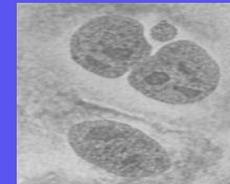




# Altadis

## Biological Assessment of Cigarette Whole Smoke



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Tobacco Smoke Department,  
Biology Toxicology  
ALTADIS, France

*Coresta meeting October 19th, 2006*

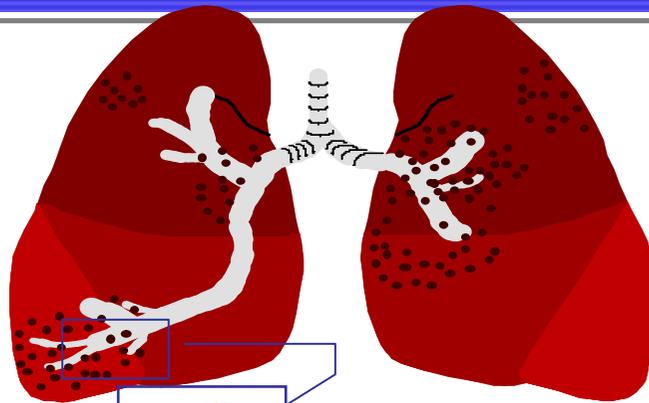


# Biological Assessment of Cigarette Whole Smoke

- ↪ Introduction
- ↪ Description of the main components of the Cultex system
- ↪ Results of Cytotoxicity according to the Neutral Red test
- ↪ Results of Genotoxicity according to the Comet assay
- ↪ Conclusion



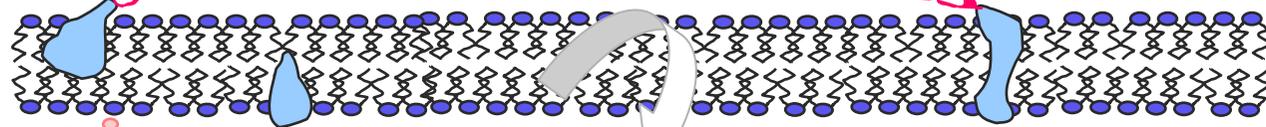
# Mainstream Smoke



Toxic Metabolites and/or  
Reactive Oxygen Species  
 $O_2^{\cdot-}$  ,  $OH^{\cdot}$



Membrane Alteration



Action on the  
cellular viability

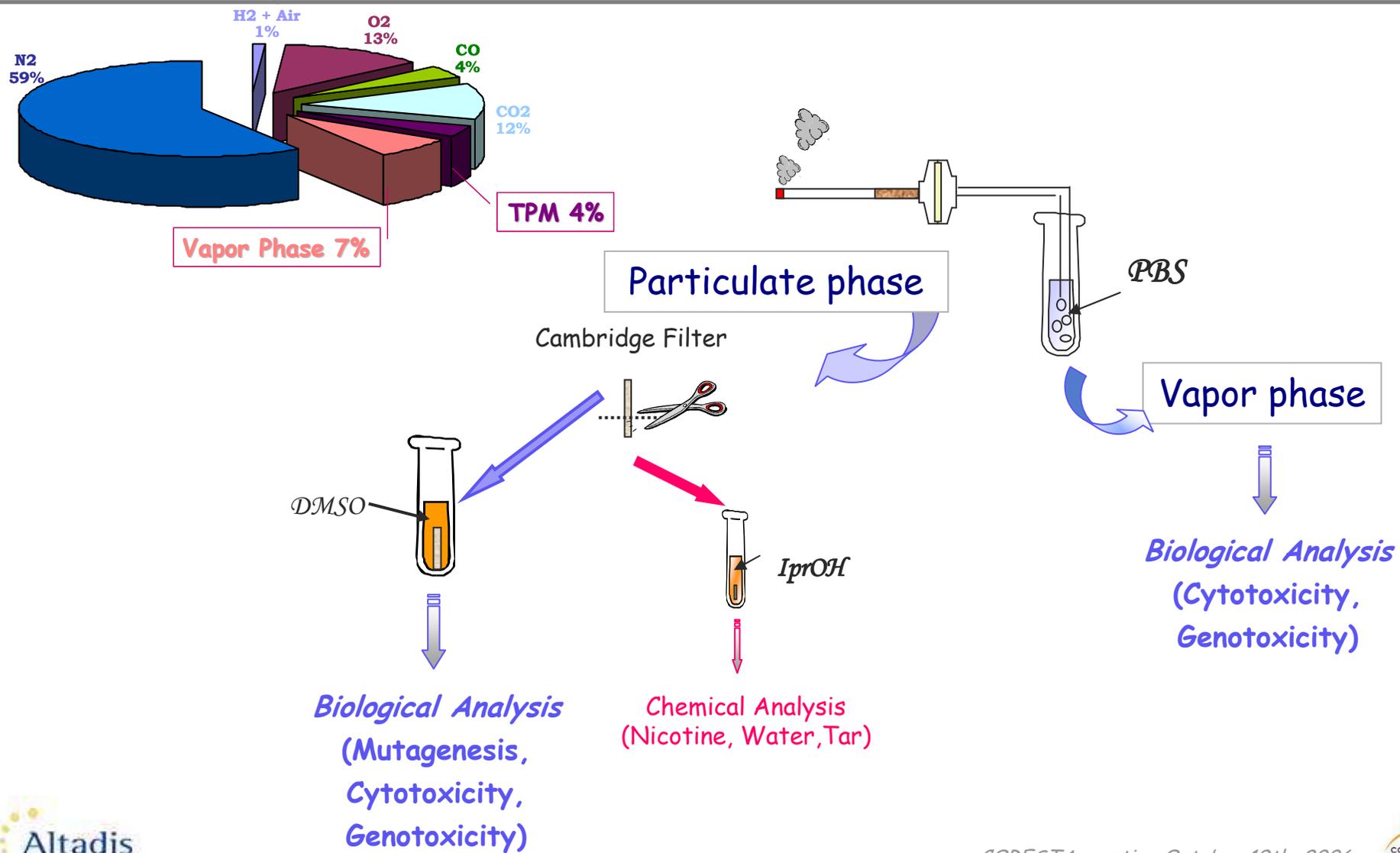
Action on the nucleic acids  
DNA damage





