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The Comet Assay – How to recognise "Good Data"

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4th September 2015

ICAW

Content

- Regulatory Genetic Toxicology
- JaCVAM trial overview and results
- Protocols
- Historical control data
- Statistics
- Power curves
- Control charts

Regulatory Genotoxicity Testing

- Revision, designated S2(R1), November 2011
- Replaces and combines the previous S2A and S2B guidelines, issued in 1995 and 1997 respectively.
- Strategy to reduce delays due to high frequency of non relevant in vitro positive results thus reducing animal use

ICH S2(R1) – 2 Options

- Option 1
  - Ames
  - Chromosome aberration assay

  or
  - Mouse lymphoma assay

  or
  - In vitro micronucleus assay
  - In vivo chromosome aberration assay

  or
  - In vivo micronucleus assay (Acute or repeat dose)

ICH S2(R1) – 2 Options

- Option 1
  - 2nd In vivo test?
  - Comet assay

  - Negative results in two appropriate in vivo assays with adequate exposure are sufficient to demonstrate lack of genotoxicity

ICH S2(R1) – 2 Options

- Option 2
  - Ames
  - In vivo micronucleus assay (Acute or repeat dose)
  - In vivo Comet assay

ICH S2(R1) - Comet

- ICH S2(R1) released in 2011
- Comet OECD guideline released in 2014

- Although there was no OECD guideline for the comet assay at the time of release of ICH S2(R1) it was considered a suitable 2nd in vivo assay.

  - UDS assay was losing popularity due to poor sensitivity

JaCVAM validation trial

- Within and or between-lab variability of assay results examined in phases 1-3
- Variability disappeared with protocol refinement, confirmed in phase 4.1
- Criteria for negative and positive controls confirmed
- % tail intensity 1 – 8% Liver, 1- 20% Glandular stomach
- In validation study no clear false positives

Phase 4.2 – Results

- Final judgement
  - All results discussed under a blind condition with coded chemicals
  - Statistics and histopathology

- Predictive capability determined
  - Carcinogens – Positive sensitivity
  - Non carcinogens – Negative specificity

Phase 4.2 – Results

- Genotoxic carcinogens
  - Expect positive judgement
  - 13/19 = Positive sensitivity 68%

- Non genotoxic – non carcinogens
  - Expect negative judgement
  - 7/8 = Negative specificity 88%

Phase 4.2 – Results

- Genotoxic non carcinogens
  - Prefer negative judgement
  - 5/6 = Negative specificity 83%

- Non genotoxic carcinogens
  - Prefer negative judgement
  - 7/7 = Negative specificity 100%

Phase 4.2 – Results

- CONCORDANCE
  - 32/40 = 80%

JaCVAM trial concluded comet assay could be at least equal or more sensitive to detect genotoxic carcinogens which could be detected in the UDS assay.

Life before the guideline

Not sensitive enough!

What does a positive look like?

It's too sensitive!

What does it mean?

What measurement to use?

What statistics ?

What does it measure?

What protocol to use?

What tissues – are they the same?

What pH?

Image analysis or eye?

Cell or nucleus?

What about Apoptosis, Necrosis , hyperplasia etc?

It doesn't detect mutagens!

What end point?

What on earth are hedgehogs and ghost cells!

Protocol - JaCVAM

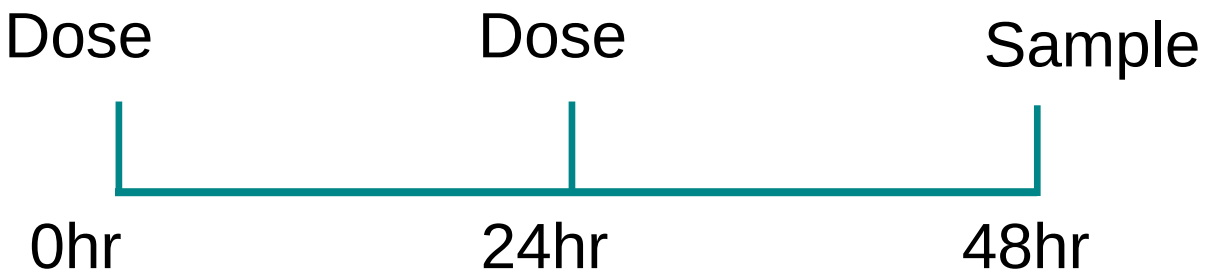
- Design
  - Rat: Crl:CD (SD), 7-9 weeks at dosing, 5 per group
  - Vehicle control and 3 dose levels
  - Dosing 0hrs, 24hrs, 45hrs sampling at 48hrs
  - Positive control EMS at 200 mg/kg/day
  - Tissues liver and stomach
  - Electrophoresis 0.7 V/cm, voltage 0.30A, 2-8°C
  - Measured % tail DNA

