Does interdisciplinary work between toxicologists and statisticians improve the understanding of the comet assay?

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1. Introduction

Toxicologists

Statisticians

1. Introduction

GUM working group “Statistics”

GUM
Gesellschaft für Umwelt-Mutationsforschung e.V.

- **Headed by**: Dr. Christina Ziemann, Dr. Bernd-Wolfgang Igl
- **Founded**: 2016
- **Member**: 25 statisticians and toxicologists from academia, industry, regulatory body

INTEREST TO JOIN???
Feel free to contact us!

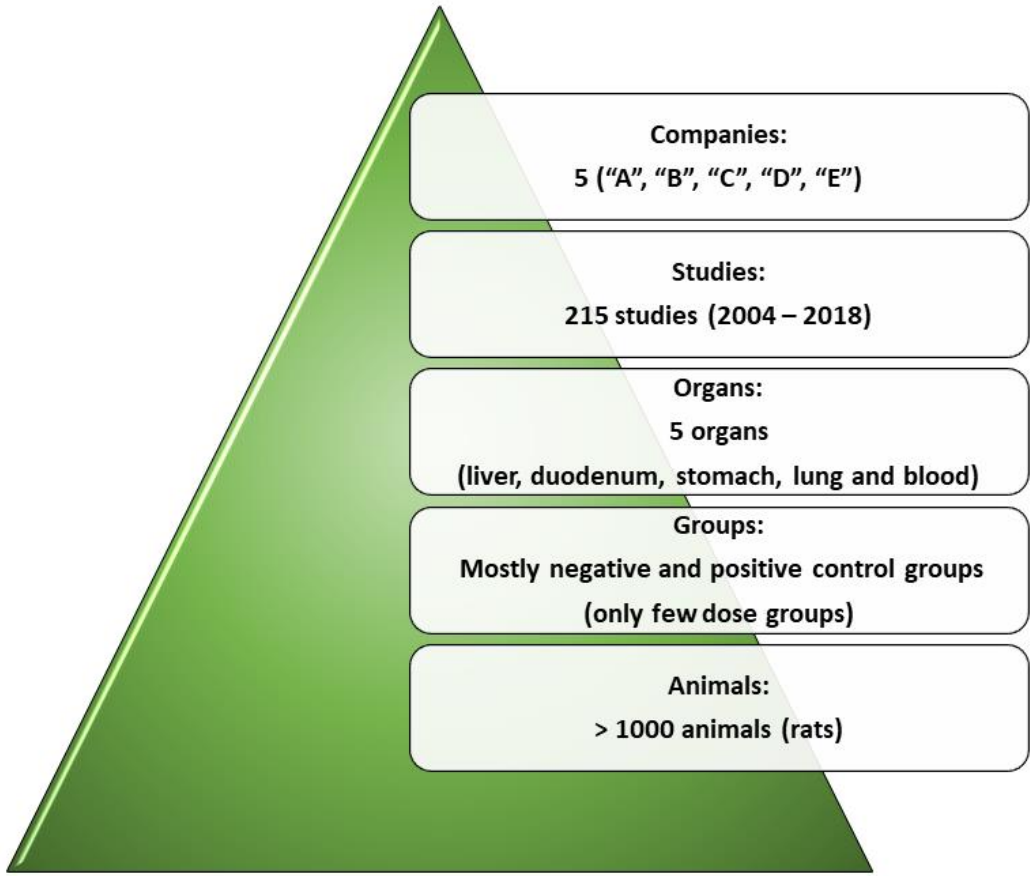
Aim: Providing a platform for an open and in-depth discussion of various statistical topics in Genetic Toxicology

Current Focus: In Vivo Mammalian Alkaline Comet Assay

1. Introduction

Previous work on a **small data set**: Dependence of the test result on the slide summary measure (Tug et al., 2020).

Now **large data set** available:

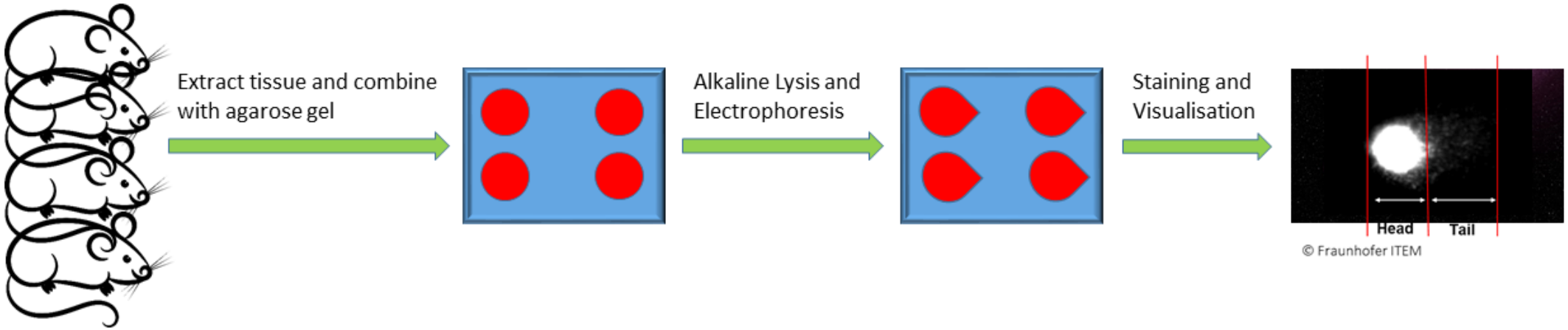


Meta-data collected by means of a questionnaire (e.g. species, exposure route, approx. 40 new variables).

1. Introduction

Current focus: ***In Vivo* Mammalian Alkaline Comet Assay** (Single Cell Gel Electrophoresis Assay)

- is a cheap, relatively easy to perform, fast and sensitive technique (traces back to the mid 1980s)
- becomes more and more popular as a standard method for testing DNA damage in mammalian tissues
- test principle:



  - DNA molecules are polar and, thus, DNA fragments migrate towards the anode during electrophoresis

