Streptococcus pneumoniae infections in a context of Chronic obstructive pulmonary disease (COPD)

Study of the factors contributing to the recurrence of the disease

Thesis public defense

Nathalie M. Vandevelde

Supervisor : Professor Françoise Van Bambeke

- Brussels, October 13, 2014 -

But ….

What is Chronic Obstructive Pulmonary Disease (COPD) ?

What are bacterial Acute Exacerbations of COPD ?

What is *Streptococcus pneumoniae* ?

INTRODUCTION - Chronic Obstructive Pulmonary Disease or Chronic Bronchitis

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  - **man**
  - **81 years old in 2010 (**birth : 1929**)**
  - **miner during 25 years**
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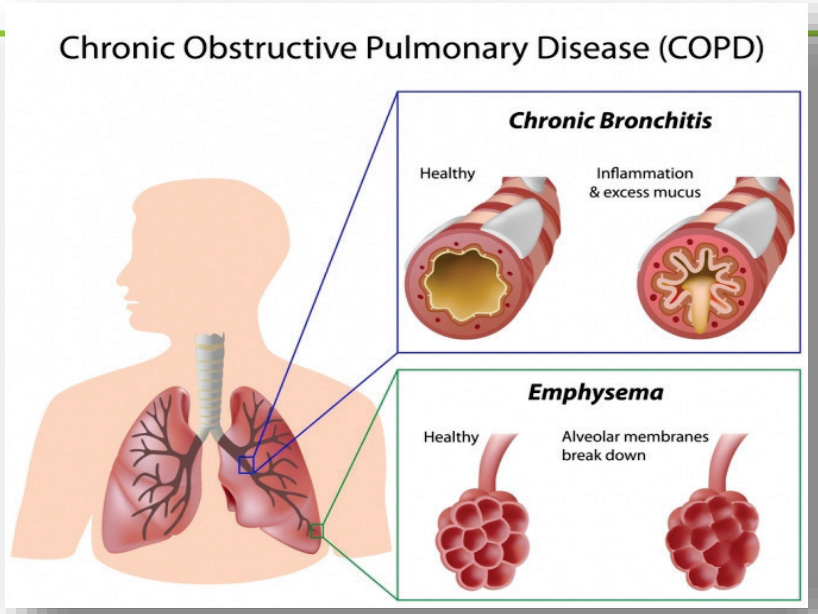
- Bronchial obstruction : ↓ 40% of the expiratory function (GOLD 2**)**

- 1-2 bacterial exacerbations/ year



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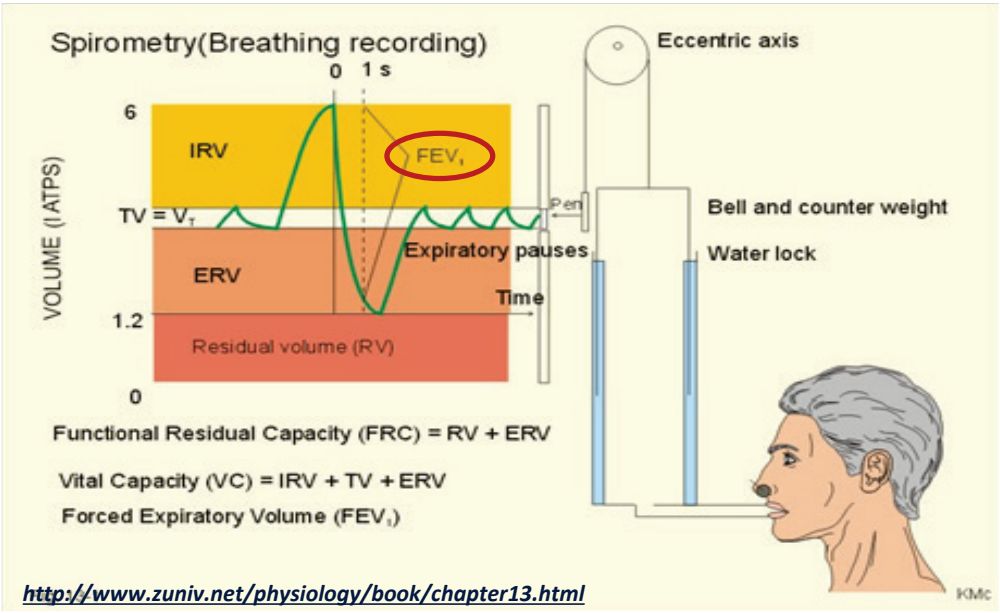

http://www.zuniv.net/physiology/book/chapter13.html

Stage	Spirometric FEV_1 measures
I	Mild COPD : $FEV_1 \geq$ 80% predicted
II	Moderate COPD : 50% $\leq FEV_1 <$ 80% predicted
III	Severe COPD : 30% $< FEV_1 <$ 50% predicted
IV	Very severe COPD : $FEV_1 <$ 30% predicted

Stratification according to the
Global Initiative for Chronic Obstructive Lung Disease (GOLD, 2014)

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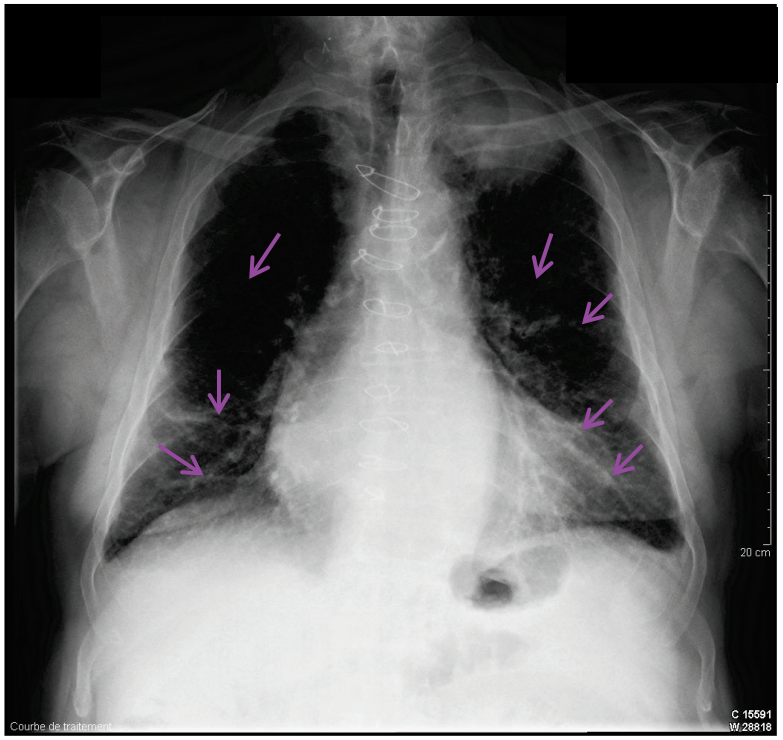

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(inflammation of the lymphatic vessels associated with carcinomal lesions and bacterial infection**)**

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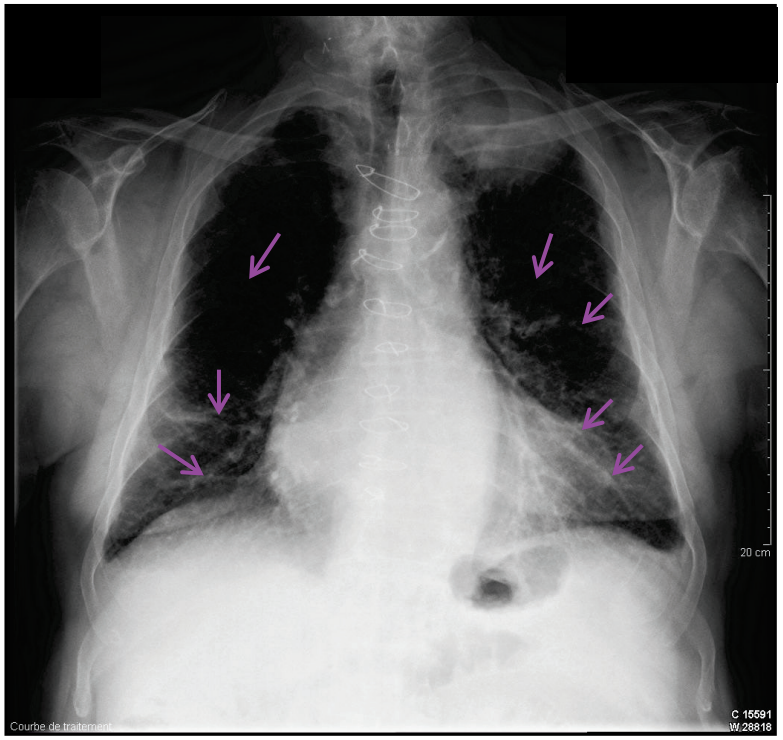

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- Hypertension (157/72 mmHg)

- Heart transplant (1992), cardiomegaly

- Hypercholesteloremia

- Moderate hyperglycemia (fasting glucose 139 mg/dl)

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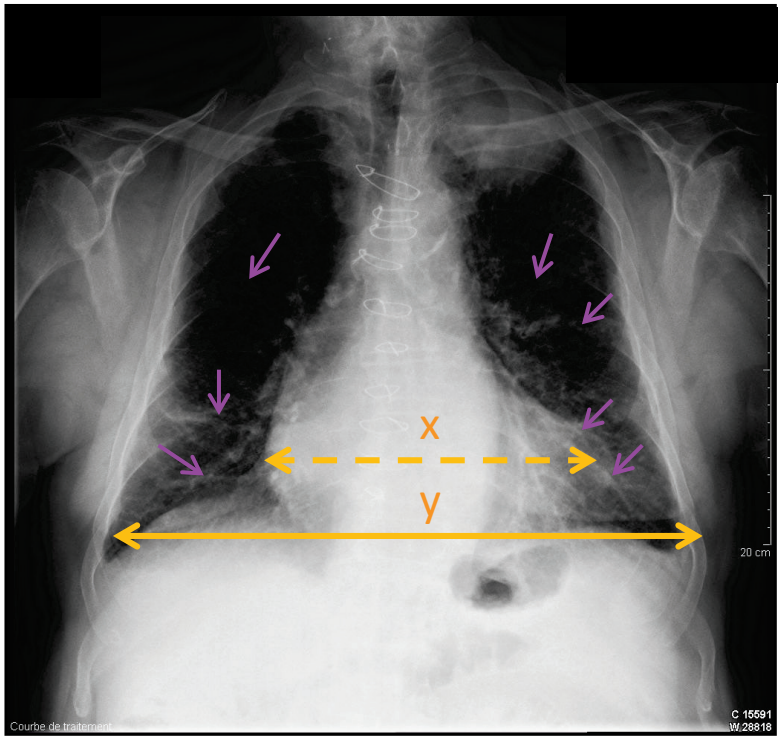
- Chronic renal failure

- Cancer



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Cardiomegaly (x/y >0.5)

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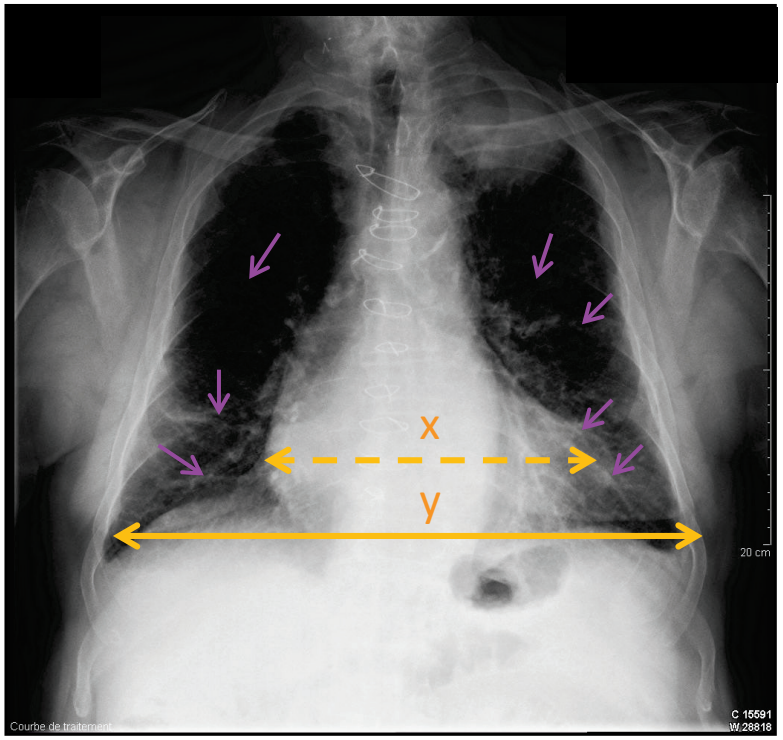

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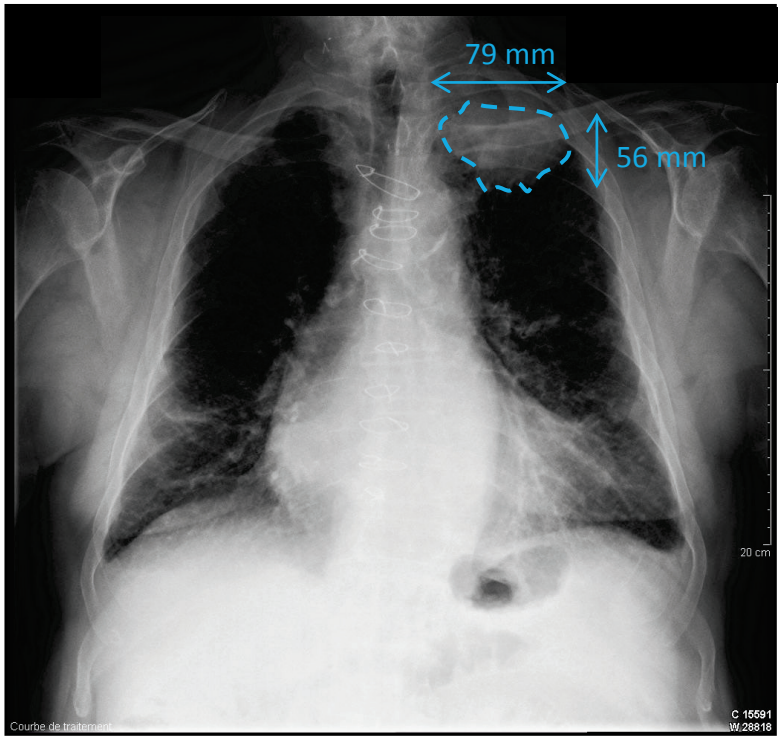

Cancerous mass (see lung upper left lobe**)**

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Take Home Message

• Who?

- Adults >50 years [a]
- Men > women [a]

- Long-term exposition to inhaled toxic substances [a,b]

- Bronchial obstruction [c]
- Chronic cough [c]
- Emphysema [c]
- Lung inflammation [c]
- Repeated bronchial infections [c]

- Cardiovascular diseases (~ 60-70%) [d]
- Cancer (~ 30%) [d]
- Diabetes (~ 20-30%) [d]

Non-reversible decrease of the respiratory function

[a] Lundback et al, 2003, Respir Med 97: 115-122; [b] Anthonisen et al, 2007, Can Respir J, 14:432-434; [c] Dos Santos et al, 2012, Am J Physiol Lung Cell Mol Physiol, 303:627-633; [d] Sin etal, 2006, Eur Respir J 28: 1245-1257

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- Bronchial obstruction : ↓ 40% of the expiratory function (GOLD 2)
  => SHORT & LONG-ACTING BRONCHODILATORS

- Chronic cough and lung inflammation

- 1-2 bacterial exacerbations/ year
  => ANTIBIOTICS

- Hypertension (157/72 mmHg)
  => 3 ANTI-HYPERTENSIVE DRUGS /day

- Heart transplant (1992), cardiomegaly
  => 3 ANTI-REJECTION DRUGS

- Hypercholesteloremia
  => CHOLESTEROL-LOWERING DRUGS

- Moderate hyperglycemia (fasting glucose 139 mg/dl)
  => ANTI-DIABETIC DRUGS

- Overweight (BMI : 33kg/m^2)

- Chronic renal failure

- Cancer

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• Who?

- Adults >50 years [a]
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- Highly polymedicated

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INTRODUCTION - Chronic Obstructive Pulmonary Disease or Chronic Bronchitis

Non reversible decrease of the respiratory function

Frequent acute exacerbations (AECB)

Comorbidities

1990 | 2000 | 2010 | **2020** | 2030 | 2040

2 millions

3.5 millions **5 millions**

Number of deaths per year worldwide [a]

3rd cause of mortality around the world [b]

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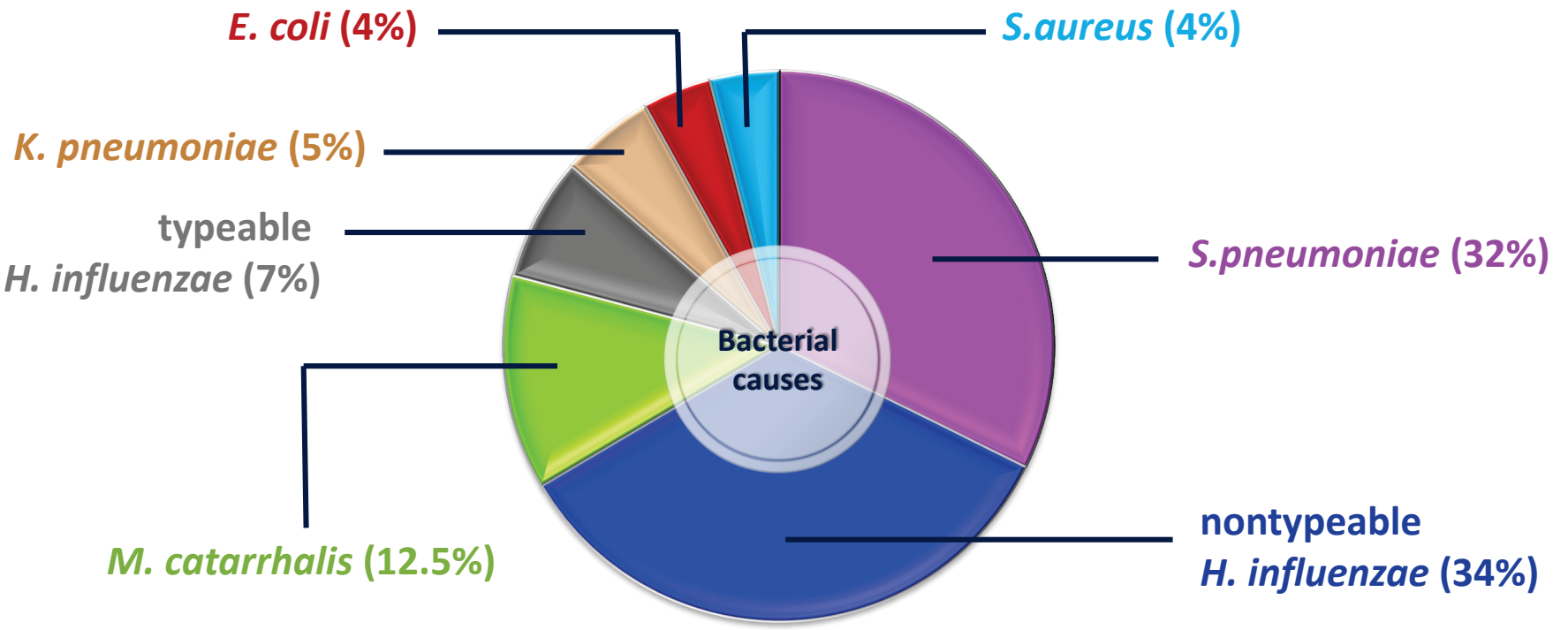
INTRODUCTION - Acute Exacerbations of Chronic Bronchitis (AECB)

Bacterial etiology in 50 to 80% of cases



Adapted from Aydemir *et al*, Pak J Med Sci, 2014, 30 (5): 1011-1016

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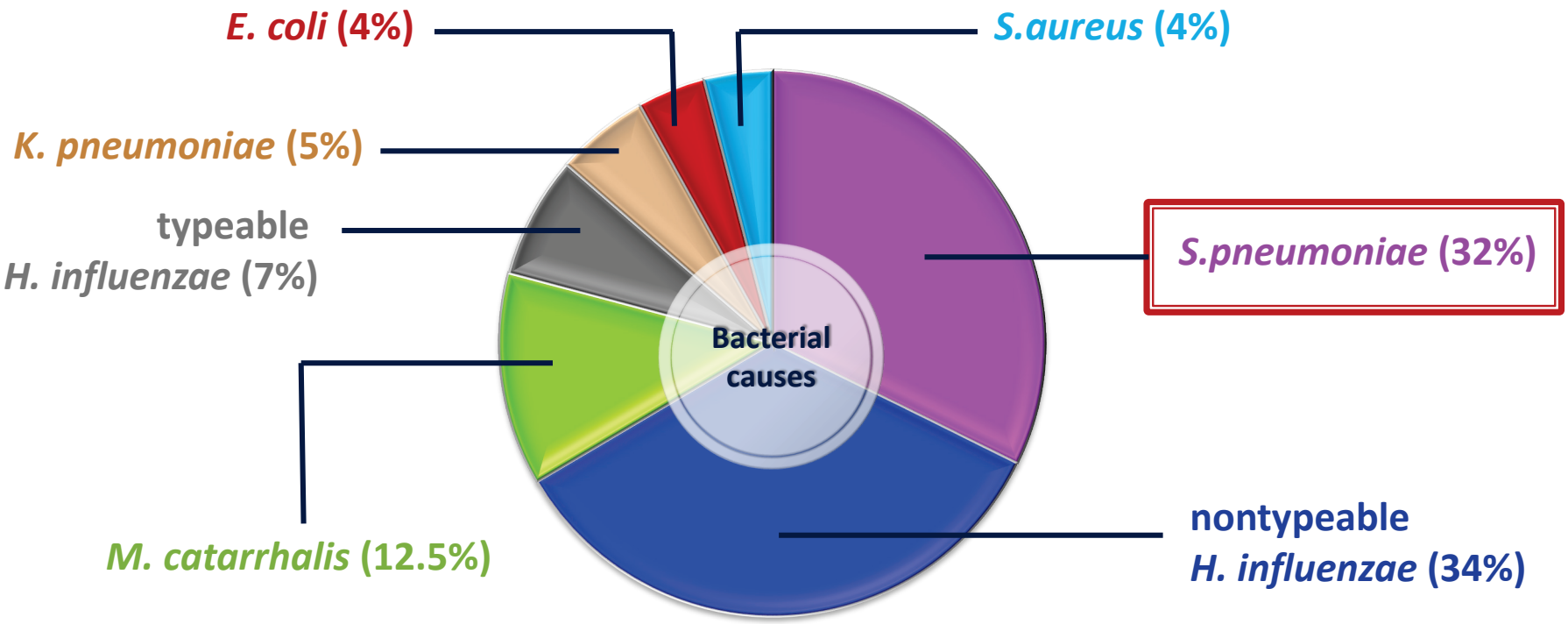

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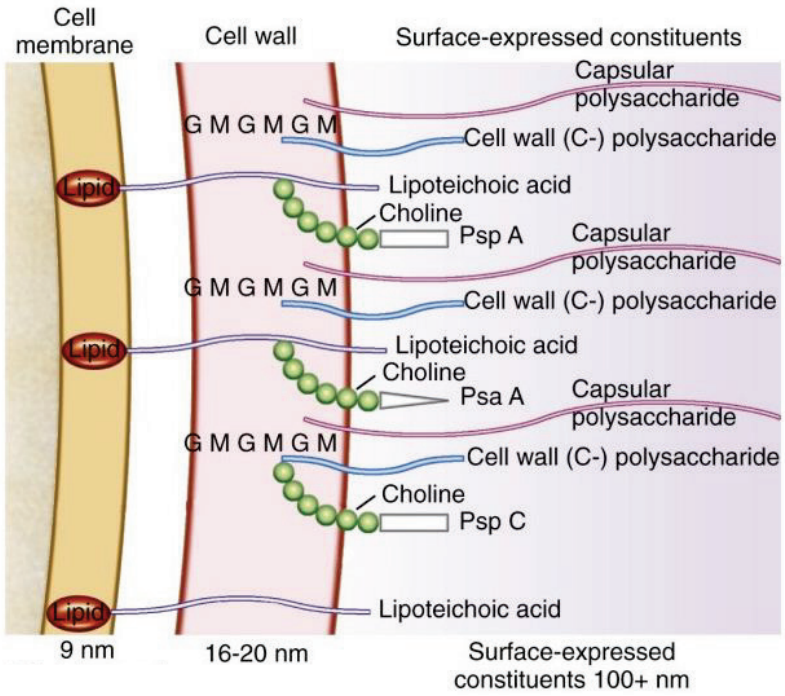
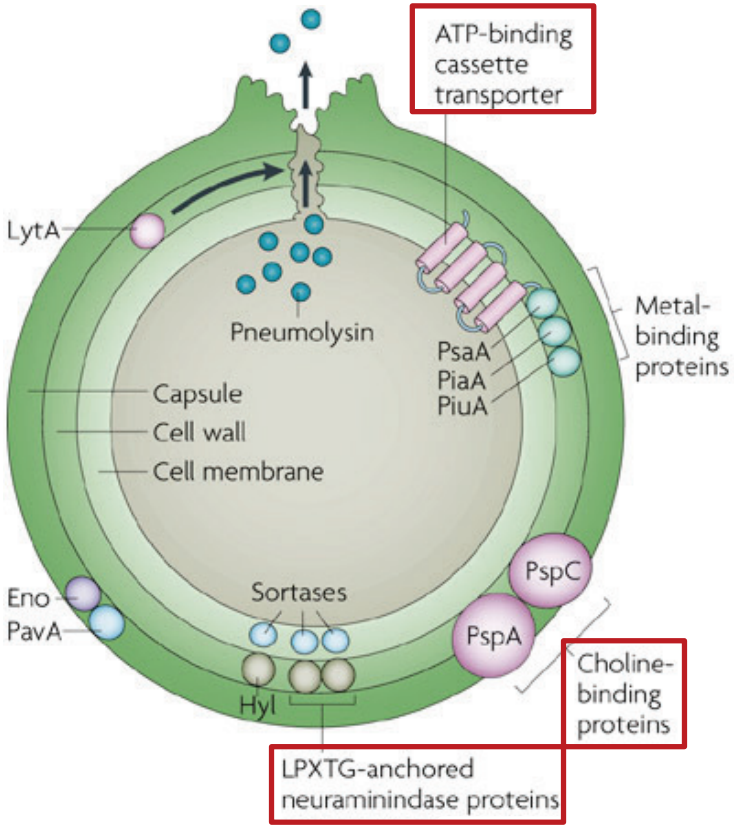
Streptococcus pneumoniae is the most predominent bacterial pathogen involved in AECOPD, **regarding to all stages of COPD patients' bronchial obstruction (GOLD 1 - 4)** [a]

[a] Eller et *al.* Chest. 1998;113 :1542-1548

INTRODUCTION - *Streptococcus pneumoniae* or Pneumococcus

- **diplococci or small chains** (diameter: 0.5 - 1 µm)
- **Gram positive bacterium** [a]
- **Several virulent factors** [a]

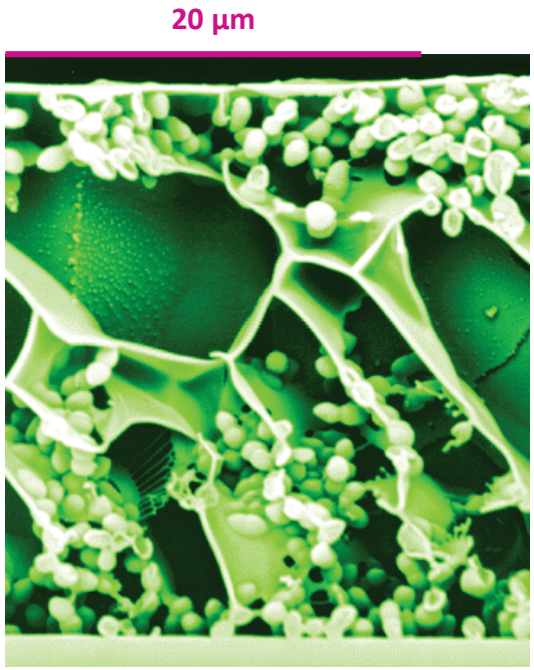




[a] Kadioglu *et al*, 2008, Nat. Rev. Microbiol. 6:288-301 ; Domenech *et al*, 2009, Environ. Microbiol. 11:2542-2555

A special life mode: growth within biofilms :

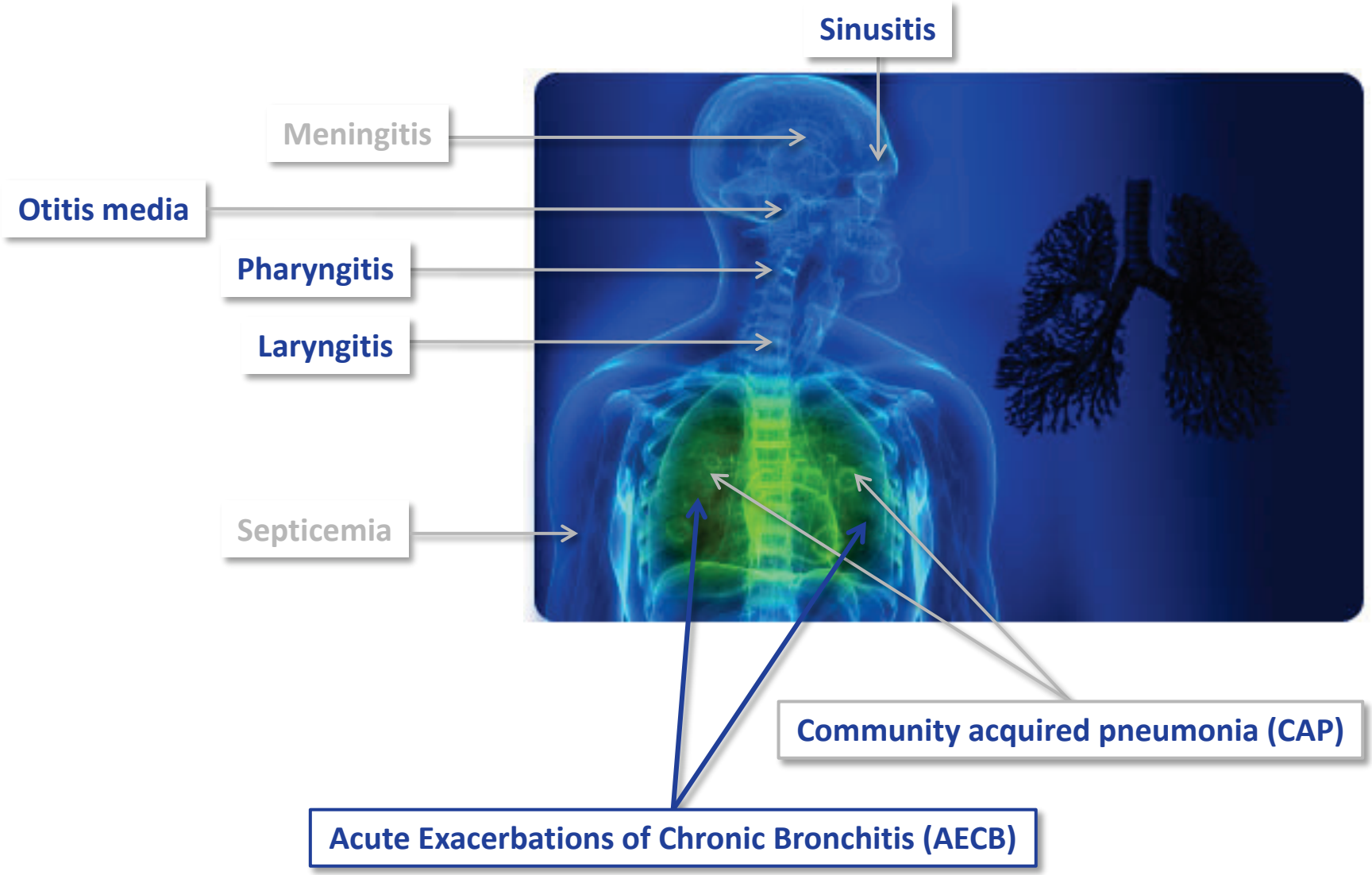
- tridimensional communities of cells embedded in a structured matrix
- adhering to inert/living surfaces
- protected from the immune system and antibiotics
- involve in up to 80% of chronic infections



Low Temperature Scanning Electron Microscopy (LTSEM) of a *S. pneumoniae* R6 biofilm formed on a glass surface (Moscoso *et al.*, 2006).