Experimental variability

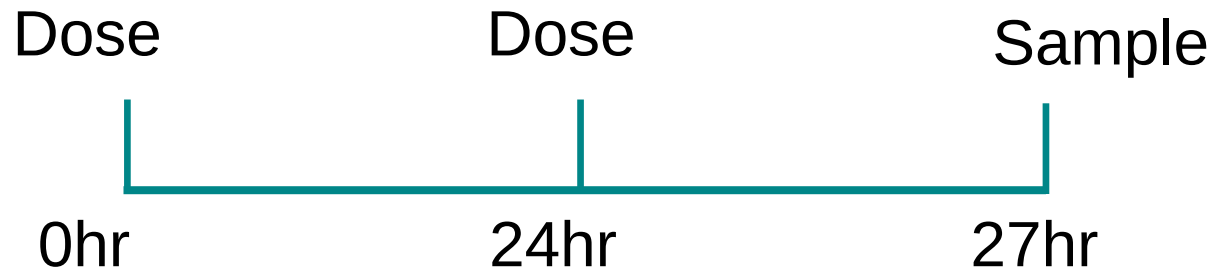
- Cell death (apoptosis, necrosis) and endonuclease activity are associated with increased DNA fragmentation
- This could be caused by a test item or inappropriate assay conditions (e.g., time and temperature during tissue processing), electrophoresis conditions → misleading data interpretation

Protocols

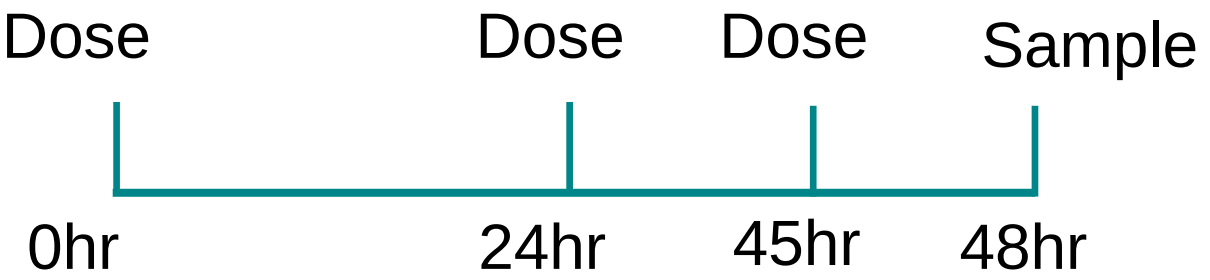
Micronucleus



Comet



Combined



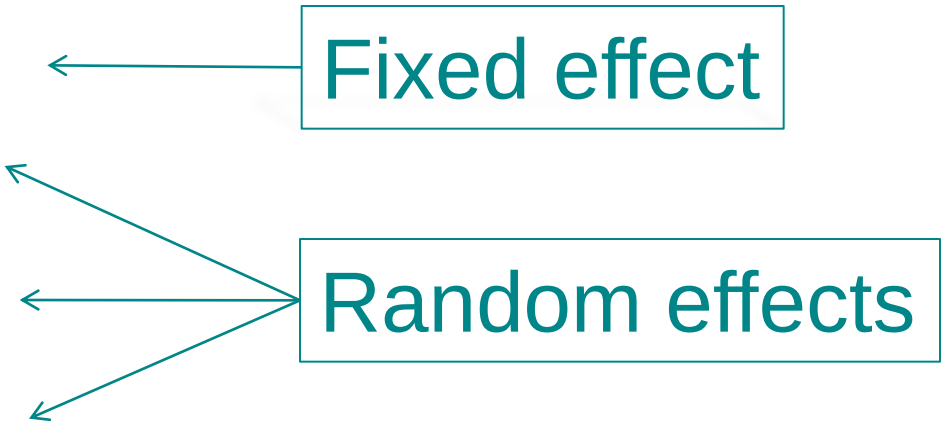
What does “good” data look like?

