# Changes of protein expression, gene methylation and DNA-damaging properties by CP-47,498-C8 a cannabinoid mimetic compound

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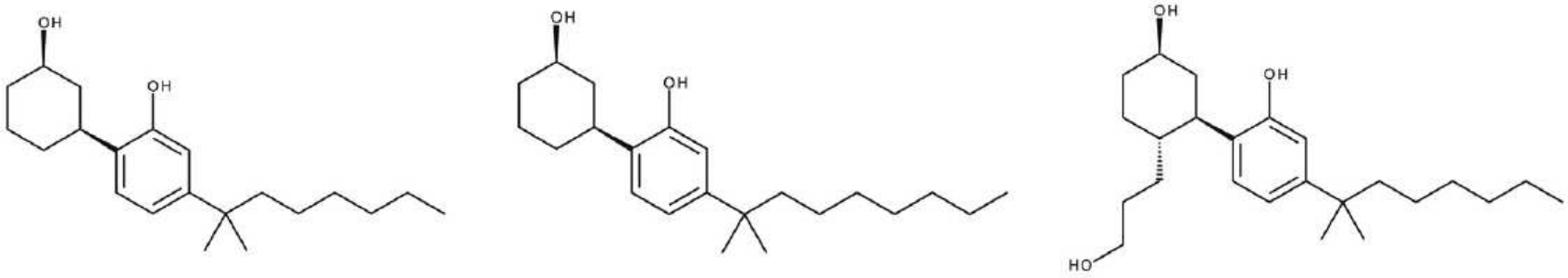
**EU-Project: SPICE II Plus**

# Topics

- Background and aims
- Proteomics
- Induction of DNA damage
- Changes of DNA methylation patterns
- Summary and conclusions

# Background

**Several cannabinoid-mimetic compounds were developed by Pfitzer. These drugs have a cyclohexyl-phenolic ring and are structurally more similar to THC as other synthetic cannabinoids with regard to the alkyl side chain attached to the central phenol moiety.**




CP 47,497 (C7) CP 47,497 (C8) CP 55,940

**Structure 2.** Cyclohexylphenols — Structures of CP 47,497 (C7), CP 47,497 (C8), and CP 55,940.

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**Presley et al. 2013, Analysis of Synthetic Cannabinoids in Botanical Material: A Review of Analytical Methods and Findings**

## So far, seven compounds of this group were found in herbal mixtures.

| | | |
|---|---|---|
| CP 47,497 (C6) | $C_{20}H_{32}O_2$ | 304.24023 |
| CP 47,497 | $C_{21}H_{34}O_2$ | 318.25588 |
| CP 47,497 (C8) | $C_{22}H_{36}O_2$ | 332.27153 |
| CP 47,497 (C8) *trans*-diastereomer | $C_{22}H_{36}O_2$ | 332.27153 |
| CP 47,497 (C8) + C2 variant | $C_{24}H_{40}O_2$ | 360.30283 |
| CP 47,497 (C9) | $C_{23}H_{38}O_2$ | 346.28718 |
| CP 55,940 | $C_{24}H_{40}O_3$ | 376.89775 |

**Presley et al. 2013, Analysis of Synthetic Cannabinoids in Botanical Material: A Review of Analytical Methods and Findings**

**We investigated the toxicological properties of a number of compounds in a panel of in vitro test systems with human derived cells. With lymphocytes, buccal and hepatic cell lines highly interesting results were obtained, i.e. the drug induced pronounced genotoxic effects (DNA migration) in human derived cell lines.**

Toxicology and Applied Pharmacology 277 (2014) 164–171

Contents lists available at ScienceDirect

Toxicology and Applied Pharmacology

journal homepage: www.elsevier.com/locate/ytaap

# Investigation of the in vitro toxicological properties of the synthetic cannabimimetic drug CP-47,497-C8

Verena J. Koller [a], Volker Auwärter [b], Tamara Grummt [c], Bjoern Moosmann [b], Miroslav Mišík [a], Siegfried Knasmüller [a,*]

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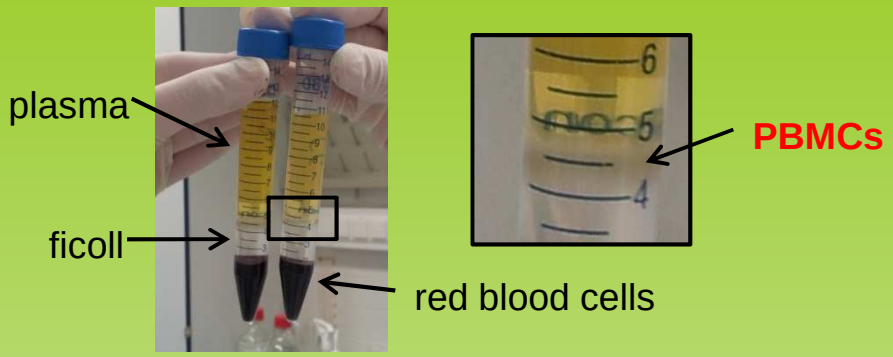
# Part I
# Results of proteome analysis

**Aim of the present study was a more detailled investigation of the molecular biological effects of the drug in human cells. Therefore we analyzed the impact of exposure of fresh peripheral lymphocytes to the drug on protein profiles in human lymphocytes.**

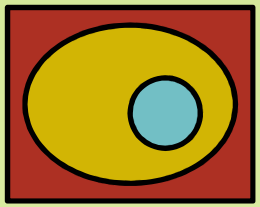
# Methods (I) – Sample Preparation

1. PBMC isolation from full blood via Ficoll Paque




2. Cultivation of PBMCs in serum-free culture medium and treatment with CP 47.497
    1. control: 3h cultivation
    2. + CP 47.497: addition of CP 47.497 (10µM) for 3h

3. Cell fractionation
    1. supernatant
    2. cytoplasm
    3. nuclear extract



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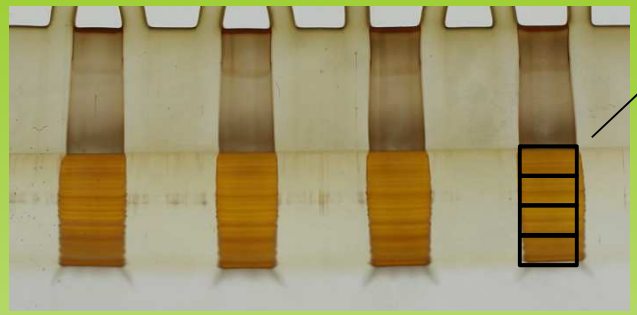
# Methods (II) – LC-MS Analysis

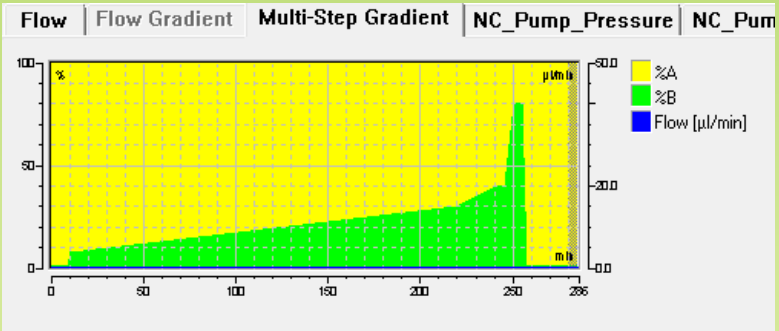


fractionation according to molecular weight

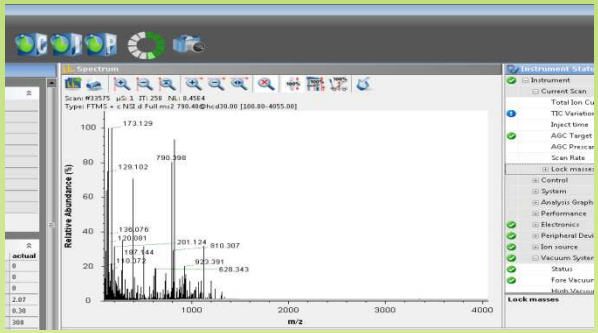
→ tryptic digest

→ 1 fraction/4 injections for MS/MS analysis

→ LC (UltiMate™ 3000 RSLCnano) – MS/MS (QExactive Orbitrap)