Moscoso et al, 2006 J. Bacteriol (188):7785-7795 ; Moscoso et al, 2009, Int. Microbiol (12):77-85 ; Vlastaralos et al, 2007, Laryngoscope (117):668-673

INTRODUCTION - *Streptococcus pneumoniae* or Pneumococcus



INTRODUCTION - *Streptococcus pneumoniae* or Pneumococcus

- **Commensal nasopharynx carriage in 5 - 10% of adults** [a]
- **Mainly pathogenic, especially in** >65 years, <2 years and immunocompromised patients [a]

=> 1.6 millions of deaths every year [b]

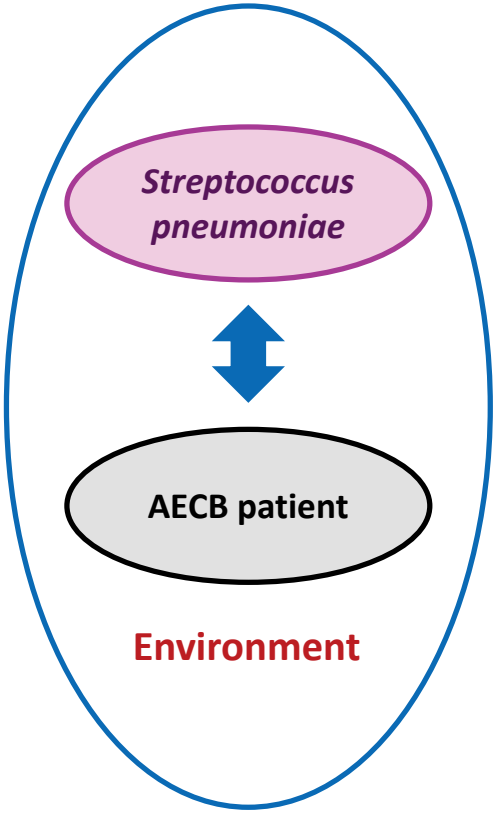
Community acquired pneumonia (CAP)

Acute Exacerbations of Chronic Bronchitis (AECB)

[a] Perez-Trallero *et al*, 2011, AAC 55:2729-2734; [b] Trappetti *et al*, 2013 Infect. Immun. 81:505-513

INTRODUCTION - *Streptococcus pneumoniae* or Pneumococcus

- Require antibiotic administration
- Especially for AECB (highly recurrent)



- **But, antibiotic choices have to be appropriate...**

=> avoid favoring/selecting resistance

=> safe for patients

=> not affected by other co-medications (drug interactions)

THESIS OBJECTIVES

Main objectives of this thesis …

… to understand HOW

… to find WAYS to RESTORE

Making Fundamental Research

Increasing global scientific knowledge

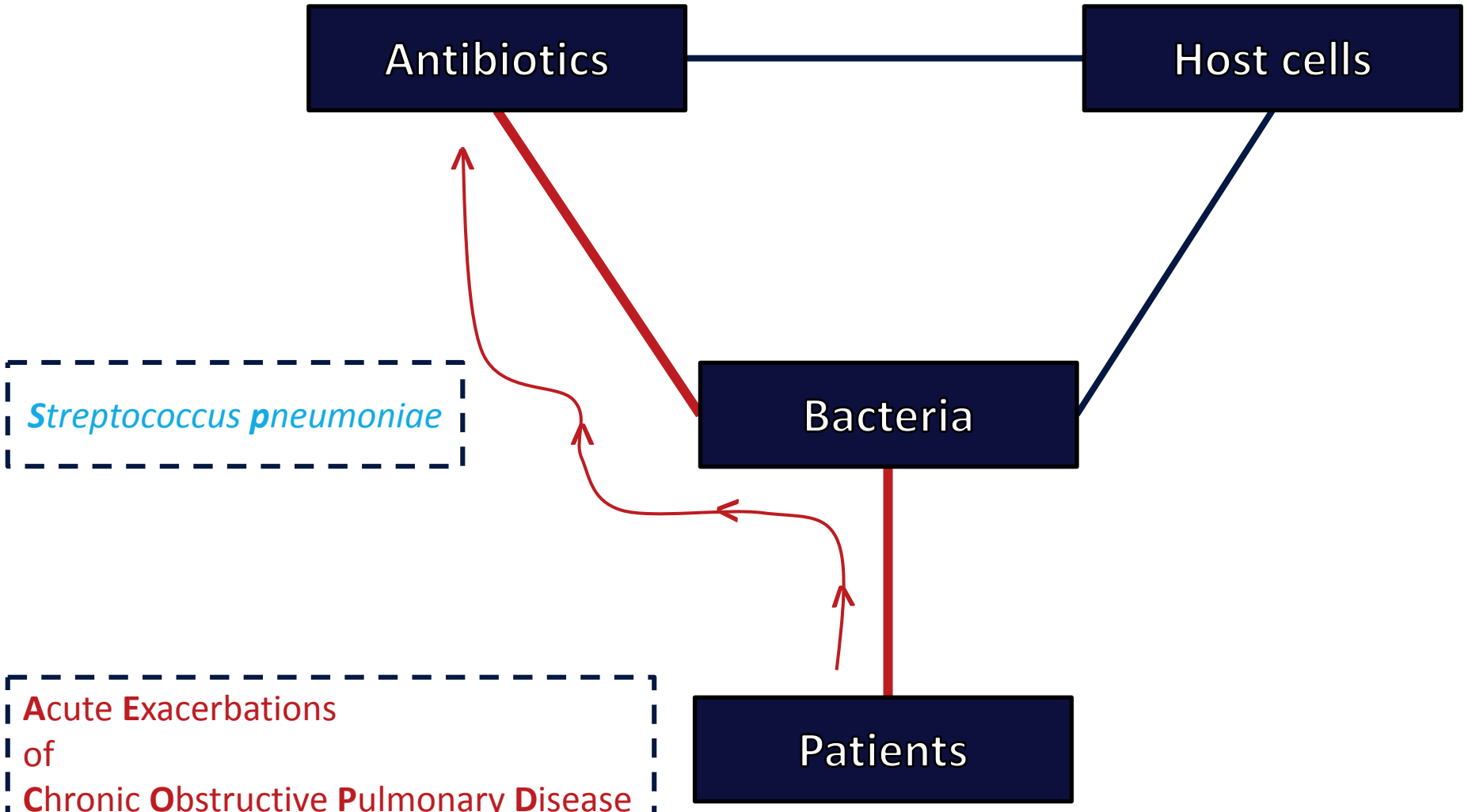
Characterizing AECB pneumococcal strains

Investigating the Etiology of Therapeutic Failures

Offering answers to clinicians

Determining what is/are the best therapeutic option(s) to treat each *S.p* AECB.

- Finding new markers to predict the resistance of *S.p.* strains to some antibiotics.
- Investigating the causes of antibiotic resistance.
- Setting-up new methods to better characterize the activity of antimicrobials.


Antibiotics
Host cells
Bacteria
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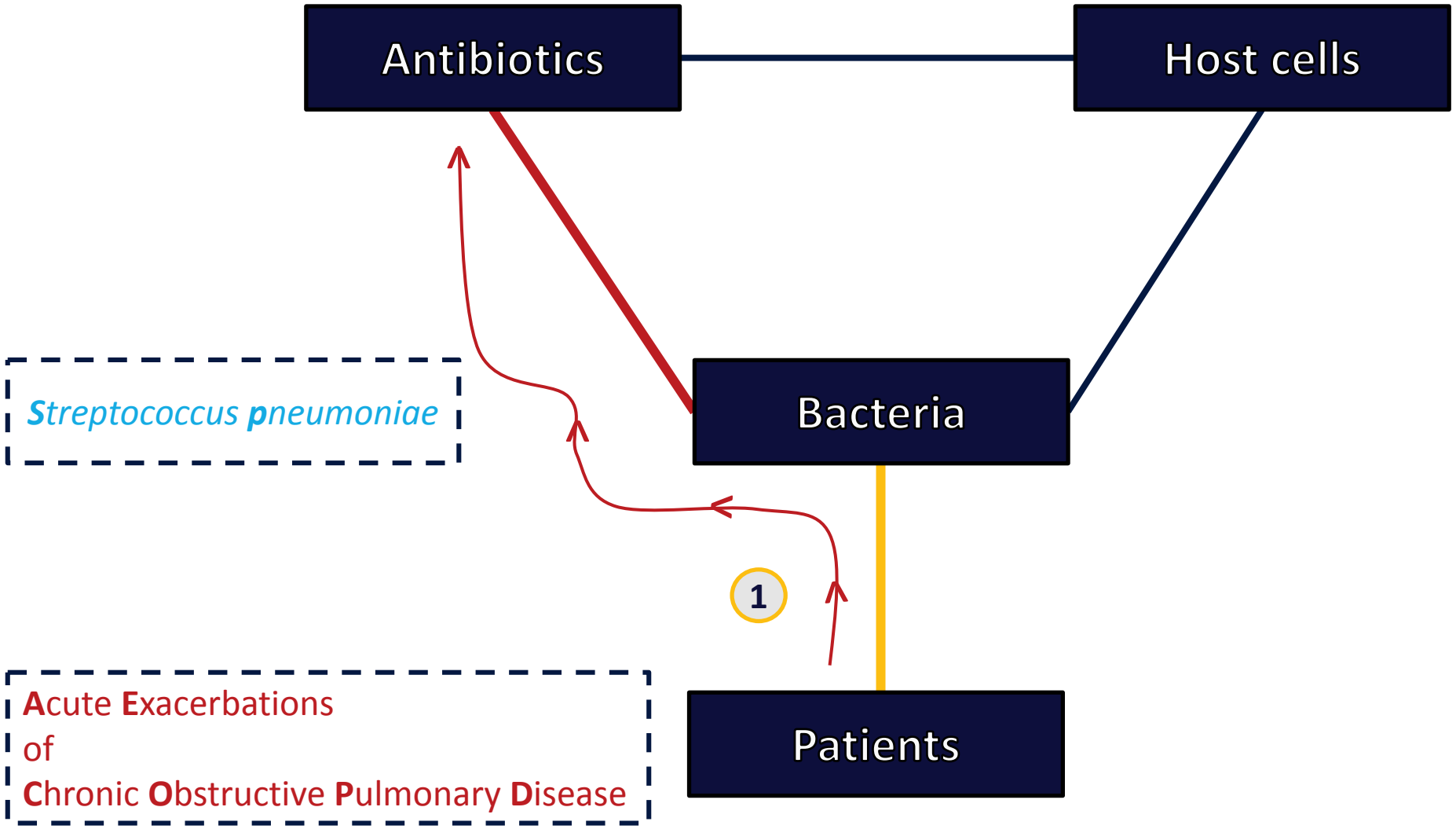
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RESULTS : CHAPTER 1

3-years multicentric epidemiological and clinical study

International Journal of Antimicrobial Agents 44 (2014) 209–217

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journal homepage: http://www.elsevier.com/locate/ijantimicag

Characterisation of a collection of *Streptococcus pneumoniae* isolates from patients suffering from acute exacerbations of chronic bronchitis: In vitro susceptibility to antibiotics and biofilm formation in relation to antibiotic efflux and serotypes/serogroups

Nathalie M. Vandevelde[a], Paul M. Tulkens[a,*], Yvan Diaz Iglesias[a], Jan Verhaegen[b], Hector Rodriguez-Villalobos[c], Ivan Philippart[d], Julie Cadrobbi[e], Nathalie Coppens[f], An Boel[g], Kristien Van Vaerenbergh[g], Hugo Francart[h], Raymond Vanhoof[i], Giuseppe Liistro[j], Paul Jordens[k], Jean-Paul d'Odemont[l], Yvan Valcke[m], Franck Verschuren[n], Françoise Van Bambeke[a]

[a] Pharmacologie cellulaire et moléculaire & Centre de Pharmacie Clinique, Louvain Drug Research Institute, Université catholique de Louvain, Brussels, Belgium
[b] Laboratorium microbiologie, Universitair Ziekenhuis Gasthuisberg, Leuven, Belgium
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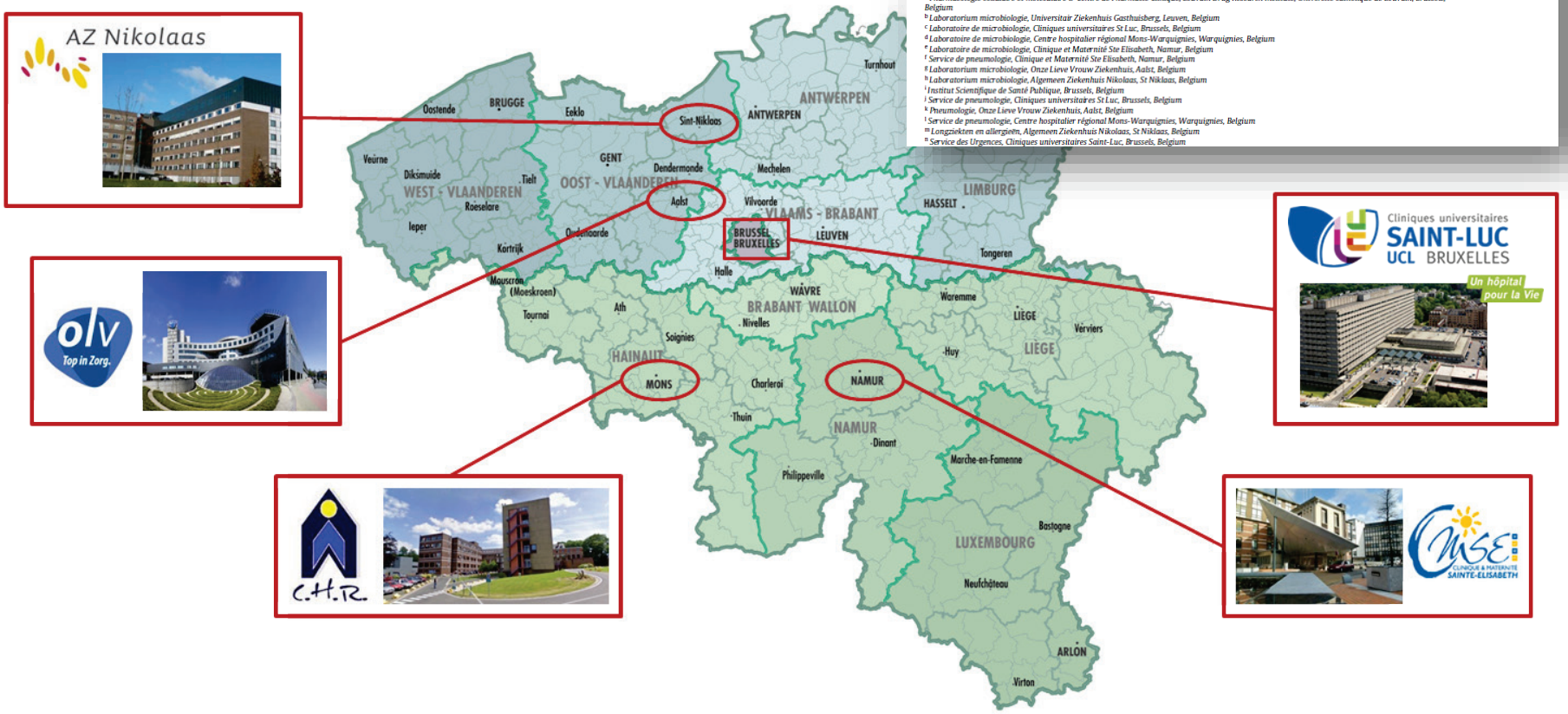
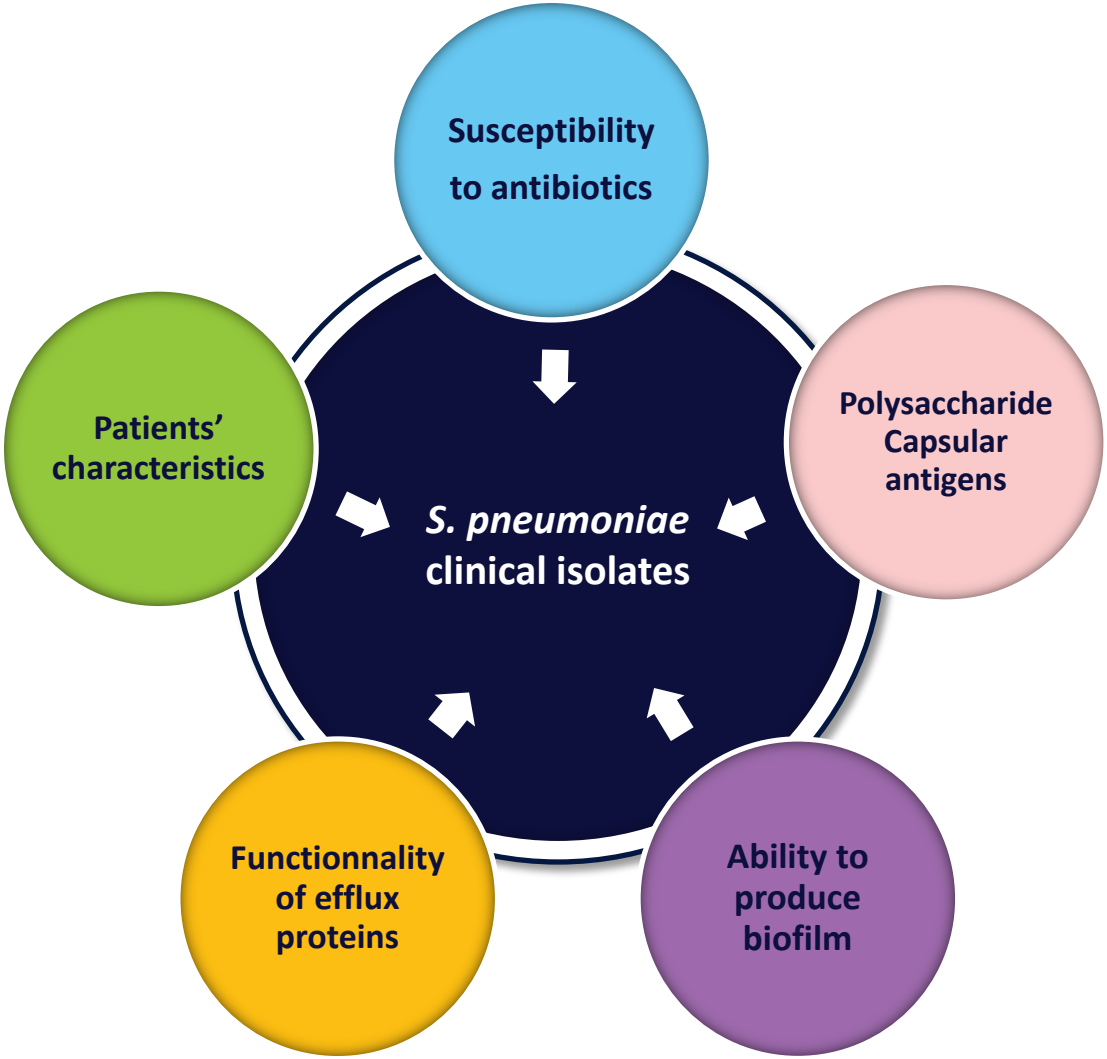


101 AECB *Streptococcus pneumoniae* clinical isolates & patients' medical data…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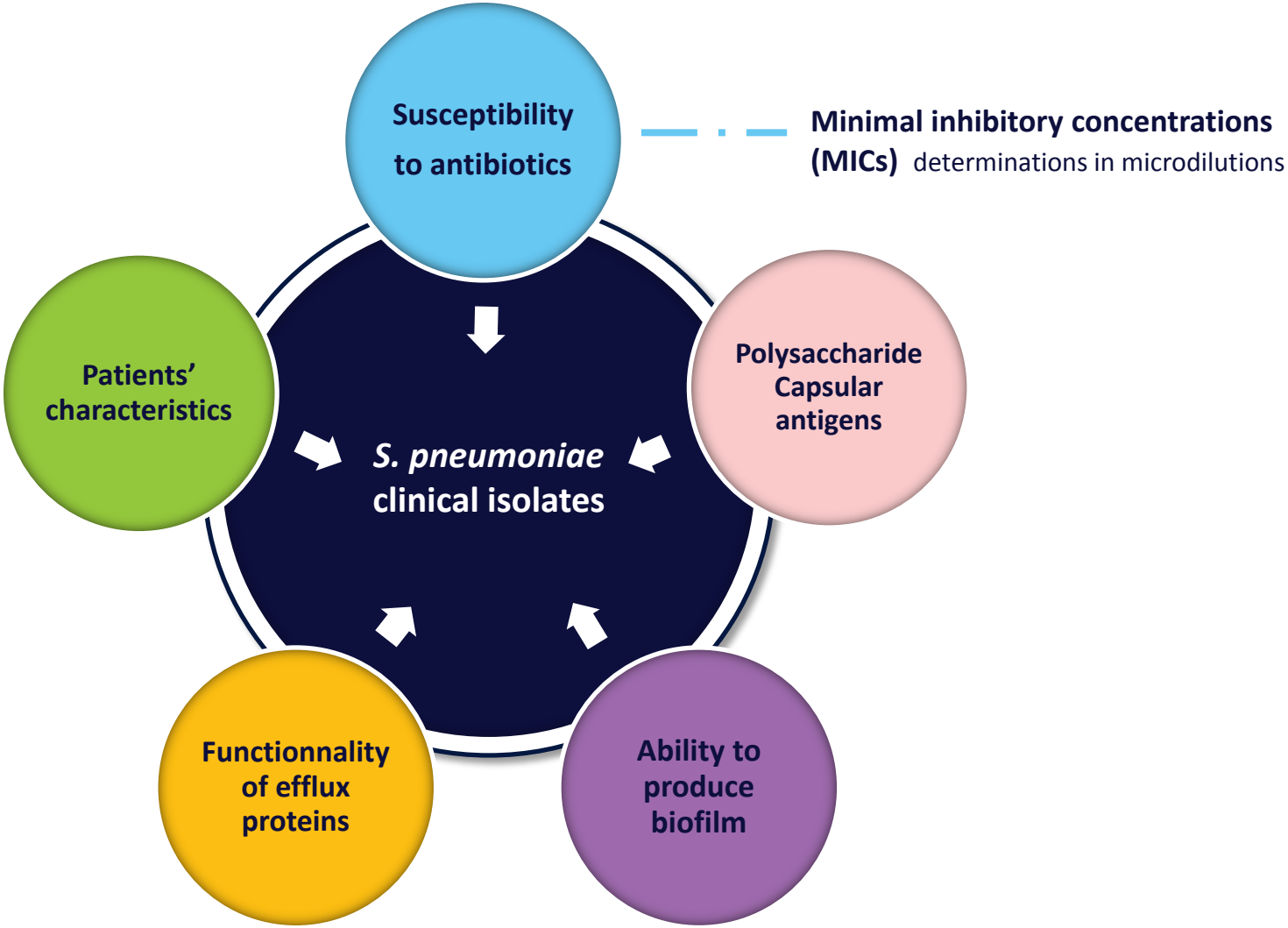
Polysaccharide Capsular antigens
Ability to produce biofilm
Functionnality of efflux proteins
Patients' characteristics
S. pneumoniae clinical isolates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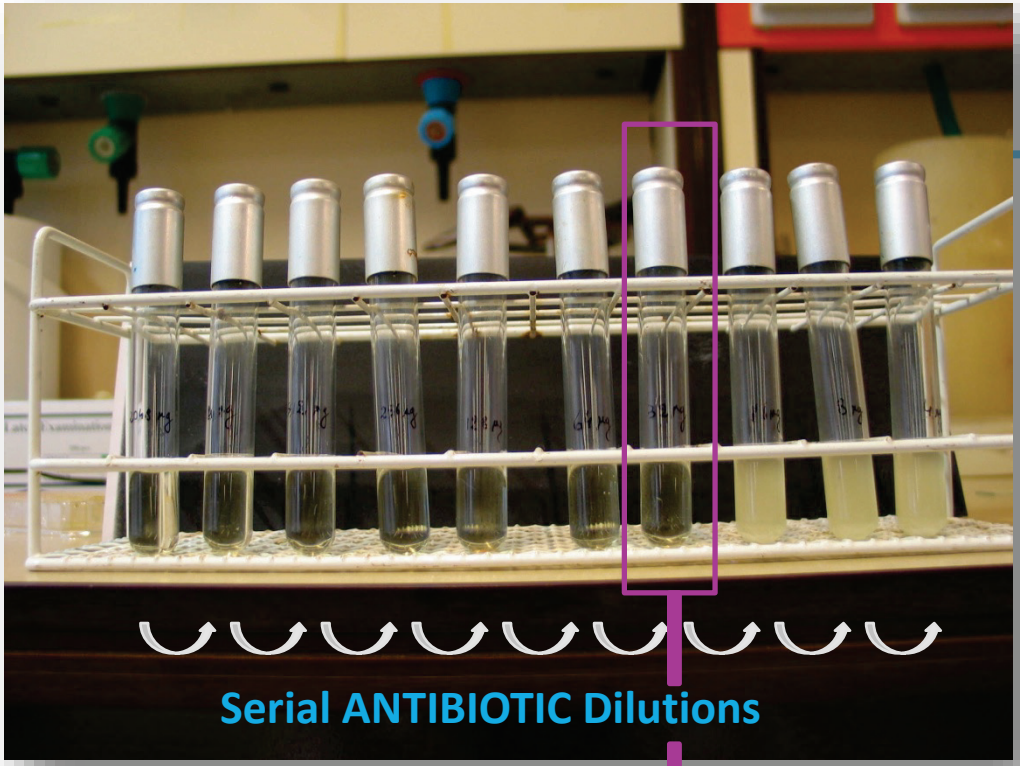

Minimal inhibitory concentrations (MICs) determinations in microdilutions

Minimal inhibitory concentrations (MICs) :

smallest antimicrobial concentration inhibiting bacterial growth

RESULTS : CHAPTER 1

Antibiotics mode of action	Antibiotic classes	Antibiotic molecules	% poorly-susceptible isolates (n=101) according to susceptibility European criteria (EUCAST) [a]		
			Breakpoint (S / R) mg/L	% Poorly susceptible strains I / R [b]	Average R %
Inhibition of bacterial wall synthesis	Betalactams	amoxicillin	≤0.5 / >2	8/8	17.5
		cefuroxime	≤0.25 / >0.5 [d]	6/13	
		ceftriaxone [c]	≤0.5 / >2	8/1	1
Inhibition of the proteic synthesis	Macrolides	clarithromycin	≤0.25 / >0.5	1/27.7	~ 33.5
		azithromycin	≤0.25 / >0.5	1/38.6	
	Ketolides	telithromycin	≤0.25 / >0.5	1/2	~ 2
		solithromycin	na	na	na
Inhibition of DNA replication	Fluoroquinolones	moxifloxacin	≤0.5 / >0.5	0/8	~ 5.5
		levofloxacin	≤2 / >2	0/3	
		ciprofloxacin [e]	≤0.125 / >2	82.2/13.8	~ 96

na: not applicable (no breakpoint defined)

[a] European Committee for Antibiotic Susceptibility Testing
[b] I: intermediate; R: resistance
[c] oral form (cefuroxime axetil)
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Good susceptibility to ceftriaxone, telithromycin, moxifloxacin & levofloxacin