- For damaged DNA, nucleic morphology resembles a “comet” with head and tail

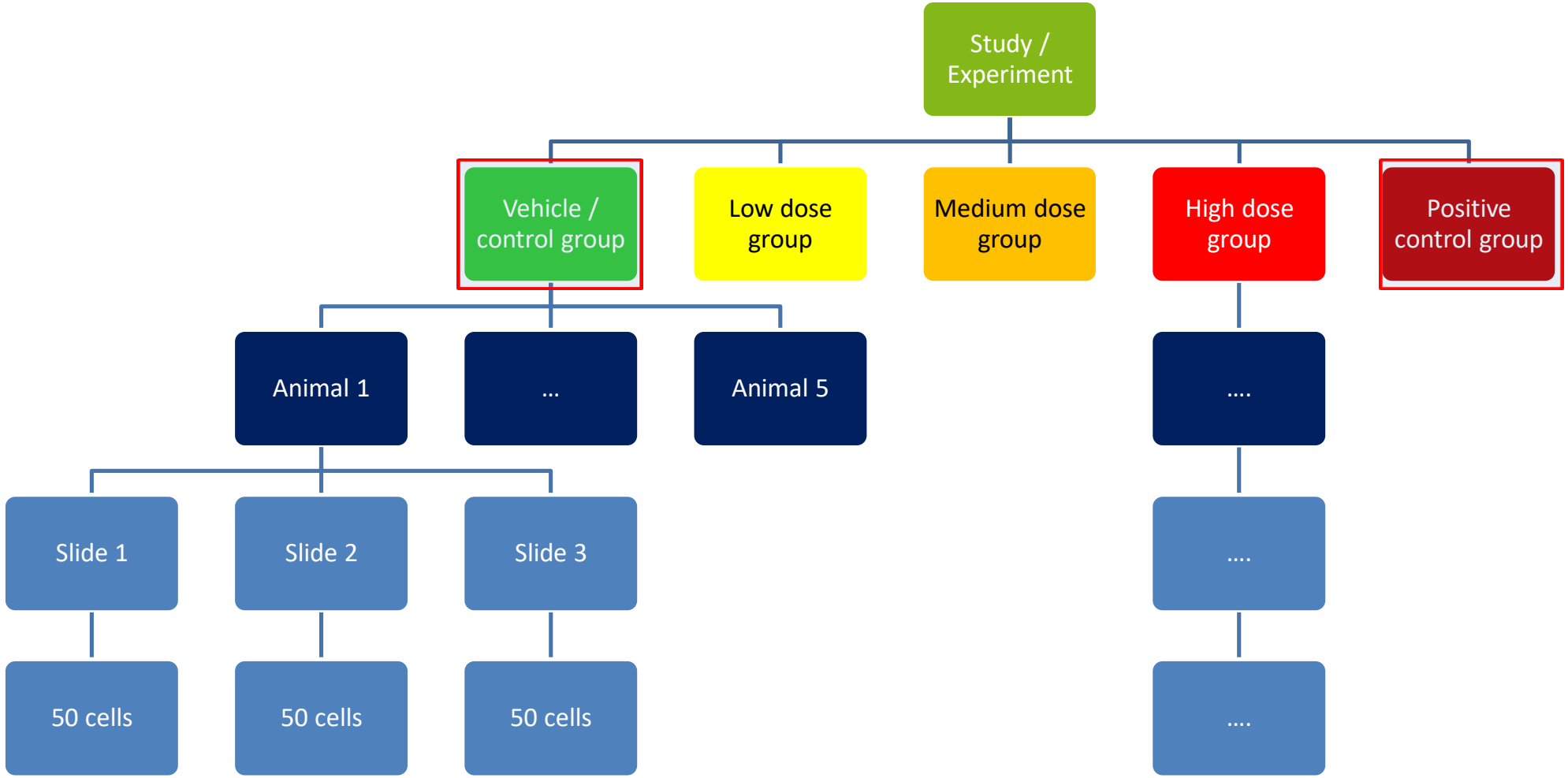
1. Introduction

Experimental design:

- 5 treatment group per experiment
- 5 animals per group
- 3 slides per animal
- 50 cells per slide

- Total:

150 cells per animal / 750 cells per group / 3750 cells per experiment



1. Introduction

Current focus: ***In Vivo* Mammalian Alkaline Comet Assay** (Single Cell Gel Electrophoresis Assay)

- At the end of gel electrophoresis, the shape of the comet is analyzed:

  primary parameter: **tail intensity**

- A new OECD guideline (TG 489) “In Vivo Mammalian Alkaline Comet Assay“ was adopted:
  29 July 2016

The corresponding OECD guideline 489 highlights the importance of statistical analyses and historical controls while no detailed procedures are given.

- Various publications have tried to make statistical statements on very small or simulated data (e.g. Wiklund & Agurell 2003, Bright et al. 2011).

1. Introduction

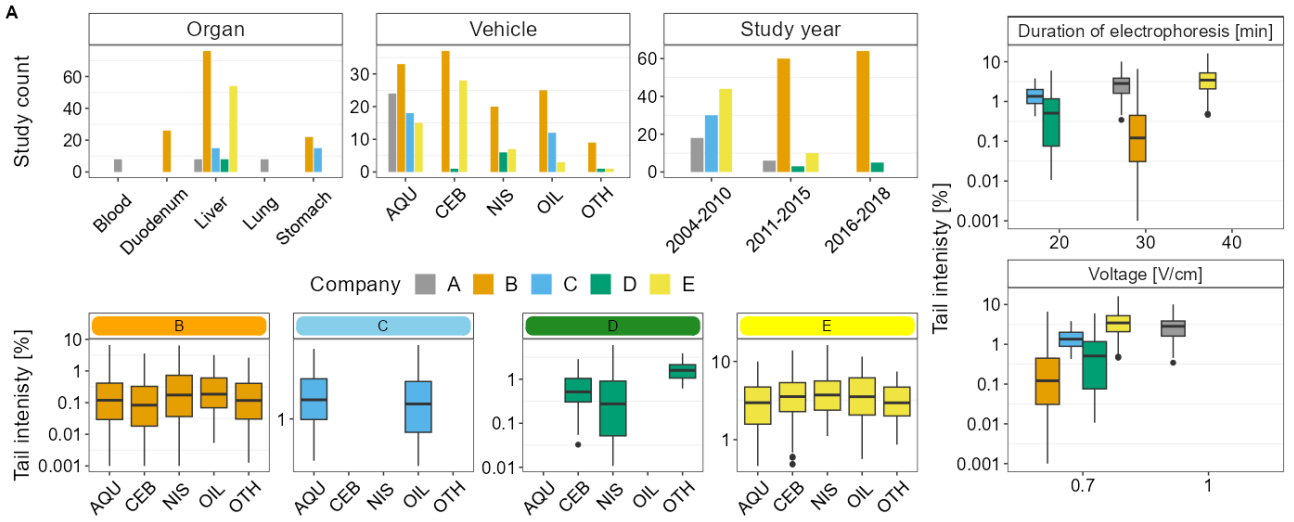
Pre-processing

- One species (rats)
- Selection of the most important and interesting variables (e.g. exposure route)
- **Problem**: Unique combinations from different variables between the companies

  ➔ Many confounded parameters

  ➔ Identification problem (fictive example)