# Whole Smoke

## Classical Smoke samples for Toxicological Evaluation





# Biological Assessment of Cigarette Whole Smoke

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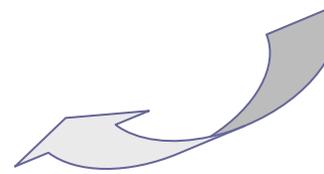
↪ Results of Genotoxicity according to the Comet assay

↪ Conclusion

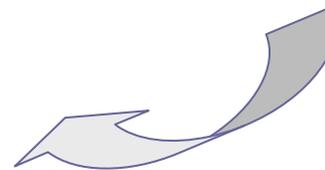


# Whole Smoke Exposure system

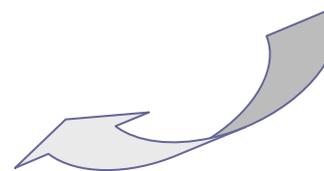
Whole smoke Generation with  
VC10 Robot  
(rotative smoking machine)



Whole smoke Dilution  
with clean Air

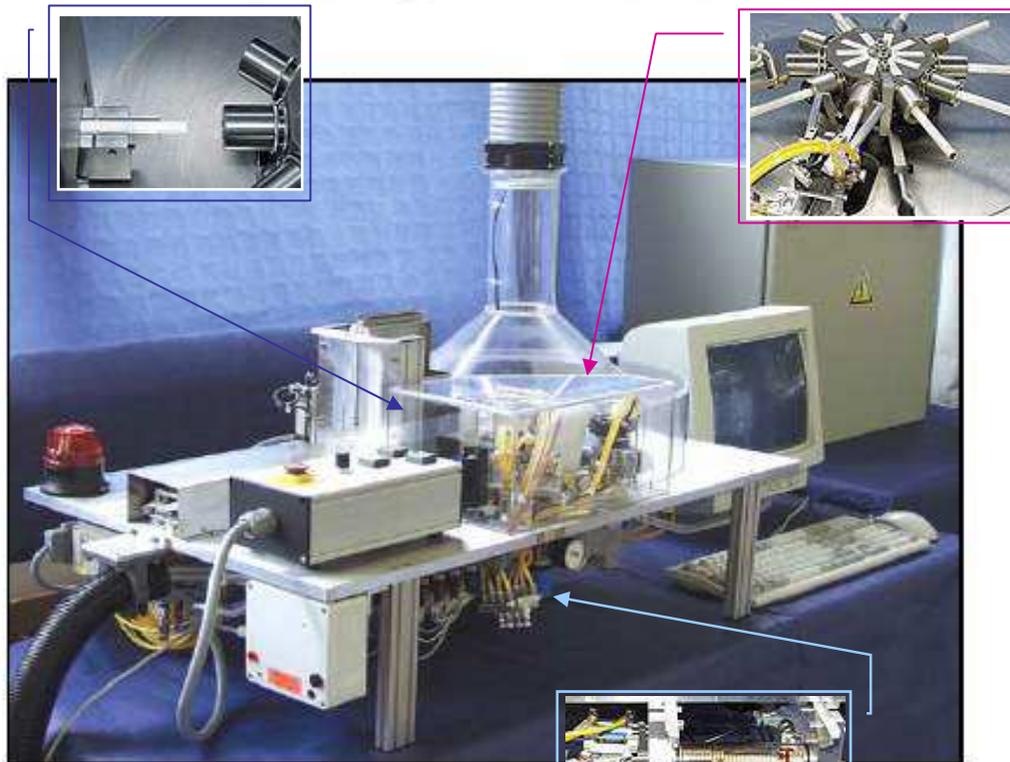


Cell's Exposure  
with  
whole smoke diluted



# The Smoking Machine

## Lab Automation Smoking Robot VC 10



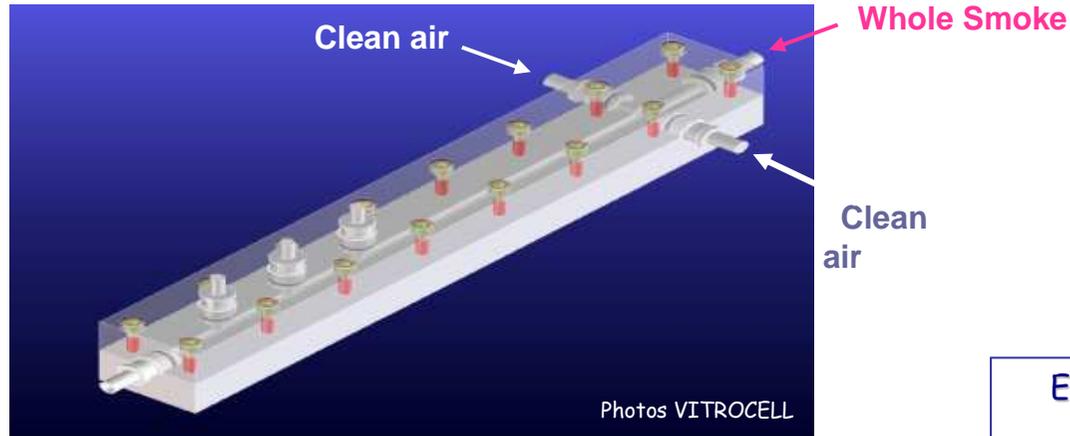
Photos VITROCELL

Generation of smoke with the shortest distance to cell cultures to avoid aging and to guarantee a smoke composition matching the real-life situation.

Programmable parameters of the smoking process: single cigarette smoke or serial smoke mode for a defined number of cigarettes, puff duration, puff volume, puff frequency and exhaust duration



# Whole smoke dilution with clean Air

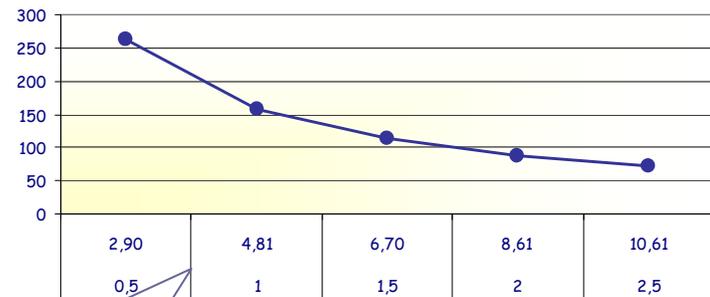


Example of calculation  