High macrolide resistance

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		ceftriaxone [c]	≤0.5 / >2	8/1	1
Inhibition of the proteic synthesis	Macrolides	clarithromycin	≤0.25 / >0.5	1/27.7	~ 33.5
		azithromycin	≤0.25 / >0.5	1/38.6	
	Ketolides	telithromycin	≤0.25 / >0.5	1/2	~ 2
		solithromycin	na	na	na
Inhibition of DNA replication	Fluoroquinolones	moxifloxacin	≤0.5 / >0.5	0/8	~ 5.5
		levofloxacin	≤2 / >2	0/3	
		ciprofloxacin [d]	≤0.125 / >2	82.2/13.8	~ 96

Belgian therapeutic guidelines [a]

Amoxicillin, in alternation with moxifloxacin

Good susceptibility to ceftriaxone, telithromycin, moxifloxacin & levofloxacin

High macrolide resistance

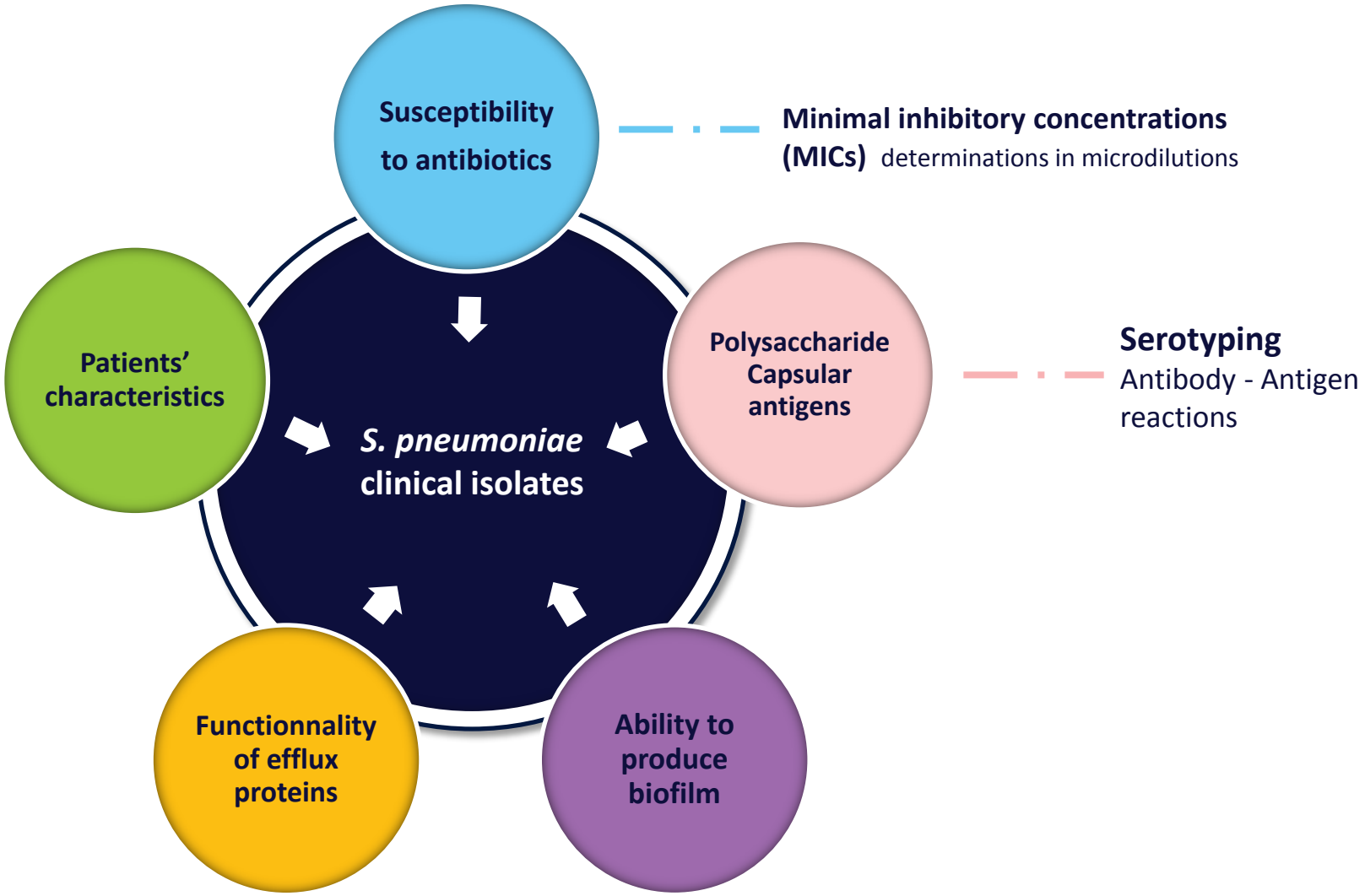
[a] Van Bambeke et al, 2007, Drugs (67):2355-2382 ; BAPCOC 2012

But…

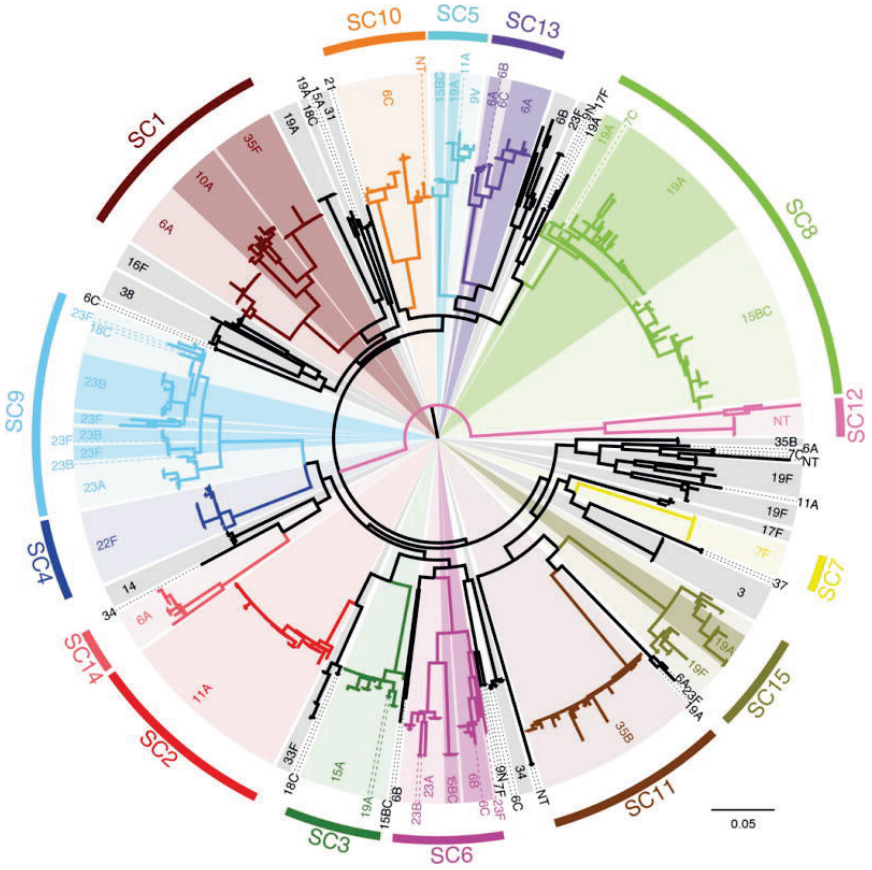
Does it mean that these molecules are clinically active in all AECB cases?

Which molecule for patients presenting allergy to penicillins ?

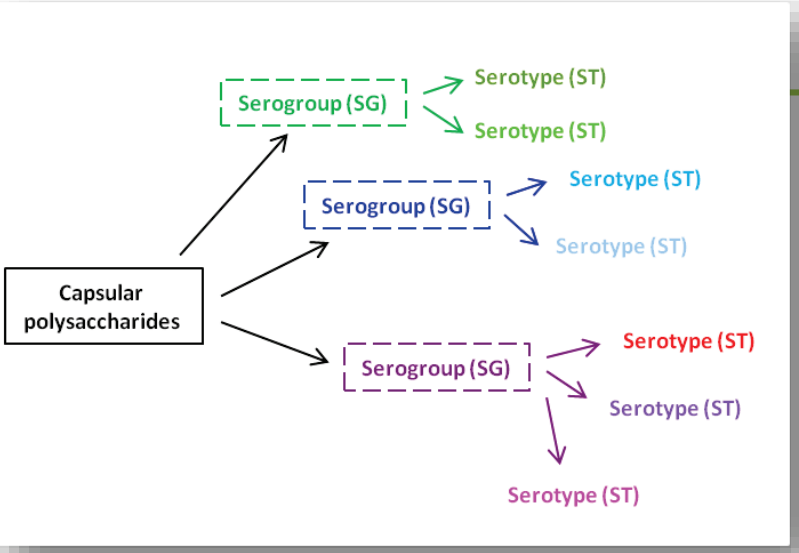
And what about the benefice-risk of fluoroquinolones in frail patients ?

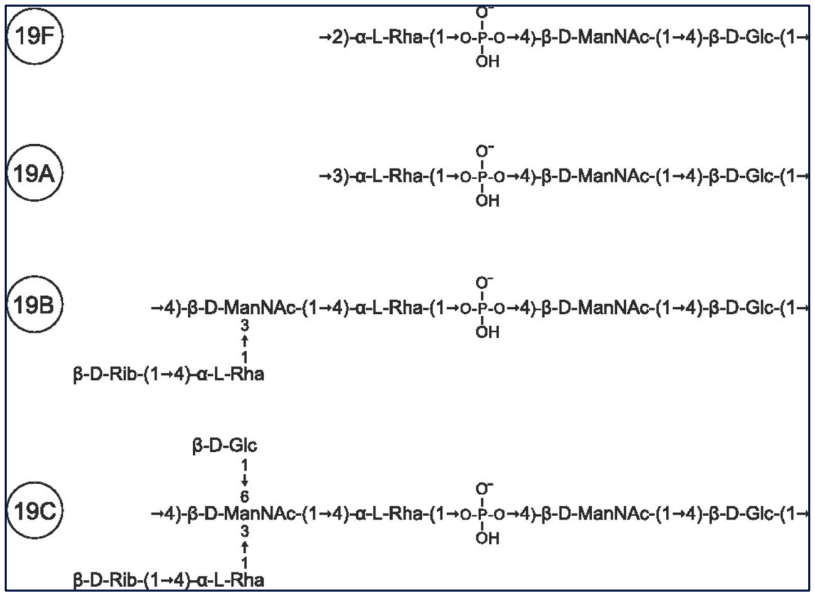

Susceptibility to antibiotics
Minimal inhibitory concentrations (MICs) determinations in microdilutions
Polysaccharide Capsular antigens
Serotyping
Antibody - Antigen reactions
Patients' characteristics
S. pneumoniae clinical isolates
Functionnality of efflux proteins
Ability to produce biofilm

Capsular polysaccharides determine the serotype



Pneumococcal phylogeny. The maximum likelihood was generated using 106,196 polymorphic sites. Fifteen monophyletic sequence clusters (SCs) are labeled, with the terminal branches of the tree colored black indicating taxa that constitute a sixteenth polyphyletic group. Within the monophyletic sequence clusters, light background shading indicates one particular serotype, with darker shading and dashed lines used to indicate groups of isolates of alternative serotypes. Croucher et al, 2013, Nat. Genet. , 45 (6): 656-63



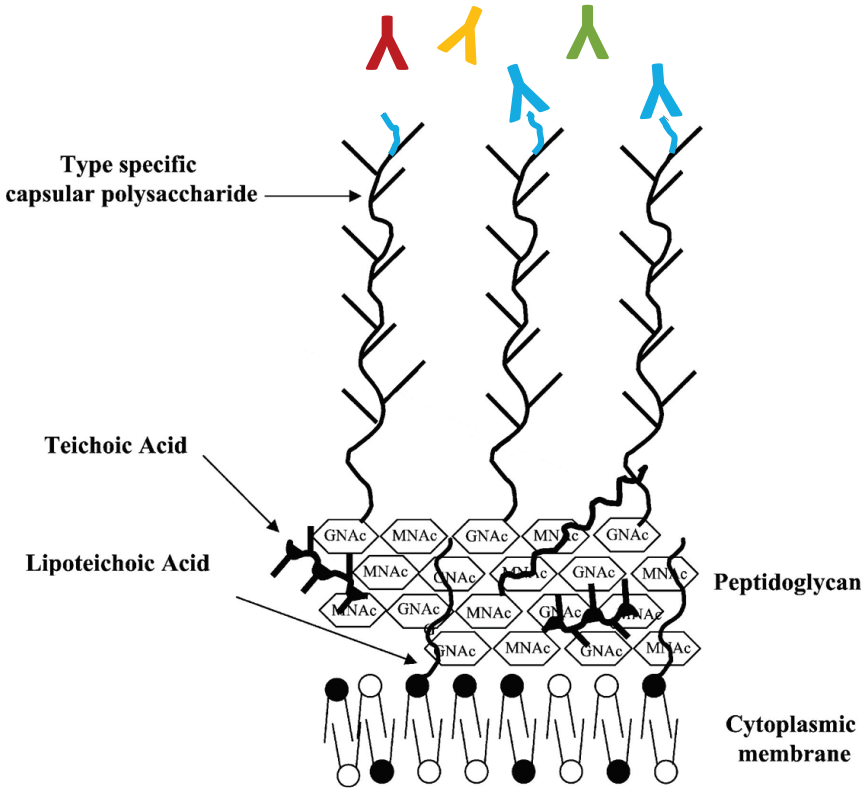


Capsular structures of S. pneumoniae SG 19. Kjaer et al, 2013, Infect. Immun. 81 (2): 452-9

KU LEUVEN

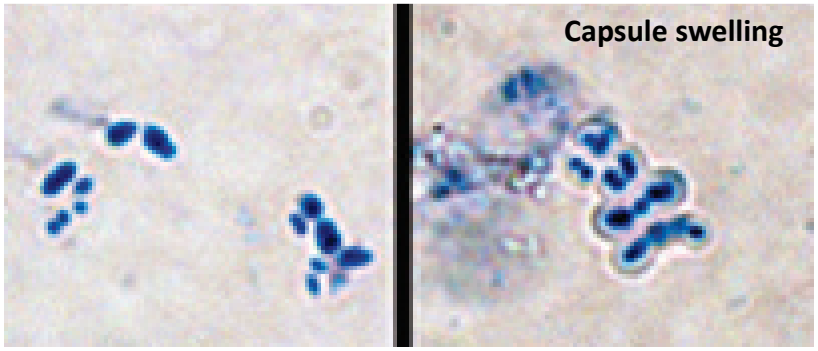
Prof. J. Verhaegen

Capsular polysaccharides determine the serotype



Hancock L E , and Gilmore M S PNAS 2002;99:1574-1579

Immuno-precipitation using antibodies specific of each serotype

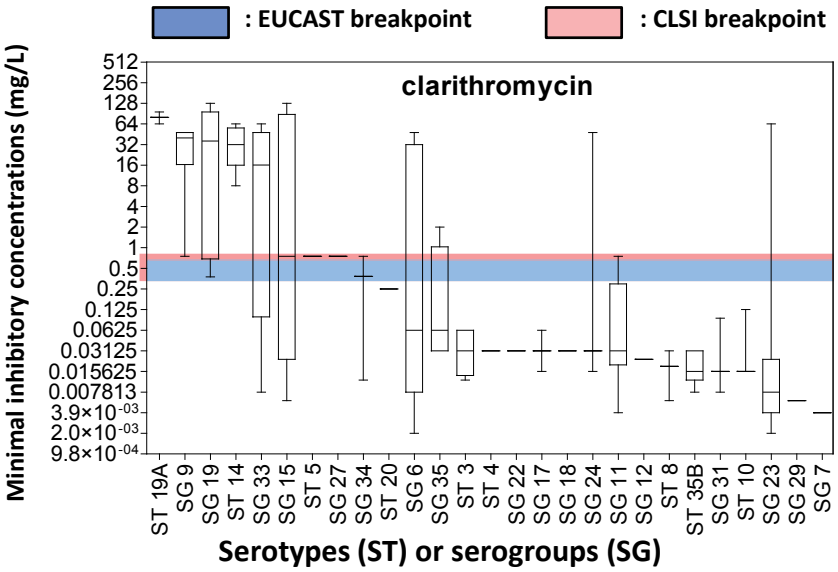


Negative **Positive**

The AECB clinical isolates collected were exposed to serotype-specific antibodies

RESULTS : CHAPTER 1 : 3-years epidemiological study

Isolates susceptibility to clarithromycin as a function of their serotype/serogroup



Antibiotic susceptibility to clarithromycin for all isolates as a function of their serotype (ST) / serogroup (SG) ranked from less to more susceptible. Data are presented as "Box and whiskers plots" giving the 25, 50 and 75 quartiles (boxes and horizontal line) of the MIC distributions with the lower and upper bars extending from the lowest to the highest MIC value observed. The blue and pink horizontal ribbons show the intermediate zones of clinical susceptibility according the interpretive criteria of EUCAST (from > S to > R; [20]) and CLSI (from >S to < R [19]), respectively.

RESULTS : CHAPTER 1 : 3-years epidemiological study

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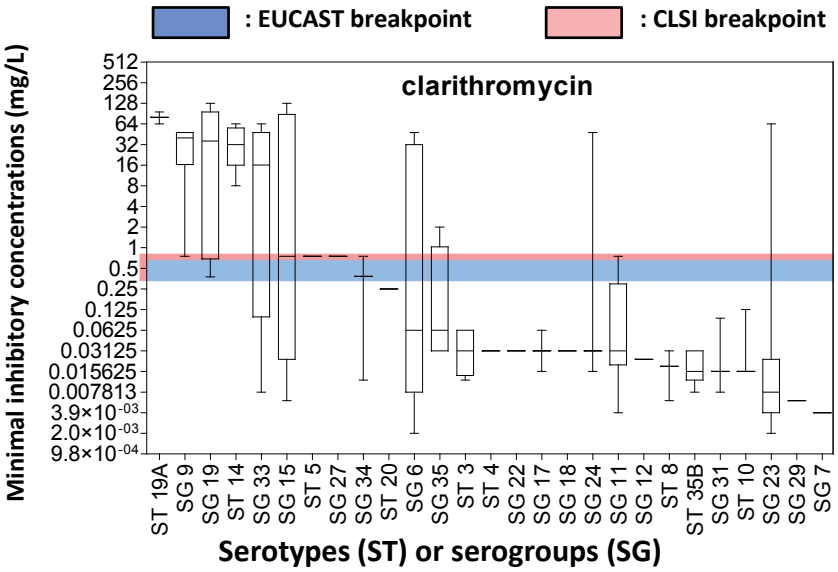

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β-lactams [a]		Macrolides [b]		Ketolides [c]		Fluoroquinolones [d]	
ST/SG [e]	Ranking [f]	ST/SG [e]	Ranking [f]	ST/SG [e]	Ranking [f]	ST/SG [e]	Ranking [f]
ST 14	5	ST 19A	2	ST 14	2	ST 19A	2
ST 19A	7	SG 9	5	ST 19A	4	SG 33	5
SG 9	10	ST 14	6	SG 19	8	ST 4	6
SG 29	10	SG 19	7	SG 9	13	ST 5	10
SG 15	23	SG 33	10	SG 17	15	SG 15	15
SG 23	61	SG 12	44	ST 35B	41	ST 8	45
SG 7	63	ST 10	44	SG 12	44	SG 18	46
SG 31	65	SG 23	48	SG 29	46	ST 20	48
ST 3	66	SG 29	53	SG 34	50	SG 7	50
ST 8	68	SG 7	53	ST 20	54	SG 29	51

[a] amoxicillin, cefuroxime and ceftriaxone
[b] clarithromycin and azithromycin
[c] telithromycin and solithromycin
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[e] serotypes (ST) and serogroups (SG)
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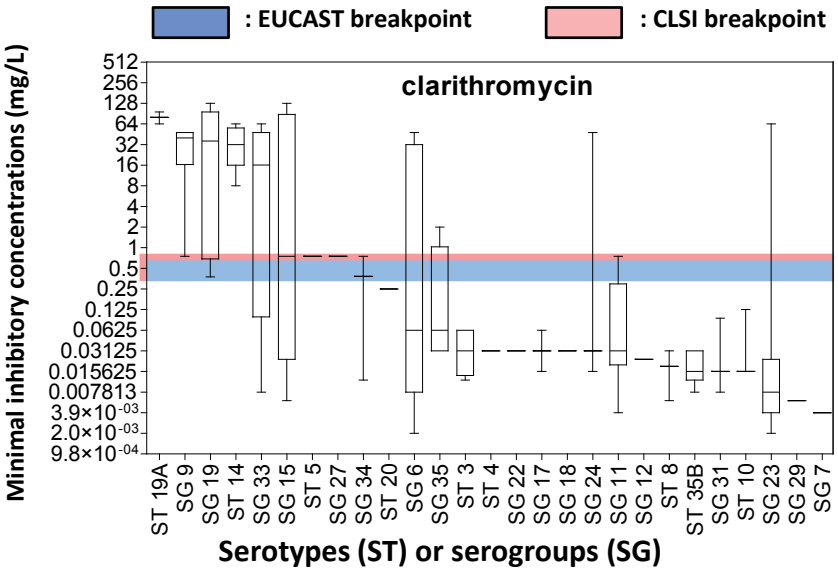
All Serotypes/serogroups do not have the same susceptibility profile to antibiotics

RESULTS : CHAPTER 1 : 3-years epidemiological study

Isolates susceptibility to clarithromycin as a function of their serotype/serogroup



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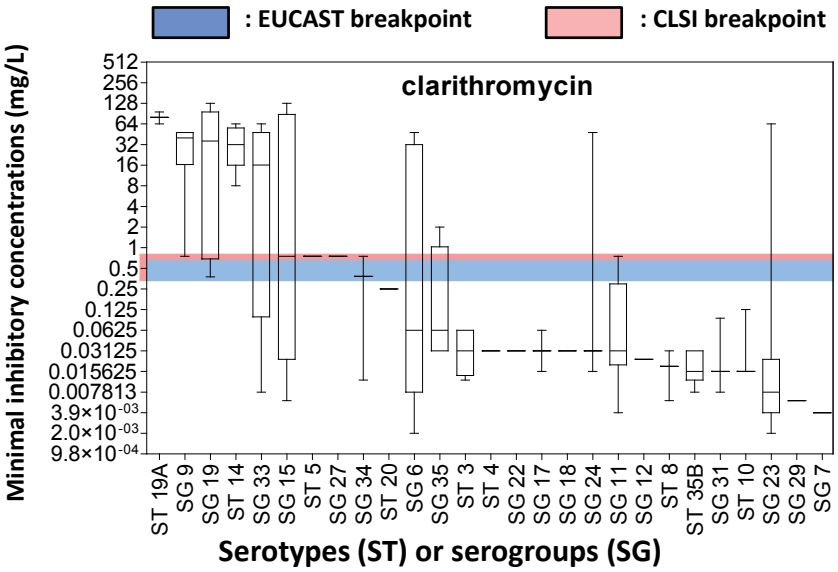

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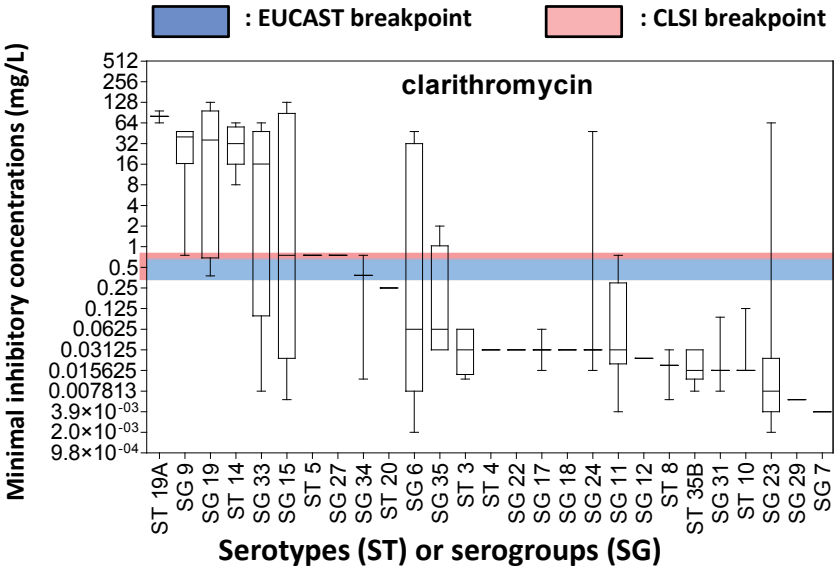

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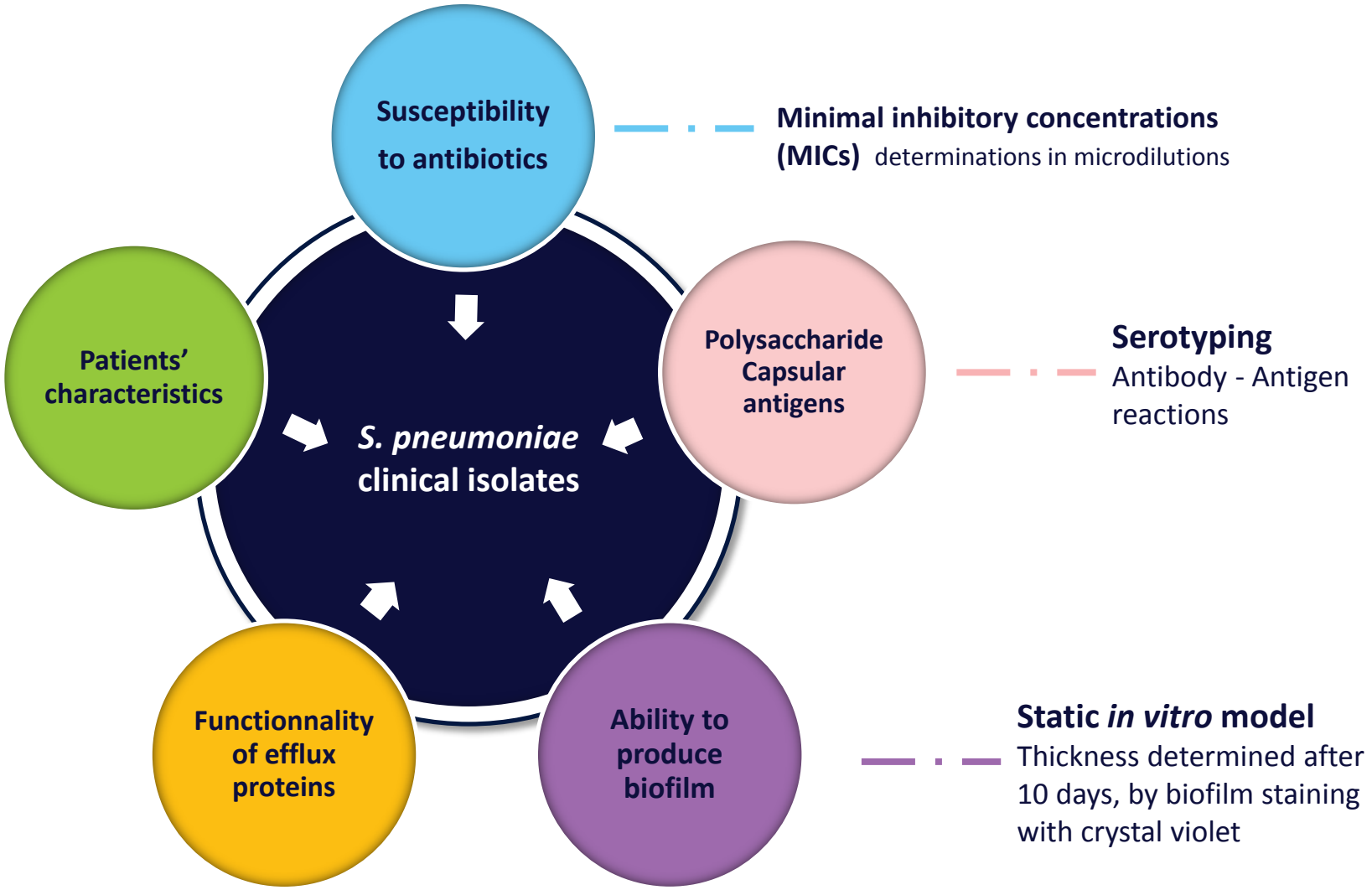
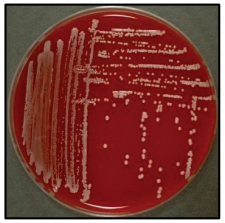
All Serotypes/serogroups do not have the same susceptibility profile to antibiotics

=> Capsular antigens may perhaps translate the strain susceptibility to AB and guide « infection-personalised » therapeutic choices

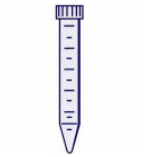

Susceptibility to antibiotics
Minimal inhibitory concentrations (MICs) determinations in microdilutions
Polysaccharide Capsular antigens
Serotyping
Antibody - Antigen reactions
Patients' characteristics
S. pneumoniae clinical isolates
Functionnality of efflux proteins
Ability to produce biofilm
Static in vitro model
Thickness determined after 10 days, by biofilm staining with crystal violet

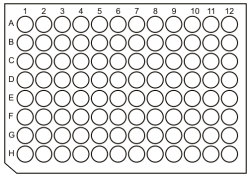
RESULTS : CHAPTER 1



Bacterial suspension

+ Medium
caMHB + 5% LHB

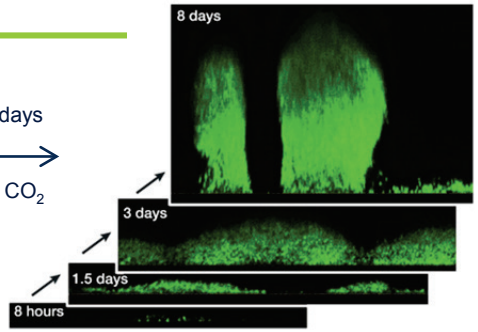




+ Medium
caMHB + 5% LHB + 2% Glucose

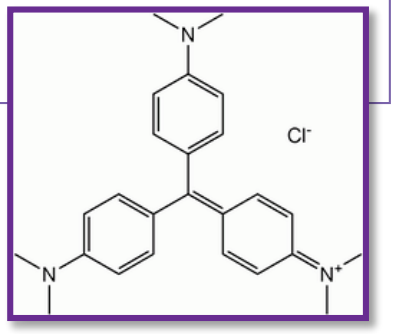
up to 20 days

37°C; 5% CO_2

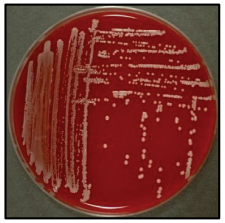


Fuqua and Greenberg, Nat Rev Mol Cell Biol. 2002;3(9):685-95

Biofilm thickness quantified over time through
- biofilm staining with Crystal violet (CV)
- spectrophotometric measures of CV absorbance (570nm)