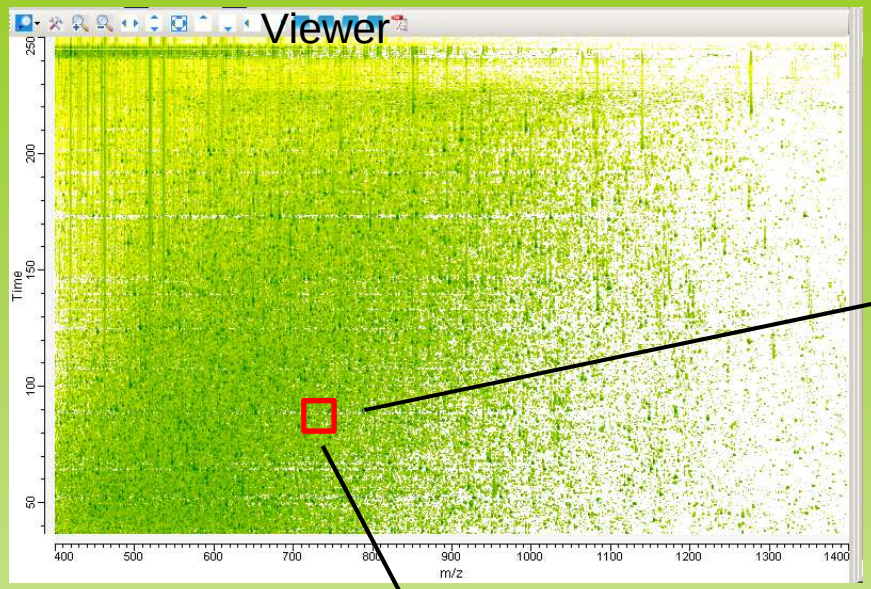

LC-Method: 280min
Gradient: 235min from 8-40% Solvent B
Solvent A: 98% $H_2O$, 2% ACN, 0,1% FA
Solvent B: 80% ACN, 20% $H_2O$, 0,1% FA
Separation Column: Dionex, Acclaim PepMap RSLC, C18, 75µm x 50cm

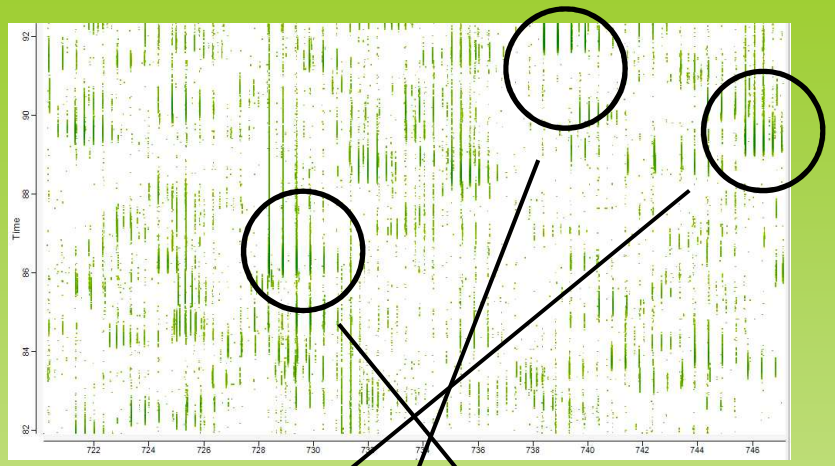


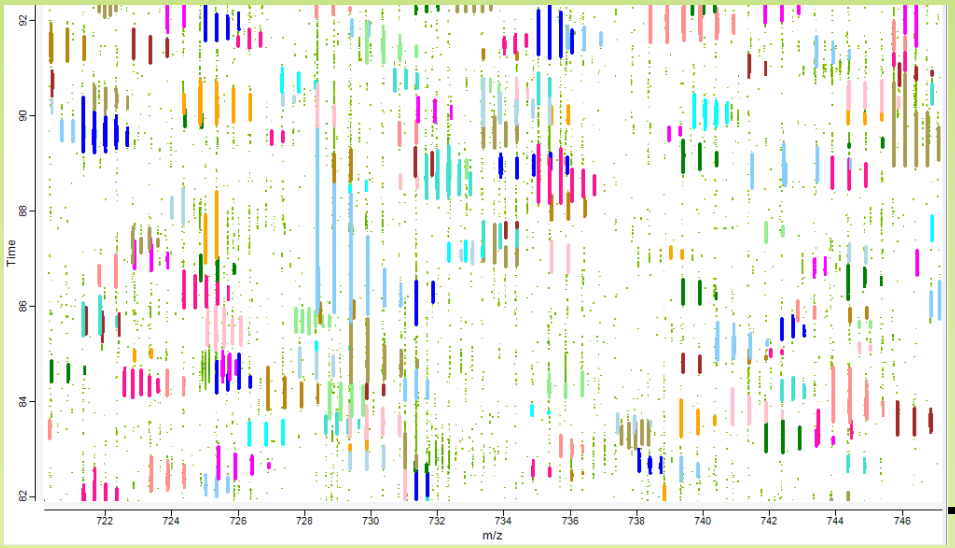
MS1: Resolution: 70.000;
max IT: 120ms;
scan-range: 400-1400m/z
MS2: Resolution: 17.500;
max IT: 250ms;
Top12

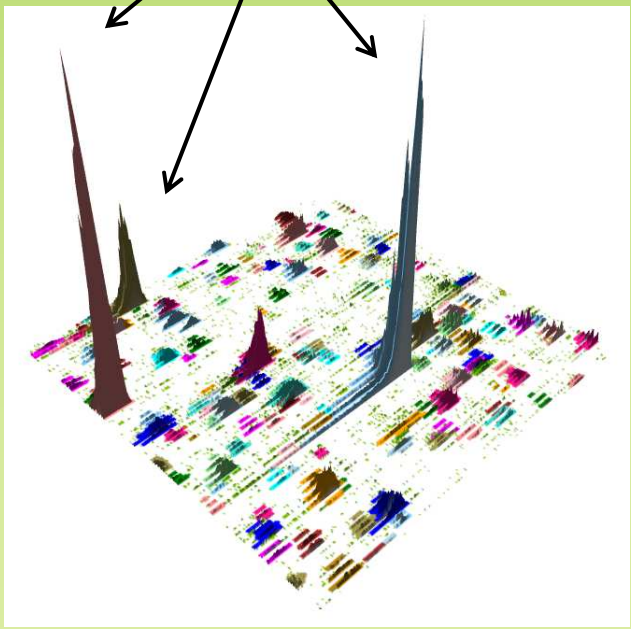
# Methods (III) – Data Analysis

MaxQuant - Viewer



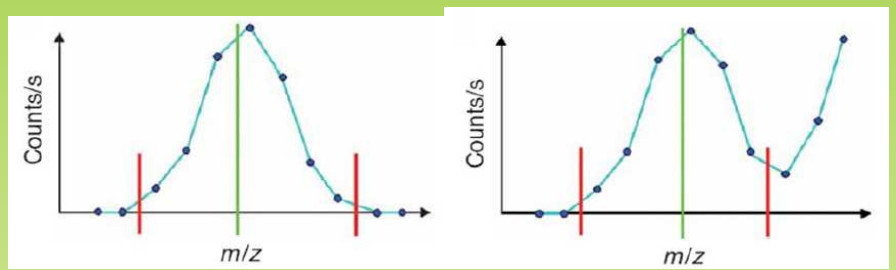



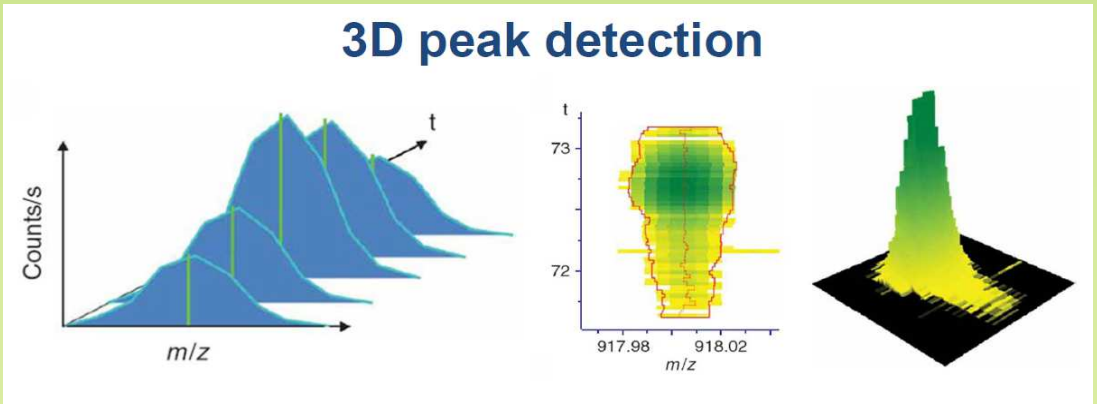



# Methods (III) – Data Analysis
# Peak detection – Peptide quantification

- First 2D peak detection in each MS scan
- Local maxima
- Boundaries: 0 intensity or local minima
- Centroid position: Gaussian fit based on five central points
- Centroid intensity: summed intensities over 2D peak