for an Exposure of  
4 cig - 10mg/cig - 8 puffs

### Calculation of dilution factor

PV	Puff volume (mL)	35,00
PED	Puff Exhaust Duration (sec)	8,00
Equals Smoke (mL/min)	$PV \cdot (60/PED)$	262,5
TS	Equals Smoke (L/min)	0,2625
DAS	Dilution Syntetic Air (L/min)	1
TV	Total Volume (smoke + air) (L/min)	DAS + TS : 1,26
	DF : Dilution Factor	TV/TS : 4,81

TPM Exposure ( $\mu\text{g}/\text{vessel}$ )



Air Flow (L/min)	Dilution Factor	Exposure ( $\mu\text{g}/\text{vessel}$ )
0,5	2,90	262
1	4,81	158
1,5	6,70	113
2	8,61	88
2,5	10,61	72

# Cultex System : Cellular Exposure

**Cell Culture :**  
**A549 Human Lung Cells**  
Minimum Essential Medium  
with fetal serum

**Exposure conditions :**  
Single mode (cig per cig)  
4 cigarettes  
Diluted whole smoke  
30 minutes

**Biological Analysis**  
Cytotoxicity  
Genotoxicity



Photos VITROCELL

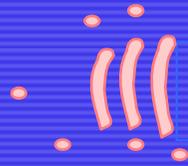


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# RESULTS



## Neutral Red Uptake

Cellular viability of A549 (Human Lung Cells) is determined with the Neutral Red test.

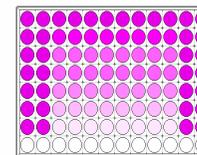
This test is based on the ability of the living cell to include a vital dye, the Neutral Red.