RESULTS : CHAPTER 1



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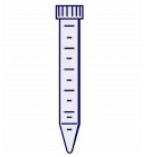

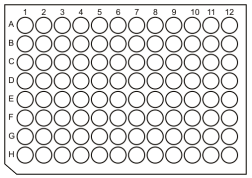

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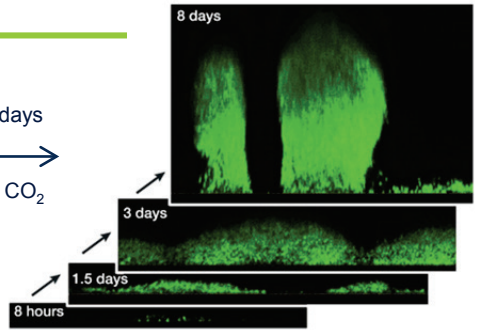
up to 20 days

37°C; 5% CO_2



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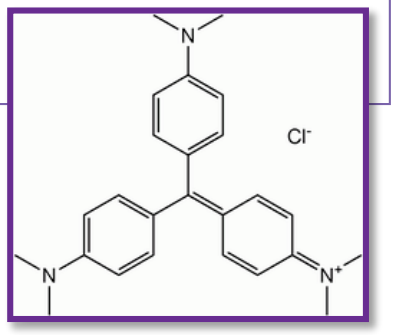
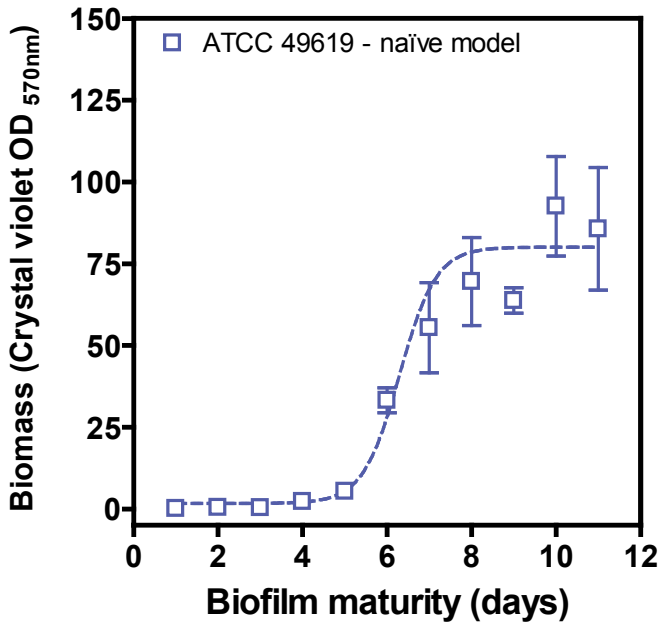
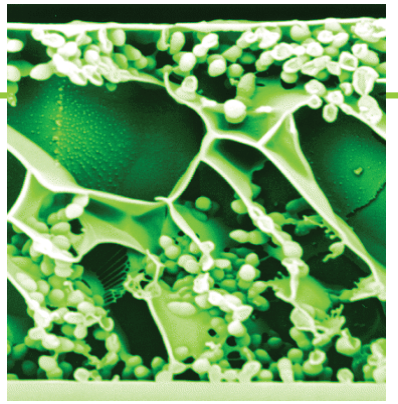
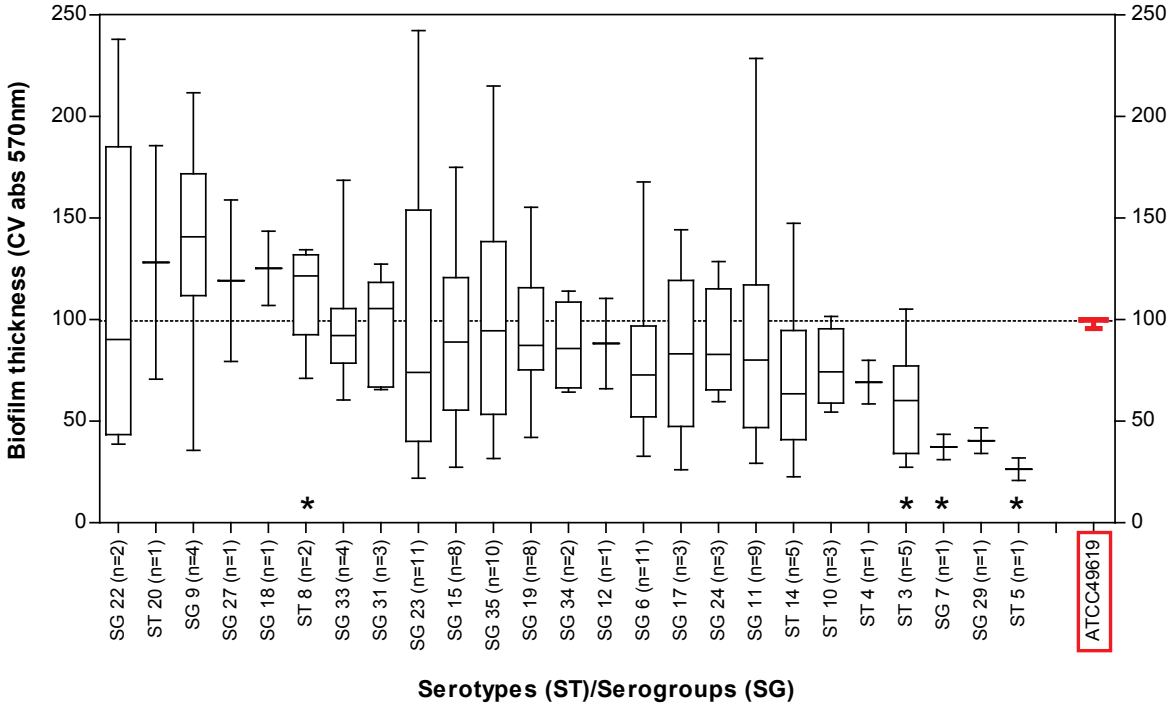




RESULTS : CHAPTER 1 : 3-years epidemiological study

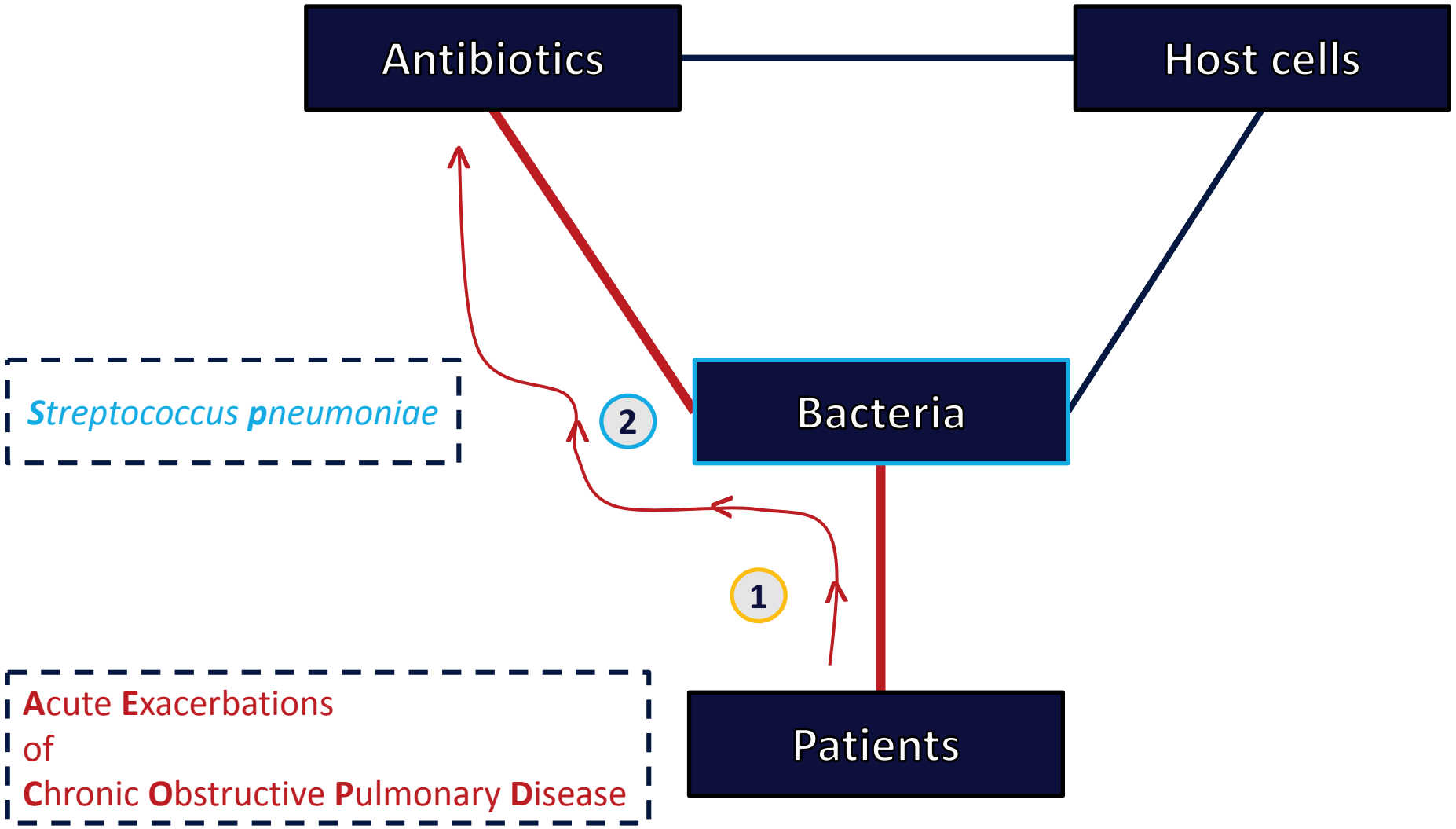


Biofilm production after 10-days for all isolates as a function of their serotype/serogroup

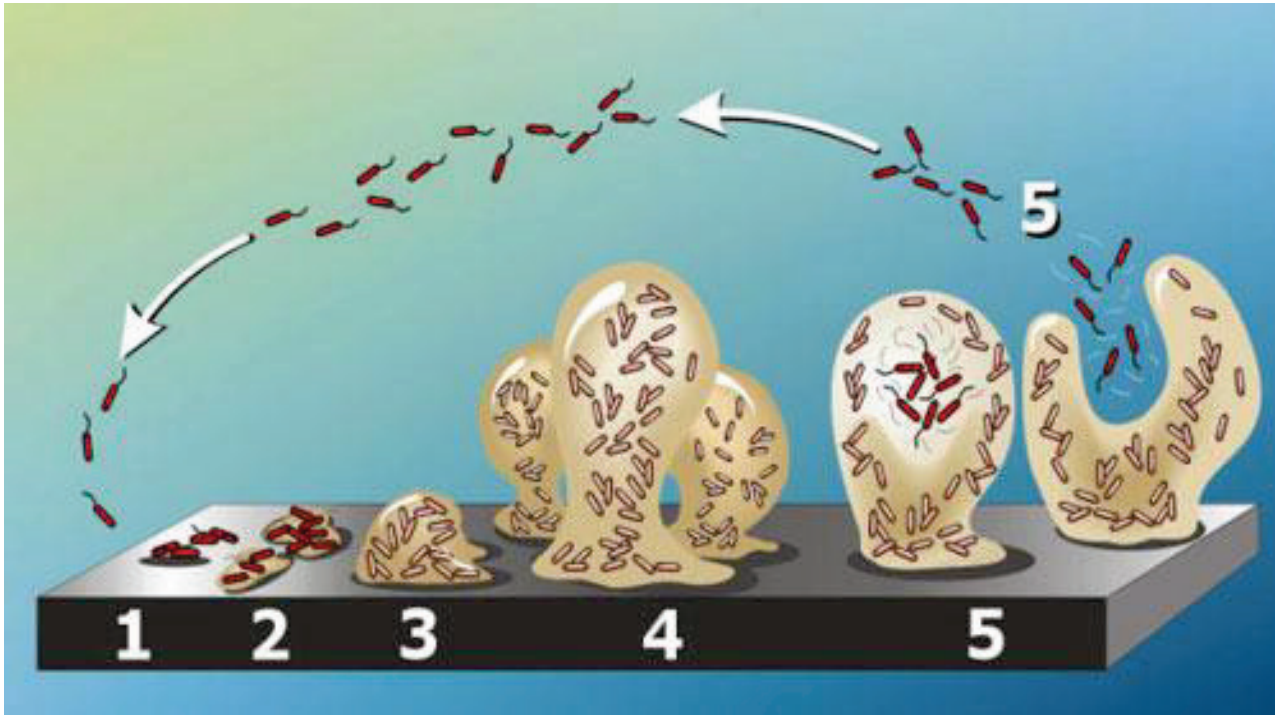


Biofilm production after 10 days of culture for all clinical isolates (as a function of their serotype (ST) / serogroup (SG) and ranked from the most to the least productive [using arithmetic means; the box and whiskers show the lowest, the 25 and 75 percentiles, the median and the highest value]; the number of isolates tested for each ST/SG is shown in the abscissa) and of the reference strain ATCC 49619 (ST19F). Each strain was tested twice in with 3 to 6 measures each time. Strains with ST belonging to a single SG have been pooled (and marked as SG) after having observed no significant differences between these serotypes. The horizontal dotted line corresponds to the mean value for the reference strain. Strains marked ST correspond to isolates where there was only one serotype. ST/SG reported in the litterature as causing acute infections are marked with an asterisk.

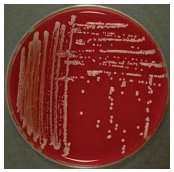
All pneumococcal serotypes/serogroups produce biofilm but some of them seem to be higher or lower producers **=> implication in cell persistence**


Antibiotics
Host cells
Bacteria
Patients
Streptococcus pneumoniae
2
1
Acute Exacerbations
of
Chronic Obstructive Pulmonary Disease

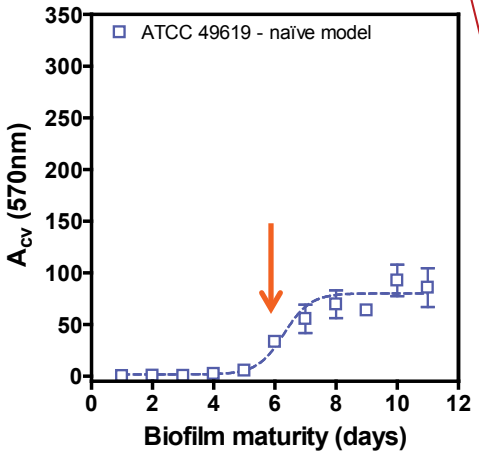
RESULTS : CHAPTER 2 : *In vitro* biofilm models

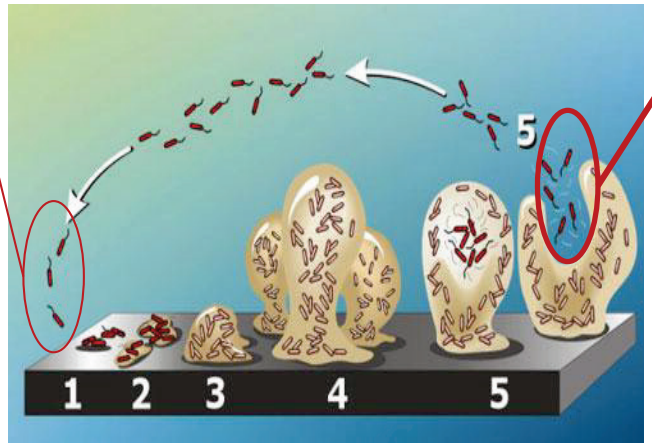


RESULTS : CHAPTER 2 : *In vitro* biofilm models

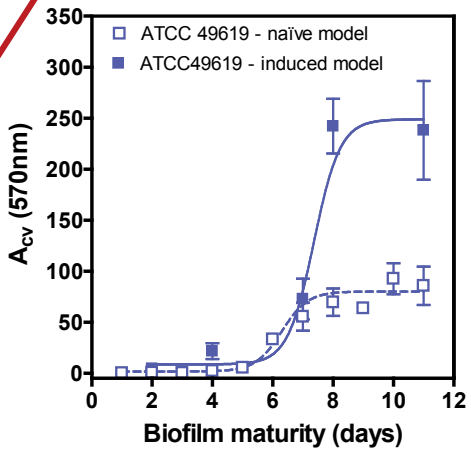


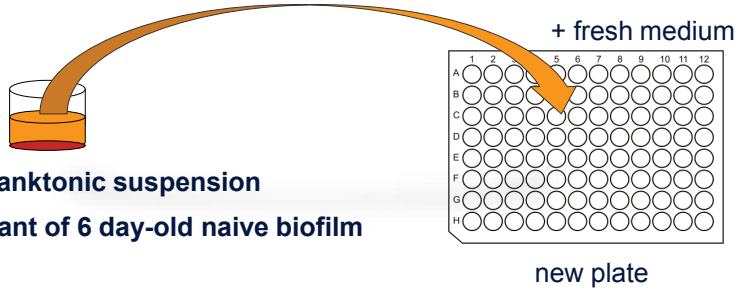
Naive model



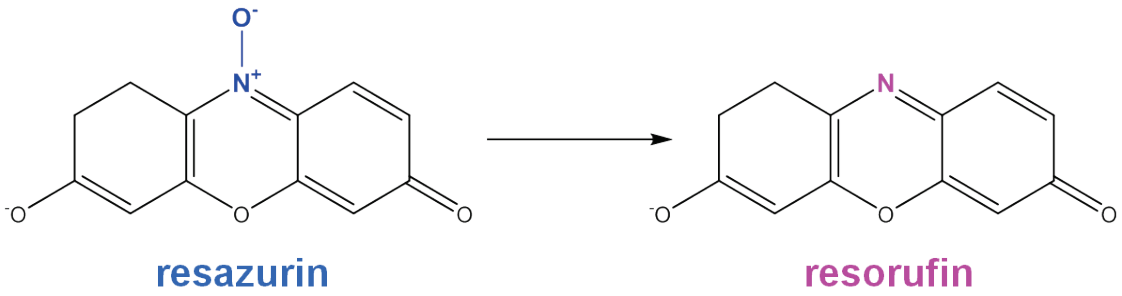


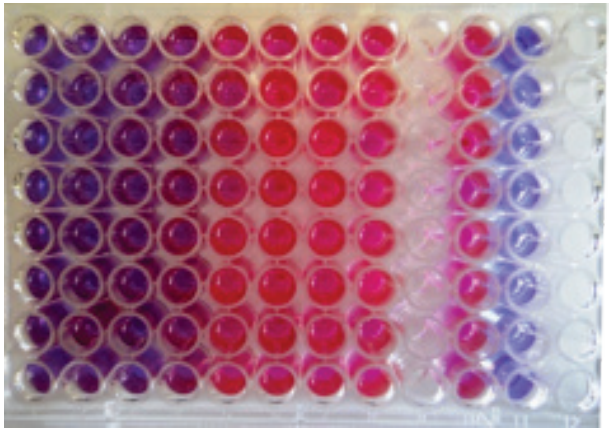
Induced model





Resazurin viability assay [a] adapted to pneumococcal biofilms





C- : medium <u>without</u> AB

C+: SDS 1%m/v aq. solution

RESULTS : CHAPTER 3

Antibiotics

Host cells

3

Streptococcus pneumoniae

2

Bacteria

1

Acute **E**xacerbations
of
Chronic **O**bstructive **P**ulmon

Antimicrobial Agents and Chemotherapy

Antibiotic Activity against Naive and Induced Streptococcus pneumoniae Biofilms in an *In Vitro* Pharmacodynamic Model

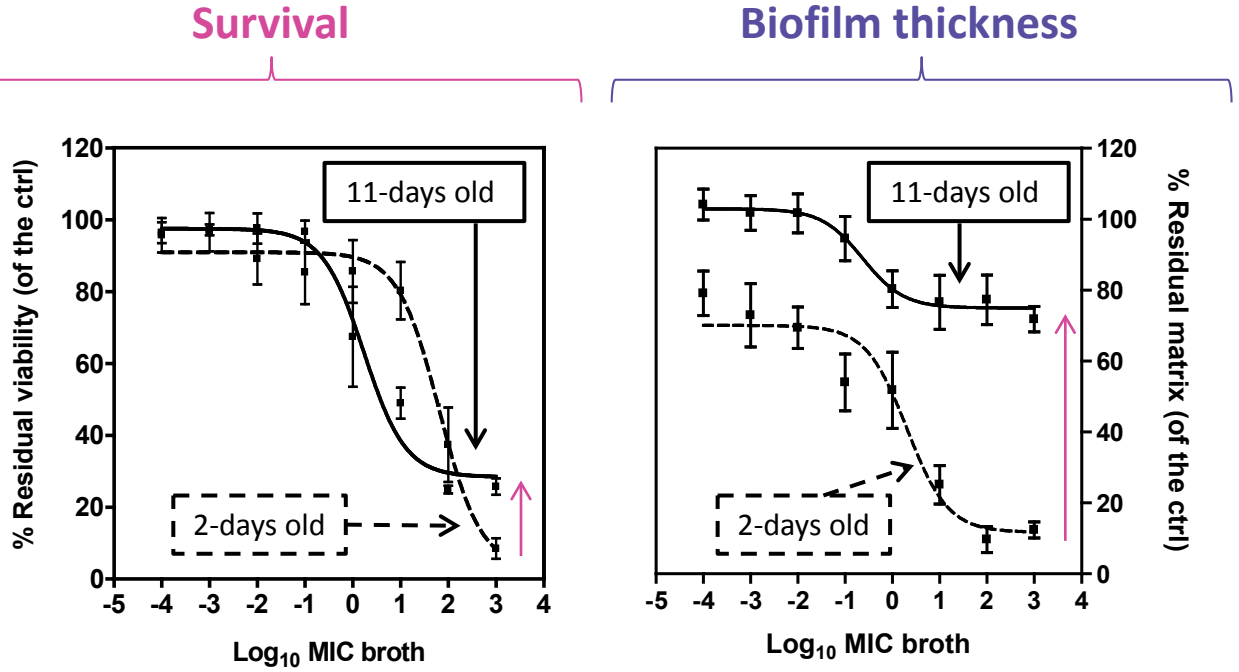
Nathalie M. Vandevelde, Paul M. Tulkens and Françoise Van Bambeke

Antimicrob. Agents Chemother. 2014, 58(3):1348. DOI: 10.1128/AAC.01858-13.
Published Ahead of Print 16 December 2013.

RESULTS : CHAPTER 3 : Pharmacodynamic studies of antibiotic activity

Ex: ATCC 49619 biofilms - MXF
- - : 2 days-old
— : 11 days-old

Moxifloxacin
Naive model



Data were used to fit a sigmoid function (Hill equation, slope factor set to 1) by non-linear regression

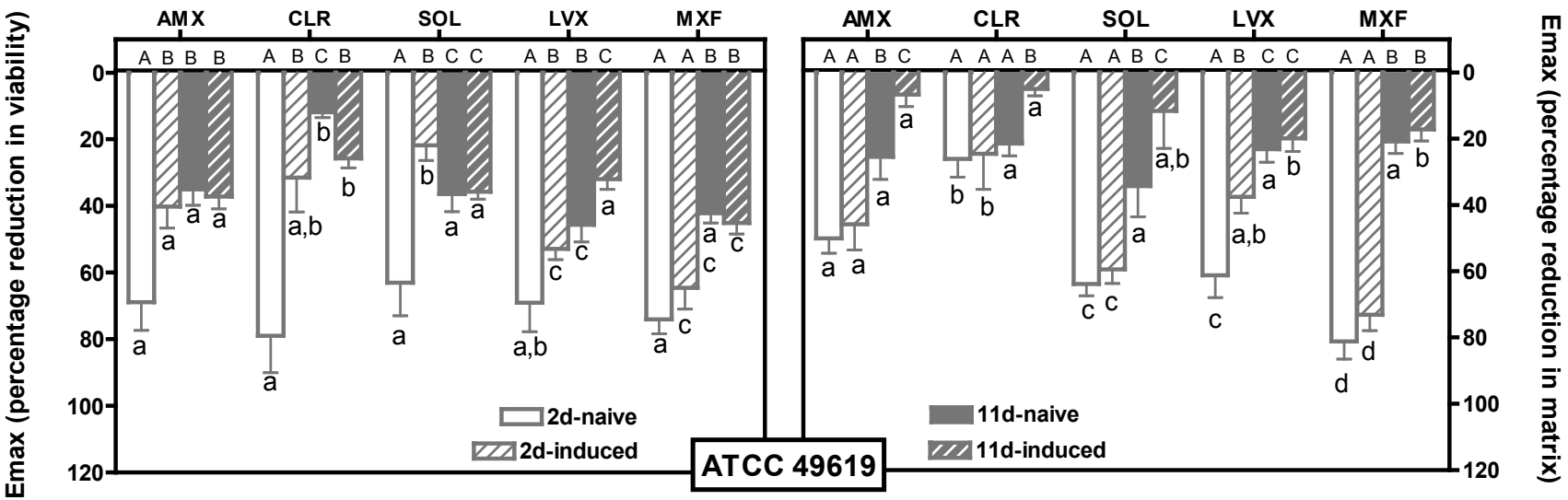
Biofilm model	Effect on bacterial survival		Effect on biofilm thickness	
	E_{max} (% loss of viability with 95% CI)	EC_{50} Concentration (X MIC [mg/L])	E_{max} (% loss of matrix with 95% CI)	EC_{50} Concentration (X MIC [mg/L])
2 days naïve	74.07 (65.42 to 82.72) /A	56,23/A	81.25 (70.63 to 91.87) /A	0.1 /A
11 days naïve	**42.18** (36.11 to 48.25) /B	3,78/B	**20.87** (13.59 to 28.15) /B	**>10^4** /B

Stat. analysis: unpaired, two-tailed t-test for comparisons between maturity stages, values with different letters are significantly different from each other (P<0.05)

Matrix effect : ↓ Efficacy & Potency

RESULTS : CHAPTER 3 : Pharmacodynamic studies of antibiotic activity

Comparison of antibiotic maximal efficacies on viability or biomass for 2-days and 11-days old naïve and induced biofilms of strain ATCC49619



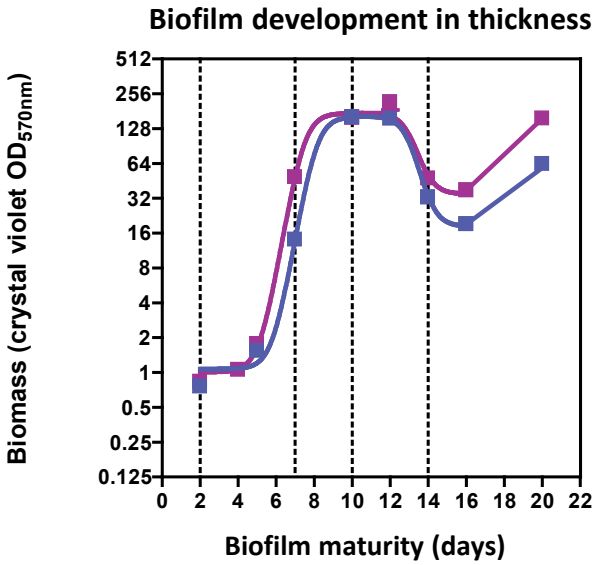
Comparison of antibiotic maximal efficacies (E_{max}) expressed as percentages reduction in viability (left panels) or biomass (right panels) as compared to the control (no antibiotic) for 2-days and 11-days old naïve and induced biofilms of strain ATCC49619. Values were calculated using the Hill equation of the concentration-response curves and are presented as means ± SEM. Statistical analyses: one-way ANOVA with Tukey post test for multiple comparisons; values with different letters are significantly different from each other ($p<0.05$). Small letters: comparison between antibiotic for each type of biofilm; caps letters: comparison between different types of biofilms for each antibiotic.

