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# Impact of vehicle exhaust exposure on respiratory epithelial and natural killer cells

VERT Focus Event

March 16th 2018

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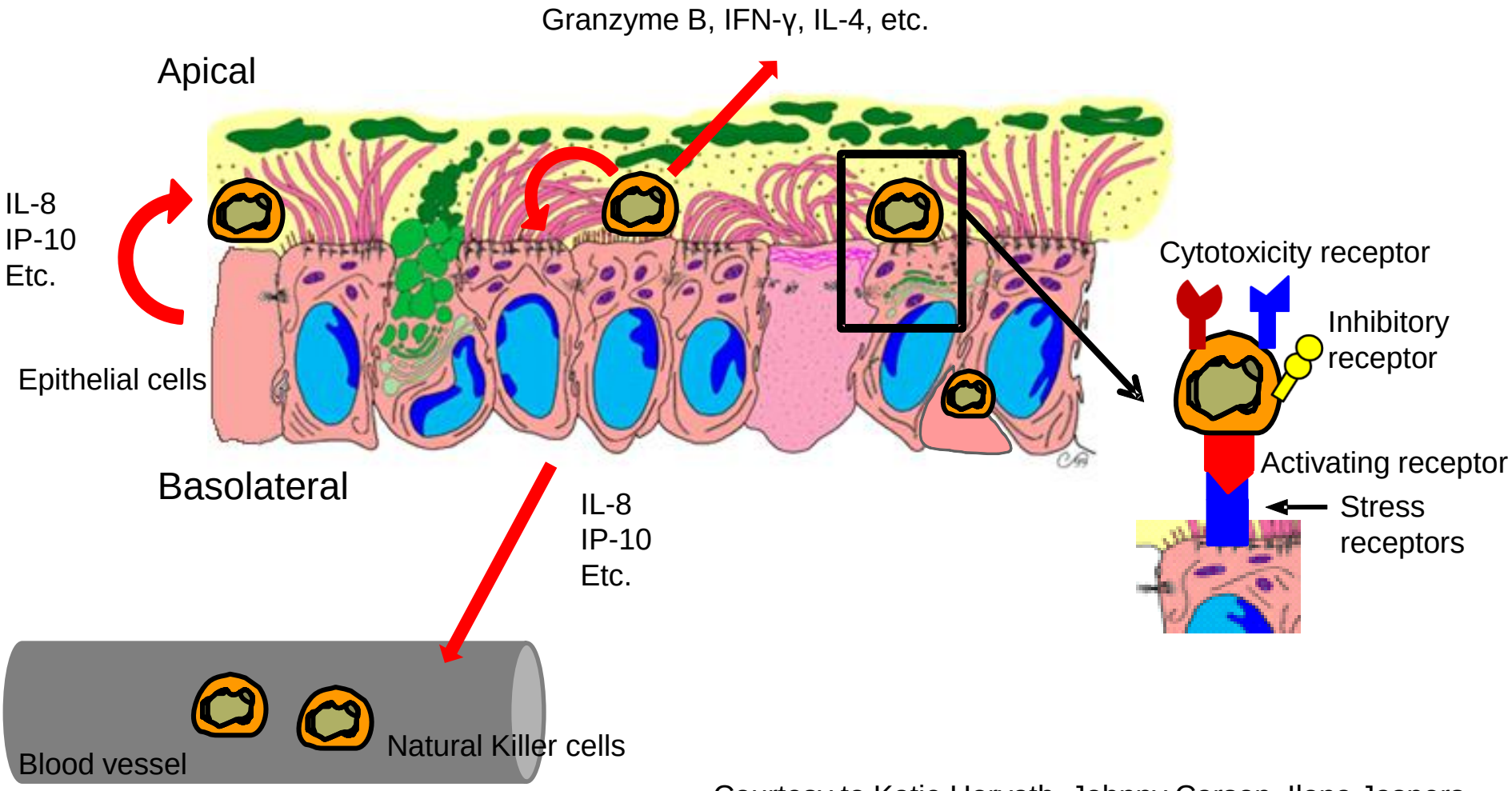
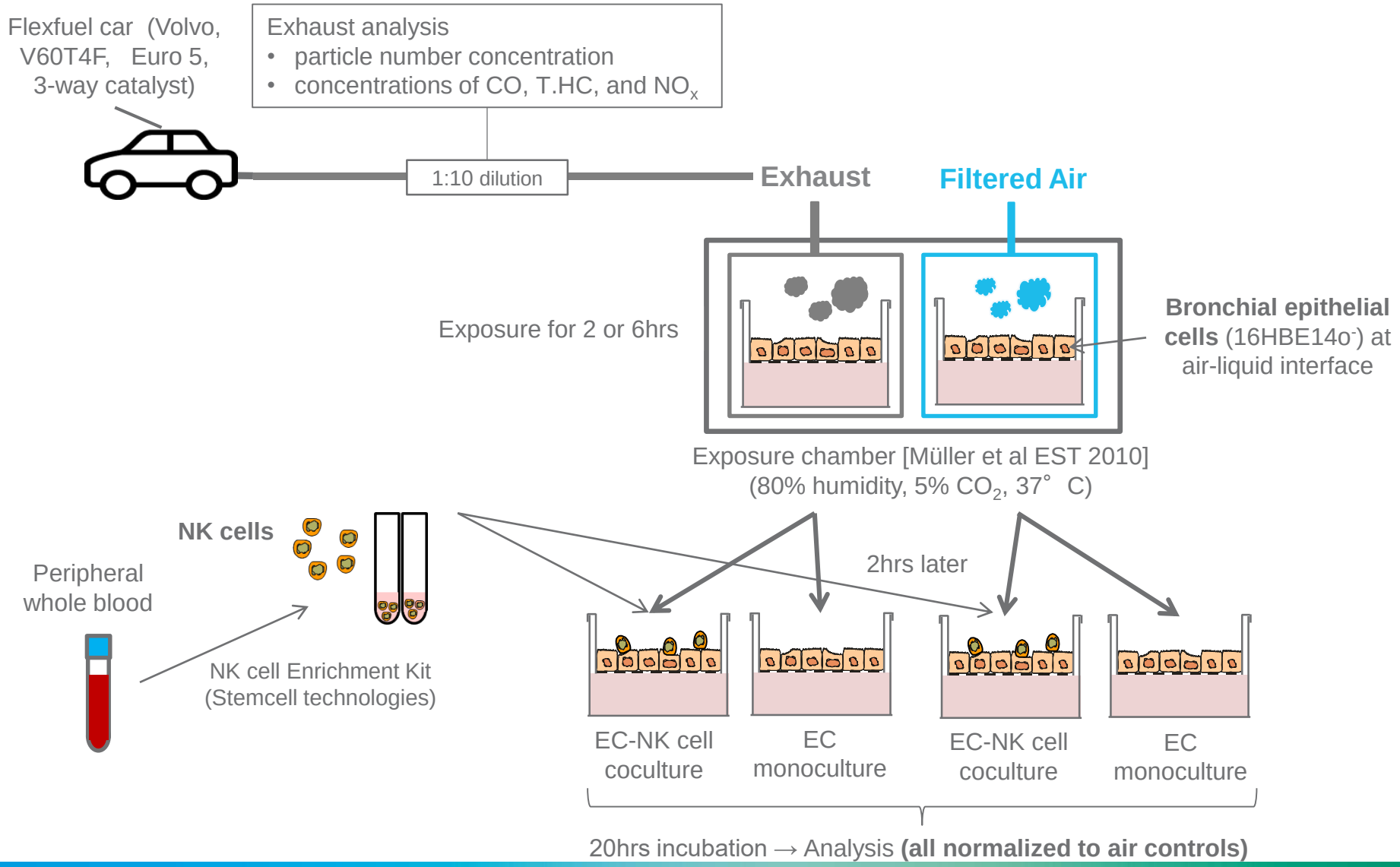
# Epithelial Cell – Natural Killer Cells Interactions