A

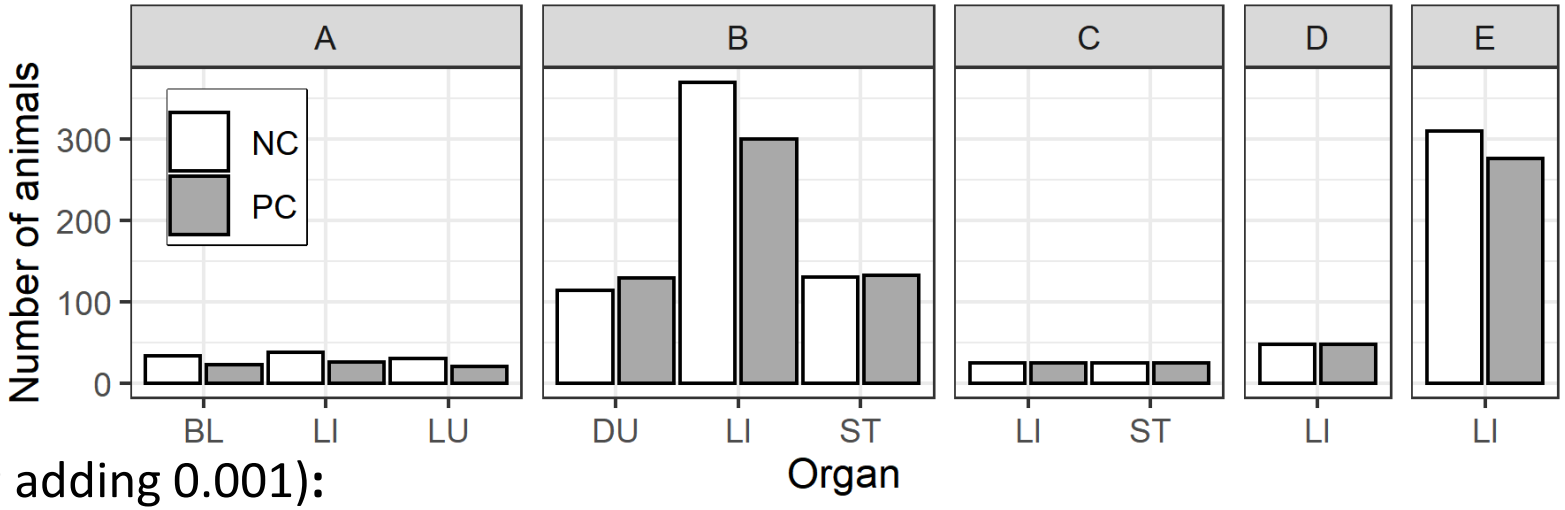


B

Technical settings	Administration settings	Sample characteristics	Other
Unwinding time	Vehicle type	Species	Analysis system
Amperage	Type of positive control	Sex	Year of performance
Voltage	Dose of positive control	Organ	
Duration of electrophoresis			

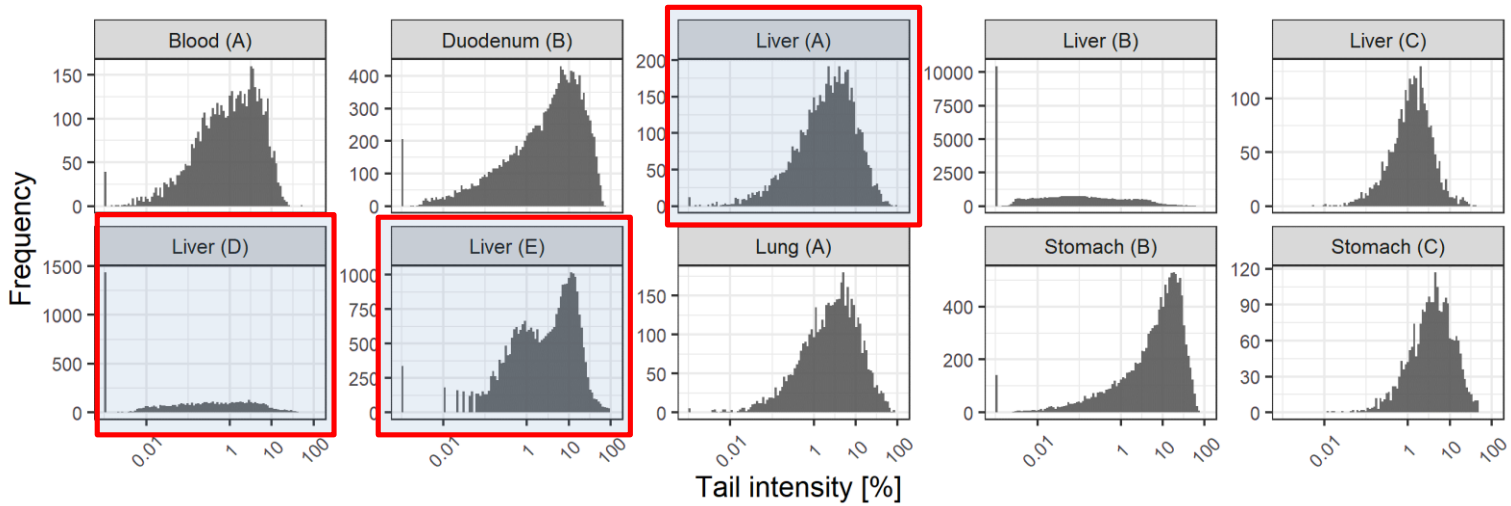
1. Introduction

General overview:

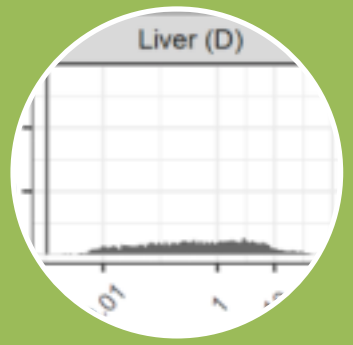


First result:
Major company effect

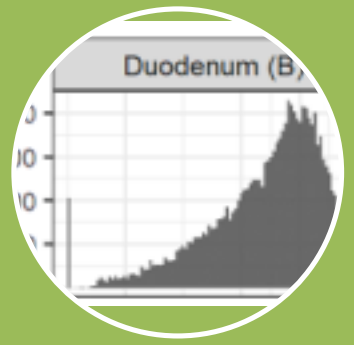
Distributions (after adding 0.001)**:**



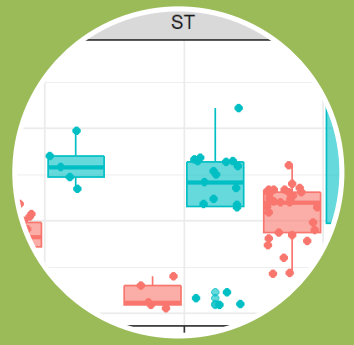
2. Results: Overview



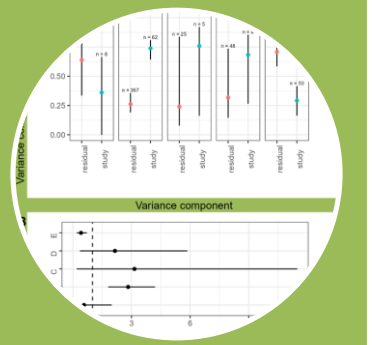
Zero handling



Slide summary handling

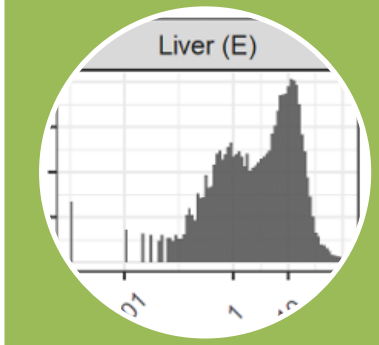


Difference between negative and positive controls



Variability component analysis

Different analysis systems



Bimodality

➔ Not shown here

2. Results: Zero proportions

Problem: Occurring zero values are critical for some statistical analyses:

- log-transformation

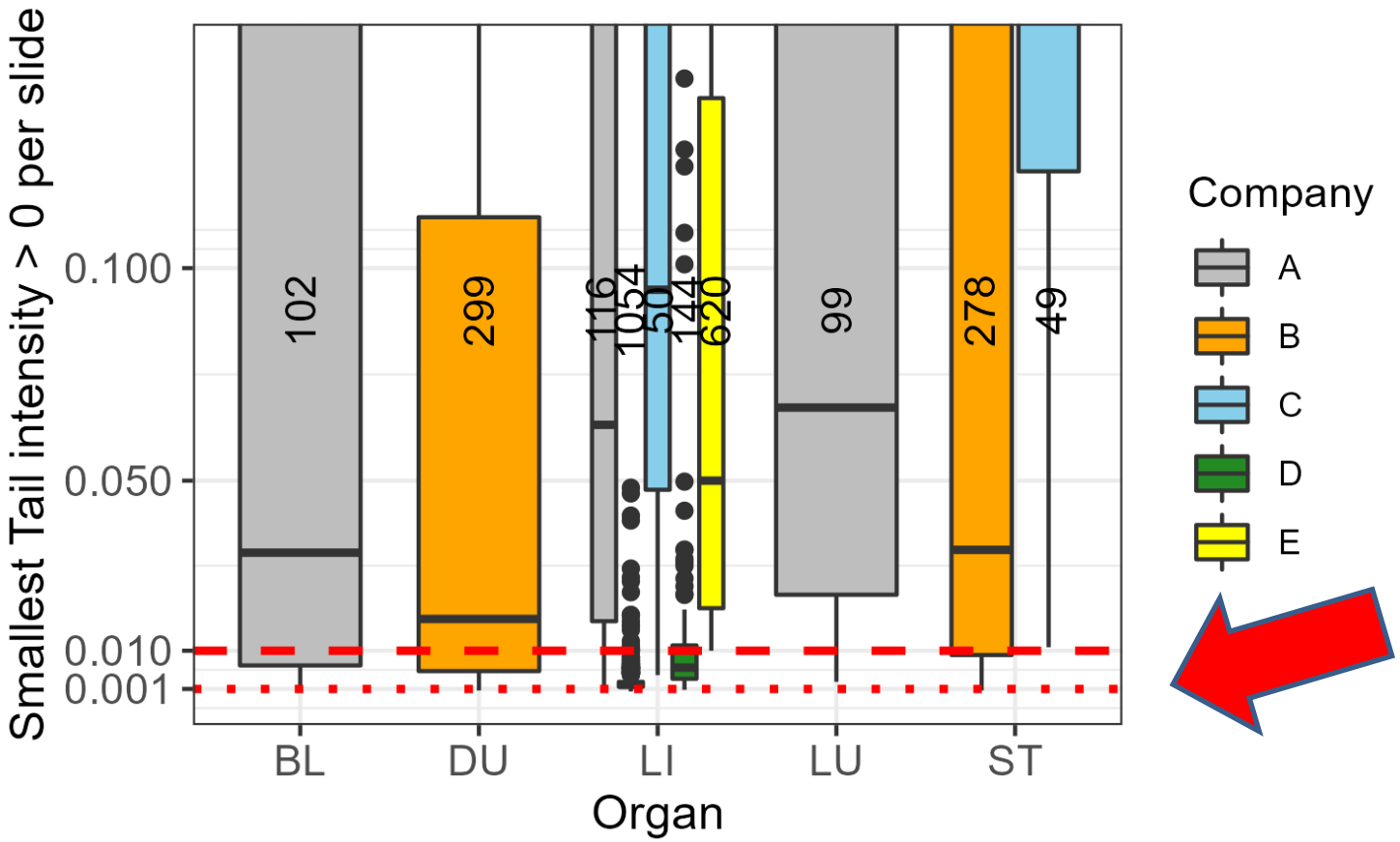
Suggestion OECD TG 489:

Tail intensity (TI) + 0.001

Question:

Is this small constant well chosen?

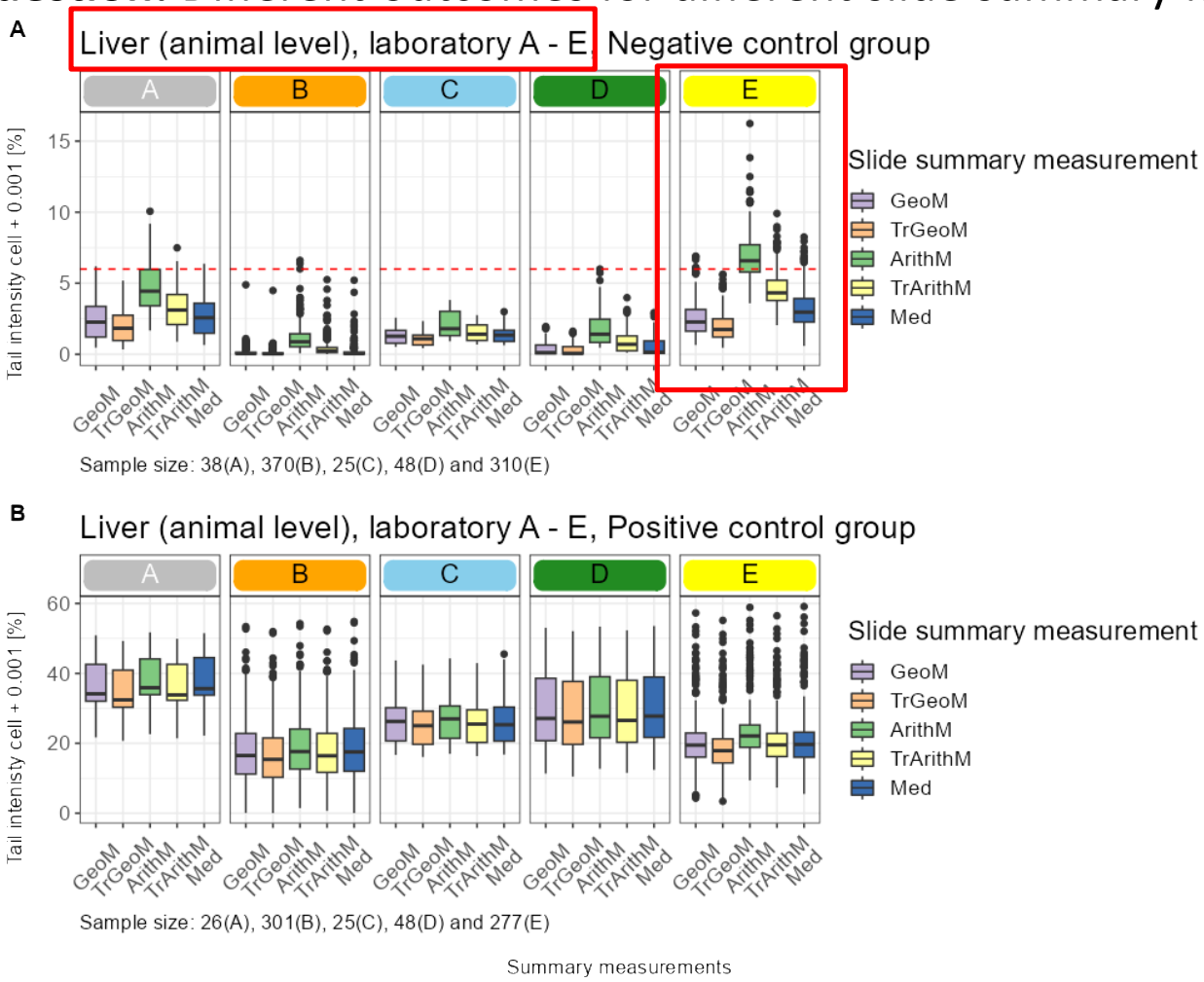
- For most slides in the data set the smallest TI values unequal 0 were > 0.001