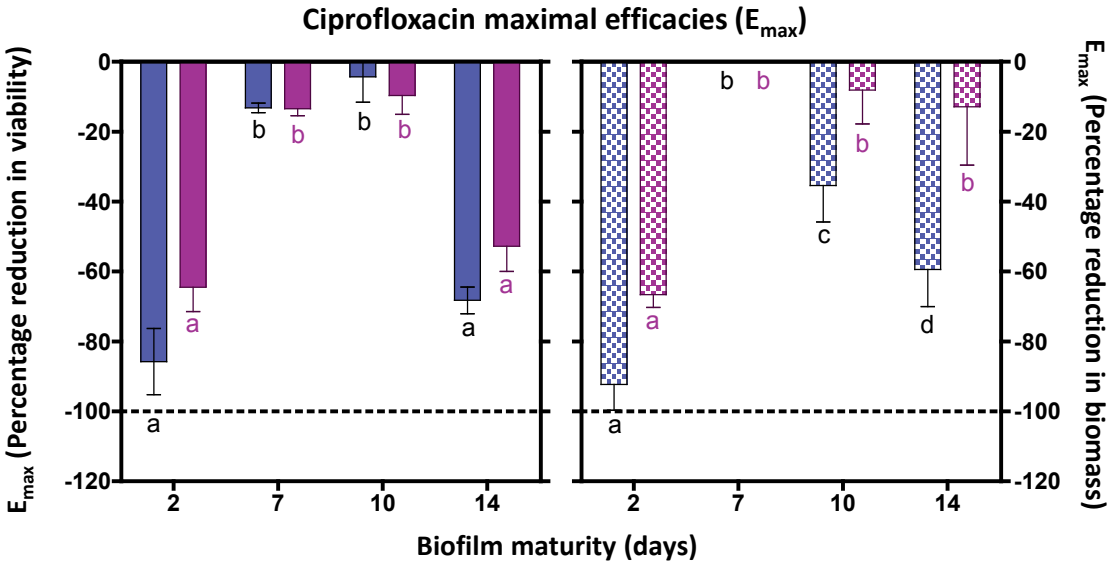
- **Matrix effect : biofilm maturity and induction**

- **More active drugs : moxifloxacin (MXF), Levofloxacin (LVX) and solithromycin (SOL)**

RESULTS

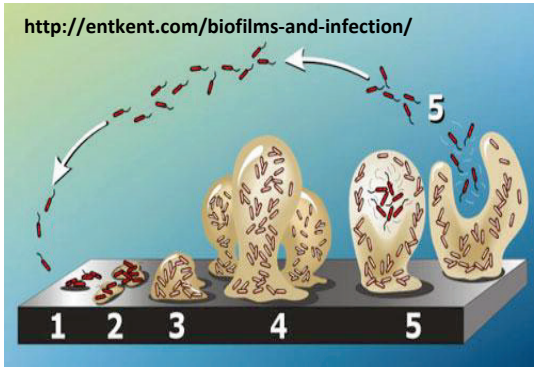
Reference strain ATCC 49619
Clinical AECB isolate N1



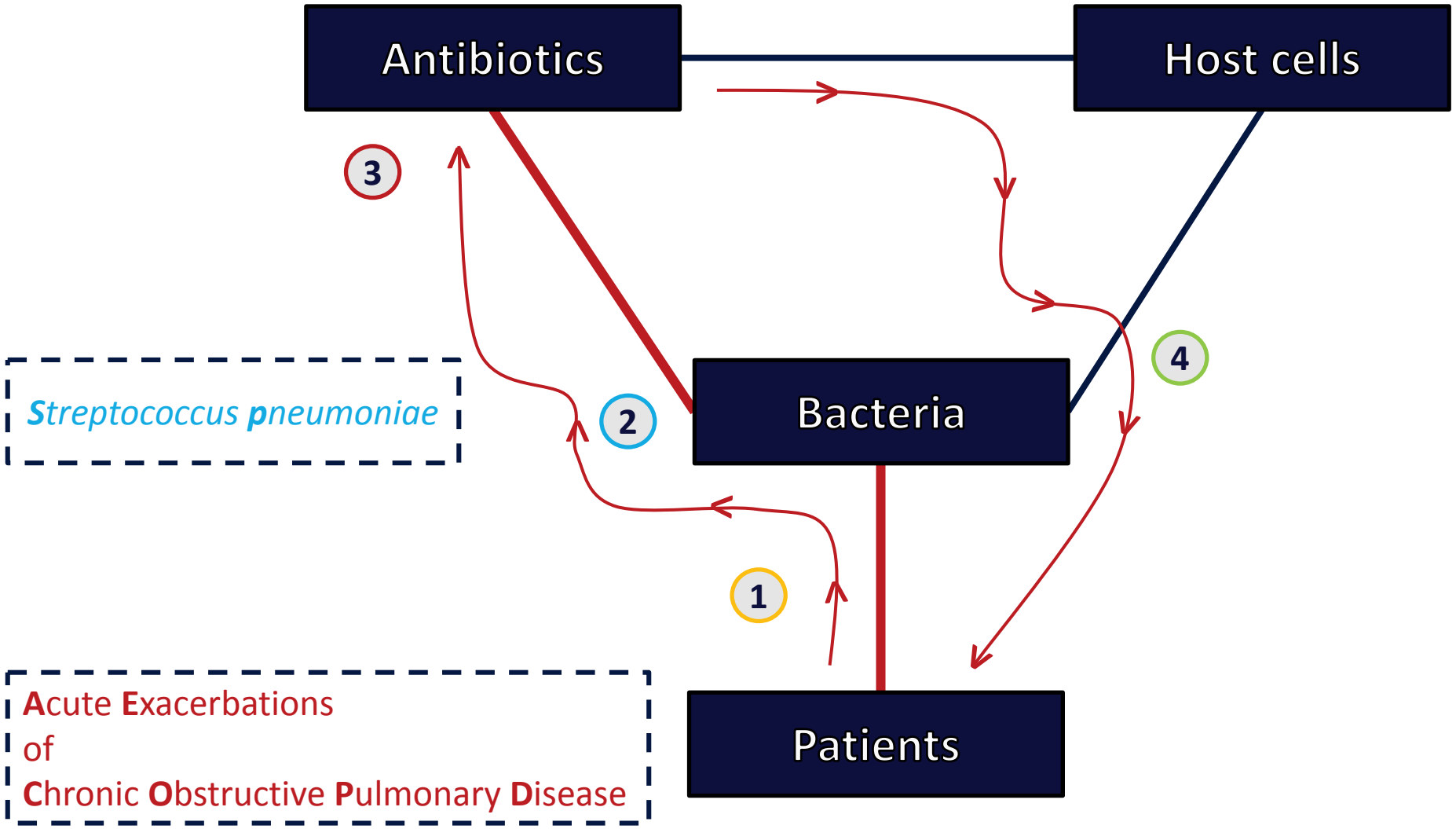


- spontaneous matrix disassembly (at old maturity stages) restores antibiotic activity, even for poorly active AB

- promoting this phenomenon may be an appealing strategy for improving antibiotic activity towards pneumococcal biofilms



Yvan Diaz Iglesias, Françoise Van Bambeke, Nathalie M. Vandevelde (ESGB 2014)


Antibiotics
Host cells
3
4
Streptococcus pneumoniae
2
Bacteria
1
Patients
Acute Exacerbations
of
Chronic Obstructive Pulmonary Disease

BRONCHODILATORS (BD)

Interactions

S. pneumoniae BIOFILMS

2 reference strains
1 AECB clinical isolate

Interactions

ANTIBIOTICS

Moxifloxacin
Levofloxacin

Solithromycin

Bronchodilators improve antibiotic activity against pneumococcal biofilms.

Nathalie M. Vandevelde, Paul M. Tulkens, Giulio G. Muccioli, Françoise Van Bambeke

RESULTS : CHAPTER 4

BRONCHODILATORS (BD)

Interactions

S. pneumoniae BIOFILMS

2 reference strains
1 AECB clinical isolate

Interactions

ANTIBIOTICS

Moxifloxacin
Levofloxacin

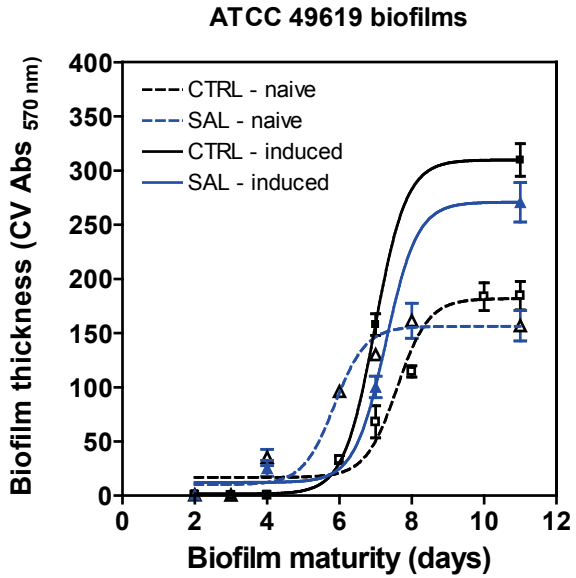
Solithromycin

1 Biofilm growth in different media

Ref. medium "m" : caMHB + 5%LHB + 2% Glc

m + SALBUTAMOL 7.25mg/L* (β2-agonist)

(*) Concentration in the Epithelial lining fluid (ELF)



Kinetics of biofilm formation (biomass, as evaluated by crystal violet absorbance OD_{570nm}) by the reference capsulated strain ATCC49619 in the naive (dotted line ; open symbols) and induced (full line ; closed symbols) models when cultivated in control conditions (black), or in medium supplemented with 7.25 mg/L salbutamol (blue). All values are means ± standard error of the mean (SEM) of 3 to 26 experiments (each made 12 times; when not visible, the SEM bars are smaller than the size of the symbols). Data were used to fit a sigmoidal dose-response function.

RESULTS : CHAPTER 4

BRONCHODILATORS (BD)

Interactions

***S. pneumoniae* BIOFILMS**

2 reference strains
1 AECB clinical isolate

Interactions

ANTIBIOTICS

Moxifloxacin
Levofloxacin

Solithromycin

1 **Biofilm growth in different media**

Ref. medium "m" : caMHB + 5%LHB + 2% Glc

m + SALBUTAMOL 7.25mg/L* (β2-Agonist)

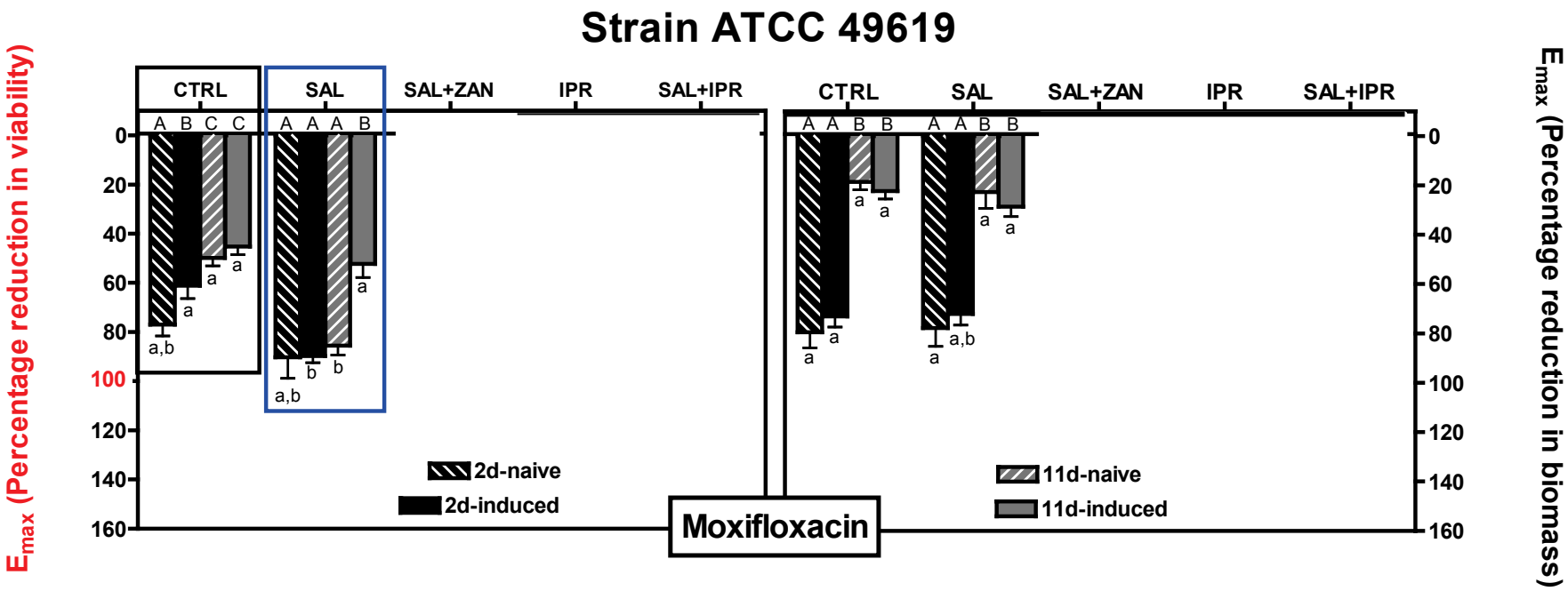
2 **Biofilm treatment in absence of BD**

ANTIBIOTICS

Ex: MOXIFLOXACIN
Fluoroquinolone >< DNA replication

(*) Concentration in the Epithelial lining fluid (ELF)

Comparison of antibiotic maximal efficacies on viability or biomass for 2-days and 11-days old naïve and induced biofilms of strain ATCC49619 grown under control conditions or in supplemented media



Comparison of antibiotic maximal efficacies (E_{max}) expressed as percentages reduction in viability (left panels) or biomass (right panels) as compared to the control (no antibiotic) for 2-days and 11-days old naïve and induced biofilms of strain ATCC49619 treated with moxifloxacin. Values were calculated using the Hill equation of the concentration-response curves and are presented as means ± SD. Statistical analyses: one-way ANOVA with Tukey post test for multiple comparisons; values with different letters are significantly different from each other ($p<0.05$). Small letters: comparison between biofilm grown conditions for each antibiotic; caps letters: comparison between different types of biofilms for each biofilm culture medium.

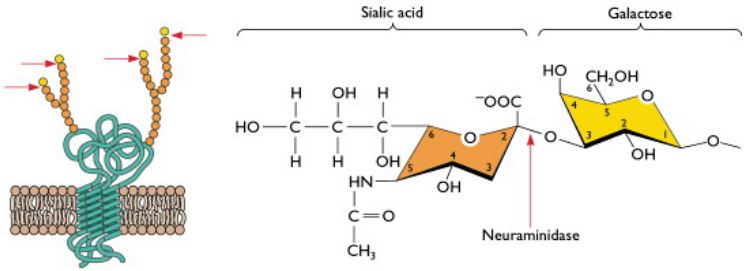
Biofilm development in the presence of SALBUTAMOL improves the antibiotic bactericidal activity towards pneumococcal sessile cells, without any effect on biomass.

Related to changes in matrix composition or cohesion ?

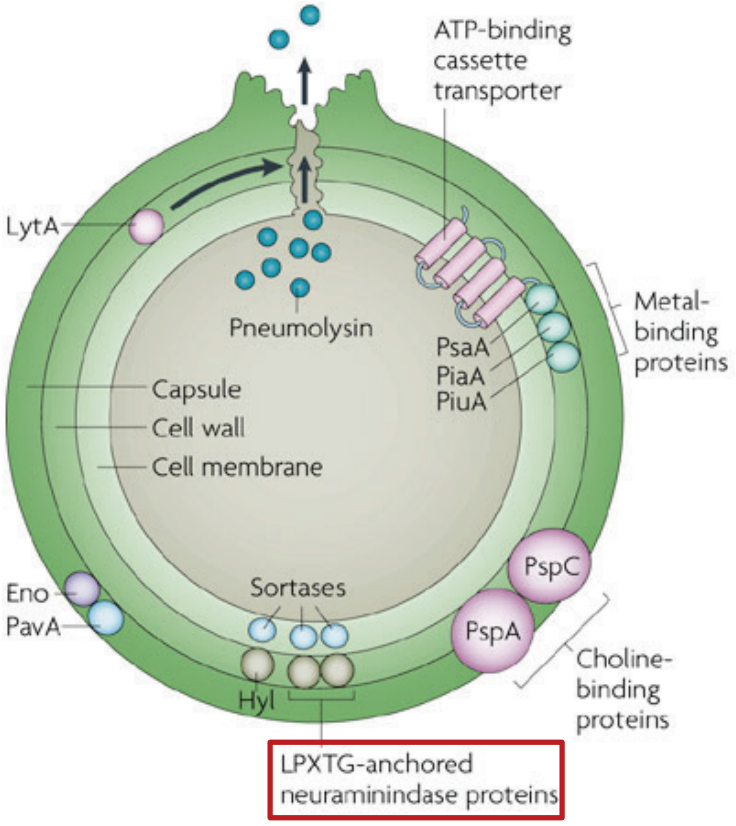
- **The Neuraminidase A:** the main enzymes involved in pneumococcal biofilm formation [a]

- Cleaving terminal sialic acid sugar residues from polysaccharides chains (at the surface of host eukaryotic cells and bacteria) [a]

  => creating receptors for prokaryote-prokaryote or prokaryote-eukaryote adhesion [b]



http://www.twiv.tv/virus-entry-into-cell

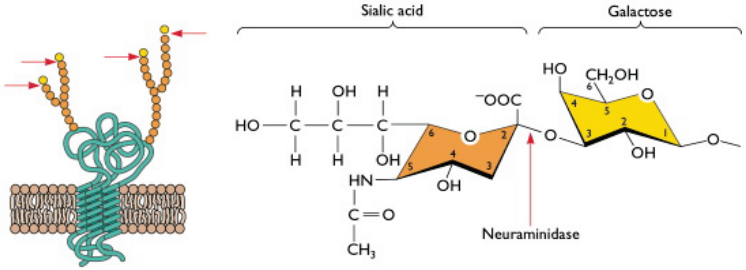


- **Changes in the enzymatic activity are translated by modifications in free sialic acid concentration in the extracellular compartment.**

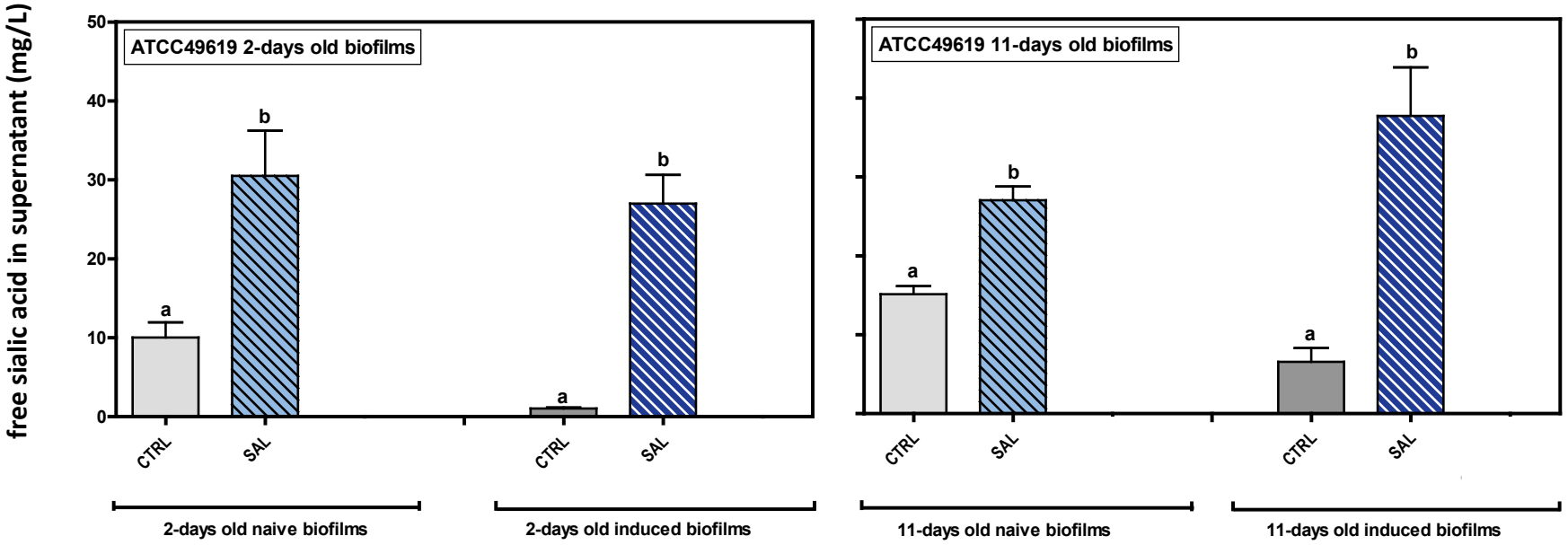
[a] Trappetti et al, 2009 (199):1497-1505; [b] Severi et al, 2007 (9):2817-2822; Hong et al, 2007 (189):8300-8307; Parker et al, 2009 (77): 3722-3730

RESULTS : CHAPTER 4

HPLC-MS (Prof. G.G. Muccioli)
& enzymatic dosage



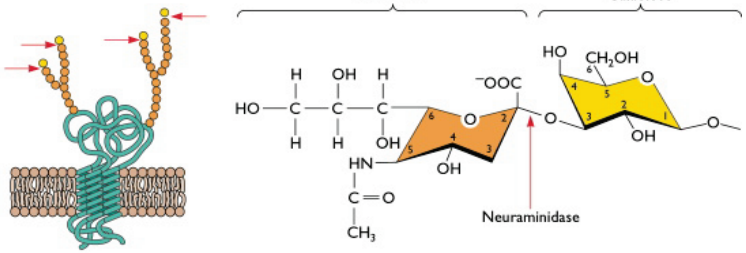
Influence of salbutamol (SAL;7.25 mg/L) or of salbutamol (7.25mg/L) combined with zanamivir (250 mg/L) (SAL+ZAN) on the concentration of free sialic acid in the biofilm supernatant as determined for each strain individually and, for each of them, for 2-days (left histogram) and 11-days (right histogram) naive and induced biofilms. Statistical analysis (one-way ANOVA with Tukey's post-test): in each group, bars with different letters indicate significant differences between media ($p < 0.05$).



Salbutamol induces a loss of matrix cohesion translated by an increases of free sialic acid levels in supernatant

RESULTS : CHAPTER 4

HPLC-MS (Prof. G.G. Muccioli)
& enzymatic dosage



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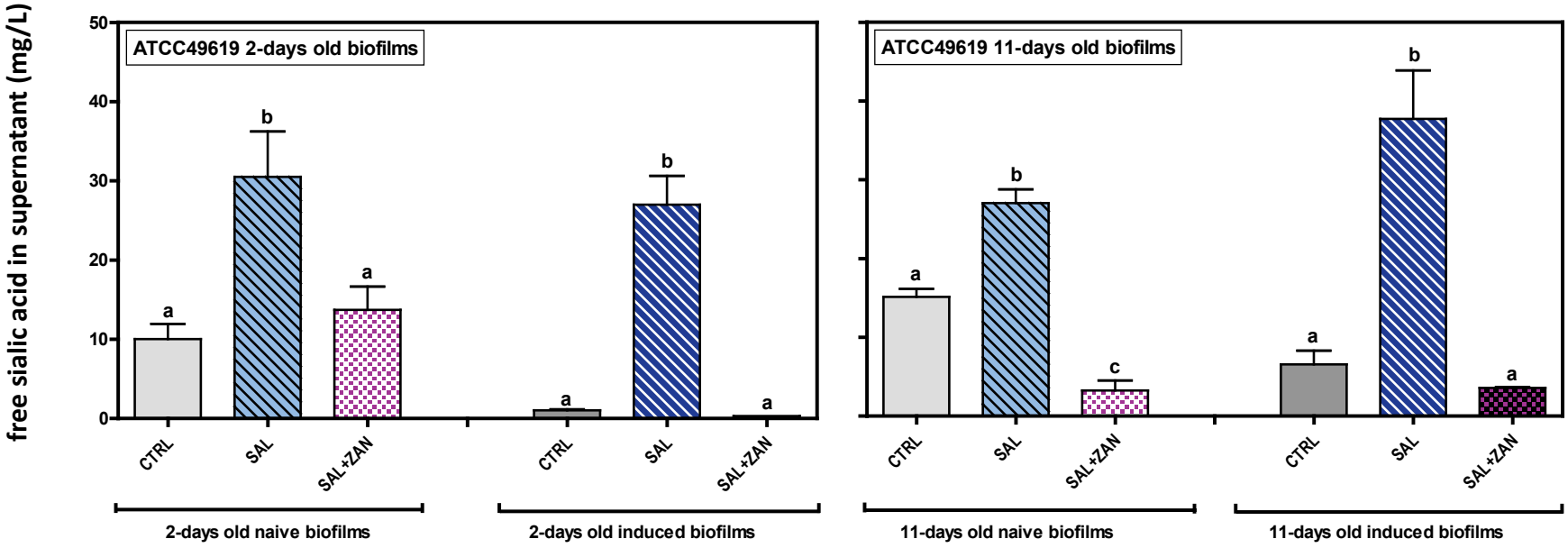
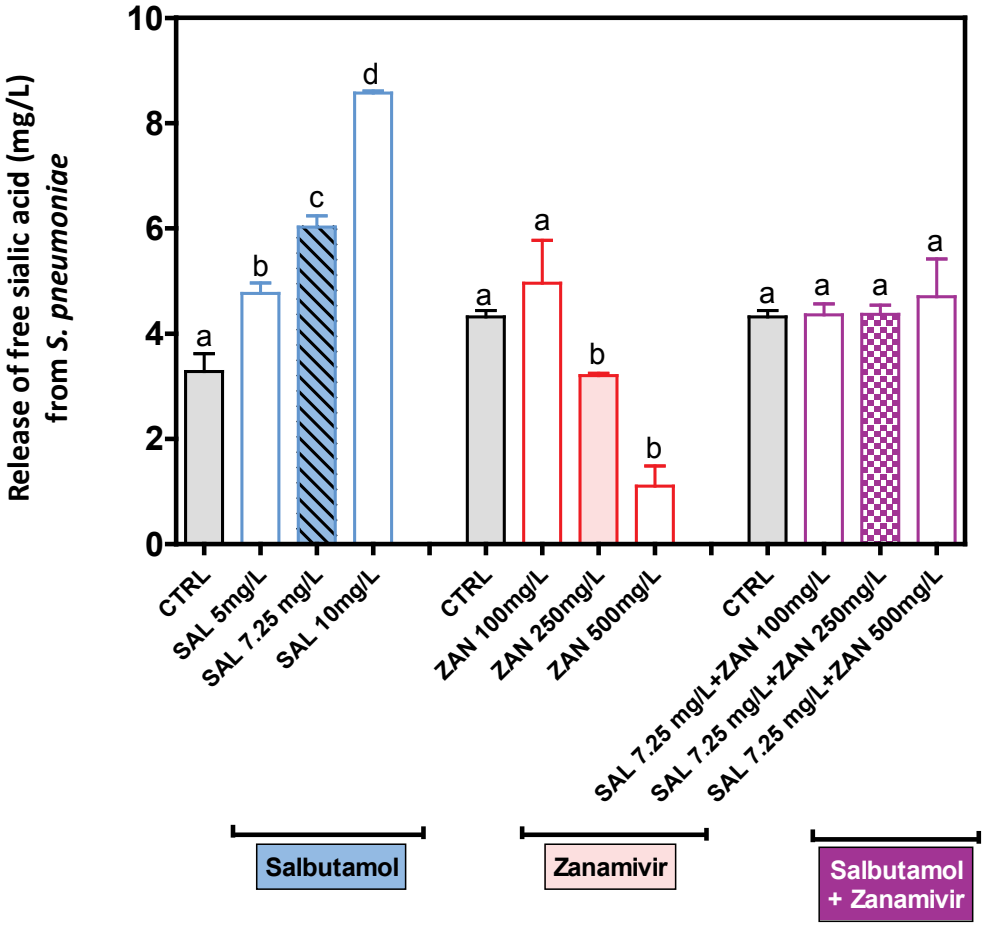


Salbutamol induces a loss of matrix cohesion translated by an increases of free sialic acid levels in supernatant

Reverted by zanamivir, an inhibitor of the neuraminidase A implicated in changes of matrix tridimensional structure



Amounts of sialic acid (mg/L) released from *S. pneumoniae* collected from the supernatant of 2-days old naive biofilms made by reference strains ATCC49619 by purified *Arthrobacter ureafaciens* α-(2→3,6,8,9)-neuraminidase alone or in the presence of salbutamol, zanamivir, or their combination. CTRL (full bars): control conditions (no addition); SAL, ZAN, SAL+ZAN: addition of salbutamol, zanamivir, or their combination at the concentrations indicated in the abscissa. Statistical analysis (one-way ANOVA with Tukey's post-test): in each group, bars with different letters indicate significant differences between conditions ($p < 0.05$). Salbutamol and/or zanamivir concentrations used for biofilm culture during studies of biofilm development and antibiotic activity are represented with stippled bars, other concentrations used in this experiment are represented with open bars.

