. Sperm viability was monitored as membrane integrity [using the fluorophore carboxyfluorescein diacetate (C-FDA) and propidium iodide (PI)], mitochondrial function (using the fluorophore R-123) and motility characteristics [using Computer Assisted Sperm Analysis (CASA)]. Substraction of the SP and extension followed by incubation in PBSm significantly ($p < 0.05$) decreased sperm viability, which could be restored by addition of autologous SP. Furthermore, exposure of the extended spermatozoa to homologous SP (from any other individual boar) significantly ($p < 0.05$) varied with the source of the sire; some boars exerting beneficial effects (even surpassing the effects of the autologous SP; $p < 0.05$) while at least one boar negatively ($p < 0.05$) influencing the viability of the incubated spermatozoa. It is concluded that SP should be present when incubating highly extended spermatozoa. As a result of the obvious differences among boars, it would be advantageous to examine the ability of SP to maintain sperm viability prior to the use of SP pools during sperm handling *in vitro*.

## Introduction

The ejaculated spermatozoa are immersed in seminal plasma (SP), a medium composed of aliquots of the fluid of the testis, epididymal tail and the secretions of the accessory sexual glands of the sire. The SP contains a wide variety of factors that influence the functionality of spermatozoa (Mann and Lutwak-Mann 1981; Rodriguez-Martinez et al. 1984; Rodriguez-Martinez 1991; Iwamoto et al. 1992; Maxwell et al. 1997; Strzezek 2002) although the biological effects of these SP factors on sperm function are complex and not well understood. A variation in the presence, absence or concentration of some components, most probably proteins (either of epididymal or accessory gland origin, Fournier-Delpech and Thibault 1993), may be responsible for the variability seen on the effects – detrimental or beneficial – on the spermatozoa (Maxwell and Johnson 1999). A high variability exists in the SP composition among species or even males within the same species, as well as between ejaculates of homologous males (Killian et al. 1993; Ashworth et al. 1994; Zhu et al. 2000).

Some SP fractions, such as the prostatic one (England and Allen 1992), the gel fraction in stallion, boar or camelids (Mann and Lutwak-Mann 1981), as well as the secretion of the seminal vesicles, are considered by some authors as detrimental for sperm survival *in vitro* (ram, Ashworth et al. 1994; bull, Way et al. 2000; buck, Azerêdo et al. 2000). Decreases in fertility have been reported following the exposure of ruminant (Dott et al. 1979) or stallion (Corteel 1980) spermatozoa to either autologous or homologous SP. Therefore, when handling spermatozoa *in vitro*, the removal of SP (by centrifugation or extension with a buffer) is praxis, and considered critical to ensuring maximal sperm viability in the majority of protocols for semen preservation.

However, the SP has also proven beneficial for spermatozoa. In pigs or bulls, incubation of spermatozoa in autologous or homologous SP at room temperature increased the sperm resistance to cold shock, a process that occurs when recently ejaculated spermatozoa are chilled (Pursel et al. 1973; Ollero et al. 1998; Eriksson et al. 2001). Viability can be restored in cold-shocked ram spermatozoa if incubated with selected portions of SP-proteins, particularly a 20-kDa band (Barrios et al. 2000). Addition of SP to thawed ram spermatozoa reverted changes seen during cryopreservation (capacitation-like; Pursel and Johnson 1975) to such an extent that motility was increased *in vitro* (Gillan and Maxwell 1999; Maxwell et al. 1999), as well as fertility post-Artificial Insemination (Maxwell et al. 1999). Similar changes have been reported in other species (for review, see Rodriguez-Martinez et al. 1998). Furthermore, the addition of SP to boar spermatozoa subjected to sperm sorting for chromosomal sex through flow cytometry is usual (Maxwell et al. 1998; Johnson and Welch 1999; Vazquez et al. 2003) where a 2000-fold extension of the ejaculate is required. Addition of 10% (v/v) of SP to the collection medium of sex-sorted spermatozoa improves their sperm viability and motility as well as stabilizes the plasma membrane thus preventing premature capacitation (Maxwell et al. 1997), and extending their fertilizing ability (Maxwell et al. 1998).

Addition of homologous SP (e.g. from different males) can even influence the fertilizing capacity of the spermatozoa (bulls: Killian et al. 1993). For instance, the addition of SP from low-fertility bulls decreased the penetration of zona-free oocytes by spermatozoa from bulls of high fertility (Henault and Killian 1996).

 