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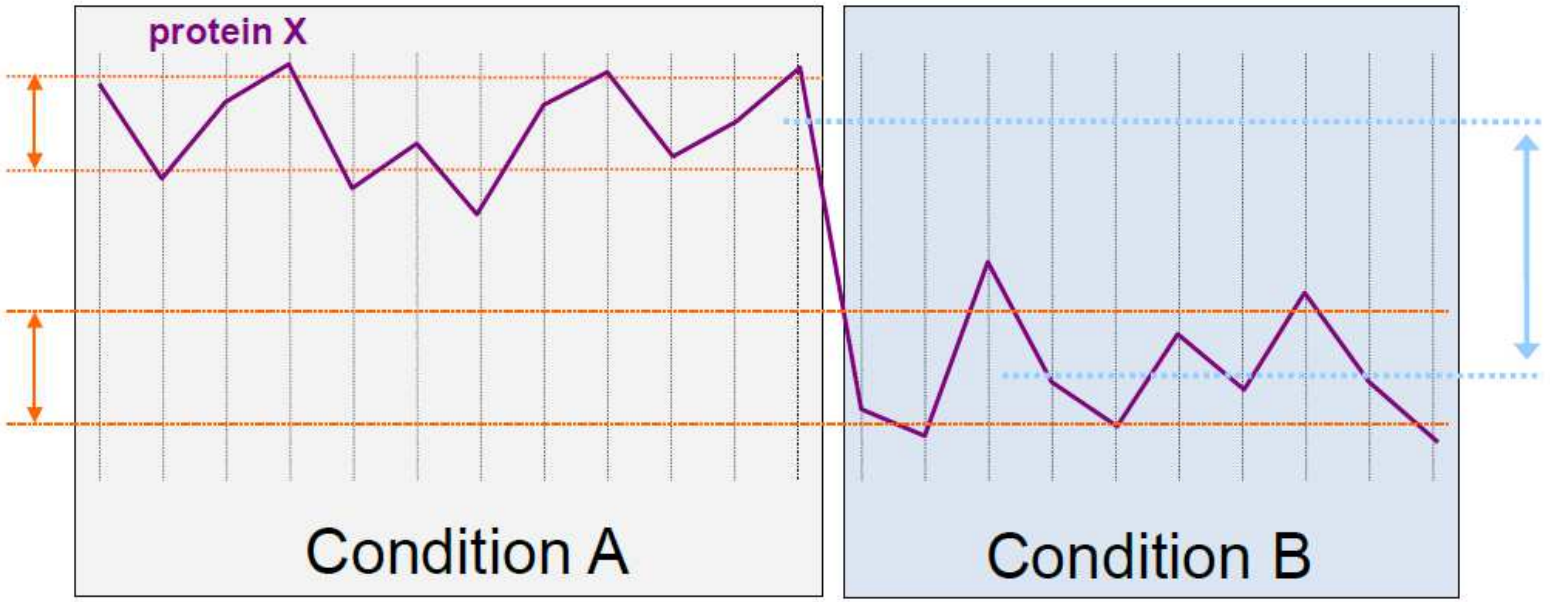
# Methods (III) – Data Analysis

## T test

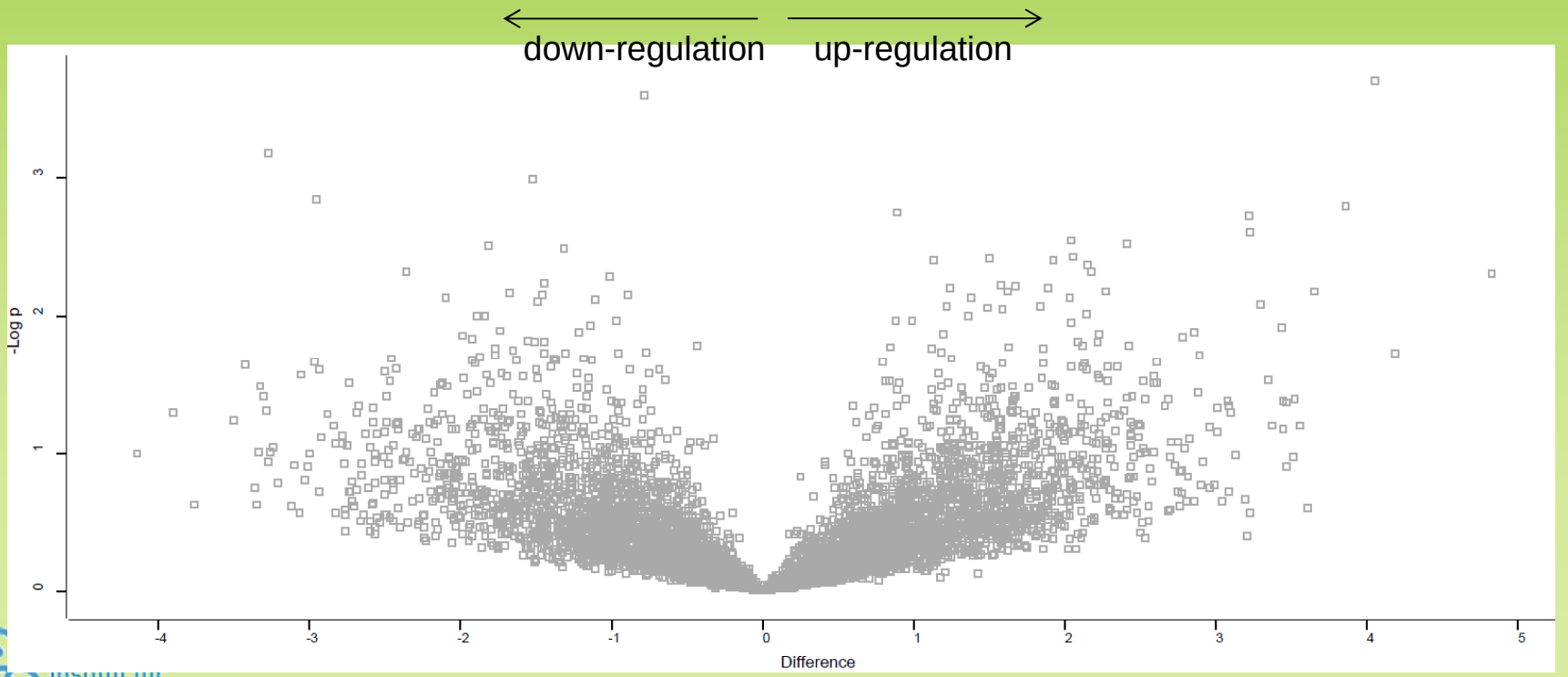



- Solution: Consider not only variance between groups (difference of means) but also variance within groups
- Test statistic:

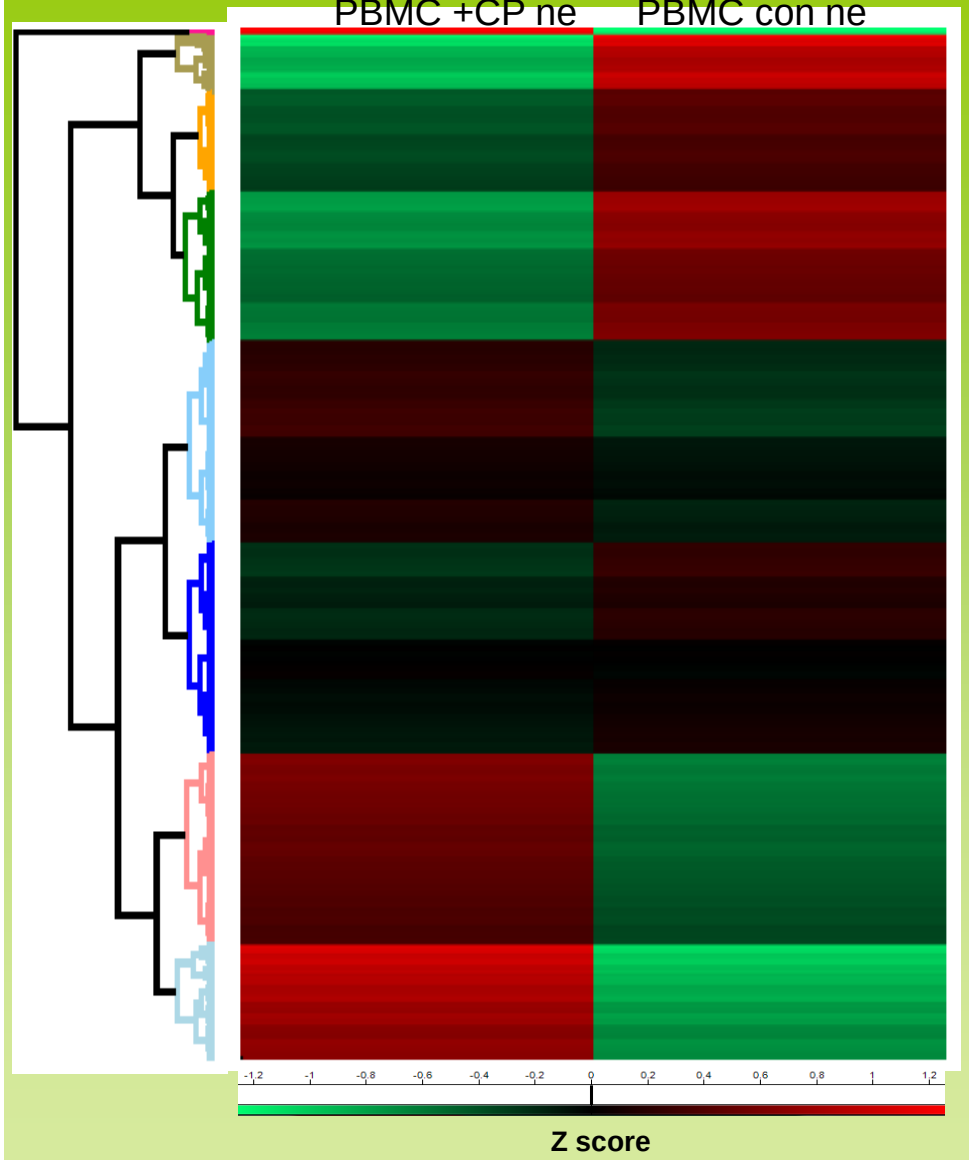
$$t^2 = \frac{\text{variance between groups}}{\text{variance within groups}}$$