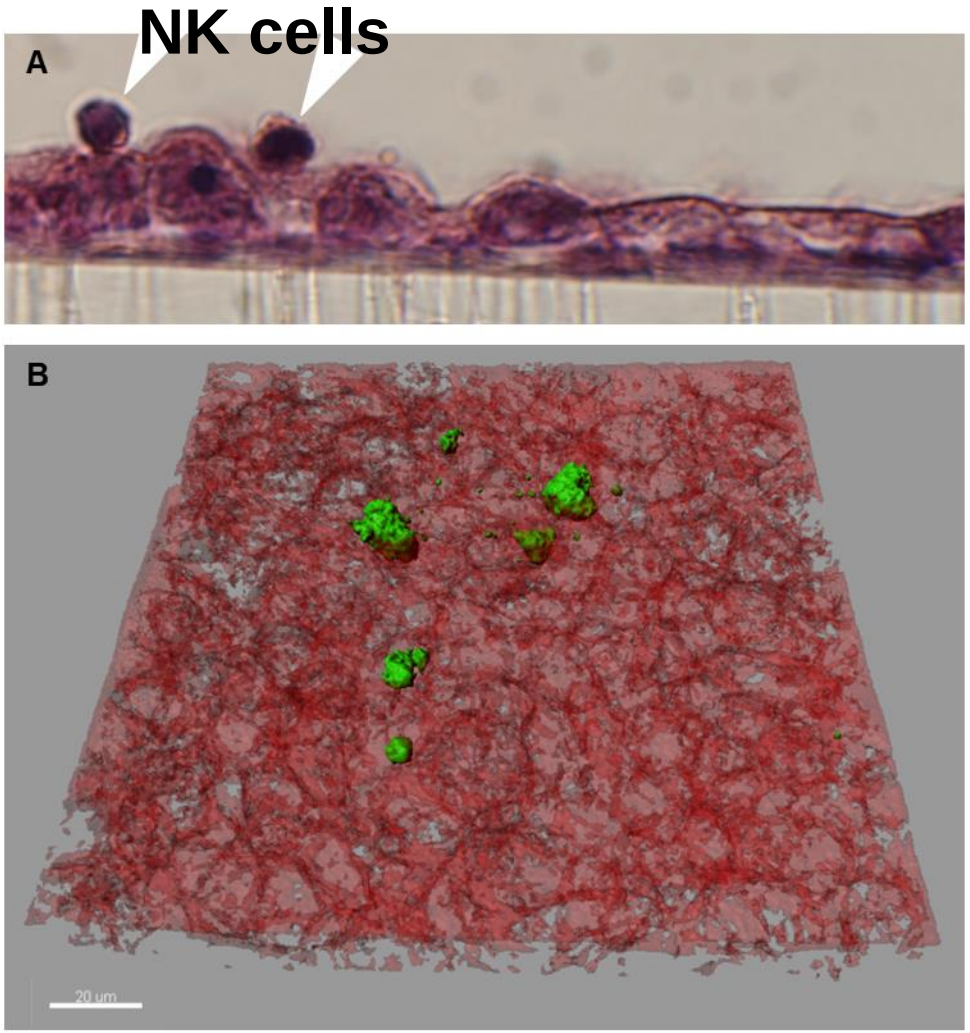



Courtesy to Katie Horvath, Johnny Carson, Ilona Jaspers

# Study Design



# Co-culture model: Epithelial and Natural Killer Cells



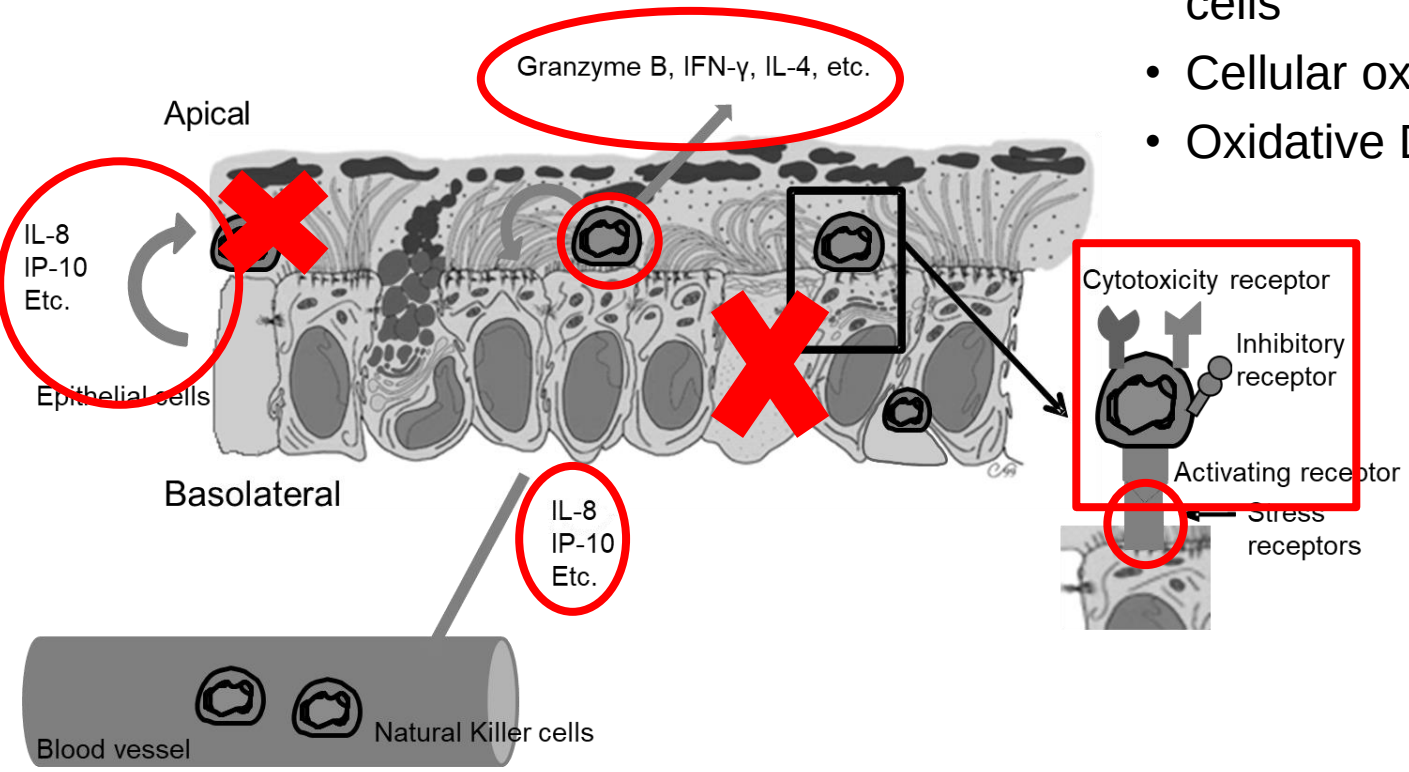

**Epithelial cells**

**Natural Killer Cells**

**16HBE14o$^{-}$ bronchial epithelial cell line**

# Endpoints

- Cytotoxicity
- Stress receptors on epithelial cells
- Phenotype & Activation of Natural Killer cells
- Cytokines
- Killing potential of Natural Killer cells
- Cellular oxidative stress
- Oxidative DNA damage



# Car Settings

- **Ethanol:** Effect of ethanol supplement
  - E0: gasoline fuel
  - E85: gasoline with 85% ethanol

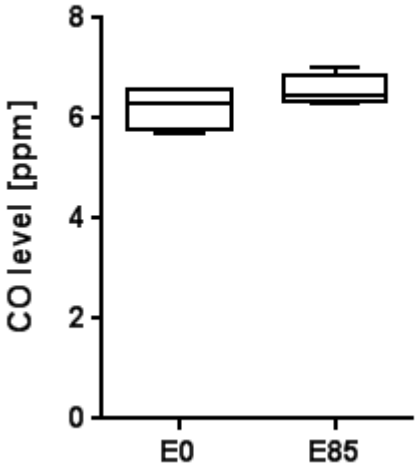
  > Steady State Driving Cycle (SSC)

- **Filter:** Effect of a gasoline particle filter
  - Reference: not filter
  - GPF: coated gasoline particle filter (GPF)

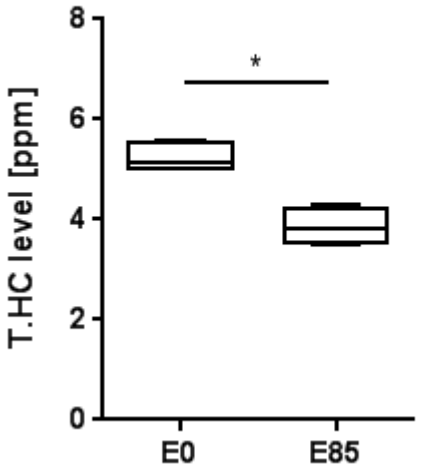
  > Worldwide Harmonized Light-Duty Vehicles Test Cycle (WLTC)

# Ethanol – Exhaust Characterization

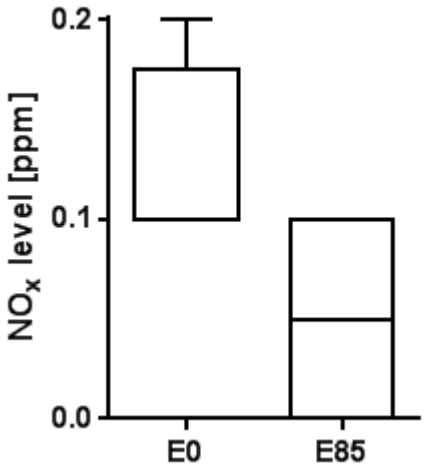
**carbon monoxide**



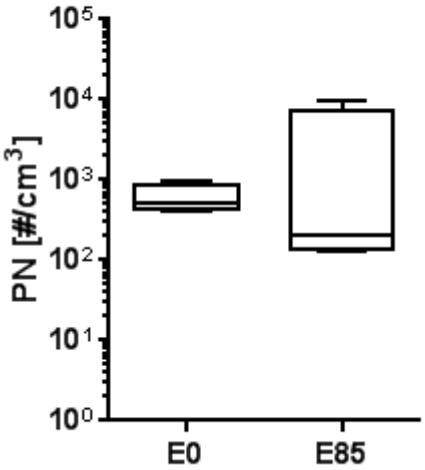

**total hydrocarbons**




**nitrogen oxides**




**particle number**



# Ethanol – Epithelial cells

**Table 4**
Comparison of effects of exposure to E0 or E85 exhaust in ECs of monocultures or cocultures.