Purified *Arthrobacter ureafaciens* α-(2→3,6,8,9)-neuraminidase

Salbutamol increases the neuraminidase activity

Zanamivir inhibits the neuraminidase activity

Salbutamol - Zanamivir antagonism

RESULTS : CHAPTER 4

BRONCHODILATORS (BD)

Interactions

S. pneumoniae BIOFILMS

2 reference strains
1 AECB clinical isolate

Interactions

ANTIBIOTICS

Moxifloxacin
Levofloxacin

Solithromycin

1 Biofilm growth in different media

Ref. medium "m" : caMHB + 5%LHB + 2% Glc

m + SALBUTAMOL 7.25mg/L* (β2-Agonist)
+/- ZANAMIVIR 250 mg/L** (NanA inhibitor)

(*) Concentration in the Epithelial lining fluid (ELF)

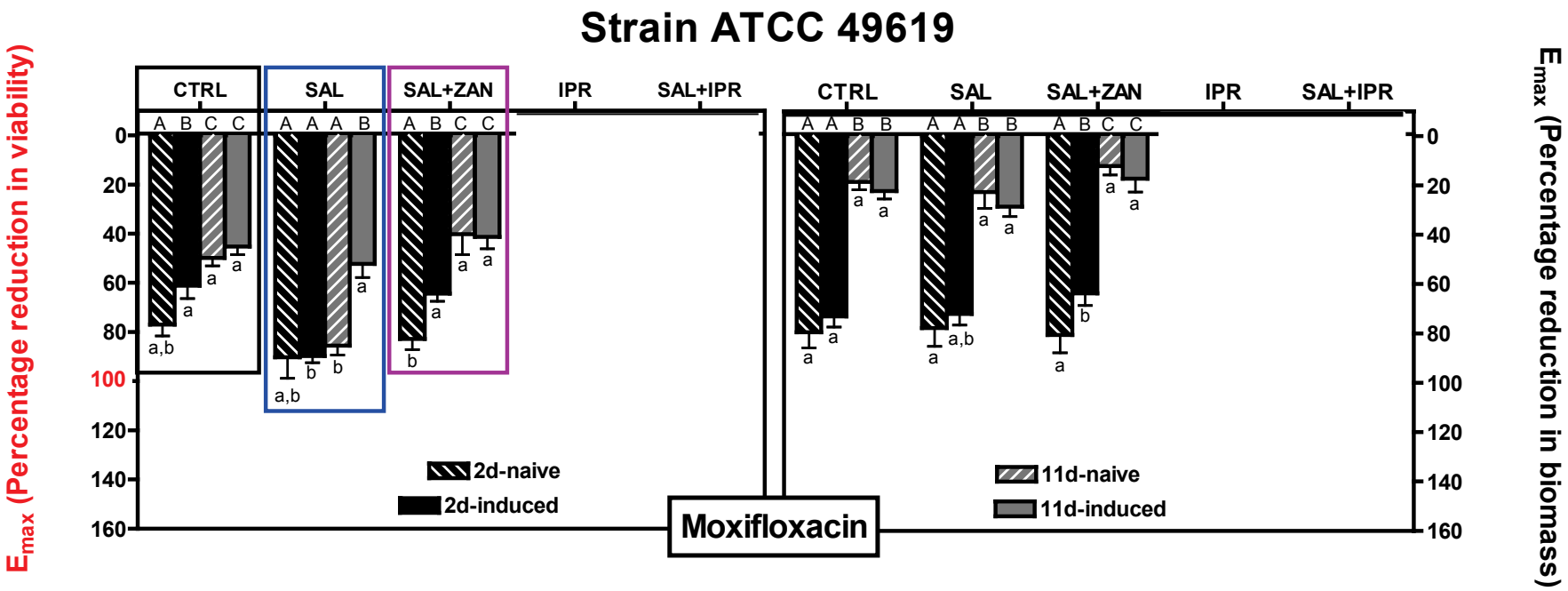
(**) Neuraminidase inhibitory concentration

2 Biofilm treatment in absence of BD

ANTIBIOTICS

Ex: MOXIFLOXACIN
Fluoroquinolone >< DNA replication

Comparison of antibiotic maximal efficacies on viability or biomass for 2-days and 11-days old naïve and induced biofilms of strain ATCC49619 grown under control conditions or in supplemented media



Comparison of antibiotic maximal efficacies (E_{max}) expressed as percentages reduction in viability (left panels) or biomass (right panels) as compared to the control (no antibiotic) for 2-days and 11-days old naïve and induced biofilms of strain ATCC49619 treated with moxifloxacin. Values were calculated using the Hill equation of the concentration-response curves and are presented as means ± SD. Statistical analyses: one-way ANOVA with Tukey post test for multiple comparisons; values with different letters are significantly different from each other ($p<0.05$). Small letters: comparison between biofilm grown conditions for each antibiotic; caps letters: comparison between different types of biofilms for each biofilm culture medium.

The SALBUTAMOL-mediated improve of antibiotic bactericidal activity is probably related to loss of matrix cohesion, dependent on the NanA activity.

RESULTS : CHAPTER 4

BRONCHODILATORS (BD)

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S. pneumoniae BIOFILMS

2 reference strains
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ANTIBIOTICS

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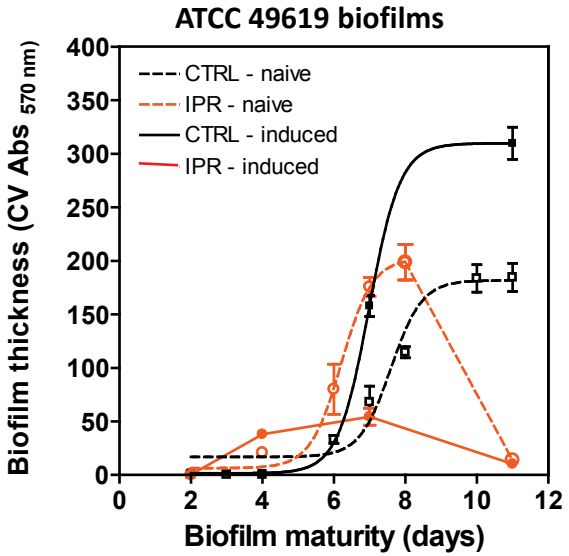
Ref. medium "m" : caMHB + 5%LHB + 2% Glc

m + IPRATROPIUM 1.45mg/L* (musc. antag.)

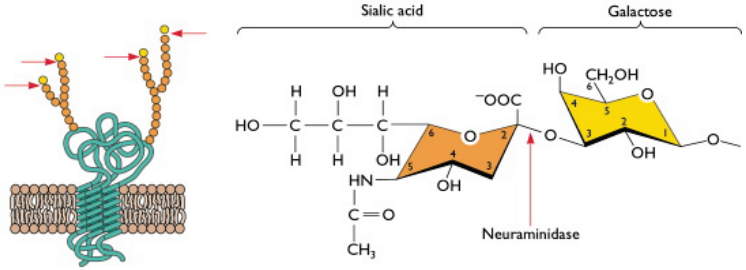
(*) Concentration in the Epithelial lining fluid (ELF)



Kinetics of biofilm formation (biomass, as evaluated by crystal violet absorbance OD_{570nm}) by the reference capsulated strain ATCC49619 in the naive (dotted line ; open symbols) and induced (full line ; closed symbols) models when cultivated in control conditions (black), or in medium supplemented with 1.45 mg/L ipratropium (orange). All values are means ± standard error of the mean (SEM) of 3 to 26 experiments (each made 12 times; when not visible, the SEM bars are smaller than the size of the symbols). Data were used to fit a sigmoidal dose-response function whenever possible (dotted straight lines are used when changes in OD_{570nm} occurred abruptly).

Biofilm disassembly with matrix loss from day 8 in the naive model and from day 7 in the induced model

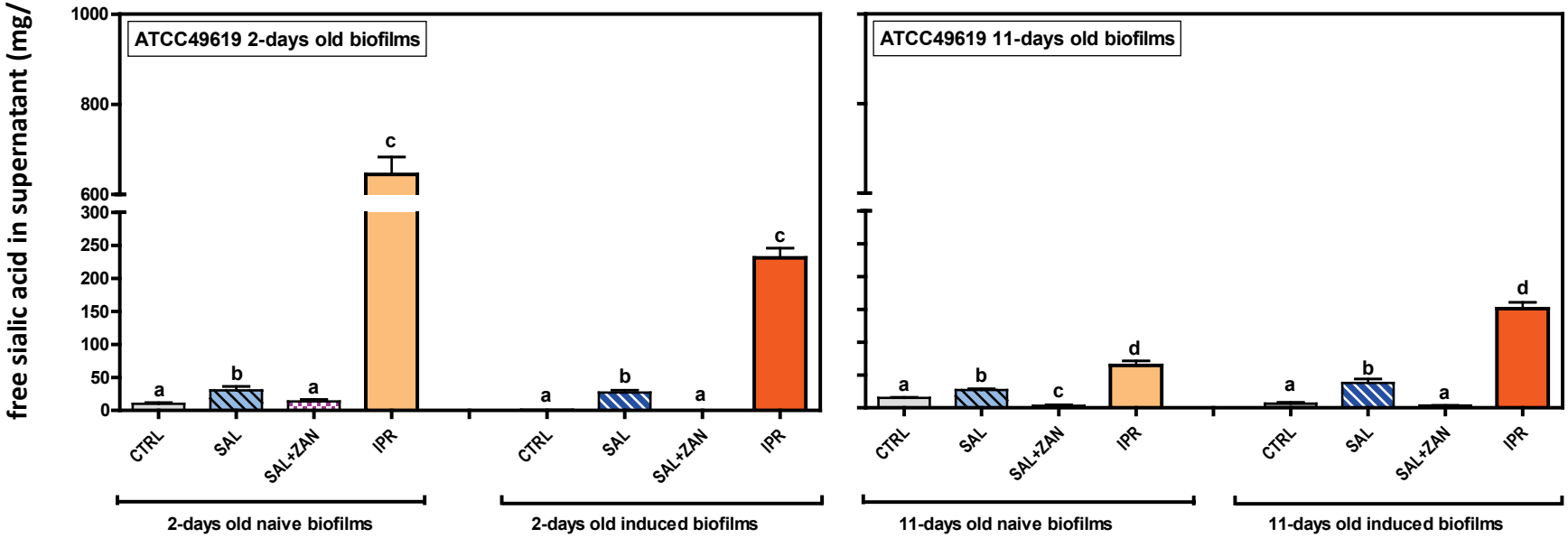
RESULTS : CHAPTER 4



Sialic acid cleavage by the neuraminidase enzyme

http://www.twiv.tv/virus-entry-into-cell

Influence of salbutamol (SAL;7.25 mg/L), salbutamol (7.25mg/L) combined with zanamivir (250 mg/L) (SAL+ZAN) or of ipratropium (IPR 1.45 mg/L) on the concentration of free sialic acid in the biofilm supernatant as determined for each strain individually and, for each of them, for 2-days (left histogram) and 11-days (right histogram) naive and induced biofilms. Statistical analysis (one-way ANOVA with Tukey's post-test): in each group, bars with different letters indicate significant differences between media ($p < 0.05$).



The ipratropium-mediated biofilm disassembly is accompanied by a major release of free sialic acid levels in supernatant.

Free sialic acid may be considered as a marker of matrix cohesion.

RESULTS : CHAPTER 4

BRONCHODILATORS (BD)

Interactions

***S. pneumoniae* BIOFILMS**

2 reference strains
1 AECB clinical isolate

Interactions

ANTIBIOTICS

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Levofloxacin

Solithromycin

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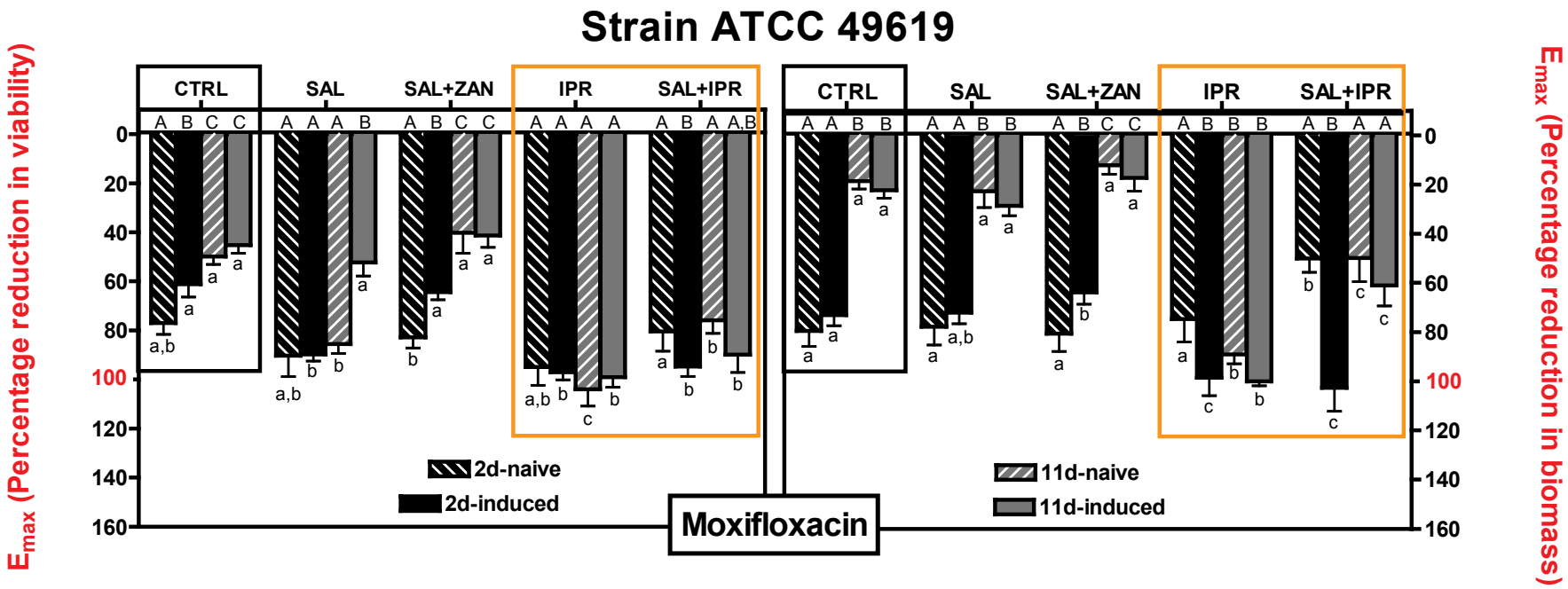
Fluoroquinolone >< DNA replication

(*) Concentration in the Epithelial lining fluid (ELF)

Comparison of antibiotic maximal efficacies on viability or biomass for 2-days and 11-days old naïve and induced biofilms of strain ATCC49619 grown under control conditions or in supplemented media



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DISCUSSION

the FEBS Journal

Inhibition of pneumococcal choline-binding proteins and cell growth by esters of bicyclic amines

Beatriz Maestro[1], Ana González[2], Pedro García[2] and Jesús M. Sanz[1]

1 Instituto de Biología Molecular y Celular, Universidad Miguel Hernández, Elche, Spain
2 Departamento de Microbiología Molecular, Centro de Investigaciones Biológicas, Consejo Superior de Investigaciones Científicas, Madrid, Spain

Keywords
antibiotic resistance; circular dichroism (CD); inhibition of bacterial growth; repeat proteins; *Streptococcus pneumoniae*

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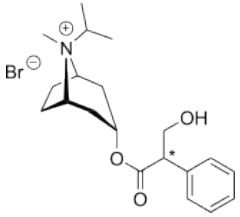
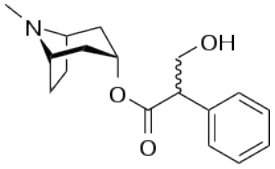
doi:10.1111/j.1742-4658.2006.05584.x

Streptococcus pneumoniae is one of the major pathogens worldwide. The use of currently available antibiotics to treat pneumococcal diseases is hampered by increasing resistance levels; also, capsular polysaccharide-based vaccination is of limited efficacy. Therefore, it is desirable to find targets for the development of new antimicrobial drugs specifically designed to fight pneumococcal infections. Choline-binding proteins are a family of polypeptides, found in all *S. pneumoniae* strains, that take part in important physiologic processes of this bacterium. Among them are several murein hydrolases whose enzymatic activity is usually inhibited by an excess of choline. Using a simple chromatographic procedure, we have identified several choline analogs able to strongly interact with the choline-binding module (C-LytA) of the major autolysin of *S. pneumoniae*. Two of these compounds (atropine and ipratropium) display a higher binding affinity to C-LytA than choline, and also increase the stability of the protein. CD and fluorescence spectroscopy analyses revealed that the conformational changes of C-LytA upon binding of these alkaloids are different to those induced by choline, suggesting a different mode of binding. *In vitro* inhibition assays of three pneumococcal, choline-dependent cell wall lytic enzymes also demonstrated a greater inhibitory efficiency of those molecules. Moreover, atropine and ipratropium strongly inhibited *in vitro* pneumococcal growth, altering cell morphology and reducing cell viability, a very different response than that observed upon addition of an excess of choline. These results may open up the possibility of the development of bicyclic amines as new antimicrobials for use against pneumococcal pathologies.

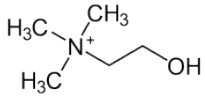
Ipratropium Br



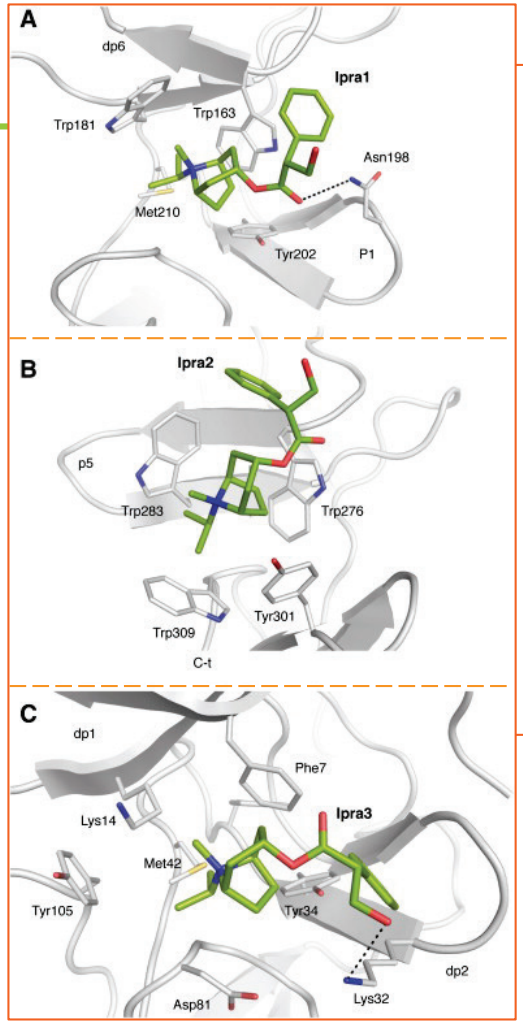
Atropine



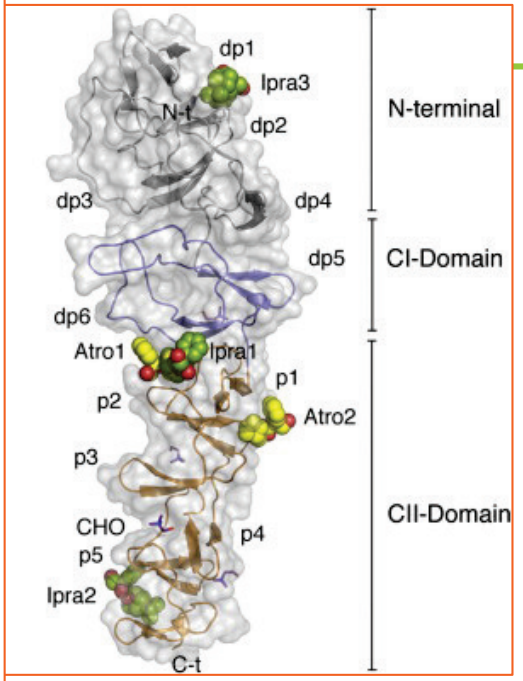
Choline



LytA amidase, LytC Lysozyme and Pce Phosphorylcholinesterase inhibition (Maestro et *al*, 2007**)**



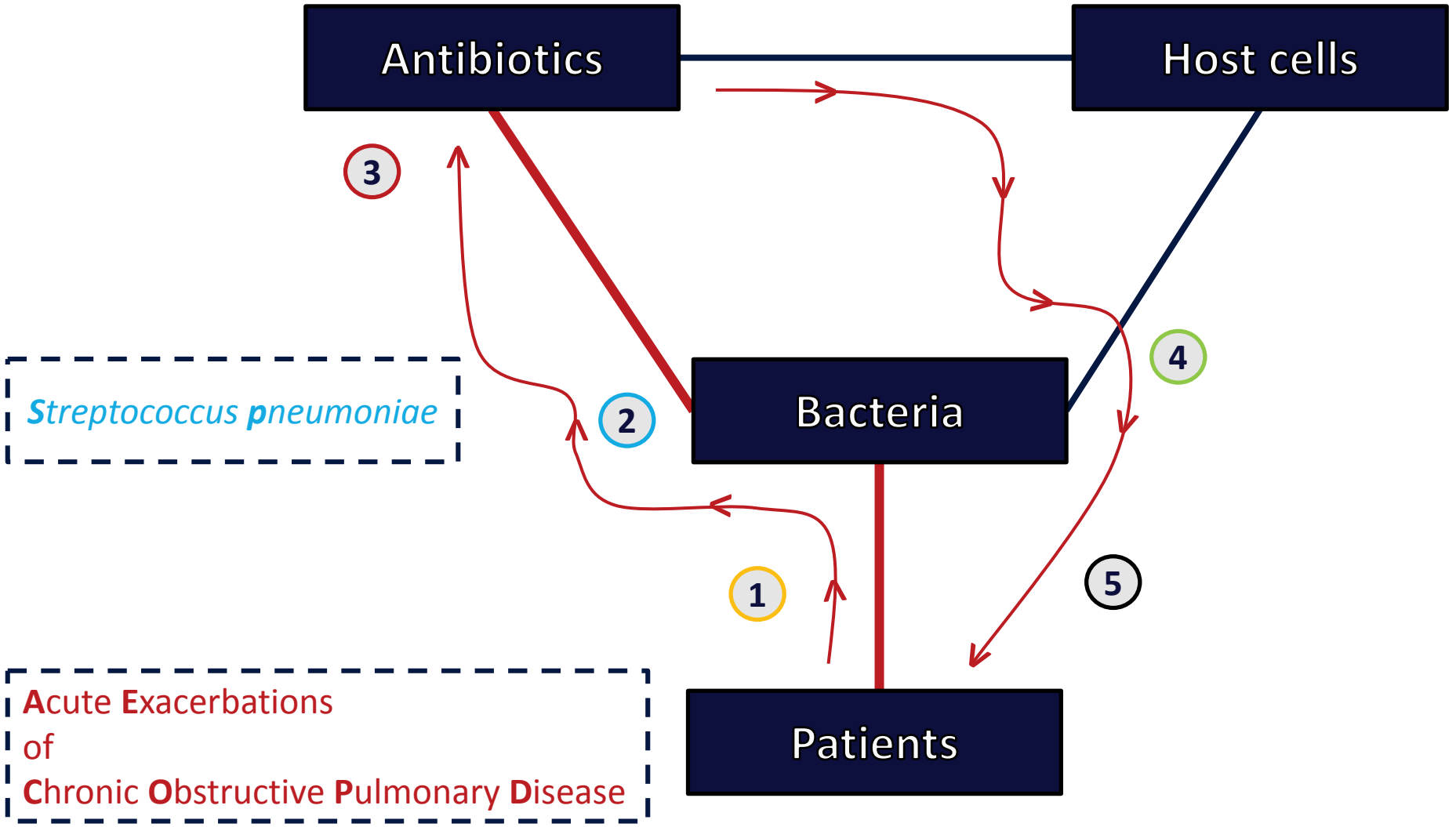
Details of the ipratropium recognition by CbpF. The views show the interactions between CbpF and ipratropium. The residues forming the binding site are drawn as capped sticks. Carbon atoms of the ligand are in green. Hydrogen bonds are shown as dashed lines. (A) Ipratropium recognition at the cavity between dp6 and p1 repeats. (B) Ipratropium recognition at the cavity between p5 and the C-terminal. (C) Ipratropium recognition at the cavity between dp1 and dp2 repeats.



Atropine and ipratropium ligands attached to pneumococcal CbpF. Stereoview representation of the general structure of CbpF in complex with atropine and ipratropium; complexes are superimposed to compare localization of both ligands. The atropine molecules are drawn as space-filled models in yellow and ipratropium molecules are drawn as space-filled models in green. The amino-terminal module of CbpF is in gray, the CI domain in blue and CII domain in orange. Choline molecules bound to the choline-binding sites are drawn as sticks. CbpF, choline-binding protein F. CHO, choline. p1–p5, canonical choline-binding repeats. dp1–dp6, non-canonical choline-binding repeats. Atro, atropine molecule. Ipra, ipratropium molecule.

Crystal structures of CbpF complexed with atropine and ipratropium reveal clues for the design of novel antimicrobials against *Streptococcus pneumoniae* (Silva-Martin et *al*, 2014**)**

DISCUSSION


Antibiotics
Host cells
3
Streptococcus pneumoniae
2
Bacteria
4
1
5
Acute Exacerbations
of
Chronic Obstructive Pulmonary Disease
Patients

DISCUSSION

Are some non-antibiotic drugs deleterious for anti-bacterial therapy ?

antihypertensive calcium channel blocker (verapamil)	↓ *in vitro E.coli* susceptibility to ampicillin (Gunics et al, 2000)
Mucolytic agent (N-acetylcystein)	↓ *in vitro S. aureus, P. aeruginosa, K. pneumoniae, E. coli* susceptibilities to many AB (Goswani et al, 2010 ; Garcia et al, 2012)
non-steroidal anti-inflammatory drugs (salicylates)	↓ *in vitro S. aureus, K. pneumoniae, E. coli* susceptibilities to fluoroquinolones, through the induction of efflux (Riordan et al, 2007; Tavio et al, 2004)
Benzodiazepines (diazepam)	↓ *in vitro S. aureus, K. pneumoniae, E. coli* susceptibilities to fluoroquinolones, through the induction of efflux (Tavio et al, 2004 ; Tavio et al, 2012; Vandevelde NM., Van Obbergh M. et al, ICAAC 2013)
Antipsychotics (haloperidol)	↓ *in vitro E. coli* susceptibilities to fluoroquinolones, through the induction of efflux (Tavio et al, 2012)

CONCLUSION

• My first COPD patient...

- **man**
- **81 years old in 2010 (**born in 1929**)**
- **miner during 25 years**
- **former smoker (**stopped in 1990, total 45 UAP**)**

- **Bronchial obstruction : ↓ 40% of the expiratory function (**GOLD 2**)**
  => SHORT & LONG-ACTING BRONCHODILATORS

- **Chronic cough and lung inflammation**

- **1-2 bacterial exacerbations/ year**
  => ANTIBIOTICS

- **Hypertension** (157/72 mmHg)
  => 3 ANTI-HYPERTENSIVE DRUGS /day

- **Heart transplant (1992), cardiomegaly**
  => 3 ANTI-REJECTION DRUGS

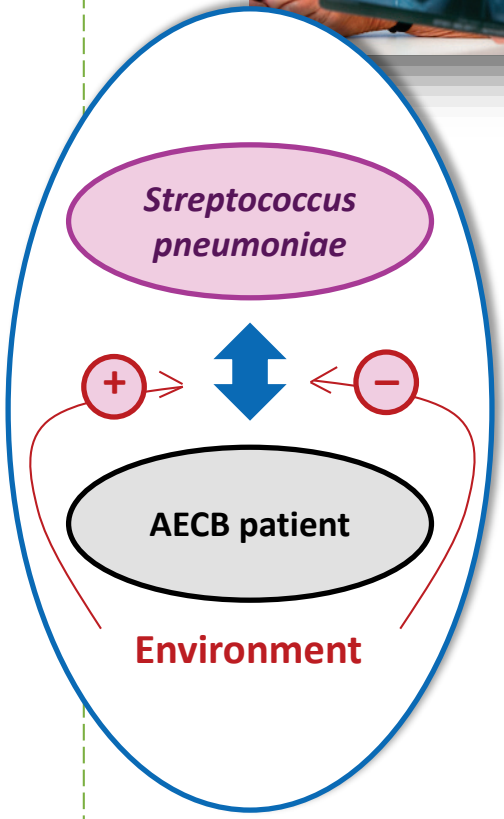
- **Hypercholesteloremia**
  => CHOLESTEROL-LOWERING DRUGS

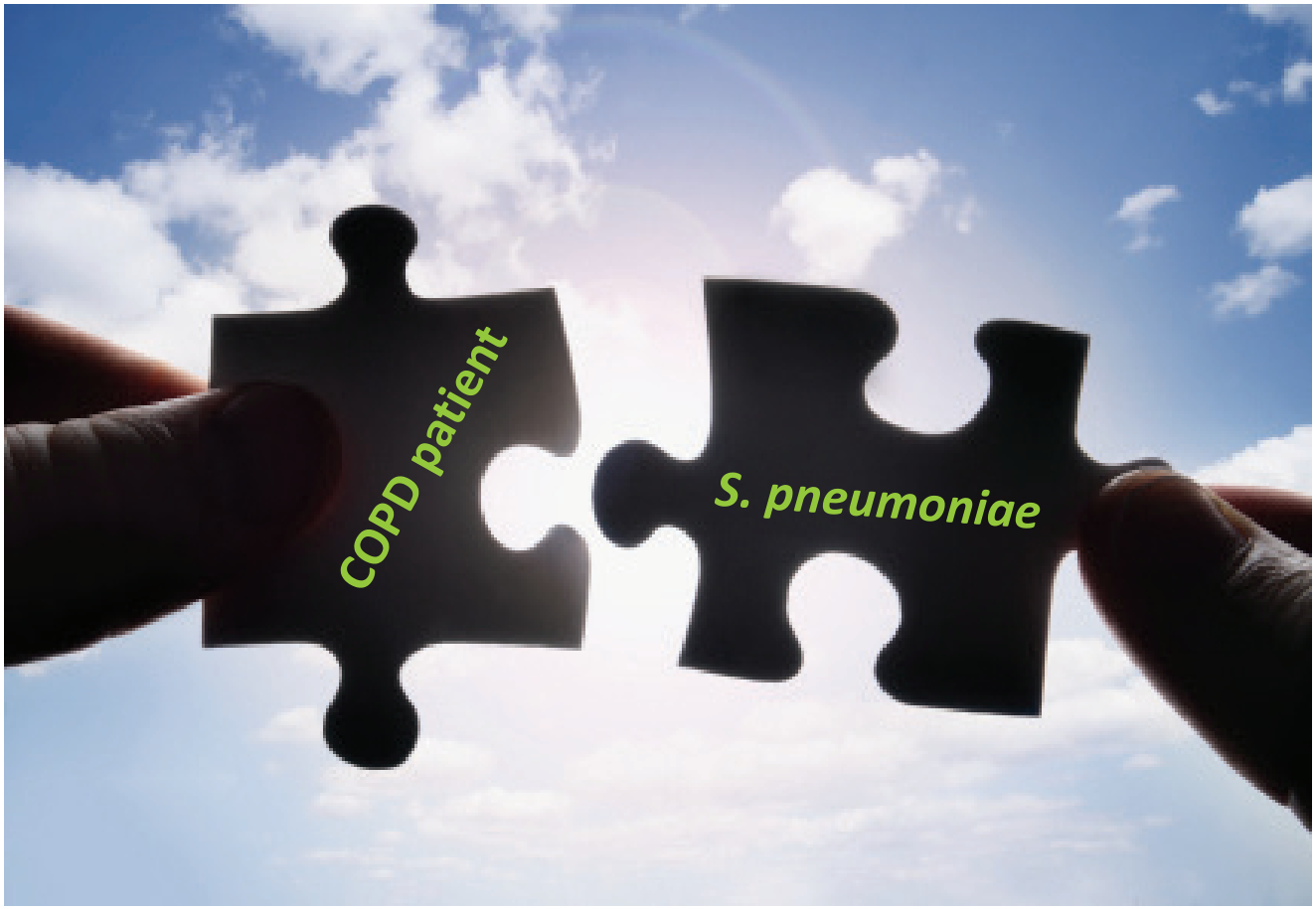
- **Moderate hyperglycemia (** fasting glucose 139 mg/dl**)**
  => ANTI-DIABETIC DRUGS

- **Overweight** (BMI : 33kg/m^2)

- **Chronic renal failure**

- **Cancer**





Thank you !!