- How many animals per group? >=5
- How many slides per animal? 3
- How many cells per slide? 50
- Which parameter to measure? % tail intensity
- What does vehicle or positive control data look like?
- Is it the same for all tissues?

Dose (mg/kg/day)	Animal number	Slide Number	Number of cells scored	Single slide mean tail intensity (%)	Animal mean tail intensity (%)	Group mean tail intensity (%)	Single slide median tail intensity (%)	Animal mean of median tail intensity (%)	Group mean of median tail intensity (%)

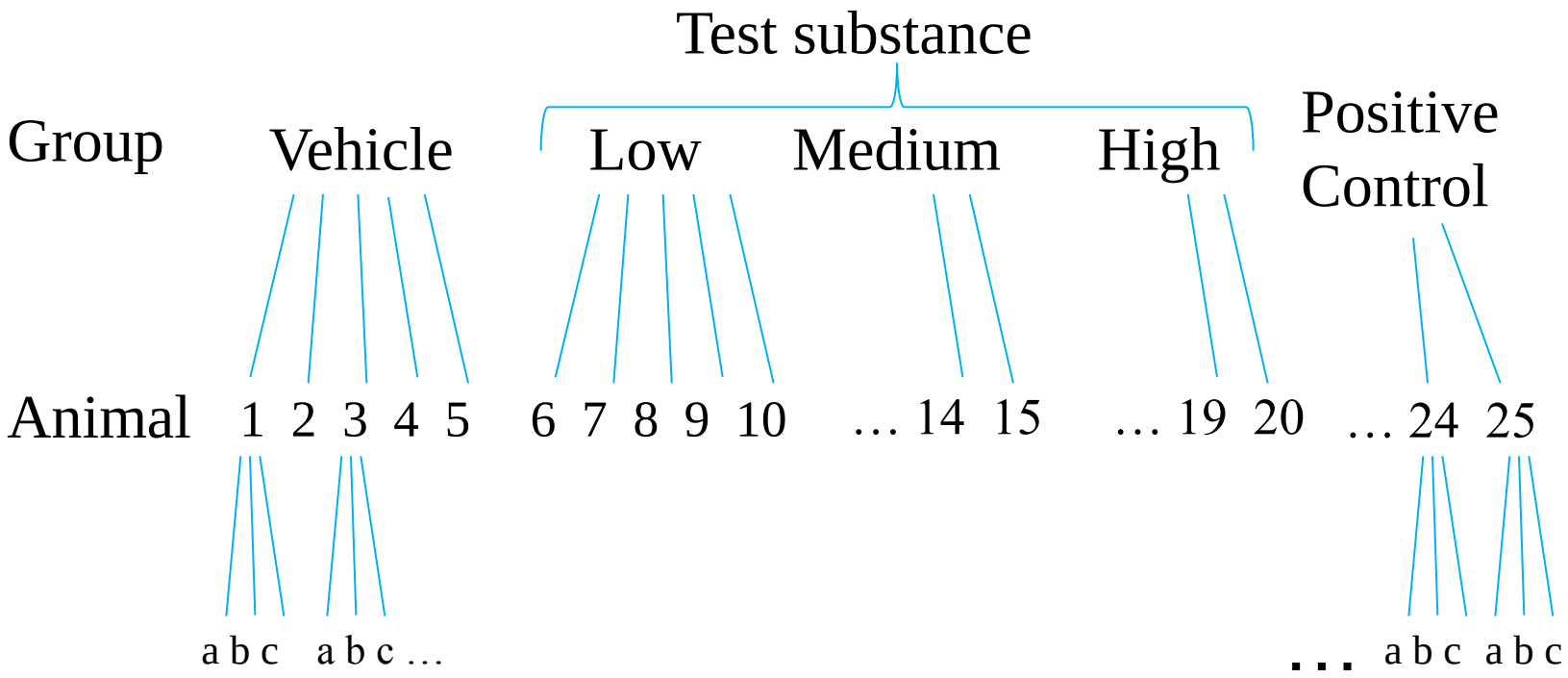
What does “good” data look like?

