A Combined Morphometric and Statistical Approach to Assess Nonmonotonicity in the Developing Mammary Gland of Rats in the CLARITY-BPA Study.

Montévil M, Acevedo N, Schaeberle CM, Bharadwaj M, Fenton SE, **Soto AM**

Malka, watercolor ©
by Luisa Soto

The CLARITY BPA study

- The “Consortium Linking Academic and Regulatory Insights on BPA Toxicity” (CLARITY-BPA) was a comprehensive “industry-standard” Good Laboratory Practice-compliant 2-year chronic exposure study of BPA toxicity that was supplemented by hypothesis-driven independent investigator-initiated studies.
- There was a “core” regulatory study –a typical US-FDA study.
- There were 14 investigator-initiated studies that focused on disease-associated, morphological, functional and molecular endpoints. The purpose of this design was:

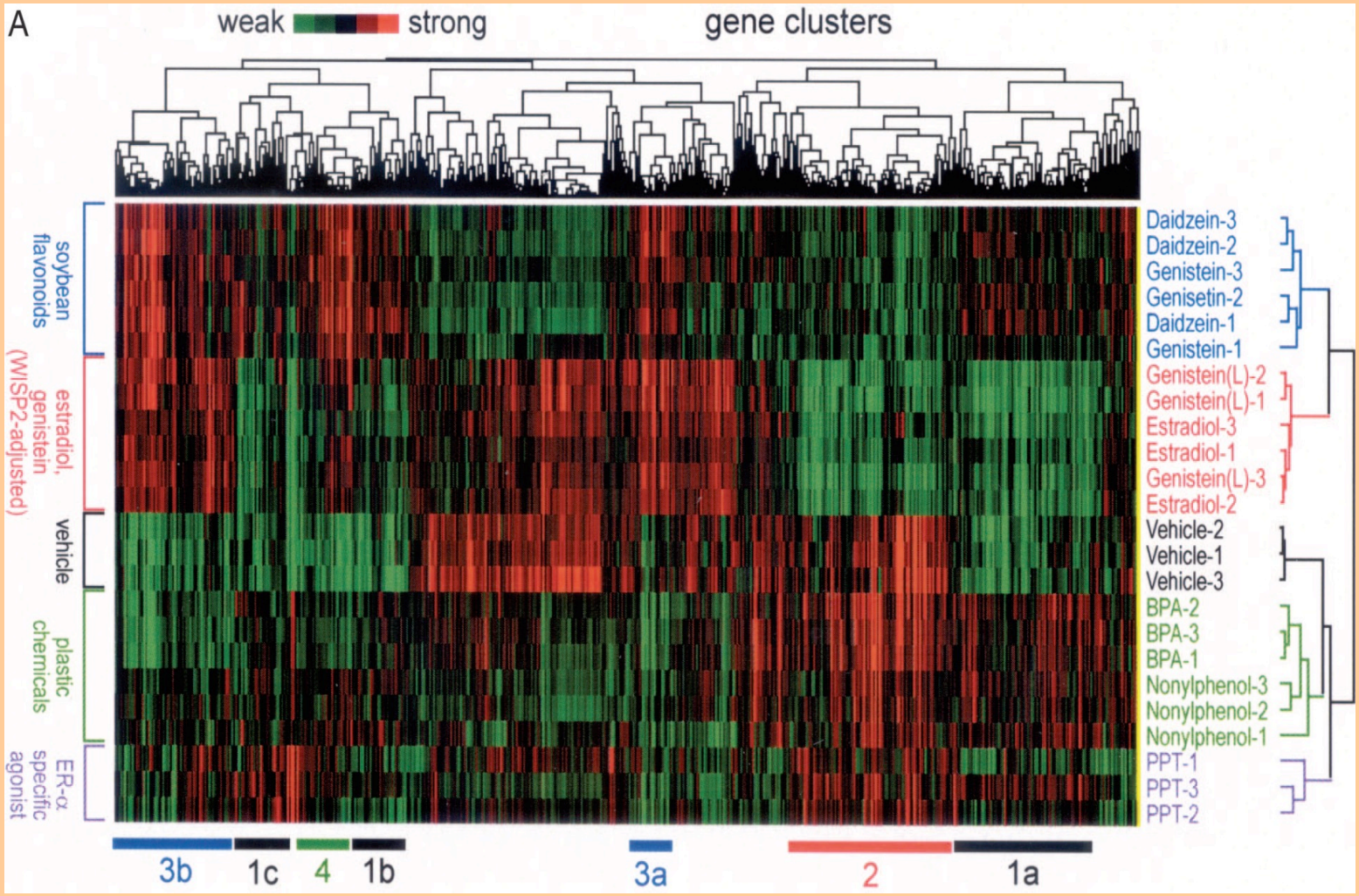
  - 1) that the results from the independent studies would be incorporated into a guideline-compliant study
  - 2) that these state of the art additions to the guideline study would provide a stronger dataset for regulatory agencies and
  - 3)to test whether industry standard endpoints are sensitive, specific and predictive for agents that interfere with hormone systems