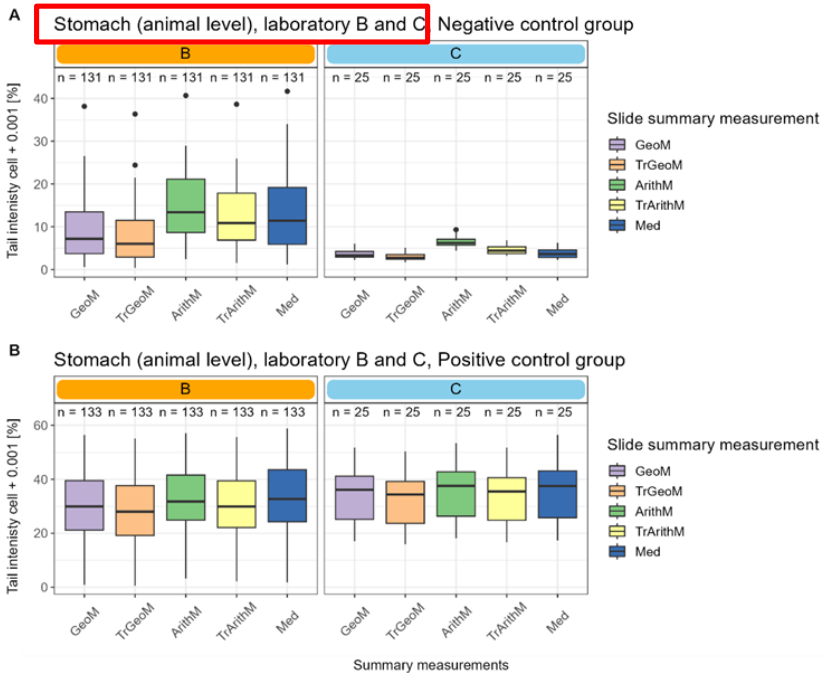
Recommendation: Always include at least 3 decimal places, when measuring the TI.

2. Results: Summary handling

Question: Different outcomes for different slide summary measures? (Tug et al., 2020)



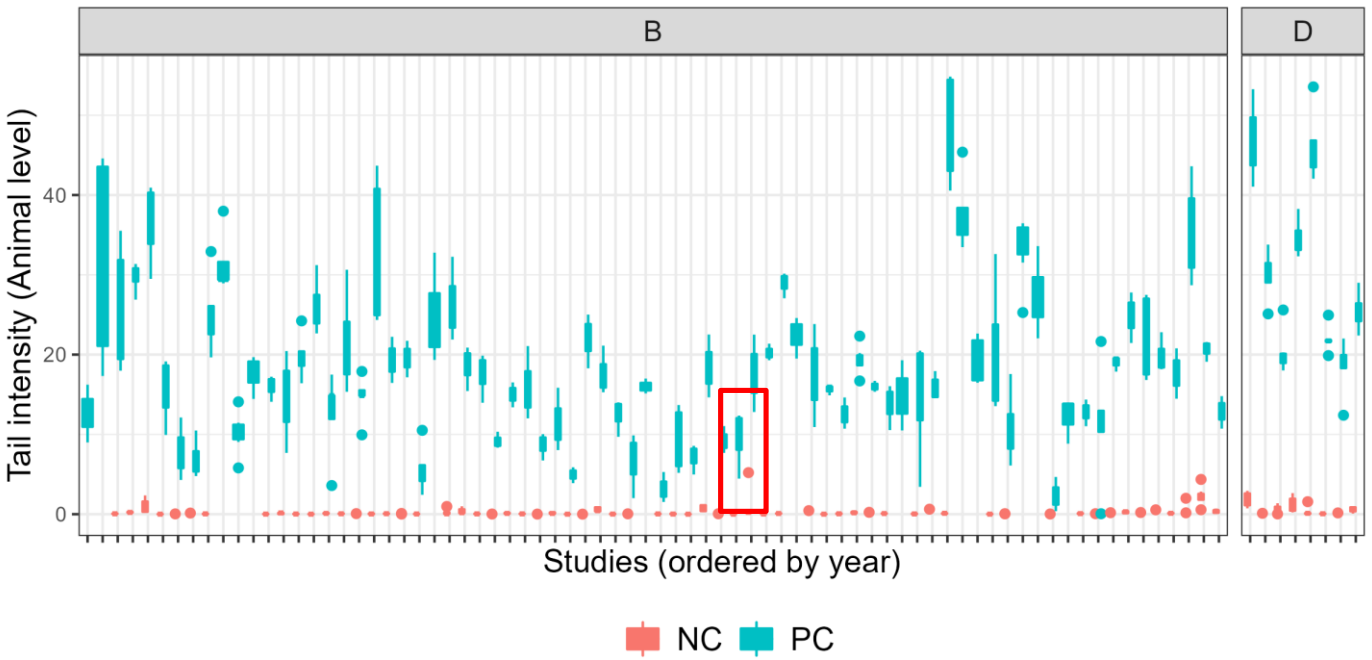
- **Negative controls**: Effects of slide summary measure noted for every company and organ
- Arithmetic mean oversensitive
- **Positive controls**: No effect of slide summary measure