The optical density is proportional to the number of living cells, and the inhibited concentration can be evaluated.

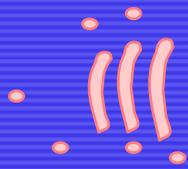
**Classically**, this is the Inhibited Concentration 50 ( $IC_{50}$ ) that is used, the concentration of tobacco smoke leading to 50% of cell death.



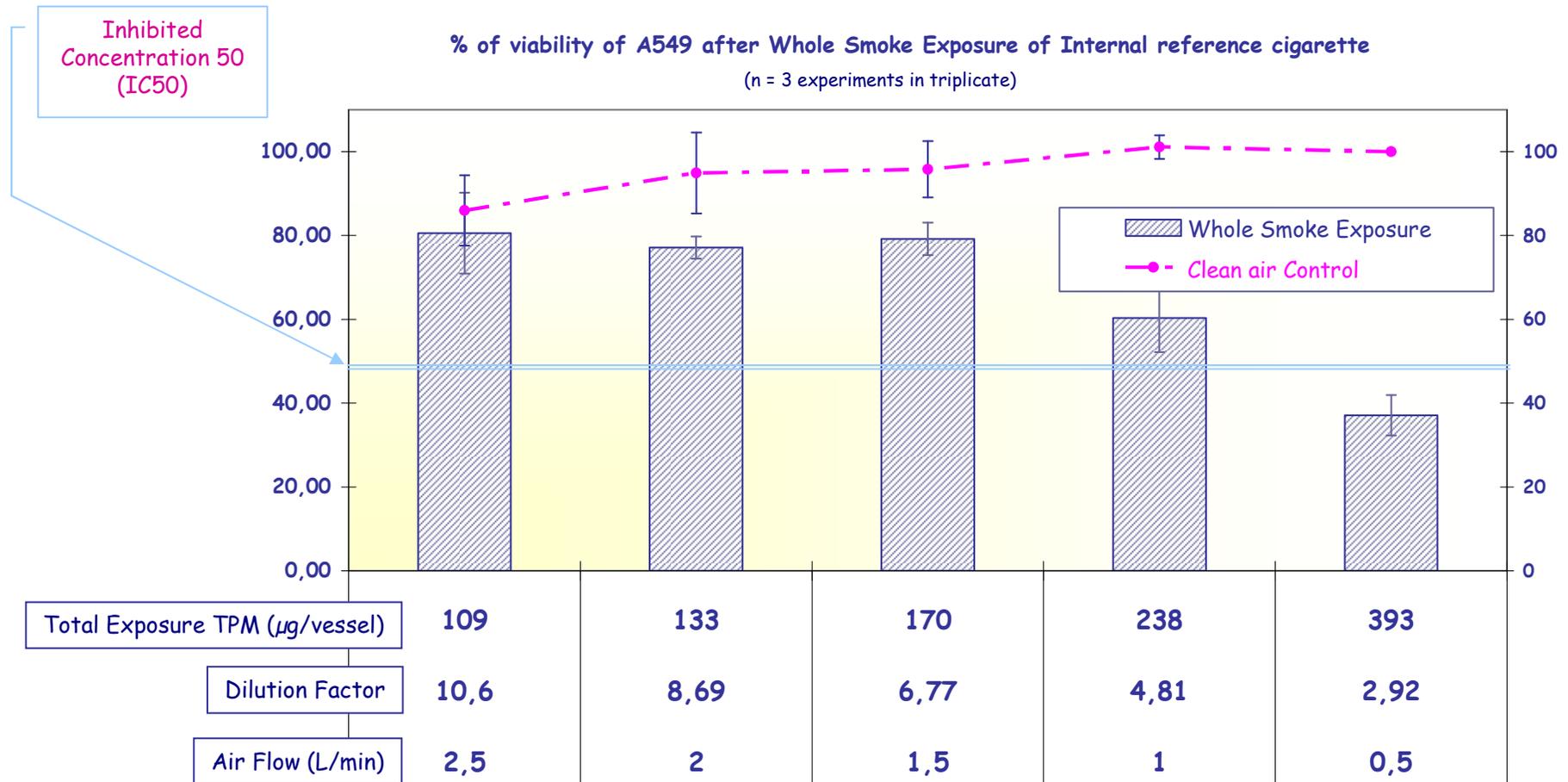
- 20 hours after exposure, Cells were incubated with the Neutral Red
- Neutral Red Extraction after cellular lysis, and
- Transfer of Neutral Red on 96-well plate