| | | | E0 | E85 | p-Value |
|---|---|---|---|---|---|
| Flow cytometry measurement | | | | | |
| Cytotoxicity (% dead EC) | | MC | 0.93 [0.8–0.99] | 1.02 [0.88–1.06] | 0.23 |
| | | CC | 0.81 [0.25–0.98] | 1.09 [0.84–1.26] | 0.38 |
| EC surface markers (MFI) | MICA/B | MC | 1.70 [1.06–2.52] | 1.06 [0.82–1.33] | 0.11 |
| | | CC | 1.57 [1.03–19.17] | 1.13 [0.30–1.35] | 0.25 |
| | ULBP2/5/6 | MC | 1.40 [1.12–1.51] | 1.24 [0.89–1.63] | 0.66 |
| | CD183 | | | | |
| DNA damage (MFI) | | MC | 1.62 [0.89–10.7] | 0.82 [0.41–1.07] | 0.06 |
| | | CC | 1.25 [0.94–2.43] | 0.97 [0.79–0.99] | 0.20 |
| Quantitative real-time RT-PCR | | | | | |
| ULBP2 | | MC | 0.88 [0.44–2.77] | 0.70 [0.06–0.79] | 0.57 |
| | | CC | 1.05 [0.49–4.68] | 1.23 [0.28–3.95] | > 0.99 |
| MICA | | MC | 0.83 [0.59–3.44] | 1.22 [0.05–33.69] | 0.83 |
| | | CC | 1.10 [0.94–4.89] | 1.55 [0.46–7.11] | > 0.99 |
| IL-8 | | MC | 0.49 [0.28–0.91] | 0.71 [0.06–20.48] | 0.57 |
| | | CC | 0.80 [0.60–4.11] | 1.33 [0.22–14.88] | 0.25 |
| IP-10 | | MC | 0.71 [0.16–0.81] | 18.78 [0.69–33.06] | 0.23 |
| | | CC | 1.30 [0.96–1.72] | 2.02 [0.05–4.7] | > 0.99 |
| Colorimetric assay | | | | | |
| Oxidative Stress (GSH/total protein) | | MC | 1.06 [1.02–1.08] | 1.19 [0.92–1.20] | 0.34 |
| | | CC[a] | 0.99 [0.78–1.31] | 0.99 [0.96–1.00] | > 0.99 |