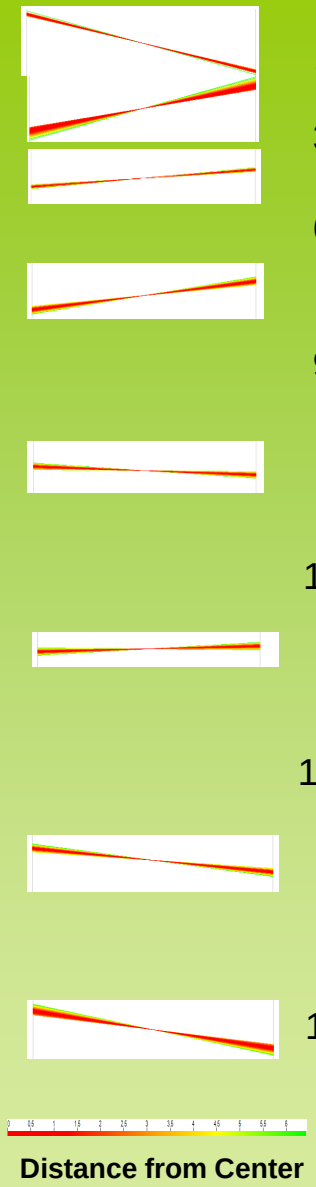
# Results – Data Evaluation by Vulcano Plot

- In total 6883 proteins identified in control and CP-treated PBMCs (non-redundant, without isoforms)
- FDR < 0.01 for peptides and proteins, only swissprot entries
- A minimum of 2 peptides identified per protein




Difference in $2^n$




38 proteins

361 proteins (Cluster 2)

684 proteins

993 proteins

1345 proteins

1409 proteins

1290 proteins

763 proteins (Cluster 1)

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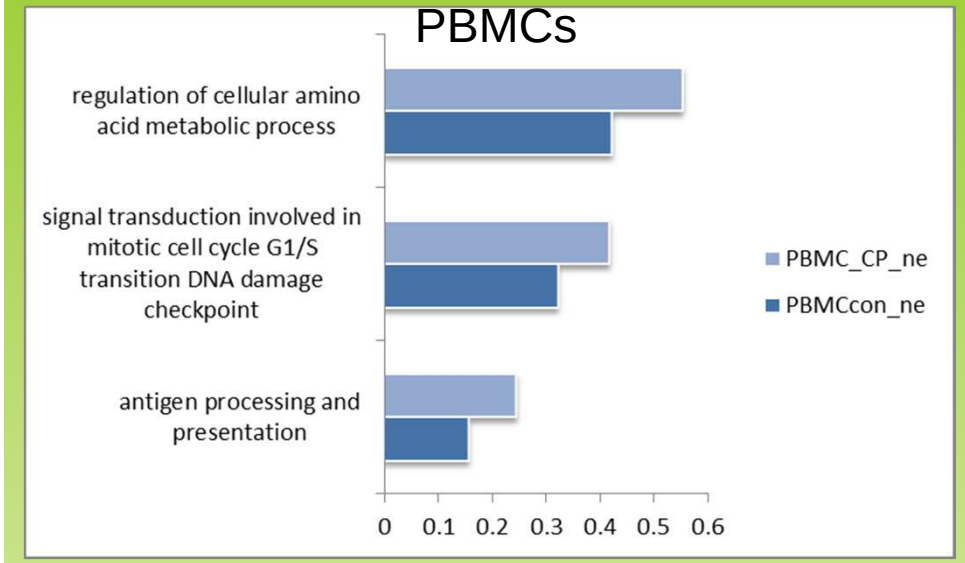
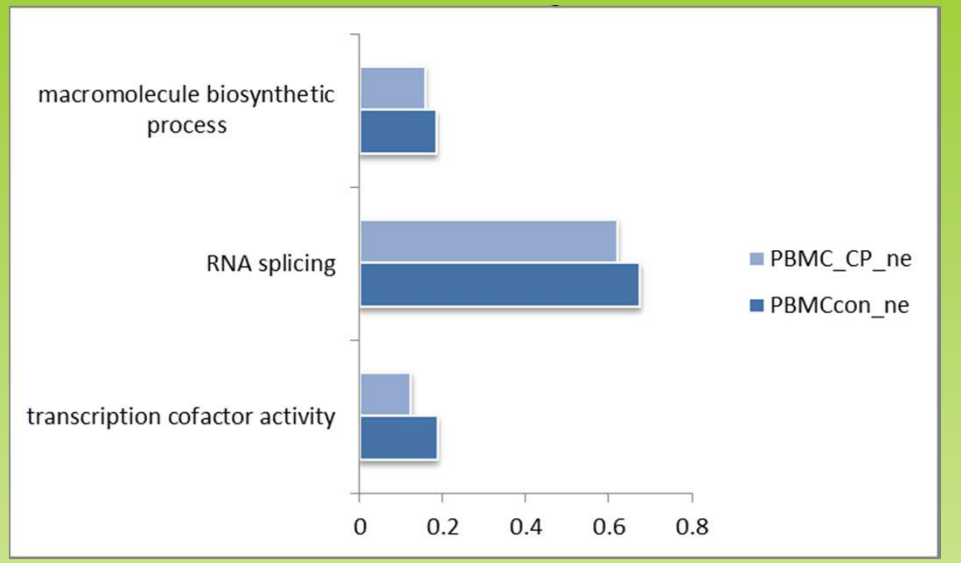
**In total: 6883 protein idendifications**

# Results – Cluster Analysis

Cluster 1 – up-regulation in CP-treated PBMCs




Cluster 2 – down-regulation in CP-treated

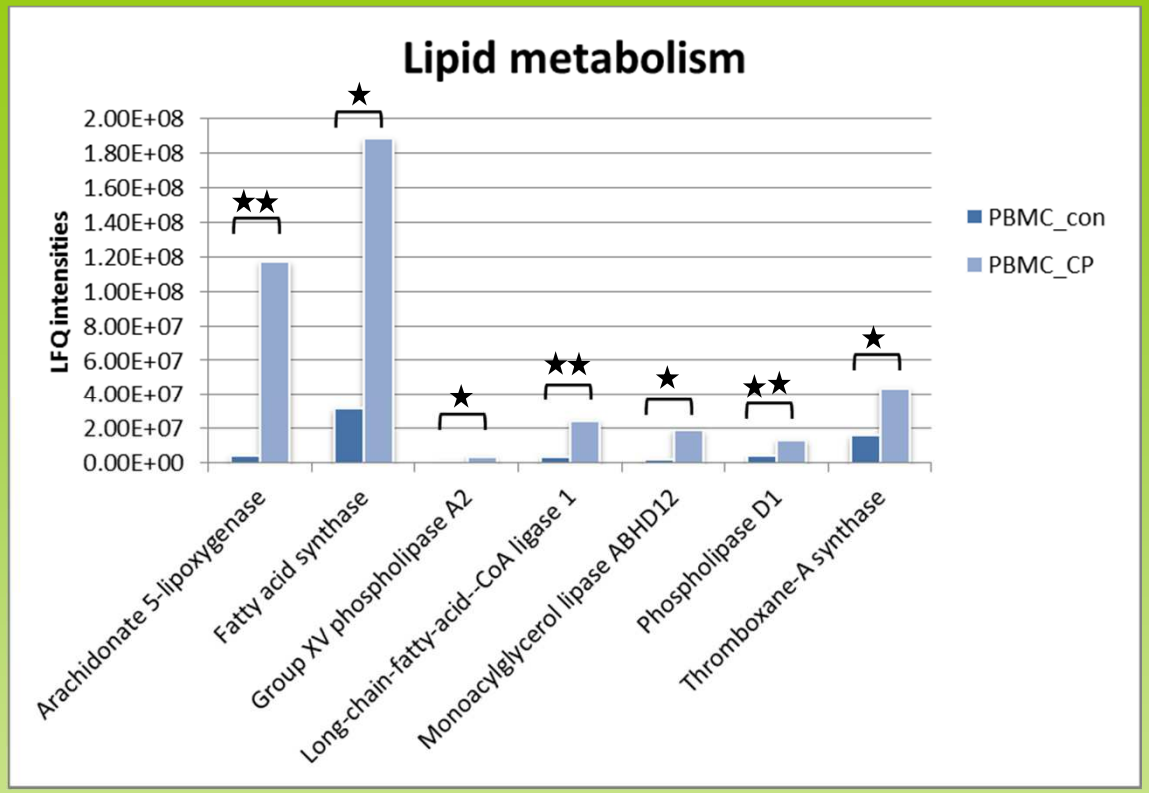



## Cluster 1

| Type | Name | P value | Enrichment | Total | In cluster | Cluster size | Ben. Ho. FDR |
|---|---|---|---|---|---|---|---|
| GOBP name | **antigen processing and presentation** | 3.22E-12 | 3.1612 | 117 | 41 | 763 | 3.68E-09 |
| GOBP name | **regulation of cellular amino acid metabolic process** | 3.30E-10 | 4.5105 | 42 | 21 | 763 | 1.88E-07 |
| GOBP name | **signal transduction involved in mitotic cell cycle G1/S transition DNA damage checkpoint** | 6.40E-09 | 3.8166 | 52 | 22 | 763 | 1.63E-06 |

## Cluster 2

| Type | Name | P value | Enrichment | Total | In cluster | Cluster size | Ben. Ho. FDR |
|---|---|---|---|---|---|---|---|
| GOMF name | **transcription cofactor activity** | 1.76E-06 | 2.4978 | 229 | 30 | 361 | 0.00074544 |
| GOBP name | **RNA splicing** | 5.80E-08 | 1.9525 | 213 | 60 | 993 | 9.95E-05 |
| GOBP name | **macromolecule metabolic process** | 2.07E-06 | 1.1674 | 2565 | 432 | 993 | 0.0015754 |




★ t-test significance: p<0.05

★★ t-test significance: p<0.01

- These proteins are important regulators of inflammatory processes
- Monoacylglycerol lipase ABHD12 → regulator of endocannabinoid signaling pathways (!)