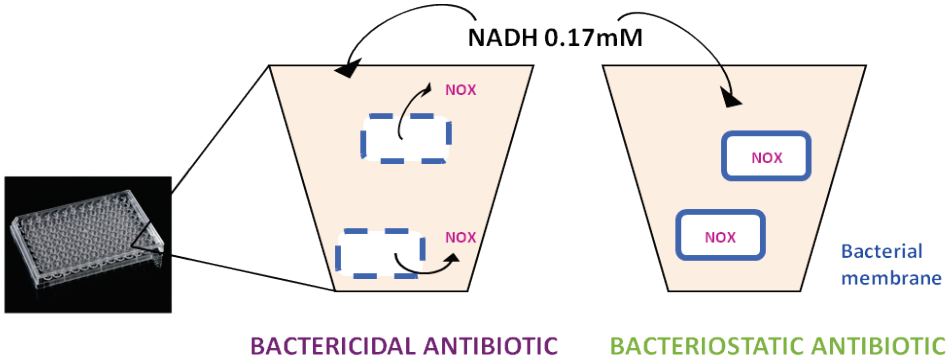
- *Ma promotrice de thèse, le Professeur Françoise Van Bambeke*
- *The members of the jury and thesis committee*
- *Le Professeur Paul Tulkens*
- *Le Professeur Marie-Paule Mingeot*
- *Les cliniciens et microbiologistes ayant participé à mon étude clinique*
- *L'ensemble des membres du laboratoire FACM*
- *Les membres du groupe de recherche CLIP*
- *Les autres doctorants et post-doctorants du LDRI*
- *Les chercheurs avec qui j'ai travaillé:*

  Dr. Julia Bauer
  Professeur Giulio G. Muccioli

- *Ma famille*

Merci à tous du fond du cœur !! ☺

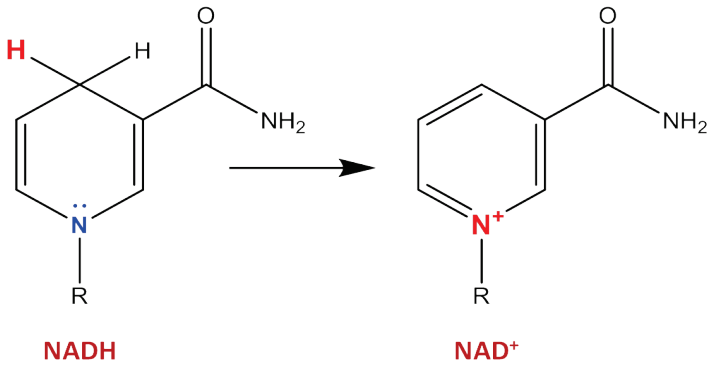
RESULTS : CHAPTER 5 : Set up of an NADH oxidation-based viability assay



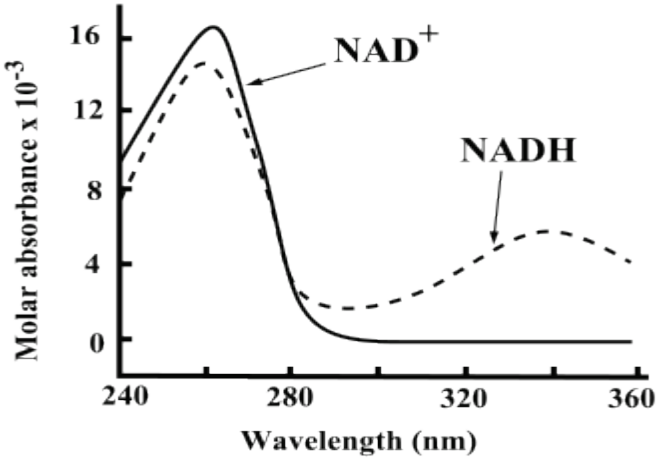
A/Schematic representation of the differences existing between NADH oxidase (NOX) release in the case of bactericidal versus bacteriostatic antibiotic activities.

B/NADH and NAD^+ chemical structures.

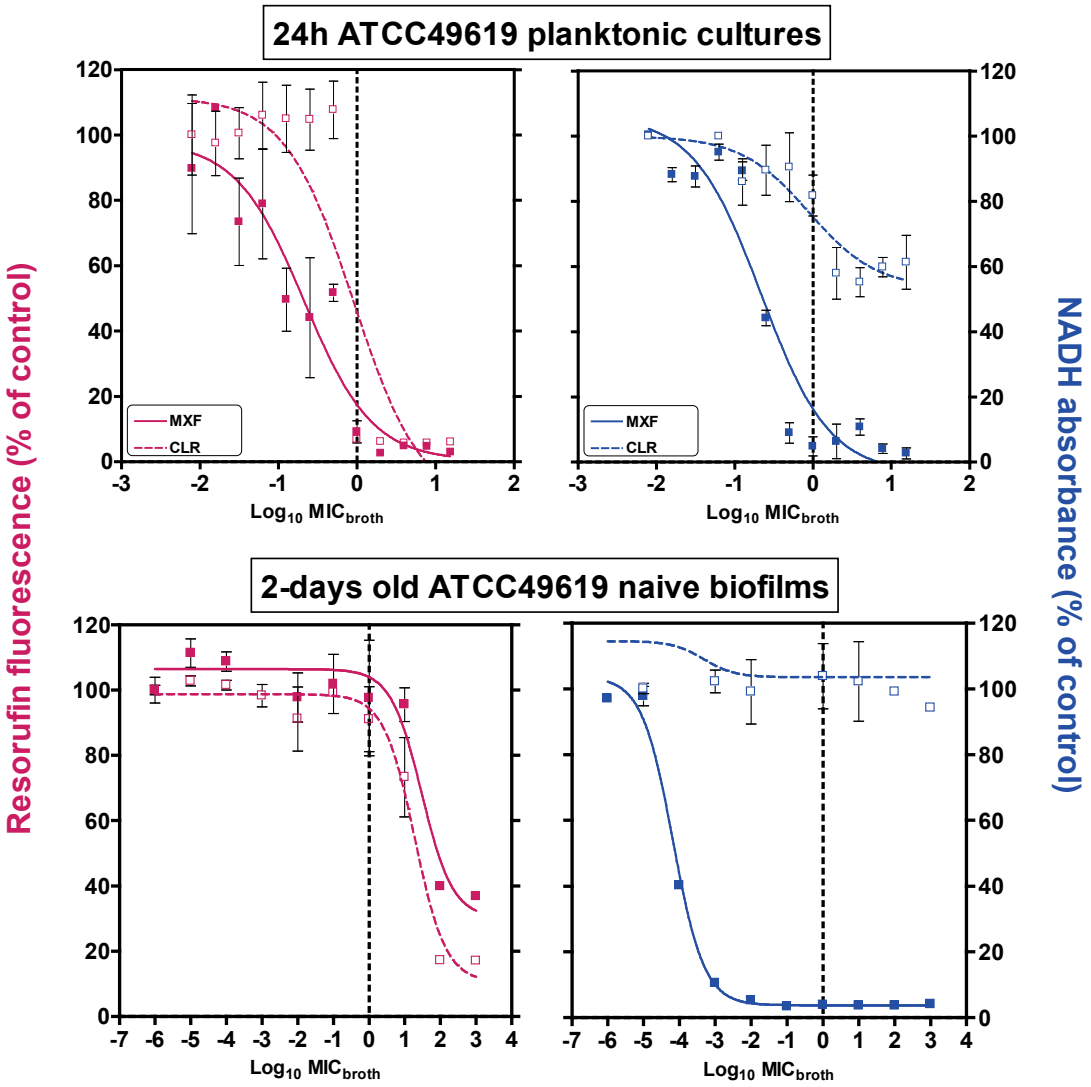
C/Schematic representation of NAD^+ and NADH UV absorbance spectra with maximal absorbances values measured at respectively 260 and 339nm.

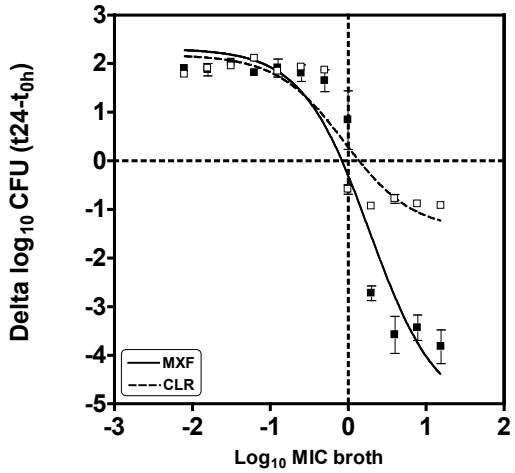


R = ribose-P-P-ribose-adenine



RESULTS : CHAPTER 5 : Set up of an NADH oxidation-based viability assay

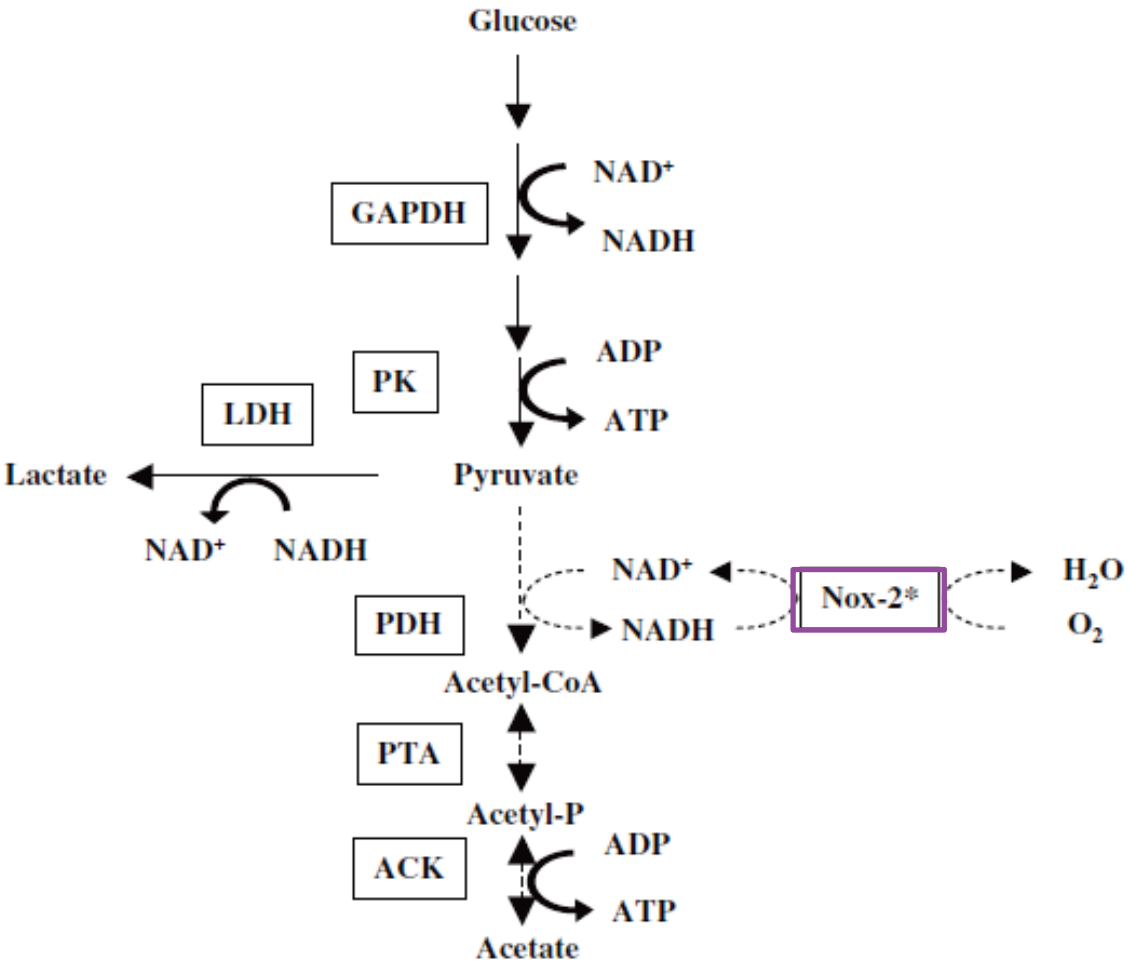




Dose-response curves of moxifloxacin (MXF, full symbols) and clarithromycin (CLR, open symbols) activities on bacterial survival in planktonic cultures and 2-days old biofilms made with strain ATCC 49619, after 24hours of incubation with increasing concentrations of antibiotics. The ordinate shows the change in viability as a percentage of the control value (no antibiotic present) and measured by the decrease in resorufin fluorescence, in NADH absorbance and in CFU. All values are means ± SD of 2 to 6 independent experiments. When not visible, the error bars are smaller than the size of the symbols.

RESULTS : CHAPTER 5 : Set up of an NADH oxidation-based viability assay

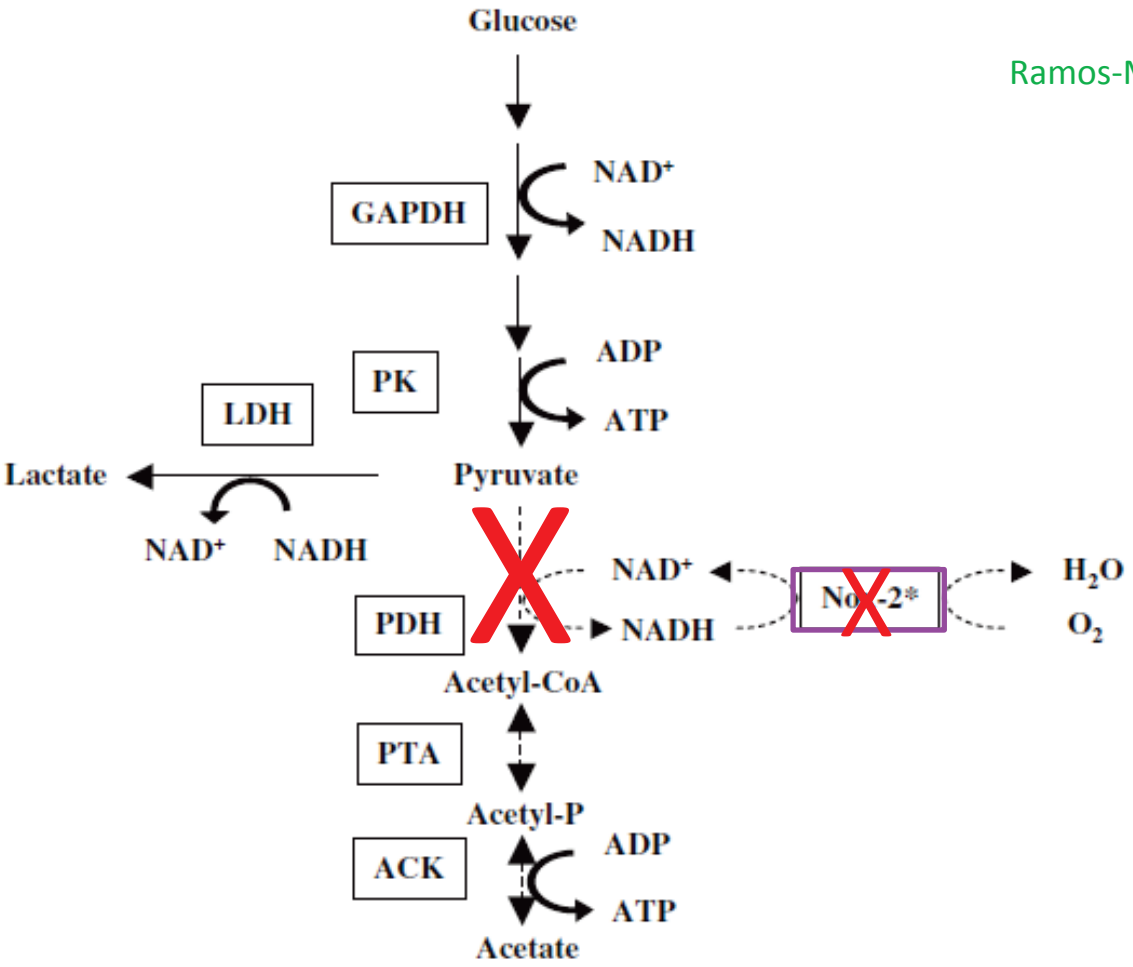
Yamamoto et al, 2006 - *S. agalactiae*



GAPDH : glyceraldéhyde –phosphate dehydrogenase
PK : pyruvate kinase
LDH : lactate dehydrogenase
PDH : pyruvate dehydrogenase
NOX: NADH oxidase
PTA : phosphate acetyltransferase
ACK : acetylphosphokinase

RESULTS : CHAPTER 5 : Set up of an NADH oxidation-based viability assay

Yamamoto et al, 2006 - *S. agalactiae*
Yesilkaya et al, 2009 - *S. pneumoniae*
Ramos-Montanez et al, 2010 - *S. pneumoniae*



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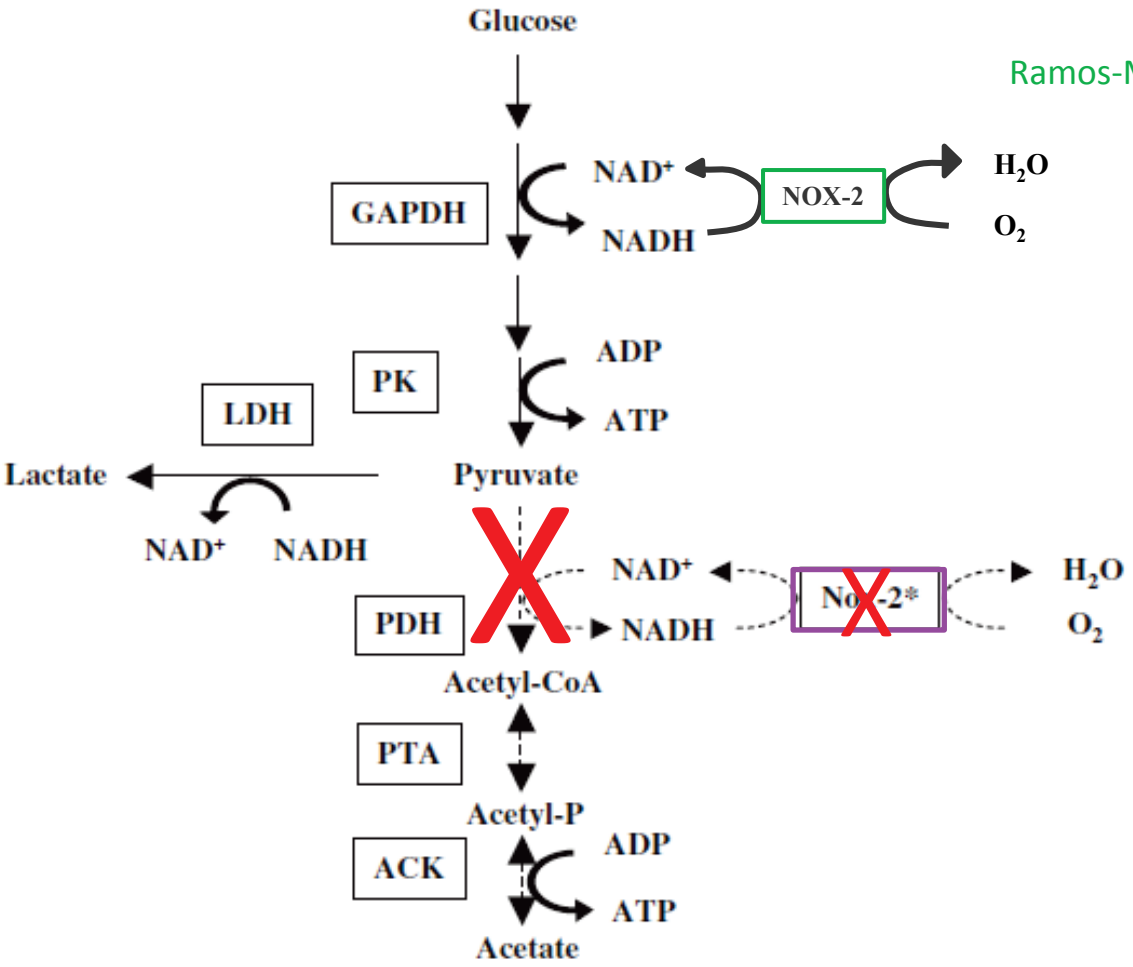

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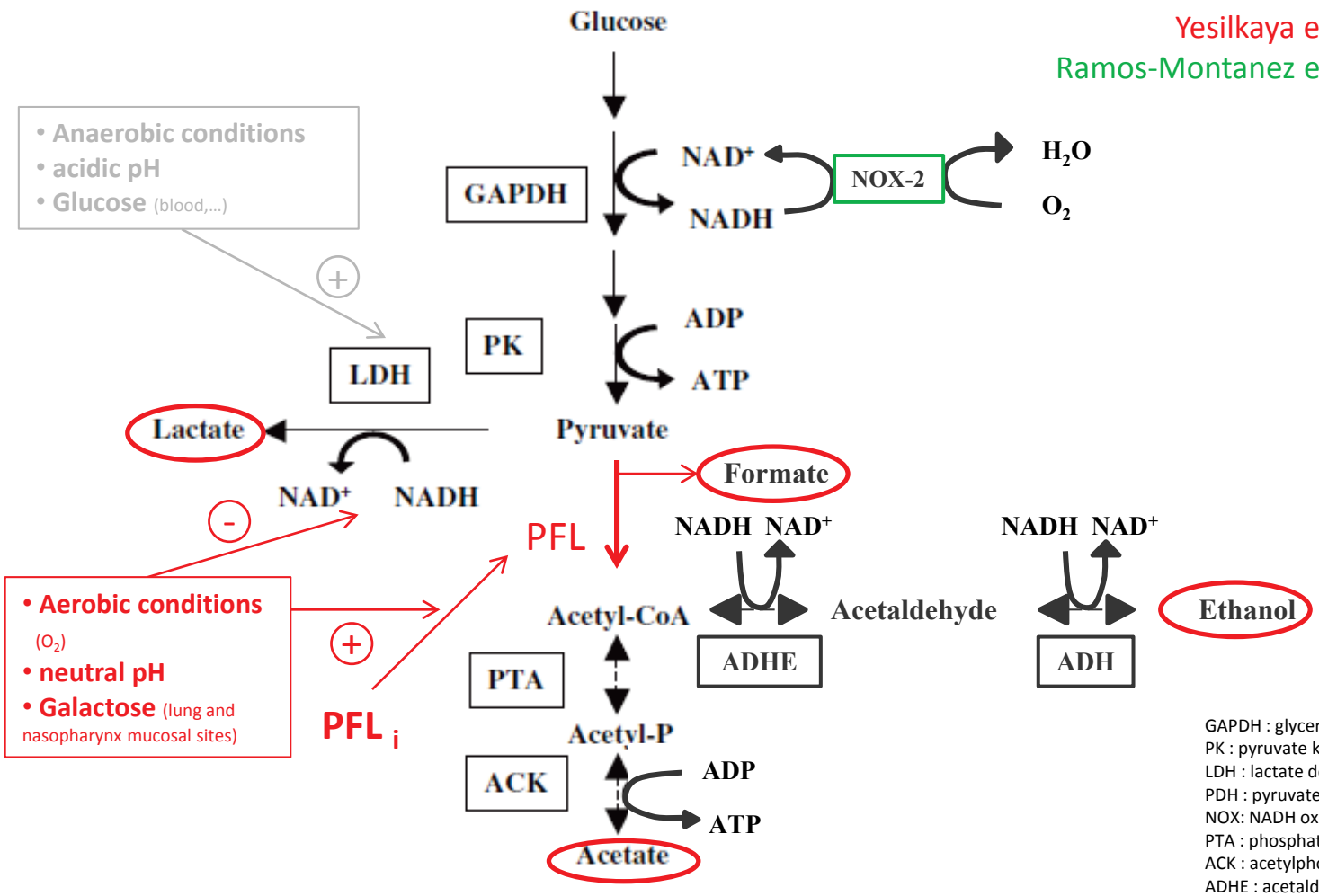
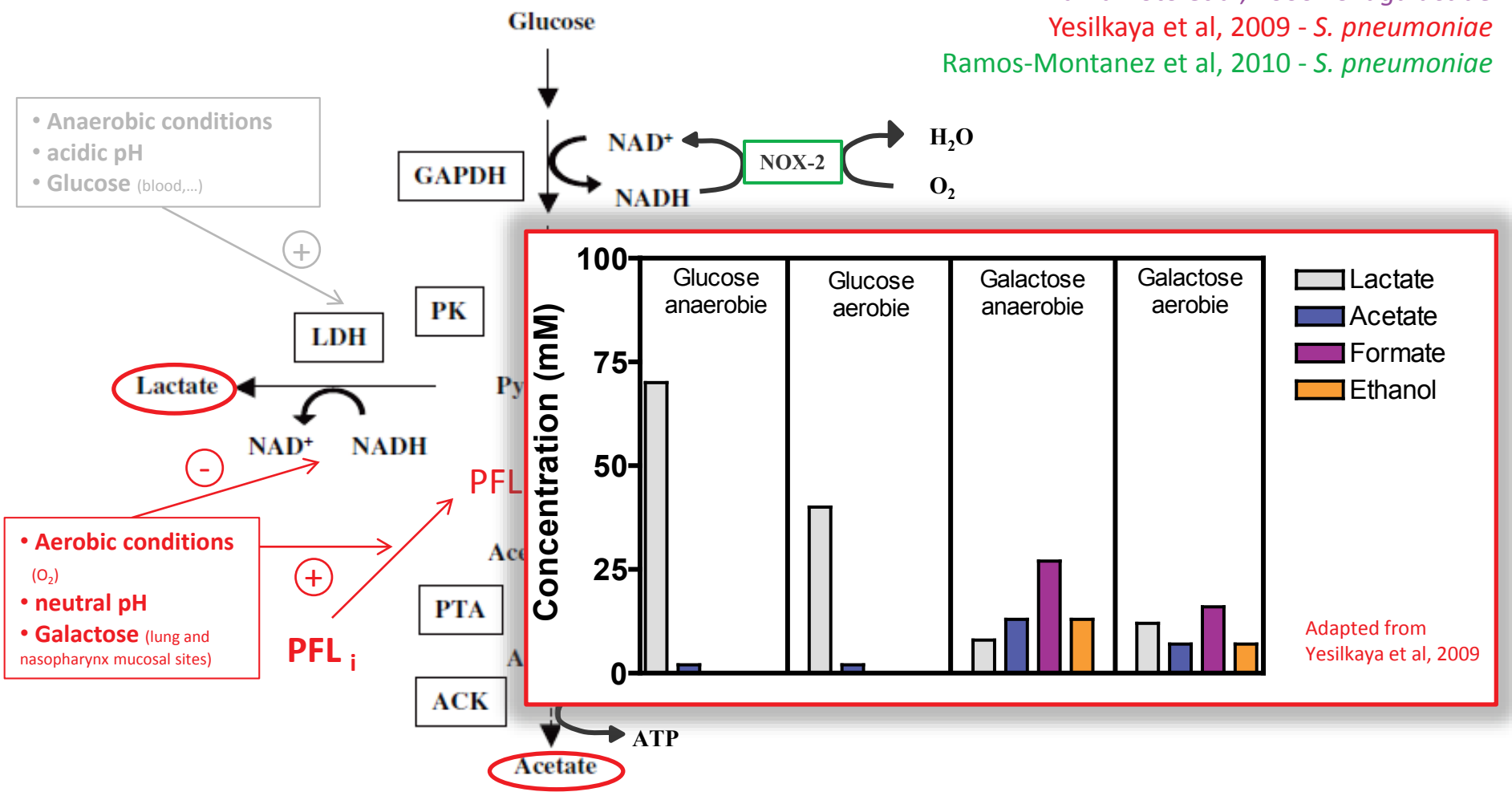


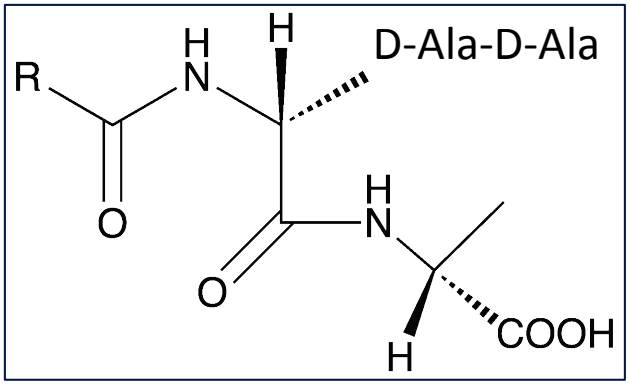
GAPDH : glyceraldéhyde –phosphate dehydrogenase
PK : pyruvate kinase
LDH : lactate dehydrogenase
PDH : pyruvate dehydrogenase
NOX: NADH oxidase
PTA : phosphate acetyltransferase
ACK : acetylphosphokinase
ADHE : acetaldehyde dehydrogenase
ADH : alcohol dehydrogenase