- Four-way hierarchical nested design:
  - Treatment
  - Animal
  - Gel (slide)
  - Cell



- Treatments tested against the animal variability

What does “good” data look like?



Historical control

As more experimental data are added to the historical control distribution, concurrent negative controls should ideally be within the 95% control limits of that distribution. The laboratory's historical negative control database should be statistically robust to ensure the ability of the laboratory to assess the distribution of their negative control data. The literature suggests that a minimum of 10 experiments may be necessary but would preferably consist of at least 20 experiments conducted under comparable experimental conditions.

Historical control

Animal reference ranges Mean % tail intensity

Species/strain	Control	Tissue	Mean	SD	Max	Med	Min
Rats Crl:CD(SD)	Vehicle	Liver	2.67	0.68	4.18	2.54	1.56
	Positive	Liver	49.90	11.19	72.10	46.48	36.94

Animal reference ranges Median % tail intensity

Species/strain	Control	Tissue	Mean	SD	Max	Med	Min
Rats Crl:CD(SD)	Vehicle	Liver	0.58	0.54	2.39	0.37	0.06
	Positive	Liver	49.86	12.27	74.22	45.37	35.07

Historical control

Group reference ranges Mean % tail intensity

Species/strain	Control	Tissue	Max	Med	Min
Rats Crl:CD(SD)	Vehicle	Liver	3.29	2.43	2.30
	Positive	Liver	61.65	48.63	39.41

Group reference ranges Median % tail intensity

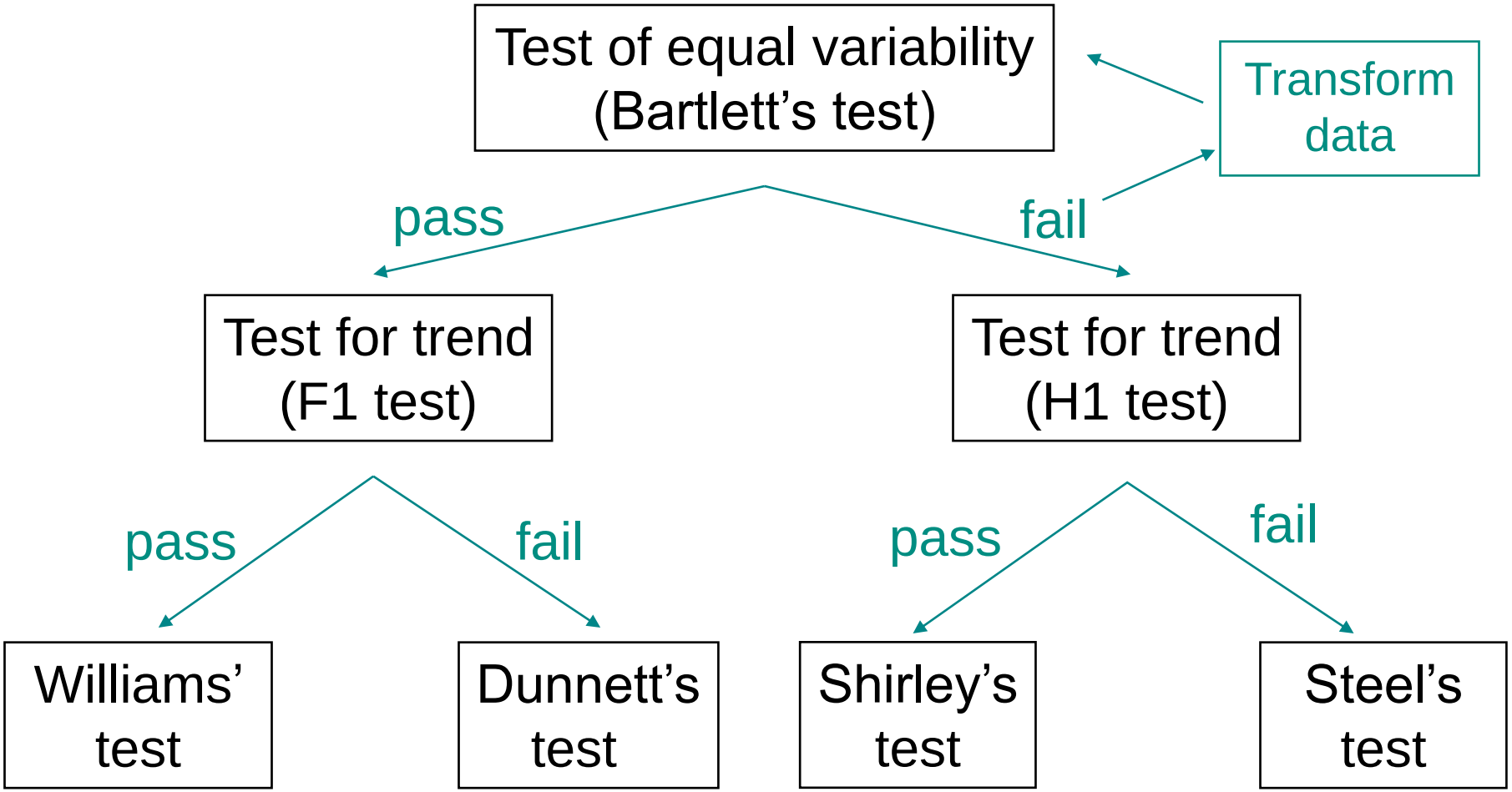
Species/strain	Control	Tissue	Max	Med	Min
Rats Crl:CD(SD)	Vehicle	Liver	0.99	0.45	0.32
	Positive	Liver	62.66	48.47	38.45