Data are normalized to corresponding air controls (resulting in 1 = no effect). Values are presented as median [range] of percentage of dead cells, MFI, relative gene expression or ratio of GSH relative to total protein. Data of EC monocultures were analyzed by Mann-Whitney test and those of cocultures by Wilcoxon signed-rank test. Abbreviations: CC, coculture; GSH, glutathione; MC, monoculture, MFI, mean fluorescence intensity.

[a] Includes ECs and NKs, since the cells cannot be separated for this assay. Boarderline significant p-values are marked in bold number.

No cytotoxicity

No effect on stress receptors

Trend for less DNA damage in monocultures

No effect on stress receptors

No effect on cytokines

No oxidative stress

# Ethanol – Natural Killer cells

**Table 5**
Comparison of effects of exposure to E0 and E85 in NKs cocultured with ECs.

| | | E0 | E85 | p-Value |
|---|---|---|---|---|
| Cytotoxicity (% dead NKs) | | 0.76 [0.52–1.67] | 1.05[1.01–1.39] | 0.38 |
| NK cell surface markers (MFI) | CD16 | 1.02 [0.98–1.04] | 0.97 [0.96–1.02] | 0.25 |
| | | 1.01 [0.91–1.08] | 0.99 [0.92–1.09] | > 0.99 |
| | | 1.01 [0.95–1.15] | 1.02 [0.90–1.13] | 0.63 |
| | | 0.99 [0.81–1.07] | 1.15 [1.03–1.92] | 0.13 |
| | CD183 | 1.39 [0.86–1.48] | 1.27 [0.97–1.29] | 0.50 |
| | CD314 | 1.97 [0.10–6.70] | 1.20 [0.90–1.34] | 0.50 |
| | CD335 | 1.22 [0.92–1.48] | 1.28 [1.10–1.41] | 0.63 |
| NK cell intracellular markers (MFI) | grzB | 1.00 [0.92–1.13] | 1.05 [0.88–1.12] | > 0.99 |
| | IFN-γ | 0.96 [0.88–1.00] | 1.08 [0.85–1.79] | 0.38 |
| | IL-4 | 1.00 [0.88–1.60] | 0.92 [0.86–1.03] | 0.38 |
| Killing potential (% dead target cells) | | 0.92 [0.77–1.14] | 0.92 [0.86–1.09] | 0.88 |
| DNA damage (MFI) | | 1.13 [0.87–1.84] | 0.71 [0.59–1.04] | 0.13 |

Data are normalized to corresponding air controls (resulting in 1 = no effect, negative values were adjusted to positive values by adding the same fixed value to all data points of the same endpoints). Values are presented as median [range] of percentage of dead cells, or MFI. Data were analyzed by Wilcoxon signed-rank test. Abbreviations: grzB, granzyme B; MFI, mean fluorescence intensity.

No cytotoxicity

No change of phenotype & activation

No effect on cytokines

No effect on killing potential

No DNA damage

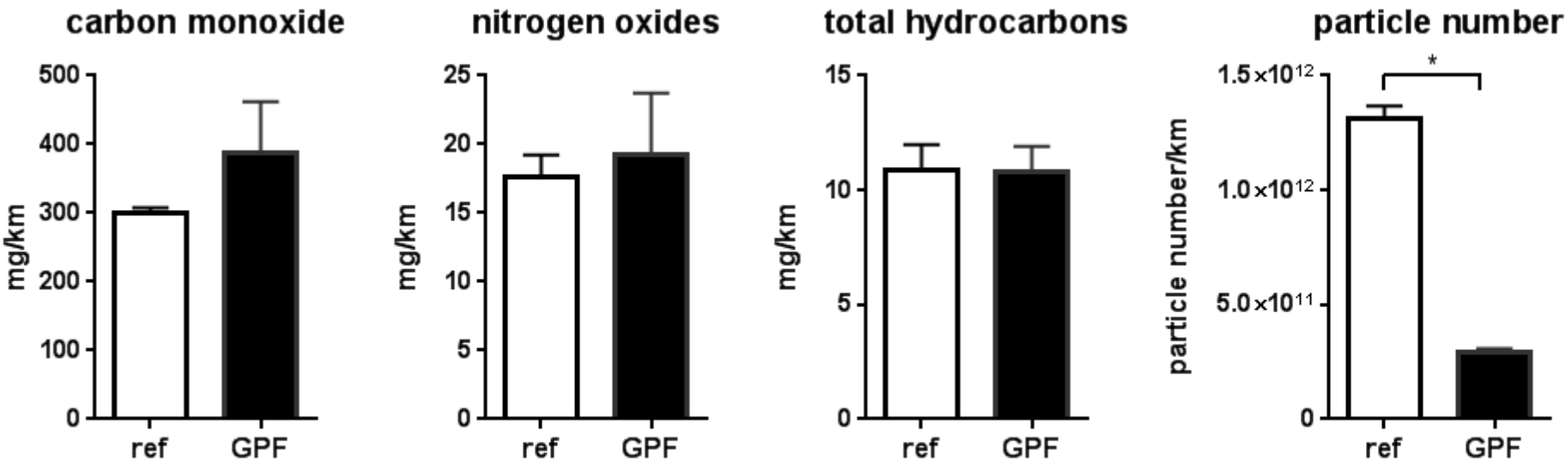
# Ethanol – Conclusion

- No major toxic effects of pure gasoline or ethanol gasoline exhaust from a modern flex-fuel car in epithelial cell monocultures or cocultures of epithelial and natural killer cells