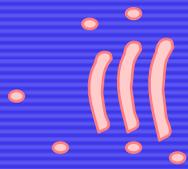


- Reading at 540 nm
- Viability Determination



# Feasibility of the Neutral Red test





# Comparative effect : Acetate *versus* Charcoal Filter

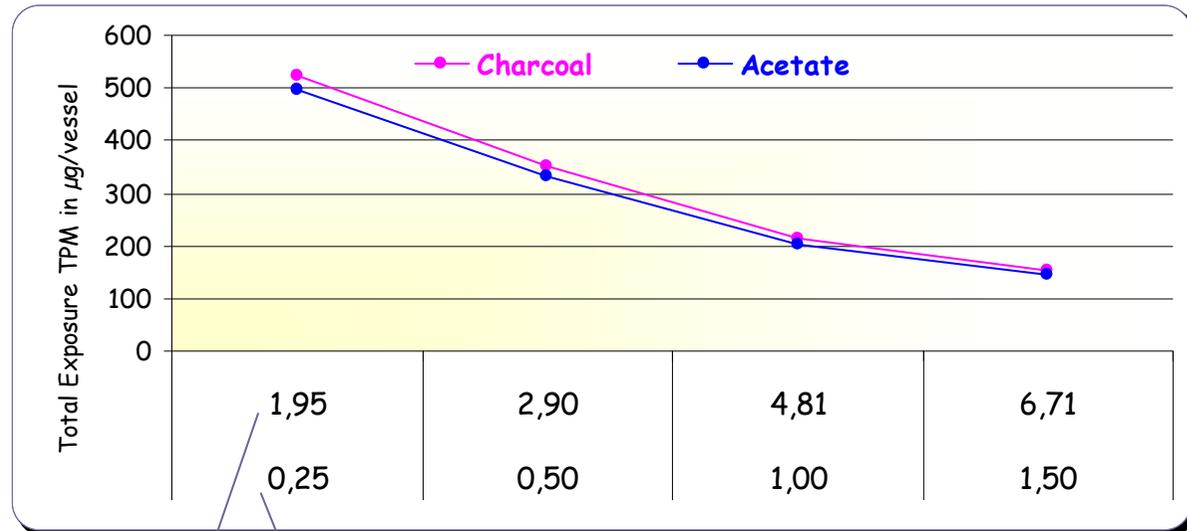
## Cigarette Characteristics

Acetate	Charcoal
Same Blend	
Same Paper	
Pressure drop : 112	
Filter	
Acetate	Charcoal
Puff Number	
7,1	7,4
TPM (mg/cig)	
12,73	13,44
Tar (mg/cig)	
10,04	10,21