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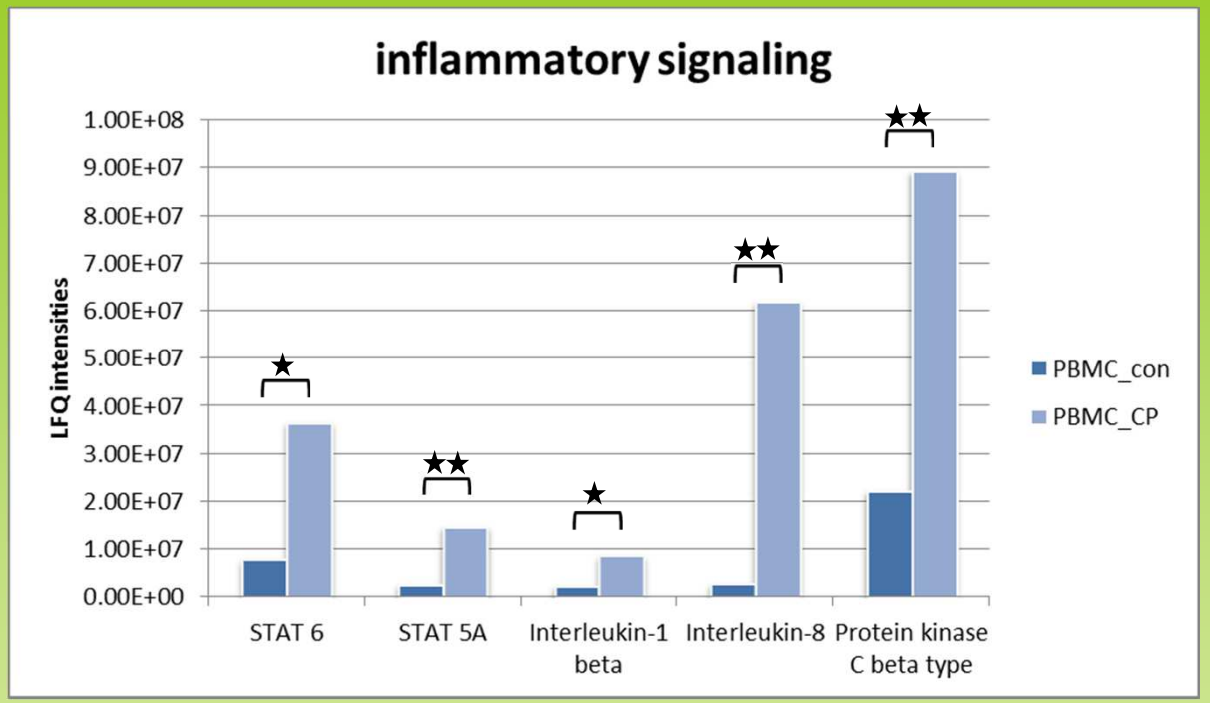


★ t-test significance: p<0.05

★★ t-test significance: p<0.01

- Confirming significant effects of CP 47.497 on inflammation

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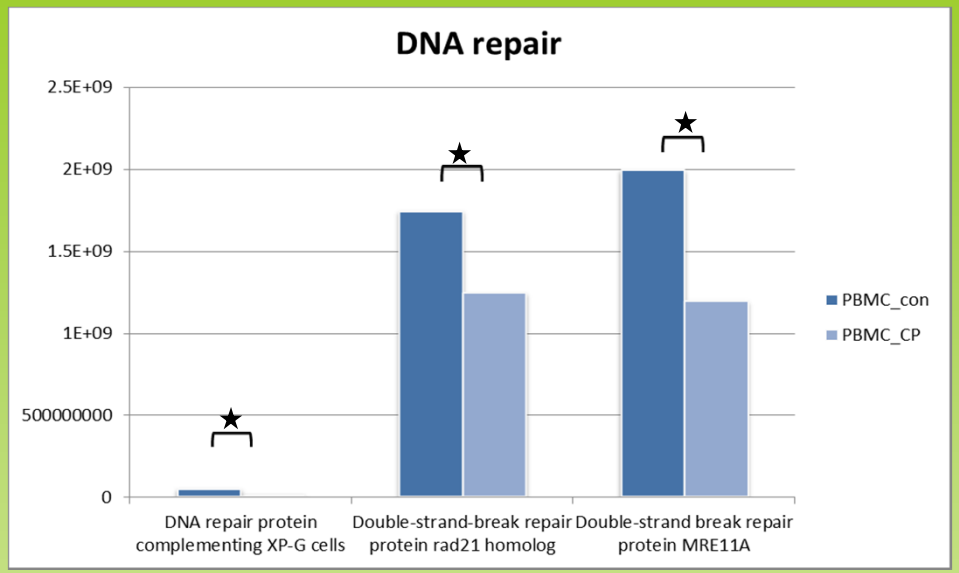
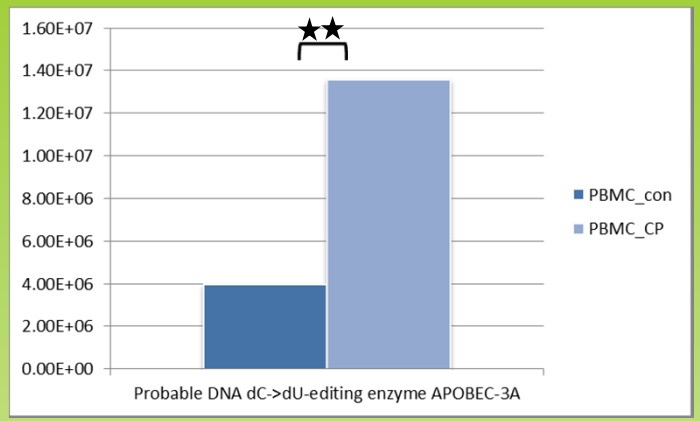
# Results - DNA integrity and DNA-methylation




★ t-test significance: p<0.05

★★ t-test significance: p<0.01

Example:

- Apparent down-regulation of the cell's machinery for DNA repair
- Probable DNA dC->dU-editing enzyme APOBEC-3A → epigenetic regulation of gene expression through the process of active DNA demethylation

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# Summary: Results of the proteome analysis

**The results indicate that**

**(i) CP causes alterations of proteins which are involved in inflammatory processes and lipid metabolism.**

**(ii) some proteins were altered which play a role in DNA repair processes.**

**(iii) finally an alteration was seen in a gene which plays a role in DNA methlyation.**

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# Part II

**The second series of experiments concerned the impact of the drug on DNA stability. Damage of the genetic material causes diseases (cancer, reduced life span, ect…) reduces the fertility and may lead to malformations in the offspring.**

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**Two test systems were used**

1) **Comet assays (single cell gel electrophoresis tests)**

2) **Micronucleus assays**

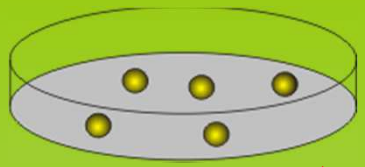
**Comet assays are based on the determination of DNA migration in an electric field. The size and intensity of the "COMETS" reflects the extent of damage. The test is increasingly used as a fast screening assay.**

**Disadvantage: comets disappear as a result of DNA repair processes, and it is not known if they have biological consequences.**