- Use of ethanol as part of fuel for gasoline cars is probably not harmful

- Further studies (chronic, in vivo) are needed

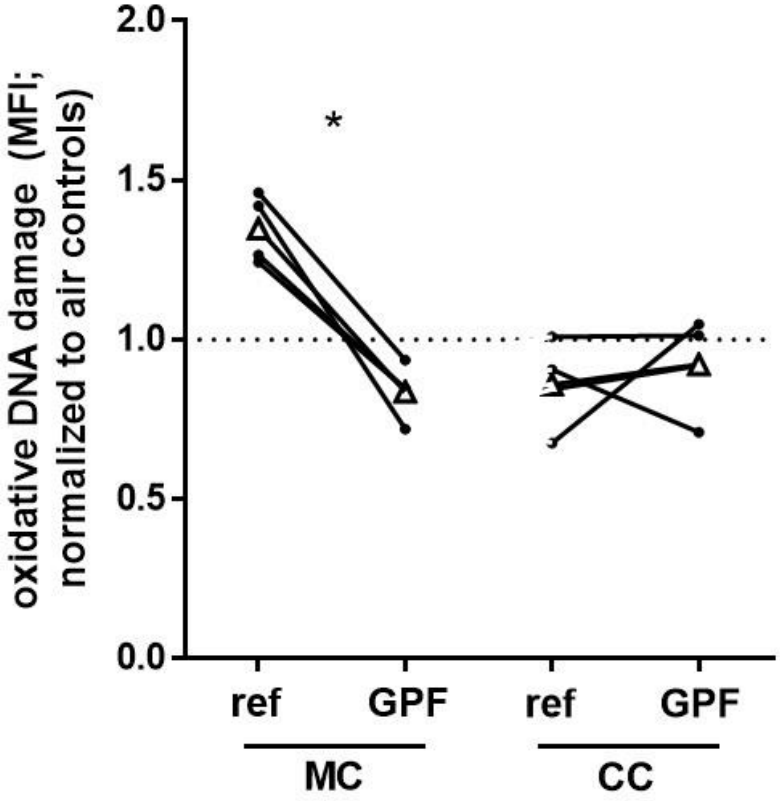
# Gasoline Particle Filter (GPF) – Exhaust (1)




**Increase of particle size**
**mean diameter: 64nm → 107nm**

# GPF – Epithelial cells

- No cytotoxicity
- No effect on stress receptors
- Reduction of DNA damage in monocultures
- No effect on cytokines (gene expression and protein)
- No oxdiative stress



# GPF – Natural Killer Cells

| | | Reference | | p-value |
|---|---|---|---|---|
| Cytotoxicity (% dead NKs) | | 0.90 [0.76–1.4 | –1.05] | >0.99 |
| NKs surface markers (MFI) | CD16 | 0.98 [0 | 0 [0.93–1.07] | 0.25 |
| | CD158b | | 1.02 [0.94–1.90] | 0.38 |
| | CD159a | –1.12] | 1.00 [0.91–1.27] | 0.38 |
| | CD | / [0.86–1.02] | 1.08 [0.89–1.20] | 0.13 |
| | | 0.98 [0.96–1.18] | 1.03 [0.93–1.45] | 0.38 |
| | | 1.02 [1.01–1.05] | 1.00 [0.99–1.06] | 0.63 |
| NKs intracellul | grzB | 1.01 [0.86–1.13] | 1.04 [0.92–1.08] | 0.88 |
| | IFN-γ# | 0.92 [0.23–1.53] | 0.90 [0.82–2.26] | 0.88 |
| | IL-4 | 1.07 [0.89–1.47] | 0.98 [0.86–1.12] | 0.63 |
| Killing potenti | % dead target cells) | 1.03 [0.97–1.10] | 0.88 [0.71–1.06] | 0.25 |
| DNA damage (MFI) | | 0.89 [0.55–1.10] | 0.93 [0.76–1.04] | 0.88 |

No effects in Natural killer cells

# GPF - Conclusion

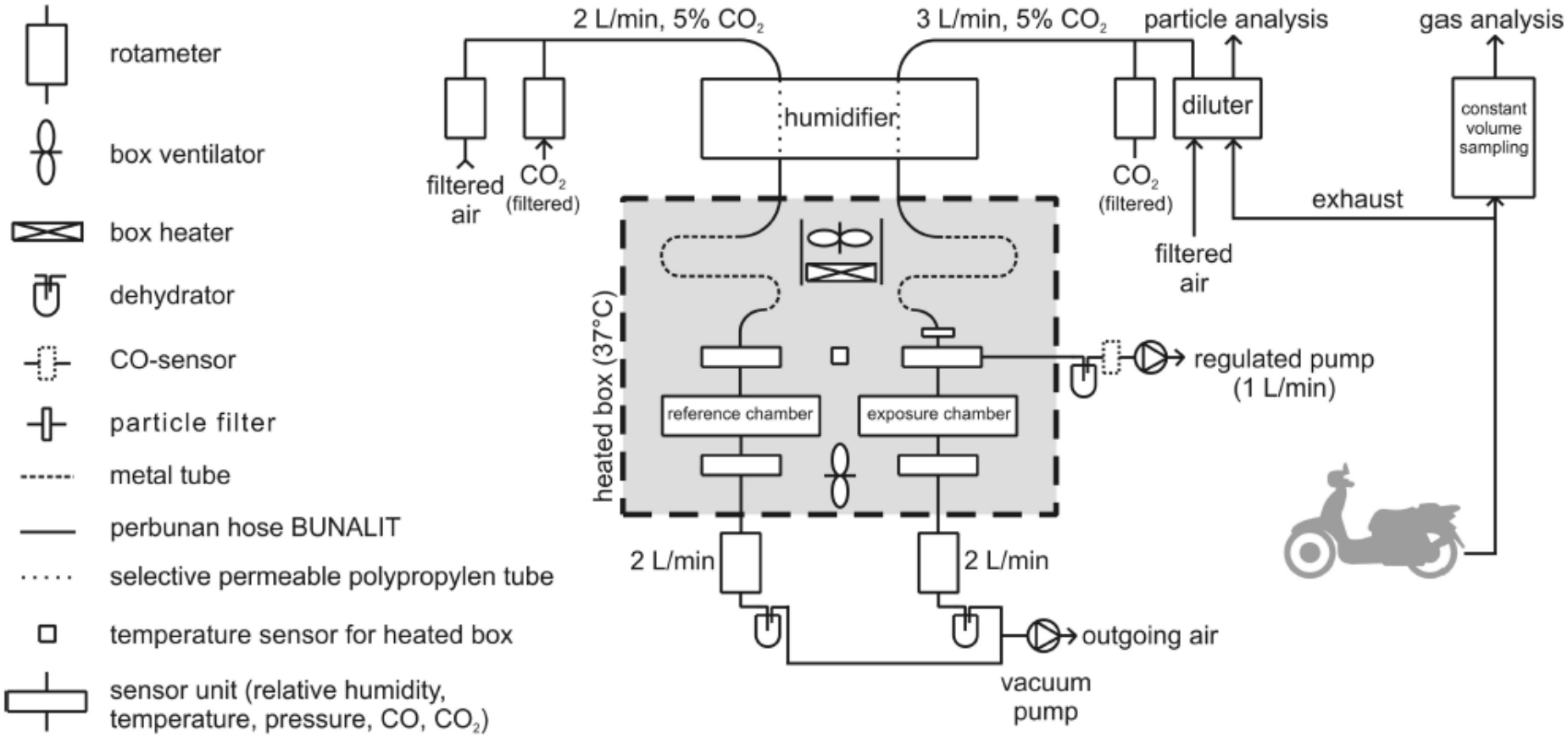
- Use of a GPF decreases oxidative DNA damage, and thus potentially reduces the carcinogenic potential of the exhaust