**Smoke generation**  
4 cigarettes - 7 puffs per cigarette

**Duration of exposure**  
30 minutes

## Total Exposure (TPM $\mu\text{g}/\text{vessel}$ )



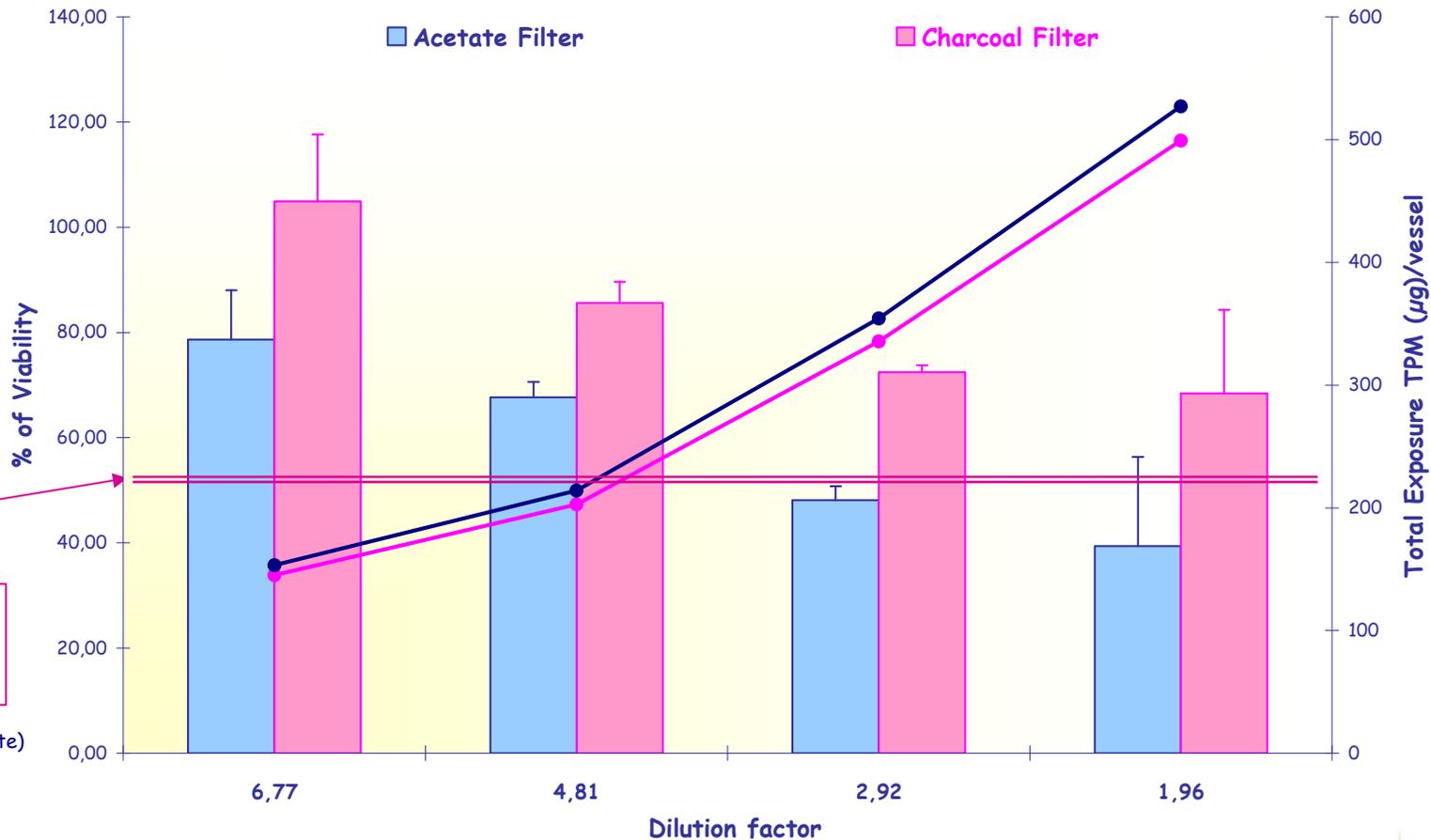
Dilution Factor

Air Flow (L/min)



# Comparative effect : Acetate *versus* Charcoal Filter

Charcoal  
Smoke  
is less  
cytotoxic  
than  
Acetate  
Smoke



Inhibited  
Concentration 50  
(IC50)

(n = 3 experiments in triplicate)





# Biological Assessment of Cigarette Whole Smoke

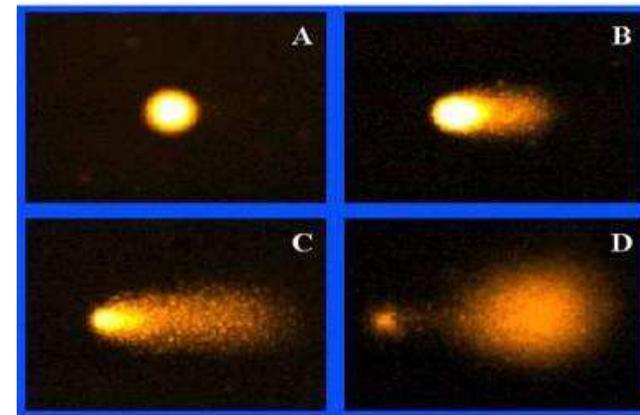
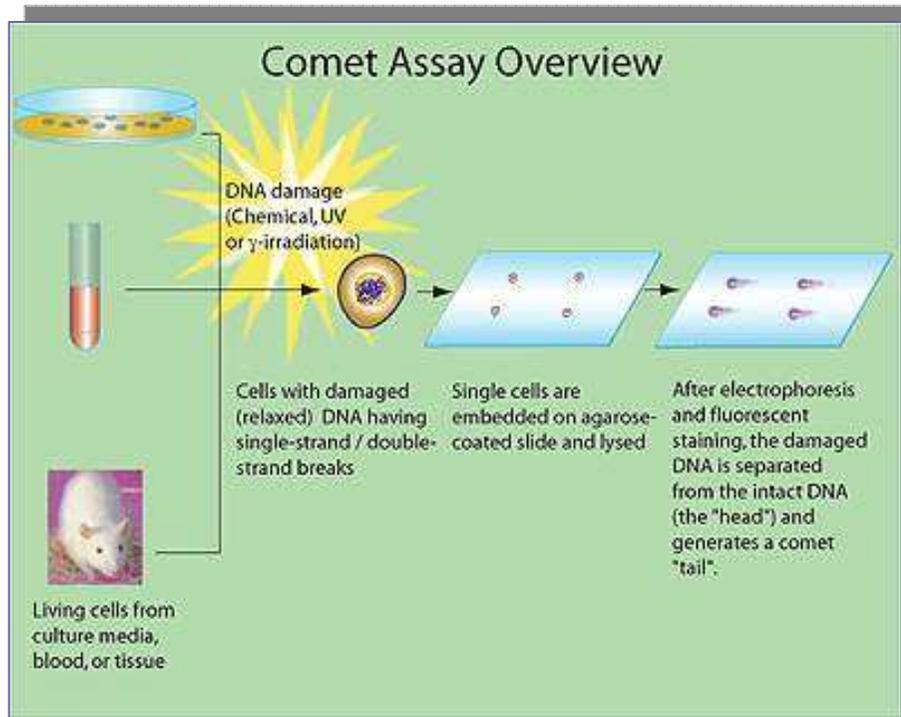
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# RESULTS



