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Novel Antimicrobial Agents and Therapeutic Approaches for Nosocomial pneumonia Caused by *Pseudomonas aeruginosa*

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Novel Antimicrobial Agents and Therapeutic Approaches for Nosocomial pneumonia Caused by *Pseudomonas aeruginosa*

Doctoral thesis presented by

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Supervised by:

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AUTORITZACIÓ DELS DIRECTORS DE LA TESI DOCTORAL

EL PROF. ANTONI TORRES MARTÍ, METGE CONSULTOR SENIOR DEL SERVEI DE PNEUMOLOGIA DE L'HOSPITAL CLÍNIC DE BARCELONA I CATEDRÀTIC DE MEDICINA DE LA UNIVERSITAT DE BARCELONA

I LA DRA. LAIA FERNÁNDEZ-BARAT, INVESTIGADORA CIBER ACREDITADA I PROFESSORA ASSOCIADA DE LA UNIVERSITAT DE BARCELONA

CERTIFIQUEN:

Que la Tesi Doctoral que duu per títol "**Novel Antimicrobial Agents and Therapeutic Approaches for Nosocomial Pneumonia Caused by *Pseudomonas aeruginosa***", presentada per l'Ana Motos Galera per optar al grau de Doctor en Medicina i Recerca Translacional amb menció Internacional, ha estat realitzada sota la seva supervisió.

I AUTORITZEN:

La seva presentación per ser jutjada pel tribunal corresponent.

Per tal de que quedi constancia, signen la present a Barcelona, Juliol del 2022

Prof. Antoni Torres, MD, PhD

Dra. Laia Fernández-Barat, PhD

And once the storm is over you won't remember
how you made it through, how you managed to survive.
You won't even be sure, in fact, whether the storm is really over.
But one thing is certain. When you come out of the storm
you won't be the same person who walked in.
That's what this storm's all about.

Haruki Murakami, Kafka on the Shore

A mis padres

A la meva família

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INTRODUCTION

Developement of this doctoral thesis has taken place during 2016-2022 at the *Institut d'Investigacions Biomèdiques August Pi I Sunyer* (IDIBAPS - Hospital Clínic de Barcelona - CIBERES), the University of Barcelona, and Center for Anti-Infective Research and Development at Hartford Hospital. Both Prof. Antoni Torres and Dr. Laia Fernández-Barat provided guidance and oversight of such thesis. To be submitted for the degree of Doctor, this dissertation has been prepared in accordance with regulations established by the University of Barcelona, including a compilation of four separate studies accepted for publication in peer-reviewed scientific journals. This doctoral dissertation received approved by the *Comissió Acadèmica del Programa Doctorat* of the Faculty of Medicine of the University of Barcelona.

The studies presented in this dissertation form part of an animal experimentation research line within the research group called "Applied research in respiratory infections and critically ill" and led by Dr. Antoni Torres. These laboratory studies aim to test the efficacy of new antibiotics and novel therapeutic approaches in animal models of nosocomial pneumonia. The studies herein reported were conducted at both the Division of Animal Experimentation, Department of Pulmonology, Hospital Clinic – IDIBAPS – University of Barcelona, Barcelona, Spain and the Center for Anti-Infective Research and Development, Hartford Hospital, Hartford, CT, USA. All the presented studies in this dissertation acquired the ethical approval from the Animal Experimentation Ethics Committee of the University of Barcelona and the Hartford Hospital Institutional Animal Care and Use Committee, respectively.

The thesis includes a general introduction, hypothesis and objectives, results, a general discussion and final conclusions. The results presented in this dissertation have conferred pioneering knowledge onto the field and been published in internationally renowned medical journals. The cumulative impact factor of these publications is 36.613, based on the 2021 Journal Citations Reports ® Science Edition (Clarivate Analytics). All tables and figures, except Figure 1, were originally created by the PhD candidate with the BioRender.com and GraphPad Prism (version 8.0; GraphPad Software, La Jolla, CA, USA) software.

Apart from the work performed within the scope of this doctoral thesis, the PhD student has served as principal investigator and collaborator of various other projects related to the line of research of respiratory infections and critically illness. Such undertakings and investigation have resulted in the production of other original manuscripts, included in **Appendix 1**.

FINANCIAL SUPPORT AND CONFLICT OF INTEREST

Financial support was provided by the Institut d'Investigacions Biomèdiques August Pi I Sunyer (IDIBAPS); Ministerio de Ciencia e Innovación; Centro de Investigación Biomédica en Red- Enfermedades Respiratorias (CIBERES, CB06/06/0028); Quality Research Group from Generalitat de Catalunya (SGR/944); University of Barcelona; "ICREA Academia 2019" award to Prof. Antoni Torres from the University of Barcelona; La Marató de TV3 (201831-10); "La Caixa" Foundation (LCF/PR/HR19/52160019); and the Instituto de Salud Carlos III de Madrid (COV20/00110, ISCIII); Fondo Europeo de Desarrollo Regional (FEDER) "Una manera de hacer Europa".

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A. Torres and G. Li Bassi received an unrestricted grant from Cardeas Ltd, Seattle, WA, USA, through their affiliated institution. Cardeas Ltd., the manufacturer of amikacin/fosfomycin, did not participate in the conduct of the study, data collection and management, analysis, interpretation of data or preparation of the manuscript. Cardeas Ltd. reviewed the final manuscript.

A. Torres and G. Li Bassi received an unrestricted grant from Merck & Co, Kenilworth, NJ, USA, through their affiliated institution. Merck & Co, the manufacturer of ceftolozane-tazobactam, did not participate in the conduct of the study, data collection and management, analysis, interpretation of data or preparation of the manuscript. Merck & Co reviewed the final manuscript.

D.P. Nicolau received an unrestricted grant from F. Hoffmann-La Roche Ltd., Basel, Switzerland, through their affiliated institution. F. Hoffmann-La Roche Ltd, the manufacturer of nacubactam, did not participate in the conduct of the study, data collection and management, analysis, interpretation of data or preparation of the manuscript. F. Hoffmann-La Roche Ltd reviewed the final manuscript.

A. Motos, the PhD candidate, does not have any conflict of interest related to this doctoral dissertation.

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ABBREVIATIONS

ACM	All-cause mortality
ALAT	Asociación Latinoamericana de Tórax
ATS	American Thoracic Society
AUC	Area Under the Curve
BAL	Bronchoalveolar lavage
C/T	Ceftolozane-tazobactam
CFU	Colony-forming unit
CI	Confidence interval
Cmax	Maximum concentration
ELF	Epithelial Lining Fluid
EMA	European Medicine Agency
ERS	European Respiratory Society
ESBL	Extended spectrum β-lactamases
ESCMID	European Society of Clinical Microbiology and Infectious Diseases
ESICM	European Society of Intensive Care Medicine
ETT	Endotracheal tube
***f*T>MIC**	Free time above MIC
HAP	Hospital-acquired pneumonia
ICU	Intensive Care Unit
ICUAP	ICU-acquired pneumonia
IDSA	Infectious Diseases Society of America
IEAT	Inappropriate empirical antimicrobial treatment
IMV	Invasive mechanical ventilation
IV	Intravenous
KPC	*Klebsiella pneumoniae* carbapenemase
MDR	Multidrug-resistant
MIC	Minimum Inhibitory Concentration
MBL	Metallo-β-lactamase

MRSA	Methicillin-resistant *S. aureus*
OXA	Oxacillin-type carbapenemase
PBP	Penicillin-binding proteins
PD	Pharmacodynamics
PDR	Pandrug-resistant
PK	Pharmacokinetics
PTA	Probability of Target Attainment
RCT	Randomized Controlled Trial
VAP	Ventilator-associated pneumonia
v-HAP	Ventilated hospital acquired pneumonia
XDR	Extensively drug-resistant

PHD THESIS FORMAT

Thesis in the form of a collection of published articles. This thesis comprises four main objectives and four articles. The main objectives were:

1. To clarify whether pharmacokinetic models built with full local level (ELF) profiles can lead to more precise estimates.

   Motos A, Kuti JL, Li Bassi G, Torres A, Nicolau DP. Is One Sample Enough? beta-Lactam Target Attainment and Penetration into Epithelial Lining Fluid Based on Multiple Bronchoalveolar Lavage Sampling Time Points in a Swine Pneumonia Model. Antimicrob Agents Chemother. 2019;63(2).

   Impact Factor 5.938 (Q1- 51/279 Pharmacology & Pharmacy) based on the 2021 Journal Citation Reports ® Science Edition (Clarivate Analytics).

2. To elucidate the benefits of appropriate empiric antimicrobial therapy with ceftolozane-tazobactam compared to inappropriate empiric therapy.

   Motos A, Li Bassi G, Pagliara F, Fernandez-Barat L, Yang H, Aguilera Xiol E, et al. Short-Term Effects of Appropriate Empirical Antimicrobial Treatment with Ceftolozane/Tazobactam in a Swine Model of Nosocomial Pneumonia. Antimicrob Agents Chemother. 2021;65(2).

   Impact Factor 5.938(Q1 – 51/279 Pharmacology & Pharmacy) based on the 2021 Journal Citation Reports ® Science Edition (Clarivate Analytics).

3. To evaluate the efficacy of the antimicrobial combination of meropenem-nacubactam in strains of *Pseudomonas aeruginosa t*hat express KPC and overproduce AmpC.

   Asempa TE, **Motos A**, Abdelraouf K, Bissantz C, Zampaloni C, Nicolau DP. Meropenem-nacubactam activity against AmpC-overproducing and KPC-expressing Pseudomonas aeruginosa in a neutropenic murine lung infection model. Int J Antimicrob Agents. 2020;55(2):105838.

   Impact Factor 15.441 (Q1- 7/279 Pharmacology & Pharmacy) based on the 2021 Journal Citation Reports ® Science Edition (Clarivate Analytics).

4. To analyze the role of nebulized amikacin/fosfomycin as adjuvant therapy, compared to intravenous administration of meropenem in nosocomial pneumonia caused by *P. aeruginosa*.

Li Bassi G, **Motos A**, Fernandez-Barat L, Aguilera Xiol E, Chiurazzi C, Senussi T, et al. Nebulized Amikacin and Fosfomycin for Severe Pseudomonas aeruginosa Pneumonia: An Experimental Study. Crit Care Med. 2019;47(6):e470-e7.

Impact Factor 9.296(Q1- 7/35 Critical Care Medicine) based on the 2021 Journal Citation Reports ® Science Edition (Clarivate Analytics).

RESUMEN

Introducción: La neumonía nosocomial causada por *Pseudomonas aeruginosa* está asociada a una alta mortalidad morbilidad. Además, la elevada incidencia de multirresistencias a los antimicrobianos, hacen que el tratamiento tanto empírico como dirigido sea una decisión compleja para los clínicos. Diversas estrategias se han planteado entre las que figuran la optimización de la concentración de los antimicrobianos vía la nebulización o través de un mejor estudio de la penetración a nivel pulmonar; así como el uso de nuevas combinaciones antimicrobianas como son ceftolozane-tazobactam, meropenem-nacubactam.

Objetivos: Los objetivos fundamentales fueron: (i) esclarecer si los modelos farmacocinéticos construidos con perfiles completos a nivel local (ELF, por sus siglas en inglés) pueden conducir a estimaciones más precisas; (ii) elucidar los beneficios del tratamiento antimicrobiano empírico apropiado con ceftolozane-tazobactam en comparación con el tratamiento empírico inapropiado; (iii) evaluar la eficacia de la combinación antimicrobiana de meropenem-nacubactam en cepas de *P. aeruginosa* que expresan KPC y sobreproducen AmpC; (iv) analizar el papel de la amikacina/fosfomicina nebulizada como terapia adyuvante, en comparación con la administración de intravenosa de meropenem en la neumonía nosocomial causada *por P. aeruginosa.*

Materiales y métodos: Los estudios incluidos en esta tesis doctoral se basaron fundamentalmente en un modelo porcino de neumonía grave y en un modelo de infección pulmonar en ratones neutropénicos. Los animales se inocularon con diferentes cepas de *P. aeruginosa*, para posteriormente ser randomizados y tratados en función del diseño de cada estudio. Se analizaron los resultados microbiológicos, histológicos, inflamatorios y parámetros clínicos. Además, se realizaron análisis farmacocinéticos y farmacodinámicos.

Principales resultados y conclusiones: Los principales hallazgos fueron que: (i) los modelos ELF construidos con concentraciones en puntos dispersos dan como resultado estimaciones similares a los construidos a partir de perfiles concentrados; (ii) el tratamiento inicial apropiado con ceftolozane-tazobactam disminuyó la carga bacteriana en las secreciones respiratorias, evitó el desarrollo de resistencias y logró el objetivo terapéutico a nivel farmacodinámico; (iii) la adición de nacubactam a meropenem resultó en una reducción bacteriana sustancial en los recuentos de *P. aeruginosa*; (iv) y se corroboró que la amikacina/fosfomicina nebulizadas reduce la presencia de *P. aeruginosa* en las secreciones traqueales y limita el desarrollo de resistencias, pero tiene una eficacia insignificante en el tejido pulmonar.

BACKGROUND

1. NOSOCOMIAL PNEUMONIA

Pneumonia is one of the most common nosocomial infections to occur in hospitalized patients (1, 2). Nosocomial pneumonia or hospital-acquired pneumonia (HAP) is defined as infection of the pulmonary parenchyma ≥48 hours after admission in patients who did not present any signs of antecedent infection at hospital admission (3). Among nosocomial pneumonias, there is also ventilator-associated pneumonia (VAP). VAP develops in patients admitted to the intensive care unit (ICU) who have undergone invasive mechanical ventilation (IMV) for at least 48 h (4).

Definitions for nosocomial pneumonia, HAP and VAP are not homogeneous throughout literature (5). The complexity of nosocomial pneumonia subsets in both the ward and ICU is shown as a diagram in **Figure 1**. In the following doctoral thesis, nosocomial pneumonia is used to refer to the onset of pneumonia 48 hours or more after hospital admission, either in the ICU (ICU-acquired pneumonia; ICUAP) or conventional ward (HAP), and irrespective of IMV (5). While, VAP appears after 48 hours of IMV (6), patients with severe nosocomial pneumonia who require IMV during their treatment after infection onset do not meet the definition of VAP (7). Nonetheless, ventilated HAP (v-HAP) warrants consideration, given that this specific subtype is associated with increased severity due to respiratory failure when compared against non-ventilated HAP (8). Other conditions such as nosocomial tracheobronchitis are not detailed in this doctoral thesis.

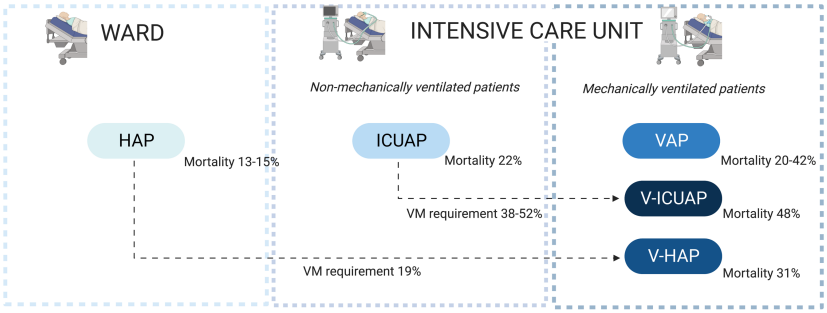

Figure 1. Complexity of nosocomial pneumonia from wards to ICU settings.

Color intensity shows the progression of nosocomial pneumonia severity. Crude mortality data are shown for each entity. ICU, intensive care unit; ICUAP, ICU-acquired pneumonia; HAP, hospital-acquired pneumonia; VAP, ventilator-acquired pneumonia; V-ICUAP, ventilated ICU-acquired pneumonia; V-HAP, ventilated hospital-acquired pneumonia. Source: Adapted from Ibn Saied W Intensive Care Med, 2020 (9). Data from Melsen WG *et al.* Lancet Infect Dis, 2013 (10), Magill SS *et al.* N Engl J Med, 2014 (11), Micek ST *et al.* Chest, 2016 (12), Giuliano KK *et al.* Am J Infect Control, 2018 (13), Ibn Saied W *et al.* Crit Care Med, 2019 (14), Gonçalves-Pereira J *et al.* J Hosp Infect, 2021 (15).

In the following paragraphs, we will summarize the current understanding of the epidemiology, physiopathology, diagnosis, prevention, and treatment of nosocomial pneumonia, focusing specifically on novel antimicrobials agents and new therapeutic strategies for this hospital-acquired infection.

1.1 Incidence, outcomes, and economic impact

Nosocomial pneumonia constitutes an important health problem worldwide, causing high morbidity and mortality among hospitalized patients (2, 16, 17). In Europe, nosocomial pneumonia is the most frequent health care infection, accounting for up to 40% among all such infections (18). Within the last decade, its incidence of has ranged between 12-33% in the United States, and several series have reported this infection as the second most common nosocomial infection (1, 11, 18, 19). Most nosocomial pneumonia cases occur in non-ventilated patients. The incidence of HAP in patients admitted to the conventional ward ranges between 1.6-6.2 cases per 1,000 admissions (20). Mortality ranging between 11-18% has been consistently reported for HAP in non-ICU-admitted patients. Among this subset of patients, though, 19% will require IMV, thereby increasing mortality risk to 28-31% (15). The highest risk for nosocomial pneumonia is in patients requiring IMV (VAP or v-HAP); mortality can reach up to 50% (8).

In the ICU, VAP represents more than 80% of pneumonia cases due to a 6-20-fold increased risk of pneumonia onset in patients receiving IMV (19, 21). Overall, incidence density of VAP ranges between 2-7 episodes per 1,000 ventilator days, with significant differences present between the United States and Europe (22, 23). While the US observes a VAP incidence between 1.9 and 3.8 per 1000 days of IMV (22), the figure increases to 6.6 per 1,000 days in Europe (23). Nevertheless, this index is in continual decline due to the implementation of bundled measures aimed at reducing nosocomial pneumonia incidence (24). Indeed, according to the ENVIN-HELICS report, incidence in Spain is considerably lower than 10 years ago (14.9 versus 5.41 episodes per 1,000 days of IMV in 2009 and 2019, respectively) (25). Attributable mortality of VAP remains controversial and highly depent on. A recent systemic meta-analysis including data from 24 randomised prevention study trials showed overall attributable mortality of VAP to be 13% (10).

As nosocomial pneumonia lengthens hospital stays by 7-9 days (12, 13), healthcare costs in patients with nosocomial pneumonia increase, especially if VAP develops. The excess of unadjusted costs associated with VAP was estimated to be approximately 40,000-49,000 US dollars per patient (26, 27),

while estimated non-ventilated HAP acute care costs were reported as ranging between 28,000-40,000 US dollars (11, 13).

1.2 Etiology and resistance mechanisms

Microorganisms responsible for nosocomial pneumonia differ according to geographic areas, hospital location, patients' specific characteristics, hospital and ICU stay duration, and risk factors for multidrug-resistant (MDR) pathogens (28). However, large series have shown that the most frequent causative pathogens are aerobic, Gram-negative bacilli like *Pseudomonas aeruginosa*, *Escherichia coli*, *Klebsiella pneumoniae* or *Acinetobacter* species; *Staphylococcus aureus* is the predominantly isolated Gram-positive pathogen (**Table 1**) (29-31). Certainly, these pathogens, also named ESKAPE by their acronym, are responsible for more than 80% of nosocomial pneumonia cases (29, 32). It is rare that causative agents for nosocomial pneumonia are fungi and viruses, even though cases of caused by *Candida spp* and *Aspergillus fumigatus* have been reported particularly in immunocompromised patients (2, 33).

Pathogens	Europe (n=2,393)(30)		USA (n=1,334) (29)		Asia (n=2,530) (31)		Latin America (29)	All regions (n=7,496) (29)	
	HAP	VAP	HAP	VAP	HAP	VAP	HAP/VAP	HAP	VAP
Staphylococcus aureus	20.9	16.2	48.6	34.4	15.8	12.2	20.1	26.6	19.5
Pseudomonas aeruginosa	**21.6**	**22.6**	**18.4**	**21.2**	**15.6**	**25.9**	**28.2**	**22.4**	**26.6**
Klebsiella species	11.0	15.8	7.1	8.4	12.0	16.7	12.1	10.5	10.2
Enterobacter species	5.7	4.8	4.3	5.6	4.1	4.2	6.2	7.5	7.0
Acinetobacter species	5.4	16.3	2.0	3.0	13.5	36.5	13.3	8.3	14.3
Serratia species	3.8	3.2	5.5	6.5	1.2	0.7	2.4	4.1	4.1
Escherichia coli [a]	12.0	9.2	-	-	6.9	3.4	5.5	-	-
Other CAP pathogens [b]	8.2	4.4	3.3	6.6	6.7	2.2	3.7	2.6	4.1

Table 1. Frequency of bacterial pathogens associated with hospital-acquired pneumonia and ventilator-associated pneumonia across geographic areas.

Data is reported in percentages. [a] *E. coli* frequency was not reported for USA and all regions. [b] CAP pathogens included *Haemophilus influenzae, Streptococcus pneumoniae* and *Moraxella catarrhalis*. CAP, community-acquired pneumonia; HAP, hospital-acquired pneumonia; VAP, ventilator-associated pneumonia. Source: Compiled by the PhD candidate. Data extracted from Flamm RK *et al.* Int J Antimicrob Agents, 2016 (30), Jones RN *et al.* Clin Infect Dis, 2010 (29), and Chung DR *et al.* Am J Respir Crit Care Med, 2011(31).

1.2.1 *Pseudomonas aeruginosa*

Among the aforementioned nosocomial pathogens, *P. aeruginosa* is the most common Gram-negative bacterial pathogen to cause life-threatening nosocomial pneumonia (29, 34). It has intrinsic resistance to many antimicrobial agents, increasing over the last decades due to selection pressure exerted by the inappropriate and indiscriminate use of broad-spectrum antibiotics (e.g. empirical therapies for infections in critically ill patients) (35).

	Biochemical characteristics	Main involved enzymes	Substrates
β-lactamases	Enzymatic inactivation of β-lactam antibiotics		
Ambler class C	Chromosomally located. Cephalosporinases	AmpC	Cephalosporins
Ambler class A	Extended-spectrum β-lactamases. Penicillinases inhibited by clavulanic acid and carbenicillinases	PER, TEM, SHV, CTX-M, GES, CARB, VEB, KPC	Cephalosporins, penicillin, monobactams
Ambler class B	Metallo-β-lactamases conferring resistance to most of -β-lactams except monobactams	IMP, VIM, SPM	Penicillin, cephalosporins, carbapenems
Ambler class D	Oxacillinases	OXA-	Penicillin, oxacillin
Efflux systems	Increased efflux pump expression (i.e., antibiotic efflux)	MexAB-OprM, MexCD-OprJ MexEF-OprN MexXY-OrpM	Fluoroquinolones, β-lactams, tetracycline, tigecycline, chloramphenicol
Outer membranes	Decreased of porin expression	OrpD	β-lactams, tetracycline, aminoglycosides, chloramphenicol, ciprofloxacin
LPS modification	Modification or loss of LPS	pmrAB and phoPQ	Colistin
Topoisomerase IV and DNA gyrase	Mutation in critical genes for bacterial DNA replication	parC, gyrA, gyrB, parE	Fluoroquinolones
16S rRNA methylases	Methylation of 16S rRNA	RmtA, RmtD, and ArmA	Aminoglycosides
Aminoglycoside-modifying enzymes	Enzymatic inactivation by aminoglycoside-modifying enzymes	AAC(6')-I, AAC(6')-II, ANT(2'')-I, APH(3')-VI	Aminoglycosides

Table 2. Mechanisms of *P. aeruginosa* antimicrobial resistance.

Classification of mechanisms of *P. aeruginosa* antimicrobial resistance, their major biochemical characteristics, main involved enzymes or proteins and substrates. Source: Compiled by the PhD candidate. Data extracted from Zavascki AP *et al.* Expert Rev Anti Infect Ther, 2010 (36); El Zowalaty ME *et al.* Future Microbiol 2015 (37); Eichenberger EM *et al.* Antibiotics, 2019 (38).

Standardized definitions for MDR (i.e., nonsusceptibility to at least one antibiotic in at least three classes of antibitotics), extensively drug-resistant (XDR) (i.e., nonsusceptibility to at least one agent in all but two or fewer classes of antibiotics) and pandrug-resistant (PDR; i.e., nonsusceptibility to all agents) were proposed for *P. aeruginosa* infections in 2012 (39).

Prevalence of MDR among patients with HAP/VAP due to *P. aeruginosa* is high (40), and have been associated with increased in-hospital mortality (41). In fact, the percentage of MDR and XDR *P. aeruginosa* isolates vary depending on the setting, local epidemiology and if HAP or VAP, with figures reaching 20-43% and 5-21%, respectively (31, 42-44). The 2022 annual report by the European Antimicrobial Resistance Surveillance Network reported that 30.1% of the *P. aeruginosa* isolates were resistant to at least one of the antimicrobial groups under surveillance, while 12.1% were resistant to three or more antimicrobial groups (45). The main resistance mechanisms of *P. aeruginosa* include hyperexpression of chromosomal AmpC β-lactamases, loss of outer membrane channel OprD, increased efflux pump systems, production of carbapenemases, and lipopolysaccharide modification (46). Other mechanisms comprise extended spectrum β-lactamases (ESBL), topoisomerase IV/DNA gyrase mutation, methylation of 30S RNA and PmrA-PmrB two-component system genetic modification(35, 47). A summary of resistance mechanisms, major characteristics and their substrates is displayed in **Table 2**.

1.3 Pathogenesis and risk factors

Development of pneumonia depends on the virulence of pathogens; size of the inoculum reaching the lung; and degree of alteration of host defense responses (48, 49). Mechanisms for nosocomial pneumonia consists of aspiration of the pathogen into the upper respiratory tracts; inhalation of contaminated aerosols via the respiratory tract or the endotracheal tube (ETT) if the patient is intubated; and, more rarely, bacterial translocation by hematogenous dissemination (**Figure 2**) (49, 50). Endogenous colonization is the primary source of pathogens (51, 52). However, exogenous flora may also play a significant role, irrespective of preventive strategy implementation (51). In the subset of mechanically ventilated patients, the presence of the ETT facilitates microaspirations of oropharyngeal secretions and bacteria into the lungs via the folds in the ETT cuff (53). Additionally, the ETT completely impairs anatomical barriers, creating a direct canal to the lungs. The formation of biofilm (i.e., aggregated microorganisms within an exopolysaccharide matrix) on the inner surface of the ETT also entails an

important risk factor as a persistent source of colonization (54, 55). Finally, given its influence on pulmonary immunity, the "gut-lung axis" dysbiosis in intestinal microbiota has been highlighted as a potential risk factor for nosocomial pneumonia (56).

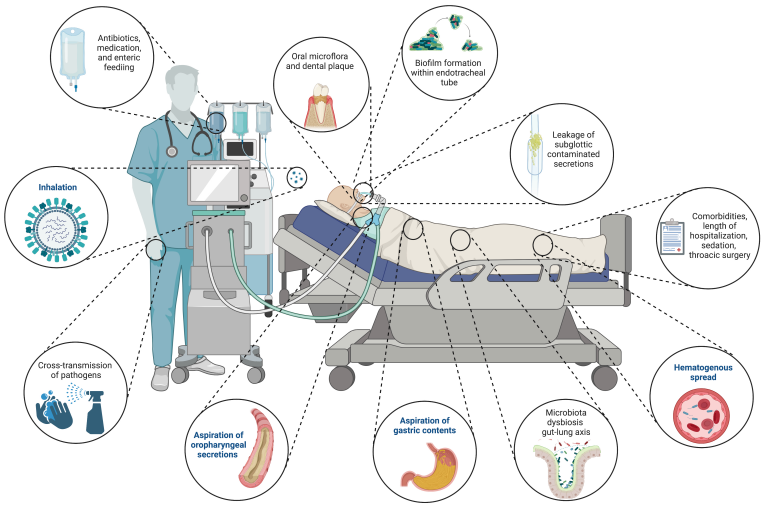

Figure 2. Main mechanisms of pathogenesis and risk factors for nosocomial pneumonia

Main pathogenic mechanisms are highlighted in blue while patient-related, while procedure-related and intervention-related risk factors are colored in black. Source: Own illustration.

1.4 Diagnostics

The presence of leukocytosis, fever, and purulent secretions; the appearance of a new infiltrate on a chest radiograph or extension of existing ones; and a deterioration in gas exchange constitute clinical signs to suspect pneumonia (57, 58). Nevertheless, these are not specific enough, especially in critically ill and mechanically ventilated patients in whom multiple conditions may present same signs and symptoms (59). In this context, it is highly recommended to obtain respiratory samples prior to any antimicrobial therapy to confirm the diagnosis, identify the pathogen responsible for the infection and thus, adapt the initial empirical antibiotic treatment accordingly (57, 60). For non-ventilated patients, non-invasive sampling (i.e., spontaneous expectoration, sputum induction, nasotracheal suctioning) is recommended (57, 60). Non-invasive sampling (i.e., tracheal aspirate) with semiquantitative cultures is also the preferred methodology for VAP or nosocomial pneumonia diagnoses in patients requiring IMV, given such approach helps avoid unnecessary harm and cost (57). However, invasive sampling (i.e., bronchoalveolar lavage

(BAL), protected specimen brush, mini-BAL) can occasionally be performed; it may help decrease antibiotic exposure (60, 61). Current defined thresholds are 10^5 colony-forming unit (CFU)/mL for tracheal aspirate; $\geq 10^4$ CFU/mL for BAL; and $\geq 10^3$ CFU/mL for protected specimen brush (59). Moreover, the determination of antimicrobial resistance is crucial to guide the antimicrobial therapy. Mostly, the minimum inhibitory concentration (MIC), the lowest concentration of an antimicrobial that will inhibit the visible growth of a microorganism, is used to grade into susceptible, increased exposure, or resistant to a particular antimicrobial by using a breakpoint (62, 63).

In the last decades, several different molecular methods have been developed for more rapid identification, including resistance genes, and therefore, to improve overall utilization of antimicrobials (64). In this scenario, the impact of choosing the adequate antimicrobial by obtaining the microbiological identification earlier may be notorious.

1.5 Prevention

Many elements have been considered to have a significant impact on reducing nosocomial pneumonia incidence worldwide. Besides the common practices of hand hygiene and use of protective gloves and gowns (65), some other measures have strong evidence to support their routine use and consideration as key components in prevention bundles (60, 66, 67). Those bundles focus on preventing aspiration of contaminated subglottic secretions and stomach contents. To avoid gastro-esophageal reflux, a fundamental element is the semi-recumbent position (i.e., bed elevation to 30-45º) (68, 69). Selective digestive decontamination and selective oral decontamination have been designed to reduce the contamination of both subglottic secretions and gastric contents (70, 71). Although these strategies were promising at first, the unclear, long-term impact of their routine use on antimicrobial resistance (72) has led current international guidelines to recommend the use of selective oral decontamination, albeit not selective digestive decontamination, in ICUs with low resistance rates (60).

In mechanically ventilated patients, ETTs with subglottic drainage may decrease the leakage of contaminated secretions via the cuff, and thereby, VAP incidence and mortality (73). The use of coated tubes to prevent biofilm formation and cuff pressure monitoring have also been largely investigated; however, high initial costs in the former and inconclusive benefits in the latter make their routine use

ambiguous (74-76). In contrast, reducing ventilator circuit manipulations and suctions may also protect against unneeded contamination of lower airways (77). Given the strong correlation between VAP incidence and prolonged IMV, other strategies focusing on reducing IMV duration have been implemented. Among them, spontaneous breathing trials (78), daily awakening trials (79) and early mobilization (80) have shown positive impact on shortening IMV duration, and thereby, a decreased number of VAP episodes.

2. CURRENT TREATMENT FOR NOSOCOMIAL PNEUMONIA

Treatment for nosocomial pneumonia includes comprehensive measures such as antimicrobial therapy, the use of respiratory support devices (i.e., oxygen mask, high flow nasal cannular, non-invasive ventilation and IMV), non-antimicrobial agents or adjunctive therapies, and other organ function support strategies. Among them, though, antimicrobial treatment, including both empirical and pathogen-targeted treatments, is the most important (57).

2.1 Empirical therapy for nosocomial pneumonia

Hasty initiation of empirical antimicrobial therapy may be a key factor in improving clinical outcomes of patients with nosocomial pneumonia. However, antibiotic selection is intricate for physicians. There must be a balance between selecting adequate empirical treatment to cover potential MDR pathogens and minimizing the risk of future resistance due to an overuse of the most effective antibiotics and avert adverse events related to the use of multiple broad-spectrum agents.

Selection of appropriate empirical treatment should be based on local etiology and the presence of risk factors for MDR/XDR pathogens. Indeed, hospitals and ICUs are highly recommended to be in possession of their own updated data of local antibiotic resistance, as it may change across units (60, 81). Furthermore, physicians should also consider the patient's severity of illness, clinical characteristics, presence of severe sepsis or septic shock, other organ function status and prior antibiotic use (57, 60, 82). European Respiratory Society, European Society of Intensive Care Medicine, European Society of Clinical Microbiology and Infectious Diseases and Asociación Latinoamericana del Tórax (ERS/ESICM/ESCMID/ALAT) guidelines recommend including late-onset HAP/VAP (i.e., $\geq$ 5 days of

hospitalization) as a risk factor for MDR pathogens (60). In patients clinically suspected of HAP/ VAP, empirical antimicrobial treatment should be started as soon as possible after clinical diagnosis of HAP/VAP and the retrieval of respiratory secretions for microbiological cultures have been performed (3). Even in cases wherein the drug is selected properly, mortality and hospitalization can increase if empirical treatment is delayed (83).

Empirical treatment recommendations by both international guidelines are summarized in **Figure 3** (57, 60). In patients with suspected nosocomial pneumonia and low risk factors, a narrow-spectrum antibiotic that covers methicillin-susceptible *S. aureus* is suggested. For Gram-negative bacilli coverage including *P. aeruginosa*, physicians should administer a narrow-spectrum single agent with activity against *P. aeruginosa*. In contrast, for patients at high risk of resistance or mortality, a combination therapy of broad-spectrum antimicrobials targeting *P. aeruginosa* and ESBL-producing pathogens, as well as an antimicrobial drug to cover methicillin-resistant *S. aureus* (MRSA) is recommended.

Inappropriate empirical treatment (IEAT) indicates that the empirical dug administered within the first three days of clinical suspicion of nosocomial pneumonia was not active against the identified pathogen. The rate of IEAT in patients with nosocomial pneumonia can reach up to 60% (84). Primarily, the increasing resistance to classical β-lactams and difficulty in achieving adequate concentrations due to high MICs drive this percentage (85). Indeed, the ENVIN-HELICS program computed a 30% likelihood of patients receiving an inadequate empirical treatment for a *P. aeruginosa* infection, including even with combination therapy (25). The impact of IEAT on mortality is still inconclusive due to conflicting results found in literature (86). For example, prospective observation study performed to define the impact of appropriate empirical antimicrobial selection on clinical outcomes of patients with VAP showed that mortality was lower in patients who received appropriate treatment versus those with inadequate therapy, including even in those who switched treatment after microbiological data became available (87). Also, in 115 patients with microbiologically confirmed cases of VAP and in whom 85% received appropriate therapy, mortality was significantly higher in those with inadequate empirical therapy than in those with appropriate therapy (47 vs. 20%, $p = 0.04$) (88). On the other hand, in a study of 758 ICU-admitted patients with nosocomial pneumonia due to MDR pathogens, investigators Vasudevan et al. reported that IEAT was not an independent risk factor for ICU mortality (89). Similarly, in a multicenter study of critically ill patients with Gram-negative lower tract respiratory infections, failure in empirical treatment selection culminated in more hospital days and thus, higher economic burden; however, there was no impact on all-

cause mortality (ACM) (90). On the other side, it is equally apparent that excessive antibiotic use promotes the emergence and spread of antibiotic-resistant pathogens from patients in ICUs (91). Nevertheless, international guidelines consider the appropriateness to be more important to the outcome, and place it therefore in higher consideration than the emergence of resistance (57, 60).

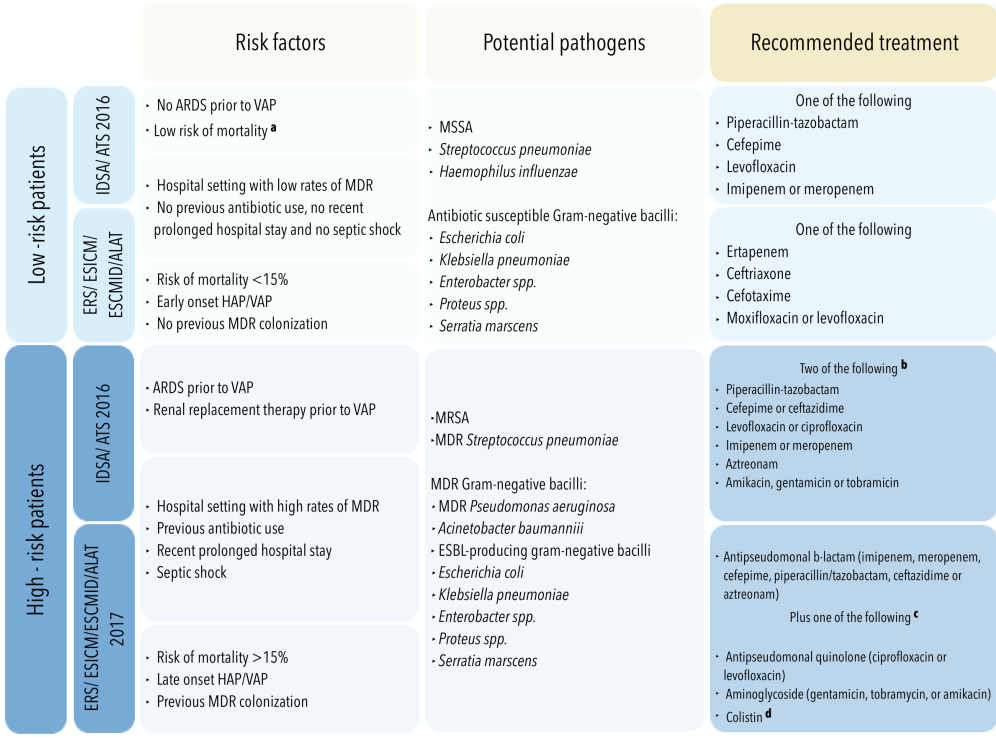

Figure 3. Recommendations for empirical treatment for clinically suspected cases of HAP and VAP per risk factors for MDR pathogens by the international guidelines.

[a] Risk factors for mortality depend on the requirement of mechanical ventilation and/or septic shock status. [b] If the patient has no factors for mortality, only one agent is recommended. In case of high-risk mortality or intravenous antibiotics, two antipseudomonal are recommended to avoid the use of two β-lactams. [c] For patients who are not in septic shock, only a single Gram-negative agent such as antipseudomonal β-lactam or quinolone is recommended. [d] Colistin may be needed in settings with a high prevalence of MDR *A. baumanniii.* Source: Compiled by the PhD candidate. Data extracted from Kalil AC *et al.* Clin Infect Dis, 2016 (57) and Torres A *et al.* Eur Respir J, 2017 (60). ARDS, acute respiratory distress syndrome; ESBL, extended-spectrum β-lactamases; HAP, hospital-acquired pneumonia; MDR, multidrug resistance; MSSA, methicillin susceptible *S. aureus*; MRSA, methicillin-resistant *S. aureus*; VAP, ventilator-associated pneumonia.

2.2 Pathogen-targeted therapy for *P. aeruginosa* nosocomial pneumonia

Efficacy evaluation should be performed within 48-72 hours of empirical treatment once microbiological cultures are available. Such evaluation should consider patient's clinical response, evolution on chest radiographs, follow-up cultures and other laboratory examinations (3, 92, 93). Thereafter, clinicians should switch to targeting the pathogen or opting for a narrower antibiotic regimen or monotherapy—also called de-escalation—based on antimicrobial susceptible testing results. Current guidelines do not recommend one agent more than the other, albeit a special statement has advised clinicians to avoid aminoglycoside monotherapy (57, 60). Mainly, the poor penetration of aminoglycosides into the lungs is the primary reason for this suggestion. Actually, to obtain microbiologically effective intrapulmonary concentrations, clinicians would have to administer high systemic concentrations that would otherwise increase risk of toxicity (94, 95).

Both Infectious Diseases Society of America – American Thoracic Society (IDSA/ATS) and European guidelines recommend 7-8 days of antibiotic therapy for most patients (57, 60); however, ERS panelists note that short-course therapy may not be possible for non-fermenting Gram-negative pneumonias. IDSA/ATS guidelines have not found differences between pneumonia patients with and without non-fermenting Gram-negative pathogens and have extended the recommendation to include longer courses only for patients with slow clinical and radiological recovery.

3. NOVEL THERAPEUTIC STRATEGIES

The high incidence of MDR microorganisms has reiterated the importance of rationally using antimicrobial agents (96). In the last decades, novel approaches have been continually developed to reduce the selective pressure of MDR expansion and associated mortality and morbidity (97, 98). These strategies range from antimicrobial optimization and breakthrough drug combination and delivery to bacteriophage therapy and immunotherapy (98, 99). In the context of this PhD thesis, here we present two examples in particular: antimicrobial optimization and nebulization of antimicrobials.

3.1 Optimizing antibiotic administration

Among the possible and recognized strategies, an antibiotic optimization approach requires the consideration of both pharmacokinetics and pharmacodynamics (100). Pharmacokinetics (PK) is the

branch of pharmacology that studies the change in drug concentrations in body compartments over time. Drug concentration variation is related to the process of absorption (i.e., transfer to central compartment); distribution (i.e., transfer among peripheral compartments); metabolism (i.e., biotransformation); and excretion or elimination of drugs. The main PK parameters that play a role in the final drug concentration that reaches the tissue include volume of distribution and clearance. Antibiotic properties are also determinant in describing the fate of administered drugs. Considering this perspective, PK is affected by drug physicochemical properties, mainly aqueous solubility, and protein binding. Hydrophilic antibiotics disseminate in intravascular and extravascular body fluids, while lipophilic drugs can reach lipid tissue and distribute intracellularly (101). The percentage in which a drug binds to protein implies how microbiologically active free drug, as protein-bound drug cannot interact with molecular targets (102).

The other pharmacological area is pharmacodynamics (PD), which assesses the effects of antimicrobial agents. Therapeutic outcomes are determined by the concentrations reached at the site of action, which is dependent on PK behavior, and antibiotic susceptibility of microorganisms, expressed as the MIC. Pathogen-drug interaction has classically been determined by *in vitro* methods; however, therapeutic success will depend also on isolate virulence, immune response, and site of infection (103). Given PD properties, antibiotics have been classified into three categories: concentration-dependent, time-dependent or a combination of concentration- and time-dependent (which is based on the concentration-time curve associated with maximal bacterial killing). The PD drivers associated with each of these groups, respectively, include the ratio of maximum free drug concentration to the MIC (fC_{max}/MIC); free time above MIC (fT>MIC); and ratio of the area under the curve to the MIC (fAUC/MIC). Among antimicrobials commonly used to treat nosocomial pneumonia, that is, β-lactams, fT<MIC is the related predictor with bacteria eradication and microbiological response (104). The fCmax/MIC is the close-fitted parameter for aminoglycosides (105), fluoroquinolones (106) and polymyxins, whereas fAUC/MIC is suitable for predicting the efficacy of vancomycin (107) and oxazolidinones (108). In each case, only free drug is considered (102).

3.1.1 Optimization in patients with nosocomial pneumonia

Mathematical relationships between dosing regimen and resultant plasma concentrations can be established and decisive, given that the concentration profile over time can affect outcomes. Furthermore, the ability of drugs optimization may help to suppress the emergence of resistance, thereby representing a critical preventive response to this current and alarming epidemiological concern (109).

Given all of the changing parameters, studies have observed inadequate concentrations of antibiotics during critical illness, which may drive to IEAT. In this context, there is a need for optimal doses in this subset of patients (110, 111). Indeed, PK parameters depend largely on the host and are subject to influence by illness severity (112). Clearance can change rapidly given the fluctuating hemodynamic state and renal function in critically ill patients (113). Similarly, volume of distribution tends to increase in those patients due to a capillary leakage, in which fluid moves from the capillaries to surrounding tissues and body cavities (114) (115). This value also depends on both the pharmacological characteristics of the drug and serum protein concentration. Alterations in these protein levels such as hypoalbuminemia – observed in approximately 40% of critically ill patients – can increase the unbound fraction of the drug, raising the volume of distribution and clearance as a result (116). This translates to lower antibacterial exposure, which could result in suboptimal treatment for the patient (114).

Pharmacodynamic parameter breakpoints have been widely identified in lower respiratory tract infections. Free concentrations in plasma are often considered as an acceptable approximation for free concentrations at the site of infection, but this is not always the case. In some cases, they may be misleading (117). Measuring antimicrobial concentrations at the site of infection might be more relevant in predicting clinical response (118). Although it is possible to include additional compartments in PK modelling phase and perform simulations for concentrations at the site of infection, sampling in each compartment is required to properly estimate exposure profiles. Determining intrapulmonary drug concentrations in the epithelial lining fluid (ELF) compartment is currently the most widely employed method to estimate antibiotic exposure for extracellular respiratory tract pathogens (**Figure 4**) (118, 119). Nevertheless, some limitations must be considered in this context. First, drug concentration is measured in BAL samples and then correlated to ELF by using urea as an endogenous marker (120). This estimation is inaccurate: it can contribute to underestimation, or conversely, overestimation of the intrapulmonary concentration (121). Other technical errors such as dwelling time of fluid during bronchoscopy or contamination of BAL with blood urea need to be considered (121).