- Application of GPFs seems to be beneficial

- Further studies (chronic, in vivo) are needed

# Retrospection – 10 year ago…

Our schematic of the exposure system looked like that:



# …we exposed the cells for 1 and 2 hours…

**TABLE 1. Physical Characterization of the Exhaust Emissions**

| | | | scooter exhaust emission |
|---|---|---|---|
| particle number [1/cm$^3$, 10-400nm] | | | $4.02 \cdot 10^6 \pm 4.75 \cdot 10^4$ |
| mean diameter [nm] | | | $111.08 \pm 3.25$ |
| surface area [µm$^2$/cm$^3$] | | | $4.11 \cdot 10^4 \pm 1.07 \cdot 10^4$ |
| particles deposited on TEM grids [1/cm$^2$] | 2 h exposure duration | exposure | $11.8 \cdot 10^7 \pm 11.0 \cdot 10^7$ |
| | | reference | $4.56 \cdot 10^7 \pm 4.22 \cdot 10^7$ |
| | 1 h exposure duration | exposure | $8.63 \cdot 10^7 \pm 8.79 \cdot 10^7$ |
| | | reference | $5.81 \cdot 10^7 \pm 7.70 \cdot 10^7$ |
| **gas phase emissions** | | **ambient air** | **scooter exhaust emission** |
| CO [ppm] | | $1.17 \pm 0.24$ | $40.2 \pm 8.9$ |
| $CO_2$ [%] | | $0.04 \pm 0.00$ | $0.124 \pm 0.002$ |
| HC [ppm] | | $12.05 \pm 8.80$ | $100.2 \pm 7.0$ |
| $NO_x$ [ppm] | | $0.05 \pm 0.03$ | $13.3 \pm 1.8$ |

…we diluted the exhaust 1:100…

# …we tested the optimal duration of after-incubation…

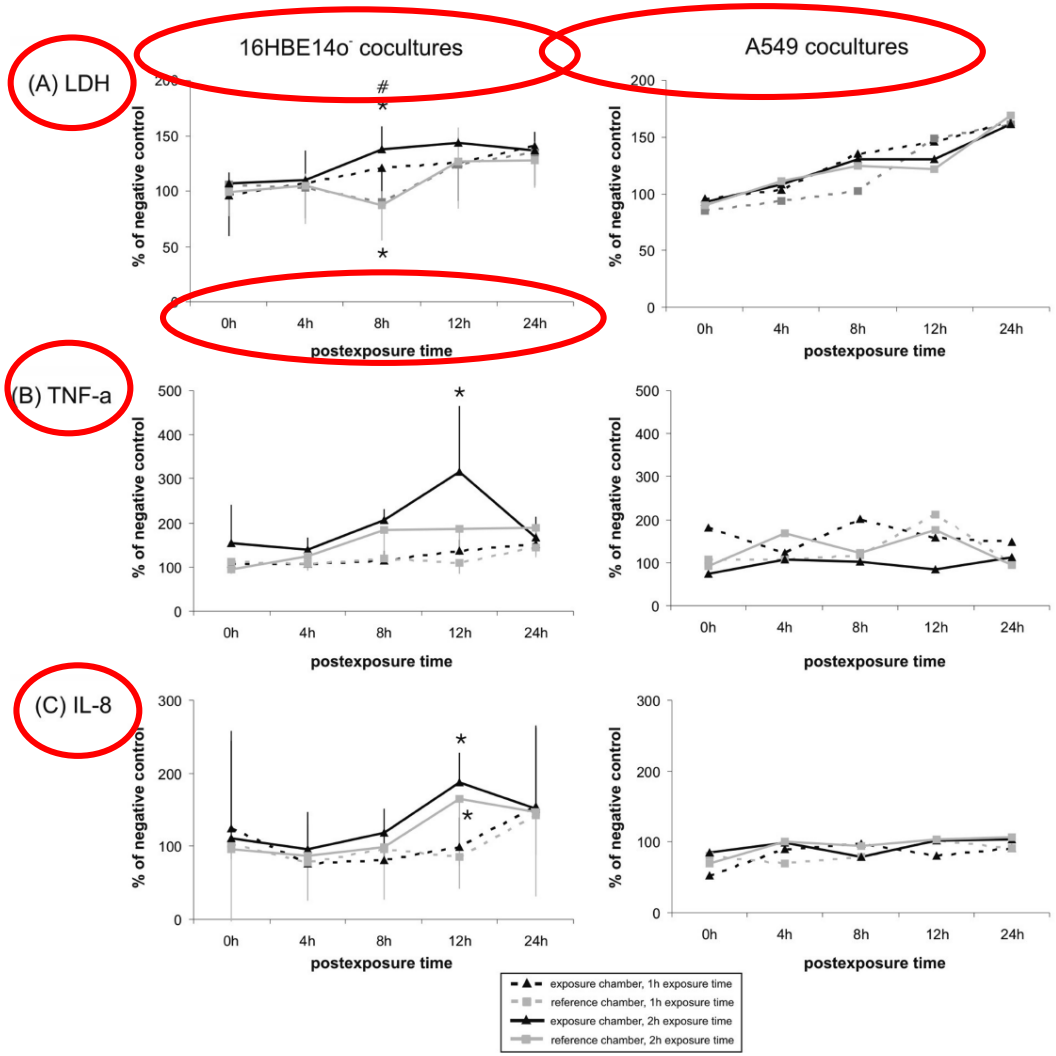



FIGURE 2. Cytotoxicity and (pro-) inflammatory response in different cell culture types after exposure for 1 and 2 h, and 0–24 h postexposure time, owing to scooter exhaust emissions. * means statistically significant difference ($p < 0.05$) compared to control and # compared to reference.