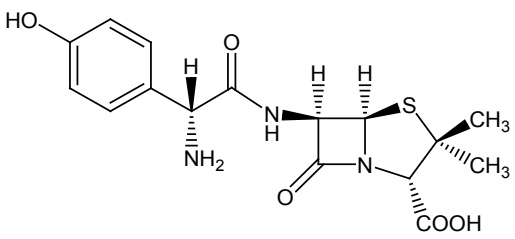
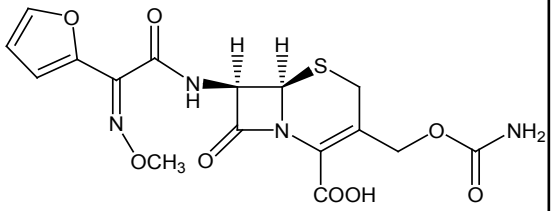
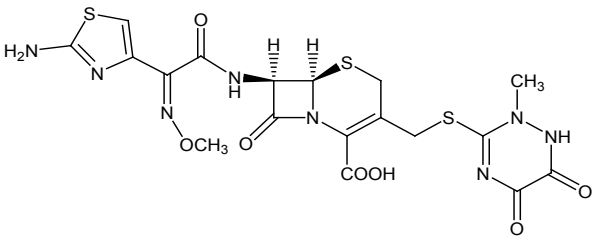
RESULTS : CHAPTER 5 : Set up of an NADH oxidation-based viability assay

Yamamoto et al, 2006 - *S. agalactiae*
Yesilkaya et al, 2009 - *S. pneumoniae*
Ramos-Montanez et al, 2010 - *S. pneumoniae*



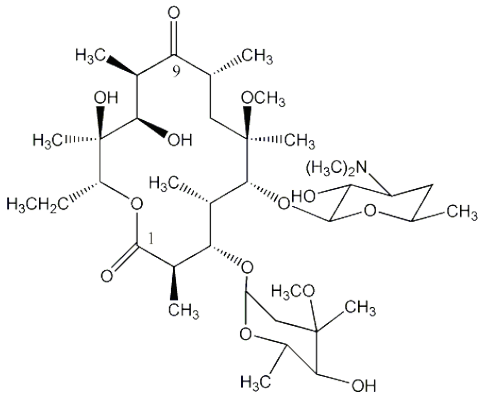
Structures – Betalactams



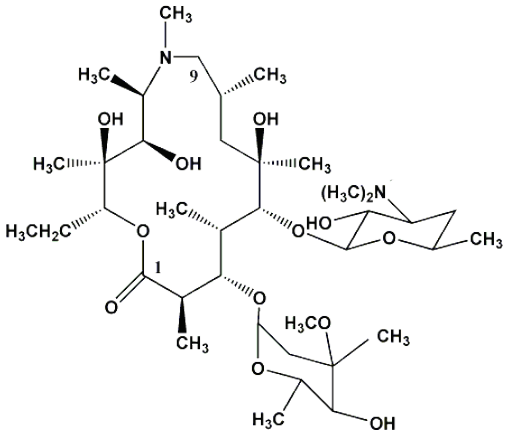
		
amoxicillin	**cefuroxime**	**ceftriaxone**

Structures – Macrolides & kétolides

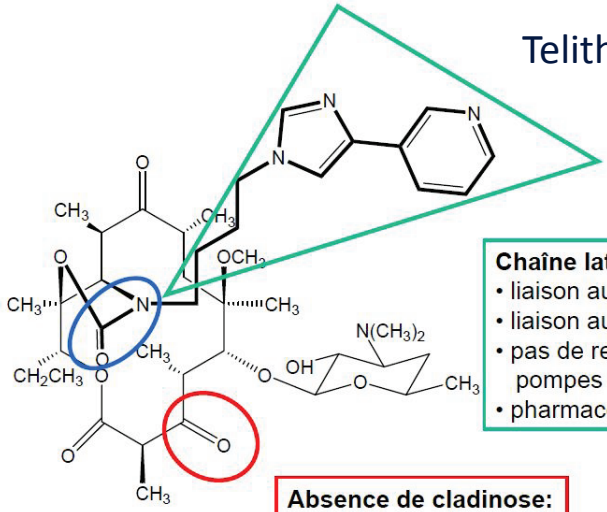
Clarithromycine



Azithromycine



Telithromycine

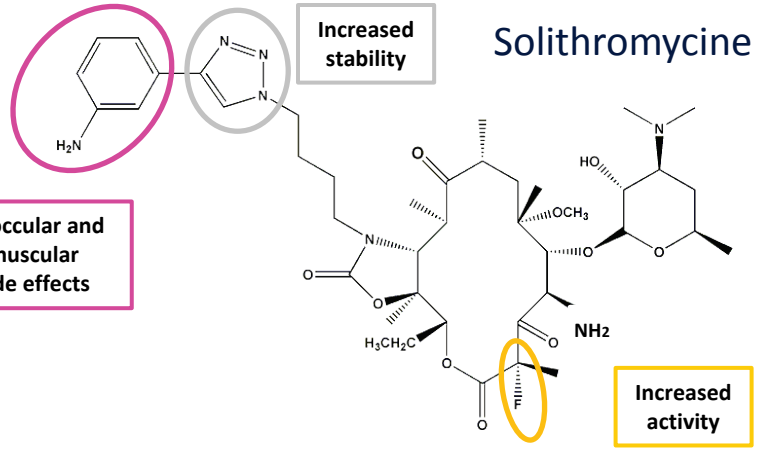


Chaîne latérale:
- liaison au domaine II
- liaison aux ribosomes méthylés
- pas de reconnaissance par les pompes à efflux de *S. pneumoniae*
- pharmacocinétique

Absence de cladinose:
- stabilité en milieu acide
- pas d'induction MLS_B

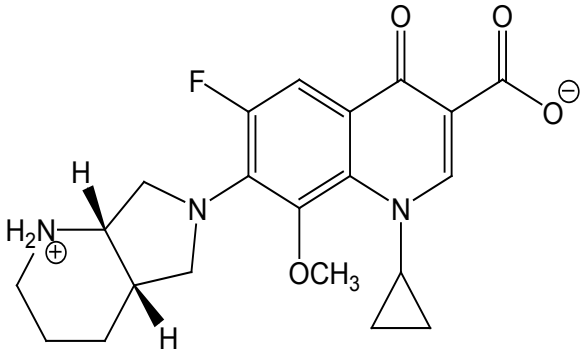
Carbamate:
- activité accrue

Solithromycine

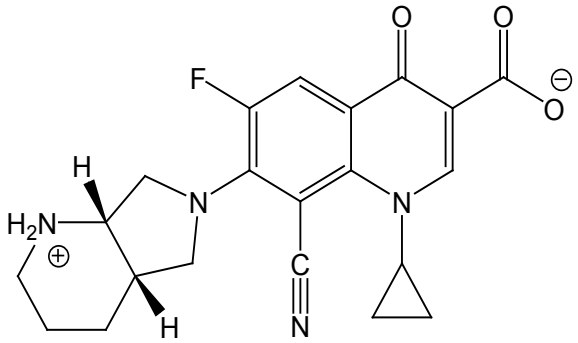


Structures - Quinolones

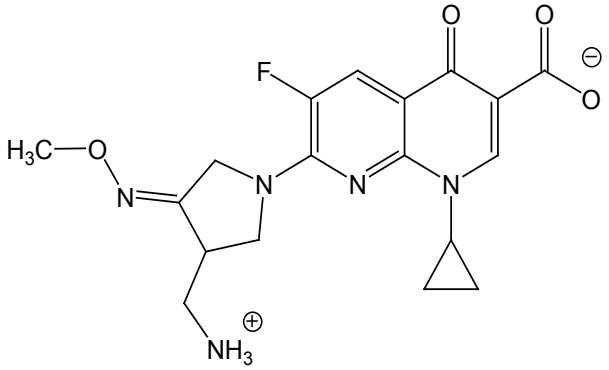
Moxifloxacine



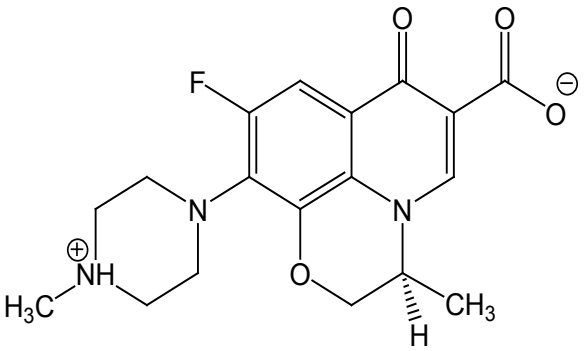
Pradofloxacine



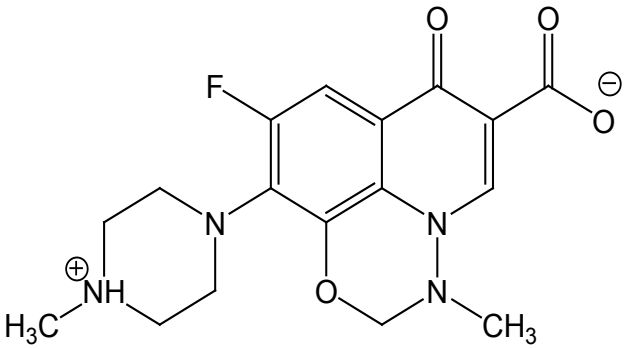
Gemifloxacine



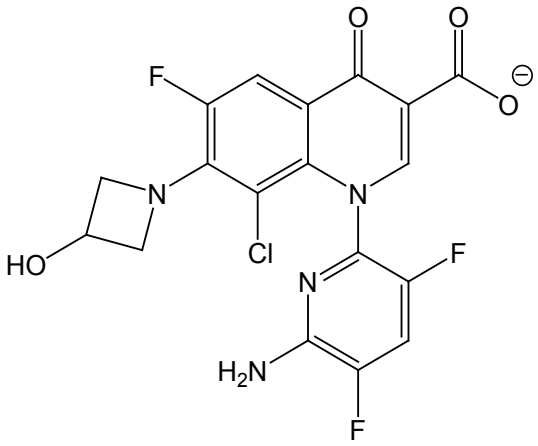
Levofloxacine



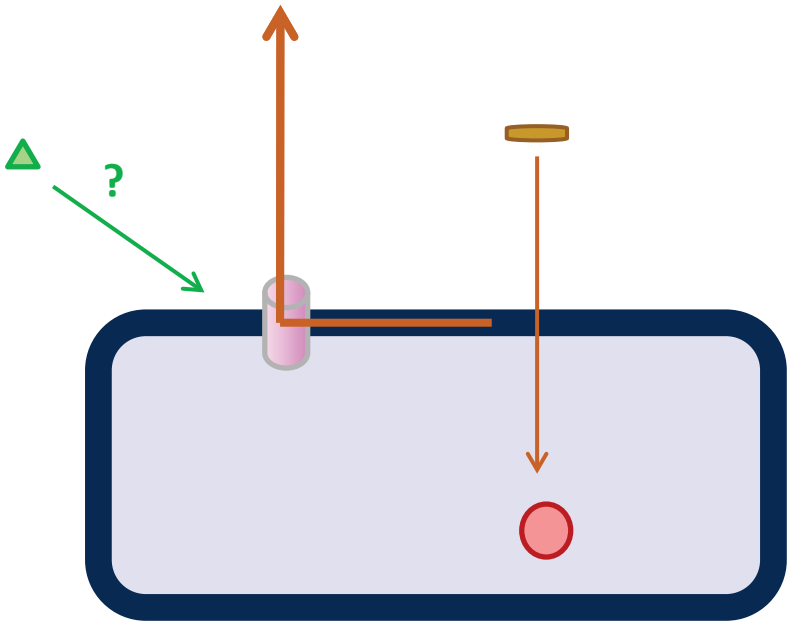
Marbofloxacine



Delafloxacine



Fluoroquinolone efflux pumps



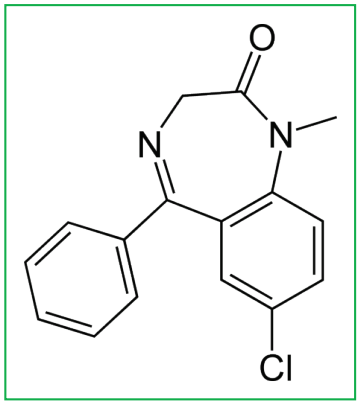
Diazepam

Antibiotic

Efflux pump

Bacterial membrane

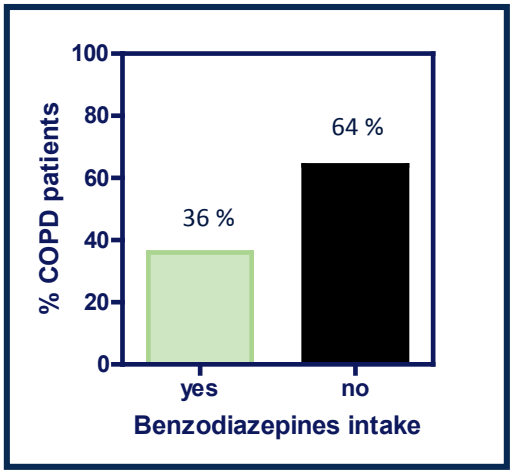
Therapeutic target



?

Diazepam :
anxiolytic & spasmolytic benzodiazepine, binding to the $GABA_A$ receptors

Efflux modulation by diazepam leading to resistance in *S. pneumoniae* ?

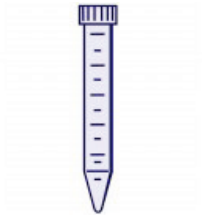


Diazepam → modifications of bacterial behaviour [a,b,c,d]

[a]Giuliodori et al, 2007; Review on bacterial stress topics *Ann N Y Acad Sci 1113: 95-104 ;* [b] Hadjivassileva et al, 2007 ; Int J Antimicrobial Agents 29:672-678; [c] Tavio et al, 2004; *J Med Microbiol 53: 1119-22 ;* [d] Tavio et al, 2012; Int J Antimicrobial Agents 39:90-94

Planctonic cultures : which impact of diazepam on bacterial efflux ?

Bacterial growth conditions :



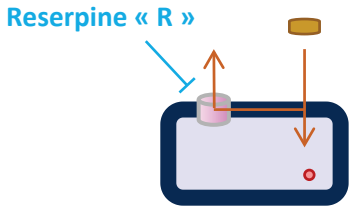
0h 3h 16h 1d 2 d 4d 8d

Medium : caMHB + 5%LHB ± DZP 1µg/ml (~ Human peak plasma concentration [0,2-1,5µg/ml][b]) ; 37°C ; 5% CO_2

Suspensions diluted everyday with fresh medium to OD_{620nm} = 0,1 (0,5 McF)

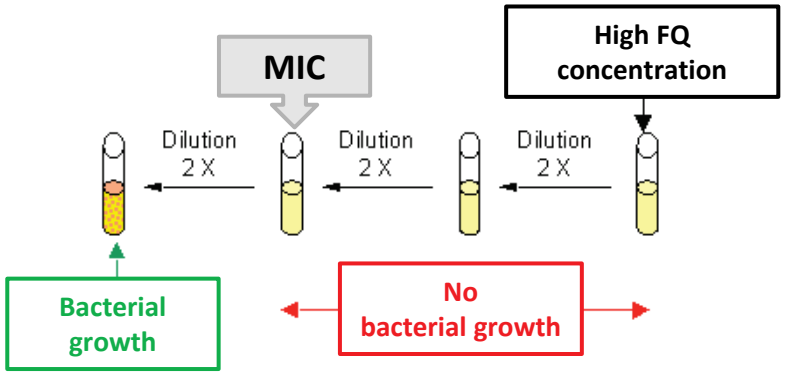
Evaluation of the bacterial susceptibility to fluoroquinolones

- **MICs (*) measures in microdilutions (96-well plates)**
- **In absence vs presence of reserpine (10mg/L) , an efflux pump inhibitor**
- **Fluoroquinolones tested : ex : - Norfloxacin (FQ substrate of efflux pumps PatA/B [a,c,d])**
  - Moxifloxacin (not substrate of efflux pumps [a,c,d])



(*) **MIC : Minimal inhibitory concentration :**

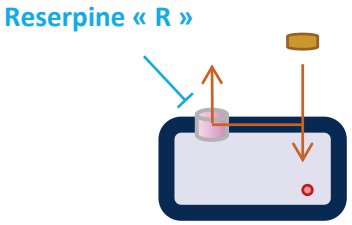
Smalest antibiotic concentation able to inhibit bacterial growth



[a] El Garch et al,2010; [b] Drug Information Handbook 18th Edition; [c] Lismond et al, 2011; [d] Piddock et al, 2002 b

Planctonic cultures : which impact of diazepam on bacterial efflux ?

Results :



Strain	Days of culture	Growth medium	Measures of the MIC (µg/ml)			
			NOR	NOR + R	MXF	MXF + R
ATCC 49619	0	CTRL m	**3**	**1.5**	**0.063**	**0.063**
		m + DZP 1µg/ml	3	1.5	0.063	0.063
	8	CTRL m	4	2	0.125	0.063
		m + DZP 1µg/ml	**32**	**4**	**0.063**	**0.063**

- **Susceptibility to NOR < MXF**

- **Significant impact on bacterial susceptibility to norfloxacin (**substrate of efflux pumps **) : induction of an efflux phenotype by Diazepam after 8 days of culture ***

- **No significant impact of Diazepam on moxifloxacin activity (**not substrate of efflux pumps**)**

(*) Also observed for other FQ substrate of efflux pumps

Antibiotic pharmacodynamic studies in polytherapies

1 Biofilm formation in different media :

- Reference medium : « m » : caMHB + 5% LHB + 2% glucose
- medium supplemented with diazepam (Human Peak Plasma Concentration [a]) :

  Medium « DZP » : m + **Diazepam 1µg/ml** (Valium® Sol. for injection i.v. / i.r)

2 Biofilms treatment with antibiotics (in absence of diazepam)

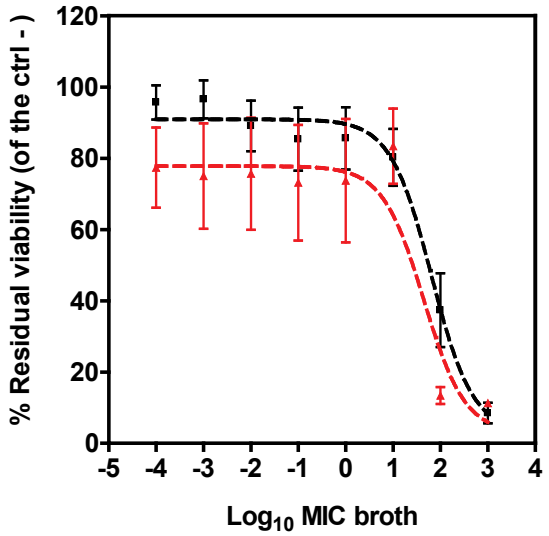
[a] Drug Information Handbook 18th Edition

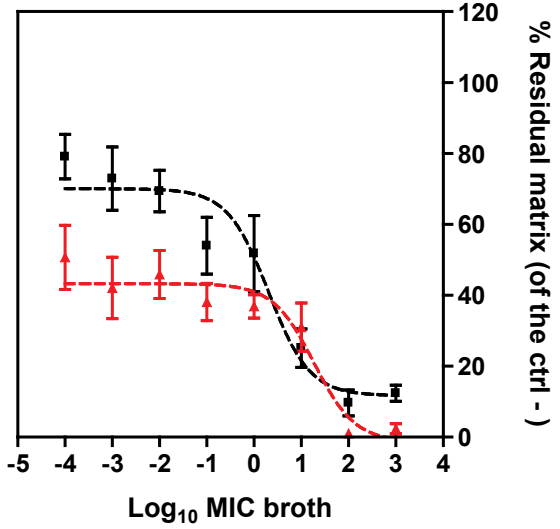
Results : Antibiotic pharmacodynamic studies in polytherapies

Ex: ATCC 49619 biofilms
- - - : 2 days-old , m
— : 11 days-old , m
- - - : 2 days-old , m+DZP 1µg/ml
— : 11 days-old , m+DZP 1µg/ml

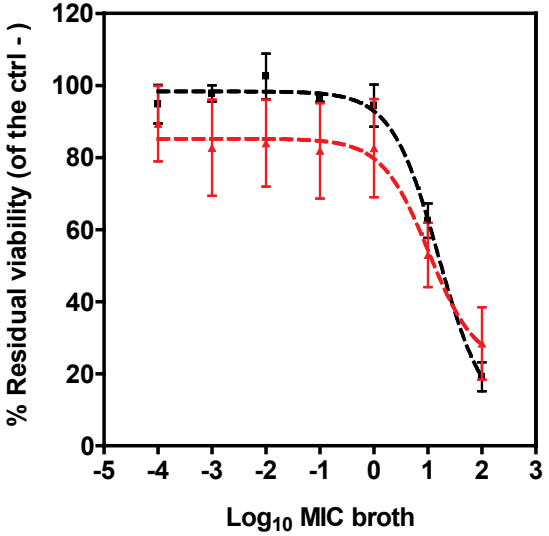
Survival | **Biofilm mass**

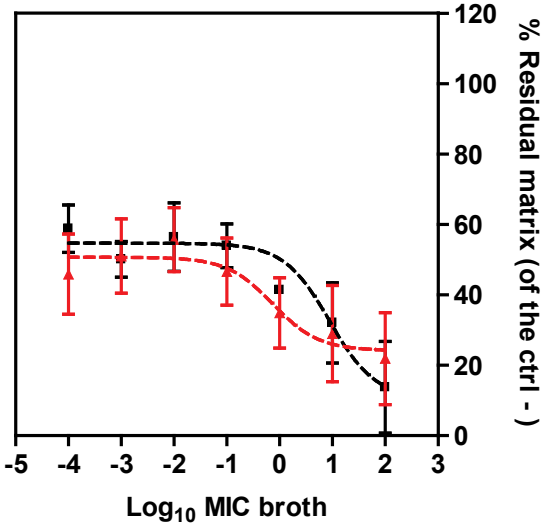
Moxifloxacin
Not substrate of efflux pumps





Norfloxacin
Substrate of efflux pumps





Results : Antibiotic pharmacodynamic studies in polytherapies

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Survival | **Biofilm mass**

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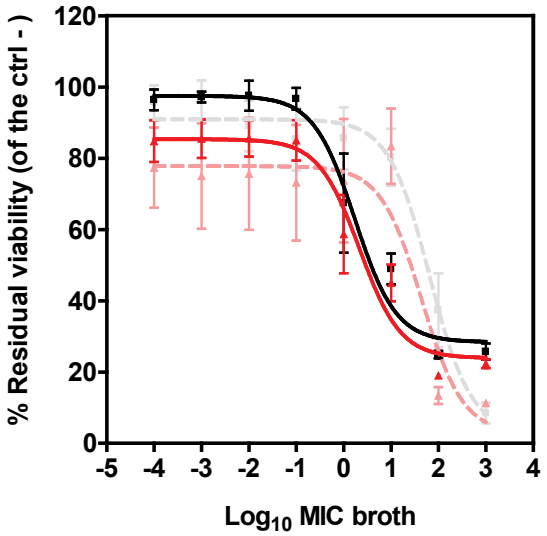
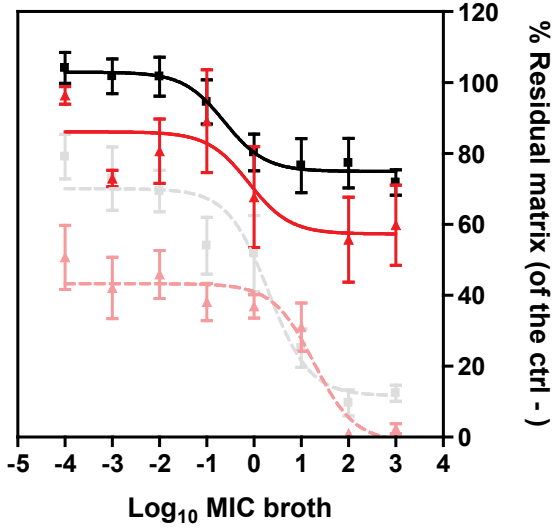
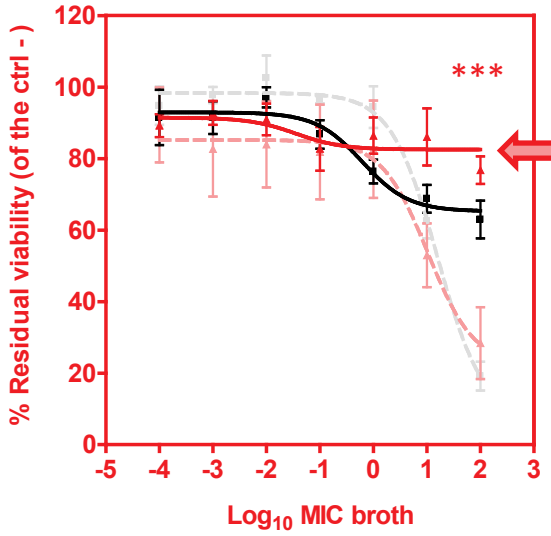
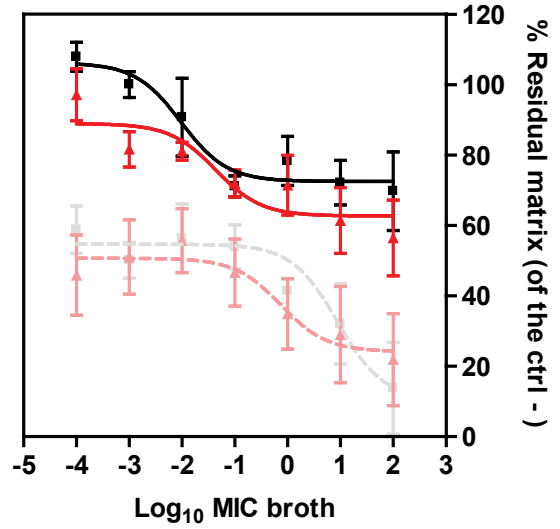




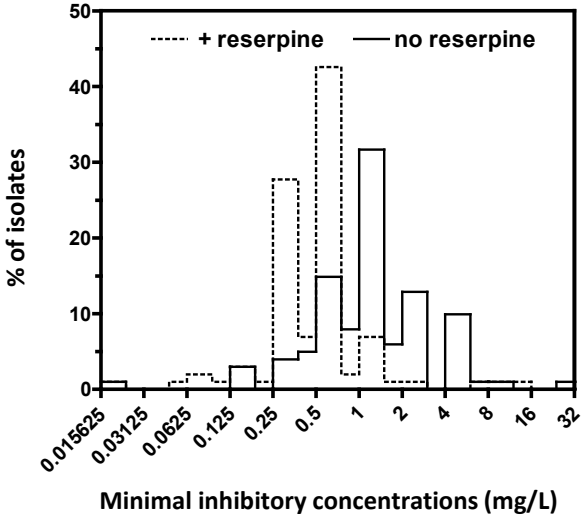
Norfloxacin
Substrate of efflux pumps





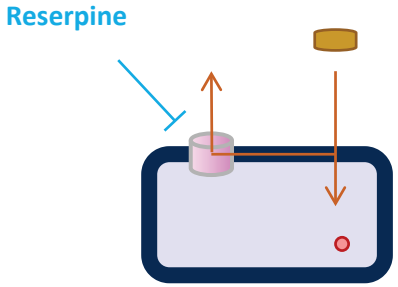
THESIS RESULTS : CHAPTER 1 : 3-years epidemiological study

Efflux to ciprofloxacin

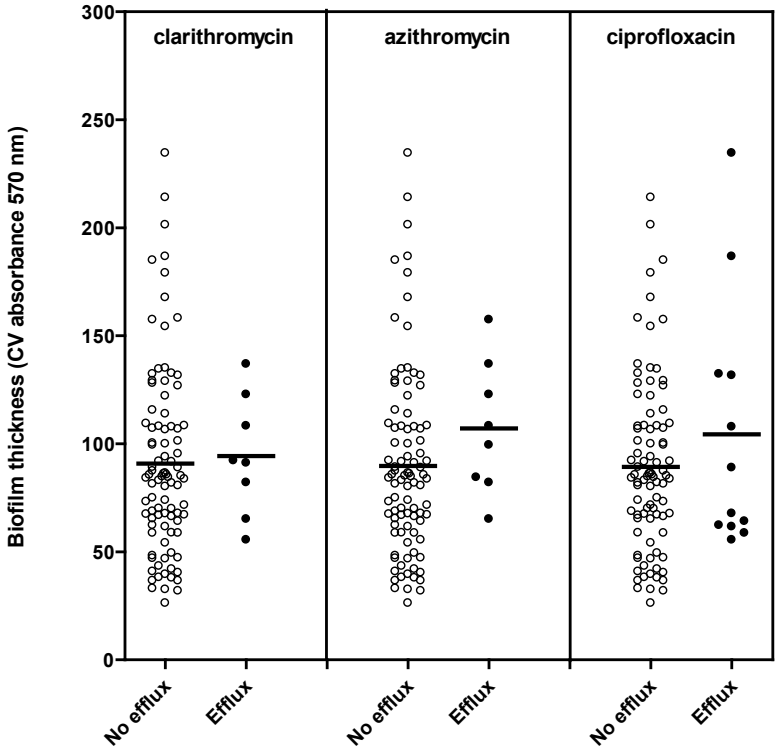


Ciprofloxacin MIC distribution for all isolates (n=101) determined in the absence (control; plain line) or in the presence (dotted line) of 10 mg/L reserpine (statistical analysis: $p < 0.0001$ when comparing distributions in the absence and in the presence of reserpine by two-tailed paired tests [Wilcoxon signed rank test (non parametric) and t test (parametric)]).

CIP efflux in >30% of isolates



Distribution of isolates biofilm production as a function of phenotypic efflux



Comparison of biofilm production after 10 days of culture for strains resistant to both clarithromycin and azithromycin and to ciprofloxacin using EUCAST interpretive criteria and grouped according to the absence (open symbols) or the presence (closed symbols) of efflux as detected for clarithromycin and azithromycin by dissociation of susceptibilities with clindamycin, and for ciprofloxacin by a 2-fold decrease in MICs upon addition of reserpine (10 mg/L). No correlation between efflux and biofilm thickness was seen unpaired t-test (with of without Welch correction).

No correlation between efflux and biofilm production