Macrolides, oxazolidines and fluroquinolones have higher ELF than serum concentrations, while β-lactam, aminoglycosides and glycopeptides showed the inverse. In all of the cases notwithstanding, the relation is linear. In contrast, carbapenems showed discordance in the form of concentration-time profiles called hysteresis (118). Therefore, penetration ratios will vary in magnitude with the sampling time(s)

chosen. To overcome this issue, it is recommended that research studies determine penetration ratios from estimates of the AUCs values obtained from plasma and ELF data.

Pooled data at each sampling time point are then averaged to estimate a concentration profile in ELF throughout the dosing interval. In more recent years, population PK modeling and Monte Carlo simulation methods have been applied to these data to assess the variability in ELF penetration and evaluate antibiotic PD and target attainment (PTA). Populations are constructed with specified demographics, relevant infection profiles and individualized antibiotic PK profiles (122). Monte Carlo simulation is a computer modelling process that incorporates both the variability in PK parameters and the natural MIC distribution within a bacterial population to create a hypothetical population of thousands of patients. For each of these hypothetical patients, a concentration-time profile is simulated and the PD target (e.g., *f*T > MIC) calculated. The PTA is an estimation of the probability that simulated subjects can achieve this predefined PD target within the entire simulated population. A PTA of 90% or higher at MIC values of interest is a widely accepted value to support a dose regimen (111). Such investigations can contribute significantly to identifying optimal antibiotic selection, alongside to dosing regimens and MIC breakpoints for new and existing agents.

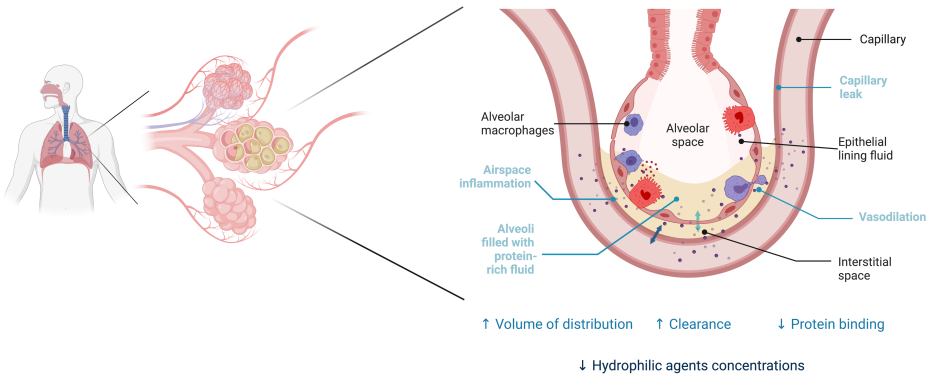

Figure 4. Pathophysiological alterations at lung level during nosocomial pneumonia and their potential effect on pharmacokinetics of antimicrobial agents.
Source: Own illustration.

3.2 Nebulized antimicrobials

The other strategy for ventilated nosocomial pneumonia (i.e., VAP or v-HAP) treatment that falls within the scope of this doctoral thesis is nebulization of antimicrobial agents. In the last decades,

nebulized antibiotics—also called inhaled or aerosolized drugs, depending on the delivery system—have been widely proposed, to respond to the increasing rates of MDR pathogens in patients with nosocomial pneumonia (123, 124). Currently, aminoglycosides and polymyxins are the drugs that have been most investigated.

As mentioned before, intravenous treatment has several limitations, including insufficient lung distribution and development of adverse side effects (123). With an established role in cystic fibrosis and bronchiectasis (125, 126), the aim of nebulized antimicrobial agents in nosocomial pneumonia is to deliver a therapeutically effective amount of the drug directly into the respiratory system, so it acts in the bronchi and reaches high deposition in the infected lung parenchyma (127). Nebulized antimicrobials could accomplish an extremely high local drug concentration capable of eradicating MDR/XDR pathogens (128). Moreover, Palmer *et al.* have demonstrated that nebulized antibiotics prevented the development of resistance to intravenous antibiotics (129). Similarly, low systemic exposure may dramatically reduce potential adverse effects, as Abdellatif and colleagues demonstrated in a randomized trial evaluating high doses of nebulized colistin versus intravenous colistin for VAP (130). In fact, patients in the nebulized group had a significantly lower incidence of acute renal failure, a higher level of oxygenation and a shortened time to bacterial eradication than those in the control group receiving intravenous colistin, although the overall clinical cure rate was not significantly different.

Some important considerations must be taken into account as it relates to this strategy and antimicrobial choice, dosing regimen, formulation and delivery system (131). With respect to drug characteristics, formulation should be between 150-1200 mOsm/kg and have a pH of 4.0-8.0 to avoid bronchial irritation, cough and/or bronchoconstriction (132, 133). Also, particle diameter size must range between 1-5 μm to prevent deposition in the circuit and, at the same time, avoid systemic absorption (134). Moreover, the nebulizer in itself and ventilator settings during nebulization are key factors for adequate drug deposition. After several technological improvements, currently available vibrating mesh nebulizers have increased aerosol delivery efficiency by up to 40-60% (135, 136). Specific setting to limit inspiratory flow turbulences that included an optimal distance from the nebulizer to Y-piece, no humidifier use, low breathing rate, low inspiratory flow and prolonged inspiratory time may facilitate the adequate drug lung deposition (124, 137). Finally, extension and severity of lung infection also affect lung distribution of aerosolized antibiotics; sufficient airway patency and alveolar opening are required for correct deposition to be achieved (135, 138).

According to current USA guidelines (57), which were written before the last failed randomized controlled trials (RCT) (139, 140), nebulized antibiotics are only recommended as adjunctive therapy for patients with VAP caused by XDR bacteria only susceptible to aminoglycosides or colistin and reject their routine use. As lung and airway concentrations may be subtherapeutic in these antibiotic classes, combined treatment with nebulized antimicrobial may be beneficial. Also, the US expert panel contemplates their use as a last resort for patients who are not responding to intravenous antibiotics alone. Finally, the European Society of Clinical Microbiology and Infectious Diseases have made a statement against the routine use of nebulized antimicrobial agents and support use of such agents only in the aforementioned conditions (141).

3.2.1 Nebulized Amikacin/Fosfomycin

Of the potential antibiotics that can be nebulized into the respiratory system, the combination of fosfomycin and an aminoglycoside could prove to confer great benefit on patients with VAP caused by either MDR Gram-negative or Gram-positive pathogens (142, 143).

Amikacin is an aminoglycoside that is active against Gram-negative aerobic bacilli, including *P. aeruginosa* (144). A bactericidal antibiotic, amikacin exhibits concentration-dependent killing (145). In other words, for therapeutic success, it is necessary to administer a large dose that is 5-10 times greater than the MIC of the target organism at the site of infection (146, 147). Severe respiratory infections due to XDR pathogens are often treated by parenteral administration of amikacin combined with other antibiotic classes (57, 60), although nephrotoxicity and ototoxicity have been commonly associated with such administration (148).

Fosfomycin is a broad-spectrum phosphonic acid antibiotic with bactericidal activity against Gram-negative bacteria and Gram-positive bacteria, including MRSA (149, 150). Fosfomycin is moderately active against *P. aeruginosa* (150). A time-dependent drug, fosfomycin inhibits bacterial cell wall synthesis and enters into the bacterial cell by two means of transport: a constitutively functional L- α -glycerophosphate transport and the hexose-phosphate uptake system (149). Fosfomycin monotherapy is commonly used to treat uncomplicated urinary tract infections caused by *E. coli* (151). A single intravenous or intramuscular dose of 2 g of fosfomycin achieves peak serum concentrations of between 25-95 µg/mL within 1-2 hours (152), while lung distribution and concentrations are very low (1-13 µg/mL) (153). These pulmonary concentrations are insufficient to kill most pathogens, in particular *P. aeruginosa*, and therefore make nebulization a good option.

In the last decades, investigators have assessed the effects of aerosolized amikacin on the treatment of Gram-negative pneumonia in *in vivo* models (127, 128, 138, 154, 155). In an animal study, Goldstein *et al.* (127) compared the deposition and the efficacy of nebulized amikacin in comparison to intravenous (IV) amikacin with ventilated piglets with *E. coli* severe pneumonia. Besides finding 30 times higher tissue concentration in nebulized pigs, they also found lower lung bacteria burden in this group in comparison to the intravenous one (127, 154).

Nebulized amikacin has been also used in a few studies to treat intubated patients with VAP – often in association with other systemic antibiotics (128, 139, 140, 156-159). Investigators Lu *et al.* (128) compared 8-day curative rates between aerosolized and intravenous ceftazidime and amikacin in patients with VAP due to *P. aeruginosa*. Results showed a similar curative rate, although antibiotic resistance developed only in patients treated with intravenous antibiotics. Similarly, Palmer *et al.* (159) showed a reduction in the resistance pressure in critically ill patients treated with nebulized antibiotics. In a later study in mechanically ventilated patients with gram-negative pneumonia, Niederman *et al.* (156) found that aerosolized amikacin distributed well throughout the lung while maintaining serum concentrations below the renal toxicity.

Despite a suggested benefit in uncontrolled observational studies, two recent double-blind RCT studies failed to meet their primary efficacy endpoints (139, 140). First, in a prospective, double-blind, placebo-controlled trial of 143 patients with Gram-negative VAP (IASIS trial, NCT01969799), Kollef *et al.* (139) tested a combination of nebulized amikacin (300 mg q12 h) and fosfomycin (120 mg q12 h) as adjunctive therapy against standard-of-care IV antibiotics plus nebulized saline for 10 days. While this RCT failed to find effects on clinical outcomes, nebulized antibiotics were associated with a faster sterilization of bronchial secretions and, once again, a significantly reduced emergence of drug-resistant bacteria. A second double-blind, placebo-controlled trial (INHALE trial, NCT01799993 and NCT00805168)(140) randomized patients with Gram-negative VAP: 725 patients received either nebulized amikacin or placebo as adjunctive therapy to standard-of-care IV antibiotics. In this case, investigators found no difference in survival at 28-32 days between both treatment groups (odds ratio 0.84, 95% confidence interval (CI) 0.55–1.28; p=0.43).

Despite these discouraging results, several factors may have had a negative influence on treatment efficacy (159-163). For instance, data differs as to whether nebulized drug reaches the terminal bronchi and alveoli, if the edema, inflammatory debris and the mucus almost entirely obstruct the distal bronchi

(**Figure 5**) (135, 164). In addition, several technical aspects and timing of the nebulization procedure could influence lung drug distribution (137). Finally, the potential methodological biases in the RCT design may have also played a role in these unsatisfactory results (160, 162). Nebulized antimicrobials as a rescue threapy could have merits in patients with difficult-to-treat infections; they could also serve a purpose in patients with PDR pathogens as per current pneumonia guidelines recommendations (57, 60, 141). However, the associated survival benefits of these treatments in these subpopulations have not yet been studied in large RCTs.

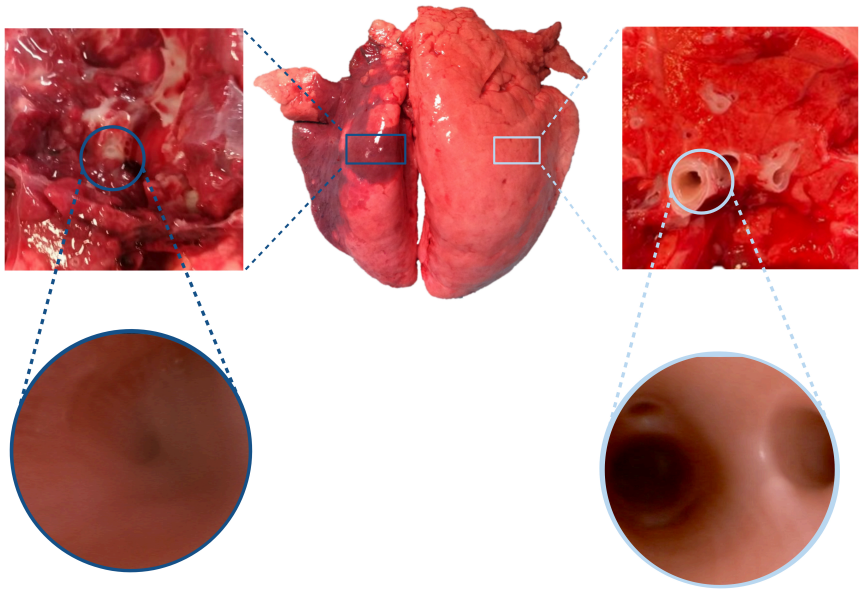

Figure 5. Lung tissue with heterogenous damage and bronchoscopy evaluation in swine *P. aeruginosa* monolateral pneumonia model.

The penetration of nebulized antibiotics into the distal pulmonary parts of highly infected regions filled with respiratory secretions could be reduced in comparison to proximal areas or healthy sections. Source: Own illustration.

4. NOVEL ANTIMICROBIAL AGENTS AGAINST *P. aeruginosa*

The likelihood of resistance to conventional antipseudomonal β-lactam, although are frequently prescribed, is high and commonly associated with resistance to other traditional β-lactams (165). In an effort to overcome the various resistance enzymes observed in *P. aeruginosa*, novel antimicrobial agents and new combinations of β-lactam/β-lactamase inhibitors have been developed (166). Some of these antibiotics have received preference based on their potential advantages reported in *in vitro* data, and

pivotal and observational studies (167). However, various experts have warrant the use of other antibiotic families in accordance with the site of infection, clinical severity and comorbidities, risk factors for MDR acquisition, and existing MDR pathogens in each unit/hospital (168, 169), as suggested by IDSA/ATS (57) and ERS/ESICM/ESCMID/ALAT (60) algorithms.

Ceftobiprole (170), ceftazidime-avibactam (171), ceftolozane-tazobactam (172), meropenem-vaborbactam (173), imipenem-relebactam-cilastin (174) and cefiderocol (175) are new molecules that recently licensed for the treatment of nosocomial pneumonia. **Table 3** displays the main RCTs carried out in the last years for nosocomial pneumonia patients. A summary of labeled indications, approved dosages, and main outcomes are presented. Moreover, ceftaroline-avibactam (176), aztreonam-avibactam (177), cefoperazone-sulbactam (178), plazomicin (179), meropenem-nacubactam (180), and murepavadin (181) are other new investigational drugs in development phases that may be approved, representing promising options to improve the antimicrobial armamentarium against *P. aeruginosa* nosocomial pneumonia.

Among all these novel antimicrobial agents, this doctoral thesis will focus on both β-lactams/β-lactamase inhibitors: ceftolozane-tazobactam and meropenem-nacubactam.

4.1 Ceftolozane-tazobactam

Ceftolozane-tazobactam (C/T) is an intravenously administered combination of novel cephalosporin ceftolozane and β-lactamase inhibitor of tazobactam. In both the European Union and United States, C/T has received approval for the treatment of complicated intra-abdominal or urinary tract infections in adults (182). Moreover, in June 2019, the Food and Drug Administration approved the use of C/T to treat HAP/VAP due to Gram-negative microorganisms in patients aged 18 years and older (182). The European Medicines Agency (EMA) also extended its indication to nosocomial pneumonia in December 2020.

4.1.1 Mechanism of action and spectrum activity

Ceftolozane, previously known as CXA-101 and FR264205, is a cephalosporin structurally like ceftazidime; however, it has a pyrazole ring that provides stability and prevents hydrolysis by AmpC β-lactamases (183, 184).

Drug name	Included Gram-negative (166, 185-187)	EMA labeled indications	Study trial and num. patients	Dosage	Comparator	Design and population	Primary outcomes Intervention vs comparator	Risk differences (95% CI)
Ceftolozane-tazobactam (172)	ESBL *Enterobacteriaceae* Limited AmpC and OXA-48 *Enterobacteriaceae* Efflux and AmpC *P. aeruginosa*	Approved in 2019 for adults with HAP/VAP at 3g q8h	ASPECT-NP (NCT02070757) 726 patients	3g q8h as 1-h IV infusion	Meropenem	Double-blind, non-inferiority RCT for ventilated HAP + VAP	ACM: 24.0% vs 25.3% CC TOC: 54.4% vs 53.3% MC TOC: 73.1% vs 68.0%	ACM: 1.1 (-5.1 to 7.4) CC TOC: 1.1 (-6.2 to 8.3) MC TOC: 4.5 (-3.4 to 12.5)
Ceftobiprole (170)	Nonextended spectrum β-lactamases, non-AmpC, and non-cabepenemases-producing *Enterobacterales*, *P. aeruginosa*	Approved in 2013 for adults with HAP excluding VAP at 500 mg q8h	NCT00210964 781 patients	500 mg q8h as 2-h IV infusion	Ceftazidime plus linezolid	Double-blind, non-inferiority RCT for HAP + VAP	ACM: 16.7% vs 18.0% CC TOC: 77.8% vs 76.2% MC TOC: 62.9% vs 67.5% [a]	ACM: -1.2(-7.4 to 5.0) CC TOC: 1.6 (-6.9 to 10.0) MC TOC: -16.7 (-38.8 to -0.4)
Ceftazime-avibactam (171)	ESBL, AmpC, KPC, OXA-48 *Enterobacteriaceae* AmpC *P. aeruginosa*	Approved in 2018 for adults with HAP/VAP at 2.5g q8h	REPROVE (NCT01808092) 879 patients	2.5g q8h as IV 2-h infusion	Meropenem	Double-blind, non-inferiority RCT for GN HAP + VAP	ACM: 9.6% vs 8.3% CC TOC: 68.8% vs 73.0% MC TOC: 55.6% vs 64.1%	ACM: NA CC TOC: -4.2 (-10.8 to 2.5) MC TOC: -8.6 (-18.7 to 1.6)
Cefiderecol (175)	ESBL, AmpC, KPC, OXA-48, MBL *Enterobacteriaceae* Efflux and AmpC *P. aeruginosa, MDR A. baumannii*	Approved in 2020 for adults with Gram-negative infections and limited options	APEKS-NP (NCT03032380) 300 patients	2g q8h as 3-h IV infusion	Meropenem	Double-blind, non-inferiority RCT for Gram-negative HAP + VAP	ACM: 21.0% vs 20.5% CC TOC: 64.8% vs 66.6% MC TOC: 47.6% vs 48.0%	ACM: 0.5 (-8.7 to 9.8) CC TOC: -2.0 (-12.5 to 8.5) MC TOC: -1.4 (-13.5 to 10.7)
Meropenem-vaborbactam (173)	ESBL, AmpC, KPC *Enterobacteriaceae* AmpC *P. aeruginosa* Limited *MDR A. baumannii*	Approved in 2018 for adult HAP/VAP at 4g q8h	TANGO-II (NTC02168946) 77 patients	4g q8h as 3-h IV infusion	Best available therapy	Open-label RCT for Carbapenem-resistant *Enterobacteriaceae* infections including HAP + VAP	ACM: 3.1% vs 33.3% CC TOC: 59.4% vs 26.7% MC TOC: 53.1% vs 33.3%	ACM: -29.0 (-5.1 to 7.4) CC TOC: 32.7 (4.6 to 60.8) MC TOC: 19.8 (-9.7 to 49.3)
Imipenem-cilastin-relebactam (174)	ESBL, AmpC, KPC *Enterobacteriaceae* Efflux and AmpC *P. aeruginosa* Limited *MDR A. baumannii*	Approved in 2020 for adults with HAP/VAP at 1.25g q6h	RESTORE-IMI2 (NCT02493764) 537 patients	1,250 mg q6h as 30-min IV infusion	Piperacillin/ tazobactam	Double-blind, non-inferiority RCT for HAP + VAP	ACM: 15.9% vs 21.3% CC TOC: 61.0% vs 55.8% MC TOC: 67.9% vs 61.9%	ACM: -5.3 (-11.9 to 1.2) CC TOC: 5.9 (-3.2 to 13.2) MC TOC: 6.2 (-2.7 to 15.0)

Table 3. List of last major randomized phase II trials of novel intravenous antimicrobial agents for the treatment of *P. aeruginosa* nosocomial pneumonia.[a] The reported primary and secondary endpoints were HAP (excluding VAP) patients. ACM, all-cause mortality; AmpC, Ambler class C β-lactamase; CC TOC, clinical cure at test-of-cure; EMA, European Medicines Agency; ESBL, extended spectrum β-lactamase; HAP, hospital-acquired pneumonia; IV, intravenous; KPC, *Klebsiella pneumoniae* carbapenemase; MBL, New Delhi metallo-β-lactamase; MC TOC, microbiological cure at test-of cure; MDR, multidrug resistance; OXA-48, oxacillin carbaepenemase 48; RCT, randomized clinical trial; VAP, ventilator-associated pneumonia. Source: Compiled by the PhD candidate.

Like other cepthalosporins, ceftolozane exerts bactericidal activity by binding to penicillin-binding proteins (PBPs), thus inhibiting cell wall biosynthesis, and inducing bacterial cell lysis and death (188). Tazobactam is a well-established β-lactamase inhibitor that inhibits most class A, including ESBLs, and a number of class C β-lactamases(189). Its addition in a 2:1 ratio therefore protects ceftolozane against hydrolysis due to β-lactamase enzymes

C/T is active against Gram-negative and Gram-positive bacteria, the greatest *in vitro* activity is that against *P. aeruginosa*, including those which are MDR and XDR (190). *In vitro* activity of C/T against *P. aeruginosa* isolates collected in recent surveillance reports are shown in **Figure 6**. Its activity is 20-25% greater than other competitor compounds, making C/T the most active compound after colistin (191). Susceptibility ranges between 81-98%, being similar across various geographic and clinical settings (192-197).

Activity against *Enterobacteriaceae* is also good, albeit more variable and dependent on the specific species and β-lactamases (195). In contrast, C/T has no activity against most carbapenemases (*Klebsiella pneumoniae* carbapenemase (KPC) and metallo-β-lactamases (MBL)); it is, however, residual against OXA-48 (198). Similarly, activity against Gram-positive is quite limited.

Current Clinical and Laboratory Standards Institute (M100 32nd edition, valid from February 2022)(62) and European Committee on Antimicrobial Susceptibility Testing breakpoints (version 12.0, valid from January 2022)(63), for C/T based on 1,000-to-500 mg dosing of C/T intravenously for *P. aeruginosa* and *Enterobacteriaceae* are ≤ 4/4 mg/L and ≤2/4 mg/L, respectively. Based on current data, potential for the selection of resistance to C/T against *P. aeruginosa* appears to be linked to intrinsic AmpC modifications and horizontally-acquired β-lactamases (e.g., OXA-14)(199, 200), while efflux pumps upregulation and structure/functional changes of porin channels have not been shown to have significant impact (201).

4.1.2 Pharmacokinetics and pharmacodynamics

In phase I studies, ceftolozane shows linear PK after 1 g dose with C_{max} up to 92.3 mg/L, plasma half-life around 2.5 hours, protein binding approximately 20% and 14L of volume of distribution (202, 203). As both ceftolozane and tazobactam are renally excreted, clearance decreases with impaired renal function (204). C/T dosages must, therefore, be adjusted according to the creatinine clearance.

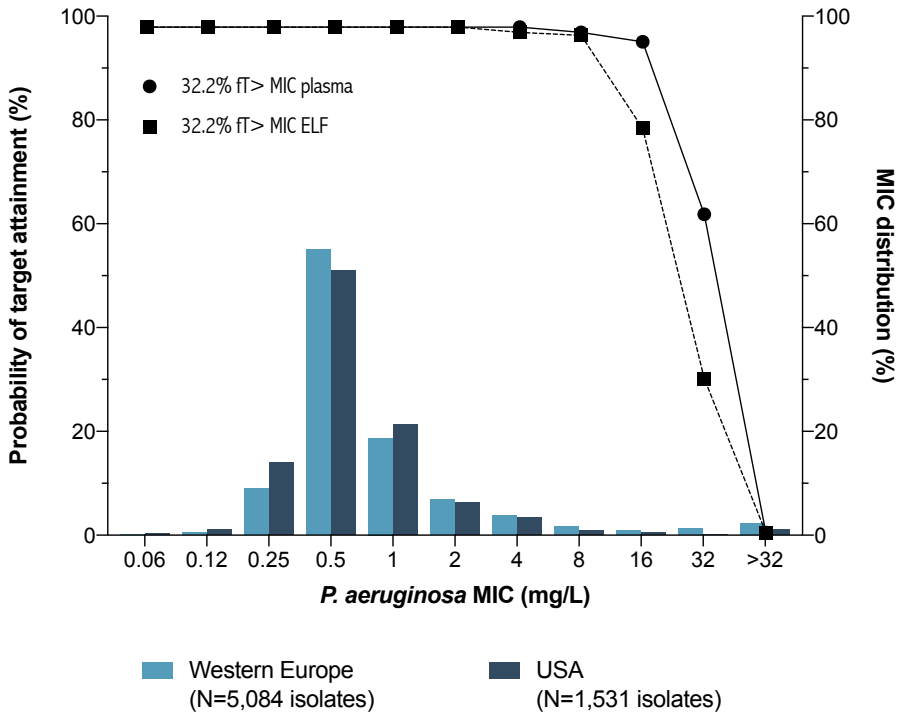

Figure 6. Distribution of *P. aeruginosa* isolates from nosocomial pneumonia patients in terms of C/T value and the PTA in both compartments.

Histograms represent the MIC distribution stratified by region. Lines represent PTA values through MIC level. PTA of ceftolozane in plasma and ELF in patients with nosocomial pneumonia and normal renal function following 3 g C/T administered as a 1-h q8h, using 32.2% *f*T>MIC as pharmacodynamic target. Source: Own illustration. Distribution of isolates were obtained from Carvalhaes CG *et al.* Diagn Microbiol Infect Dis, 2019 (196) and Sader HS *et al.* J Antimicrob Chemother, 2020 (197) for Western Europe and US data, respectively. PTA data was obtained from Xiao AJ *et al.* J Clin Pharmacol, 2016 (205). ELF, epithelial lining fluid; *f*T>MIC, free time above MIC; MIC, minimum inhibitory concentration, PTA, probability of target attainment.

Consistent with other β-lactam antimicrobials, ceftolozane exhibits time-dependent bactericidal activity (206). As such, the PD parameter best correlated to C/T antimicrobial activity is the percentage of time in which free drug concentration is above the infecting organism's MIC across a dosing interval (i.e., 40-50%*f*T>MIC) (207, 208). Neutropenic murine thigh infection model with humanized doses evaluated the bactericidal efficacy, showing that 40% *f*T>MIC is likely to achieve >1-log killing against *P. aeruginosa* isolates with MICs as high as 16 mg/L (209).

Subsequent population PK modeling analyses using human data have shown that depending on the dosing scheme, PTA might not be as high (210). Indeed, main Monte Carlo simulations with alternative dosing regimens suggested that a dose more than 1.5 g of C/T and/or continuous infusion may optimize PTA (**Figure 6**)(205), especially for those critically ill patients with augmented renal clearance, pneumonia, or with MDR *P. aeruginosa* isolates (110, 211). After intravenous administration of 1.5 g of C/T, the intrapulmonary penetration based on total ELF/plasma AUC was 48%. This is higher than piperacillin-tazobactam (i.e., 26%), hinting at appropriate penetration (210). However, when Xiao *et al.* (205) conducted Monte Carlo simulation in 25 healthy subjects, they found that doubling the approved dose of C/T (i.e., 3 g q8h) for complicated urinary tract infections and complicated intra-abdominal infections is necessary in treating nosocomial pneumonia. Specifically, at 3-g dose, PTA for 1-log kill was approximately 98.4% for pathogens with an MIC up to 8 mg/L in ELF, whereas the PTA was approximately 85% at 1.5 g dose (205). These models were confirmed by evaluating PK data for ceftolozane and tazobactam in plasma and ELF of a 3-g dose of C/T administered via a 1-hour infusion every 8 hours in adult patients with ventilated nosocomial pneumonia (212).

4.1.3 Clinical trials for nosocomial pneumonia

C/T has been evaluated in several clinical, multicenter RCT across all indications (172, 213-216). This section will only discuss the clinical trial for nosocomial pneumonia including VAP. The ASPECT-NP study (NCT02070757) (172), a multicenter phase III study, compared 2g ceftolozane/ 1g tazobactam with 1g of meropenem both as 1-h intravenous infections every 8h in patients with ventilated nosocomial pneumonia (either VAP or v-HAP) (**Table 3**). Based on the aforementioned PK data (205), the dose of C/T was the double that of approved dosing regimens for both complicated urinary tract and intra-abdominal infections. Patients received treatment for 8-14 days. This balanced randomized study of 726 patients, C/T showed non-inferiority when compared to meropenem as it relates to primary outcome of 28-day ACM in the intention-to-treat population (weighted treatment difference 1.1%, [95% CI -5.1 to 7.4]) (**Figure 7**). C/T was also non-inferior to meropenem in terms of clinical cure at test-of-cure and appeared well tolerated. Nevertheless, higher rates of treatment-related adverse events occurred in C/T than meropenem group (172). Furthermore, C/T resulted in comparable outcomes between participants with either augmented renal clearance or normal renal function (217).

Significant differences were demonstrated with respect to the non-pre-defined subgroup of patients with HAP who required IMV (218). Further analyses in this subset of patients showed—after adjusting for

variables with great impact on mortality (i.e., bacteremia and vasopressor treatment)—that the odd ratio for 28-day ACM with meropenem treatment versus C/T was 2.3 (95% CI 1.2 – 4.5) (218). While limited due to retrospective analysis, this finding suggests a potential C/T survival advantage. Although baseline pathogens in meropenem group had lower MIC values and were thus more susceptible to randomized study drug, C/T may perform better due to greatest chance of achieving the PD target associated with antibacterial activity at the site of the infection (219). Of note, meropenem dosing regimen was not optimized to extended infusions (e.g., 3-h infusion), which is recommended in critically ill patients (220, 221).

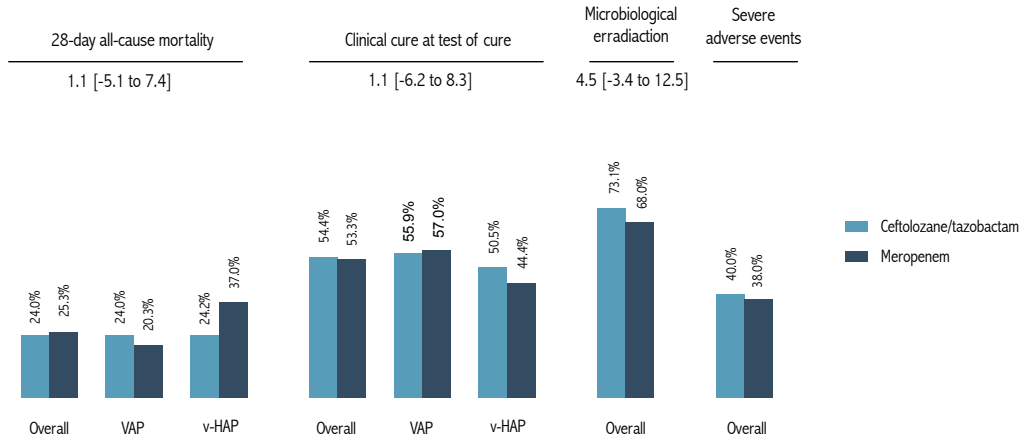

Figure 7. Primary and secondary efficacy outcomes in overall population and various subpopulations from ASPECT-NP study of ceftolozane-tazobactam against meropenem in the intention-to-treat population.

Weighted treatment differences (meropenem minus ceftolozane-tazobactam) are shown for the overall population. Percentage of patients achieving the primary and secondary outcomes are display for the overall population and VAP and ventilated-HAP subpopulations. VAP, ventilator-associated pneumonia; v-HAP, ventilated hospital-acquired pneumonia. Source: Own illustration based on data extracted from Kollef MH *et al.* Lancet Infect Dis., 2019 (172).

4.2 Meropenem-nacubactam

4.2.1 Mechanism of action and spectrum activity

Nacubactam is a novel non-β-lactam, diazabicyclooctane β-lactamase inhibitor with a triple-mechanism action (222). This inhibitor has *in vitro* activity against class A, C and some class D β-lactamases that prevent inactivation by hydrolysis due to co-administration with other β-lactam agents (222, 223). When class A serine β-lactamase hydrolyzes meropenem, nacubactam's inhibition confers

stability onto the meropenem molecule to then restore its activity in KPC presence (224, 225). Conversely, nacubactam also has affinity and inhibits PBP-2 to exert direct antibacterial effect against MBL-producing *Enterobacteriaceae* (226). Also, investigators Morinaka *et al.* described nacubactam acting as an "enhancer" of activity of several β-lactam drugs, including PBP3-targeted agents such as cefepime or piperacillin (227).

In vitro data from UK diagnostic laboratories showed that of 240 *Enterobacteriaceae* isolates, MIC for nacubactam alone ranged mostly between 1 and 4 mg/L (228). High MICs were also found among *P. aeruginosa* and *Acinetobacter baumannii* isolates. Nevertheless, approximately 80% susceptibility was achieved when nacubactam was combined with other β-lactam agents. Specifically, at higher MICs (i.e., ≥ 4 mg/L), nacubactam contributes to combination activity against bacteria with class A or class C β-lactamases, contingent on β-lactamase inhibition. In the *P. aeruginosa* strain, a 2-to-5-fold potentiation of biapenem was achieved depending on the presence and expression of OrpD porin and AmpC β-lactamase (228). In another large *in vitro* study with more than 4,000 isolates and focused on the combination of meropenem-nacubactam, presenters showed that more than 99% of *Enterobacteriaceae* were inhibited by the studied combination at 2/4 mg/L (229). Indeed, the MIC_{90} (i.e., MIC required to inhibit the growth of 90% of organisms) for meropenem was 0.03, with constant concentration of nacubactam at 4 mg/L. In contrast to the previous study, no such effect was observed in either the *Pseudomonas spp.* or *A. baummannii* isolates, remaining at similar susceptible levels than with meropenem alone. These *in vitro* results reveal the need for further *in vivo* studies assessing the efficacy of nacubactam in *P. aeruginosa* isolates.

4.2.2 Pharmacokinetics and pharmacodynamics

Studies in healthy volunteers demonstrated that meropenem and nacubactam exhibit very similar plasma protein binding, half-lives, and routes of elimination (180). Nacubactam PK were linear and comparable when it is administered alone or in combination with meropenem. Meropenem PK was also not affected by nacubactam coadministration. Like other β-lactamase inhibitors, nacubactam is predominantly renally excreted. Variation in kidney function in critically ill patients may, therefore, impact this disposition. Total nacubactam clearance in healthy volunteers ranged from 7.2 to 8.9 L/h, similar to creatinine clearance; while the volume of distribution at steady state after a dose 2,000 mg was around 26 L (180).

Single and multiple doses of nacubactam were well tolerated. Adverse events were mainly mild and resolved without sequalae (180). When nacubactam was administered in combination with meropenem, the adverse events were consistent with the known safety profile of meropenem (180). No serious adverse events, dose-limiting adverse events, or death were reported. Also, no clinically relevant dose-related trends were observed in renal biomarkers or in electrocardiogram monitoring, including QT interval.

Murine infection models were used to derive a predictive PD target of the combination efficacy (230-234). As with other inhibitors, β-lactam-β-lactamase inhibitor combinations, *f*T>MIC was an effective driver of the therapy, with half maximal inhibitory concentration value of 19.6% *f*T>MIC (234).

Based on the well-established PK/PD profile and suitability of meropenem as a potential β-lactam associate (225), the clinical development of nacubactam may be able to proceed to further clinical studies in patients with nosocomial pneumonia.

4.2.3 Clinical trials for nosocomial pneumonia

At the time of writing this dissertation, there is no registration at ClinicalTrials.gov of any phase II or phase III trials aiming to assess either the pharmacokinetics in nosocomial pneumonia or efficacy of meropenem-nacubactam in comparison to standard antimicrobial agents. Despite the paucity of *in vitro*, *in vivo* and clinical data, the combination of meropenem-nacubactam may serve as a valuable alternative in overcoming resistance emergence and treating nosocomial pneumonia.

HYPOTHESIS AND AIMS

ARTICLE 1

Is One Sample Enough? β-Lactam Target Attainment and Penetration into Epithelial Lining Fluid Based on Multiple Bronchoalveolar Lavage Sampling Time Points in a Swine Pneumonia Model

Motos A, Kuti JL, Li Bassi G, Torres A, Nicolau DP

Antimicrob Agents Chemother 2019; 63(2):e01922-18. doi: 10.1128/AAC.01922-18.

Hypothesis

Describing the disposition of antimicrobial agents at the site of infection is crucial in guiding optimal dosing for investigational agents (118). For antibiotics developed to treat patients with pneumonia, concentrations are routinely determined in the epithelial lining fluid via a collection of BAL samples (118, 121). For ethical reasons, BAL sampling in humans is routinely done at a single time point. However, this results in ambiguity in the precise ELF profile (235). Pooled data at each sampling time point are then averaged to estimate pharmacokinetic profile in ELF over the dosing interval. Pharmacokinetic modeling and Monte Carlo simulation methods have been applied to assess the estimated ELF penetration and PTA to predefined pharmacodynamics targets (204, 205, 235).

It is currently unknown if sparse sampling methodologies used in humans result in comparative penetration and pharmacodynamics exposure attainment to full ELF profiles. Thus, models constructed by full ELF profiles may lead to more accurate estimates of exposure.

Aims

The primary goal was to describe the influence of collecting sparse BAL samples from each subject on the population's pharmacokinetic profile in comparison with a full ELF profile obtained via simulated human regimens of two β-lactams, ceftolozane and piperacillin, in a swine pneumonia model (236, 237). Our secondary goals were to compare penetration ratios and the PTA achieved by different BAL sampling approaches.

ARTICLE 2

Short-term Effects of Appropriate Empirical Antimicrobial Treatment with Ceftolozane/Tazobactam in a Swine Model of Nosocomial Pneumonia

Motos A, Li Bassi G, Pagliara F, Fernandez-Barat L, Yang H, Aguilera Xiol E, Senussi T, Idone FA, Travierso C, Chiurazzi C, Amaro R, Yang M, Bobi J, Rigol M, Nicolau DP, Frigola G, Cabrera R, Ramirez J, Pelosi P, Blasi F, Antonelli M, Artigas A, Vila J, Kollef M, Torres A

Antimicrob Agents Chemother 2021; 65(2):e01899-20. doi: 10.1128/AAC.01899-20.

Hypothesis

The rising frequency of MDR pathogens is making IEAT more frequent in nosocomial pneumonia (84, 89). Indeed, the likelihood of receiving IEAT for *P. aeruginosa* infection is approximately 30% (25). The short-term effects of appropriate empirical treatment within the first 48-72h hours has not been studied yet (i.e., traditional microbiological methods take at least 48 hours to provide results). International guidelines for HAP/VAP recommend empirical therapy to cover ≥95% of pathogens in *P. aeruginosa* infections based on an institution's antibiograms (57), although due to increasing resistance it becomes arduous to achieve (238).

In this context, C/T—a novel antipseudomonal with high *in vitro* activity (191, 197)—has yet to be fully characterized against first-line empirical antibiotics for nosocomial pneumonia (239). Herein we present a prospective, randomized animal study to study the short-term benefits of appropriate empirical antimicrobial treatment C/T in comparison with IEAT with piperacillin/tazobactam, a β-lactam/ β-lactamase inhibitor commonly used for suspected cases of nosocomial pneumonia.

Aims

The primary aim of the study was to investigate bactericidal activity and lung histopathological severity during the first 48 hours of appropriate treatment with ceftolozone/tazobactam in comparison with IEAT with piperacillin/tazobactam in a pneumonia swine model due to *P. aeruginosa* (236). Secondary outcomes included *P. aeruginosa* burden in tracheal secretions and BAL fluid, the development of antibiotic resistance and inflammatory markers.

ARTICLE 3

Meropenem–Nacubactam Activity against AmpC-overproducing and KPC-expressing *Pseudomonas aeruginosa* in a Neutropenic Murine Lung Infection Model

Asempa TE, **Motos A**, Abdelraouf K, Bissantz C, Zampaloni C, Nicolau DP

Int J Antimicrob Agents. 2020; 55(2):105838. doi: 10.1016/j.ijantimicag.2019.10.019

Hypothesis

The increasing rate of MDR Gram-negative bacteria is a global concern that warrants attention as it relates to in hospitals' best practices, infection control, and the development of new antibiotics (96). Specifically, *P. aeruginosa* has a great propensity to develop antimicrobial resistance quickly (240). Its management, therefore, makes *P. aeruginosa* a serious therapeutic challenge within the clinical setting. The development of carbapenem resistance, alongside the problem of the appearance of KPC-positive appearing as an emerging resistance pattern, is compromising the use of such as antipseudomonal option (241). In this context, the need for alternative and novel therapeutic options with potent antipseudomonal activity is indisputable. Nacubactam is a breakthrough, non-β-lactam, diazabicyclooctane, and β-lactamase inhibitor with *in vitro* activity against *P. aeruginosa* isolates (227). In combination with meropenem, it may prove to be a good strategy against serious Gram-negative bacterial infections, including lung infections (223, 228).

Aims

To assess the efficacy of human-simulated ELF exposures of meropenem, nacubactam and the meropenem-nacubactam combination against chromosomal AmpC-overproducing and KPC-expressing *P. aeruginosa* in a neutropenic murine lung infection model (242).

ARTICLE 4

Nebulized Amikacin and Fosfomycin for Severe *Pseudomonas aeruginosa* Pneumonia: An Experimental Study

Li Bassi G, **Motos A**, Fernandez-Barat L, Aguilera Xiol E, Chiurazzi C, Senussi T, Saco MA, Fuster C, Carbonara M, Bobi J, Amaro R, De Rosa F, Comaru T, Yang H, Ranzani OT, Marti JD, Rinaudo M, Comino Trinidad O, Rigol M, Bringue J, Ramirez J, Nicolau DP, Pelosi P, Antonelli M, Blasi F, Artigas A, Montgomery AB, Torres A

Critical Care Medicine 2019; 47(6):e470-e7. doi: 10.1097/CCM.0000000000003724.

Hypothesis

Pneumonia caused by *P. aeruginosa* is commonly treated by IV administration of antibiotics to ensure adequacy of treatment in cases of MDR etiology (57, 60). Systemic antibiotics often achieve sub-optimal pulmonary concentrations and adverse effects, i.e., renal failure (114). The combination of amikacin/fosfomycin, delivered through a vibrating mesh nebulizer, could achieve higher pulmonary amikacin/fosfomycin concentrations and dramatically improve therapeutic efficacy and reduce antimicrobial resistance development (142, 143, 157). Yet, to date, the latest trials discourage the use of nebulized amikacin/fosfomycin for IMV patients with nosocomial pneumonia (139, 140). Moreover, the intrapulmonary distribution of nebulized amikacin/fosfomycin is not fully elucidated upon and could be affected by the extension and severity of lung infection (135), as well as the ventilator parameters used during nebulization (124, 137).

Therefore, to clarify potential benefits of nebulized amikacin/fosfomycin combined with IV meropenem, we assessed bactericidal efficacy and antibiotic resistance development in swine with severe pneumonia caused by *P. aeruginosa* resistant to amikacin and fosfomycin in comparison with systemic therapy alone.

Aims

The primary aim of this animal study of *P. aeruginosa* pneumonia swine model (236) was to evaluate the effects of nebulized amikacin/fosfomycin with IV meropenem versus IV meropenem alone on lung tissue *P. aeruginosa* burden. Furthermore, we investigated the effects of nebulized antimicrobial combinations on lung histology, pulmonary function and mechanics, antibiotic resistance acquisition, hemodynamics, and inflammation.

ORIGINAL PUBLICATIONS

The studies comprising this doctoral dissertation have been published in peer-reviewed scientific journals as detailed below.

Article 1:

Motos A. Kuti JL, Li Bassi G, Torres A, Nicolau DP. Is One Sample Enough? β-Lactam Target Attainment and Penetration into Epithelial Lining Fluid Based on Multiple Bronchoalveolar Lavage Sampling Time Points in a Swine Pneumonia Model. Antimicrob Agents Chemother 2019; 63(2):e01922-18. doi: 10.1128/AAC.01922-18.

The experiments described in article 1 were conducted at the University of Barcelona, Barcelona, Spain, *Institut d'Investigacions Biomèdiques August Pi I Sunyer*, Barcelona, Spain, and Center for Anti-Infective Research and Development, Hartford Hospital, Hartford, CT, USA.

Impact Factor 5.938 (Q1- 51/279 Pharmacology & Pharmacy) based on the 2021 Journal Citation Reports ® Science Edition (Clarivate Analytics).

Article 2:

Motos A, Li Bassi G, Pagliara F, Fernandez-Barat L, Yang H, Aguilera Xiol E, Senussi T, Idone FA, Travierso C, Chiurazzi C, Amaro R, Yang M, Bobi J, Rigol M, Nicolau DP, Frigola G, Cabrera R, Ramirez J, Pelosi P, Blasi F, Antonellu M, Artigas A, Vila J, Kollef M, Torres A. Short-term effects of appropriate empirical antimicrobial treatment with ceftolozane/tazobactam in a swine model of nosocomial pneumonia. Antimicrob Agents Chemother 2021; 65(2):e01899-20. doi: 10.1128/AAC.01899-20.

The experiments described in article 2 were conducted at the University of Barcelona, Barcelona, Spain and *Institut d'Investigacions Biomèdiques August Pi I Sunyer*, Barcelona, Spain

Impact Factor 5.938 (Q1- 51/279 Pharmacology & Pharmacy) based on the 2021 Journal Citation Reports ® Science Edition (Clarivate Analytics).

Article 3:

Asempa TE, **Motos A,** Abdelraouf K, Bissantz C, Zampaloni C, Nicolau DP. Meropenem–nacubactam activity against AmpC-overproducing and KPC-expressing *Pseudomonas aeruginosa* in a neutropenic murine lung infection model. Int J Antimicrob Agents. 2020; 55(2):105838. doi: 10.1016/j.ijantimicag.2019.10.019

The experiments described in article 3 were conducted at the Center for Anti-Infective Research and Development, Hartford Hospital, Hartford, CT, USA.

Impact Factor 15.441 (Q1- 7/279 Pharmacology & Pharmacy) based on the 2021 Journal Citation Reports ® Science Edition (Clarivate Analytics).