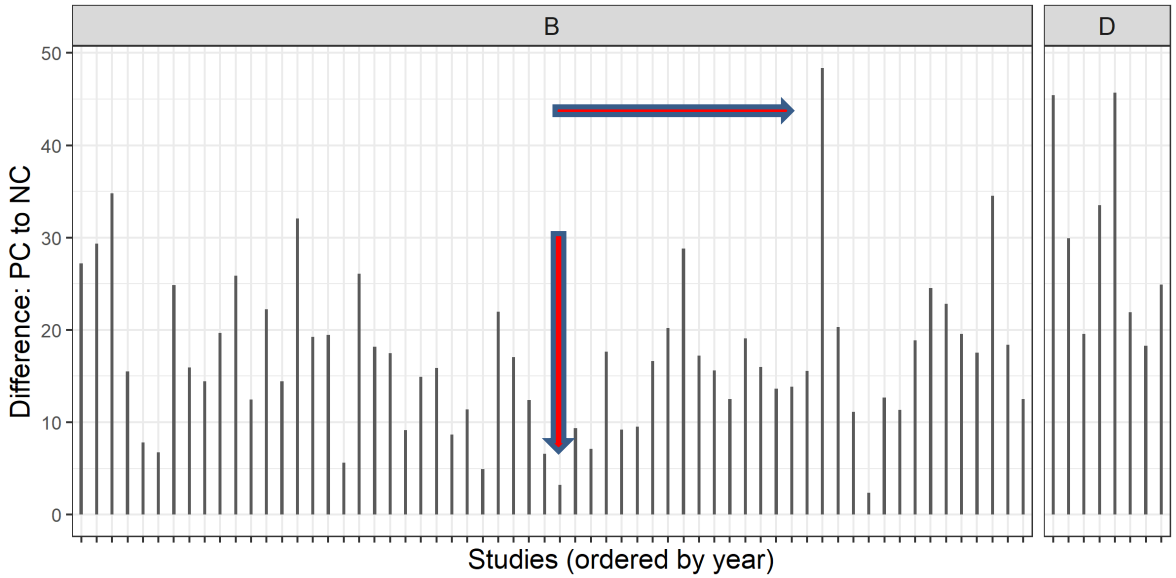


2. Results: Differences negative / positive controls

Question: Is there a **clear separation** between negative and positive control groups?

- Excerpt: Liver studies, two companies

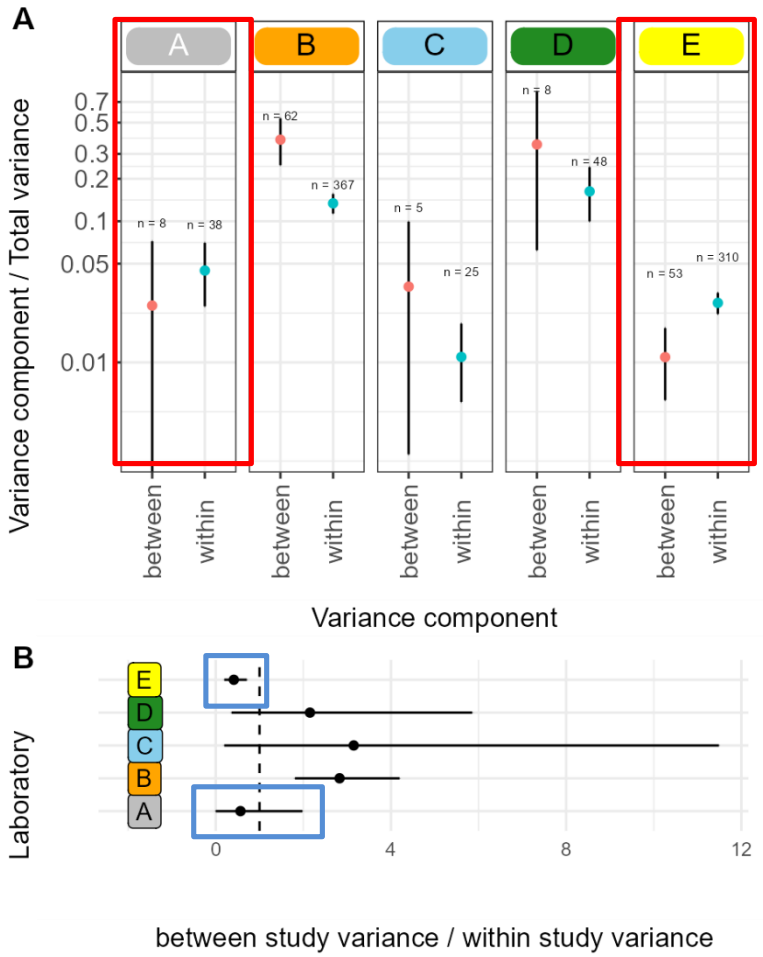




- **Yes, but differences vary from 2.3- to 48.3-fold (means of the slide medians)**
- In a few studies maximum negative control animal level > minimum positive control animal level

2. Results: Variance component analysis

Question: What is the proportion of variability at each level of the hierarchical design**?**



- Only laboratories A und E fulfil the criteria that the estimated within variance should be higher than the estimated between variance
- for laboratories A, C and D and hence the estimation of the variance components is relatively uncertain
- Hence, for the two laboratories (B and E) the proportion of variance components differs significantly from each other (but only in study E variance is smaller than the variance)

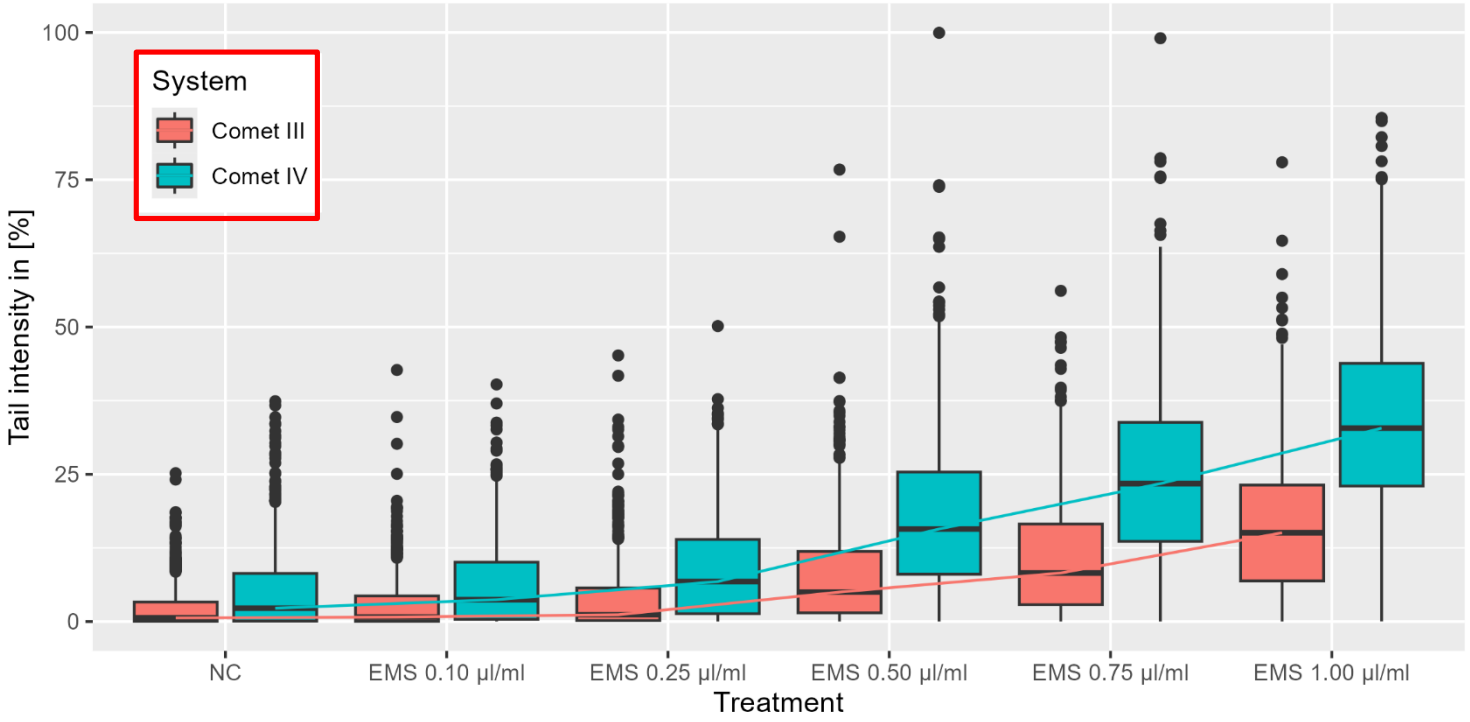
➔ simple point estimates ignoring their uncertainty can be heavily misleading