Statistical analyses

- The median value from each slide is obtained for tail intensity
- The average of the three medians is calculated and used in the statistical analysis
- For tail intensity from each organ, separate analyses are performed for comparisons of
  - Control *vs.* Increasing dose levels of Test Compound
  - Control *vs.* Positive Control
- If any variable contains any zero values, 0.001 will be added to all responses to enable a logarithmic transformation where necessary

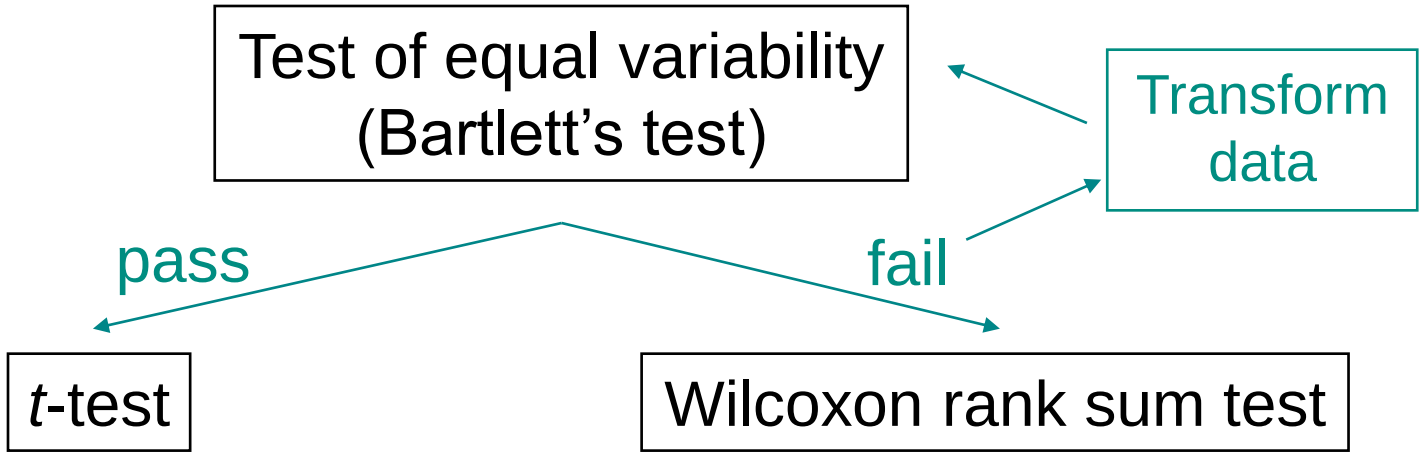
Methodology

For the comparison of test compound groups to control, the following pathway is followed:



Methodology

For the comparison of positive control to control, the following pathway is followed:

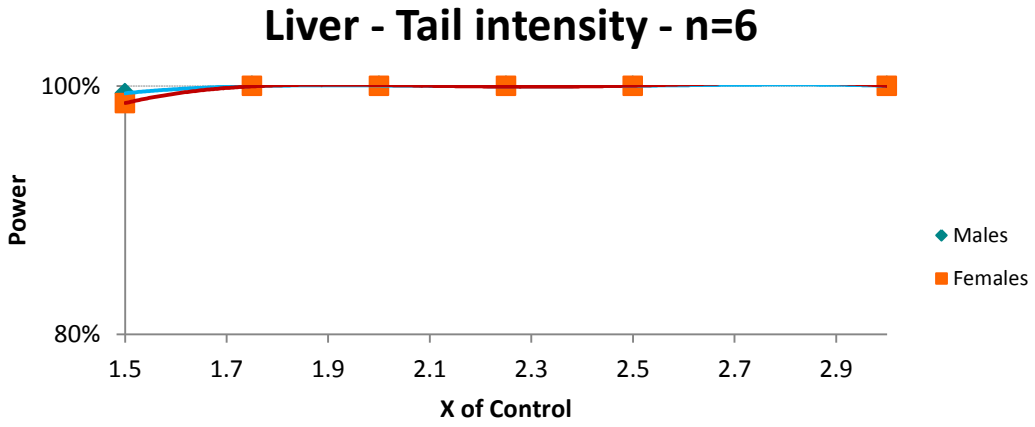


Power curves

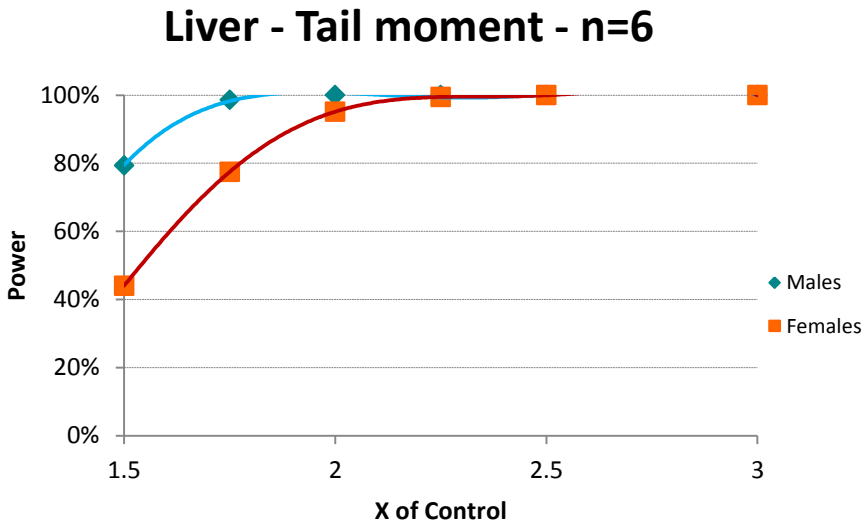
- The power has been calculated based on the power of a two sided t-test (which is equivalent to the comparison of the lowest dose group with the control using the statistical methods currently used for comet).

- Power has been calculated for group size of 6, which is standard for non positive control groups.
- Power has been calculated for the three main parameters of tail intensity, length and moment. Of these, tail intensity is the key parameter.

Power Curves, HLS Data



We could reduce the number of animals used per group



Control charts

“Laboratories should use quality control methods, such as control charts (e.g. C-charts or X-bar charts) to identify how variable their data are, and to show that the methodology is 'under control' in their laboratory.”

Control charts

- X bar chart to monitor control group means
  - Control limits are set at 3 sigma. Means probability of data falling out the limit is 0.0027 (370 points plotted before outside limits).
  - Stage 1
    - 20 studies advised to set limits (10 minimum)
      - All studies included unless extreme outlier
  - Stage 2
    - No clear guidance on limit recalculation
    - Practical advice is to recalculate limits after every 3 to 4 studies.