Article 4:

Li Bassi G, **Motos A,** Fernández-Barat L, Aguilera Xiol E, Chiurazzi C, Senussi Testa T, Saco A, Fuster C, Carbonara M, Bobi J, Amaro R, Rosa F, Comaru T, Yang H, Ranzani O, Marti J-D, Rinaudo M, Trinidad O, Rigol M, Torres A. Nebulized Amikacin and Fosfomycin for Severe *Pseudomonas aeruginosa* Pneumonia: An Experimental Study. Critical Care Medicine 2019; 47(6):e470-e7. doi: 10.1097/CCM.0000000000003724.

The experiments described in article 4 were conducted at the University of Barcelona, Barcelona, Spain and *Institut d'Investigacions Biomèdiques August Pi I Sunyer*, Barcelona, Spain.

Impact Factor 9.296(Q1- 7/35 Critical Care Medicine) based on the 2021 Journal Citation Reports ® Science Edition (Clarivate Analytics).

Editorial Article 4: Gilbert, DN. Nebulized Antibiotics for Multidrug-Resistant Ventilator-Associated *Pseudomonas aeruginosa* Pneumonia. Critical Care Medicine. 2019 47 (6):880-1. doi: 10.1097/CCM.0000000000003751

ARTICLE 1

AMERICAN SOCIETY FOR MICROBIOLOGY | Antimicrobial Agents and Chemotherapy®

PHARMACOLOGY

Is One Sample Enough? β-Lactam Target Attainment and Penetration into Epithelial Lining Fluid Based on Multiple Bronchoalveolar Lavage Sampling Time Points in a Swine Pneumonia Model

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ABSTRACT Describing the disposition of antimicrobial agents at the site of infection is crucial to guide optimal dosing for investigational agents. For antibiotics in development for the treatment of nosocomial pneumonia, concentrations in the epithelial lining fluid (ELF) of the lung are frequently determined from a bronchoscopy at a single time point. The influence of profiles constructed from a single ELF concentration point for each subject has never been reported. This study compares the pharmacokinetics of two β-lactams, ceftolozane and piperacillin, among different ELF sampling approaches using simulated human regimens in a swine pneumonia model. Plasma and ELF concentration-time profiles were characterized in two-compartment models by the use of robustly sampled ELF concentrations and by the random selection of one or two ELF concentrations from each swine. A 5,000-subject Monte Carlo simulation was performed for each model to define the ELF penetration, as described by the ratio of the area under the concentration curve (AUC) for ELF to the AUC for free drug in plasma ($AUC_{ELF}/fAUC_{plasma}$) and the probability of target attainment (PTA). Given the intersubject variability of the ELF penetrations observed, differences between the models developed using robust numbers of ELF samples versus one or two ELF samples per swine were minimal for both drugs (maximum dispersion $< 20\%$). Using a threshold exposure target of 60% of the time that the free drug concentration remains above the MIC target, the ceftolozane and piperacillin regimens achieved PTAs of $\geq 90\%$ at MICs of up to 4 and 1 μg/ml, respectively, among the different ELF sampling strategies. These models suggest that the ELF models constructed with concentrations from sparse ELF sampling time points result in exposure estimates similar to those constructed from robustly sampled ELF profiles.

KEYWORDS BAL sampling, Monte Carlo simulation, pneumonia

Hospital-acquired bacterial pneumonia (HABP) and ventilator-associated bacterial pneumonia (VABP), two of the most frequent nosocomial infections (1), remain common causes of morbidity and mortality among intensive care unit (ICU) patients (2, 3). The Gram-positive bacterium *Staphylococcus aureus* and Gram-negative bacteria, including *Pseudomonas aeruginosa*, *Acinetobacter baumannii*, and *Enterobacterales*, are among the most prevalent pathogens. The antibiotic resistance rates of these HABP/VABP pathogens vary geographically but are generally increasing worldwide (4). This

Citation Motos A, Kuti JL, Li Bassi G, Torres A, Nicolau DP. 2019. Is one sample enough? β-Lactam target attainment and penetration into epithelial lining fluid based on multiple bronchoalveolar lavage sampling time points in a swine pneumonia model. Antimicrob Agents Chemother 63:e01922-18. https://doi.org/10.1128/AAC.01922-18.

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growing antimicrobial resistance in the ICU context (5) has led to the requirement for new antimicrobial agents to treat pneumonia.

Pharmacokinetic studies to describe the disposition of a drug at the site of infection, where pharmacologic efficacy is exerted, are crucial to guide optimal dosing regimen selection for novel antibiotics (6). When combined with pharmacodynamic studies to discern the level of exposure required for bactericidal effects, an optimized dosing regimen can be designed for phase III clinical trials to increase the likelihood of success. As demonstrated previously, poor knowledge of drug disposition and the neglect of pharmacodynamics at the site of infection can lead to failure in phase II and III clinical trials (7). Therefore, when antibiotics are developed to treat patients with pneumonia, it is critical to understand the disposition at the site of infection. For most bacterial etiologies of pneumonia, the site of infection is the pulmonary epithelial lining fluid (ELF), which can be accessed via bronchoscopy and bronchoalveolar lavage (BAL) (8). Despite some drawbacks (9), estimation of the penetration ratio between ELF and free plasma is considered the benchmark for assessing the attractiveness of an antibiotic candidate for the treatment of pneumonia.

For ethical and logistical reasons, BAL sampling to determine the ELF concentration is performed only once at a defined sampling time point in healthy volunteers or patients (10–17). Pooled data at each sampling time point are then averaged to estimate a concentration profile in ELF over the dosing interval. In more recent years, population pharmacokinetic modeling and Monte Carlo simulation methods have been applied to these data to assess the variability in ELF penetration and to estimate the probability that simulated subjects can reach predefined pharmacodynamic targets (18). These data are likely to afford better estimates of the clinical outcome based on the relevant MIC distribution. Regardless of its strong clinical impact, a wide variability has been reported among studies that assessed β-lactam penetration into the ELF matrix (10–14). This dispersion is partly dependent on interpatient variability itself, the sample size of participants in the original data set, and the number of subjects simulated (19, 20). However, the influence of collecting only one BAL fluid sample from each subject has never been reported.

In order to address this question, a swine model of severe *Pseudomonas aeruginosa* VABP (21) was used to compare the penetration and the probability of target attainment (PTA) for robustly sampled ELF profiles (n = 4 to 5 BAL fluid samples from each swine over the dosing interval) of two β-lactams, ceftolozane-tazobactam and piperacillin-tazobactam, with the profiles obtained from random selection of one or two BAL fluid samples from each swine.

RESULTS

Ceftolozane robust model. For ceftolozane, 29 plasma and 29 BAL fluid samples were collected from 7 swine (n = 4 to 5 plasma samples and n = 4 to 5 BAL fluid samples per pig). A two-compartment model fitted the data the best (Akaike's information criterion [AIC] [22] score, 376.12), with one compartment representing plasma and the second compartment representing ELF. The correlation between the observed and the individual predicted ceftolozane concentrations in plasma and ELF is provided in Fig. 1A and B. The final population pharmacokinetic parameter values from the analysis are displayed in Table 1.

Piperacillin robust model. For piperacillin, a total of 34 plasma and 33 BAL fluid samples were collected from 8 swine (n = 4 to 5 plasma samples and n = 4 to 5 BAL fluid samples per pig) for robust model development. A two-compartment model fitted the data the best (AIC score, 508.79), with one compartment representing plasma and the second compartment representing ELF. The correlation between the observed and the individual predicted piperacillin concentrations in plasma and ELF is provided in Fig. 1C and D. The final pharmacokinetic estimates for the population are presented in Table 1.

Random BAL selected models. For ceftolozane, the 1-BAL model (i.e., the model in which a value from a single time point for each pig was randomly selected for model

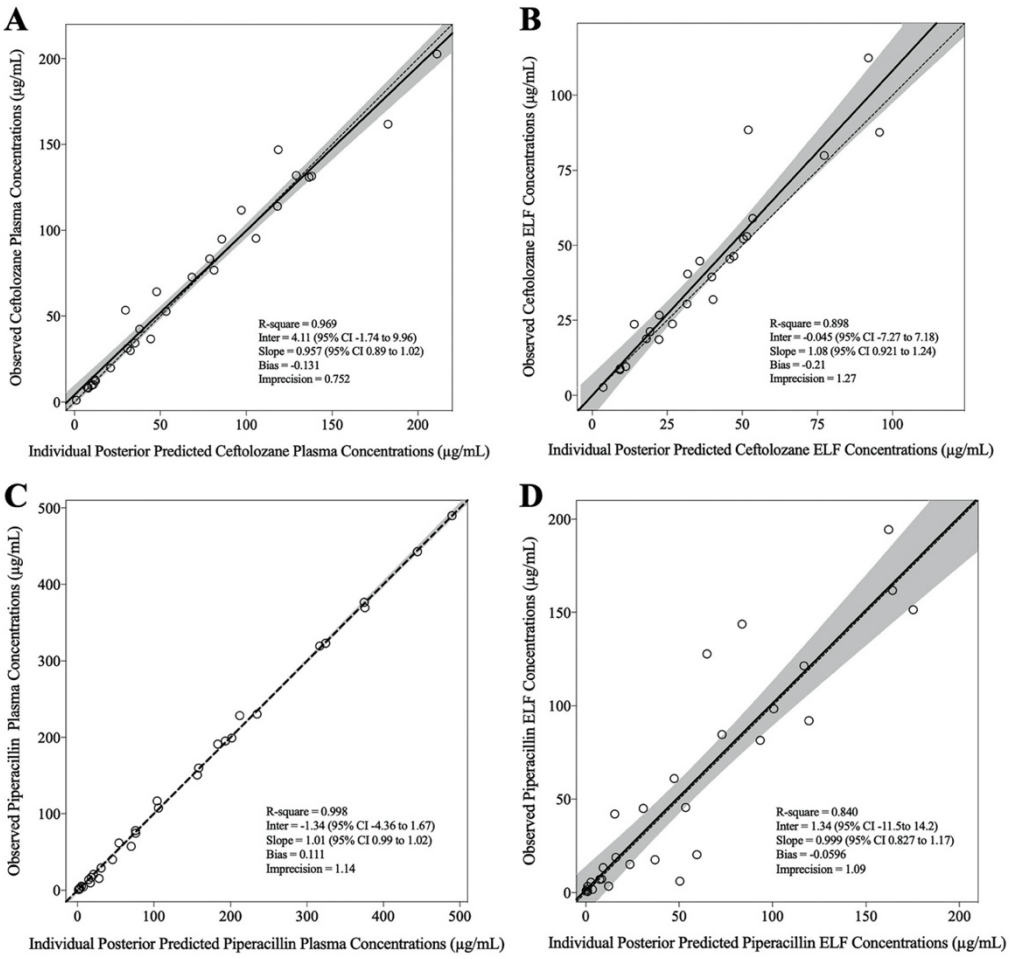

FIG 1 Observed versus maximum *a posteriori* Bayesian individual predicted probability determined using median population parameter estimates for ceftolozane concentrations in plasma (A) and ELF (B) and piperacillin concentrations in plasma (C) and ELF (D) for the robust final models. The solid lines are the regression lines, and the dashed lines are the lines of the unity. Gray bands are 95% confidence intervals (95% CI). Inter, intercept.

development) consisted of 29 plasma and 7 random ELF concentrations for model fitting. For piperacillin, the 1-BAL model included 34 plasma and 8 random ELF concentrations for model development. Similarly, 29 or 34 plasma concentrations and 14 or 16 random ELF concentrations (i.e., concentrations in ELF from 2 randomly selected time points per pig) of each drug, respectively, were used for the construction of the 2-BAL model. The profiles from the robust model were considered the real plasma and ELF profiles for each swine, while the profiels from the 1-BAL and 2-BAL models were considered experimental approximations.

The fit of the 1-BAL and 2-BAL models were acceptable and resulted in R^2 values similar to those from the robust models (Table 1; see also Fig. S1 and S2 in the supplemental material). The concentration profiles in plasma and ELF for each pig were

TABLE 1 Final population pharmacokinetic parameter estimates for different sampling approaches for 15 pigs infected with *P. aeruginosa* and treated with ceftolozane or piperacillin[a]

Drug and parameter	Value(s) for the following model:		
	Robust	1-BAL	2-BAL
Ceftolozane			
Plasma R^2	0.969	0.971	0.970
ELF R^2	0.898	0.992	0.992
CL_0 (liters/h)	4.4 ± 1.6 (4.3)	4.3 ± 1.4 (4.0)	4.3 ± 1.7 (4.0)
V_1 (liters)	8.9 ± 1.8 (9.6)	8.7 ± 1.9 (8.3)	8.9 ± 1.8 (9.5)
K_{12} (h^{-1})	0.2 ± 0.2 (0.2)	0.3 ± 0.3 (0.2)	0.22 ± 0.2 (0.2)
K_{21} (h^{-1})	0.6 ± 0.2 (0.4)	0.9 ± 0.8 (0.6)	0.5 ± 0.3 (0.5)
V_{ELF} (liters)	2.7 ± 1.5 (2.5)	3.8 ± 1.9 (3.9)	3.3 ± 1.6 (2.8)
Piperacillin			
Plasma R^2	0.998	0.990	0.999
ELF R^2	0.840	0.998	0.938
CL_0 (liters/h)	9.0 ± 2.8 (8.0)	8.7 ± 4.0 (8.1)	9.1 ± 2.8 (8.1)
V_1 (liters)	12.1 ± 4.2 (11.6)	12.4 ± 2.5 (11.6)	11.7 ± 4.4 (11.7)
K_{12} (h^{-1})	0.2 ± 0.2 (0.2)	0.2 ± 0.2 (0.2)	0.3 ± 0.3 (0.2)
K_{21} (h^{-1})	1.2 ± 0.7 (1.1)	0.4 ± 0.5 (0.2)	1.6 ± 0.7 (1.5)
V_{ELF} (liters)	10.2 ± 16.3 (2.4)	26.2 ± 29.5 (16.1)	11.9 ± 17.7 (3.1)

[a]Data for the final population pharmacokinetics parameter estimates are the mean ± SD (median) for different sampling approaches for 15 pigs infected with *P. aeruginosa* and treated with ceftolozane ($n = 7$) or piperacillin ($n = 8$). Plasma and ELF determination coefficients (R^2) between observed and individual predicted concentrations are also displayed for each model. CL_0, clearance; V_1, volume of distribution of the central compartment; K_{12}, transfer rate from the central compartment to the ELF compartment; K_{21}, transfer rate from the ELF compartment to the central compartment; V_1, volume of distribution for ELF.

resimulated using each model (the robust, 1-BAL, and 2-BAL models). No significant differences in the plasma area under the concentration curve (AUC) for free drug from 0 to 8 h ($fAUC_{0-8}$), the ELF AUC_{0-8}, and the time that the free drug concentration remained above the MIC target ($fT>MIC$) in both matrices for both antibiotics were observed between the models; however, there was visually greater variability in estimates of the ELF AUC_{0-8} and the $fT>MIC$ for ELF between the models for some pigs (Tables S1 and S2).

ELF penetration. The findings from the 5,000-subject Monte Carlo simulation based on the robust, 1-BAL, and 2-BAL models are displayed in Table 2. For the ceftolozane simulations, the median penetration into ELF was 94.3% 82.2%, and 97.0% for the robust, 1-BAL, and 2-BAL models, respectively. For the piperacillin simulations, the median penetration for the 5,000 simulated subjects was lower at 31.8%, 16.9%, and 26.9% for the robust, 1-BAL, and 2-BAL sampling approaches, respectively. The distributions for penetration were similar between sampling strategies (Fig. 2) and concordant with the estimated penetration ratio for pigs based on the robust ELF sampling profile for ceftolozane. The maximum vertical deviations (D statistic, in percent) between the robust and the 1-BAL or 2-BAL sampling approaches in the ceftolozane models were 10.2% and 9.0%, respectively. For the piperacillin models, the overlaid distributions demonstrated greater discordance; however, the maximum vertical deviations were still negligible: 17.1% for the 1-BAL sampling approach and 14.3% for the

TABLE 2 Estimation of plasma and ELF exposure and penetration of the ceftolozane and piperacillin components into ELF using Monte Carlo simulation of different BAL sampling strategies[a]

Parameter	Median (IQR) for the following drug and model:					
	Ceftolozane			Piperacillin		
	Robust	1-BAL	2-BAL	Robust	1-BAL	2-BAL
Plasma $fAUC_{0-8}$ (mg·h/liter)	377.2 (324.4–465.3)	365.0 (321.1–434.3)	381.2 (327.4– 464.0)	770.8 (593.3–906.6)	733.1 (517.4–870.4)	788.0 (620.2–937.5)
ELF AUC_{0-8} (mg·h/liter)	379.0 (217.6–493.1)	306.7 (228.7–477.7)	409.7 (238.1–514.6)	275.3 (55.0–578.2)	117.1 (31.8–444.4)	219.7 (57.7–401.3)
Penetration (%)	94.3 (61.8–134.0)	82.2 (59.7–136.2)	97.0 (62.3–143.7)	31.8 (8.8–66.6)	16.9 (5.2–48.6)	26.9 (9.1–43.6)

[a]*f*AUC, free area under the curve; penetration, $AUC_{ELF}/fAUC_{plasma}$; IQR, interquartile range (25th–75th percentiles).

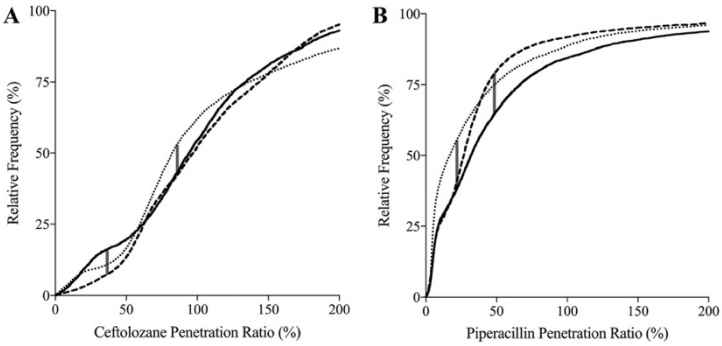

FIG 2 Cumulative distribution of the penetration ratio ($AUC_{ELF}/fAUC_{plasma}$) of ceftolozane (A) and piperacillin (B) from the Monte Carlo simulation results for different BAL sampling approaches. The black solid, dotted, and dashed lines are the penetration distributions of robust, 1-BAL, and 2-BAL models, respectively. The gray solid lines display the maximum vertical deviations.

2-BAL sampling approach in comparison with the profile obtained with robust ELF sampling. Comparable distributions of the plasma and ELF $fAUC_{0-8}$ were found between the different BAL sampling approaches (Fig.S3 and S4).

Probability of target attainment. The probability of target attainment (PTA) results for the different BAL sampling approaches are displayed in Fig. 3. For the ceftolozane simulations, a PTA of ≥90% was achieved for all the sampling strategies using an ELF *fT*>MIC of 60% at a MIC of ≤4 μg/ml, which is the susceptibility breakpoint. Specifically, PTA was 93.7, 92.9, and 95.3% for the robust, 1-BAL, and 2-BAL models at 4 μg/ml, respectively. Similarly, no differences were found between the three piperacillin models. A PTA of ≥90% was achieved, using a *fT*>MIC of 60%, at a MIC of ≤1 μg/ml. The PTA for piperacillin at the susceptibility breakpoint of 16 μg/ml was 55.8, 46.8, and 46.7% for the robust, 1-BAL, and 2-BAL models, respectively.

DISCUSSION

The pulmonary ELF penetration ratio of antimicrobial agents is essential to provide optimal dosing regimens for pneumonia (6) and for achieving a good clinical outcome. In order to describe a full population pharmacokinetic profile, samples are drawn from each study participant at various time points after antibiotic administration. However,

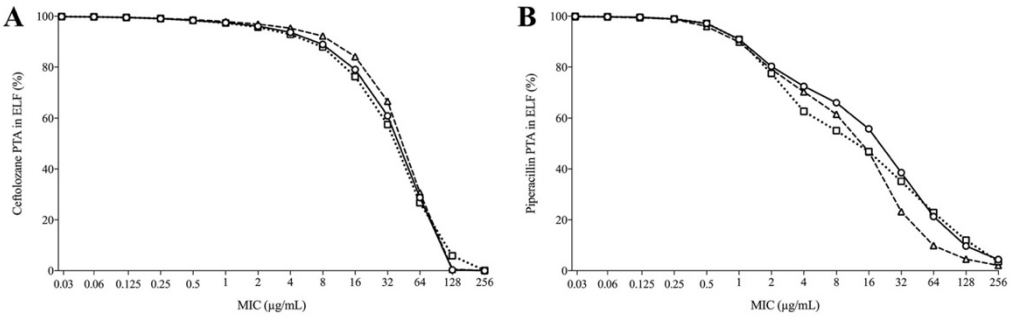

FIG 3 Probability that the ceftolozane (A) and piperacillin (B) components of ceftolozane-tazobactam (60-min infusion at 50 mg/kg q8h) and piperacillin-tazobactam (60-min infusion at 200 mg/kg q8h) will achieve an *fT*>MIC of 60% from the Monte Carlo simulation results for different BAL sampling approaches. Robust model: open circles, solid lines. 1-BAL model: open squares, dotted lines. 2-BAL model: open triangles, dashed lines.

performing multiple BALs on healthy volunteers or patients routinely after drug administration is uncommon due to ethical and logistical issues. Additionally, repeated BALs have been demonstrated to elevate morbidity in critically ill patients with pneumonia (23). To our knowledge, the effect of constructing a profile from a single BAL fluid sample per subject compared with that of constructing a profile from BAL fluid samples collected at multiple time points during a dosing interval has never been reported. In this study, we evaluated ceftolozane and piperacillin plasma and ELF concentrations from 15 swine with severe pneumonia which underwent bronchoscopy 4 to 5 times over 8 h following the first antibiotic dose. To determine the influence of different BAL sampling approaches, Monte Carlo simulations with data from a greater to a smaller number of BAL sampling time points (i.e., four or five compared with one and two BALs) were conducted.

Importantly, model development for both drugs resulted in similar plasma pharmacokinetic parameter estimates (clearance and volume of distribution for compartment 1) and AUC_{0-8} values (Table 1; see also Tables S1 and S2). However, the different sampling strategies did result in varying volumes of the ELF compartment, thereby producing nonsignificant but notable differences in AUC_{0-8} for ELF for each pig. Despite this, we found no remarkable differences in penetration ratios between the different sampling approaches when the 5,000-subject simulations were performed. As expected, the penetration distributions obtained with the 2-BAL model were closer to those obtained with the robust model than the distributions obtained when only one randomly selected BAL fluid sample was utilized (Fig. 2). When the penetration ratio was high (e.g., in the case of ceftolozane), the pharmacokinetic profiles were more similar between the models, while in the case of lower piperacillin penetration, some notable differences between the robust and 1-BAL approaches were found (Table 2).

The depositions into the ELF compartment resulted in similar *fT*>MIC values between the different sampling strategies for each pig regardless of the sampling approach (Table S1 and S2). Using an *fT*>MIC of 60% as the β-lactam pharmacodynamic driver to measure the drug exposure needed to optimize the killing of Gram-negative bacteria (24), PTA analyses were done over a range of MICs in doubling dilutions. Target attainments in ELF were quite similar across all MICs for both drugs (Fig. 3). The largest differences in PTA were observed for piperacillin at MICs of between 8 and 64 μg/ml. Notably, PTA was already quite low in these simulated pigs at these MICs, and these observations did not change the conclusions about the MIC threshold at which greater than 90% attainment was achieved (i.e., 1 μg/ml).

A wide variability of the deposition into ELF fluid was also observed for both drugs. This phenomenon is not surprising, as outliers are represented in the final Monte Carlo 5,000-subject simulation, and similar observations have been reported in several pharmacokinetic studies of β-lactams (11, 13, 14, 25). Simulated pharmacokinetic profiles diametrically characterize different physiological conditions across a patient population displaying an extensive range of different capabilities of drug distribution and elimination. In fact, dispersions in penetration ratios as large as 51% (interquartile range [IQR], 30%), 54% (IQR, 51%), and 25% (IQR, 61%) have been reported for ceftolozane (11), piperacillin (14), and meropenem (13) in critically ill patients, respectively. Lodise and colleagues (20) have already discussed the influence of the number of simulated and support patients on the piperacillin penetration ratios observed using different iterations in Monte Carlo simulations. In their letter, they demonstrated that when the number of simulated subjects increased, the penetration distribution flattened out.

Similar to our study, sparse and dense sampling approaches were compared for pharmacokinetic profiles constructed only on the basis of plasma data. Those studies found that increasing the sampling rate substantially reduced the measurement error (26, 27). Nevertheless, a complete *D*-optimal stochastic design for plasma sampling should guarantee that the variance result is minimum (28). Moreover, our current simulated ELF profiles are also supported by the profiles obtained with sparse plasma concentrations, which facilitates the characterization of the pharmacokinetic parame-

ters in the models. Indeed, an increase in sampling from one to two time points did not produce a large improvement in our analysis.

A number of limitations of this study should be noted. First, the swine VABP model is not as rigorous a test of drugs as the actual treatment of patients with ventilator-associated pneumonia but allowed the opportunity to explore the effects of sampling at multiple time points on predicted ELF exposures. Second, the drug disposition in ELF of this swine VABP model may be different from that observed in humans enrolled in studies. For example, the observed median ceftolozane ELF penetration in this model was much higher than that in healthy volunteers receiving ceftolozane at 2,000 mg every 8 h (q8h) (94% versus 51%, respectively) (11), as well as ventilated patients with suspected pneumonia (50% penetration) (L. Caro, D. P. Nicolau, J. J. De Waele, J. L. Kuti, K. B. Larson, E. Gadzicki, B. Yu, Z. Zeng, A. Adedayo, and E. G. Rhee, submitted for publication). In contrast, the ELF penetration of piperacillin in these pigs was lower than that reported in critically ill patients treated with piperacillin at 4,000 mg q8h (32% versus 54%, respectively) (14). We speculate that the differences may be attributed to the severity of illness of the swine versus that of the patients in the clinical studies. Although the patient studies for both drugs included critically ill subjects, the participants had to be clinically stable to undergo the BAL procedure, whereas the swine in the VABP model were severely ill, requiring pressor support throughout the performance of the model. Nevertheless, the MICs for PTA of ≥90% were 4 and 1 μg/ml for ceftolozane and piperacillin, respectively, using a threshold exposure of an $fT \geq$MIC of 50%, in the aforementioned studies. These values are was similar to the observations made here using a slightly stricter target of an $fT >$MIC of 60%. Second, only two β-lactams were included in this assessment, and our conclusions may not be applicable to other antibiotic classes, particularly for agents noted to have poor ELF penetration (e.g., tobramycin [29]) or for agents where the AUC/MIC best predicts pharmacodynamic activity (e.g., fluoroquinolones [30]). The human dosing regimen of piperacillin-tazobactam at 4,500 mg (4,000 mg piperacillin, 500 mg tazobactam) q8h as 60-min infusions selected for use in swine did not provide sufficient lung exposure in this swine VABP model to justify treating infections caused by pathogens with higher MICs, even in the susceptible range; a higher dose of 4,500 mg every 6 h (q6h) would increase the PTA results, presumably across all models, but should not change the relative penetration, which is not affected by dose. Finally, this study included only 7 to 8 pigs for each drug, which may limit the ability to extrapolate our results. However, even with models with small sample sizes, the results obtained with only one BAL fluid sample per subject remained analogous to those obtained with the robust model. During human clinical studies assessing pulmonary disposition and ELF penetration, ≥20 subjects are often enrolled; this is primarily to generate a composite curve for the concentration in ELF (~5 subjects per single BAL sampling time point, 4 to 5 time points during a dosing interval). Even with the sparse sampling population modeling methodologies used herein, we encourage the conduct of studies with larger subject numbers (i.e., $n \geq 20$) to best assess intersubject variability.

In conclusion, these data suggest that models of β-lactam concentrations in ELF constructed with data from sparse sampling time points, including a single BAL sampling time point, result in estimates of median penetration and pharmacodynamic exposure similar to those achieved with ELF concentration profiles created with a robust sampling strategy. These observations support current ELF sampling procedures in pharmacokinetic studies in humans.

MATERIALS AND METHODS

Swine pneumonia model. The swine model of severe *Pseudomonas aeruginosa* VABP previously described by Luna et al. (21) was used for this study. All animal experimentation was approved by the local Institutional Ethics Committee at the Hospital Clinic of Barcelona, Barcelona, Spain. Animals were managed according to National Research Council guidelines for the use and care of animals (31) at the Division of Animal Experimentation, Department of Pulmonary and Critical Care. Fifteen Large White-Landrace female pigs (weight, 32.2 ± 1.6 kg; Specipig, Barcelona, Spain) were induced, orotracheally intubated, and mechanically ventilated for 76 h. After surgical preparation and stabilization, the animals were challenged with a clinical isolate of *P. aeruginosa*. This strain was isolated from a patient with a

wound infection in 2013 and was selected based on its growth in the swine VABP model and its phenotypic profile; the strain was susceptible to ceftolozane-tazobactam (MIC, 4/4 μg/ml) but intermediate to piperacillin-tazobactam (MIC, 64/4 μg/ml) and nonsusceptible to aztreonam, cefepime, ceftazidime, imipenem, and meropenem.

Fifteen milliliters of 10^7 CFU/ml of log-phase *P. aeruginosa* culture was instilled into each pulmonary lobe using a fiber bronchoscope, as previously reported (21). Following pneumonia diagnosis at 24 h, the animals were randomized into two treatment groups: intravenous (i.v.) treatment with 50 mg/kg of body weight of ceftolozane-tazobactam q8h (n = 7) or i.v. treatment with 200 mg/kg of piperacillin-tazobactam q8h (n = 8). Both treatments were infused over 60 min. These dosing regimens were selected on the basis of the findings of preliminary studies (32) to provide plasma concentrations in the pig similar to those in the plasma of humans receiving ceftolozane-tazobactam at 3,000 mg (2,000 mg ceftolozane, 1,000 mg tazobactam) (11) and piperacillin-tazobactam at 4,500 mg (4,000 mg piperacillin, 500 mg tazobactam) q8h as 60-min infusions (33). Sedation and analgesia were maintained during the study as previously reported (34).

Blood and BAL sampling and storage. Arterial blood and BAL fluid samples were collected before the first antibiotic dose and at 1, 2, 4, 6, and 8 h after that dose in each swine. Blood samples were collected from a femoral artery catheter and placed into lithium heparin Vacutainer tubes (Becton, Dickinson, Franklin Lakes, NJ, USA), and the tubes were immediately centrifuged at 3,000 rpm for 10 min at 4°C. BALs were performed separately by instilling two 10-ml aliquots of 0.9% sodium chloride in the right middle lobe, and each aliquot was directly aspirated using a fiber optic bronchoscope (Pentax SAFE-3000; Ricoh Imaging Deutschland GmbH). The first retrieved BAL fluid aliquot was discarded to avoid bronchial fluid contamination. BAL fluid samples were collected into 15-ml tubes and centrifuged at 3,000 rpm for 10 min at 4°C. The resultant plasma and BAL fluid supernatant were separately transferred to polypropylene tubes and immediately stored frozen at −80°C until analysis. Frozen plasma and BAL fluid samples were shipped on dry ice to the Center for Anti-Infective Research and Development (CAIRD), Hartford Hospital (Hartford, CT, USA), for quantification of antibiotic concentrations.

Protein binding. Protein binding was assessed in duplicate at 1 and 2 h after the first dose. Plasma samples were transferred into ultrafiltration devices (Centrifree centrifugal filters; Millipore Corporation, Billerica, MA, USA) with a molecular mass cutoff of 30 kDa and centrifuged at 2,000 × g using a fixed-angle rotor for 45 min at 10°C to obtain the unbound drug. The unbound fraction was calculated as follows: percentage of free drug = ($C_{ultrafiltrate}/C_{plasma}$) · 100, where $C_{ultrafiltrate}$ is the unbound concentration in the ultrafiltrate and C_{plasma} is the total concentration in plasma.

Antibiotic concentration determination. Ceftolozane concentrations in plasma were determined by the high-performance liquid chromatography (HPLC) technique at CAIRD, as previously described (35). Ceftolozane BAL fluid concentrations were determined by an HPLC-tandem mass spectrometry (MS) method at Pure Honey Technologies (Billerica, MA, USA) (36). The standard curve for plasma ranged from 1 to 50 μg/ml, and the lower limit of detection was 0.9 μg/ml, whereas the range for the standard curve for BAL fluid was 0.02 to 0.5 μg/ml, and the lower limit detection was 0.02 μg/ml. The coefficients of correlation (R) were ≥0.998 and ≥0.999, respectively. For the ceftolozane plasma assay, the mean interday coefficients of variation for low (2 ng/ml) and high (40 ng/ml) quality control (QC) samples were 5.9% and 3.9%, respectively, whereas those for low (0.03 μg/ml), medium (0.09 μg/ml), and high (0.40 μg/ml) QC BAL fluid samples were 6.7%, 6.3%, and 2.2%, respectively. The mean intraday coefficients of variation for these five QC samples were 3.1%, 3.8%, 4.6%, 4.2%, and 5.8%, respectively.

Piperacillin plasma concentrations were determined by a validated high-performance liquid chromatography (HPLC) method at CAIRD (37). Piperacillin BAL concentrations were determined by a validated rapid-fire mass spectrometry method at Pure Honey Technologies. The standard curve for plasma ranged from 2 to 100 μg/ml, and the lower limit detection was 1.8 μg/ml, whereas the standard curve for BAL fluid ranged from 0.2 to 5 μg/ml, and the lower limit of detection was 0.02 μg/ml. The coefficients of correlation for plasma and BAL fluid were ≥0.996 and ≥0.997, respectively. For the piperacillin plasma assay, the mean interday coefficients of variation for low (6 μg/ml) and high (80 μg/ml) quality control (QC) samples were 4.5% and 2.8%, respectively, whereas for the low (0.3 μg/ml), medium (0.9 μg/ml), and high (3 μg/ml) QC BAL fluid samples they were 5.9%, 2.9%, and 3.5%, respectively. The mean intraday coefficients of variations for these five QC samples were 4.2%, 1.4%, 1.3%, 1.7%, and 1.4%, respectively.

Samples were analyzed for protein binding by each of ceftolozane and piperacillin following the methodology used for BAL fluid. The amount of bound drug was considered negligible for both ceftolozane and piperacillin. Therefore, the free AUC_{0-8} ($fAUC_{0-8}$) for plasma was equal to the total AUC_{0-8} for plasma for both drugs.

Urea correction. ELF concentrations (C_{ELF}) were determined, using the urea concentration as an endogenous marker, as follows: $C_{ELF} = C_{BAL} \cdot (urea_{plasma}/urea_{BAL})$, where C_{BAL} is the concentration of drug measured in BAL fluid, $urea_{plasma}$ is the concentration of urea in plasma, and $urea_{BAL}$ is the concentration of urea in BAL fluid (38). The urea concentrations in plasma and BAL fluid samples collected simultaneously at the time of bronchoscopy were analyzed by a validated enzymatic assay (Teco Diagnostics, Anaheim, CA) via the spectrophotometer detection method (Cary 50 series; Varian, Walnut Creek, CA) at CAIRD (39). The response from the calibration standards was linear from 0.1 mg/dl to 2 mg/dl, and the coefficient of correlation was at least ≥0.997. The intra-assay and interassay accuracies for the QC samples (0.15 and 1.5 mg/dl) were ≤6.52% and ≤1.4%, respectively, for intra-assay accuracy and ≤8.1%, and ≤5.8%, respectively, for interassay accuracy. The urea ratio average was used for each animal.

Population pharmacokinetic analyses. Between 5 and 6 plasma and ELF samples were collected from each pig. All available ceftolozane and piperacillin concentration data from plasma and ELF were

fitted to a 2-compartment model for each drug using the nonparametric adaptive grid (NPAG) algorithm in the Pmetrics package (version 1.5.0) for R (Laboratory of Applied Pharmacokinetics and Bioinformatics, Children's Hospital, Los Angeles, University of Southern California, Los Angeles, CA, USA) (40). A multiplicative error model was used for weighting the concentrations of both drugs. For ceftolozane in plasma and ELF, this was the inverse of the assay standard deviation (SD) multiplied by environmental noise, represented by gamma (γ), using the following equation: SD = $(0.5 + 0.15 \cdot C_{drug})$, where C_{drug} is the drug concentration. For plasma piperacillin data, the same equation was used, whereas ELF concentrations were weighted only for process noise (SD = 2). The best-fit model was discriminated based on the lowest AIC score (22), which estimates the relative information lost by each model relative to the information in each of the other models. The general differential equation for the models was as follows:

$$dX(1)/dt = R(t) - [(CL_0/V_1) + K_{12}] \cdot X(1) + K_{21} \cdot X(2) dX(2)/dt = K_{12} \cdot X(1) - K_{21} \cdot X(2)$$

where $R(t)$ is the input rate, t is time, CL_0 is the clearance from the central compartment, V_1 is the apparent volume of distribution of the plasma compartment, $X(1)$ is the drug amount in the plasma compartment, $X(2)$ is the drug amount in the ELF compartment, and K_{12} and K_{21} are the transfer rate constants from the central compartment to the ELF compartment and from the ELF compartment to the central compartment, respectively.

After final model development, each pig was randomized to a single BAL sampling time point (1, 2, 4, 6, or 8 h), as is done in human ELF studies, and the model was refit using all plasma concentration data and only the single ELF level from each pig (the 1-BAL model). Finally, each pig was randomized to two BAL sampling time points, and the model was refit again with the full plasma profile and two ELF concentrations per included pig (the 2-BAL model). The final models were based on visual inspection of the data and the AIC. Plasma and ELF concentrations over 8 h for each pig were resimulated using the three different models based on the respective dose.

Monte Carlo simulations. A 5,000-subject Monte Carlo simulation was conducted for each model developed using the Pmetrics Monte Carlo engine. Due to the small number of subjects used to develop the original models (i.e., $n = 7$ to 8 pigs per drug), a semiparametric method was utilized, where the nonparametric support points generated from Pmetrics served as the mean of one multivariate normal distribution in a multimodal, multivariate joint distribution. The weight of each multivariate distribution was equal to the probability of the point. The median dose of each antibiotic administered to the pigs was simulated for the population. Plasma and ELF concentrations were simulated every 6 min for up to 8 h for a single dose. The simulated profiles were analyzed in two ways. First, penetration into ELF was determined by calculating the AUC_{0-8} in each simulated compartment using the trapezoidal rule. The ELF penetration for each simulated pig was equal to the AUC for ELF to the AUC for free drug in plasma ($AUC_{ELF}/fAUC_{plasma}$). Second, the probability of target attainment (PTA) in ELF was calculated using the time that the free drug concentration remained above the MIC target ($fT>$MIC) as the pharmacodynamic index. An $fT>$MIC of at least 60% of the 8-h interval was applied as the target for both antibiotics, based on a conservative estimate of the exposure necessary to achieve clinical success in patients with serious Gram-negative bacterial infections (41, 42). PTA was calculated at increasing MICs in doubling dilutions of between 0.03 and 256 μg/ml. The MICs for a PTA of $\geq$90% were compared between different models as the PTA at MICs around the susceptibility breakpoints for ceftolozane-tazobactam (4/4 μg/ml) and piperacillin-tazobactam (16/4 μg/ml).

Statistical analyses. The mean, standard deviation, median, and 25th and 75th percentiles were reported for each Monte Carlo simulation. The interquartile range (IQR) difference was determined for variability analysis. The Kolmogorov-Smirnov (KS) test (43) was used to analyze cumulative distribution differences between the resulting Monte Carlo simulation populations. Maximum vertical deviations (D; in percent) between distributions were defined, with a D of $<$20% considered negligible. One-way analyses of variance were performed to compare the pharmacokinetic profiles between different BAL sampling approaches, after normality distributions were confirmed by the Shapiro-Wilks test. All statistical analyses were conducted in R (version 3.4.4; R Development Core Team, Vienna, Austria) and IBM SPSS Statistics for Mac (version 21.0; IBM Corporation, Armonk, NY, USA).

SUPPLEMENTAL MATERIAL

Supplemental material for this article may be found at https://doi.org/10.1128/AAC.01922-18.

SUPPLEMENTAL FILE 1, PDF file, 1.4 MB.

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A.T. and G.L.B. were the principal investigators for this research.

Merck was not involved in the design or conduct of the study; collection and management, analysis, and interpretation of the data; and preparation of the manu-

script. Merck reviewed the final manuscript. No other conflicts of interest exist for any of the authors.

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Supplemental Material Article 1

Version Date: November 20, 2018

ONLINE SUPPLEMENTAL MATERIAL

Is One Sample Enough? β-lactam Target Attainment and Penetration into Epithelial Lining Fluid based on Multiple Bronchoalveolar Lavage Sampling Time Points in a Swine Pneumonia Model

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Table S1. Individual ceftolozane pharmacokinetic and pharmacodynamics parameters in each swine for the Robust, 1-BAL, and 2-BAL sampling approaches

Subject	BAL sampling approach	Plasma $fAUC_{0\text{-}8h}$ (µg*h/mL)	ELF $AUC_{0\text{-}8h}$ (µg*h/mL)	Penetration ratio (%)	Plasma *f*T>MIC (%)	ELF T>MIC (%)
1	Robust	515.78	503.89	97.69	100	97.50
	1-BAL	514.54	432.77	84.11	100	97.50
	2- BAL	514.72	506.49	98.40	100	97.50
2	Robust	370.70	403.91	108.96	100	97.50
	1-BAL	371.29	241.75	65.11	100	98.75
	2- BAL	370.74	402.44	108.55	100	97.50
3	Robust	525.58	88.86	16.91	100	91.25
	1-BAL	524.48	46.95	8.95	100	58.75
	2- BAL	519.16	150.62	29.01	100	92.50
4	Robust	370.23	301.54	81.45	100	96.25
	1-BAL	370.20	526.18	142.13	100	97.50
	2- BAL	370.49	524.72	146.49	100	95.00
5	Robust	326.16	604.78	185.42	100	97.50
	1-BAL	326.56	894.99	274.06	100	97.50
	2- BAL	328.92	561.56	170.73	100	97.50
6	Robust	346.57	234.36	67.62	100	96.25
	1-BAL	344.21	254.42	73.91	100	97.50
	2- BAL	344.75	208.70	60.54	100	96.25
7	Robust	198.06	190.52	96.19	68.75	96.25
	1-BAL	198.88	264.60	133.05	67.50	93.25
	2- BAL	198.08	244.99	123.69	68.75	96.25
Mean ± SD	Robust	379.0 ± 113.8	332.6 ± 182.0	93.5 ± 50.7	95.5 ± 11.8	96.1 ± 2.2
	1-BAL	378.6 ± 112.6	380.2 ± 273.4	111.6 ± 84.3	95.4 ± 12.3	91.5 ± 14.6
	2- BAL	378.1 ± 111.5	371.4 ± 168.4	105.3 ± 48.6	95.5 ± 11.8	96.1 ± 1.8
p-value		>0.99	0.90	0.86	>0.99	0.53

*f*AUC, free area under the curve; Penetration ratio, AUC_{ELF}/ $fAUC_{plasma}$; *f*T>MIC, free time above MIC, calculated for inoculated *P. aeruginosa* isolate (ceftolozane MIC 4 µg/mL).

Table S2. Individual piperacillin pharmacokinetic and pharmacodynamics parameters in each swine for the Robust, 1-BAL, and 2-BAL sampling approaches.

Subject	BAL sampling approach	Plasma $fAUC_{0\text{-}8h}$ (µg*h/mL)	ELF $AUC_{0\text{-}8h}$ (µg*h/mL)	Penetration ratio (%)	Plasma $fT>MIC$ (%)	ELF T>MIC (%)
8	Robust	418.19	106.52	25.47	31.25	0
	1-BAL	379.45	14.81	3.90	27.50	0
	2- BAL	418.90	35.79	8.56	31.25	0
9	Robust	510.69	25.37	4.97	30.00	0
	1-BAL	505.46	27.15	5.37	31.25	0
	2- BAL	510.56	23.48	4.60	30.00	0
10	Robust	1009.07	392.17	38.86	55.00	32.50
	1-BAL	877.37	632.86	72.13	46.25	78.75
	2- BAL	1014.02	366.36	36.13	55.00	28.75
11	Robust	713.39	544.14	76.28	42.50	57.50
	1-BAL	709.47	27.31	3.85	46.25	0
	2- BAL	719.52	216.28	30.06	46.25	0
12	Robust	729.28	735.36	100.83	37.50	55.00
	1-BAL	696.42	340.60	48.91	37.50	0
	2- BAL	721.55	353.63	49.01	37.50	27.50
13	Robust	871.11	640.82	73.56	51.25	46.25
	1-BAL	853.02	519.24	60.87	46.25	60.00
	2- BAL	870.72	648.51	74.48	51.25	47.50
14	Robust	1031.87	1128.98	109.41	53.75	88.75
	1-BAL	1018.35	1291.74	126.85	51.25	87.50
	2- BAL	1032.07	1261.38	122.22	53.75	83.75
15	Robust	746.35	227.73	30.51	42.50	17.50
	1-BAL	730.94	176.84	24.19	40.00	0
	2- BAL	743.70	72.33	9.73	42.50	0
Mean ± SD	Robust	753.7 ± 217.2	475.1 ± 365.2	57.5 ± 37.9	43.0 ± 9.8	37.2 ± 30.8
	1-BAL	721.3 ± 205.0	378.8 ± 437.5	43.3 ± 43.2	40.9 ± 8.3	28.3 ± 39.8
	2- BAL	753.9± 217.9	372.2 ± 416.9	41.9 ± 40.3	43.4 ± 9.8	23.4 ± 20.4
p- value		0.94	0.85	0.70	0.83	0.72

*f*AUC, free area under the curve; Penetration ratio, AUC_{ELF}/ $fAUC_{plasma}$; *f*T>MIC, free time above MIC, calculated for *P. aeruginosa* inoculated isolates (piperacillin MIC 64 µg/mL).