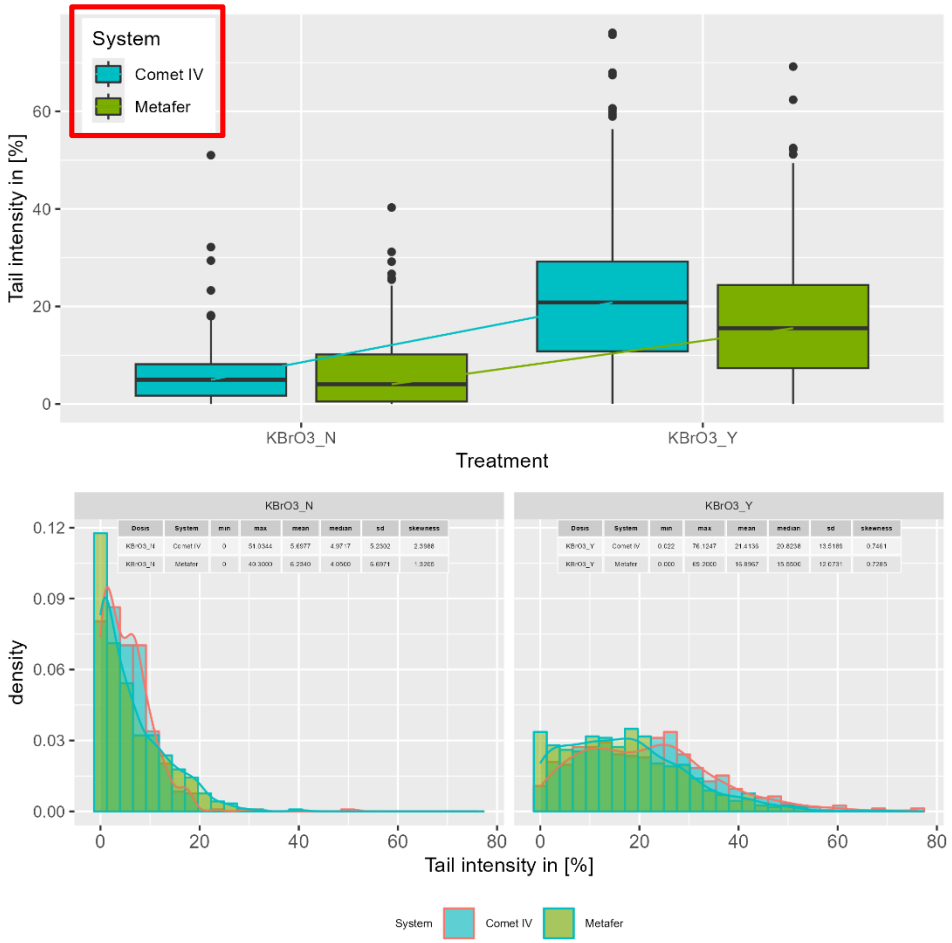
➔ **challenge**: violations of model assumptions vs. loss of information (aggregation of data)

➔ laboratories should not use their HCD for the calculation of control limits (mostly between study variation is a major source of variability, Dertinger et al., 2023)

2. Results: Different analysis system

Question: Different results with different analysis system with the same cells?

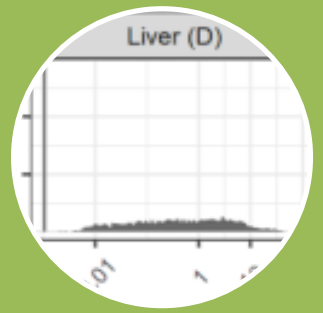




- Differences in performance and sensitivity of the Comet III and Comet IV systems (Comet IV and Metafer system similar results)
- The difference increased with increasing concentrations

3. Summary

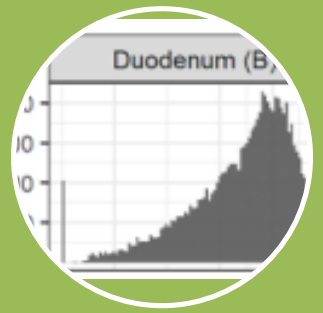
For a deeper insight into these and other results, you are welcome to read the recent publication (Tug & Duda et. al, 2024):



Zero handling:

- Based on the present data set, addition of constant (0.001), as suggested by OECD 489, is appropriate

- But, tail intensities should, therefore, be given with at least three decimal places

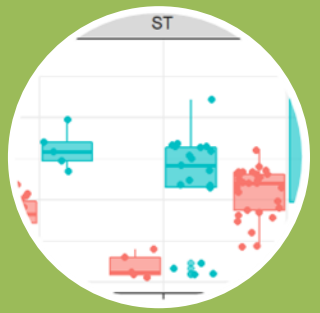


Slide summary handling:

- Different summarizing strategies lead to (extremely) different results based on the same data

- Effects in negative controls depend on company and/or organ

- Arithmetic mean oversensitive

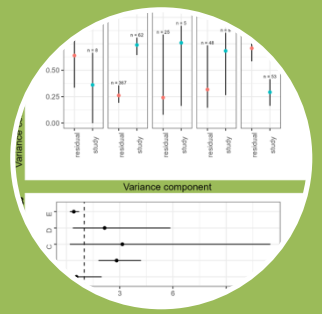


Difference between negative and positive controls:

- Clear separation between both groups in almost all studies

- Differences vary extremely (means of slide medians)

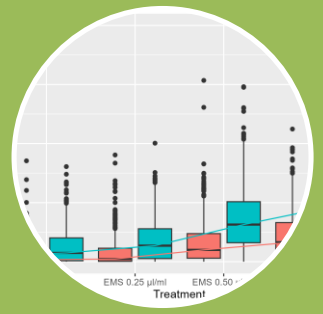
3. Summary



Variance component analysis:

- Violations of model assumptions vs. loss of information

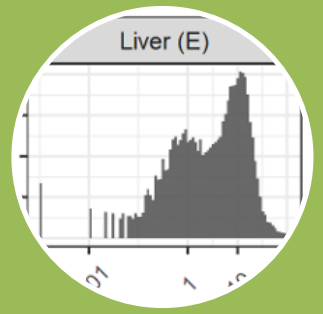
- Laboratories should not use their HCD for the calculation of control limits



Different analysis system

- Differences in performance and sensitivity of the Comet III and Comet IV systems (Comet IV and Metafer systems similar results)

- The difference increased with increasing concentrations



Bimodality:

- Different statistical approaches with not clear results (more research)

- Zero values should be avoided with longer electrophoresis time

4. Outlook

- Development of a corresponding R-Shiny app
- Further historical control analyses
- Different intervals (linear mixed model - confidence, prediction and tolerance intervals)
- Collecting further treatment data

Acknowledgement

- GUM working group „Statistics“ (https://gum-net.org/gum-ag-statistik/)
  - for providing data sets and
  - for many stimulating, informative and fruitful discussions.
- Department of Statistics, TU Dortmund University for the opportunity to explore this topic (Prof. Dr. Katja Ickstadt and Prof. Dr. Jörg Rahnenführer)*

- Contact to join GUM WG statistics:

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  bernd-wolfgang.igl@boehringer-ingelheim.com

Thank you for your attention!

Questions, comments, suggestions…?

E-Mail: tug@statistik.tu-dortmund.de

References

- Bright, J., Aylott, M., Bate, S., Geys, H., Jarvis, P., Saul, J., & Vonk, R. (2011): Recommendations on the statistical analysis of the Comet assay. Pharmaceutical Statistics, 10, 485–493, https://doi.org/10.1002/pst.530

- Dertinger, S. D., Li, D., Beevers, C., Douglas, G. R., Heflich, R. H., Lovell, D. P., . . . Zhou, C. (2023): Assessing the quality and making appropriate use of historical negative control data: A report of the International Workshop on Genotoxicity Testing.Environmental and Molecular Mutagenesis, https://doi.org/10.1002/em.2254

- OECD (2016), *Test No. 489: In Vivo Mammalian Alkaline Comet Assay*, OECD Guidelines for the Testing of Chemicals, Section 4, OECD Publishing, Paris, https://doi.org/10.1787/9789264264885-en.

- Tug T. et al. (2020): “Statistical analysis of in vivo alkaline comet assay data - Comparison of median and geometric mean as centrality measures”, Regulatory Toxicology and Pharmacology, Volume 118, https://doi.org/10.1016/j.yrtph.2020.104808

- Tug, T. & Duda, J.C. et al. (2024). In vivo alkaline comet assay: Statistical considerations on historical negative and positive control data. Regulatory toxicology and pharmacology : RTP, 148, 105583. https://doi.org/10.1016/j.yrtph.2024.105583

- Wiklund S. J., Agurell E. (2003): “Aspects of design and statistical analysis in the Comet assay”, Mutagenesis vol.18 no.2, pp.167–175, https://doi.org/10.1093/mutage/18.2.167

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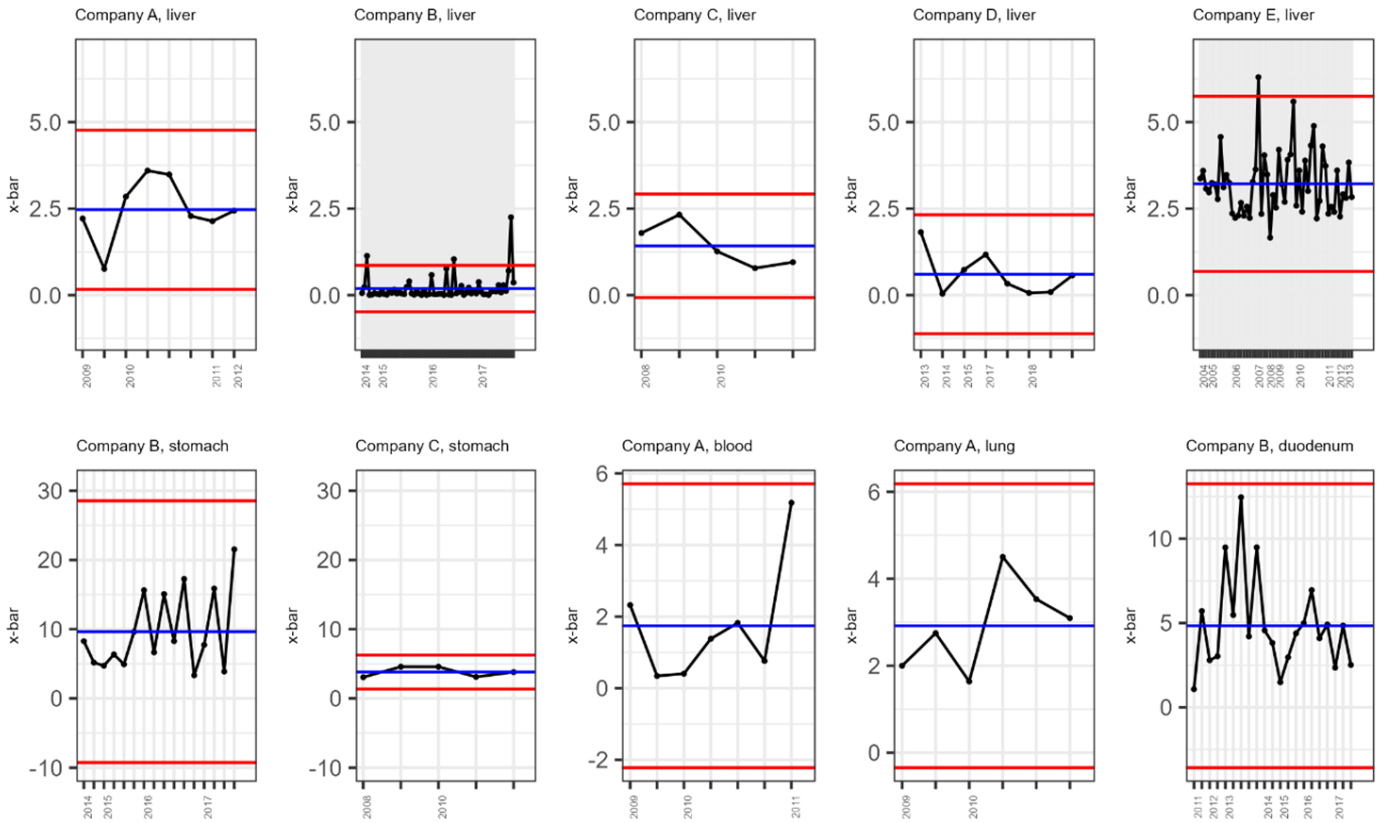
2. Results: Quality control

Control charts of raw tail intensity values for the negative control for each organ and laboratory (A-E).



For each combination, we calculated the lower/upper control limits (mean plus/minus three standard deviations, red lines) to see the longitudinal behaviour.