…compared two cell lines…

…studied cytotoxicity and (pro-) inflammation…

# …using scooters & cardborad…

**…and being a PhD student and a lot younger!**

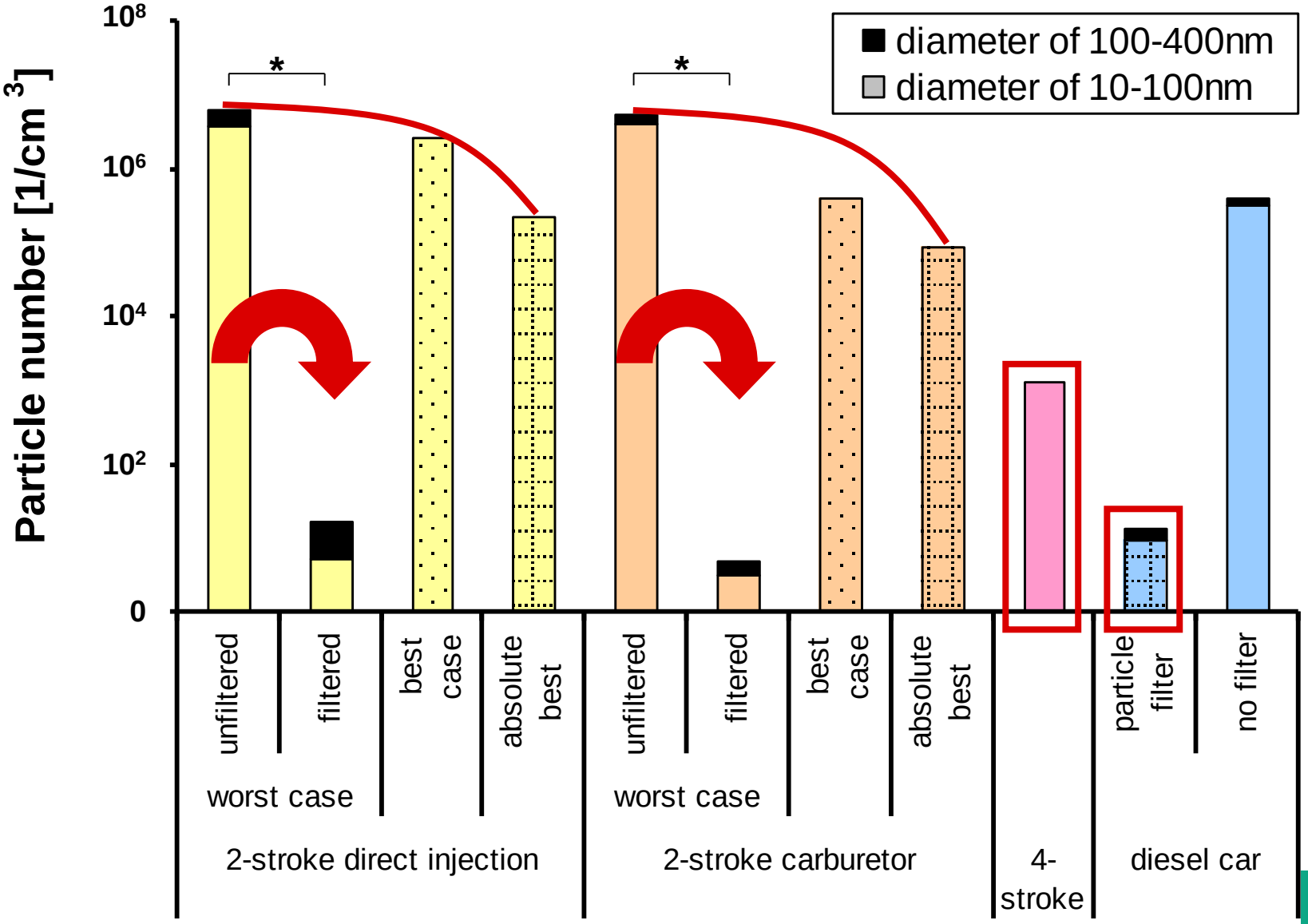
# Scooter Toxicity – Set-up

- 3D model with 16HBE14o-, macrophages and dendritic cells
- 2h exposure, 1:100 dilution, 8h &12h after-incubation
- Each 3 exposures with 3 different cells cultures (“n”=9)
- Cytotoxicity and the (pro-)inflammatory responses (TNF-α, IL-8)
- Exhaust characterization
  - Particle number
  - Nitrogen oxides ($NO_x$)
  - Total hydrocarbons
  - Carbon monoxide (CO)
- vehicles:

# Scooter Toxicity – Vehicles & Specifications

<table>
<tr><td>Scooter Peugeot 2-stroke direct injection</td><td rowspan="2">worst case<br>• unleaded fuel<br>• Swiss army oil<br>• 100% oil ratio<br>• dummy muffler</td><td rowspan="2">worst case – filtered</td><td rowspan="2">best case<br>• Aspen fuel<br>• Motorex oil<br>• 50% oil ratio<br>• oxi cat & wire mesh filter catalyst</td><td rowspan="2">absolute best case<br>• Aspen fuel<br>• Motorex oil<br>• 50% oil ratio<br>• coated particle filter</td></tr>
<tr><td>Scooter Peugeot 2-stroke carburetor</td></tr>
<tr><td>Aprilia 4-stroke scooter</td><td colspan="4">normal conditions (original, without catalyst)</td></tr>
<tr><td>Diesel car</td><td colspan="2">with particle filter</td><td colspan="2">without particle filter</td></tr>
</table>

# Scooter – Exhaust Characterization (1)



# Scooter – Exhaust Characterization (1)

- **Nitrogen oxides**
  - lower for best & absolute best cases
  - High for TSDI
  - Low for TScarb
- **Carbon monoxide**
  - Reduction for absolute best cases
  - High for 4-stroke scooter
- **Hydrocarbons**
  - Reduction for absolute best cases
  - High for 4-stroke scooter

# Scooter Toxicity – Biological Endpoints

- No effects on cytotoxicity
- TNF-α concentration reduced in TSDI absolute best case compared to 2-stroke direct injection worst case
- Concentration of nitrogen oxides negatively correlates with the TNF-α and IL-8
- Main influence on toxic potential assigned to particle number 10-100nm

# Scooter Toxicity – Conclusion

- Big differences in physical parameters
- Small biological differences
- Reduction of toxicological potential due to technical optimizations
- Main influence on the toxic potential in lung cells *in vitro* for particle number between 10-100nm

# Exposure System – Conclusion

- Increase exposure duration: from 1h or 2h → 6h or 3x6h
- Reduce dilution: from 1:100 → 1:10
- Change driving cycle: from steady state driving → dynamic cycles
- Increase number of endpoints: from cytotoxicity → cytotoxicity
  TNF-α → cytokines
  IL-8 → gene expression
  → oxidative stress
  → DNA damage
  → stress receptors

> changes introduced for studies about car exhaust (diesel and gasoline)

# Acknowledgement

**Former Histology Group of Peter Gehr, University of Bern**
Peter Gehr
Barbara Rothen-Rutishauser
Barbara Tschirren
Sandro Steiner (following PhD student)
And many more!