FIGURE LEGENDS:

Fig S1. Observed versus maximum a posteriori probability Bayesian individual-predicted using median population parameter estimates ceftolozane plasma (A) and ELF (B) and piperacillin plasma (C) and ELF (D) concentrations for the 1-BAL models. The solid lines are the regression lines and the dashed lines are the lines of the unity. Grey bands are 95% confidence intervals (95% CI).

Fig S2. Observed versus maximum a posteriori probability Bayesian individual-predicted using median population parameter estimates ceftolozane plasma (A) and ELF (B) and piperacillin plasma (C) and ELF (D) concentrations for the 2-BAL models. The solid lines are the regression lines and the dashed lines are the lines of the unity. Grey bands are 95% confidence intervals (95% CI).

Fig S3. Relative frequency distributions (%) of ceftolozane plasma $fAUC_{0\text{-}8h}$ (A) and ELF $AUC_{0\text{-}8h}$ (B) and piperacillin plasma $fAUC_{0\text{-}8h}$ (C) and ELF $AUC_{0\text{-}8h}$ (D) of Monte Carlo simulation results for each BAL sampling approach model.

Fig S4. Paired unbound plasma and ELF $fAUC_{0\text{-}8h}$ for ceftolozane (top) and piperacillin (bottom) for each sampling approach. Black dots are simulated pigs and small grey dots represents 5,000 simulated subjects.

Fig S1. Observed versus maximum a posteriori probability Bayesian individual-predicted using median population parameter estimates ceftolozane plasma (A) and ELF (B) and piperacillin plasma (C) and ELF (D) concentrations for the 1-BAL models. The solid lines are the regression lines and the dashed lines are the lines of the unity. Grey bands are 95% confidence intervals (95% CI).

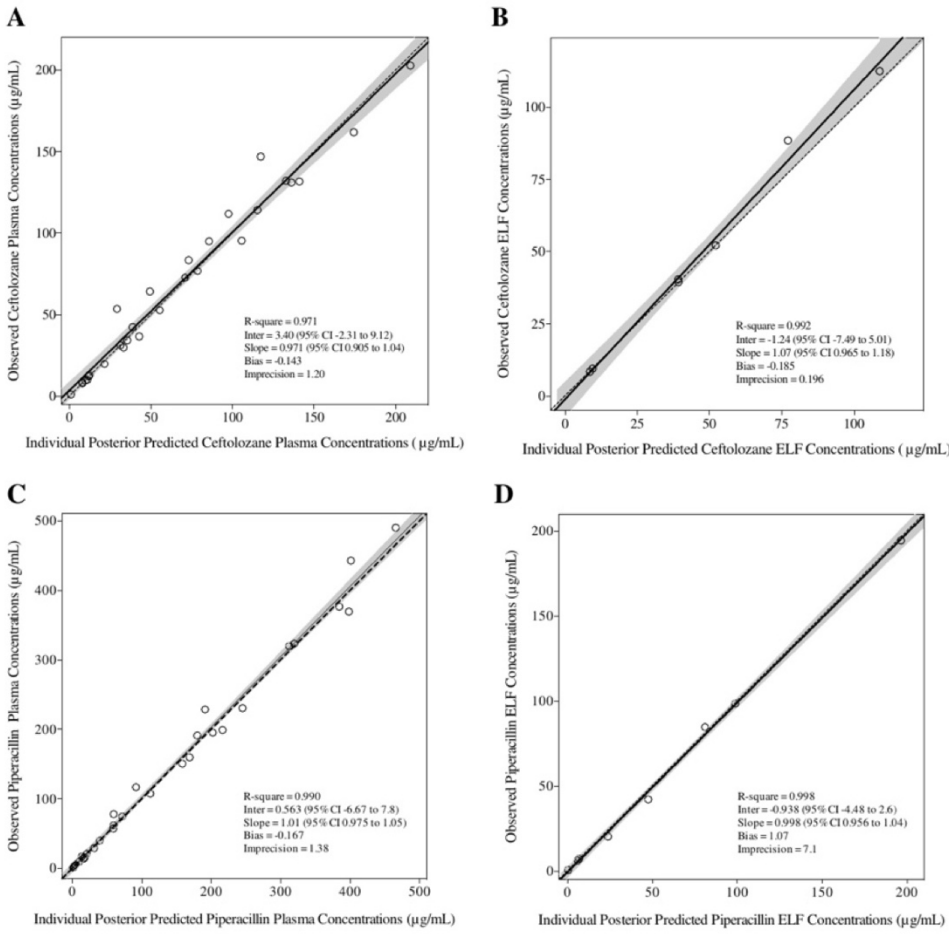


Fig S2. Observed versus maximum a posteriori probability Bayesian individual-predicted using median population parameter estimates ceftolozane plasma (A) and ELF (B) and piperacillin plasma (C) and ELF (D) concentrations for the 2-BAL models. The solid lines are the regression lines and the dashed lines are the lines of the unity. Grey bands are 95% confidence intervals (95% CI).

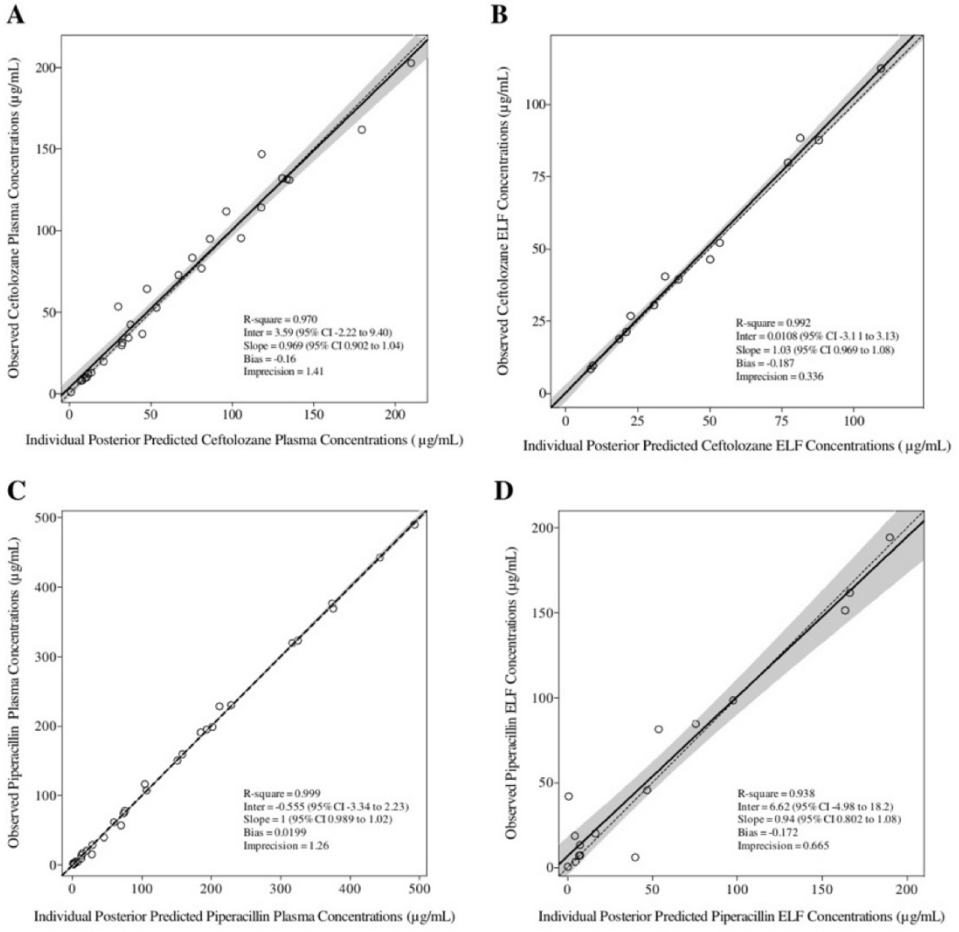


Fig S3. Relative frequency distributions (%) of ceftolozane plasma *f*$AUC_{0\text{-}8h}$ (A) and ELF $AUC_{0\text{-}8h}$ (B) and piperacillin plasma *f*$AUC_{0\text{-}8h}$ (C) and ELF $AUC_{0\text{-}8h}$ (D) of Monte Carlo simulation results for each BAL sampling approach model.

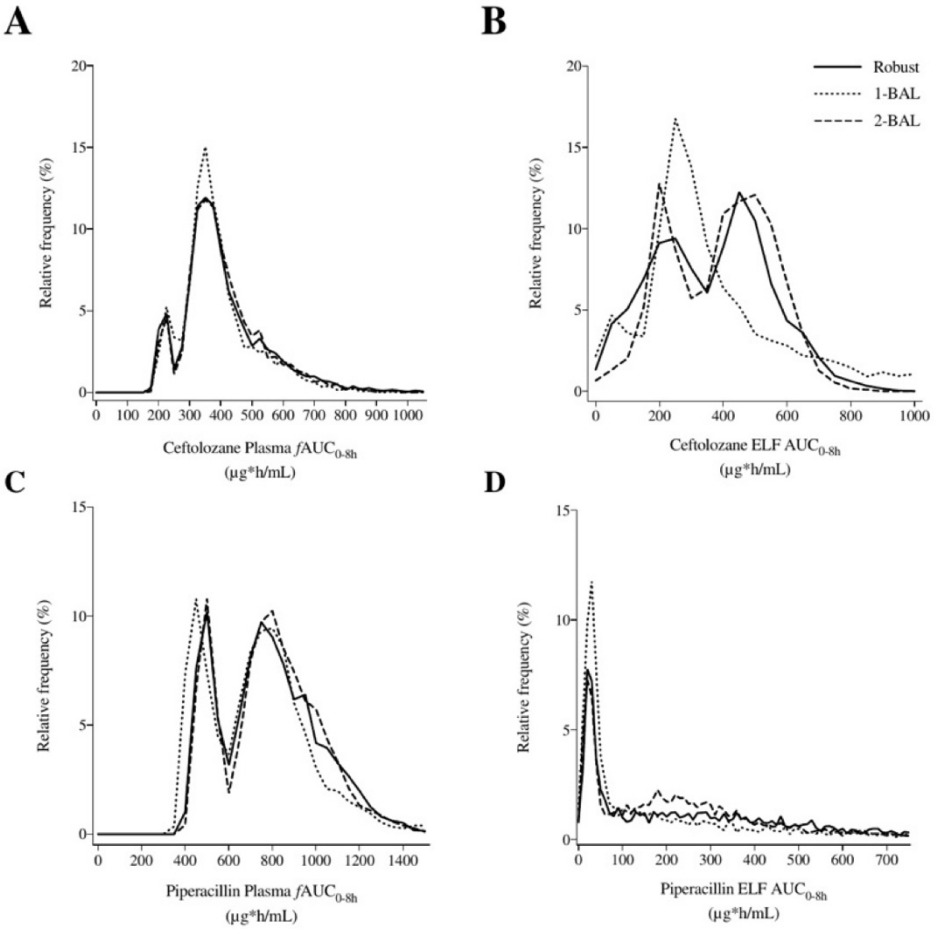
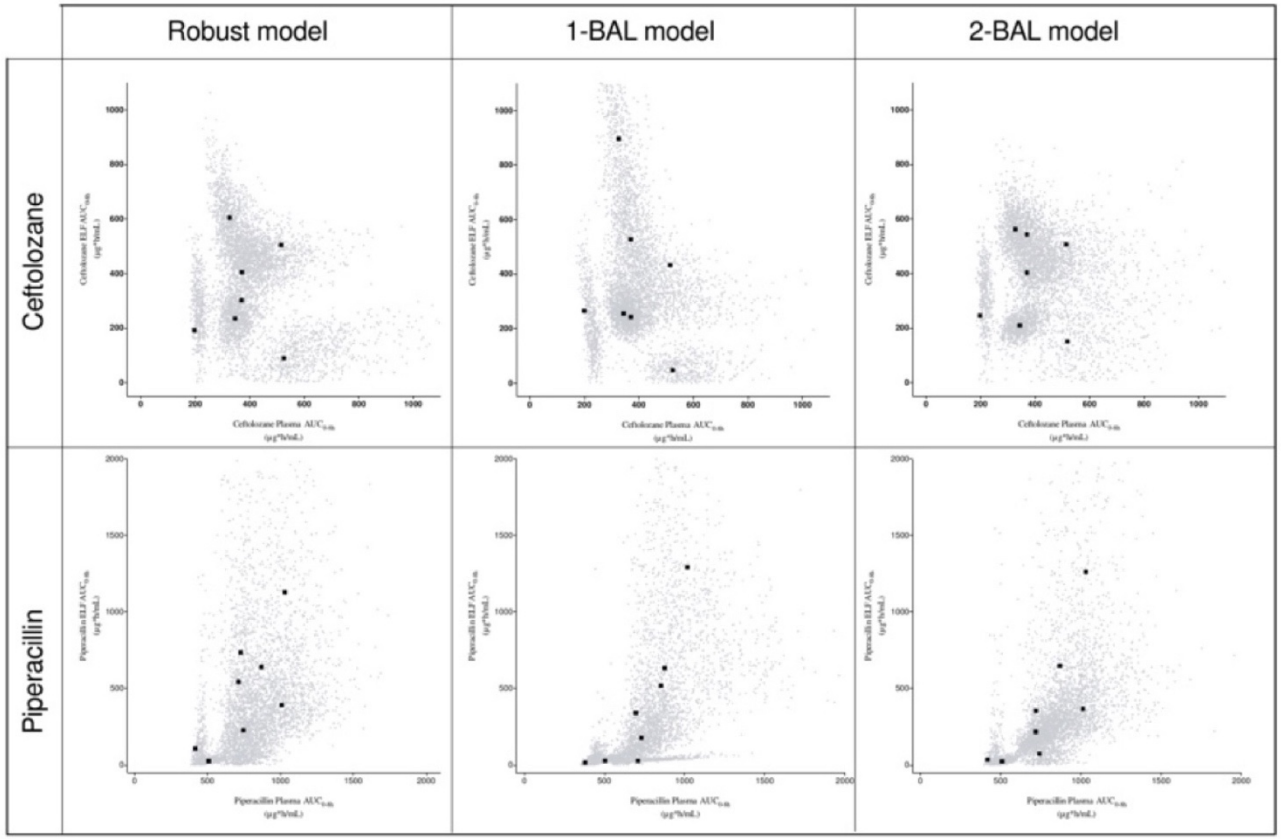


Fig S4. Paired unbound plasma and ELF $fAUC_{0\text{-}8h}$ for ceftolozane (top) and piperacillin (bottom) for each sampling approach. Black dots are simulated pigs and small grey dots represents 5,000 simulated subjects.



ARTICLE 2

AMERICAN SOCIETY FOR MICROBIOLOGY | Antimicrobial Agents and Chemotherapy®

EXPERIMENTAL THERAPEUTICS

Short-Term Effects of Appropriate Empirical Antimicrobial Treatment with Ceftolozane/Tazobactam in a Swine Model of Nosocomial Pneumonia

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ABSTRACT The rising frequency of multidrug-resistant and extensively drug-resistant (MDR/XDR) pathogens is making more frequent the inappropriate empirical antimicrobial therapy (IEAT) in nosocomial pneumonia, which is associated with increased mortality. We aim to determine the short-term benefits of appropriate empirical antimicrobial treatment (AEAT) with ceftolozane/tazobactam (C/T) compared with IEAT with piperacillin/tazobactam (TZP) in MDR *Pseudomonas aeruginosa* pneumonia. Twenty-one pigs with pneumonia caused by an XDR *P. aeruginosa* strain (susceptible to C/T but resistant to TZP) were ventilated for up to 72 h. Twenty-four hours after bacterial challenge, animals were randomized to receive 2-day treatment with either intravenous saline (untreated) or 25 to 50 mg of C/T per kg body weight (AEAT) or 200 to 225 mg of TZP per kg (IEAT) every 8 h. The primary outcome was the *P. aeruginosa* burden in lung tissue and the histopathology injury. *P. aeruginosa* burden in tracheal secretions and bronchoalveolar lavage (BAL) fluid, the development of antibiotic resistance, and inflammatory markers were secondary outcomes. Overall, *P. aeruginosa* lung burden was 5.30 (range, 4.00 to 6.30), 4.04 (3.64 to 4.51), and 4.04 (3.05 to 4.88) $\log_{10}$CFU/g in the untreated, AEAT, and IEAT groups, respectively ($P = 0.299$),

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without histopathological differences ($P = 0.556$). In contrast, in tracheal secretions ($P < 0.001$) and BAL fluid ($P = 0.002$), bactericidal efficacy was higher in the AEAT group. An increased MIC to TZP was found in 3 animals, while resistance to C/T did not develop. Interleukin-1β (IL-1β) was significantly downregulated by AEAT in comparison to other groups ($P = 0.031$). In a mechanically ventilated swine model of XDR *P. aeruginosa* pneumonia, appropriate initial treatment with C/T decreased respiratory secretions' bacterial burden, prevented development of resistance, achieved the pharmacodynamic target, and may have reduced systemic inflammation. However, after only 2 days of treatment, *P. aeruginosa* tissue concentrations were moderately affected.

KEYWORDS *Pseudomonas aeruginosa*, animal models, appropriate empirical antimicrobial treatment, mechanical ventilation, pneumonia

Nosocomial pneumonia is one of the most common hospital-acquired infections, associated with substantial morbidity and attributable mortality higher than 10% (1–3). *Pseudomonas aeruginosa* is one of the most common causative pathogens, causing life-threatening conditions (4). The latest guidelines strongly recommend appropriate empirical treatment based on local etiology and the presence of risk factors for multidrug-resistant and extensively drug-resistant (MDR/XDR) organisms (2, 5). In patients with suspected nosocomial pneumonia, recommended empirical therapy includes coverage for *P. aeruginosa* with an antipseudomonal β-lactam and/or a fluroquinolone (2). Nevertheless, due to increasing resistance to fluroquinolones and traditional β-lactams, appropriate empirical therapy is increasingly difficult. Specifically, inappropriate empirical antimicrobial therapy (IEAT) indicates the empirical antimicrobial regimen administered during the first 48 to 72 h after suspecting nosocomial pneumonia that was not active against the identified pathogen. The rate of IEAT for the treatment of nosocomial pneumonia is up to 60% (6), and it is associated with increased mortality and length of stay (7). Furthermore, achieving adequate antimicrobial pulmonary concentrations is challenging (8), due to high MICs and pharmacokinetic variations among patients with acute illnesses (9, 10).

In this scenario, ceftolozane/tazobactam (C/T) is a novel β-lactam/β-lactamase inhibitor combination antimicrobial agent which has been approved for the treatment of complicated urinary tract and intraabdominal infections in adults (11, 12) and was recently approved by the American Food and Drug Administration for the treatment of nosocomial pneumonia (12). Ceftolozane is a fifth-generation cephalosporin that is active against *P. aeruginosa* and has a notable stability against pseudomonal AmpC-mediated resistance (13, 14), while tazobactam extends efficacy against many extended-spectrum β-lactamase-producing *Enterobacteriaceae* (15). Preliminary *in vitro* studies have shown activity against up to 85% of *P. aeruginosa* isolates that are nonsusceptible to ceftazidime, meropenem and piperacillin/tazobactam (16). The drug primarily distributes into the extracellular fluid with good lung penetration (17, 18). While the approved dose for other infections is 1 g, with 0.5 g tazobactam, every 8 h (12), a larger dose of up to 3 g (1 g tazobactam) every 8 h has been approved for nosocomial pneumonia in order to achieve >90% probability of target attainment against pathogens with a MIC up to 8 mg/liter (19). A recently concluded large multicenter, randomized, controlled phase III (ASPECT-NP) trial in ventilated patients with nosocomial pneumonia compared the antibacterial efficacy of C/T and meropenem. C/T was noninferior to meropenem in treating pneumonia (weighted treatment difference (1.1%; [95% confidence interval (CI) −6.2 to 8.3]) (20). Although a novel antimicrobial with a higher susceptibility rate, such as C/T, may improve clinical outcome, further preclinical and clinical evaluations are essential to outline the role in empirical antimicrobial therapy for nosocomial pneumonia in comparison to other first-line antipseudomonal antibiotics.

Therefore, herein, we present a prospective randomized study in a validated animal model of severe *P. aeruginosa* pneumonia to study the short-term benefits of appro-

priate empirical antimicrobial treatment (AEAT) with C/T in comparison with IEAT with piperacillin/tazobactam (TZP), a β-lactam/β-lactamase inhibitor commonly used for suspected nosocomial pneumonia (2, 5). The primary aim of the study was to investigate bactericidal activity and lung histopathological severity during the first 48 h of treatment (i.e., traditional methods take at least 48 h to provide a final results) and to develop further insights into the benefits after a short period of AEAT to life-threatening pulmonary infections.

RESULTS

Preliminary study. As shown in Fig. S1 in the supplemental material, clinical, microbiological, and histological findings confirmed severe pneumonia in animals included in preliminary analyses. We initially assessed C/T concentrations of 30/15 and 60/30 mg/kg, and TZP of 100/12.5 mg/kg and 200/25 mg/kg, as 1-h infusion every 8 h (q8h), in healthy animals (Table S1). Following dose adjustment, confirmatory pharmacokinetic studies in infected animals showed that 60 mg/kg of ceftolozane achieved epithelial lining fluid (ELF) area under the concentration-time curve from 0 to 8 h (AUC_{0-8h}) slightly higher than 200 mg/h/liter, while 200 mg/kg of piperacillin achieved 100 to 140 mg/h/liter (Table S2). Therefore, doses of 50 mg/kg of ceftolozane and 200 mg/kg of piperacillin were selected to provide an ELF exposure similar to that achieved in humans following a dose of C/T of 3 g and TZP of 4.5 g every 8 h.

Main study. Out of 23 animals, 21 completed the study. Two animals were euthanized shortly after the first administration of antibiotics, for severe respiratory and hemodynamic instability, and were not included in the analysis.

Primary outcome. A total of 105 pulmonary lobes were analyzed. Qualitative and quantitative lung culture results are summarized in Fig. 1. After 48 h of treatment, the median (interquartile range [IQR]) *P. aeruginosa* tissue concentrations were 4.04 (range, 3.64 to 4.51; AEAT animals), 4.04 (range, 3.05 to 4.88; IEAT animals), and 5.30 (range, 4.00 to 6.30; untreated animals) $\log_{10}$ CFU per ml ($P = 0.299$) (Fig. 1A). Notably, animals with appropriate empirical C/T therapy presented the highest number of uncolonized lobes (20%), while the percentage of lung tissue samples with positive cultures for *P. aeruginosa* in the untreated and IEAT groups was 97.14% and 88.57%, respectively ($P = 0.033$) (Fig. 1B). Figure 1 also shows the results of histopathological analysis of the 105 lung tissue samples evaluated. No significant differences were found between histological features among therapeutic groups ($P = 0.556$). The composite histological and bacterial burden score was 6.71 (range, 5.00 to 8.36], 5.86 (range, 5.36 − 6.86), and 5.14 (4.29 to 6.57) in the untreated, appropriate, and inappropriate groups, respectively ($P = 0.460$). Lung appearance and lung/body weight ratio are reported in Fig. S2.

Secondary outcomes of microbiology assessments. Figure 2 depicts tracheal secretions and bronchoalveolar lavage (BAL) fluid *P. aeruginosa* burden throughout the study. *P. aeruginosa* colonization within tracheal secretions differed among study groups ($P < 0.001$). Specifically, appropriate empirical treatment with C/T caused a significant reduction in *P. aeruginosa* concentrations in tracheal secretions in comparison to untreated ($P < 0.001$) and TZP-treated animals ($P = 0.048$) at 48 h and at the end of the study ($P < 0.001$). IEAT with TZP had a marginal effect versus control animals after 48 h of treatment ($P = 0.002$). *P. aeruginosa* concentration in BAL fluids varied among study groups ($P = 0.002$). Indeed, AEAT with C/T yielded improved antipseudomonal effects in BAL fluid in comparison to those in the untreated ($P = 0.004$) and IEAT groups ($P = 0.018$), while no differences were found between untreated and inappropriately TZP-treated animals throughout the experiment. *P. aeruginosa* bacteremia was detected in only one, untreated animal.

Importantly, *P. aeruginosa* augmented its resistance to TZP following 48 h of treatment; in particular, a 4-fold increase in the TZP MIC was found in *P. aeruginosa* isolates from 3 animals (42.9%) (Fig. 2C). Conversely, *P. aeruginosa* isolates under appropriate initial therapy with C/T did not yield any increase in *P. aeruginosa* resistance ($P = 0.030$).

Secondary outcomes of inflammatory markers. The development of pneumonia substantially affected systemic and pulmonary cytokines. Initial *P. aeruginosa* challenge

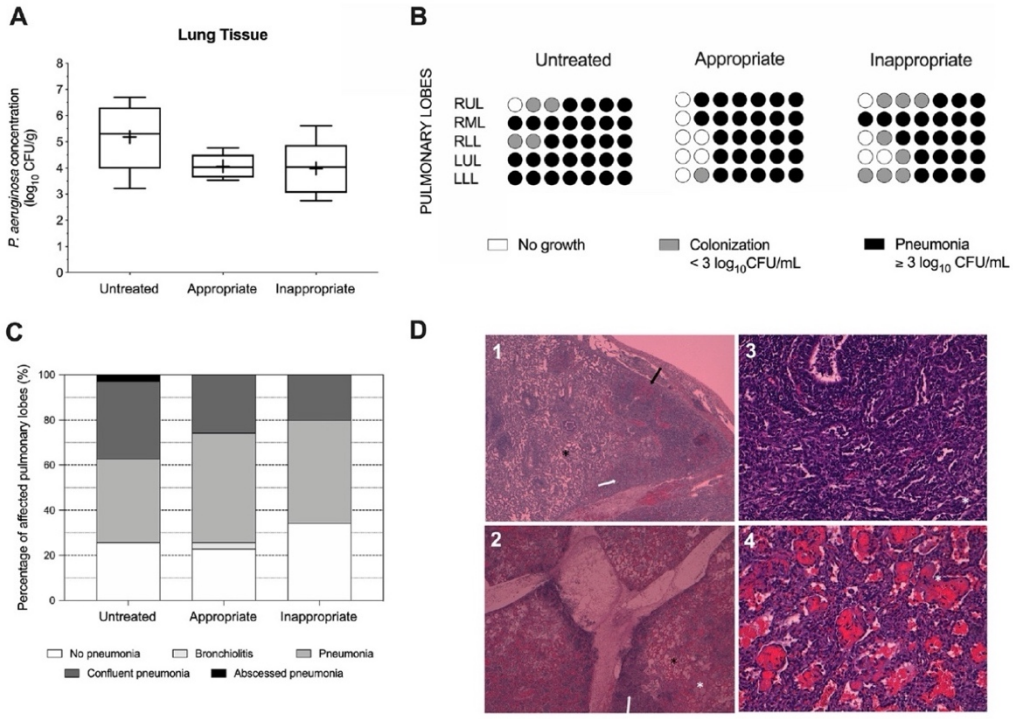

FIG 1 Pulmonary burden and severity of histopathological findings among treatment groups. (A) Box plots showing the *P. aeruginosa* concentration in lung tissue among study groups. There was no statistically significant difference in bacterial burden between study groups ($P = 0.299$). Horizontal bars represent the median, boxes represent the interquartile range, whiskers represent the range, and the plus sign denotes the mean. (B) Semiquantitative microbiological assessment of lung tissue among study groups. Each dot represents the degree of *P. aeruginosa* colonization in each lobe, defined as no growth, *P. aeruginosa* colonization < 3 $\log_{10}$ CFU/g, and pneumonia with histological confirmation and *P. aeruginosa* concentration ≥ 3 $\log_{10}$ CFU/g. Of note, significant differences were found between study groups (21 pigs; 105 lobes; $P = 0.033$). In particular, the percentage of colonization in the AEAT group was significantly lower than that of the untreated ($P = 0.028$) and IEAT groups ($P = 0.045$). In contrast, no differences in colonization proportions were found between study groups ($P = 0.194$). No lobe correlation was found. (C) Results are displayed as the percentage of scores of the five lobes per animal. No differences were found between study groups (21 pigs; 105 lobes; $P = 0.556$). (D) Three specific histopathological patterns were found only in untreated and IEAT groups as follows: the histopathology pattern characterized by pathogens and inflammatory cells within the alveolar space (D1 and D2), organizing pneumonia (D3), and alveolar diffuse damage (D4). (D1) An inflammatory infiltrate composed of polymorphonuclear leukocytes is observed, located adjacent to the interlobular septa (white arrow), preserving the centrilobular zone (asterisk). The affected areas showed an effacement of the alveolar architecture, with hemorrhagic foci (black arrow) (×4 magnification). (D2) The edematous interlobular septum separates four congestive lobules. In the lower two, an inflammatory infiltrate composed of polymorphonuclear leukocytes is observed, which tends to be located adjacent to the interlobular septa (white arrow). The centrilobular zone shows a milder acute inflammatory infiltrate that occupies the alveolar spaces, preserving the alveolar septa (black asterisk). Areas of alveolar edema can be seen (white asterisk) (×10 magnification). (D3) Dense interstitial proliferation of fibroblastic appearance that caused a decrease of the alveolar lumina, which appeared to be occupied by polymorphonuclear leukocytes and histiocytes. The foci of interalveolar fibroblast buds are spotted (white asterisk) (×20 magnification). (D4) The presence of fibrinoid material intermingled with blood (white arrow) suggested an initial stage of organization of alveolar hemorrhage (×20 magnification). AEAT, appropriate empirical antimicrobial therapy; IEAT, inappropriate empirical antimicrobial therapy; RUL, right upper lobe; RML, right medium lobe; RLL, right lower lobe; LUL, left upper lobe; LLL left lower lobe.

resulted in a significant increase in all assessed serum cytokines, except interleukin-8 (IL-8), while in BAL fluid, IL-1β and IL-8 were the only upregulated cytokines (Fig. S3). Antibiotic treatments decreased IL-1β and IL-6 (Fig. 3A and B). In particular, serum IL-1β was significantly downregulated by appropriate C/T therapy ($P = 0.031$), returning to baseline levels after 48 h of treatment, compared to untreated ($P = 0.081$) and IEAT animals ($P = 0.049$). Likewise, serum IL-6 was upregulated upon pneumonia diagnosis and showed a downward trend throughout the treatment period ($P < 0.001$) but without showing significant differences between groups.

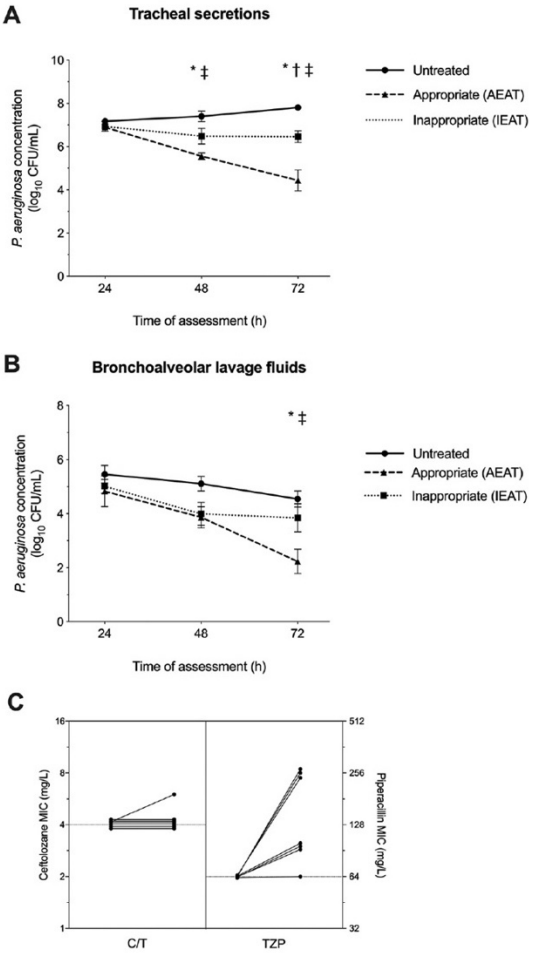

FIG 2 Tracheal secretions and bronchoalveolar lavage fluid *P. aeruginosa* burden and resistance development after antimicrobial exposure. *P. aeruginosa* concentrations ($\log_{10}$ CFU/ml) are plotted as line graphs, reporting means and standard errors of the means (SEM). (A) Tracheal secretions. *P. aeruginosa* concentrations differed among study groups ($P < 0.001$) and throughout the experiment ($P < 0.001$). *Post hoc* comparisons showed a significant reduction compared to controls at 48 h ($P < 0.001$) and at the end of the study ($P < 0.001$). The double dagger shows a significant reduction of *P. aeruginosa* burden in AEAT with C/T versus IEAT with TZP at 48 h ($P = 0.048$) and 72 h ($P < 0.001$). (B) Equally, *P. aeruginosa* concentrations in BAL fluids varied among treatment groups and times of assessments ($P = 0.002$). Essentially, the *P. aeruginosa* concentration was significantly decreased with AEAT compared to the untreated ($P = 0.0004$) and IEAT ($P = 0.018$) groups at 72 h. Before treatment started, all depicted means were not statistically different in both matrixes. Of note, the statistical significance of AEAT and IEAT groups against the untreated group is shown by an asterisk and a dagger, respectively. Differences between AEAT and IEAT are displayed by the double dagger. (C) Changes in ceftolozane MIC (left) and piperacillin MIC (right) are shown in this aligned dot before-and-after graph. Each dot represents the MIC of *P. aeruginosa* isolates at pneumonia diagnosis and after treatment for each subject in each study group. A significant effect of piperacillin exposure was observed in isolates from the IEAT group compared with those from the AEAT group. The dashed line displays the ceftolozane and piperacillin MIC of the inoculated strain. AEAT, appropriate empirical antimicrobial therapy; IEAT, inappropriate empirical antimicrobial therapy; C/T, ceftolozane/tazobactam; TZP, piperacillin/tazobactam.

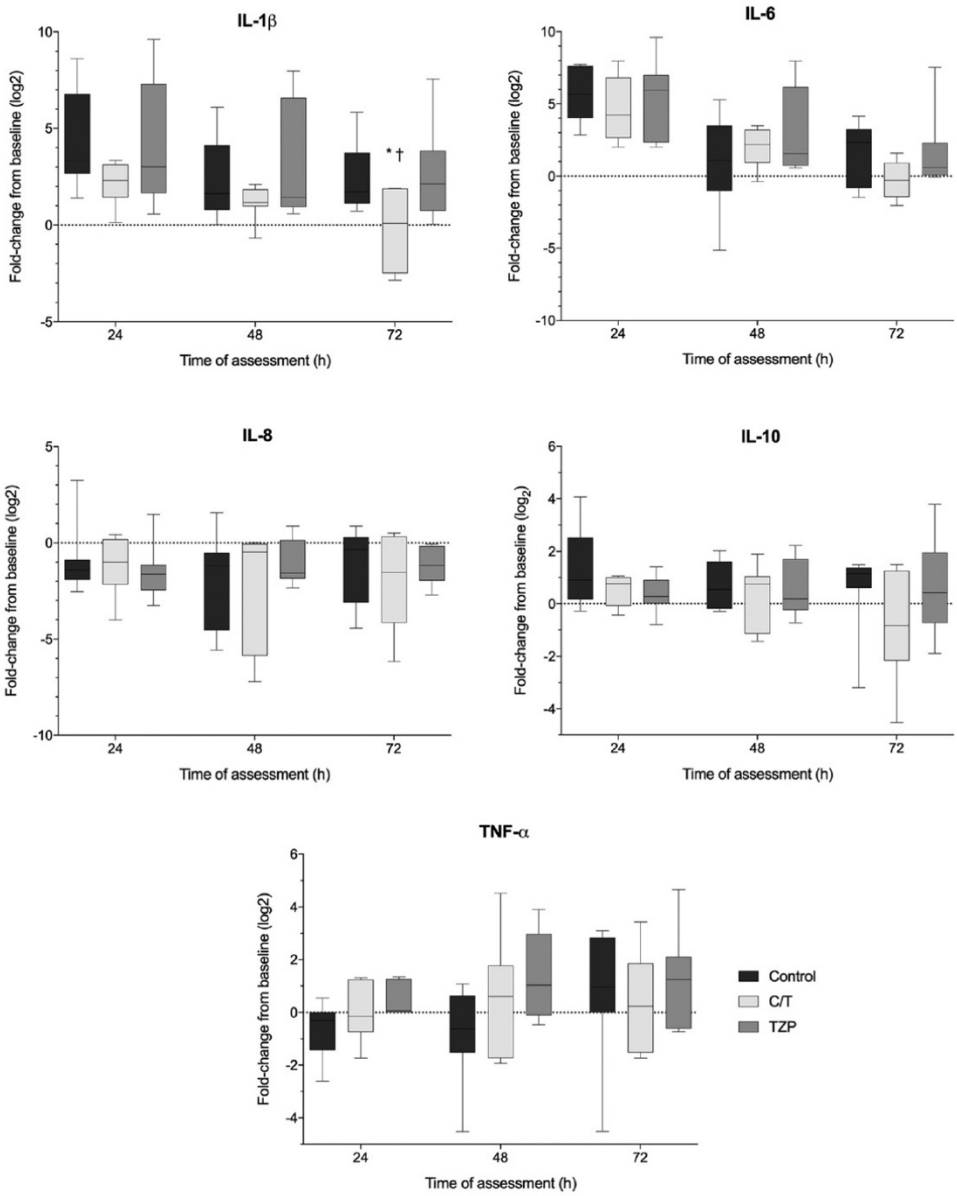

FIG 3 Serum inflammatory markers. Box plots show the fold change from baseline (log_2) among study groups. Horizontal bars represent the median, boxes represent the interquartile range, and whiskers represent the range. IL-1β varied significantly among study groups ($P = 0.031$) and throughout the study time ($P < 0.001$). Indeed, *post hoc* comparisons confirmed that IL-1β was downregulated by AEAT with C/T at 72 h in comparison with untreated ($P = 0.081$) and IEAT TZP-treated animals ($P = 0.049$). Similarly, although no statistical significance was found among study groups, IL-6 showed a downward trend throughout the study time ($P < 0.001$). In contrast, IL-8, IL-10, and TNF-α did not vary among study groups and times of assessments. AEAT, appropriate empirical antimicrobial therapy; IEAT, inappropriate empirical antimicrobial therapy; IL, interleukin; TNF-α, tumor necrosis factor alpha; C/T, ceftolozane/tazobactam; TZP, piperacillin/tazobactam.

TABLE 1 Ceftolozane and piperacillin pharmacokinetics and pharmacodynamics in infected animals[a]

	Ceftolozane (AEAT) ($n = 6$; 50 mg/kg)	Piperacillin (IEAT) ($n = 6$; 200 mg/kg)
Pharmacokinetic parameters		
CL (liters/h)	4.33 (4.06–4.57)	7.62 (6.48–8.11)
V_c (liters)	9.78 (9.40–10.34)	10.35 (9.07–12.50)
V_{ELF} (liters)	2.06 (1.48–2.71)	2.42 (1.35–7.85)
K_{cp} (h^{-1})	0.10 (0.05–0.16)	0.16 (0.10–0.23)
K_{pc} (h^{-1})	0.58 (0.36–0.83)	0.88 (0.52–1.68)
Pharmacodynamic indices		
Plasma *f*AUC (mg/h/liter)	358.40 (331.26–370.58)	808.73 (733.55–974.58)
ELF *f*AUC (mg/h/liter)	267.95 (201.48–378.32)	592.48 (430.16–711.73)
Penetration (%)	88.82 (71.08–105.77)	74.92 (47.45–94.69)
Plasma *f*T > MIC (%)	100.00 (100.00–100.00)	46.88 (42.50–53.13)
ELF *f*T > MIC (%)	96.25 (96.25–97.19)	50.63 (35.94–56.88)

[a]Data are reported as median and interquartile range (IQR) (25th to 75th percentile). CL, clearance; V_c, volume of distribution of the central compartment; V_{ELF}, volume of distribution of the peripheral epithelial lining fluid (ELF) compartment; K_{cp}, transfer rate constant from the central compartment to the peripheral ELF compartment; K_{pc}, transfer rate constant from the peripheral ELF compartment to the central compartment; *f*AUC, free area under the curve to MIC ratio over first 8 h; *f*T > MIC, free time above the MIC over first 8 h.

BAL fluid IL-1β, IL-6, and IL-8 (Fig. S4) peaked post-bacterial burden and remained relatively upregulated thereafter, without differences between groups. Of note, in BAL fluid, IL-8 presented a higher concentration than in serum, while IL-6 showed the opposite trend.

Secondary outcomes of pharmacokinetics. Antibiotic concentrations were quantified in blood and BAL fluid in all treated animals. Table 1 and Fig. S5 describe the plasma and ELF pharmacokinetic profiles of ceftolozane and piperacillin. As expected, due to MIC disparities, ceftolozane achieved a higher percentage of time above MIC (%T > MIC) in both matrixes than piperacillin.

Clinical variables, hemodynamics, and biochemistry. Table 2 depicts the dynamics of clinical, hemodynamics, and biochemistry variables. Neither main clinical nor hemodynamics variables were affected by antimicrobial treatments, yet those parameters changed significantly over the course of the study. The quantity and presence of purulent tracheal secretions were significantly lower in the AEAT group. A trend toward a higher vasopressor dependency index was found in the IEAT with TZP and untreated groups. No differences were found in creatinine levels among study groups, while liver enzymes were significantly higher in the control group, and gamma-glutamyl transferase slightly increased in the AEAT with C/T group.

Pulmonary mechanics and gas exchange. Figure S6 shows changes in pulmonary variables throughout the study period. Oxygenation differed between groups and throughout the study period ($P < 0.001$). In particular, the ratio of partial pressure of oxygen per inspiratory fraction of oxygen was drastically impaired at 24 h in all groups ($P < 0.001$) and differed between study groups at the end of the study ($P = 0.018$). This variation was mainly driven by the unresolved impairment in gas exchange in untreated animals. Other variables, except for the peak airway pressure, were not affected by study treatments.

DISCUSSION

In this randomized experimental study in animals with severe pneumonia caused by XDR *P. aeruginosa*, we demonstrated that in comparison with IEAT with TZP, appropriate empirical antimicrobial therapy with humanized regimens of C/T for 48 h only achieved the following results: (i) enhanced bactericidal effect in tracheal secretions and BAL fluids, (ii) hindered emergence of resistance, (iii) achieved pharmacodynamic target, and (iv) diminished systemic inflammation, as specifically shown by reduced IL-1β. However, the short course of therapy did not significantly reduce lung tissue burden among the study groups. Similarly, both antimicrobial treatments had marginal effects on clinical variables.

Severe *P. aeruginosa* pneumonia is a life-threatening infection most commonly

TABLE 2 Clinical variables, pulmonary mechanics, and hemodynamic parameters during 48 h of treatment[a]

Variable	Baseline $n = 21$	Untreated ($n = 7$)	Appropriate (AEAT) ($n = 7$)	Inappropriate (IEAT) ($n = 7$)	*P* value: Effect group	*P* value: Effect time
Clinical signs						
Body temp (°C)	37.7 ± 0.3	38.3 ± 0.2	38.1 ± 0.2	38.2 ± 0.3	0.680	0.400
WBC (× 10^9/liter)	9.4 ± 0.8	21.7 ± 3.3	18.7 ± 4.8	18.5 ± 4.9	0.822	0.002
Semiquantitative tracheal secretions	0.3 ± 0.7	1.7 ± 0.4	1.2 ± 0.3[b]	1.4 ± 0.2	0.018	0.560
Purulent secretions (%)	4.8	92.9	73.2[c]	92.9	0.002	
Hemodynamics						
Heart rate (beats per minute)	74.0 ± 5.6	68.0 ± 11.8	68.4 ± 12.3	76.7 ± 11.7	0.427	<0.001
Mean arterial pressure (mm Hg)	85.8 ± 3.7	74.1 ± 4.4	71.7 ± 3.1	72.6 ± 3.3	0.815	0.032
Mean pulmonary arterial pressure (mm Hg)	16.1 ± 2.2	22.3 ± 1.9	21.7 ± 0.9	22.1 ± 1.2	0.936	<0.001
Cardiac output (liters/min)	2.8 ± 0.1	4.0 ± 0.3	3.8 ± 0.6	4.0 ± 0.6	0.926	0.008
VDI (mm Hg^{-1})	0	0.43 ± 0.13	0.55 ± 0.32	0.91 ± 0.31	0.472	<0.001
SVR (dynes/s/cm^{-5})	2450 ± 165	1442 ± 102	1550 ± 361	1393 ± 247	0.860	0.002
PRV (dynes/s/cm^{-5})	284.7 ± 15.1	214.5 ± 25.4	231.2 ± 31.3	227.2 ± 25.4	0.653	0.390
Biochemistry analysis						
Creatinine (mg/dl)	1.2 ± 0.02	1.3 ± 0.03	1.2 ± 0.05	1.4 ± 0.06	0.347	0.243
ALT (IU/liter)	34.7 ± 1.7	31.6 ± 2.8	21.8 ± 1.8[b]	24.1 ± 3.6	0.021	0.394
GGT (IU/liter)	69.9 ± 16.5	50.5 ± 5.3	51.7 ± 3.9	36.6 ± 7.7[d]	0.020	0.212
Alkaline phosphatase (IU/liter)	178.0 ± 25.4	135.5 ± 25.7	159.5 ± 28.4	158.0 ± 36.0	0.371	<0.001
Total bilirubin (mg/dl)	0.20 ± 0.03	0.27 ± 0.08	0.20 ± 0.07	0.39 ± 0.15	0.133	<0.001

[a]Data are reported as the mean ± standard deviation of the level from each variable during 48 h of treatment. Clinical and hemodynamics values were recorded every 6 h, while biochemistry analyses were performed every 12 h. The *P* value stands for the probability of differences between treatment groups (i.e., untreated, AEAT, and IEAT groups). Intergroup comparisons with Bonferroni corrections AEAT, appropriate empirical antimicrobial therapy; IEAT, inappropriate empirical antimicrobial therapy; WBC, white blood cells; VDI, vasopressor dependency index; SVR, systemic vascular resistance; PVR, pulmonary vascular resistance; ALT, alanine aminotransferase; GGT, gamma-glutamyl transferase.