Our mammary gland study

- Our overarching goal was to obtain morphological markers of altered mammary gland development that could be used as indicators of an increased propensity for cancer and to provide a quantitative assessment of mammary gland development. We explored the following hypotheses:

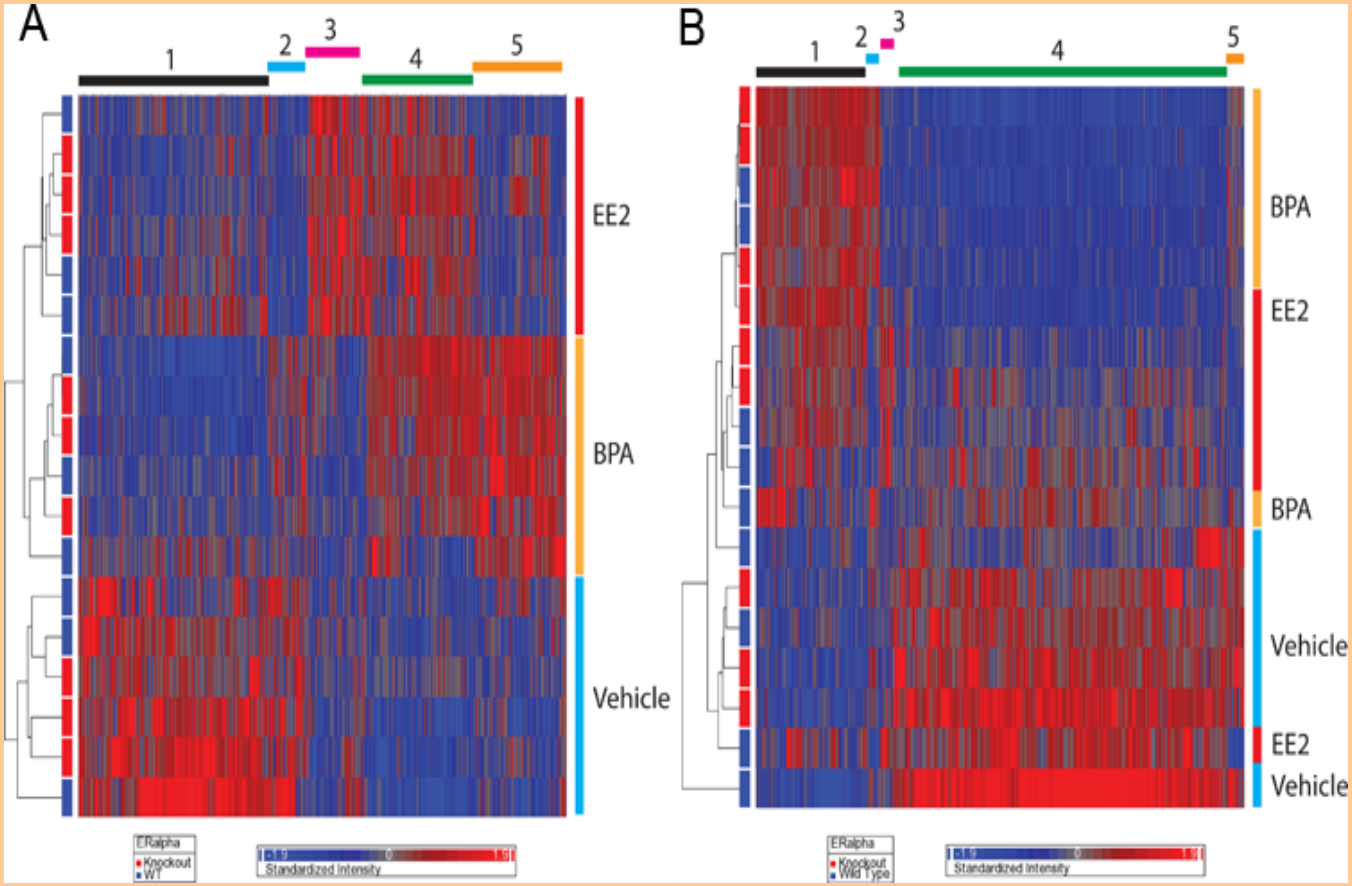
- (1) pre-pubertal mammary gland morphology at PND 21 is an excellent predictor of pathological outcomes that manifest during adulthood, based on data obtained independently in our laboratory (mostly in mouse models) and that of Dr. S. Fenton (National Toxicology Program) (mostly using rat models);
- (2) perinatal exposure to BPA induces abnormal post-pubertal/adult development of the mammary gland;
- (3) BPA generates non-monotonic dose-response curves
- (4) Like in our previous studies, not all estrogens were born equal (sometimes BPA=EE2, sometimes BPA≠EE2).