## Genotoxicity testing : Comet Assay or Single Cell Gel Electrophoresis



A : Normal cell

B : Weak Damage

C : Strong Damage

D : Apoptosis

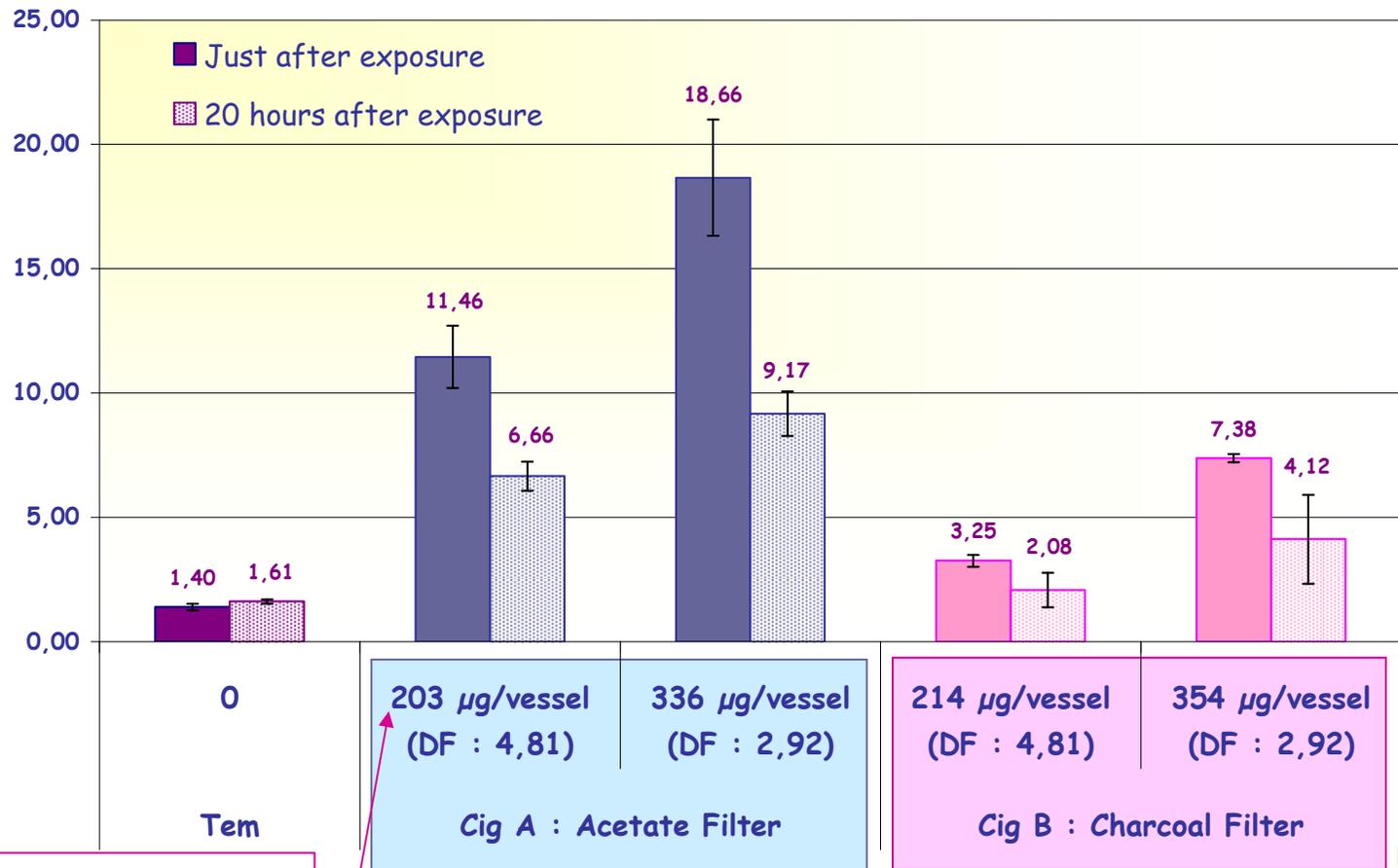
Results are expressed in Tail Moment (TM) :  
The Percentage of fluorescence in the Tail is closely correlated with the level of DNA damage



Charcoal Smoke  
less  
Genotoxic  
than Acetate  
Smoke

Genotoxicity  
observed 20h  
after exposure  
is lower than  
right after  
exposure  
(DNA repair process)

Tail Moment (TM)