Control charts – Rules

- **Nelson rules** are a method in process control of determining if some measured variable is out of control (unpredictable versus consistent)

- One point is more than 3 sd from the mean.

- Nine (or more) points in a row are on the same side of the mean.

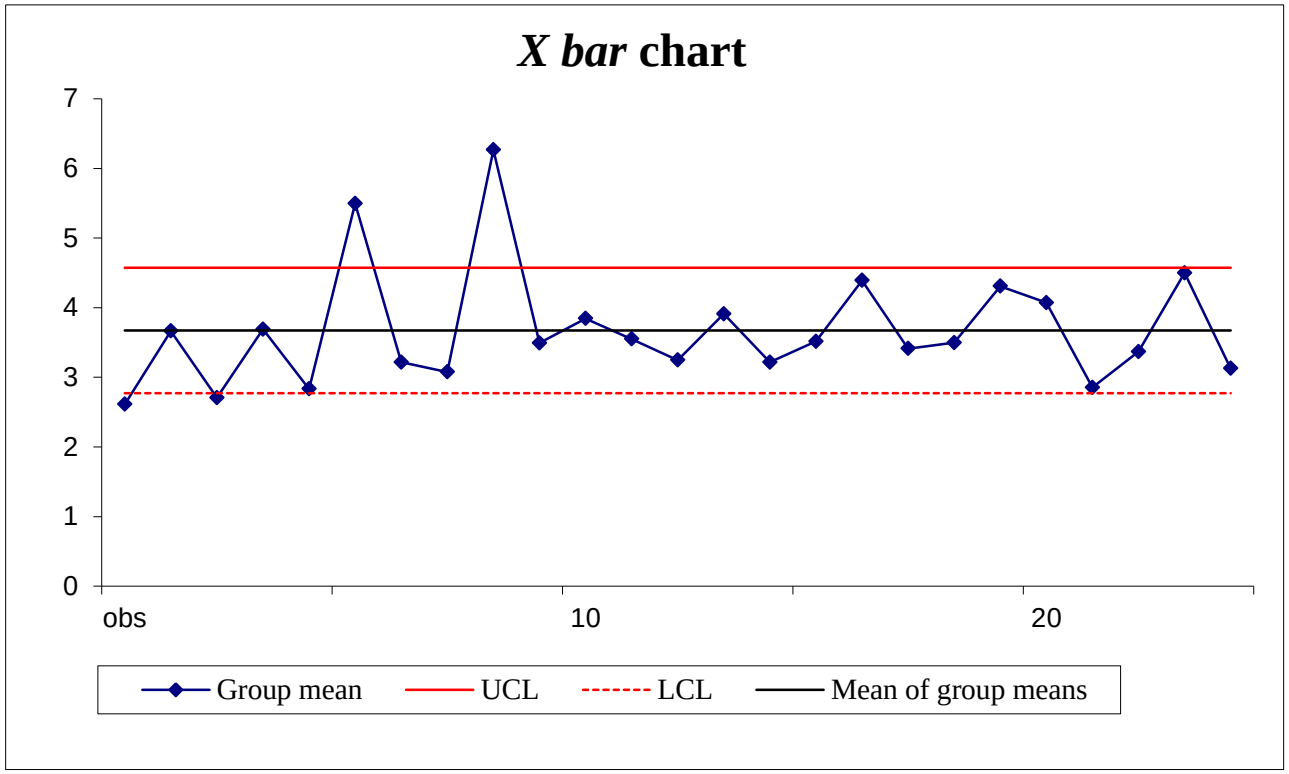
- Six (or more) points in a row are continually increasing (or decreasing)

- Fourteen (or more) points in a row alternate in direction, increasing then decreasing.