# Comet assay

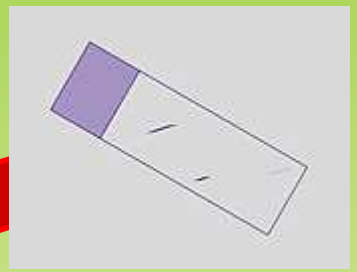
**CP-47,498-C8 + lymphocytes**

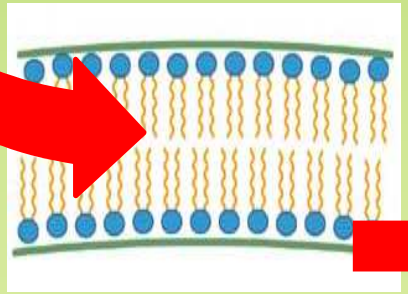
(buccal derived -TR146, liver derived - HepG2 or lymphocytes)



incubation

washing

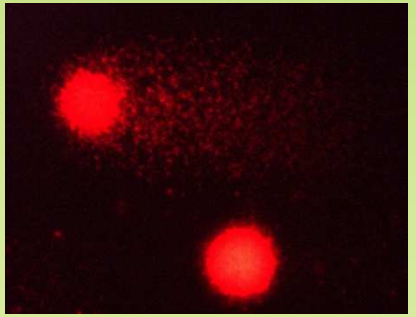




lysis

unwinding and electrophoresis

Analysis of the comets



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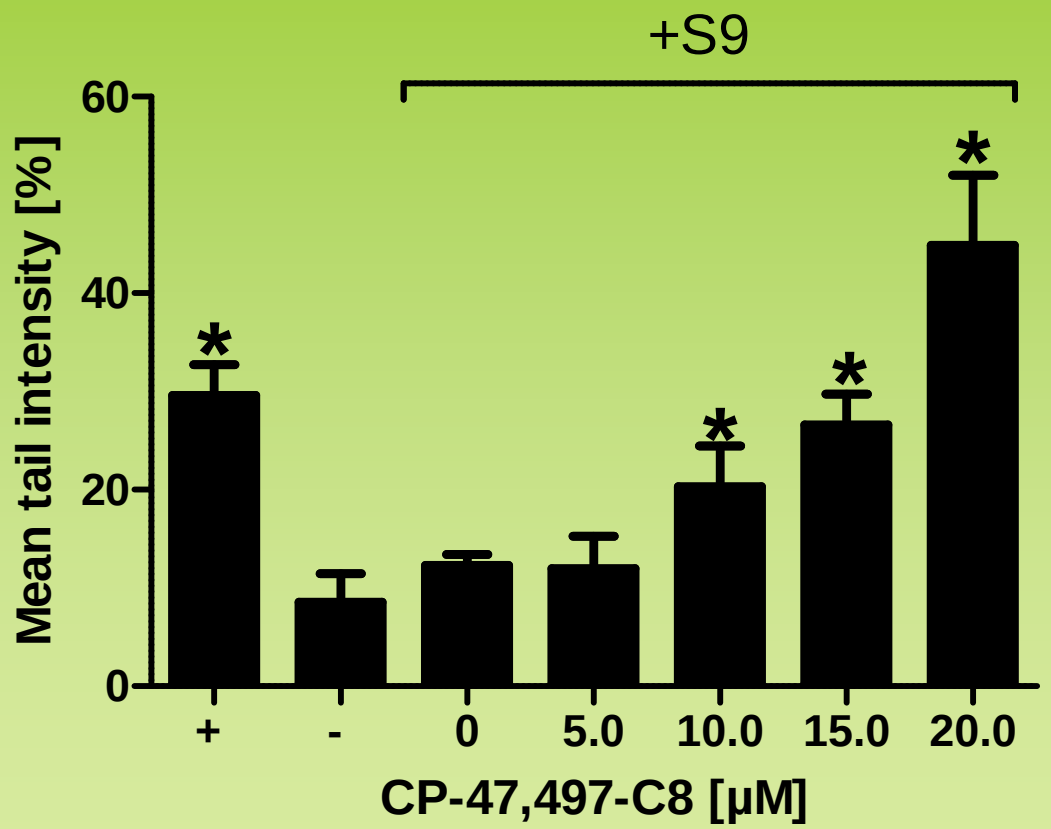
# Results with CP-47,497-C8

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# Do these changes lead to chromosomal damage which is directly associated with adverse health effects ?

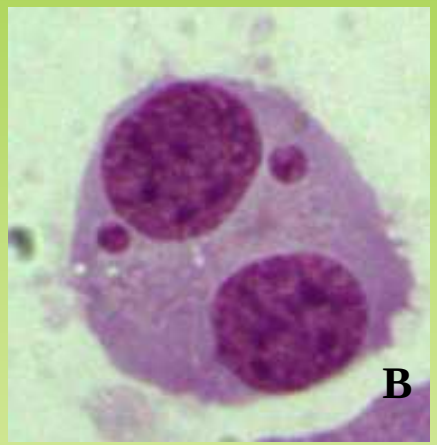
**To clarify this question micronucleus assays were conduced. MN which reflect structural and numerical chromosomal aberrations. It is known that increased MN rates in humans are a biomarker of increased cancer rates** (Bonassi et al. 2011, Mutagenesis 26, 93-100)

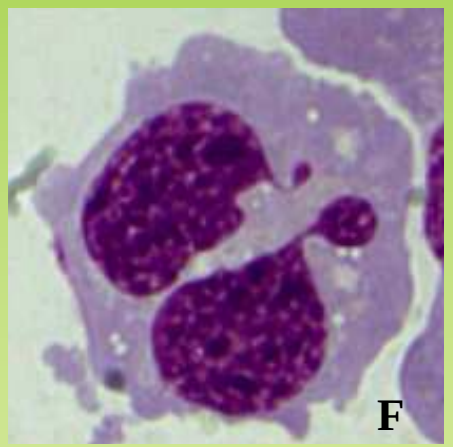
**Mutagenesis** vol. 26 no. 1 pp. 93–100, 2011 doi:10.1093/mutage/geq075

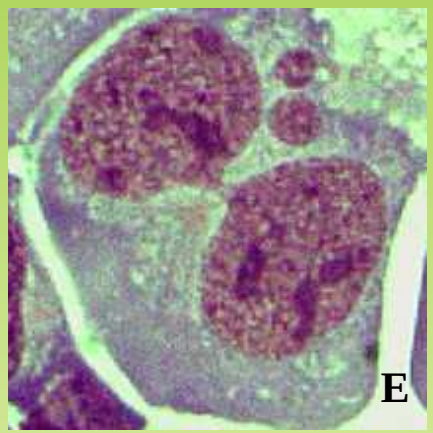
**REVIEW**

**Micronuclei frequency in peripheral blood lymphocytes and cancer risk: evidence from human studies**

**In addition to micronuclei also other nuclear aberrations were evaluated which are indicative for DNA damage.**




**B: Binucleated cells**

**F: Nuclear buds reflect gene amplification**

**E: Nuclear bridges are dicentric chromosoms**

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# Standard guidlines for MN Test with lymphocytes

PROTOCOL

## Cytokinesis-block micronucleus cytome assay

Michael Fenech

NATURE PROTOCOLS | VOL.2 NO.5 | 2007 | 1085

29 October 2012

**OECD GUIDELINE FOR THE TESTING OF CHEMICALS**

**Proposal for updating Test Guideline 487**

**In Vitro Mammalian Cell Micronucleus Test**

# Results of MN assay

**A significant increase of the MN and of other nuclear anomalies was induced by the drug.**

| Compound | NDI Mean [‰] ± SD | BN-MN Mean [‰] ± SD | MN Mean [‰] ± SD | Nbuds Mean [‰] ± SD | NPB Mean [‰] ± SD |
|---|---|---|---|---|---|
| Pos. Ctrl | **1,74 ± 0,06** | **48,36 ± 9,37** | **50,62 ± 10,10** | **12,44 ± 5,92** | **2,66 ± 1,17** |
| Neg. Ctrl | 2,04 ± 0,12 | 4,14 ± 0,52 | 4,26 ± 0,50 | 2,96 ± 1,56 | 1,67 ± 0,76 |
| 1.0 µM | 1,99 ± 0,14 | 4,40 ± 0,79 | 4,40 ± 0,79 | 3,91 ± 2,08 | 2,10 ± 1,67 |
| 2.5 µM | 2,02 ± 0,12 | 4,89 ± 1,01 | 4,89 ± 1,01 | 4,33 ± 1,61 | 2,05 ± 0,79 |
| 5.0 µM | 2,01 ± 0,14 | 5,26 ± 0,98 | 5,37 ± 1,20 | 2,57 ± 1,90 | 2,24 ± 2,79 |
| 7.5 µM | 2,01 ± 0,15 | **6,03 ± 1,28** ! | **6,03 ± 1,28** ! | 3,97 ± 1,45 | 2,45 ± 2,41 |
| 10.0 µM | 1,93 ± 0,19 | **6,37 ± 1,83** | **6,58 ± 2,03** | 4,48 ± 2,24 | 1,89 ± 0,96 |