[b]$P < 0.05$ versus untreated.

[c]$P < 0.05$ versus untreated and IEAT.

[d]$P < 0.05$ versus untreated and AEAT.

encountered in intensive care unit (ICU) patients (21). The empirical antimicrobial regimen (that is, therapy administered for 48 to 72 h until pathogen identification and *in vitro* susceptibility data are available) is usually categorized as inappropriate when it did not include any antibiotic showing *in vitro* activity against the isolated bacteria. Some authors have included dosing, route, or duration considerations within the definition. In these settings, the growing prevalence of antibiotic-resistant *P. aeruginosa* strains is posing as a major threat for initial antimicrobial treatment accuracy (22). Indeed, the frequency of IEAT for the treatment of nosocomial pneumonia is up to 60% (6), and in the subpopulation of pneumonia caused by MDR *P. aeruginosa*, it is up by 70% (23).

Early initiation of appropriate antibiotic therapy might be a key factor in improving outcomes in patients with nosocomial pneumonia. However, antibiotic selection is challenging, given the aim to strike a balance among administering adequate empirical antibiotic treatment, minimizing the risk of increasing ecological pressure for resistance selection, and decreasing the likelihood of side effects. International guidelines for nosocomial pneumonia consider the appropriateness of the empirical treatment to be important to the outcome, though, and place it in higher consideration as a result compared to the emergence of resistance or side events (2, 5).

Nevertheless, the degree of influence of IEAT on mortality risk from MDR/XDR infections in critically ill patients remains controversial; conclusions from clinical studies have left an unanswered question. Claeys et al. recently reported that 44.6% patients with ICU-acquired lower respiratory infections caused by Gram-negative pathogens were administered IEAT (24). In this study, cefepime (45.1%) and TZP (36.8%) were the most frequent empirical treatments, and the lack of *in vitro* susceptibility was the primary cause of IEAT (24). As a consequence, IEAT translated into significantly higher lengths of stay and an associated economic burden; however, clinical failure and all-cause mortality were not significantly higher than compared to patients with

appropriate empirical treatment (24). Vasudevan and colleagues presented similar findings, reporting that IEAT was not an independent risk factor for ICU mortality among critically ill patients with pneumonia caused by MDR/XDR pathogens (25). In contrast, a prospective cohort study comparing appropriate treatment and IEAT in patients with a strong suspicion of ventilator-associated pneumonia (VAP) showed that the mortality rate (38%) was lower in the former group compared to those receiving IEAT (91%) (26). A separate prospective cohort of patients with VAP reported similar findings, with the mortality rate lower in patients undergoing appropriate treatment (20%) than that of patients receiving IEAT (47%) (27).

As a result, association between IEAT and mortality in patients with nosocomial pneumonia continues to be counterintuitive (28). Additionally, the beneficial impact on outcomes in patients with nosocomial pneumonia within the first 48 to 72 h of admission has not been studied yet. We therefore aimed to analyze what happened during this window, that is, between first sampling and the determination of microbiological results dependent on the appropriateness of an empirical treatment. Our results strengthen the hypothesis that early initiation of appropriate antibiotic therapy is a fundamental factor for improved outcomes in nosocomial pneumonia. Compounding this is a study by Mortensen et al., in which they reported that AEAT was associated with decreased mortality at 48 h in patients with community-acquired pneumonia (29). Although differences in mortality were not found in our study, perhaps due to a small sample size, significant burden reduction in tracheal secretions and BAL fluids were detected when animals received AEAT. These reductions may indicate the first visible step of infection eradication during the administration of appropriate empirical therapy, particularly before any observation of a decrease in lung tissue burden can be made.

As mentioned above, short-term benefits of appropriate empirical treatment included the attainment of a pharmacodynamics target, as well as the prevention of resistance development. Ceftolozane has been demonstrated to be perhaps more stable against the most common resistance mechanisms of *P. aeruginosa*, which are driven by mutation, upregulation, or hyperproduction, i.e., AmpC, efflux pumps, or OprD (14, 30). Remarkably, in our study, C/T prevented resistance development in the AEAT group, whereas the MIC increased substantially after only 48 h of treatment with TZP. Differences between the AEAT and IEAT groups in target attainment for pharmacodynamics (i.e., %T $>$ MIC), which is also directly related to bactericidal efficacy, may also explain disparities in resistance development dynamics. Moreover, the mutation frequency for TZP was considerably higher than for C/T in our strain, which might also be linked to the TZP MIC increase (see "Additional Methods" in the supplemental material). It is of equal importance to highlight that using broad-spectrum antibiotics for initial therapy in order to avoid IEAT may indeed lead to a worsening antimicrobial resistance burden due to selection of even more resistant pathogens. The development of novel antibiotics is therefore necessary if clinicians are to have an increased likelihood of choosing an active, effective agent for empirical therapy of nosocomial pneumonia. Similarly, the development of rapid, low-cost diagnostic microbiological tools that allow the prompt use of narrow-spectrum antibiotics is equally important.

In addition, our study sheds light on the effects of C/T in a large animal model that closely resembles critically ill patients with severe MDR/XDR *P. aeruginosa* pneumonia. Currently, therapeutic options for MDR/XDR Gram-negative pathogens are extremely limited (31). C/T treatment, however, appears to be a promising option with excellent *in vitro* (32) and *in vivo* efficacy, enabling the attainment of pharmacodynamic targets in central and peripheral compartments (19). Ceftolozane has shown excellent antipseudomonal efficacy, even against MDR/XDR strains (13, 33). Interestingly, in hospitalized patients with pneumonia, C/T inhibited 94% of *P. aeruginosa* isolates obtained from these individuals, while TZP demonstrated activity against only 69% (33). These observations highlight current clinical limitations of the latter, relatively longstanding, antibiotic. Moreover, an increase in carbapenem-resistant *P. aeruginosa* isolates has been observed, comprising 26% of isolates nonsusceptible to meropenem. In this

context, C/T is likely to be selected for achieving AEAT and should be preserved for MDR/XDR pathogens.

This study presents some limitations that deserve further discussion, though. First, TZP could have yielded subinhibitory concentrations in ELF and ultimately facilitated emergence of resistance. Our methods nevertheless attempted to replicate current clinical conditions; in IEAT cases, especially, the attainment of pharmacodynamic targets in central and peripheral compartments was usually unexpected. The rationale behind selecting a particular strain in our study was to represent this phenotypic profile for which C/T is likely to be chosen for empirical treatment in patients with resistance risk factors and in those individuals admitted to ICUs with high MDR/XDR prevalence (i.e., nonsusceptibility to β-lactams, including carbapenems). Second, the corroboration of secondary outcomes was limited by the use of only one *P. aeruginosa* strain and the length of the therapy. Even though both antimicrobials adequately penetrated lung tissue, pulmonary infection was exceedingly severe and marginally affected by the short course of treatment. We may therefore lack accuracy in detecting potential differences in lung tissue between study groups. Nevertheless, we wanted to reproduce the clinical setting, where 48 h after initiation of the empirical treatment, pathogen identification and *in vitro* susceptibility data would be available, and the clinician would have the possibility to switch the antibiotic therapy. Moreover, a major strength of our study was the survival rate of more than 90% of the animals evaluated. This fact afforded comprehensive appraisal of infection dynamics and response to treatment. Third, in comparison with phase I studies of healthy volunteers, ceftolozane penetration into ELF of our animals achieved greater figures (17); however, as demonstrated in our preliminary analysis, a C/T dosage of 50 mg/kg achieved similar results as those reported in humans. Differences in C/T pharmacokinetics in severely infected lungs could explain these findings, which are likely to be reproducible in critically ill patients with severe pneumonia. Indeed, the C/T concentrations in ELF of our swine model exceeded the MIC for 100% of the dosing interval, with a MIC of 4 mg/liter, analogous to previous observations in humans (34). Similarly, the piperacillin ELF AUC_{0-8h} showed greater figures than expected based on preliminary studies. This unexpected finding could be explained by highly variable intrapulmonary exposure, unrelated to plasma exposure, as previously detailed by Felton et al. (35). Finally, within our setting, animals did not have comorbidities and were in deep sedation throughout the study. These dissimilarities when considering critically ill patients with nosocomial pneumonia are noteworthy to mention.

Conclusions. In a mechanically ventilated swine model of XDR *P. aeruginosa* pneumonia, appropriate initial treatment with C/T decreased respiratory secretions' bacterial burden, prevented development of resistance, achieved the pharmacodynamic target, and may reduce systemic inflammation. However, after only 2 days of treatment, *P. aeruginosa* tissue concentrations were moderately affected. These data imply several potential benefits of AEAT and call for further experimental and clinical studies to fully determine the short-term implications of IEAT. The translation of our findings to clinical practice is obviously encouraging the use of new antibiotics against MDR/XDR bacteria as soon as possible. This problem is be solved not with conventional cultures but probably with the implementation of rapid molecular techniques that can detect resistance.

MATERIALS AND METHODS

This study was conducted at the Division of Animal Experimentation, Hospital Clinic, Barcelona, Spain. The study protocol was approved by the Animal Experimentation Ethics Committee of the University of Barcelona (reference number 9772).

Preliminary studies. We employed a porcine model of severe *P. aeruginosa*, as previously described (36). In order to catch the potential scenario of empirical antimicrobial therapy failure, we selected an XDR (β-lactam nonsusceptible, including carbapenems) *P. aeruginosa* strain not susceptible to TZP (MIC, 64/4 mg/liter) and at the upper range of the C/T susceptibility profile (MIC, 4/4 mg/liter) (33). Full antimicrobial susceptibility is presented in Table S3. Resistance mechanisms, mutation frequencies, and clinical sources are also described (see "Additional Methods" in the supplemental material). Two animals were used to confirm the pneumonia clinically, microbiologically, and histologically. Single-dose phar-

macokinetic studies of C/T and TZP were performed in healthy animals to identify humanized doses. In particular, we aimed at achieving ELF ceftolozane AUC_{0-8h} of about 150 to 175 mg/h/liter (i.e., 3 g in humans) (19) and ELF piperacillin AUC_{0-8h} of about 100 to 140 mg/h/liter (i.e., 4.5 g in humans) (37). The pharmacokinetic parameters were derived individually for each pig, and the AUC_{0-8h} was calculated by using the linear trapezoidal rule. Confirmatory pharmacokinetic studies were performed in infected animals.

Main study. Twenty-three large white Landrace female pigs (32.9 ± 1.7 kg; Specipig, Barcelona, Spain) were intubated and mechanically ventilated up to 76 h. Sedatives and analgesics were administered as previously described (38). Pneumonia was developed by intrabronchial inoculation of 15 ml of 7 log_{10} CFU/ml of the aforementioned *P. aeruginosa* strain (36). After 24 h, pneumonia was confirmed (see "Additional Methods" in the supplemental material) and treatment commenced. Based on the results of pharmacokinetic studies, animals were randomized to receive, every 8 h, intravenous saline solution (untreated) or 50 mg/kg of ceftolozane and 25 mg/kg of tazobactam (AEAT) or 200 mg/kg of piperacillin and 25 mg/kg of tazobactam (IEAT) over 1 h. Figure S7 displays the study design and assessment plan.

Primary outcome. The animals were euthanized 76 h after tracheal intubation (4 h after the last antimicrobial dose), and quantitative pulmonary cultures were performed (38). Furthermore, each lobe was biopsied, and the pneumonia severity score was computed (39). Semiquantitative evaluation of each specimen was derived from the sum of the worst histological and bacterial burden scores (40). Investigators were blinded to the treatment allocation.

Secondary outcomes. Every 24 h, we cultured tracheal secretions, BAL fluid, and blood. In addition, *P. aeruginosa* resistance to C/T and TZP was quantified. Prior to bacterial challenge, and every 24 h thereafter, interleukin-1β (IL-1β), IL-6, IL-8, IL-10, and tumor necrosis factor-α (TNF-α) were quantified in serum and BAL fluids by bead-based multiplex assays with Luminex technology (Millipore Iberica, S.A., Madrid, Spain) (41). The antimicrobial concentration was measured in plasma and BAL fluids through high liquid chromatography at baseline and at 1, 2, 4, 6, and 8 h thereafter (42–44). Protein binding was assessed in duplicate, and ELF concentrations were determined using urea concentration as an endogenous marker (45). A 2-compartment model for each drug was performed using the nonparametric adaptive grid algorithm (46, 47). Hemodynamic parameters, pulmonary variables, gas exchange, and urinary output were evaluated throughout the study; ventilator settings were adjusted and clinical sepsis guidelines applied to achieve ventilatory and hemodynamic stability (38).

Statistical analysis. Continuous variables were described as means and standard deviation (SD) or median (interquartile range [IQR]; 25th to 75th percentile), while categorical variables were described as counts and percentages. The normality of the residuals of the mixed models was assessed. In the case of normal distribution, differences among study groups and/or times of assessments of continuous variables were analyzed through a linear mixed-effects models (MIXED) procedure based on a repeated measures approach (restricted maximum likelihood analysis). For nonparametric distributions, the Kruskal-Wallis test was used. Categorical variables were analyzed using the Chi-square test. Each pairwise comparison was corrected using the Bonferroni test. A two-sided P value of ≤ 0.05 was considered statistically significant. All statistical analyses were performed using IBM SPSS Statistics 21.0 (Armonk, NY, USA).

SUPPLEMENTAL MATERIAL

Supplemental material is available online only.

SUPPLEMENTAL FILE 1, PDF file, 1.1 MB.

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A.M., G.L.B., and A.T. participated in protocol development, study design, study management, statistical analysis, and data interpretation and wrote the first draft of the report. F.P., L.F.-B., H.Y., E.A.X., T.S., F.A.I., C.T., C.C., R.A., M.Y., J.B., M.R., G.F., R.C., and J.R. participated in data collection and interpretation and critically reviewed the first draft of the report. D.P.N., P.P., F.B., M.A., J.V., and M.K. participated in the study design and reviewed the report.

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Supplemental Material Article 2

SUPPLEMENTAL DIGITAL CONTENT

SHORT-TERM EFFECTS OF APPROPRIATE EMPIRICAL ANTIMICROBIAL TREATMENT WITH CEFTOLOZANE/TAZOBACTAM IN A SWINE MODEL OF NOSOCOMIAL PNEUMONIA

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ADDITIONAL METHODS

Strain characterization

The strain used in this study was isolated from a patient with a wound infection in 2013 and was selected based on its growth in the swine VABP model and its phenotypic profile [Table S3].

For the presence of metallo-β-lactamases (bla_{IMP}, bla_{VIM}, bla_{SPM}, bla_{SIM}, bla_{NDM}, and bla_{GIM}), serine-carbapenemases (bla_{KPC}, bla_{IMI}, bla_{GES}, $bla_{CTX\text{-}M}$), cephalosporinases (bla_{AmpC}), and oxacillinases ($bla_{OXA\text{-}50}$, $bla_{OXA\text{-}48}$) was determined by PCR (1). The QRDRs of the four quinolone target genes (*gyrA, gyrB, parC and parE*) were analysed for the presence of mutations after PCR amplification and sequencing as previously described (2). Moreover, AME genes were screened using the custom primers: *aac(3)-Ia, aac(3)-Ib, aac(3)-Ic, -aac(3)-Id, ant(2'')-Ia, aac(6')-Ib,* and *aph(3)-V (3)I.* The *P. aeruginosa* isolate harbored the genes encoding β-lactamase ($bla_{OXA\text{-}50}$), aminoglycoside-modifying enzymes (*aac (3)-Ic* and *ant (2'')-Ia*, and mutations in *GyrA* (T83I), *GyrB* (F516S) and *ParC* (S87L).

Resistance mutant frequencies were determined in the *P. aeruginosa* isolate for both antimicrobials following previously established procedures (4). Experiments were done in triplicate and repeated three times with similar results. Mean values are reported. The mutation frequency for CT was $<10^{-9}$ when the isolate was exposed to 4 mg/L of C/T (i.e., C/T MIC). The mutation frequency for TZP was $2.15 \cdot 10^{-6}$ when the isolate was exposed to 64 mg/L of TZP (i.e., TZP MIC).

Pneumonia confirmation criteria

Based on our previous studies (5), *P. aeruginosa* pneumonia after 24 hours from bacterial inoculum was suspected if three of the following clinical criteria were encountered:

1. Body temperature > 38.5°C or < 36°C
2. White blood count > 14,000/mm3 or < 4000/mm3
3. Respiratory system compliance ≤ 20 ml/cm H2O
4. A decrease in PaO2/FIO2 ≥ 90 from baseline values
5. Presence of purulent secretions
6. Mean arterial pressure ≤ 65 mm Hg without the use of vasoactive drugs

Table S1. Ceftolozane and piperacillin pharmacokinetics and pharmacodynamics in healthy animals

	Ceftolozane		Piperacillin
	30 mg/kg (n=2)	**60 mg/kg (n=2)**	**200 mg/kg (n=2)**
Pharmacokinetic parameters			
Plasma C_{max} (mg/L)	84.06; 91.54	201.04; 172.43	370.18; 356.48
Elimination half-life (h)	3.39; 3.56	5.45; 3.53	1.09; 0.79
Unbound fraction (%)	100; 100	100; 100	100; 100
Pharmacodynamic indices			
Plasma *f*AUC(mg*h/L)	196.7; 212.90	484.27; 453.69	662,67; 559.38
ELF AUC (mg*h/L)	8.05; 9.20	104.93; 69.64	261.19; 106.77

Table S1 caption: Data are as individual values for each animal. Piperacillin plasma concentrations in animals receiving 100/12.5 mg/kg were below detection limit. C_{max}, maximum (or peak) serum concentration; T_{max}, time at which the C_{max} is observed; *f*AUC, free area under the curve to minimum inhibitory concentration ratio over first 8 h; ELF, epithelial lining fluid.

Table S2. Ceftolozane and piperacillin pharmacokinetics and pharmacodynamics in infected animals

	Ceftolozane	Piperacillin
	60 mg/kg (n=2)	**200 mg/kg (n=2)**
Pharmacokinetic parameters		
Plasma C_{max} (mg/L)	151.34; 202.75	199.06; 319.61
Elimination half-life (h)	3.85; 2.62	0.89; 0.88
Unbound fraction (%)	100; 100	100; 100
Pharmacodynamic indices		
Plasma *f*AUC (mg*h/L)	534.61; 570.42	414.63; 533.42
ELF AUC (mg*h/L)	130.04; 306.09	134.55; 145.49

Table S2 caption: Data are as individual values for each animal. C_{max}, maximum (or peak) serum concentration; T_{max}, time at which the C_{max} is observed*f*AUC, free area under the curve to minimum inhibitory concentration ratio over first 8 h; ELF, epithelial lining fluid.

Table S3. Antibiotic susceptibility profile of *Pseudomonas aeruginosa* strain used in our experimental studies

Full antimicrobial susceptibility, resistance mechanisms, mutation frequencies, and clinical source are presented in Table S3

Antimicrobial	MIC (mg/L)	Susceptibility by CLSI (6)	Susceptibility by EUCAST (7)
Gentamicin	8	I	R
Tobramycin	1	S	S
Amikacin	24	I	R
Imipenem	16	R	R
Meropenem	128	R	R
Ceftazidime	16	I	R
Cefepime	64	R	R
Ciprofloxacin	32	R	R
Levofloxacin	>32	R	R
Piperacillin/tazobactam	64/4	I	R
Ceftolozane/tazobactam	4/4	S	S
Aztreonam	>32	R	R
Colistin	1	S	S

Table S3 caption: MIC, minimum inhibitory concentration; CLSI, Clinical and Laboratory Standards Institute; EUCAST, European Committee on Antimicrobial Susceptibility Testing; S, susceptible; I, intermediate; R, resistant.

FIGURE LEGEND

Figure S1. Clinical, microbiological and histological confirmation of severe pneumonia. Two animals were intubated and mechanically ventilated up to 76 hours. Animals were challenge with extensively drug-resistant *Pseudomonas aeruginosa* strain. Data are reported as mean and standard deviation. Main clinical (A-C) microbiological (D,E) and histological (F) results are displayed in the figure. Following bacterial challenge (displayed by the vertical dashed line) significant increase in temperature (A) was observed. Similarly, ratio between arterial pressure of oxygen and inspiratory fraction of oxygen (PaO_2/FiO_2) significantly decreased (B) after inoculation. In contrast, white blood cells (C) varied between both animals and no differences were found throughout the study (D) *P. aeruginosa* burden within tracheal secretions and bronchoalveolar lavage upon diagnosis of pneumonia. (E) Upon autopsy, *P. aeruginosa* tissue concentration was higher than 3 log_{10} CFU/g in all lobes of all animals. (F) Histopathological pattern of pneumonia consistently found in all the lobes of all animals (x20 magnification). PaO_2/FiO_2, ratio between arterial pressure of oxygen and inspiratory fraction of oxygen; CFU, colony-forming unit; BAL, bronchoalveolar lavage; RUL, right upper lobe; RML, right medium lobe; RLL, right lower lobe; LUL, left upper lobe; LLL left lower lobe.

Figure S2: Lung tissue macroscopic findings. Upon autopsy, lungs were excised and macroscopically analyzed during biopsies collection. Lobes were defined as potentially infected if purulent secretions or abscessual areas were found with any of the following concomitant signs: edema, extensive atelectasis. In untreated group, 80.0% of lobes were potentially infected, while 57.1% and 65.7% in AEAT with C/T and IEAT with TZP, respectively (p=0.12). Lung/Body weight ratio of untreated, AEAT and IEAT groups were 1.48±0.23, 1.41±0.22, and 1.32±0.23, respectively, (p=0.43). Each lung/body weight ratio is display in the respectively picture.

Figure S3: Impaired cytokine production after bacterial challenge. The concentration of cytokines (solid line) in 42 samples of serum and bronchoalveolar lavage fluids (dashed line) was measured in duplicates at baseline and 24h after bacterial challenged. IL-1β, IL-6 and IL-10

concentrations significantly increased in serum samples, whilst IL-8 was downregulated after PA inoculation. In BAL fluids, IL-1β and IL-8 were upregulated at pneumonia diagnosis in comparison with baseline levels. Significant differences in serum between time of assessments are displayed by asterisk, while in BAL fluids by dagger. IL, interleukin; TNF-α, tumor necrosis factor alpha; BAL, bronchoalveolar lavage.

Figure S4. Inflammatory markers in bronchoalveolar lavage fluids. Boxplots showing fold change from baseline (log2) among study groups. Horizontal bars represent the median, boxes represent the interquartile range and whiskers the range. IL-1β and IL-8 increased throughout the study time. Nevertheless, IL-1β, IL-6, and IL-8 did not significantly changed either among study groups or times of assessment. IL-10 and TNF-α concentrations were below the detection limit and no comparisons among groups were performed. IL, interleukin.

Figure S5. Pharmacokinetic studies. Mean plasma (solid lines) and ELF (dashed lines) concentrations versus times of assessment of ceftolozane (A) and piperacillin (B) dosage regimens, post single antibiotic administration. Grey bands display the area within error bands (standard error of the mean, SEM) and the horizontal dotted lines are the minimum inhibitory concentrations (MIC) for each antimicrobial. Of note, a dose of 50 mg of ceftolozane and 200 mg of piperacillin achieved ELF concentrations above *Pseudomonas aeruginosa* MIC for more than 50% of the time. ELF, epithelial lining fluid.

Figure S6. Gas exchange and pulmonary mechanics. Mean values per each timepoint among study groups. (A) Partial pressure of oxygen and inspiratory fraction of oxygen ratio differed among study groups at the end of the study (p=0.018). (B) Pulmonary shunt was similar among study groups (p=0.69). (C) Peak airway pressure differed among study groups (p=0.049). In particular, untreated animals showed the highest figures. In contrast, plateau airway pressure (D), respiratory system compliance (E) were similar among study groups. Of note, statistically significances of post-hoc comparisons with Bonferroni correction between AEAT and IEAT groups against untreated group are shown by asterisk and dagger, respectively. Differences between

AEAT and IEAT are displayed by double dagger. PaO_2/FiO_2, ratio between arterial pressure of oxygen and inspiratory fraction of oxygen.

Figure S7: Main 76-hour study sequential assessments. Antimicrobials were administered every 8h. Microbiology and inflammation assessments were performed every 24h. The pharmacokinetics assessments were conducted only in animals enrolled into the AEAT and IEAT groups. Upon the first administration of antibiotics, analysis of plasma and epithelial lining fluid antibiotic concentrations at pre-dose, 1, 2, 4, 6- and 8-hours post-dosing were carried out. Clinical variables, hemodynamic parameters, pulmonary variable and gas exchange were measured every 6h. Necropsy was performed after 76 hours from tracheal intubation and four hours after last antimicrobial dose.

Figure S1

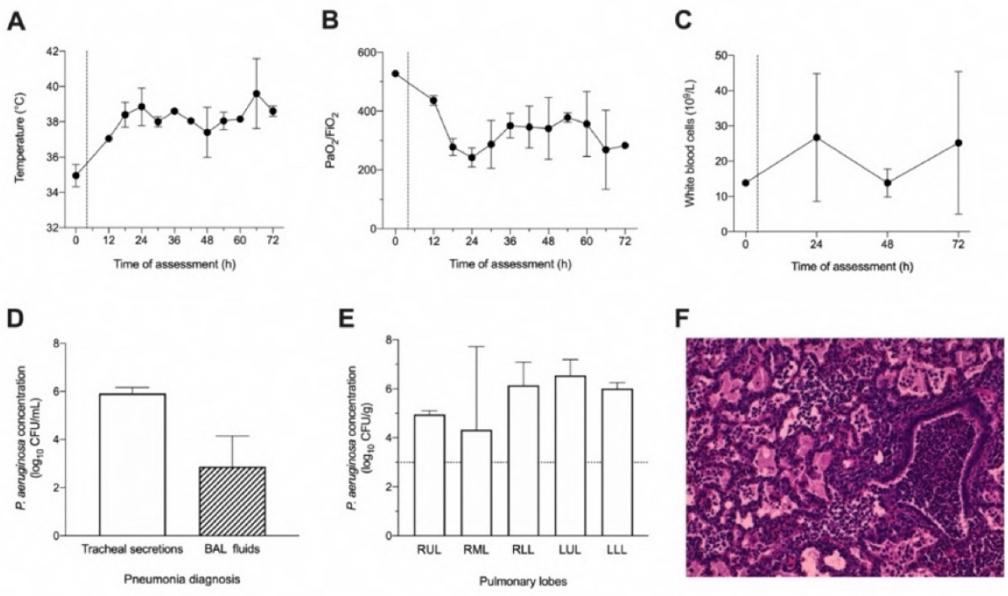

A
Temperature (°C)
Time of assessment (h)
B
PaO2/FiO2
Time of assessment (h)
C
White blood cells (10⁹/L)
Time of assessment (h)
D
P. aeruginosa concentration (log10 CFU/mL)
Tracheal secretions
BAL fluids
Pneumonia diagnosis
E
P. aeruginosa concentration (log10 CFU/g)
RUL
RML
RLL
LUL
LLL
Pulmonary lobes
F

Figure S2

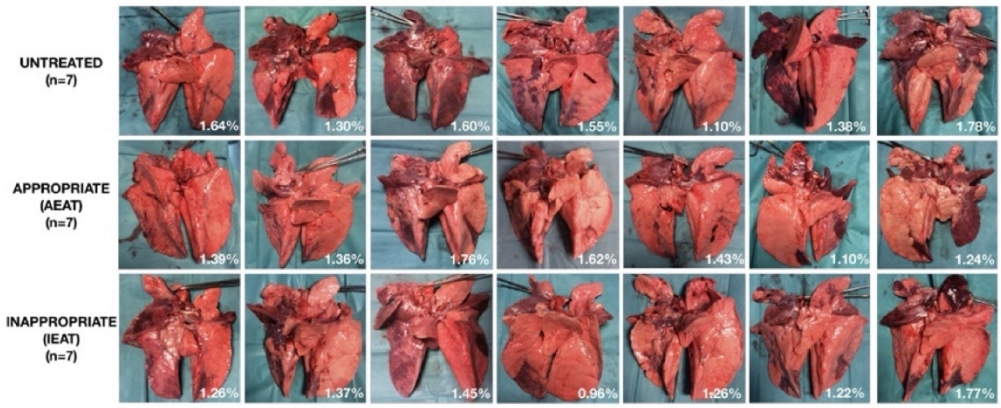

UNTREATED
(n=7)
1.64%
1.30%
1.60%
1.55%
1.10%
1.38%
1.78%
APPROPRIATE
(AEAT)
(n=7)
1.39%
1.36%
1.76%
1.62%
1.43%
1.10%
1.24%
INAPPROPRIATE
(IEAT)
(n=7)
1.26%
1.37%
1.45%
0.96%
1.26%
1.22%
1.77%

Figure S3

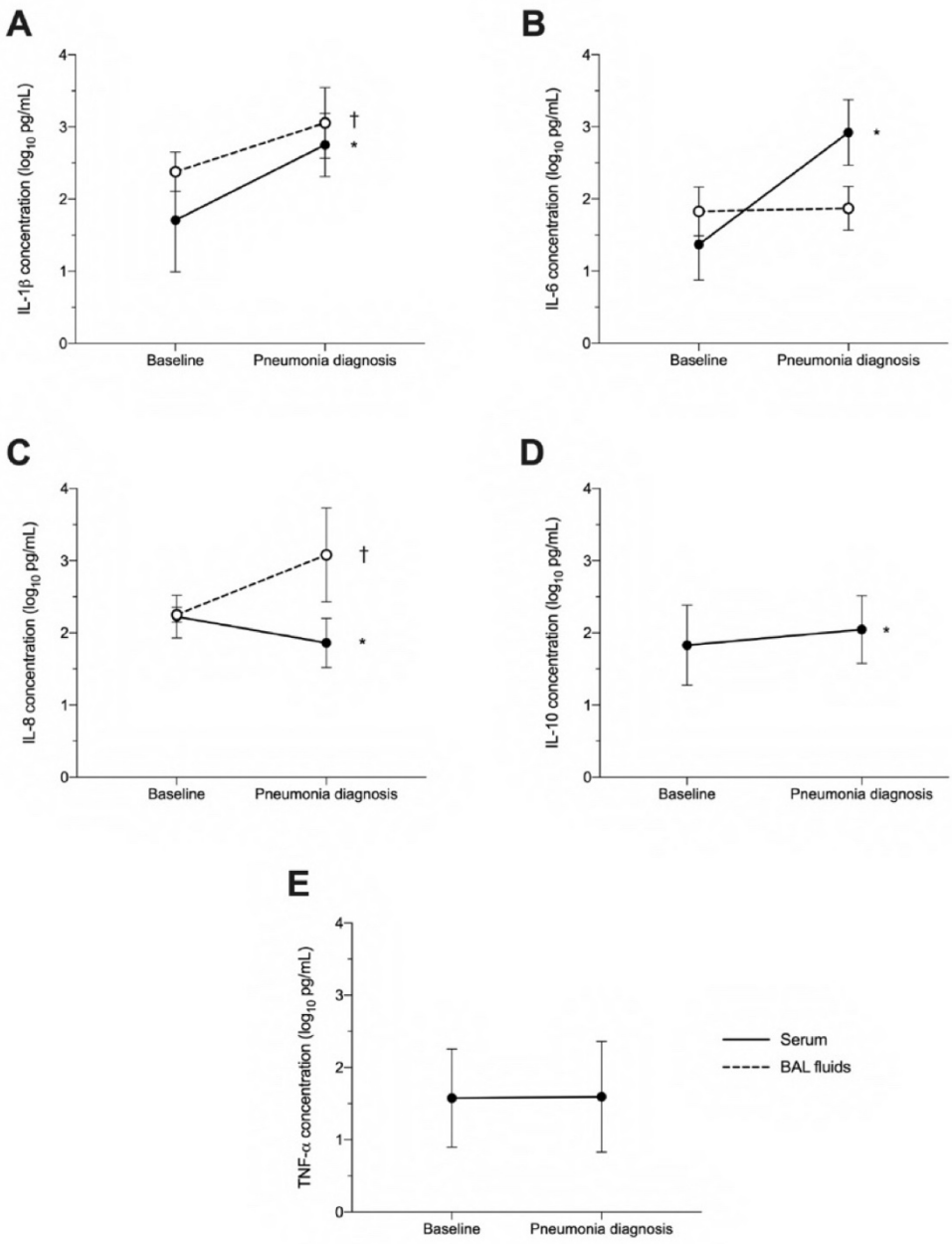

A
IL-1β concentration (log10 pg/mL)
†
*
Baseline
Pneumonia diagnosis
B
IL-6 concentration (log10 pg/mL)
*
Baseline
Pneumonia diagnosis
C
IL-8 concentration (log10 pg/mL)
†
*
Baseline
Pneumonia diagnosis
D
IL-10 concentration (log10 pg/mL)
*
Baseline
Pneumonia diagnosis
E
TNF-α concentration (log10 pg/mL)
Baseline
Pneumonia diagnosis
Serum
BAL fluids

Figure S4

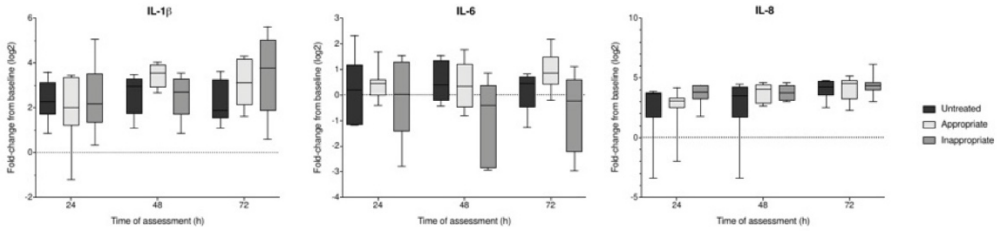

IL-1β
IL-6
IL-8
Fold-change from baseline (log2)
Time of assessment (h)
24
48
72
Untreated
Appropriate
Inappropriate

Figure S5

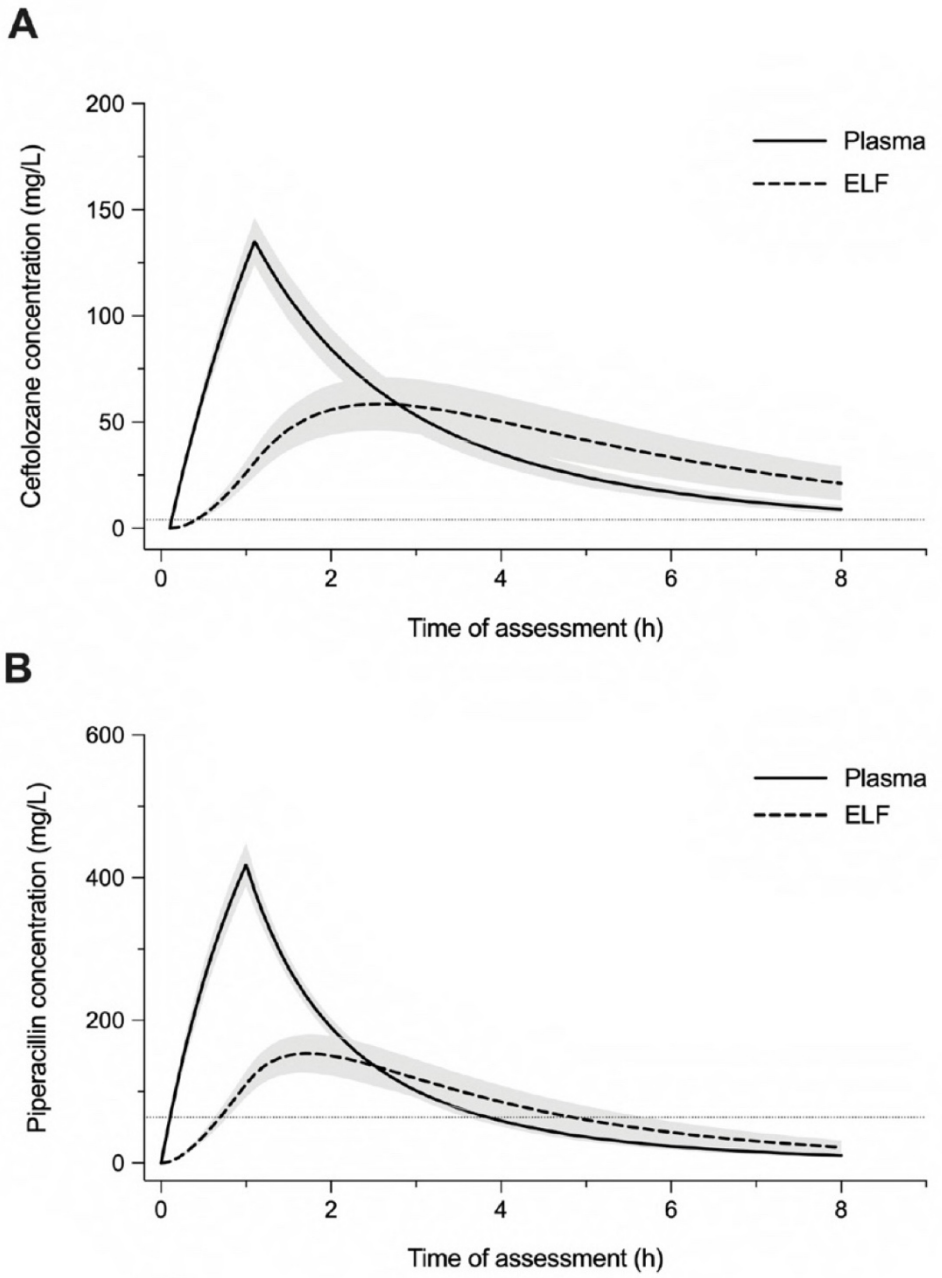

A
Ceftolozane concentration (mg/L)
200
150
100
50
0
Plasma
ELF
0
2
4
6
8
Time of assessment (h)
B
Piperacillin concentration (mg/L)
600
400
200
0
Plasma
ELF
0
2
4
6
8
Time of assessment (h)

Figure S6

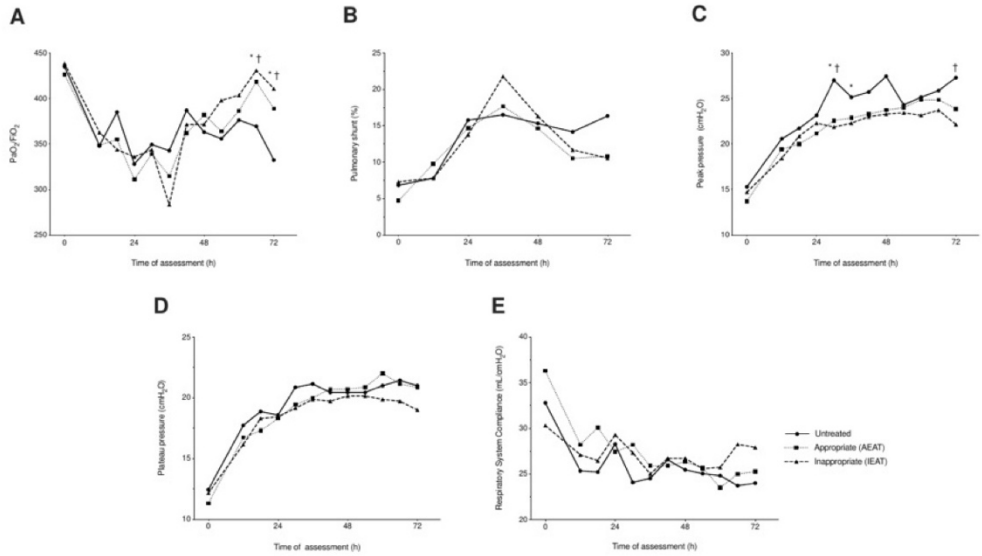
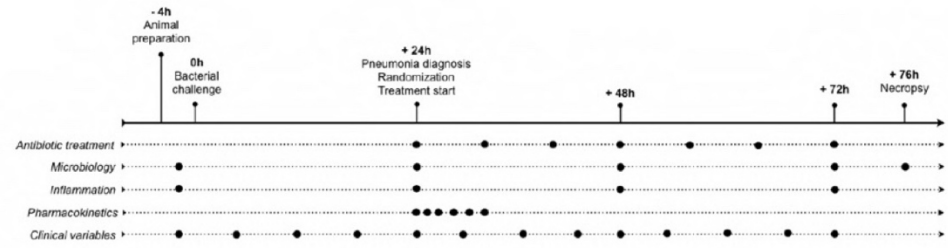

A
B
C
D
E
Time of assessment (h)
Untreated
Appropriate (AEAT)
Inappropriate (IEAT)

Figure S7


- 4h
Animal preparation
0h
Bacterial challenge
+ 24h
Pneumonia diagnosis
Randomization
Treatment start
+ 48h
+ 72h
+ 76h
Necropsy
Antibiotic treatment
Microbiology
Inflammation
Pharmacokinetics
Clinical variables

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ARTICLE 3

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Meropenem–nacubactam activity against AmpC-overproducing and KPC-expressing *Pseudomonas aeruginosa* in a neutropenic murine lung infection model

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ABSTRACT

Nacubactam is a novel non-β-lactam diazabicyclooctane β-lactamase inhibitor under development for the treatment of serious Gram-negative infections. This study assessed the efficacy of human-simulated epithelial lining fluid (ELF) exposure of nacubactam in combination with meropenem against AmpC-overproducing (n=4) and *Klebsiella pneumoniae* carbapenemase (KPC)-expressing (n=3) *Pseudomonas aeruginosa* isolates in the neutropenic murine lung infection model. Meropenem, nacubactam and meropenem–nacubactam (1:1 concentration ratio) minimum inhibitory concentrations (MICs) were determined in triplicate using broth microdilution. Regimens that provided ELF profiles mimicking those observed in humans given nacubactam 2 g q8h (1.5-h infusion) alone and in combination with a subtherapeutic ELF exposure of meropenem were administered 2 h after inoculation. Efficacy was assessed as the change in $\log_{10}$ colony-forming units (CFU)/lung at 24 h compared with 24-h meropenem monotherapy. Meropenem, nacubactam and meropenem–nacubactam MICs were 8–>64, 128–>256 and 2–16 mg/L, respectively. Meropenem and nacubactam monotherapy groups demonstrated bacterial growth over 24 h for each isolate. Against AmpC-overproducing and KPC-expressing *P. aeruginosa* isolates, meropenem–nacubactam resulted in −2.73±0.93 and −4.35±1.90 $\log_{10}$CFU/lung reduction, respectively, relative to meropenem monotherapy. Meropenem–nacubactam showed promising in-vivo activity against meropenem-resistant *P. aeruginosa*, indicative of a potential role for the treatment of infections caused by these challenging pathogens.

1. Introduction

Pseudomonas aeruginosa is one of the most common pathogens implicated in healthcare-associated pneumonia, representing a significant public health burden [1,2]. For years, carbapenems have been utilized successfully to treat infections caused by multidrug-resistant *P. aeruginosa*; however, the development of carbapenem resistance is compromising this antipseudomonal option. Carbapenem resistance in *P. aeruginosa* is multi-factorial and mediated by hyperproduction (stable derepression) of AmpC β-lactamase, mexAB-oprM up-regulation, OprD porin loss and expression of carbapenemases [3,4]. In *P. aeruginosa*, these carbapenemases are mostly metallo-β-lactamases; however, several case reports from diverse geographic regions have documented *Klebsiella pneumoniae* carbapenemase (KPC)-positive *P. aeruginosa* as an emerging resistance pattern [5,6].

Nacubactam is a novel non-β-lactam diazabicyclooctane β-lactamase inhibitor with in-vitro activity against class A β-lactamases such as KPC, class C and some class D β-lactamases [7,8]. The purpose of this study was to evaluate the efficacy of human-simulated epithelial lining fluid (ELF) exposure of nacubactam in combination with meropenem against chromosomal AmpC-

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0924-8579/

Table 1
Phenotypic profiles and resistance mechanisms for the *Pseudomonas aeruginosa* isolates utilized in the in-vivo efficacy studies.

Organism ID	β-lactamase	Genes with detected resistance mutations[a]	Modal MIC (μg/mL)			
			MEM	NAC	MEM-NAC (1:1)[b]	MEM–NAC (4 mg/L)[c]
PSA C29-3	AmpC[d]	*oprD, mexT, PA4179, PA5160*	8	>256	4	2
PSA C7-18	AmpC[d]	*mexT*	16	256	16	16
PSA C14-22	AmpC[d]	*oprD, mexT, nalC*	16	256	16	16
PSA C28-5	AmpC[d]	*PA2020, PA2213*	32	256	16	16
PSA 1602	KPC-5, OXA-50, PAO	ND	64	128	2	0.5
PSA 1593	KPC-2	ND	>64	128	8	4
PSA 1663	KPC-2	ND	>64	128	4	0.5

MIC, minimum inhibitory concentration; MEM, meropenem; NAC, nacubactam; ND, not determined.
[a] Reference sequence: NC_002516.2 *P. aeruginosa* PAO1 chromosome.
[b] 1:1 MEM and NAC concentration ratio.
[c] MEM in combination with a fixed NAC concentration of 4 mg/L.
[d] Chromosomal AmpC overproduction.

overproducing and KPC-expressing *P. aeruginosa* in a neutropenic murine lung infection model.