**Pediatric Pulmonology UKBB/Kinderklinik Bern**
Philipp Latzin
Jakob Usemann
Michèle Roth
Selina Steiner
Andrea Zelmer

**BioNanomaterials Adolphe Merkle Institute University Fribourg**
Barbara Rothen-Rutishauser
Christoph Bisig

**Laboratory for IC-Engines & Exhaust Emission Control Bern University of Applied Sciences**
Jan Czerwinski
Pierre Comte
Philipp Willi

Peter und Traudl Engelhorn Stiftung zur Förderung der Lebenswissenschaften
EV|UP Erdöl-Vereinigung Union Pétrolière
bernische krebsliga ligue bernoise contre le cancer
Schweizerische Eidgenossenschaft Confédération suisse Confederazione Svizzera Confederaziun svizra
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# Questions?


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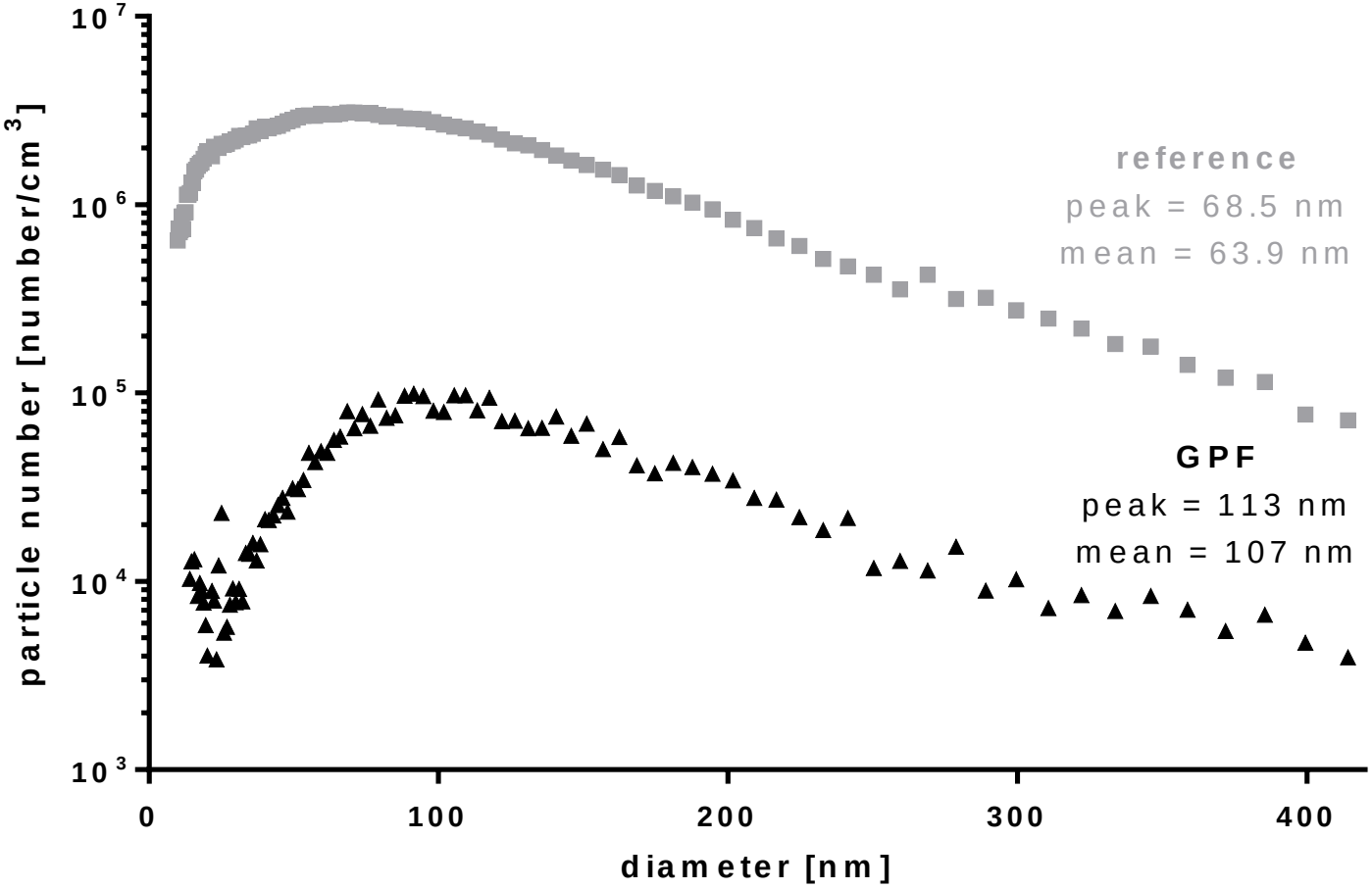
# GPF – Exhaust (2)



# Scooter Toxicity – Concept of «toxic potential»

**Summary of biological analysis:**

- **significant difference between control & exposure: 2+**
- **tendency: 1+**

| | TDSI | | | | carburetor | | | | 4-stroke | diesel car | |
|---|---|---|---|---|---|---|---|---|---|---|---|
| | worst case | best case | filtered | absolute best | worst case | best case | filtered | absolute best | | particle filter | without particle filter |
| cytotoxicity (LDH) | 1+ | 2+ | 1+ | 0 | 4+ | 2+ | 0 | 0 | 3+ | 2+ | 0 |
| pro-inflammatory response (TNFa) | 4+ | 4+ | 1+ | 1+ | 6+ | 4+ | 4+ | 1+ | 2+ | 1+ | 1+ |
| inflammatory response (IL-8) | 3+ | 4+ | 1+ | 4+ | 3+ | 4+ | 2+ | 2+ | 2+ | 2+ | 4+ |
| **total biological** | **8+** | **10+** | **3+** | **5+** | **13+** | **10+** | **6+** | **3+** | **7+** | **5+** | **5+** |