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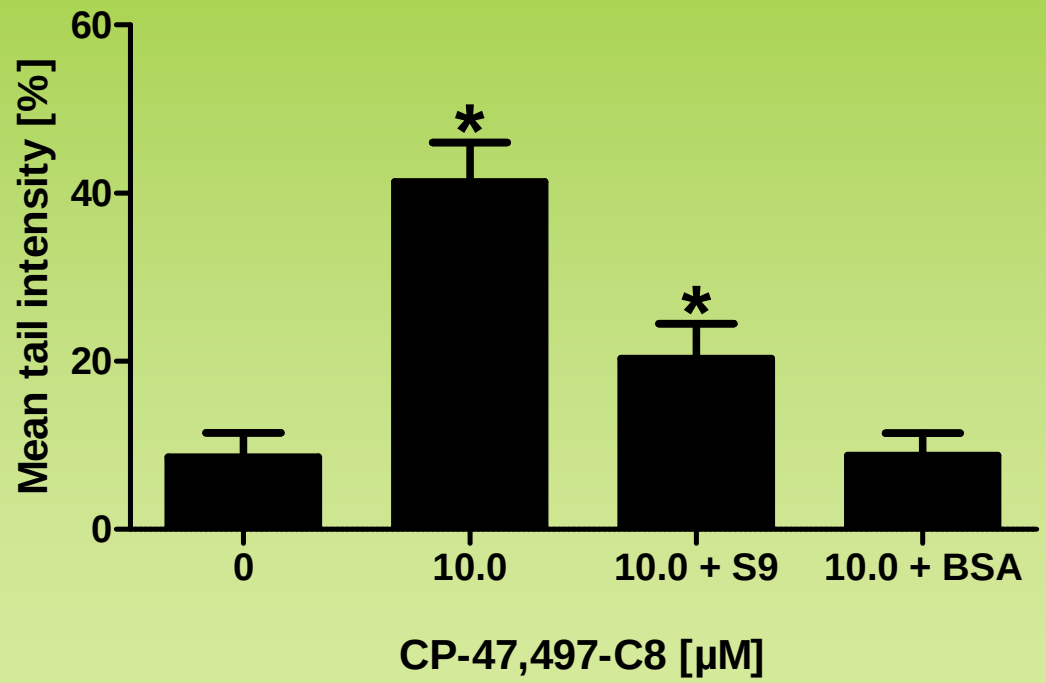
# What are the underlying mechanisms ?

**Many genotoxins are activated by phase I enzymes which are not active in human lymphocytes therefore we conducted an experiment in which a liver enzyme homogenate (S9) was added which activates procarcinogens. With this mix, a decrease of MN formation was seen, therefore we conclude that the compound is "directly acting" and does not require activation; this observation is in agreement with the observation in that the drug induces pro-inflammatory proteins (interleucin 9) which cause release of radicals.**

# What are the underlying mechanisms ?

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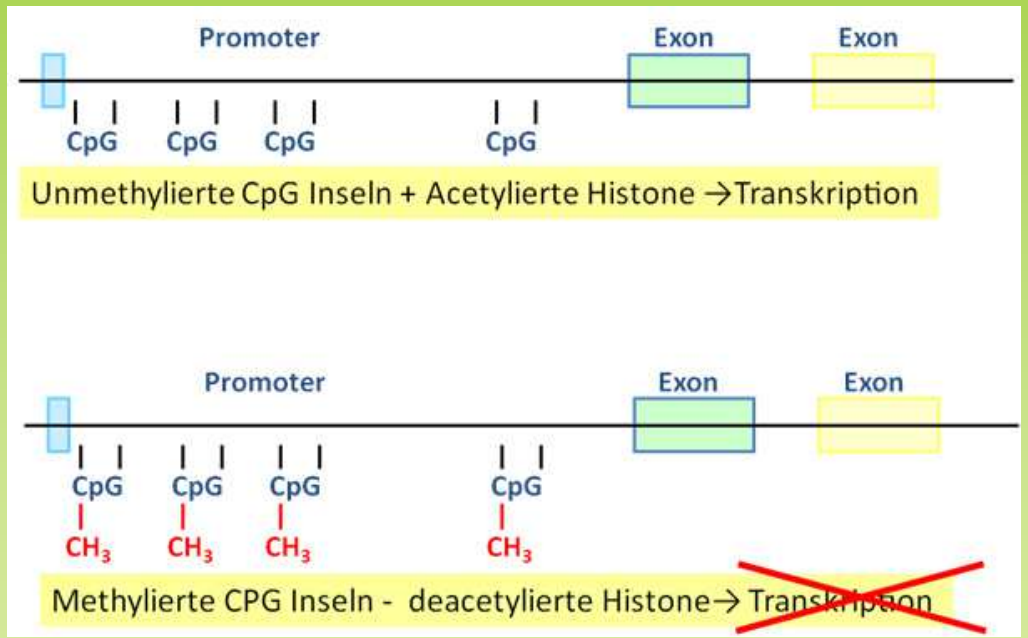
Hypermethylation_ less transcription
Hypomethylation mor tzranscription

# Part III
# Alterations of methylation patterns

**Methylation of genes causes changes in their transcription.**




**Hypermethylation ⟶ less transcription**

**Hypomethylation ⟶ more transcription**

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# Treatment

**Lymphocytes were treated for 3 and 6 hrs with the drug, subsequently the changes of the methylation pattern were analyzed.**

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# Methods

Bisulfite Conversion (EpiTect® Bisulfite Kit )

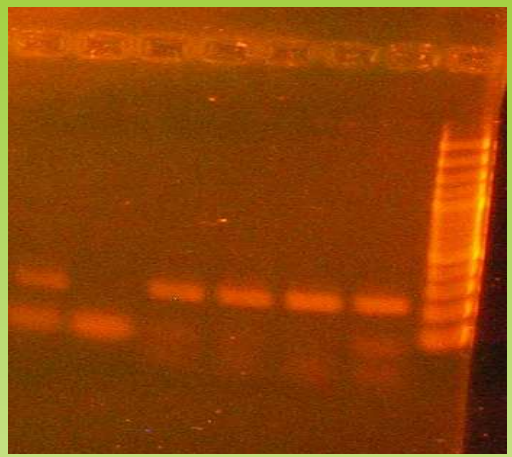
Picodrop (Picodrop Limited, Hinxton, UK)

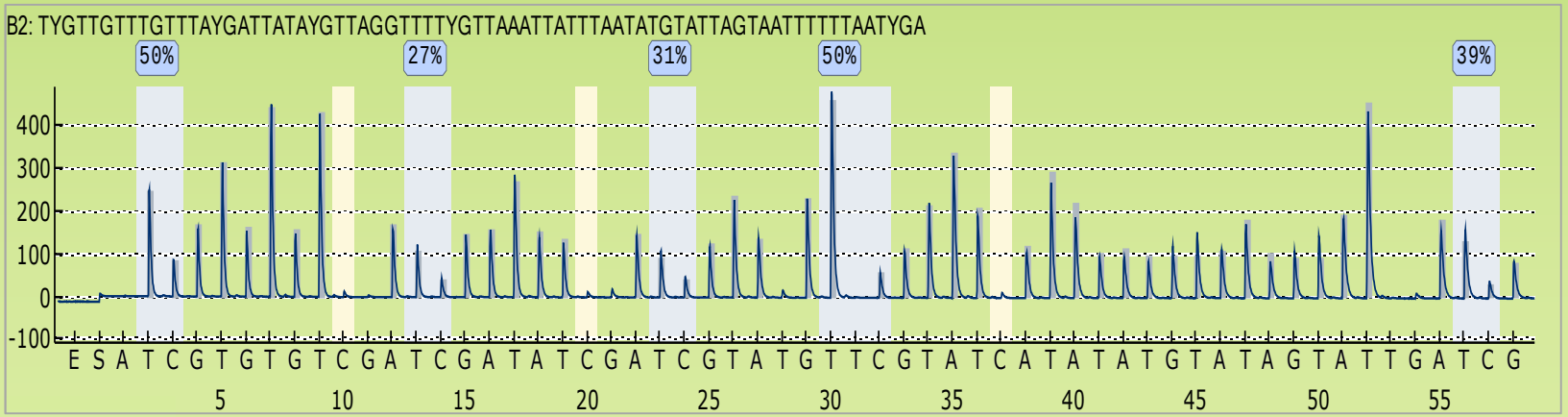
Endpoint PCR

Agarosegel (2%)

Pyrosequencing (Qiagen, Hilden, Germany)