2. Methods

2.1. Bacterial isolates and susceptibility testing

Seven meropenem-resistant *P. aeruginosa* clinical isolates [AmpC-overproducing (n=4), KPC-expressing (n=3)] were utilized in this study; one isolate (PSA 1602, CDC #0090) was obtained from the Food and Drug Administration/Centers for Disease Control and Prevention Antimicrobial Resistance Isolate Bank (Atlanta, GA, USA) and the remaining six isolates were obtained from the Center for Anti-Infective Research and Development isolate repository. All isolates were maintained in skimmed milk (BD Biosciences, Sparks, MD, USA) at −80°C. Each isolate was subcultured twice on trypticase soy agar with 5% sheep blood (BD Biosciences), and grown for 18–20 h at 37°C under 5% CO_2 prior to use in the experiments. Gene sequencing and expression was performed via Acuitas whole-genome sequence analysis and reverse transcriptase polymerase chain reaction using methods consistent with published reports [9]. For meropenem–nacubactam minimum inhibitory concentration (MIC), doubling dilutions of meropenem were utilized in a 1:1 concentration ratio with nacubactam as well as a fixed nacubactam concentration of 4 mg/L. The MICs of meropenem, nacubactam and meropenem–nacubactam were determined in triplicate for all isolates using the broth microdilution methodology as outlined by the Clinical and Laboratory Standards Institute [10].

2.2. Neutropenic lung infection model

Pathogen-free, female ICR mice weighing 20–22 g (Envigo RMS, Inc., Indianapolis, IN, USA) were utilized in the study, and the protocol was approved by the Hartford Hospital Institutional Animal Care and Use Committee. Mice were rendered transiently neutropenic by intraperitoneal injection of cyclophosphamide (250 mg/kg on Day-4, 100 mg/kg on Day-1). Uranyl nitrate (5 mg/kg on Day-3) was administered to produce a controlled degree of renal impairment. The mice were anesthetized using vaporized isofluorane (2–3% v/v in an oxygen carrier), and lung infection was produced by intranasal inoculation of 0.05 mL of inoculum [suspension of 10^7 colony-forming units (CFU)/mL bacteria in 3% hog gastric mucin]. Antimicrobial therapy was initiated 2 h after lung inoculation. Treated and 24-h control mice were killed at the end of the study period, and lungs were harvested aseptically and processed as described previously [11].

2.3. In-vivo efficacy studies

For assessment of efficacy, three treatment arms were utilized: meropenem monotherapy, nacubactam monotherapy and meropenem–nacubactam in combination. Previously developed human-simulated murine dosing regimens of nacubactam were utilized [11]. The developed nacubactam regimen provided %T >ELF concentration and ELF area under the curve similar to those achieved in an open-label, intrapulmonary lung penetration healthy volunteer study (Clinical Trial Registration No. NCT03182504) following a dose of nacubactam 2 g q8h as 1.5-h infusion. Pilot efficacy studies showed that despite the elevated meropenem MICs of the examined isolates, ELF exposures resulting from the administration of meropenem monotherapy equivalent to 2 g q8h as 1.5-h infusion produced marked bacterial kill (data not shown). Thus, in order to demonstrate an additional benefit of nacubactam in combination with this potent β-lactam backbone, a meropenem dosing regimen that resulted in ≥2-log CFU growth among the evaluated isolates was administered as the meropenem monotherapy and combination groups. This meropenem regimen consisted of six doses every 8 h over 24 h (0 h, 3.75 mg/kg; 1.5 h, 4.75 mg/kg; 2.75 h, 4.75 mg/kg; 4 h, 4.25 mg/kg; 5.5 h, 2.25 mg/kg; 7.25 h, 1.25 mg/kg). Efficacy was quantified by the bacterial reduction with meropenem–nacubactam at 24 h relative to the 24-h meropenem monotherapy treatment group. To compare antimicrobial efficacy between regimens, Student's t-test was used and $P \leq 0.05$ was considered to indicate statistical significance.

3. Results

3.1. In-vitro susceptibility studies

MICs of meropenem, nacubactam and meropenem–nacubactam as well as known resistance profiles for the seven meropenem-resistant isolates are listed in Table 1. Meropenem–nacubactam MICs (1:1 concentration ratio) ranged from 4 to 16 mg/L and from 2 to 8 mg/L for the AmpC-overproducing and KPC-expressing isolates, respectively. Nacubactam at a fixed concentration of 4 mg/L in combination with doubling dilutions of meropenem resulted in comparable in-vitro activity for the majority of isolates.

3.2. In-vivo efficacy studies

In vivo, mean bacterial densities ($\log_{10}$ CFU/lung ± standard deviation) at 0 h were 6.17±0.21 in control mice and increased to 8.64±0.94 $\log_{10}$ CFU/lung in untreated mice at 24 h. The bacterial counts in the meropenem monotherapy and nacubactam monotherapy treatment groups at 24 h were

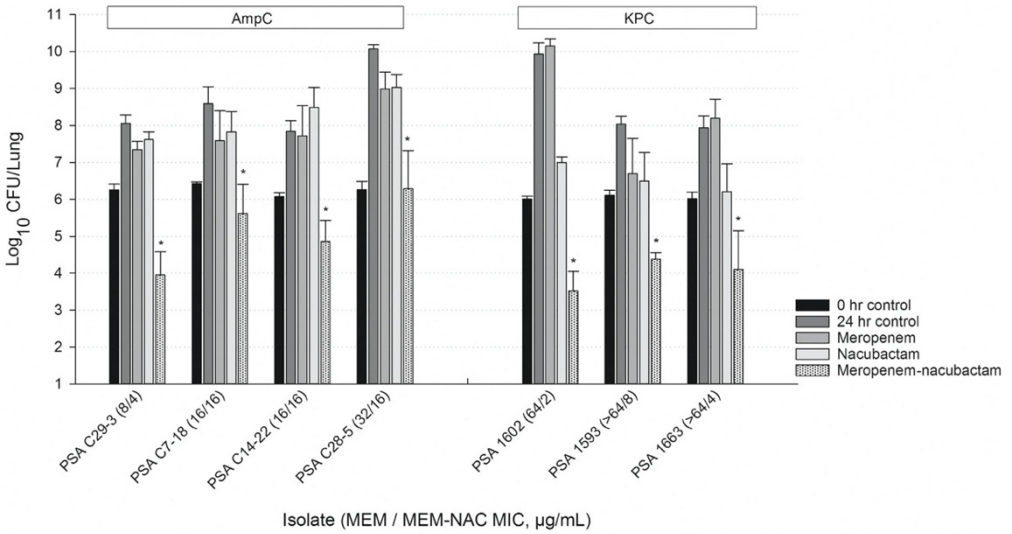

Fig. 1. Efficacy of meropenem (MEM), nacubactam (NAC) and meropenem–nacubactam (MEM-NAC) against *Pseudomonas aeruginosa* isolates. Data represent mean ± standard deviation. *Significant difference relative to 24-h meropenem monotherapy ($P \leq 0.05$). CFU, colony-forming units; KPC, *Klebsiella pneumoniae* carbapenemase; MIC, minimum inhibitory concentration.

8.08±1.24 and 7.53±1.09 $\log_{10}$ CFU/lung, respectively. Meropenem–nacubactam resulted in a mean −2.73±0.93 $\log_{10}$ CFU/lung reduction against AmpC-overproducing isolates, and a mean −4.35±1.90 $\log_{10}$ CFU/lung reduction among KPC-expressing isolates relative to the growth observed with meropenem monotherapy (Fig. 1).

4. Discussion

P. aeruginosa has emerged as one of the leading causes of nosocomial infection, especially in patients undergoing invasive procedures or on mechanical ventilation [12]. Given the propensity to quickly develop antimicrobial resistance, the management of *P. aeruginosa* poses a serious therapeutic challenge, supporting the need for alternative and novel therapeutic options with potent antipseudomonal activity. A growing number of studies have shown enhanced efficacy of meropenem–nacubactam against Enterobacteriaceae harboring a variety of β-lactamases including KPC; however, in-vivo activity against *P. aeruginosa* is limited [13,14]. In a murine thigh infection study with a single AmpC-derepressed *P. aeruginosa* isolate, the administration of cefepime alone or nacubactam (previously OP0595) alone resulted in bacterial counts comparable to the untreated control group, while cefepime-nacubactam resulted in a 2–4 $\log_{10}$ CFU/thigh reduction [15].

Despite the use of subtherapeutic meropenem exposure in this study, the addition of nacubactam to meropenem resulted in substantial bacterial reduction using the conventional assessment of efficacy (i.e. change in $\log_{10}$ CFU/lung after 24 h relative to 0-h untreated controls); bacterial reduction of >1 $\log_{10}$ CFU/lung was observed among all KPC-expressing isolates, and in two of the four AmpC-overproducing isolates. Nacubactam has been reported to show synergy, (i.e. enhancer effect) in combination with β-lactams against bacterial isolates expressing class A and C β-lactamases. For example, synergy was demonstrated *in vitro* with SHV-18-expressing *K. pneumoniae* and *P. aeruginosa* strains with high-level AmpC activity [16]. For the isolates in the current study, meropenem exposures were below the typical %*f*T>MIC threshold predictive of carbapenem efficacy [17], yet in combination with nacubactam, significant reductions in bacterial burden were observed *in vivo*, suggestive of an enhancer effect with nacubactam.

Historically, antimicrobial options for enzyme-mediated β-lactam-resistant *P. aeruginosa* were limited and associated with pharmacologic challenges including toxicity (e.g. colistin-based therapy) [8]. Fortunately, the unmet need for safe and reliable therapies for these pathogens has seen the development and approval of several β-lactam/β-lactamase inhibitor combinations (i.e. ceftazidime–avibactam, ceftolozane–tazobactam and meropenem–vaborbactam). However, the spectrum of activity of ceftolozane–tazobactam does not include KPC [8], and the activity of ceftazidime–avibactam and meropenem–vaborbactam against KPC-expressing *P. aeruginosa* has not been fully investigated. With this current observation of enhanced meropenem–nacubactam activity against carbapenem-resistant *P. aeruginosa*, future studies evaluating the relative efficacy of meropenem–nacubactam and other available therapies will provide an opportunity to delineate appropriate treatment options for these challenging pathogens.

Notably, whole-genome sequencing among the AmpC-overproducing isolates in this study revealed mutations in resistance genes such as *mexT* and *oprD*. The *MexT* gene is a known regulator of the MexEF-OprN efflux pump, while the *OprD* gene regulates the entry of carbapenems through outer membrane porins [2,3]. Carbapenem resistance due to loss of porins often occurs in conjunction with other mechanisms such as derepressed AmpC [2,3,18]. The specific impact of *mexT* and *oprD* mutations to meropenem resistance is beyond the scope of this study; however, the efficacy of meropenem–nacubactam implicates AmpC β-lactamase as a significant driver of resistance. Importantly,

differences in the extent of bacterial killing with meropenem–nacubactam against the AmpC-overproducing (2-log kill) and KPC-expressing (4-log kill) isolates in the current study may reflect contributions of porin deletions and efflux pumps among the AmpC-overproducing isolates. Furthermore, the combined presence of β-lactamases and outer membrane porin deficiency has been noted to diminish the effect of novel β-lactamase inhibitors [19,20].

5. Conclusions

In summary, a human-simulated ELF exposure of nacubactam in combination with meropenem effectively reduced the bacterial burden in the lungs of neutropenic mice infected with both AmpC-overproducing and KPC-expressing *P. aeruginosa* isolates. These data support a role for meropenem–nacubactam in the treatment of enzyme-mediated carbapenem-resistant *P. aeruginosa*.

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Ethical approval: The study protocol was approved by the Hartford Hospital Institutional Animal Care and Use Committee (Assurance #A3185-01).

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ARTICLE 4

Nebulized Amikacin and Fosfomycin for Severe *Pseudomonas aeruginosa* Pneumonia: An Experimental Study

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Objectives: Latest trials failed to confirm merits of nebulized amikacin for critically ill patients with nosocomial pneumonia. We studied various nebulized and IV antibiotic regimens in a porcine model of severe *Pseudomonas aeruginosa* pneumonia, resistant to amikacin, fosfomycin, and susceptible to meropenem.

Design: Prospective randomized animal study.

Setting: Animal Research, University of Barcelona, Spain.

Subjects: Thirty female pigs.

Interventions: The animals were randomized to receive nebulized saline solution (CONTROL); nebulized amikacin every 6 hours; nebulized fosfomycin every 6 hours; IV meropenem alone every 8 hours; nebulized amikacin and fosfomycin every 6 hours; amikacin and fosfomycin every 6 hours, with IV meropenem every 8 hours. Nebulization was performed through a vibrating mesh nebulizer. The primary outcome was lung tissue bacterial concentration. Secondary outcomes were tracheal secretions *P. aeruginosa* concentration, clinical variables, lung histology, and development of meropenem resistance.

Measurements and Main Results: We included five animals into each group. Lung *P. aeruginosa* burden varied among groups

($p < 0.001$). In particular, IV meropenem and amikacin and fosfomycin + IV meropenem groups presented lower *P. aeruginosa* concentrations versus amikacin and fosfomycin, amikacin, CONTROL, and fosfomycin groups ($p < 0.05$), without significant difference between these two groups undergoing IV meropenem treatment. The sole use of nebulized antibiotics resulted in dense *P. aeruginosa* accumulation at the edges of the interlobular septa. Amikacin, amikacin and fosfomycin, and amikacin and fosfomycin + IV meropenem effectively reduced *P. aeruginosa* in tracheal secretions ($p < 0.001$). Pathognomonic clinical variables of respiratory infection did not differ among groups. Resistance to meropenem increased in IV meropenem group versus amikacin and fosfomycin + meropenem ($p = 0.004$).

Conclusions: Our findings corroborate that amikacin and fosfomycin alone efficiently reduced *P. aeruginosa* in tracheal secretions, with negligible effects in pulmonary tissue. Combination of amikacin and fosfomycin with IV meropenem does not increase antipseudomonal pulmonary tissue activity, but it does reduce development of meropenem-resistant *P. aeruginosa*, in comparison with the sole use of IV meropenem. Our findings imply potential merits for preemptive use of nebulized antibiotics in order to reduce resistance to IV meropenem. (*Crit Care Med* 2019; XX:00–00)

Key Words: amikacin; antibiotic nebulization; bacterial pneumonia; fosfomycin; mechanical ventilation; *Pseudomonas aeruginosa*

Patients with multidrug resistant (MDR) Gram-negative pneumonia are treated with a combination of IV antibiotics (1, 2). MDR pathogens are on the rise worldwide, endangering future use of broad-spectrum antibiotics (3, 4) and among those, *Pseudomonas aeruginosa* has the ability to rapidly develop resistance (5). Carbapenems are commonly used against *P. aeruginosa*, but worrisome resistance to these antibiotics is on the rise (6, 7), further complicated by the lack of new drug development (8). In addition, carbapenems may inadequately distribute into the pulmonary tissue (9), specifically in the most severe critical patients (10), further sustaining development of bacterial resistance.

Aerosolized antibiotics could overcome these limitations (11, 12), because they may rapidly achieve significant pulmonary concentrations (12), while reducing risks of resistance. In addition, antibacterial agents that could be potentially inhaled, that is, colistin or amikacin, has been associated with serious adverse events (13, 14) when administered systemically. Among the antibiotics that can be nebulized into the respiratory system, amikacin has been vastly explored (15–18). Importantly, when fosfomycin was delivered with an aminoglycoside, killing of Gram-negative pathogens was further enhanced because fosfomycin impairs cell wall synthesis and better penetrates into bacterial biofilms, which ultimately results in increased aminoglycosides uptake (19, 20).

Recently the effects of nebulized amikacin and fosfomycin (AFA), in combination with IV antibiotics, have been tested in patients with Gram-negative pneumonia (21). AFA shortened the time to bacterial eradication but did not lead to any improvement in the Clinical Pulmonary Infection Score (CPIS), which was defined as the primary study outcome or survival. Nonetheless, several limitations in the methods and design of the study may have contributed to these discouraging results, and several critical outcomes, such as pulmonary bacterial and histologic burden and dynamics of antibiotic resistance, were not addressed. Finally, several factors such as humidification, respiratory rate, inspiratory to expiratory ratio, end-inspiratory pause drastically affect mass median aerodynamic diameter of nebulized particles and the overall risk of turbulent airflow, ultimately resulting in inadequate intrapulmonary deposition of antibiotics (22). Regrettably, during clinical trials, a strict control of these factors is challenging leading to potential decrease in efficacy.

Therefore, to elucidate crucial points overlooked in previous study and to further evaluate potential benefits of nebulized AFA, we appraised in animals with severe AFA-resistant *P. aeruginosa* pneumonia, the antibacterial effects of AFA on pulmonary tissue and antibiotic resistance. Furthermore, we investigated the effects of nebulized antibiotics on pulmonary mechanics, hemodynamics, and inflammation.

MATERIALS AND METHODS

Detailed methods are reported in the **Supplemental Digital Content** (http://links.lww.com/CCM/E474). Ethical Committee approved preliminary evaluation and study protocol of the main study, according to the National Research Council guidelines as well as Spanish regulatory principles.

Thirty-seven Large-White Landrace female pigs underwent 78 hours of mechanical ventilation. Inspiratory gases were conditioned through a heated humidifier (Fisher & Paykel, Auckland, New Zealand). As previously reported (23), animals were challenged intrabronchially with *P. aeruginosa*, resistant to AFA (minimal inhibitory concentration [MIC] > 32 mg/L), but susceptible to meropenem (MIC = 0.75 mg/L). Diagnosis of pneumonia was established based on a significant decline in oxygenation, plus an increase in temperature or leukocytosis or purulent secretions. Following diagnosis of pneumonia, animals were randomized into the following groups:

1) Control group (CONTROL): A 6-mL sterile IV solution of 0.9% NaCl was aerosolized every 6 hours. No IV antibiotics were administered.
2) Amikacin: Three-hundred milligram of amikacin diluted into 6 mL of sterile IV solution of 0.9% NaCl were nebulized every 6 hours.
3) Fosfomycin: One-hundred twenty milligram of fosfomycin diluted into 6 mL of sterile IV solution of 0.9% NaCl were nebulized every 6 hours.
4) IV meropenem (IV-MERO): Twenty-five milligram/kilogram of meropenem were administered IV every 8 hours. Additionally, a 6-mL sterile IV solution of 0.9% NaCl was aerosolized every 6 hours.
5) AFA: One-hundred twenty milligram of fosfomycin and 300 mg of amikacin diluted in 6 mL of sterile IV solution of 0.9% NaCl were aerosolized concomitantly every 6 hours.

6) Nebulized AFA and IV meropenem (AFA+IV-MERO): One-hundred twenty milligram of fosfomycin and 300 mg of amikacin diluted in 6 mL of sterile IV solution of 0.9% NaCl were aerosolized concomitantly every 6 hours. In addition, every 8 hours, 25 mg/kg of meropenem were administered IV.

Of note, doses of amikacin, fosfomycin, and IV meropenem were selected based on the results of preliminary laboratory results in three pigs (**eFig. 1**, Supplemental Digital Content 1, http://links.lww.com/CCM/E474) and previous clinical pharmacokinetics/pharmacodynamics data (24, 25). We used a vibrating mesh nebulizer (In-line eFlow Nebulizer System; PARI Respiratory Equipment, Midlothian, VA) (**eFig. 2**, Supplemental Digital Content 1, http://links.lww.com/CCM/E474) and specific ventilatory settings to improve delivery and retaining of nebulized antibiotics. Adverse events were recorded.

Primary Outcome

Upon autopsy, after 6 hours from last antibiotic treatment, we collected and cultured pulmonary biopsies from each lobe to quantify lung tissue *P. aeruginosa* concentration and corroborate differences among groups. In addition, we planned to investigate intergroup dissimilarities, specifically between groups undergoing IV-MERO.

Secondary Outcomes

Microbiology Assessments. Tracheal secretions were collected to quantify *P. aeruginosa* concentration prior to *P. aeruginosa* inoculation (baseline), upon the clinical diagnosis of pneumonia and 24 and 48 hours thereafter. Bronchoalveolar lavage (BAL) was performed prior to *P. aeruginosa* inoculation (baseline), upon the clinical diagnosis of pneumonia, and 48 hours thereafter. We also assessed, in IV-MERO and AFA+ IV-MERO groups, *P. aeruginosa* resistance to meropenem.

Pathology Studies. Upon autopsy, we took a sample from the most affected region, that is, with extensive atelectasis, severe edema, abscess of each of the five lobes for histologic assessment. Lung histology was evaluated according to previously published methods using a six-point injury score (23).

Clinical Variables. Pulmonary mechanics, gas exchanges, and hemodynamics were assessed after surgical preparation (baseline) and every 24 hours thereafter as previously reported (26).

Inflammatory Markers. We collected blood and performed a BAL, after surgical preparation (baseline) and every 24 hours thereafter to quantify systemic and pulmonary tumor necrosis factor–α, interleukin (IL)–6, IL-8, and IL-10 levels.

Pharmacokinetics. In animals enrolled into the amikacin, fosfomycin, and IV-MERO groups, meropenem and amikacin concentration was quantified in plasma at pre dose, 10 minutes, 1, 2, and 4 hours post dosing, in tracheal secretions at pre dose, 10 minutes, 2, and 4 hours, and in BAL at pre dose, 2, and 4 hours post dosing.

Statistical Analysis. Sample size analysis to detect differences in *P. aeruginosa* lung burden is reported in the Supplemental Digital Content (http://links.lww.com/CCM/E474). Continuous variables were analyzed using a restricted maximum likelihood analysis, based on repeated measures approach. Normality of the model residuals was assessed, and in case of not-normal distribution, we used Kruskal-Wallis, Wilcoxon Mann-Whitney *U* and McNemar tests. Multiple comparisons among groups were adjusted through Bonferroni methods. Categorical variables were analyzed using Fisher exact test. All statistical analyses were performed using SAS software (Version 9.4; SAS Institute, Cary, NC).

RESULTS

Thirty of 37 pigs (32.3 ± 2.1 Kg) were included into the study groups (five per group), whereas seven animals were euthanized, before any treatment, for severe hemodynamic/respiratory instability. Pneumonia was clinically diagnosed following 13.6 ± 5.9 hours from the beginning of the study, without differences among groups ($p = 0.610$). **eFigure 3** (Supplemental Digital Content 1, http://links.lww.com/CCM/E474) depicts dynamics of pathognomonic clinical signs of pneumonia. Upon pneumonia diagnosis, secretions were purulent in all animals.

Nebulization

Median number of nebulizations per animal, throughout the study time, was 11 (minimal and maximal number of nebulizations 9–11), without differences among study groups ($p = 0.087$). **eTable 1** (Supplemental Digital Content 1, http://links.lww.com/CCM/E474) reports ventilatory settings during nebulizations and highlights higher F_{IO_2}, respiratory rates (RRs), and levels of positive end-expiratory pressure (PEEP) in the CONTROL group. **eFigure 4** (Supplemental Digital Content 1, http://links.lww.com/CCM/E474) shows changes in critical pulmonary and hemodynamic variables during nebulization among groups, with no effects associated with the nebulization procedure. As shown in **eTable 2** (Supplemental Digital Content 1, http://links.lww.com/CCM/E474), the most common adverse effect of nebulization was an increase in the production of respiratory secretions, detected in more than 70% of the nebulization events in all groups, but IV-MERO ($p < 0.001$).

Primary Outcome

Figure 1*A* shows *P. aeruginosa* burden in lung tissue differed among groups ($p < 0.001$). In particular, median lungs *P. aeruginosa* colonization was higher than 6 log CFU/g in all groups, except in AFA+ IV MERO and IV-MERO, which presented median [interquartile range] of 4.45 [3.70] and 4.39 [2.07], respectively. Of note, intergroup comparisons corroborated statistically significant differences in favor of AFA+ IV MERO and IV-MERO groups ($p < 0.05$), whereas no difference were found between the groups undergoing IV-MERO ($p = 1.00$), irrespective of the use of AFA.

Secondary Outcomes

Microbiological Studies. As shown in **Figure 2*A***, *P. aeruginosa* tracheal secretions colonization differed among study groups ($p < 0.001$). Differently, BAL fluids *P. aeruginosa* concentrations were similar among study groups ($p = 0.153$) (**Fig. 2*B***). In animals treated only with IV-MERO, *P. aeruginosa* progressively became resistant to meropenem versus AFA+IV-MERO ($p = 0.004$) (**Fig. 3**).

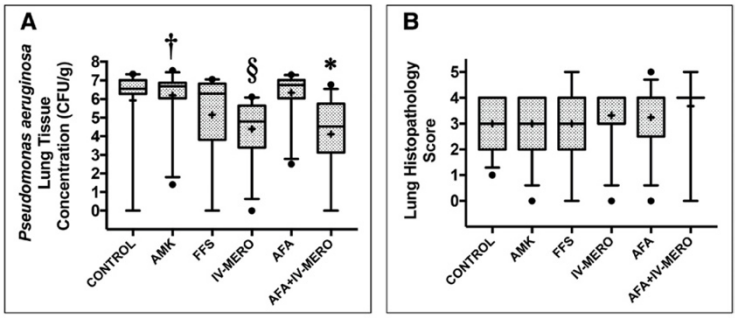

Figure 1. Lung burden upon autopsy after antibiotic treatment. In each box plot, the median value is indicated by the horizontal *line*, the 25th and 75th percentiles are indicated by the lower and the upper hinges of the *box*, whereas *whiskers* represent the fifth and 95th percentiles. Outliers are depicted by the upper and lower *dots*. **A**, The nebulized amikacin + fosfomycin (AFA) + IV meropenem (IV-MERO), and IV-MERO groups showed the highest antipseudomonal activity in pulmonary tissue ($n = 150$; $p < 0.001$) but without statistically significant antibacterial benefits with the use of AFA. *Intergroup comparisons with Bonferroni corrections, $p < 0.05$ versus AFA, nebulized amikacin (AMK), nebulized saline (CONTROL), nebulized fosfomycin (FFS); †Intergroup comparisons with Bonferroni corrections, $p < 0.05$ versus FFS; §Intergroup comparisons with Bonferroni corrections, $p < 0.05$ versus AFA, AMK, CONTROL. **B**, Conversely, Lung Histopathology Score was quite consistent among study groups corroborating on average a score of 3 (pneumonia) in all groups ($n = 150$; $p = 0.186$). CFU = colony-forming unit.

Histology Studies. **Figure 1*B*** shows Lung Histopathology Score among study groups. Lung appearance upon autopsy retrieval and lung/body weight ratio are reported in **eFigures 5** and **6** (Supplemental Digital Content 1, http://links.lww.com/CCM/E474), respectively. A histopathology pattern of bacterial and acute inflammatory infiltration, mainly at the edges of the interlobular septa (**Fig. 4*A***), was found in 60%, 84%, 61%, 26%, 69%, and 9% of the CONTROL, amikacin, fosfomycin, IV-MERO, AFA, and AFA+IV-MERO groups, respectively ($p < 0.001$) (**eTable 3**, Supplemental Digital Content 1, http://links.lww.com/CCM/E474). Another predominant pattern, mainly in animals treated with IV-MERO, was characterized by pathogens and inflammatory infiltrates localized within the alveolar spaces (**Fig. 4*B***) or a mixed pattern in which both components were variably present (**Fig. 4*C***).

Clinical Variables. Clinical variables, averaged throughout the study, are reported in **eTable 4** (Supplemental Digital Content 1, http://links.lww.com/CCM/E474). Irrespective of the aforementioned bactericidal effects of AFA+ IV-MERO and IV-MERO groups, clinical variables were not affected by study treatments, except for the quantity of pulmonary secretions. In particular, the CPIS did not differ among study groups ($p = 0.179$) (**eFig.7**, Supplemental Digital Content 1, http://links.lww.com/CCM/E474). The highest creatinine level was found in the IV-MERO group.

Pulmonary Mechanics and Hemodynamics. RR and PEEP levels were greater in the control group (**eTable 5**, Supplemental Digital Content 1, http://links.lww.com/CCM/E474). In all groups, oxygenation drastically impaired at 24 hours, and the drop was consistent throughout the study, more prominently in the control and AFA groups (**eFig. 8**, Supplemental Digital Content 1, http://links.lww.com/CCM/E474). This was related to a comparable increase in pulmonary shunt. Nebulized or IV antibiotic therapies did not affect pulmonary mechanics (eFig. 8 *D–F*, Supplemental Digital Content 1, http://links.lww.com/CCM/E474). Likewise, study treatments poorly affected hemodynamic variables, except for a more pronounced hyperdynamic status encountered in the fosfomycin group (**eTable 6**, Supplemental Digital Content 1, http://links.lww.com/CCM/E474).

Inflammatory Markers. **eFigure 9** (Supplemental Digital Content 1, http://links.lww.com/CCM/E474) reports

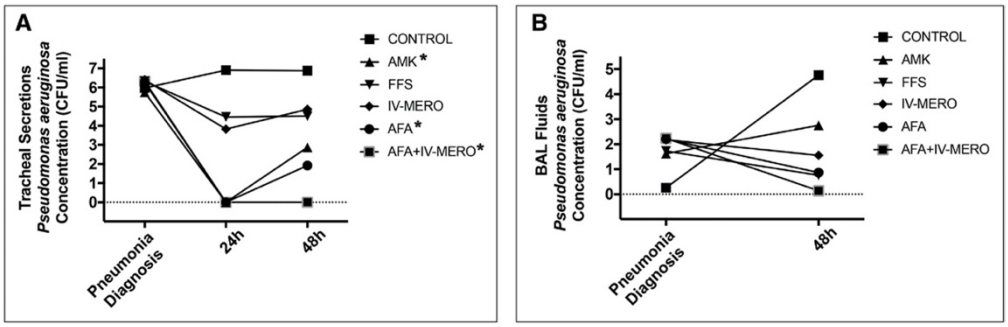

Figure 2. Tracheal secretions and bronchoalveolar lavage (BAL) fluids microbiology studies. Data report median values per each time point among study groups. **A**, Tracheal secretions *Pseudomonas aeruginosa* concentration differed among study groups ($n = 88$; $p < 0.001$). *Intergroup comparisons with Bonferroni corrections, $p < 0.01$ versus nebulized saline (CONTROL), nebulized fosfomycin (FFS), IV meropenem (IV-MERO). **B**, In contrast, BAL fluids *P. aeruginosa* concentration did not differ among study groups ($n = 57$; $p = 0.153$). AFA = nebulized amikacin + fosfomycin, AMK = nebulized amikacin. CFU = colony-forming unit.

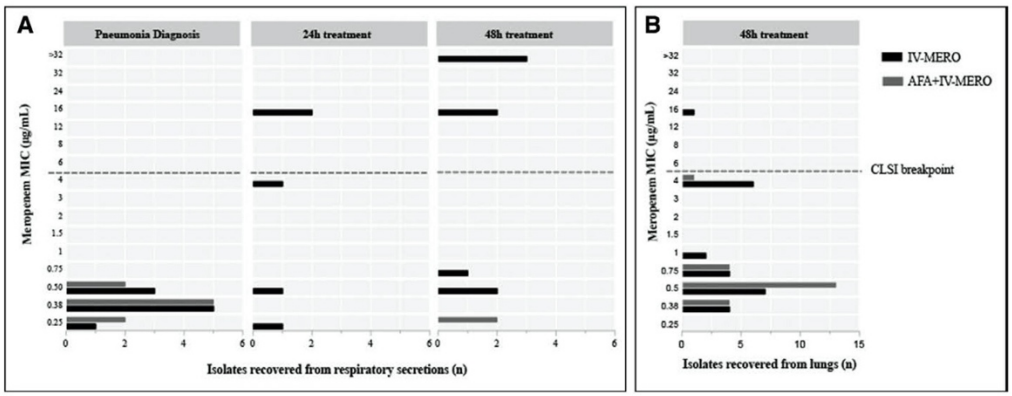

Figure 3. Dynamics of *Pseudomonas aeruginosa* resistance to meropenem. Bar charts depict number of *P. aeruginosa* isolates, yielding the reported meropenem minimal inhibitory concentration (MIC), cultured from tracheal aspirate and bronchoalveolar lavage fluids samples (**A**) and from lung tissue upon autopsy (**B**). The horizontal *line* highlights Clinical and Laboratory Standards Institute (CLSI) breakpoint MIC value for *P. aeruginosa* resistance to meropenem. **A**, Median (interquartile range) meropenem MIC of *P. aeruginosa* isolated in tracheal secretions and bronchoalveolar lavage fluids of animals treated with meropenem alone was 0.38 (0.38–0.50), 4.00 (0.50–16), and 16 (0.62–64) at pneumonia diagnosis, 24, and 48 hr thereafter, respectively, in comparison with animals treated with meropenem and adjuvant inhalation of amikacin and fosfomycin, 0.38 (0.38–0.38), no isolates, and 0.25 (0.25–0.25), respectively ($n = 33$; $p = 0.004$). **B**, Likewise, median meropenem MIC of *P. aeruginosa* isolates in lung tissue of animals treated with meropenem alone was 0.75 (0.50–4.00) in comparison with 0.50 (0.50–0.50) in animals treated with meropenem and adjuvant inhalation of amikacin and fosfomycin ($n = 46$; $p = 0.046$). AFA+IV-MERO = nebulized amikacin + fosfomycin + IV meropenem, IV-MERO = IV meropenem.

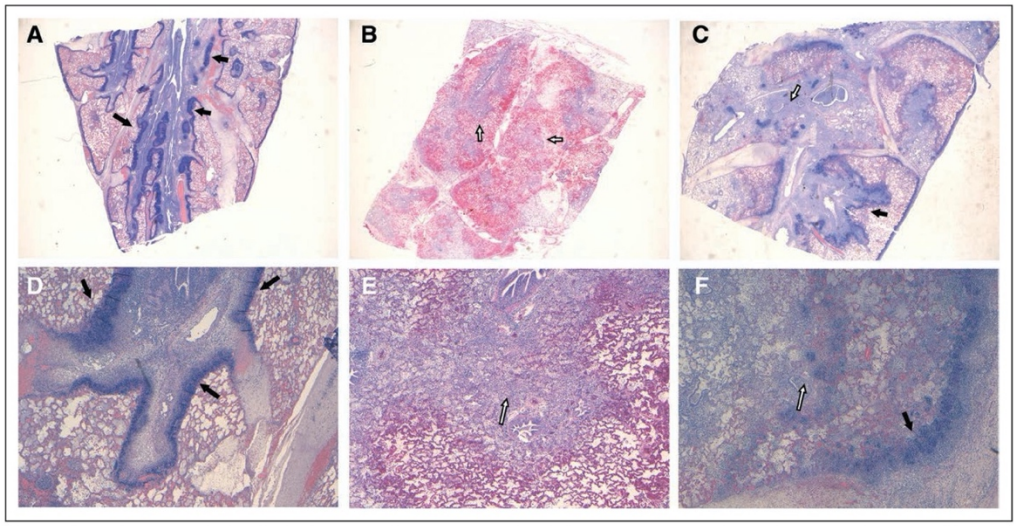

Figure 4. Lung tissue histopathology studies. Representative photomicrographs of pulmonary tissue retrieved upon autopsy. **A** and **D**, Pattern of bacteria and inflammatory cells prominently present within alveolar regions adjacent to interlobular septa is clearly noticeable and highlighted by the *black arrows*. **D**, Greater magnification highlighting profuse infiltration of bacteria and inflammatory cells along the interlobular septal regions (*black arrows*). **B** and **E**, Regular histologic pattern of pneumonia with extensive infiltration by pathogens and inflammatory cells within the centrilobular alveolar regions, highlighted by *white arrows*. **C** and **F**, Show a mixed histologic pattern with both features described above patchily present. *White arrows* depict centrilobular infiltrates, whereas *black arrows* show infiltrates adjacent to intralobular septa. Hematoxylin & eosin staining. Magnification: A–C = ×40, D–F= ×200.

concentration of systemic inflammatory markers in serum. Although the majority of tested cytokines decreased over time, study treatments did not significantly impact any of these inflammatory markers. Similarly, concentration of cytokines tested in BAL fluids (**eFig. 10**, Supplemental Digital Content 1, http://links.lww.com/CCM/E474) marginally varied among groups.

Antibiotics Pharmacokinetics. The highest concentrations of nebulized amikacin were found in tracheal secretions and BAL fluids, whereas marginal figures were found in plasma (eFig. 5 and **eTable 7**, Supplemental Digital Content 1, http://links.lww.com/CCM/E474). In contrast, IV-MERO reached the highest levels in plasma. Importantly, in the IV-MERO group, there was a delay in achieving maximal concentration in tracheal secretions and BAL fluid. Furthermore, we did not find any linear association between MIC of *P. aeruginosa* cultured from lung tissue upon autopsy and meropenem concentrations in tracheal secretions ($p = 0.252$), BAL fluids ($p = 0.342$), and blood ($p = 0.137$).

DISCUSSION

Our experimental study in a porcine model of severe pneumonia by *P. aeruginosa*, resistant to amikacin and susceptible to meropenem, demonstrated that IV-MERO was essential to reduce lung tissue *P. aeruginosa* concentration, whereas the bactericidal efficacy of nebulized antibiotics was most prominent in tracheal secretions. Importantly, the combination of AFA and IV-MERO reduced the risk of developing resistance to the IV antibiotic.

Latest clinical guidelines marginally supported the use of nebulized antibiotics in mechanically ventilated patients (2, 27) owing to the lack of reliable evidence. In this context, animal models of severe pneumonia could provide valuable endpoints, while limiting confounding factors encountered in critically ill patients. Indeed, one of the major strengths of our study was the use of a model of highly severe pneumonia, which was confirmed by severe respiratory/hemodynamic instability, leading to early termination in 20% of the tested animals. Furthermore, we challenged the animals with a *P. aeruginosa* strain resistant to amikacin. Following nebulization, we found amikacin concentrations 1,500-fold and 80-fold the *P. aeruginosa* MIC in tracheal secretions and BAL fluids, respectively, similarly to previous clinical findings (21, 26). Another merit of the animal model used in our experiments was the possibility to sample lung tissues, which would be unfeasible in clinical studies, allowing us to achieve the most critical results of our experiments.

Bactericidal Effects of Nebulized Versus IV Antibiotics

Previous clinical studies (17, 18, 21, 28) failed to convincingly demonstrate merits of the use of amikacin for pneumonia in mechanically ventilated patients. In a phase II clinical trial, Lu et al (18) demonstrated that clinical cure was comparable between nebulized or IV ceftazidime and amikacin, but antibiotic resistance was reduced using nebulized antibiotics. In more recent studies, nebulized amikacin increased pathogens eradication in patients with MDR pneumonia (28); furthermore, in postcardiac surgery patients with MDR Gram-negative pneumonia, nebulized amikacin improved clinical cure (17). Finally, in a recent phase II, multicenter, double-blind trial (21), more than 140 patients with Gram-negative pneumonia received IV meropenem or imipenem, and either AFA, bid for up to 10 days, or nebulized placebo. Although AFA swiftly eradicated Gram-negative bacteria from tracheal secretions, the primary outcome (CPIS score) did not differ between groups.

In order to appropriately interpret our findings in the context of those previous clinical studies, it is important to emphasize that incomplete bacterial tissue clearance could be expected considering the very short course of treatment of our study, even though we doubled the frequency of dosing applied in the clinical trial. Also, in the last decade, several studies have demonstrated that fosfomycin enhances the activity of aminoglycosides (19, 20, 29–31). Mechanistic studies (20) showed that the bactericidal effects of AFA are caused by inhibition of *P. aeruginosa* protein biosynthesis and that fosfomycin increases the uptake of aminoglycosides into *P. aeruginosa*. Nevertheless, in our study AFA showed marginal bactericidal differences in tracheal secretions in comparison with amikacin alone and, in lung tissue, in comparison with IV-MERO.

Meropenem Resistance

Some studies suggested that nebulized antibiotics could also be applied as prophylactic measure to reduce selection pressure by broad-spectrum antibiotics (18, 32). In an interesting in-vitro study, simulating epithelial lining fluid (ELF) exposures of inhaled amikacin and IV-MERO, inhaled amikacin was associated with stability in the IV-MERO MIC of various *P. aeruginosa* strains (33). In line with these studies, we found that even during a 3-day course of IV-MERO, *P. aeruginosa* progressively increased resistance to meropenem, when selection pressure was not modulated by AFA. From a clinical standpoint, these results are important for several reasons. First, we used a *P. aeruginosa* strain resistant to AFA, but susceptibility to IV antibiotic was still ensured. Second, meropenem achieved concentrations above MIC for an extended period between subsequent doses, as clinically recommended (34). Nevertheless, as reported in **Figure 5**, in BAL fluids meropenem overtook *P. aeruginosa* MIC after 3 hours. Furthermore, we failed to find any association between increased meropenem concentrations in blood, BAL fluids, or tracheal secretions and *P. aeruginosa* MIC. This is in line with previous studies demonstrating that *P. aeruginosa* carbapenem resistance is caused by complex and rapidly evolving interactions among several cellular systems, rather than individual mutations (35).

Pathology Studies

Histopathology analysis of lung tissue demonstrated two distinct patterns, suggesting different therapeutic resolutions of the infection. As a matter of fact, we found in animals treated with IV antibiotics pathognomonic signs of pneumonia within the centrilobular alveoli, but bacteria and inflammatory cells were marginally present at alveolar regions close to

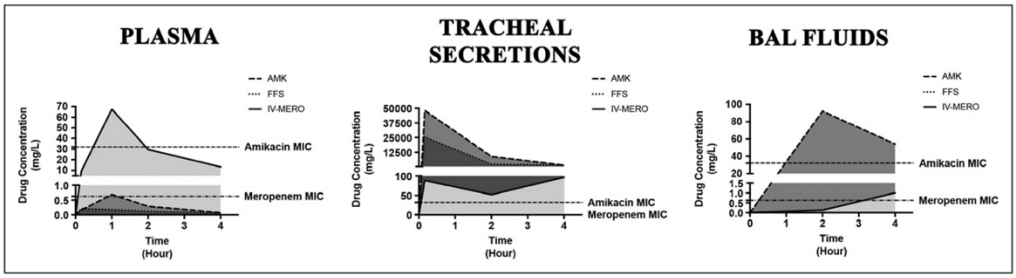

Figure 5. Area under antibiotics concentration time curve from time 0 to 4 hr in various biological matrixes. Of note, horizontal *lines* report minimal inhibitory concentration (MIC) of amikacin (32 mg/L) and meropenem (0.62 mg/L) against *Pseudomonas aeruginosa*. AFA = nebulized amikacin + fosfomycin, AMK = nebulized amikacin, FFS = nebulized fosfomycin, IV-MERO = IV meropenem.

the interlobular septa, from where antibiotics were supplied through blood vessels. The opposite scenario was found during nebulization of antibiotics, advocating that the bactericidal effects of nebulized antibiotics, as expected, first cleared pathogens within the airways and alveoli, but removed incompletely, at least during the very short course of treatment of our study, bacterial reservoirs close to the interlobular septa.

Clinical Variables

As for the effects of the study treatments on other clinical variables, we found that even the most efficacious treatment elusively affected clinical variables and systemic inflammation. Indeed, CPIS marginally varied even in AFA+IV-MERO group, questioning the value of this outcome, which was originally designed as a diagnostic tool for ventilator-associated pneumonia (36). Finally, we found very low plasma amikacin concentrations, and the highest creatinine levels were reported in the IV-MERO group, confirming safety of nebulized amikacin. Furthermore, the only adverse effect, slightly more pronounced in the AFA group, was increased production of mucus during nebulization, without any major adverse effect. Our results and previous clinical findings (32) imply that nebulized antibiotic increase bactericidal efficacy when used concomitantly with IV antibiotics and could theoretically in mechanically ventilated patients reduce relapse by MDR bacteria, which is common and often associated with inappropriate antibiotic therapy (37, 38). Our findings challenge the design of previous randomized clinical trials and imply antibiotic resistance as a primary outcome to enhance the impact and value of future clinical studies. Indeed, in the era of MDR, this would be certainly a crucial improvement, but challenging hurdles should be addressed to meet requirements by regulatory agencies.

Future Research and Clinical Implications

Our findings shed some light on the value of nebulized antibiotics during mechanical ventilation. First, based on aforementioned microbiological and histologic results, the bactericidal synergy of nebulized and IV antibiotics for severe pulmonary infections is arguable and should be potentially reconsidered. In addition, previously reported in-vitro benefits of amikacin-fosfomycin combination are not corroborated by latest findings (21). Second, in our 72-hour study, the hasty development of resistance to IV-MERO is thought-provoking and stimulates further exploratory analysis on the use of nebulized antibiotics to primarily hinder resistance to IV antibiotics, in selected high-risk patients or ICUs. Third, in future clinical studies, judicious selection of efficacy measures will be essential to advance this field of investigation and generate reliable and applicable results.

Limitations

Although we comprehensively evaluated the distinctive effects of AFA, amikacin and fosfomycin, we lacked similar comparisons in animals treated with IV-MERO. Therefore, uncertainty remains on the potential merits of aforementioned antibiotics when applied with IV-MERO. Differently than in previous clinical trials (21), we applied a shorter course of therapy, potentially affecting the efficacy and safety of tested treatments. Second, we evaluated bactericidal and clinical response to treatment only for approximately 65 hours post development of pneumonia; hence, we might have missed long-term benefits or disadvantage of the tested treatments. Third, we did not quantify antibiotics in ELF. ELF antibiotics concentration could provide crucial information to fully understand pulmonary bactericidal efficacy and development of antibiotic resistance and should be prioritized in future laboratory and clinical studies. Finally, our animals did not have comorbidities and were deeply sedated throughout the study. These dissimilarities in comparison with the ICU patient should be pondered for an appropriate translation of our findings.

CONCLUSIONS

In conclusion, in a model of severe *P. aeruginosa* pneumonia resistant to the nebulized antibiotic and susceptible to the IV antibiotic, we found that AFA alone did not efficiently clear pathogens from the lung tissue. In addition, when AFA was used with IV-MERO, increased antipseudomonal activity was only evident in tracheal secretions, without augmented *P. aeruginosa* clearance in lung tissue. Nevertheless, AFA did reduce selective pressure for developing *P. aeruginosa* strains resistant to IV-MERO. Clinical and laboratory research will be essential to confirm the value of AFA to reduce antibiotic resistance.