Total Exposure TPM  $\mu\text{g}/\text{vessel}$



## Take-home messages : Scientific

- Assessment of Biological Activity of Whole Smoke according to the **Neutral Red for the Cytotoxicity testing**, and the **Comet assay for the Genotoxicity testing** is feasible
- Biological assessment of Whole Smoke is **an essential and complementary approach** to evaluate the toxicity of cigarette smoke

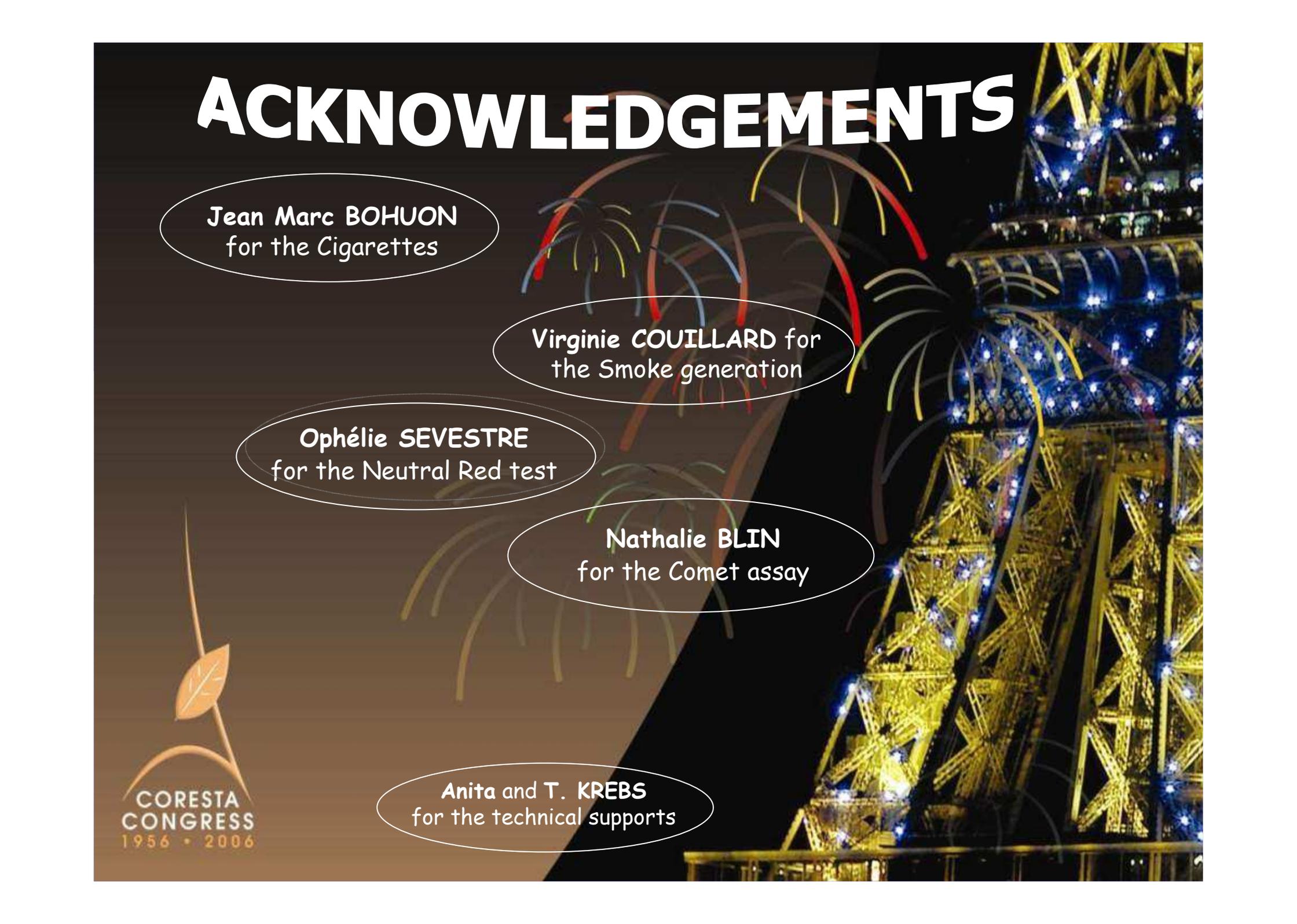


## Take-home messages : Practical

- *In vitro* Toxicological Testing of Whole Smoke is longer than *In vitro* testing of Smoke Condensate :
  - One smoke sample = One smoking
  - One analyze (Neutral Red or Comet assay)
    - ⇒ One dose-response curve
- Requirements : to develop tests using **small cellular samples**

Continue to look for more effective biomarkers and to optimize our biological tools

# ACKNOWLEDGEMENTS



**Jean Marc BOHUON**  
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**Nathalie BLIN**  
for the Comet assay

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