Statistical analysis (IBM® SPSS® Version 20)






pyrogramm

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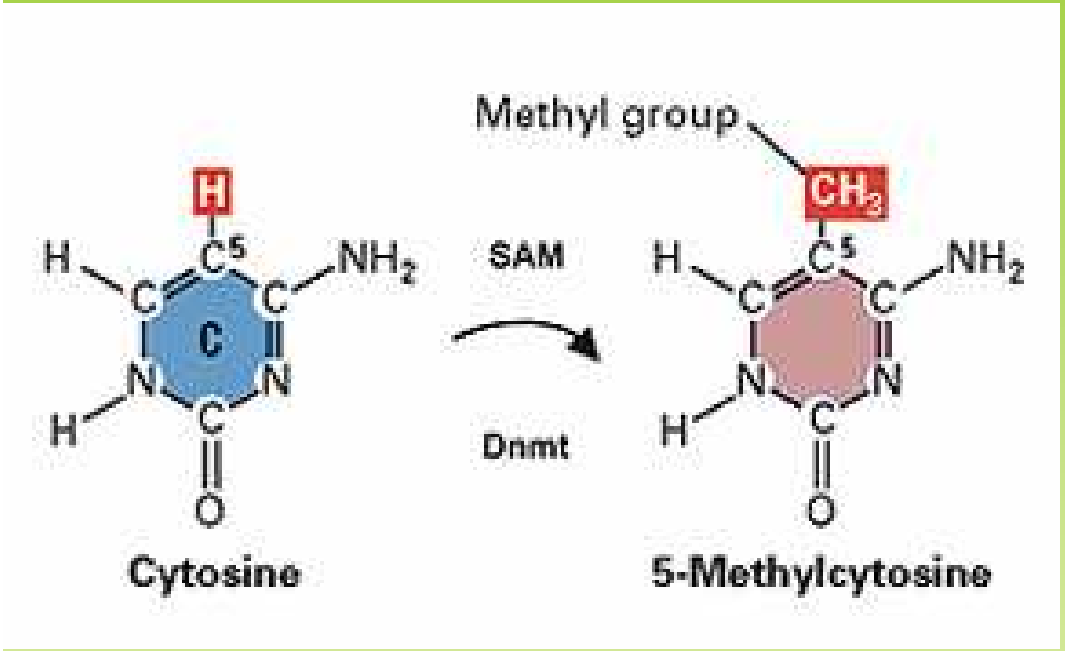
# DNA-Methylation




Hypermethylation = ↓Expression

In promoter region

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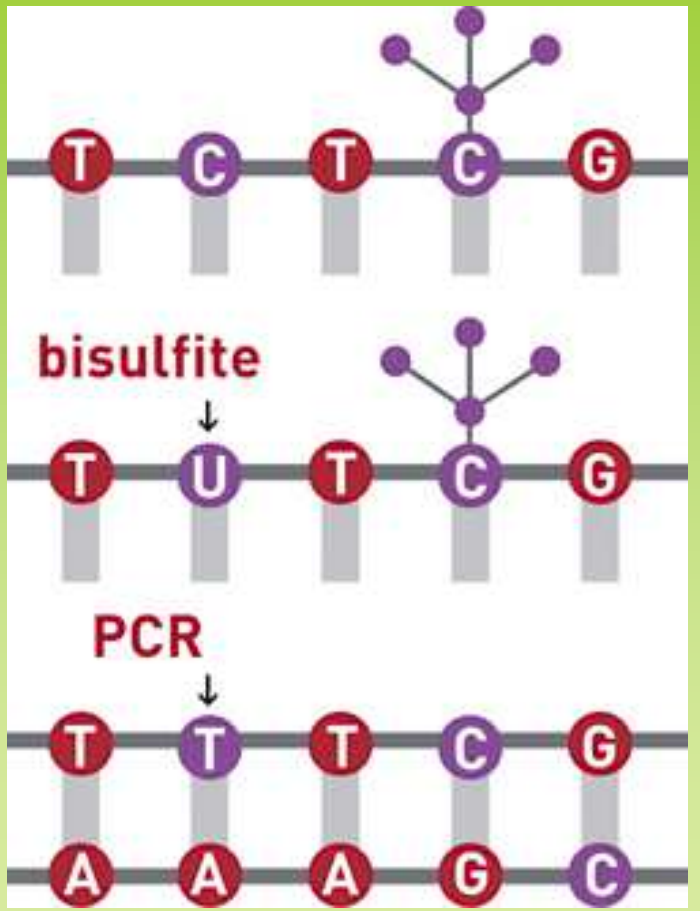
→unmethylated cytosine to uracil

→methylated cytosine remains unchanged

→detection by pyrosequencing

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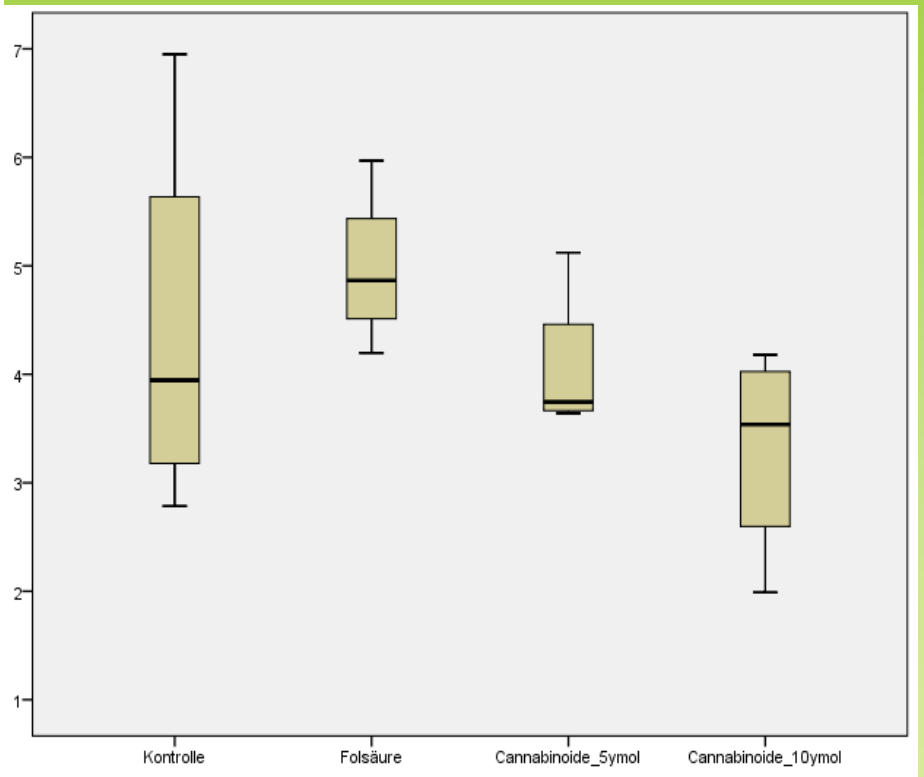
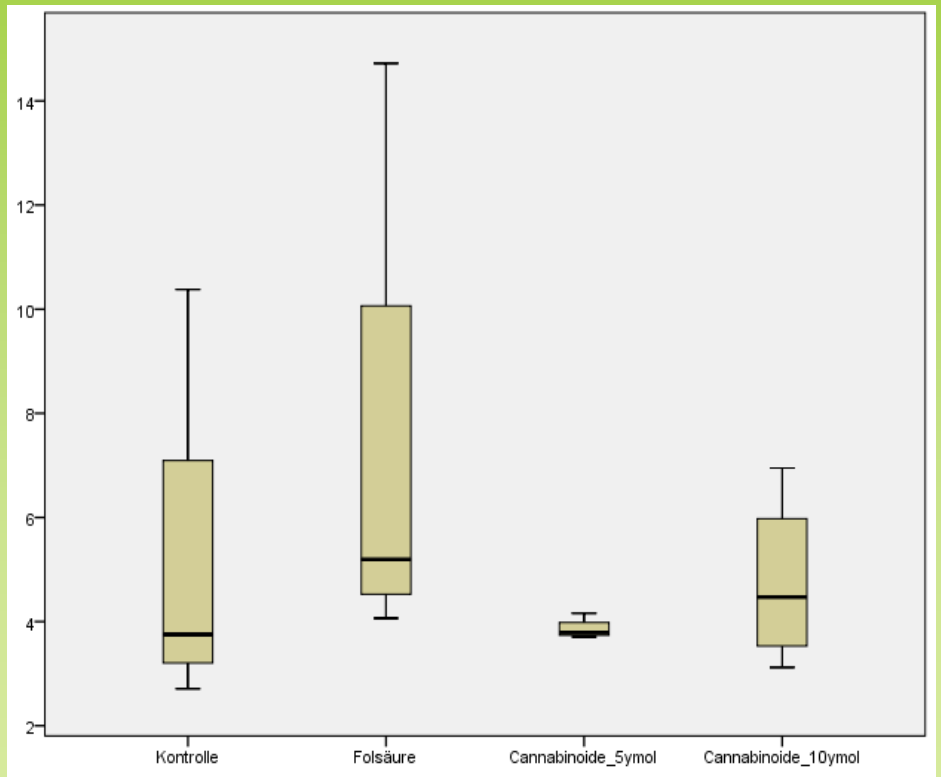
# Methylation of 2 CpGs in promotor region of Il-6 after incubation with cannabinoids (5, 10 µM). Folic acid was used as standard. lymphocytes from 4 volunteers




CpG 1




CpG 2

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# Conclusions

- **We identified a number of proteins which are up- and down-regulated by the drug, the most pronounced effects were seen in regard to genes involved in lipid metabolism and inflammatory signaling.**
- **Furthermore, changes of proteins were detected which play a role in DNA repair and DNA methylation.**
- **These alterations lead to DNA instability, we showed that CP-47,497-C8 causes COMET as well as micronucleus formation.**
- **Furthermore we found also some evidence for changes of gene methlyation as a first indication that the drug cause epigenetic alterations in human cells.**

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# Summary

**The consequences of these biological effects (which we detected in human cells) in drug users may be induction of inflammatory responses as well as induction of DNA instability (in particular in cells of the respiratory tract which come in direct contact with the drug). These effects play a key role in the etiology of cancer, therefore the findings are indicative for carcinogenic properties of the drug.**

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# Thank you for your attention

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