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Supplemental Material Article 4

Supplemental Digital Content

Li Bassi G et al. NEBULIZED AMIKACIN AND FOSFOMYCIN FOR SEVERE *PSEUDOMONAS AERUGINOSA* PNEUMONIA: AN EXPERIMENTAL STUDY

Table of Contents

Materials and Methods

Preliminary studies

Prior to the commencement of the main study, we conducted preliminary evaluations in three pigs, anesthetized, on mechanical ventilation and challenged with *Pseudomonas aeruginosa*, as reported below, and randomized as follows:

1. Control group (CONTROL): A 6-mL sterile IV solution of 0.9% NaCl was aerosolized every 6h. No intravenous antibiotics were administered.
2. Amikacin/fosfomycin (AFA): 120 mg of fosfomycin and 300 mg of amikacin diluted in 6 ml of sterile IV solution of 0.9% NaCl were aerosolized concomitantly every 6 hours.
3. Nebulized amikacin/fosfomycin and intravenous meropenem (AFA+IV-MERO): 120 mg of fosfomycin and 300 mg of amikacin diluted in 6 ml of sterile IV solution of 0.9% NaCl were aerosolized concomitantly every 6 hours. In addition, every 8 hours, 25 mg/kg of meropenem were administered intravenously.

Doses of nebulized AFA were based on the results of previous pharmacokinetics/pharmacodynamic clinical studies (1, 2). After 78 hours of mechanical ventilation, pigs were euthanized and lungs harvested and biopsied for *Pseudomonas aeruginosa* quantitative cultures to evaluate preliminary efficacy of the interventions. Preliminary results are shown in Figure 1, Supplemental digital content. In particular, the AFA and AFA + IV-MERO pigs were studied for up to 78 hours. In contrast, CONTROL animal was euthanized after only 36 hours for severe haemodynamic and respiratory instability refractory to maximal doses of vasoactive drugs.

Animal preparation and general settings

We carried out a laboratory *in-vivo* study in Large-White Landrace pigs, undergoing 78 hours of mechanical ventilation. Each pig of approximately 30-35 Kg was induced with 2-2.5 mg/Kg of propofol and orotracheally intubated with a 7.5 mm I.D ETT (Hi-Lo®, Covidien, Boulder, CO). Following intubation, pigs were ventilated through a SERVO-i mechanical ventilator (Maquet, Wayne, NJ. USA). Ventilatory parameters were initially set as follows: volume-control, tidal volume (V_T) 8 ml/Kg, pressure trigger sensitivity of -2 cm H_2O, inspiratory fraction of oxygen 0.4, duty cycle 0.33, inspiratory rise time 5%, inspiratory pause 10%, PEEP 3 cm H_2O and respiratory rate (RR) adjusted to maintain normocapnia. Inspiratory gases were conditioned through a heated humidifier (Fisher & Paykel, Auckland, New Zealand). The humidifier was set to maintain the airway temperature proximal to the 'Y' piece at 37ºC. The inspiratory line was heated. Throughout the study, internal endotracheal tube (ETT) cuff pressure was maintained at 28 cm H_20. Midazolam and fentanyl were administered as previously reported (1) to ensure absence of response to painful stimulation. Boluses of 2 mg/kg of propofol were administered as needed.

Ultrasound-guided cannulation of the femoral artery was performed for systemic arterial pressure monitoring and collection of blood samples. We surgically cannulated the jugular vein to insert an 8-Fr introducer and a 7-Fr Swan-Ganz

catheter (Swan-Ganz PAC, Edwards Lifesciences, Irvine, CA) for advanced hemodynamic monitoring. A no. 8 Foley catheter was introduced into the urinary bladder through surgical mini-pelvectomy.

Following surgical preparation, the pig was placed in the prone position. Fluid balance was maintained through infusion of lactate Ringer's and 0.9% NaCl solutions. In order to prevent pneumonia caused by endogenous oropharyngeal flora, 1 gr. of ceftriaxone was administered intravenously 30 min before intubation and then 50 mg/Kg every 12 hours for the entire duration of the study. Every 24 hours, arterial and mixed venous blood gases, hemodynamics, urine output and ventilatory settings were assessed. Every 24 hours, complete blood count, biochemistry and coagulation studies were carried out and reviewed.

Bacterial Challenge

Animals were challenged immediately after stabilization from surgical preparation – approximately 4 hours from the beginning of mechanical ventilation. Fifteen mL of 10^7 cfu/mL of a log-phase culture of *P. aeruginosa* was instilled into each pulmonary lobe of the animals. During instillation, the animals were kept in prone, slight Trendelenburg position. A clinical isolate of *P. aeruginosa* was employed expressing high-level resistance to both ceftriaxone (minimal inhibitory concentration (MIC) > 256 mg/L) and amikacin (MIC > 32 mg/L), but with susceptibility to meropenem (MIC = 0.75 mg/L). After 20 hours from the inoculation, 24 hours from the beginning of the study, we clinically confirmed pneumonia based on a decline in the ratio of arterial partial pressure and inspiratory fraction of oxygen (PaO_2/FiO_2), plus one of the following signs of infection: increase in body temperature, leukocytosis and purulent secretions.

Randomization

Following diagnosis of pneumonia, animals were randomized into the following groups. Randomization schedule was developed through SAS software (SAS version 9.4, 100 SAS Campus Drive Cary, NC, USA) clustered in randomization blocks of 6. Five pigs were assigned into each of the following groups:

1. CONTROL: As reported in methods of preliminary studies
2. Amikacin (AMK) 300 mg of amikacin diluted into 6 ml of sterile IV solution of 0.9% NaCl were nebulized every 6 hours.
3. Fosfomycin (FFS): 120 mg of fosfomycin diluted into 6 ml of sterile IV solution of 0.9% NaCl were nebulized every 6 hours.
4. Intravenous meropenem (IV-MERO): 25 mg/kg of meropenem were administered intravenously every 8 hours. Additionally, a 6-mL sterile IV solution of 0.9% NaCl was aerosolized every 6h.
5. AFA: As reported in methods of preliminary studies
6. AFA+IV-MERO: As reported in methods of preliminary studies

Doses of AMK, FFS and IV-MERO were selected based on the results of preliminary laboratory results in three pigs, as reported in Figure 1-Supplemental Digital Content, and previous clinical pharmacokinetics/pharmacodynamics data (24, 25).

Nebulization of antibiotics

Upon diagnosis of pneumonia, and every 6 hours thereafter, nebulization of antibiotics or saline was carried out through a vibrating mesh nebulizer (In-line eFlow Nebulizer System, PARI Respiratory Equipment, Midlothian, VA) positioned at the inspiratory limb, 15 cm from the Y-piece(2,3) (Supplemental Digital Material, Figure 1). During aerosolization, specific ventilatory settings were applied to optimize delivery and reduce expiratory-flow clearance. First, we increased the dose of sedatives and analgesics by 20%. Lack of cough reflex was corroborated prior to the beginning of nebulization. We applied volume-controlled ventilation, RR was reduced by 40%, the inspiratory rise time was decreased to 0% and the inspiratory pause was increased to 20%. The inspiratory-expiratory (I:E) was increased through sequential steps, until the difference between peak expiratory flow and inspiratory flow was $\leq$ 2 L/min. Throughout the nebulization procedure, in case of ventilatory discoordination, 2 mg/kg of propofol were administered. All adverse events associated with the intervention, such as significant bronchospasm or oxygen desaturation were recorded. Following activation of the nebulizer electronic controller, nebulized vapor at the inspiratory limb was corroborated and continuously monitored. Upon consistence disappearance of vapor at the inspiratory limb, we deactivated the electronic controller and we recorded the duration of nebulization.

Respiratory Measurements

Pulmonary mechanics and gas exchanges were assessed after surgical preparation (baseline) and every 24 hours thereafter. Airway pressure and respiratory flow rates were measured as previously reported (4). Flow and pressure signals were recorded on a personal computer for subsequent analysis with dedicated software (Colligo; Elekton, Milan, Italy). Tidal volumes were obtained by mathematical integration of the measured flow signal. The static elastance of the respiratory system, lung and chest wall; total inspiratory resistance; inspiratory airflow resistance and inspiratory tissue resistance were calculated through the rapid occlusion method using standard formulae (5).

Hemodynamic Measurements

Hemodynamics were assessed after surgical preparation (baseline), and every 24 hours thereafter. Arterial and venous pressures were measured with disposable pressure transducers (TrueWave Pressure Transducer, Edwards Lifescience, Irvine, CA, USA). Pulmonary artery pressure, central venous pressure, pulmonary artery wedged pressure, core blood temperature, and cardiac output were measured using a Swan–Ganz catheter. The systemic and pulmonary vascular resistances and venous admixture were calculated using standard formulae (4).

Microbiological Evaluations

Tracheal Secretions

Tracheal secretions were aspirated to quantify *P. aeruginosa* concentration upon the clinical diagnosis of pneumonia, and 24 and 48 hours thereafter. Quantitative *P.aeruginosa* cultures of tracheal secretions was performed using standard methods (1). Ultimately, bacteria were identified by mass spectrometry through a Microflex LT (BrukerDaltonics GmbH, Leipzig, Germany) and bacterial identification was performed using the MALDI BioTyper 2.0 software (BrukerDaltonics).

Bronchoalveolar lavage

Bronchoalveolar lavage (BAL) was performed in the right medium lobe with three 10-mL aliquots of sterile saline solution taken prior to PA inoculation (baseline), upon the clinical diagnosis of pneumonia, and 48 hours thereafter. The first aliquot was discarded upon recollection. *P. aeruginosa* concentration in BAL fluids was quantified as reported above.

Pseudomonas aeruginosa resistance to meropenem

In AFA+IV-MERO and IV-MERO groups we compared resistance against meropenem of *P.aeruginosa* strains isolated from tracheal secretions, BAL fluids and lung tissue. Minimal inhibitory concentration (MIC) testing was conducted by Etest (bioMérieux Inc., Hazelwood, MO). MICs were determined in triplicate for each strain. Meropenem MIC results were classified as susceptible intermediate or resistant based on the 2018 CLSI breakpoints (susceptible, ≤2 μg/ml; intermediate, 4 μg/ml; resistant, ≥8 μg/ml) (CLSI M100, 28th ed. January 2018)

Systemic inflammatory markers

Upon the clinical diagnosis of pneumonia, and 24 and 48 hours thereafter, blood was collected to quantify serum tumor necrosis factor, interleukin-6, interleukin-8 and interleukin-10 levels using the enzyme-linked immunosorbent assay method in specific porcine kits (R&D Systems, Minneapolis, MN). At the same timepoints, aforementioned cytokines were quantified in the BAL supernatant using the enzyme-linked immunosorbent assay method in specific porcine kits (R&D Systems, Minneapolis, MN).

Pharmacokinetics/pharmacodynamics studies

In animals enrolled into the amikacin, fosfomycin and meropenem alone groups (15 animals), upon the first aerosolization, or the first administration of intravenous meropenem, concentrations of antibiotics were measured from blood samples, taken at pre-dose, 10 minutes, 1, 2, 4 and 4 hours post dosing. Lithium heparin tubes were used for collecting blood. The blood samples were centrifuged at 3000 rpm for 10 min, in a refrigerated centrifuge set at 4°C. The resultant plasma was separated, transferred to uniquely labelled, clear, polypropylene tubes (2 tubes per animal per time point), frozen immediately over dry ice and then transferred to a freezer set to maintain -80°C. The urea concentration in plasma was also assayed. Antibiotics were also quantified in tracheal aspirate at pre-dose, 10 minutes, 2, and 4 hours post

dosing and in BAL at pre-dose, 2 and 4 hours post dosing. Frozen plasma, tracheal aspirate, BAL and tissue samples of amikacin and fosfomycin groups were sent to an external laboratory for quantification of antibiotic concentrations (Charles River Laboratories, Tranent, Edinburgh). Meropenem in plasma, tracheal aspirates, BAL and right lower lobe were measured through high-performance liquid chromatography (HPLC) with ultraviolet-visible detection (UV-visible) at Hartford Hospital. Individual plasma drug concentration-time data were used to calculate meropenem pharmacokinetics parameters.

Stopping rules and autopsy

Following 6 hours from last antibiotic administration, pigs were euthanized with 60 mEq of potassium chloride when PaO_2/FIO_2 was less than 70 mmHg, irrespective of maximal ventilatory support, when septic hemodynamic instability was un-responsive to high doses of inotropes. Given that our primary objective was the reduction in *P.aeruginosa* count in lung tissue, which is a parameter highly associated with the time on mechanical ventilation and duration of antibiotic treatment, the experiment was repeated in case of early study discontinuation – within 24 hours after the bacterial inoculum – owing to respiratory or hemodynamic instability not responding to maximal treatment.

Upon autopsy, animals were positioned supine, the lungs were exposed under strict asepsis, excised and placed on sterile drapes. Two samples were taken from the most affected region of each of the five lobes for histological and microbiological assessments. Lung histology was evaluated according to previously published methods using a 6-point injury score.(6) We biopsied each lobe and an experienced pathologist evaluated the lung tissue by using a validated pneumonia severity score (from 0-5 points): 0 point = no pneumonia; 1 point = purulent mucous plugging; 2 points = bronchiolitis; 3 points = pneumonia; 4 points = confluent pneumonia; and 5 points = abscessed pneumonia.(1) Classification of each specimen was based upon the worst category observed. Furthermore, quantitative cultures were performed using standard methods. We assessed bacteria growth by mass spectrometry (Microflex-LT, BrukerDaltonics) and automated bacterial identification (MALDI-BioTyper, BrukerDaltonics).

Statistical analysis

Based on previous studies (6), it was expected *P. aeruginosa* lung tissue concentration greater than 4 log cfu/gr in the CONTROL group. Furthermore, we estimated in the AFA, IV-MERO and AFA+IV-MERO groups, *P. aeruginosa* lung tissue concentration ≤ 2 log cfu/gr. Finally, in the FSF and AMK groups, we estimated *P. aeruginosa* lung tissue concentration of 3 log cfu/gr. We expected that the fixed standard deviation of *P. aeruginosa* lung tissue concentration for each of the six groups were 1 log cfu/gr. Therefore, for a desired statistical power of 80% and type 1 bias of 5%, a sample size of 5 pigs in each group was required to demonstrate significant difference in *P. aeruginosa* lung tissue concentrations among the six groups. Continuous variables were described as means and standard deviations or median [interquartile range]. Categorical variables were described as frequencies and percentages. Continuous variables were

analyzed using a restricted maximum likelihood (REML) analysis, based on repeated measures approach (PROC MIXED), including study groups, times of assessment and their interaction as factors. A compound symmetry or univariate (co)variance structure was used to model the within-patient errors and the Kenward-Roger or Between-Within approximations to estimate denominator degrees of freedom. For each continuous variable, the overall F test was first assessed for significance ($p \leq 0.05$). Two-sided comparisons among groups was also performed and a given comparison was considered significant if its p-value was ≤ 0.05. Each pair-wise comparison was corrected using Bonferroni test, in order to control for the experiment-wise error rate. We tested the assumption in PROC MIXED about normality of the model residuals, in case of not-normal distribution we used non-parametric tests. In particular, we used Kruskal-Wallis test for comparisons among study groups, with post-hoc two-sided comparisons through Wilcoxon Mann-Whitney test with Bonferroni correction. We used instead McNemar test for comparisons among times of assessment without post-hoc comparisons. Categorical variables were analyzed using Fisher's exact test. Finally, regression analysis was performed to appraise association between clinical pulmonary infection score and pulmonary burden. Overall, a two-sided p-value ≤ 0.05 was considered statistically significant. All statistical analyses were performed using SAS software (version 9.4; SAS Institute, Cary, NC, USA).

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Additional Results

eTable 1: Ventilatory settings during nebulization

	CONTROL (N. 50)	AMK (N. 55)	FFS (N. 54)	IV-MERO (N. 52)	AFA (N. 42)	AFA+IV-MERO (N. 54)	P-value
F_IO_2 (%)	70.4±15.6[b]	55.8±7.2	58.2±6.5	53.9±5.8	64.3±20.7[a]	57.1±6.9	<0.001
RR (breaths/min)	18.2±3.1[c]	15.5±3.3	15.1±4.1	15.8±2.8	16.5±2.5	16.1±3.5	0.001
Duty Cycle (%)	5.9[2.9]	5.2[2.8]	5.9[2.8]	5.9[2.8]	5.9[1.2]	5.9[2.8]	0.890
Inspiratory Flow (L/min)	83.9±24.7	85.9±24.7	78.6±17.6	84.6±22.2	76.6±23.6	81.2±18.1	0.332
Expiratory-Inspiratory Flow Difference (L/min)	-4.0[36.0]	-12.0[36.0]	-12.0[31.0]	-18.0[37.0]	-7.0[16.0]	-11.0[23.0]	0.210
PEEP (cmH_2O)	9.1±2.1[d]	8.3±1.2	8.3±1.0	7.7±1.5	9.2±1.2†	8.4±1.3	<0.001

Data are reported as means ± standard deviation or median [IQR] for normally and non-normally distributed variables. AFA, nebulized amikacin+fosfomycin; AFA+IV-MERO, nebulized amikacin+fosfomycin+intravenous meropenem; AMK, nebulized amikacin; FFS, nebulized fosfomycin; IV-MERO, intravenous meropenem; F_IO_2, inspiratory

fraction of oxygen; RR, respiratory rate; MPAP, mean pulmonary arterial pressure; PEEP, positive end-expiratory pressure. [a] post-hoc analysis with Bonferroni correction, p<0.05 vs AMK, IV-MERO; [b] post-hoc analysis with Bonferroni correction, p<0.05 vs AFA+IV-MERO, AMK, FFS, IV-MERO; [c] post-hoc analysis with Bonferroni correction, p<0.05 vs AMK, FFS, IV-MERO; [d] post-hoc analysis with Bonferroni correction, p<0.05 vs IV-MERO

eTable 2 Adverse effects during nebulization procedure

	CONTROL (N. 50)	AMK (N. 55)	FFS (N. 54)	IV-MERO (N. 52)	AFA (N. 42)	AFA+IV-MERO (N. 54)	P-value
Coughing (%)	6.4	1.9	0.0	4.3	5.1	0.0	0.226
Bronchoconstriction (%)	0.0	0.0	0.0	0.0	2.6	0.0	0.138
Oxygen desaturation (%)	0.0	0.0	0.0	0.0	2.6	0.0	0.138
Increased mucus production (%)	76.6	77.4	75.0	39.1	79.5	74.0	<0.001

Data are reported as incidence of adverse effects (%). AFA, nebulized amikacin+fosfomycin; AFA+ IV-MERO, nebulized amikacin+fosfomycin+intravenous meropenem; AMK, nebulized amikacin; FFS, nebulized fosfomycin; IV-MERO, intravenous meropenem.

eTable 3 Histopathology pulmonary patterns

Histological areas with predominant presence of bacteria and neutrophils	CONTROL (N. 25)	AMK (N. 25)	FFS (N.21)	IV-MERO (N.23)	AFA (N. 23)	AFA+IV-MERO (N. 21)
Centri-lobular alveoli (%)	40	16	38.1	73.9	30.4	90.5
Alveoli adjacent to intralobular septa (%)	60	84	61.9	26.1	69.6	9.5

Data are depicted in percentages of analyzed lung tissues. AFA, nebulized amikacin+fosfomycin; AFA+ IV-MERO, nebulized amikacin+fosfomycin+intravenous meropenem; AMK, nebulized amikacin; FFS, nebulized fosfomycin; IV-MERO, intravenous meropenem. Histology studies of lung tissue after 2.5 days of treatment showed bacterial and neutrophilic infiltrates predominantly present in different regions ($p<0.001$). Of note, when mixed patterns were found only the most predominant of aforementioned patterns was reported.

eTable 4 Clinical parameters throughout the study

	CONTROL (N. 20)	AMK (N. 20)	FFS (N. 20)	IV-MERO (N. 20)	AFA (N. 20)	AFA+MERO (N. 20)	P-value
Body Temperature (ºC)	38.1[37.1-38.6]	38.0[36.9-38.6]	37.9[37.6-38.8]	38.0[36.6-38.2]	38.0[37.9-38.7]	38.5[37.8-38.8]	0.063
Tracheal Secretions Quantity	2[1.5-2.5][a]	1.0[0.0-2.0]	1.0[0.0-2.0]	1.0[0.0-2.0]	1.0[0.0-2.0]	1.0[0.0-2.0]	0.014
Purulent Tracheal Secretions (%)	88.2	100	92.8	92.3	85.7	85.7	0.887
WBC (10^9/L)	11.1[9.4-22.7]	16.0[9.3-24.2]	16.0[11.1-27.1]	15.4[9.7-25.9]	14.5[8.6-22.6]	9.9[5.8-15.3]	0.129
Hb (g/L)	8.8[7.7-10.0]	8.4[6.9-10.0]	9.1[7.7-10.1]	8.8[7.3-10.5]	9.2[7.9-10.5]	9.3[7.7-11.6]	0.812
Platelets (10^9/L)	219.5[126.0-326.0]	173.0[144.5-353.0]	171.0[137.0-288.0]	178.0[117.5-261.0]	175.0[89.5-291.0]	143.5[94.0-289.0]	0.070
Creatinine (mg/dL)	0.9[0.7-1.1]	0.9[0.8-1.1]	1.1[0.9-1.4]	1.1[1.1-1.5][b]	0.9[0.8-1.0]	1.2[0.9-1.3]	0.001
PT (sec)	12.6[11.7-13.7]	11.8[11.4-12.7]	12.1[11.2-12.7]	12.3[11.3-13.1]	12.0[11.5-13.6]	12.4[11.3-14.4]	0.644
PTT (sec)	22.0[22.0-22.0]	22.0[22.0-22.0]	22.0[22.0-22.0]	22.0[22.0-22.0]	22.0[22.0-22.0]	22.0[22.0-22.0]	0.506

Data are reported as median [interquartile range]. AFA, nebulized amikacin+fosfomycin; AFA+IV-MERO, nebulized amikacin+fosfomycin+intravenous meropenem; AMK, nebulized amikacin; FFS, nebulized fosfomycin; IV-MERO, intravenous meropenem. Body temperature, white blood cells (WBC), hemoglobin (Hb), platelets, creatinine, prothrombin time (PT) and thromboplastin time (PTT) varied among study times (0, 24, 48 and 72 hours) ($p<0.001$). Of note, in pigs, PT reference range is between 9 and 12 sec, instead PTT reference range varies between 20 and 30 sec. [a] post-hoc comparison, $p<0.05$ vs IV-MERO; [b] post-hoc analysis with Bonferroni correction, $p<0.05$ vs AFA, AMK, CONTROL.

eTable 5: Additional ventilatory and blood gas parameters throughout the study

	CONTROL (N. 20)	AMK (N. 20)	FFS (N. 20)	IV-MERO (N. 20)	AFA (N. 20)	AFA+MERO (N. 20)	P-value
RR (breaths/min)	29.0[23.0-34.5][a]	24.0[20.0-26.5]	22.5[20.0-28.5]	27.0[24.0-33.0]	24.0[20.0-30.0]	24.0[21.0-28.0]	0.001
PEEP (cmH_2O)	9.0[5.5-10.0][b]	8.0[5.5-8.5]	8.0[4.0-9.0]	7.0[4.5-8.0]	8.0[3.0-10.0]	8.0[6.0-9.0]	0.017
pH	7.49[7.41-7.52]	7.51[7.46-7.55]	7.49[7.43-7.54]	7.51[7.45-7.56]	7.48[7.42-7.54]	7.54[7.52-7.55]	0.262
$PaCO_2$ (mmHg)	40.9[38.7-43.9]	37.4[36.3-41.5]	39.5[35.8-46.8]	37.9[35.4-42.9]	38.7[35.7-44.5]	37.8[35.0-43.6]	0.618

Data are reported as median [interquartile range]. AFA, nebulized amikacin+fosfomycin; AFA+IV-MERO, nebulized amikacin+fosfomycin+intravenous meropenem; AMK, nebulized amikacin; FFS, nebulized fosfomycin; IV-MERO, intravenous meropenem; RR, respiratory rate; PEEP, positive end-expiratory pressure; [a] post-hoc analysis with Bonferroni correction, $p<0.05$ vs AFA, AFA+IV-MERO, AMK, FFS, IV-MERO. [b] post-hoc analysis with Bonferroni correction, $p<0.05$ vs IV-MERO.

eTable 6: Hemodynamic parameters

	CONTROL (N. 20)	AMK (N. 20)	FFS (N. 20)	IV-MERO (N. 20)	AFA (N. 20)	AFA+MERO (N. 20)	P-value
HR (beats/min)	66.5[55.0-91.5]	65.0[56.5-77.5]	68.5[54.5-82.0]	55.0[45.0-70.0]	65.0[48.0-86.0]	66.0[53.5-99.0]	0.110
MAP (mmHg)	75.5[66.8-83.2]	72.6[66.5-79.0]	69.2[67.0-75.0]	74.8[68.7-82.0]	76.0[70.0-79.0]	80.5[69.5-87.0]	0.296
MPAP (mmHg)	21.8[18.2-25.0]	19.7[14.5-21.3]	19.0[15.0-26.8]	20.2[17.7-22.0]	21.7[23.7-17.0]	19.8[17.5-24.5]	0.767
CVP (mmHg)	8.0[4.0-9.0]	5.0[8.0-10.0]	8.5[4.0-10.0]	9.0[7.5-11.0]	9.0[5.0-11.0]	9.0[5.0-13.0]	0.360
PCWP (mmHg)	10.0[7.0-12.0]	10.0[7.5-11.5]	11.0[7.5-12.0]	11.0[9.0-13.5]	9.0[6.0-14.0]	11.0[7.5-14.0]	0.653
CO (L/min)	3.5[2.7-4.2]	3.2[2.6-4.8]	3.7[2.9-5.3][a]	2.8[2.3-3.5]	2.9[2.5-3.9]	3.2[2.6-4.8]	0.025
SVR (dynes/sec/cm^{-5})	1661[1177-2029]	1621[1362-1826]	1390[843-1976][b]	1977[1554-2595]	1806[1888-2190]	1700[1241-2244]	0.017
PVR (dynes/sec/cm^{-5})	253[217-300]	224[180-268]	185[154-229]	230[186-300]	248[224-317]	203[169-271]	0.054
Fluid Balance (mL)	-131[-466-377]	107[-560-1106]	-58[-565-1038]	-145[-131-577]	311[-155-1398]	264[-172-1136]	0.655
Vasopressor Dependency Index (µg/Kg/min)	0.3[0.0-6.4]	0.9[0.0-3.3]	0.4[0.0-7.8]	0.0[0.0-2.5]	0.0[0.0-1.5]	1.1[0.0-4.7]	0.503

Data are reported as median [interquartile range]. AFA, nebulized amikacin+fosfomycin; AFA+IV-MERO, nebulized amikacin+fosfomycin+intravenous meropenem; AMK, nebulized amikacin; FFS, nebulized fosfomycin; IV-MERO, intravenous meropenem; HR, heart rate; MAP, mean arterial pressure; MPAP, mean pulmonary arterial pressure; CVP, central venous pressure; PCWP, pulmonary capillary wedge pressure; CO, cardiac output; SVR, systemic vascular resistance; PVR, pulmonary vascular resistance. Of note, normal cardiac output in pigs of 30-35 Kg ranges between 2.0-3.0 L/min, while systemic and pulmonary vascular resistance range 1600-2400 and 500-600 dynes/sec/cm, respectively. [a] post-hoc analysis with Bonferroni correction, $p<0.05$ vs AFA, IV-MERO. [b] post-hoc analysis with Bonferroni correction, $p<0.05$ vs IV-MERO.

eTable 7: Pharmacokinetic parameters

	AMK (N. 5)	FFS (N. 5)	IV-MERO (N. 5)	P-value
Plasma				
Tmax (min)	10[10-60]	35[10-60]	60[60-60]	0.072
Cmax (mg/L)	0.00[0.00-0.81]	0.14[0.00-0.45]	76.40[57.24-78.55][a]	<0.001
AUC_{0-4} (mg*h/L)	0.00[0.00-1.78]	0.12[0.00-1.01]	143.8[102.8-154.8][a]	<0.001
AUC_{0-4}/MIC (mg*h/L)	0.00[0.00-0.00]	NA	230[164-248] [b]	<0.001
Tracheal Secretions				
Tmax (min)	10[10-10]	35[10-10]	120[120-240][a]	<0.001
Cmax (mg/L)	16000[7057-109791]	26008[7025-44000]	116[39-194]	0.062
AUC_{0-4} (mg*h/L)	16384[7227-155604]	33574[7791-78102]	428[76-586]	0.060
AUC_{0-4}/MIC (mg*h/L)	512[225-4862]	NA	686[122-937]	0.902
BAL Fluids				
Tmax (hour)	120[120-240]	NA	240[240-240]	0.001
Cmax (mg/L)	60.8[16.2-145.6]	NA	0.1[0.0-1.1]	0.001
AUC_{0-4} (mg*h/L)	127[16.3-333]	NA	0.1[0.0-2.4]	0.001
AUC_{0-4}/MIC (mg*h/L)	3.9[0.5-10.4]	NA	0.2[0.0-3.8]	0.142

Data are reported as median values and interquartile range. AMK, nebulized amikacin; FFS, nebulized fosfomycin; IV-MERO, intravenous meropenem. Tmax, time to reach peak maximal concentration; Cmax, maximal concentration; AUC_{0-4}, area under the concentration time curve from time 0 to 4 hr; AUC_{0-4}/MIC, ratio between area under the concentration time curve from time 0 to 4 hr and minimal inhibitory concentration of reported antibiotic against P. aeruginosa used in this study. [a]post-hoc analyses with Bonferroni correction vs. AMK and FFS; [b] post-hoc analyses with Bonferroni correction vs. AMK.

FIGURE LEGENDS

eFigure 1: Lung *Pseudomonas aeruginosa* concentration during preliminary evaluations

AFA and AFA+IV-MERO pigs survived up to 78h, while the animal in the control group was studied only up to 36 hours (24 hours post development of pneumonia) for severe hemodynamic and respiratory instability. AFA, nebulized amikacin + fosfomycin; AFA+IV-MERO, nebulized amikacin + fosfomycin + intravenous meropenem.

eFigure 2: Nebulization system

Antibiotics were delivered via the PARI eFlow Inline Nebulizer Inline System comprising an electronic controller (A left section) and a vibrating plate nebulizer (A right section). The vibrating mesh of the nebulizer contains small laser-drilled holes; thus, droplets in the range of 3.5 microns are produced. The respiratory circuit is depicted at the B section, depicting the nebulizer appropriately placed at the inspiratory limb. Nebulization was not synchronized with the inspiratory phase of the breathing cycle.

eFigure 3: Clinical signs of pneumonia

Data are reported as median. A, Ratio of partial pressure of oxygen and inspiratory fraction of oxygen (PaO_2/FIO_2) varied between times of assessments (N. 60, $p<0.001$) without differences among study groups (N. 60, $p=0.450$). B, Body temperature significantly increased between assessments at baseline and upon diagnosis of pneumonia (N. 60, $p<0.001$), without differences among study groups (N. 60, $p=0.476$). C, White blood cell count (WBC) significantly increased between assessments at baseline and diagnosis of pneumonia (N. 60, $p<0.001$), in a homogenous fashion among study groups (N. 60, $p=0.920$). AFA, nebulized amikacin + fosfomycin; AFA+IV-MERO, nebulized amikacin + fosfomycin + intravenous meropenem; AMK, nebulized amikacin; FFS, nebulized fosfomycin; IV-MERO, intravenous meropenem.

eFigure 4: Changes in critical variables during nebulization

Data reports median values per each time point among study groups. AFA, nebulized amikacin + fosfomycin; AFA+IV-MERO, nebulized amikacin + fosfomycin + intravenous meropenem; AMK, nebulized amikacin; FFS, nebulized fosfomycin; IV-MERO, intravenous meropenem. A, Peak airway pressure marginally increased during nebulization (N. 56, $p=0.142$) without any significant difference among study groups (N. 56, $p=0.244$). B, Respiratory system compliance differed among study groups (N. 56, $p<0.001$), but we did not find any difference related to the use of nebulization (N. 56, $p=0.693$). C, Heart rate was different among groups (N. 56, $p<0.001$), without differences among times of assessment (N. 56, $p=0.844$). Similarly, E, mean arterial pressure (MAP) varied among groups (N. 56, $p=0.002$), but it did not fluctuate among times of assessment (N. 56, $p=0.646$).

eFigure 5: Gross examination of lungs after dissection

Each picture represents the lungs of each pig included into the various study group. We found pathognomonic signs of severe pneumonia heterogeneously presented among study groups, i.e. atelectatic areas of various sizes, purulent airways secretions and abscessed areas. Of note, pictures of one of the pigs included into FFS groups were not taken. AFA, nebulized amikacin + fosfomycin; AFA+IV-MERO, nebulized amikacin + fosfomycin + intravenous meropenem; AMK, nebulized amikacin; FFS, nebulized fosfomycin; IV-MERO, intravenous meropenem.

eFigure 6: Lung/Body weight ratio

Each dot represents lung/body weight ratio of each pig included into the study groups. Central bar depicts mean value, while upper and lower bars shows standard deviation. Control group lung/body weight ratio was higher, although shy of statistical significance (N.30, p=0.193 among all study groups). AFA, nebulized amikacin + fosfomycin; AFA+IV-MERO, nebulized amikacin + fosfomycin + intravenous meropenem; AMK, nebulized amikacin; FFS, nebulized fosfomycin; IV-MERO, intravenous meropenem.

eFigure 7: Clinical pulmonary infection score

A, Data reports clinical pulmonary infection score (CPIS) median values per each time point fosfomycin; IV-MERO, intravenous meropenem. CPIS varied throughout study time (N. 120, $p<0.001$) without any statistically significant difference among groups (N. 120, p=0.179). B, Relationship between CPIS at the end of the study and lung *P. aeruginosa* burden. n = 30, r: 0.198; y-intercept: 3.37, slope: 0.42, p=0.013.

eFigure 8: Pulmonary variables

Data reports median values per each time point among study groups. AFA, nebulized amikacin + fosfomycin; AFA+IV-MERO, nebulized amikacin + fosfomycin + intravenous meropenem; AMK, nebulized amikacin; FFS, nebulized fosfomycin; IV-MERO, intravenous meropenem. A, Ratio of partial pressure of oxygen per inspiratory fraction of oxygen differed among study groups (N. 120, p=0.002). This variation was mainly driven by the progressive impairment of CONTROL group. * post-hoc comparisons with Bonferroni corrections, $p<0.05$ vs AFA+IV-MERO, AMK, IV-MERO. B, Pulmonary shunt was different among study groups (N. 120, p=0.020). In particular, CONTROL group showed the highest figures. † post-hoc comparisons with Bonferroni corrections, $p<0.05$ vs IV-MERO. C, Minute ventilation was not different among study groups (N. 120, p=0.565). Likewise, D, lung elastance (N. 120, p=0.497), E, inspiratory airflow

resistance (N. 120, p=0.536) were similar among study groups. Finally, F, pattern of tissue resistance favored AMK, AFA+IV-MERO and IV-MERO groups, but shy of statistical significance (N. 120, p=0.073).

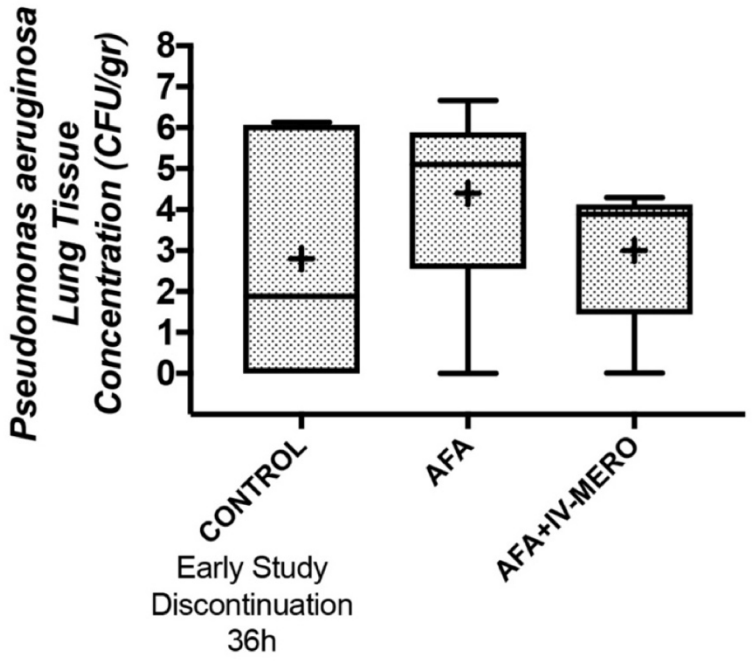
eFigure 9: Serum inflammatory markers

Data reports median values per each time point among study groups. AFA, nebulized amikacin + fosfomycin; AFA+IV-MERO, nebulized amikacin + fosfomycin + intravenous meropenem; AMK, nebulized amikacin; FFS, nebulized fosfomycin; IV-MERO, intravenous meropenem. A, interleukin (IL) 1β did not vary significantly among study groups (N. 120, p=0.152) but decreased significantly throughout the study time (N. 120, p<0.001). B, IL-6 was similar among study groups (N. 120, p=0.271) but decreased significantly throughout the study time (p<0.001). C, Likewise, IL-8 was similar among study groups (N. 120, p=0.703) and decreased significantly throughout the study time (N. 120, p<0.001). D, There was a trend towards significant difference in IL-10 among study groups (N. 120, p=0.069) and among study times (N. 120, p=0.068). E, Finally, tumor necrosis factor alpha (TNF-α) was equally similar among study groups (N. 120, p=0.161) and did not change throughout the times of assessments (N. 120, p=0.681).

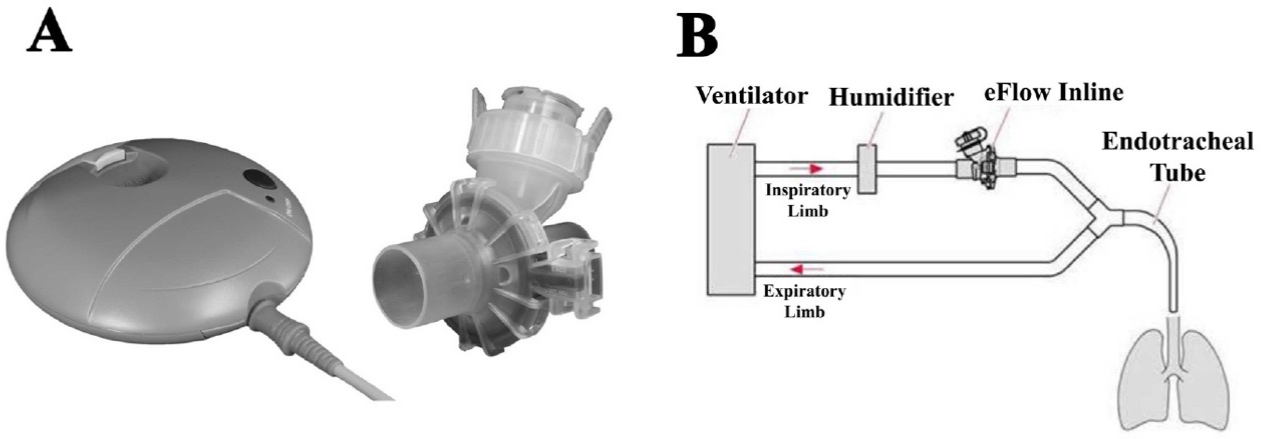
eFigure 10: Bronchoalveolar lavage fluids inflammatory markers

Data reports median values per each time point among study groups. AFA, nebulized amikacin + fosfomycin; AFA+IV-MERO, nebulized amikacin + fosfomycin + intravenous meropenem; AMK, nebulized amikacin; FFS, nebulized fosfomycin; IV-MERO, intravenous meropenem. A, interleukin (IL) 1β neither significantly among study groups (N. 90, p=0.460) nor changed between diagnosis of pneumonia and end of the study (N. 90, p=0.066). B, IL-6 was similar among study groups (N. 90, p=0.716) but decreased significantly throughout the study time (N. 90, p<0.001). C, Finally, IL-8 in BAL fluids was similar among study groups (N. 90, p=0.185) and decreased significantly throughout the study time (N. 90, p=0.025).