Control charts – Rules - continued

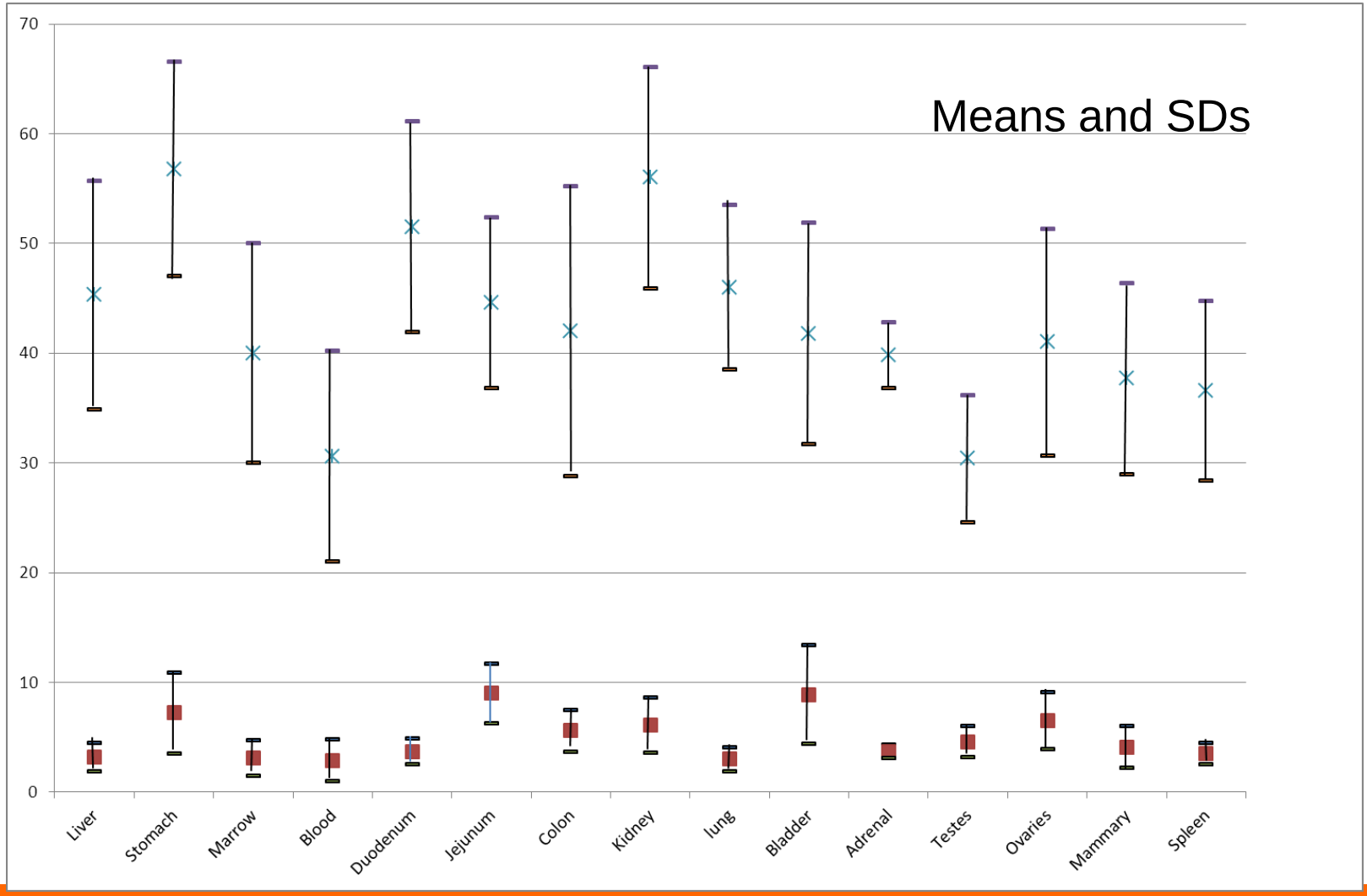
- Two (or three) out of three points in a row are more than 2 sd from the mean in the same direction.
- Four (or five) out of five points in a row are more than 1 sd from the mean in the same direction.
- Fifteen points in a row are all within 1 sd of the mean on either side of the mean.
- Eight points in a row exist with none within 1 sd of the mean and the points are in both directions from the mean.

Liver % tail intensity



X bar control limits	
UCL	4.58
LCL	2.77

HLS data various tissues



Thank you for listening-
Any Questions?