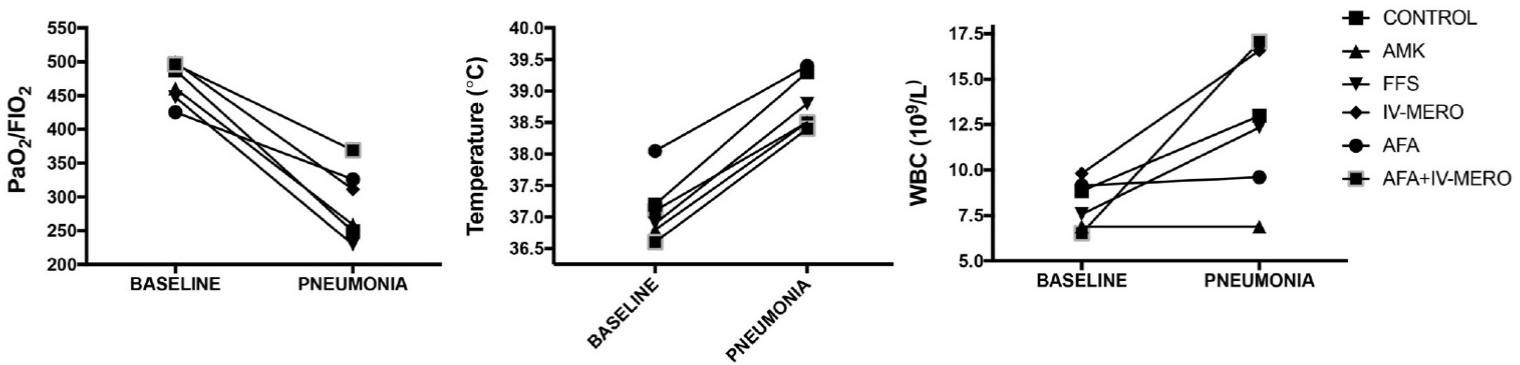
eFigure 1


Pseudomonas aeruginosa
Lung Tissue
Concentration (CFU/gr)
8
7
6
5
4
3
2
1
0
CONTROL
AFA
AFA+IV-MERO
Early Study
Discontinuation
36h

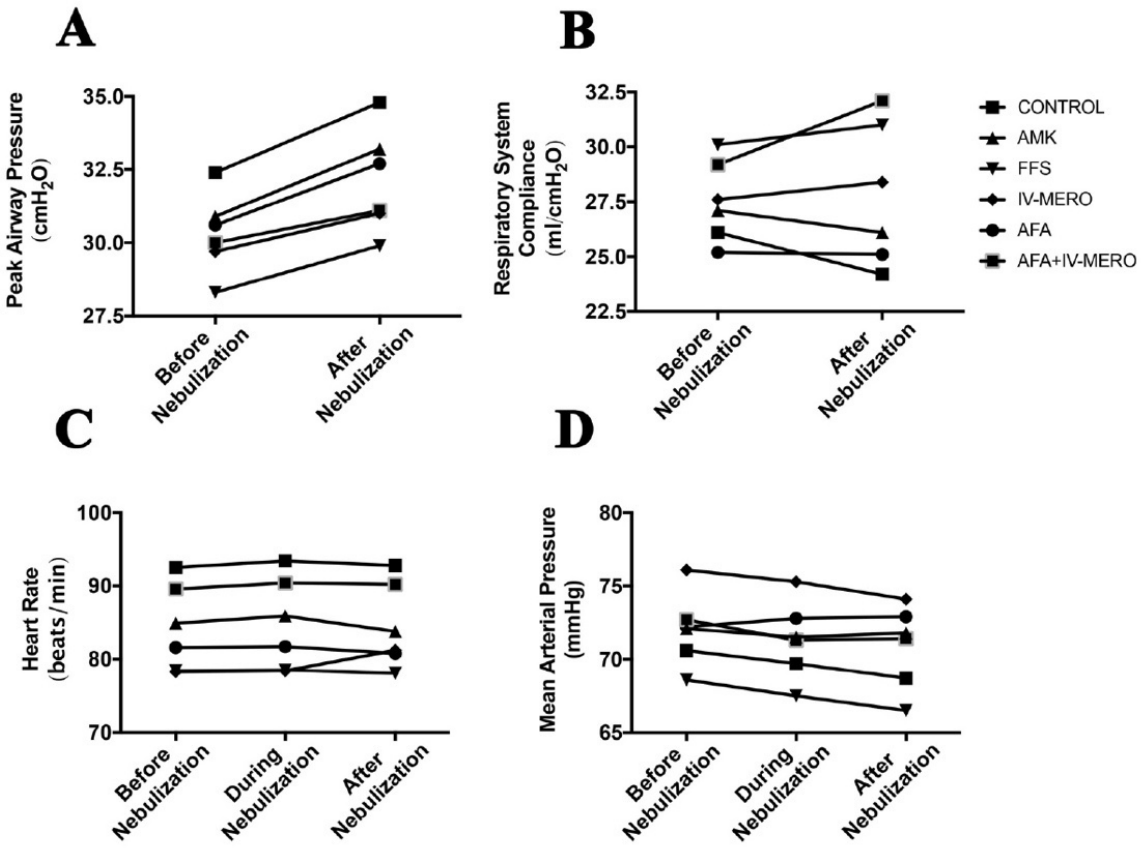
eFigure 2



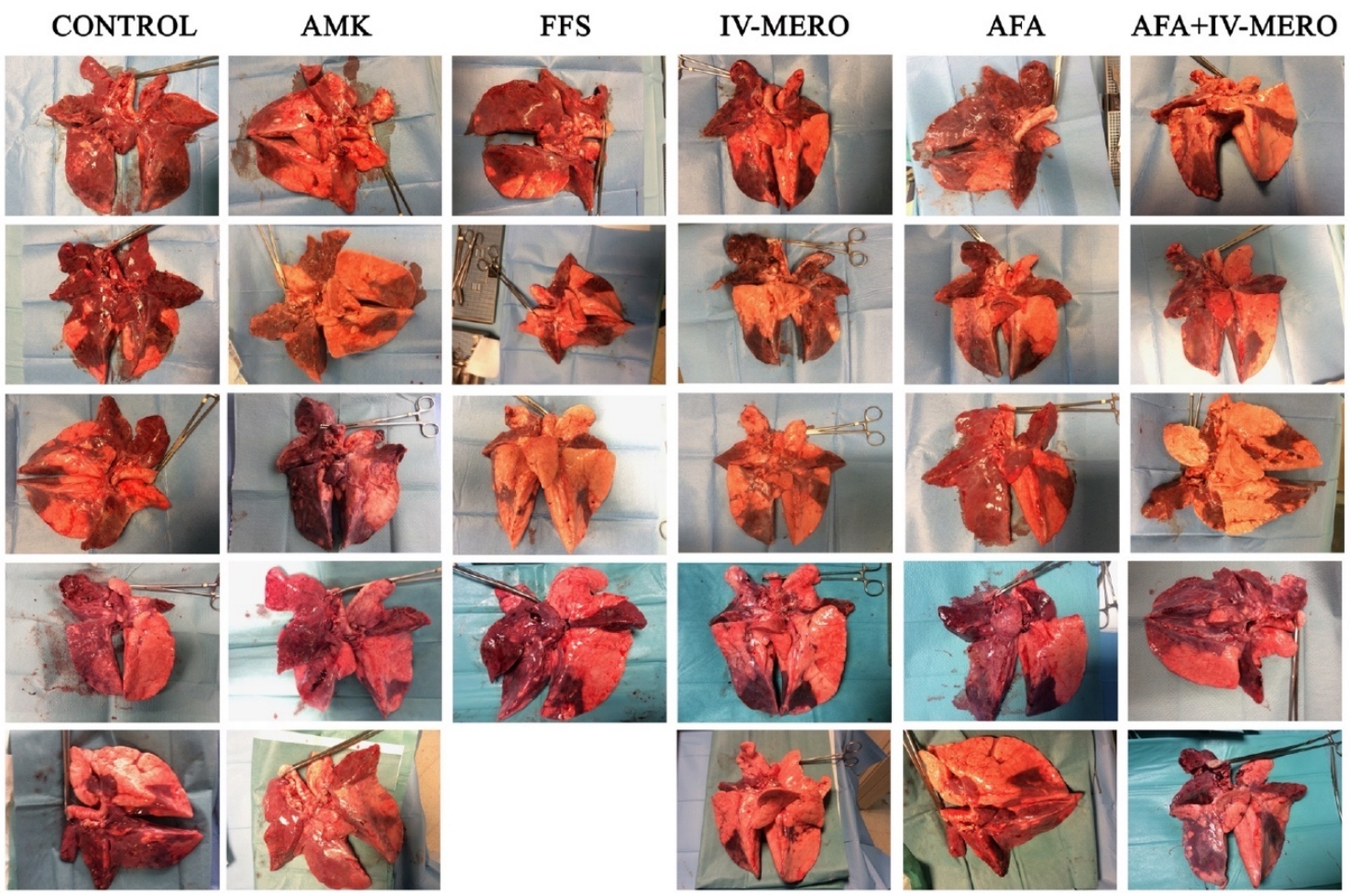
eFigure 3



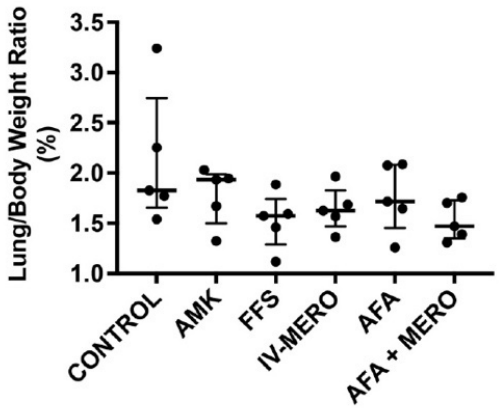
eFigure 4


A
Peak Airway Pressure (cmH_2O)
35.0
32.5
30.0
27.5
Before Nebulization
After Nebulization
B
Respiratory System Compliance (ml/cmH_2O)
32.5
30.0
27.5
25.0
22.5
Before Nebulization
After Nebulization
CONTROL
AMK
FFS
IV-MERO
AFA
AFA+IV-MERO
C
Heart Rate (beats/min)
100
90
80
70
Before Nebulization
During Nebulization
After Nebulization
D
Mean Arterial Pressure (mmHg)
80
75
70
65
Before Nebulization
During Nebulization
After Nebulization

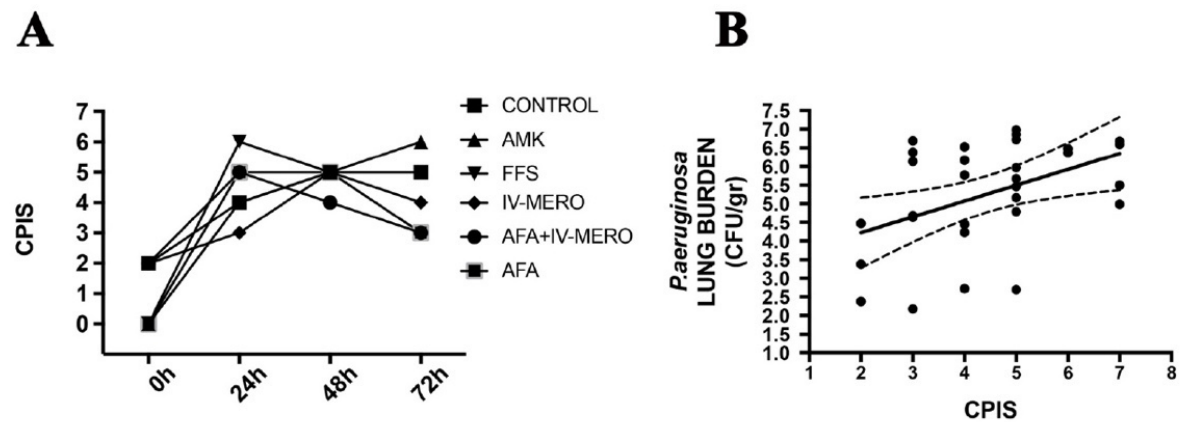
eFigure 5



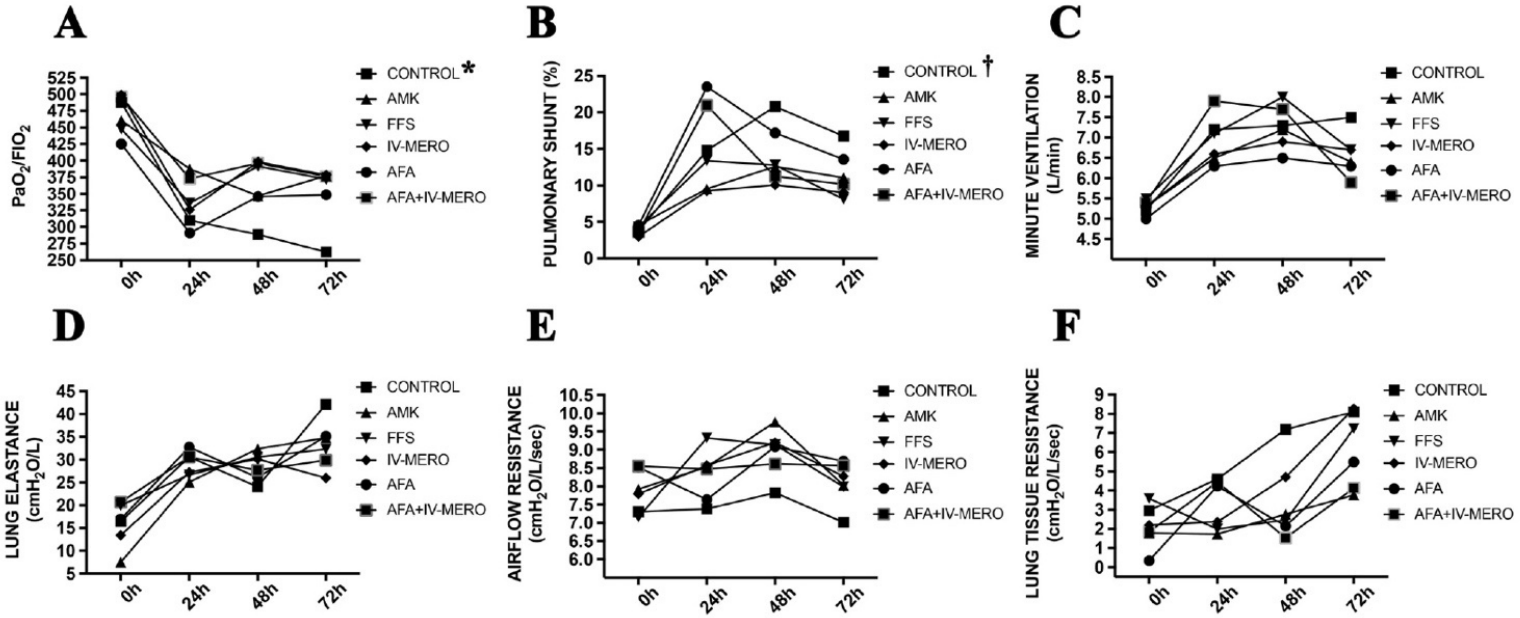
eFigure 6



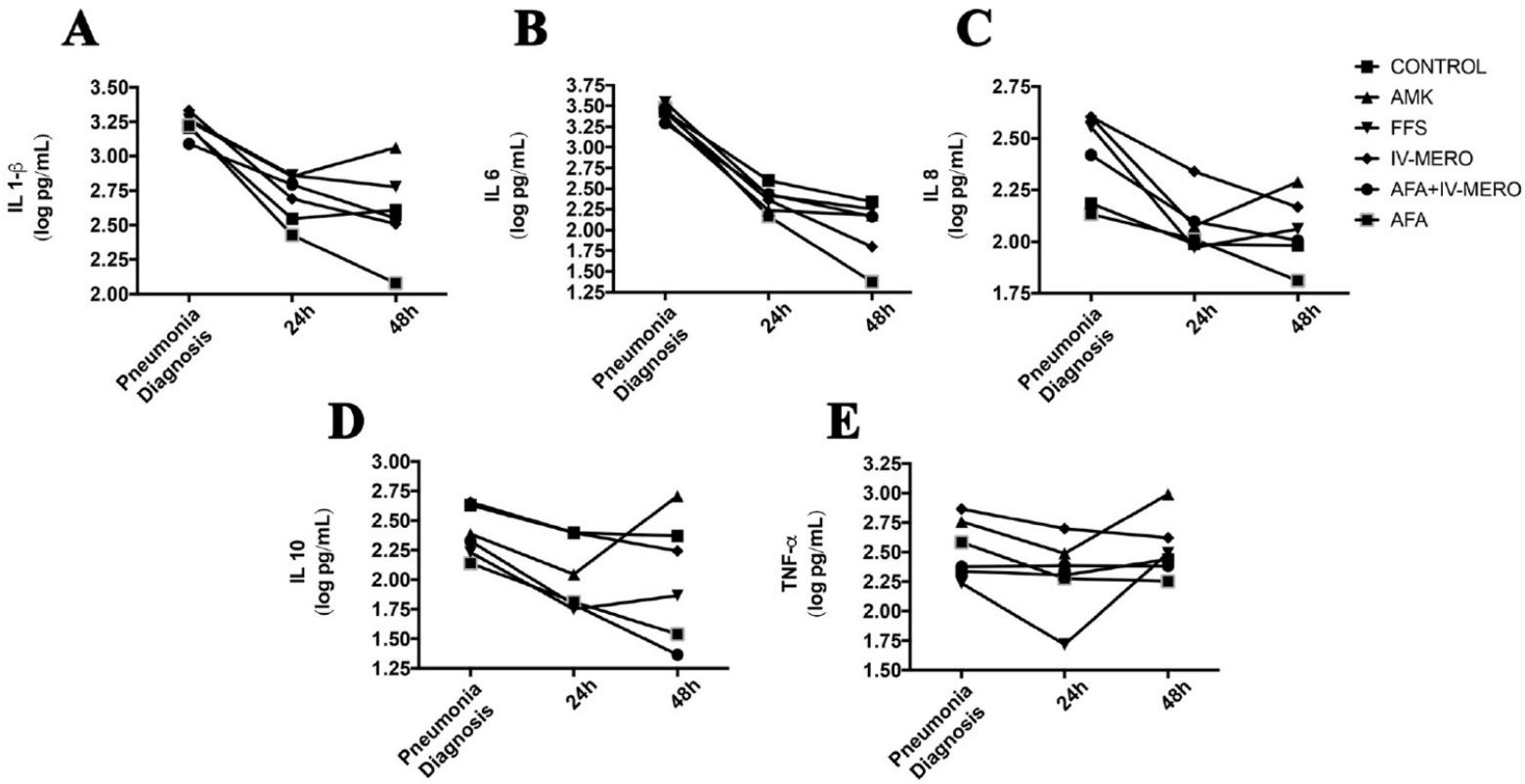
eFigure 7



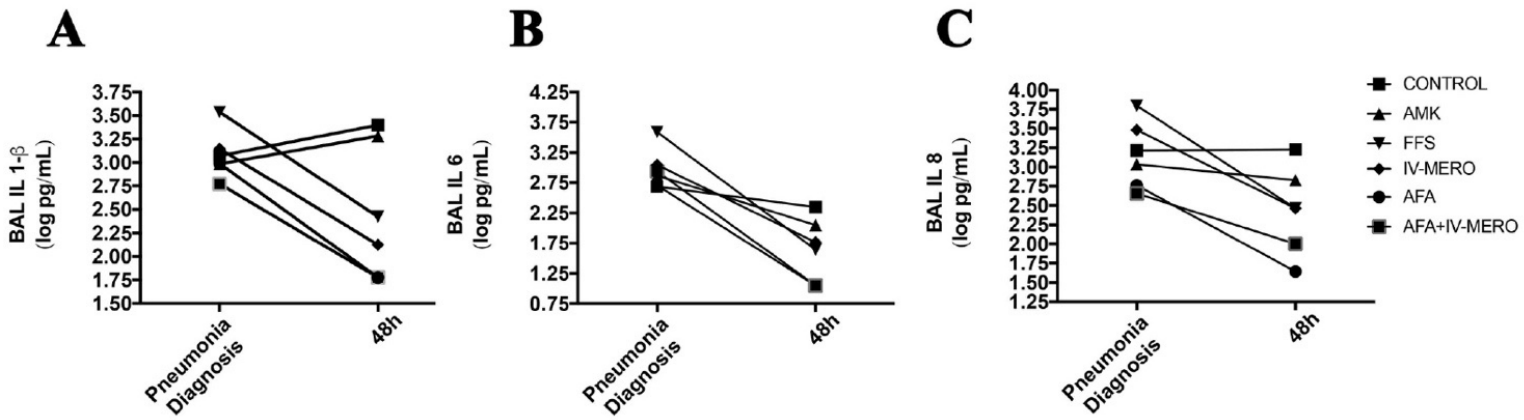
eFigure 8


A
PaO_2/FIO_2
525
500
475
450
425
400
375
350
325
300
275
250
0h
24h
48h
72h
CONTROL *
AMK
FFS
IV-MERO
AFA
AFA+IV-MERO
B
PULMONARY SHUNT (%)
25
20
15
10
5
0
CONTROL †
C
MINUTE VENTILATION (L/min)
8.5
8.0
7.5
7.0
6.5
6.0
5.5
5.0
4.5
D
LUNG ELASTANCE (cmH_2O/L)
45
40
35
30
25
20
15
10
5
E
AIRFLOW RESISTANCE ($cmH_2O/L/sec$)
10.5
10.0
9.5
9.0
8.5
8.0
7.5
7.0
6.5
6.0
F
LUNG TISSUE RESISTANCE ($cmH_2O/L/sec$)
9
8
7
6
5
4
3
2
1
0

eFigure 9


A
IL 1-β (log pg/mL)
3.50
3.25
3.00
2.75
2.50
2.25
2.00
Pneumonia Diagnosis
24h
48h
B
IL 6 (log pg/mL)
3.75
3.50
3.25
3.00
2.75
2.50
2.25
2.00
1.75
1.50
1.25
Pneumonia Diagnosis
24h
48h
C
IL 8 (log pg/mL)
2.75
2.50
2.25
2.00
1.75
Pneumonia Diagnosis
24h
48h
CONTROL
AMK
FFS
IV-MERO
AFA+IV-MERO
AFA
D
IL 10 (log pg/mL)
3.00
2.75
2.50
2.25
2.00
1.75
1.50
1.25
Pneumonia Diagnosis
24h
48h
E
TNF-α (log pg/mL)
3.25
3.00
2.75
2.50
2.25
2.00
1.75
1.50
Pneumonia Diagnosis
24h
48h

eFigure 10


A
B
C
BAL IL 1-β (log pg/mL)
BAL IL 6 (log pg/mL)
BAL IL 8 (log pg/mL)
Pneumonia Diagnosis
48h
CONTROL
AMK
FFS
IV-MERO
AFA
AFA+IV-MERO

DISCUSSION

Nosocomial pneumonia is one of the most common hospital-acquired infections. It is associated with substantial morbidity and crude mortality that could reach 70% (3, 60). In the last decade, an increase in the prevalence of MDR microorganisms has been observed due to selection pressure exerted by the inappropriate and indiscriminate use of broad-spectrum antibiotics (243). This is becoming an emerging problem due to the lack of new antimicrobial strategies (244). *P. aeruginosa* is one of the most common causative pathogens, responsible for many life-threatening conditions (29, 34). Indeed, MDR/XDR *P. aeruginosa* is a potentially challenging pathogen, being associated with an even higher mortality rate and worse clinical outcomes when compared to non-MDR (244).

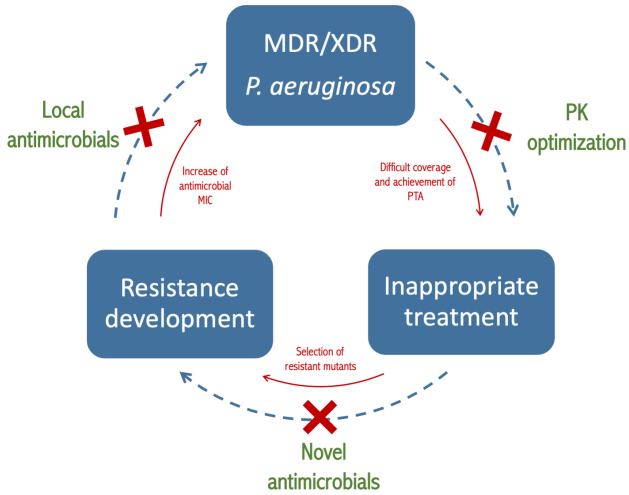


Figure 8. An integrative approach of novel antimicrobial treatments and strategies for *P. aeruginosa* nosocomial pneumonia. A summary of current setbacks and potential strategies discussed in this PhD thesis that could affect the outcomes of patients with *P. aeruginosa* nosocomial pneumonia, especially in those who required mechanical ventilation. Source: Own illustration. MDR, multidrug resistant; MIC, minimum inhibitory concentration, PK, pharmacokinetics; PTA, probability of target attainment; XDR, extensively drug resistant.

For the aforementioned reasons, an integrative approach in managing nosocomial pneumonia is a must. In this PhD thesis, the candidate has tried to elucidate some of the potential problems and solutions among novel strategies and antimicrobial therapies aimed at treating nosocomial pneumonia caused by

P. aeruginosa (**Figure 8**). Specifically, we have found that: (i) the ELF models constructed with concentrations from sparse ELF sampling time points result in exposure estimates similar to those constructed from robustly sampled ELF profiles; (ii) the appropriate initial treatment with C/T decreased respiratory secretions' bacterial burden, prevented the development of resistance, achieved the pharmacodynamic target, and possibly reduced systemic inflammation; (iii) the addition of nacubactam to meropenem resulted in substantial bacterial reduction in KPC-expressing and AmpC-overproducing *P. aeruginosa* isolates; (iv) and corroborate that nebulized amikacin and fosfomycin alone efficiently reduced *P. aeruginosa* in tracheal secretions and hindered development of meropenem-resistant P. aeruginosa, with negligible effects on pulmonary tissue.

BAL sampling for constructing pharmacokinetic antimicrobial profiles

The emergence of MDR or XDR pathogens makes antimicrobial therapy a challenge, both in ensuring adequate likelihood of efficacy and in preventing the inappropriate use of broad-spectrum antimicrobials. In this context, defining the disposition of antimicrobial agents at the site of infection is essential for guiding optimal dosing for antimicrobials targeting pneumonia (118, 204). In fact, antimicrobials developed for pneumonia are typically dosed more aggressively than the doses used for complicated nosocomial infections, given that pulmonary concentrations are generally diminished when compared to serum levels (205). The likelihood of PK parameters in critically ill patients is high, requiring dose adjustment (110). Moreover, the poor knowledge of drug disposition and the neglect of PD at the site of infection can lead to failure in phase II and III clinical trials (245). Therefore, studying the pulmonary penetration is crucial in providing optimal dosing regimens and conferring good clinical outcomes.

Drug concentrations are routinely determined in the ELF via the collection of BAL fluids (118, 246). Due to ethical and logistical issues, BAL is performed only once in healthy volunteers or patients at a defined sampling time point (247). Although bronchoscopies are widespread, effective, and generally safe; repetition of such a procedure has been shown to elevate morbidity in critically ill patients (248). Pooled data are, therefore, at each averaged to estimate pharmacokinetic profile in ELF over the dosing interval (118). Consequently, the impact of collecting only one BAL sample from each subject in the population pharmacokinetic profile was unknown.

We studied the pharmacokinetics of ceftolozane and piperacillin in a swine model of severe *P. aeruginosa* pneumonia (237). We used this database to delineate a simple approach that would determine

the impact of different sampling approach on the population PK profile. We successfully constructed population pharmacokinetic models for ceftolozane and piperacillin using robust (i.e., concentrations in ELF from 4-5 time point per pig), 1-BAL (i.e., concentrations in ELF from one randomly selected time point per pig) and 2-BAL sampling approaches (i.e., concentrations in ELF from two randomly selected time points per pig). Astonishingly, related drug models resulted in similar pharmacokinetic parameter estimates. Furthermore, no remarkable differences were found in plasma and ELF AUC, nor in penetration ratios between sampling approaches were found when the 5,000-subject simulations were run. As expected, the penetration distributions obtained with the 2-BAL model were closer to those obtained with the robust model when compared to the 1-BAL model. Nevertheless, the increase from one to two time points did not confer a large improvement. PTAs in ELF displayed across all MICs for both drugs were consistently similar among different sampling approaches.

Similar to our study, sparse and dense sampling approaches were compared for PK profiles constructed only with plasma concentrations (249, 250). For instance, Choi *et al.* found that increasing the sampling frequency reduce the bias due to time measurement error radically (249). However, a complete D-optimal informative design for plasma sampling should generate good parameter estimates (251). Thus, someone might argue that a similar situation may then occur with BAL sampling. As the number of sampling times increases, we might find ourselves obtaining larger pieces of information. Nevertheless, unless an inconceivably large number of samples were obtained (ethical issues aside), the models would still show dispersion for patients who are in the outlier part of the distribution (251). Our data suggests that a single BAL sampling timepoint per each subject would be sufficient in predicting the median penetration and variability for both β-lactam drugs. Additionally, sparse ELF models result in similar exposure estimates to robustly sampled BAL profiles. Therefore, this study validates current ELF sampling procedures in PK studies done in humans. However, even with the endorsement of sparse sampling methodologies, we encourage that studies be conducted with larger subject numbers (i.e., $n \geq 20$ subjects) to best assess intersubject variability.

Several limitations of this study should be noted. First, the swine pneumonia model is not as rigorous as the actual treatment of patients with ventilator-associated pneumonia. However, it did allow for the opportunity to explore the effects of sampling at multiple time points on predicted ELF exposures. Second, the drug disposition in ELF of this swine model may be different from that observed in humans enrolled in studies, even though the PK profile comparison was out of the study scope. Third, only two β-lactams

were included in this assessment. Our conclusions may not be applicable in other antibiotic classes particularly for agents noted to have poor ELF penetration (e.g., aminoglycosides). Finally, this study included only 7 – 8 pigs for each drug, which may limit the ability to extrapolate results.

Short-term effects of appropriate empirical treatment with ceftolozane-tazobactam

The global dissemination of antimicrobial resistance complicates empirical antibiotic therapy decisions, which are essential in patients with a suspected infections Moreover, high MICs and PK variations among patients with acute illnesses also threaten adequate antimicrobial pulmonary concentrations (110, 113). An empirical antimicrobial regimen is usually categorized as inappropriate when it did not include any antibiotic showing *in vitro* activity against the isolated bacteria. However, the lack of PD target attainment, even when the pathogen is susceptible to the antimicrobial, should begin to be considered as part of IEAT.

In the hospital and ICU settings, the high prevalence of MDR/XDR *P. aeruginosa* strains is posing as a major threat when it comes to decisions regarding appropriate initial antimicrobial treatment (35). In fact, the frequency of IEAT is up by 70% (238). Although clinical practice universally assumes that an overall beneficial outcome due to appropriate antibiotic therapy, some studies showing not impact on mortality continued to be published (89, 90).

Our study on a swine model of severe and XDR *P. aeruginosa* pneumonia corroborates that appropriate empirical treatment with human-simulated C/T regimen yields higher bactericidal efficacy in tracheal secretions and BAL fluids. Importantly, C/T averts the development of *P. aeruginosa* resistance and lessens systemic inflammation in comparison with IEAT. Yet, due to short antimicrobial course, *P. aeruginosa* tissue burden was moderately affected. The results suggest that C/T may serve as a useful empirical therapeutic strategy in ICU-admitted patients when there is a high likelihood that MDR *P. aeruginosa* is the causative pathogen.

Ceftolozane has been shown to possibly more stable against the most common resistance mechanisms of *P. aeruginosa*, driven by mutation, upregulation or hyperproduction, i.e., AmpC, efflux pumps or OprD (252). Remarkably, in our study C/T avoided the development of resistance. In contrast, after only 48 hours of treatment with piperacillin-tazobactam, MIC increased substantially. Nevertheless, it is also important to emphasize that Hadair *et al.* recently published a small case series of sixteen patients with MDR *P. aeruginosa* pneumonia. These patients underwent longer treatment with C/T, on average 20 days, and 12.5% developed resistance to C/T (199). Authors identified AmpC overexpression and

mutations as potential resistance mechanisms in those isolated strains. Therefore, using broad-spectrum antibiotics for initial therapy in order to avoid IEAT may indeed lead to a worsening in antimicrobial resistance burden due to the selection of even more resistant pathogens (91). The development of novel antibiotics is therefore necessary if clinicians are to have a higher chance of choosing an active, effective agent for empirical therapy of nosocomial pneumonia (96). Similarly, the development of rapid, low-cost diagnostic microbiological tools that allow the prompt use of narrow-spectrum antibiotics is equally significant (64).

Among recently developed antimicrobials, C/T efficacy has been judged in comparison to other antibiotics, especially ceftazidime-avibactam – a combination of a third-generation cephalosporin and a novel β-lactamase inhibitor (166). Both drugs demonstrated its efficacy, presenting great *in vitro* activity and less resistance development; they can be used to limit carbapenems use (97). In a recent metanalysis of clinical outcomes using C/T and ceftazidime-avibactam for the treatment of MDR Gram-negative infections, the researchers found similar clinical success rates among them, with a pooled rate of 73.3% (95% CI, 68.9%–77.5%)(253). Nevertheless, *in vitro* data suggested that C/T may have enhanced activity against *P. aeruginosa* and may, therefore, be preferred for hospital settings with a higher MDR frequency (254, 255). Also, the activity of imipenem-relebactam-cilastatin, a carbapenem combined with cilastatin and a novel β-lactamase inhibitor, appears to be slightly lower than C/T (194). Unfortunately, those agents have never been compared, and real-world data rivalling novel antimicrobial agents is needed in order to resolve C/T place in therapy.

In conclusion, our experimental study is the first study to shed some light on comparisons with another first-line antibiotic and comprehensively assessing the effects of C/T in a large animal model that closely resembles critically ill patients with severe *P. aeruginosa* pneumonia. Our findings are also in line with the *in vitro* data and clinical studies (172, 195, 196, 255, 256), and further emphasize the value of C/T for nosocomial pneumonia.

A number of limitations of this study should be noted. First, piperacillin-tazobactam could yielded subinhibitory concentrations in ELF and therefore facilitated the development of resistance. However, our methods aimed to simulate clinical conditions. In this context, the attainment of PD targets in both systemic and pulmonary compartments is usually unexpected, in cases of IEAT. Second, the validation of the outcomes was restricted due to one *P. aeruginosa* strain and therapy duration. Finally, animals in our

setting did not have comorbidities and were deeply sedated throughout the study. These dissimilarities – when considering critically ill patients with nosocomial pneumonia – are noteworthy to mention.

Efficacy of meropenem-nacubactam against *P. aeruginosa* pneumonia

Investing in developing novel antibiotics can help reduce the impact of resistance and lower IEAT rates in hospital settings. The limited armamentarium against MDR *P. aeruginosa* has led to the development of several novel β-lactam-β-lactamase inhibitor combinations. Among them, the combination of meropenem-nacubactam may appear a potential option for treating the nosocomial pneumonia caused by *P. aeruginosa*.

In contrast to other β-lactamase inhibitors, such as avibactam and vaborbactam, nacubactam possesses a multiple mechanism of action – first, as a β-lactamase inhibitor against organisms with Class A and C enzymes. The second is its intrinsic antimicrobial activity against *Enterobacteriaceae*. There, it enhances of the activity of various β-lactam agents including carbapenem-resistant *P. aeruginosa* by AmpC-derepressed β-lactamase (222). Therefore, combinations of nacubactam with β-lactam agents boast the a potential to overcome resistance (225, 226).

A growing number of studies have reported enhanced efficacy of different β-lactam drugs and nacubactam against *Enterobacteriaceae* harboring a variety of β-lactamases including KPC (225, 226). Nevertheless, *in vitro* and *in vivo* activity against *P. aeruginosa* are still limited. In a recent abstract including 203 *P. aeruginosa* isolates, Sader *et al.* reported an inhibition rate of 82.2% when nacubactam was combined with meropenem, even though higher figures were found when the combination was with cefepime or piperacillin (257).

In the present study, the availability of data on the meropenem-nacubactam bronchopulmonary PK in healthy adults allowed for the efficacy of the combination to be evaluated using the human-simulated ELF exposures in a murine lung infection model. This improves the translation application of study outcomes to the clinic. Despite the use of subtherapeutic meropenem exposure, the addition of human-simulated nacubactam dosing regimen show a synergistic effect against *P. aeruginosa* isolates. Indeed, meropenem-nacubactam combination achieved significant bacterial killing among KPC-expressing and AmpC-overproducing *P. aeruginosa* isolates in this neutropenic lung model in mice. Based on the study results, meropenem-nacubactam appears to be an option to treat enzyme-mediated carbapenem-

resistant *P. aeruginosa* pneumonia. This may be clinically relevant as some of the novel β-lactams-β-lactamase inhibitor combinations do not include KPC-expressing *P. aeruginosa*, especially as the kind of resistance mechanism start to be alarmed (241). Indeed, C/T spectrum does not cover KPC-expressing *P. aeruginosa* (190). Thus, this combination could provide an opportunity to add another treatment option against these challenging pathogens.

Similar results were obtained by Morinaka and collegues when they tested the efficacy of nacubactam in combination with cefepime in in a neutropenic murine thigh AmpC-derepressed *P. aeruginosa* infection model (227). Interestingly, administration of either cefepime or nacubactam alone showed a bacterial count similar to the controls; howver, in combination, bacterial counts decreased up to 4 log CFU.

All results together suggest that nacubactam, which alone has no antibacterial activity *in vivo*, works as an AmpC- and KPC-expressing *P. aeruginosa* isolates. Future studies are needed to compare the activity of meropenem-nacubactam to those of ceftazidime-avibactam and meropenem-vaborbactam in order to examine whether meropenem-nacubactam offers a potential alternative to ceftazidime-avibactam in resistant strains. Other combinations of β-lactams and nacubactam should be tested *in vivo*, as current *in vitro* data suggests higher efficacy than the actual combination (257). Finally, other novel antimicrobial agents still in development phases such as murepavadin (181), cefoperazone-sulbactam (178) or plazomizin (179), may be in a better position for the future treatment of *P. aeruginosa* nosocomial pneumonia.

Nebulization of amikacin/fosfomycin for ventilated *P. aeruginosa* nosocomial pneumonia

The rising rates of MDR and a paucity of treatment options have also stimulated interest in nebulized antimicrobials as adjunctive therapy to traditional systemic monotherapy in patients with ventilated nosocomial pneumonia, especially VAP (258). Theoretically, through nebulization, antimicrobial efficacy would be optimized, guaranteeing adequate drug levels at the site of infection; reducing the risk of the appearance of resistance, and avoiding the risk of systemic toxicity (129, 156, 158). In this context, *in vivo* and observational studies conducted during the early 2000s reinforced this idea. They found that nebulized antimicrobials reached high intra-pulmonary concentrations and have benefits in terms of resolution of signs and symptoms of pneumonia in comparison to systemic therapy alone (128, 135, 154,

158). Nevertheless, the two recent RCTs (IASIS and INHALE trials)(139, 140) did not show benefits when nebulized antimicrobial therapy was used as adjunctive treatment for VAP or v-HAP.

In this context, and in line with IASIS trial, we set out to investigate the efficacy of nebulized amikacin/fosfomycin with IV-meropenem in comparison to IV-meropenem alone in animals with severe amikacin/fosfomycin-resistant yet meropenem-susceptible *P. aeruginosa* pneumonia (259). We also added nebulized antibiotics alone and as a combination to the study treatment groups. We appraised the bactericidal effects in pulmonary tissue and secretions, the potential emergence of antimicrobial resistance, lung histology, and drug distribution in a controlled setting with limited confounding factors, otherwise unfeasible in clinical studies

We have demonstrated that the potential benefits of nebulized amikacin/fosfomycin as adjunctive therapy in bacterial eradication are ephemeral at best. Indeed, in our model using a *P. aeruginosa* strain resistant to amikacin yet susceptible to meropenem, IV-meropenem drove the reduction of the lung tissue *P. aeruginosa* concentration. In contrast, nebulized amikacin/fosfomycin had a great effect on *P. aeruginosa* burden in tracheal secretions, showing bactericidal synergy when combined with systemic treatment. Those results are in line with the latest RCT trials (139, 140). Indeed, IASIS trial, which use the same drug combination for nebulization, found significantly fewer positive tracheal cultures on days 3 and 7 than placebo. The reason behind the fact that nebulized antibiotics did not prove beneficial, neither in the RCT nor in our *in vivo* study, appears related to the susceptibility of the infected microorganisms (162). All enrolled patients in both trials were infected by susceptible pathogens to intravenous antibiotics as *P. aeruginosa* was susceptible to meropenem in our model. Therefore, any adjunctive therapy, even if effective, was unlikely to have a detectable effect. Remarkably, Kollef *et al.* (139) reported clinical cure rates among PDR cases of 67% versus 25% in treatment and placebo groups, respectively. This context defends the standpoint that inhaled antibiotics may be only beneficial in the management of VAP due to difficult-to-treat organisms (260). Indeed, in a meta-analysis including 11 studies of which six were RCTs, aerosolized therapy led to higher resolution rates for patients with resistant pathogens, albeit not in those with susceptible bacteria (261).

Also, we found that even with 48-h course of IV-meropenem, nebulized amikacin/fosfomycin also suppressed the emergence of meropenem-resistant subpopulation in contrast with only IV-meropenem. Among the last clinical studies that evaluated post-treatment isolated microorganisms, none found an increase in resistance in patients treated with nebulized therapy (139, 140). Indeed, some studies showed

that nebulized treatment may hinder the development of resistance to the IV therapy (129). In the IASIS trial, only one patient in the nebulized amikacin/fosfomycin group compared to eight in the placebo group showed a fourfold or greater increase in MICs. Unfortunately, in the INHALE trial the emergence of resistance was not evaluated. In this scenario, nebulized antibiotics such as amikacin could have a greater window for efficacy and help prevent resistance development (**Figure 9**). Indeed, the RCT should consider other endpoints like the antibiotic side effects and the overuse of systemic antibiotics. If nebulized antimicrobials were used during 7 – 10 days of systemic therapy, being longer for nonresponders, this adjunctive therapy could reduce the amount of systemic antimicrobial prescribed (260). Given the promising results as it relates to increasing the barrier to antibiotic resistance, future trials should also include this important metric. Clinical and laboratory research will be essential in confirming the value of nebulized antimicrobial agents as it concerns the reduction of resistance development to systemic therapy and the determination of related mechanisms.

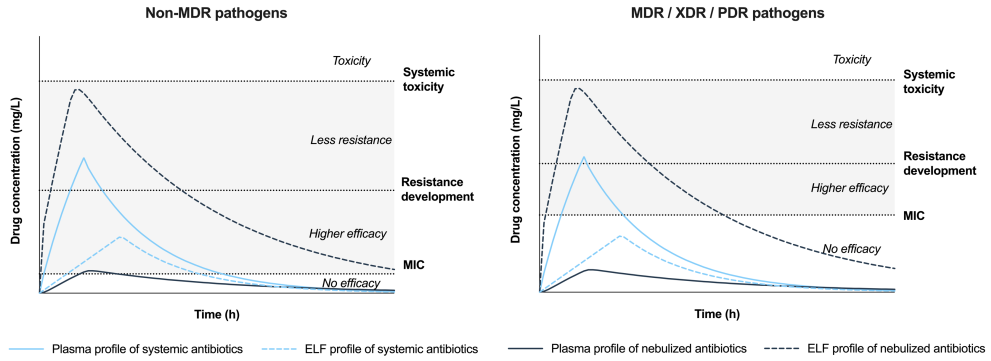


Figure 9. Theorical differences between systemic and nebulized antibiotics and potential microorganisms targeted in ventilated nosocomial pneumonia.

Differences of efficacy, drug distribution, resistance emergence, and systemic toxicity between nebulized and intravenous antimicrobials for various levels of antimicrobial resistance are displayed. The solid and dashed lines show theoretical concentration-time drug profile for plasma and ELF, respectively. The grey area displays the desirable concentration to achieve high efficacy and prevent resistance development. Source: Own illustration. MDR, multidrug-resistant; MIC, minimum inhibitory concentration; PDR, pandrug-resistance; XDR, extensively drug-resistance.

As expected, high concentrations of nebulized drugs were found in tracheal secretions and ELF with marginal figures in plasma. This reinforces the idea for using such drugs to prevent systemic toxicities. Similar results had already been published in both animal and clinical studies; however, the impact on the histopathology was never clearly assessed (127, 138, 154, 157, 158). Remarkably, a histological analysis

of lung tissue revealed that nebulized antibiotics first cleared pathogens within the airways and alveoli; however, it incompletely removed the bacterial reservoirs in the interlobular septa. The IV-meropenem treatment group presented pathognomonic signs of pneumonia within the centrilobular alveoli but marginally at alveolar regions close to the interlobular septa. Importantly, the bronchial contamination may overestimate ELF concentration, that stated, PK data should be interpreted with caution(164) and microdialysis sampling may be more accurate (262).

Other antimicrobial agents have been proposed for nebulization administration in v-HAP and VAP. Among them, other aminoglycosides and colistin were the most investigated (263). In a recent meta-analysis including 11 RCTs using nebulized amikacin, tobramycin, vancomycin, colistin or gentamicin, the use of adjunctive nebulized antibiotic therapy improved the rates of clinical cure (1.13 [95% CI 1.02 to 1.26]) and microbiological eradication (1.45 [95% CI 1.19 to 1.76]). Mortality did not, however, decreased (1.00 [95% CI 0.82 to 1.21]) for VAP patients (264). Other alternatives such as a combination of aztreonam and tobramycin with promising synergistic effects (265), nebulized arbekacin (266), a broad-spectrum aminoglycoside, and an inhaled liposomal ciprofloxacin that may allow for a slow release (267), are under study evaluation. Nevertheless, the issues of these novel approaches may be similar to those observed in nebulized amikacin/fosfomycin. The *in vitro* efficacy of all of these drugs is undeniable, but future trials could also fail if the aforementioned issues (i.e., study design, dosing and nebulization technique) are not properly addressed (**Figure 10**)(161).

In summary, nebulized antibiotics may have a place for patients with difficult-to treat pathogens and either v-HAP or VAP. In those patients with XDR or PDR pathogens, systemic treatment options are limited to IV antimicrobials with poor lung penetration (e.g., colistin or aminoglycosides) or systemic toxicities, which prevents later escalation of intravenous dosing. In fact, the use of nebulized antimicrobial agents as a rescue therapy for MDR pulmonary infections might be considered when systemic therapy fails. Also, its use for preventing biofilm formation, frustrating VAP relapses, should be investigate. High tracheal secretion drug concentration may play a significant role (268). Future studies should also compare delivery devices and settings to define the optimal method of nebulized antibiotic administration when it relates to reaching distal portions of highly infected pulmonary regions. This could be done radio-labeled drugs trackable by gamma scintigraphy or position emission tomography (269)(**Figure 7**).

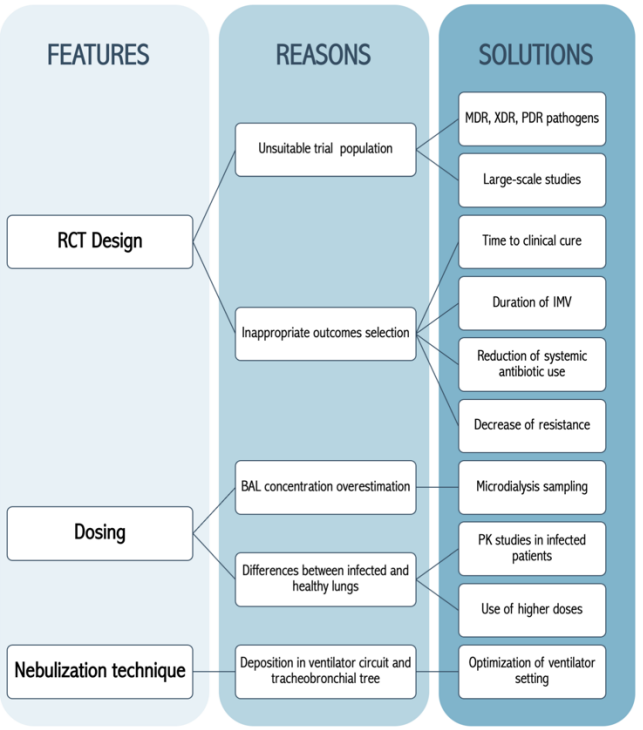


Figure 10. Features of RCTs for nebulized antimicrobials.

Summary of methodological characteristics that likely explain the negative results in RCTs for nebulized antimicrobials and potential remedies. Source: Own illustration. BAL, bronchoalveolar lavage; IMV, invasive mechanical ventilation; MDR, multidrug-resistant; PDR, pandrug-resistance; PK, pharmacokinetics; RCT, randomized clinical trials; XDR, extensively drug-resistance.

This study presents some limitation that deserve further discussion. First, unlikely in the most probable clinical scenario, we used a short therapy course. This may have influenced the efficacy of the treatments when considering long-term outcomes. Second, in our study, animals did not have comorbidities, and were young and deeply sedated. These are noteworthy dissimilarities when we considered the profile of critically ill patients with nosocomial pneumonia. Finally, we did not evaluate the mechanism of meropenem resistance.

CONCLUSIONS

In this PhD thesis, we have studied the benefits of novel treatments and strategies against *P. aeruginosa* nosocomial pneumonia in well-standardized swine and murine models of pneumonia. Indeed, we have assessed the benefits of three different and novel treatments. First, we have elucidated the influence of collecting sparse or dense BAL samples from each subject on the population's PK profile, Second, we have assessed the consequences of appropriate treatment with ceftolozane-tazabactam – a novel β-lactam-β-lactamase inhibitor combination – in comparison with IEAT. Third, we have tested the novel β-lactam/ β-lactamase inhibitor combination, meropenem-nacubactam. Finally, we have compared nebulized amikacin/fosfomycin with system therapy alone. Specifically, we can conclude that:

- The ELF models constructed with concentrations from sparse ELF sampling time points result in exposure estimates similar to those constructed from robustly sampled ELF profiles. Indeed, a single BAL sampling time point may be enough to determine median penetration and pharmacodynamic exposure.
  Thus, this study validates current ELF sampling procedures in pharmacokinetic studies in humans.

- In a mechanically ventilated swine model with XDR *P. aeruginosa* pneumonia, appropriate initial treatment with C/T decreased respiratory secretions' bacterial burden, prevented the development of resistance, achieved the pharmacodynamic target, and possibly reduced systemic inflammation. However, after only 2 days of treatment, *P. aeruginosa* tissue concentrations were moderately affected.
  This data implies the benefits of appropriate empirical treatment and calls for further clinical studies to be done to fully elucidate the short-term implications of inappropriate empirical antimicrobial treatment.

- The addition of nacubactam to meropenem resulted in substantial bacterial reduction in KPC-expressing and AmpC-overproducing *P. aeruginosa* isolates, despite the use of subtherapeutic meropenem exposure.

Therefore, meropenem-nacubactam showed promising *in vivo* activity against meropenem-resistant *P. aeruginosa*, which is indicative of its potential role in treating infections caused by these challenging pathogens.

- Our findings corroborate that nebulized amikacin and fosfomycin alone efficiently reduced *P. aeruginosa* in tracheal secretions, with negligible effects in pulmonary tissue. Combination of nebulized amikacin and fosfomycin with IV meropenem does not increase antipseudomonal pulmonary tissue activity; however, it does reduce the development of meropenem-resistant *P. aeruginosa* when compared to sole use of IV meropenem.

  Our findings imply potential merits for the preemptive use of nebulized antibiotics to reduce resistance to IV meropenem.

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