MCF7 transcriptome (XE dose producing comparable proliferative responses)



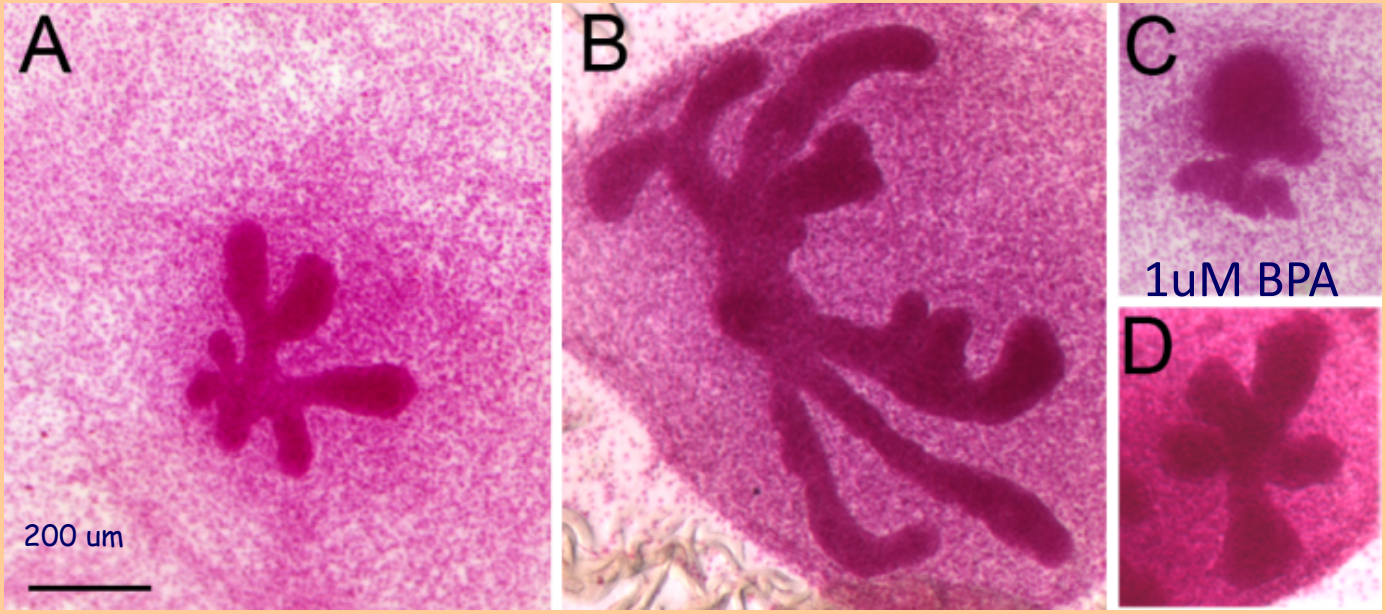
Shioda, T et al, PNAS, 2006

Mammary gland transcriptome at E19



Heat Map: Clustering analysis of an E19 mammary glands. Stroma (A) and Epithelium (B) was separated by laser capture microdissection. On the left axis, Red (KO animals) blue (WT)- from *Wadia et al, PLOS ONE 2013*

Environmentally relevant doses of BPA increase ductal area in an *ex vivo* assay



Control
FUL

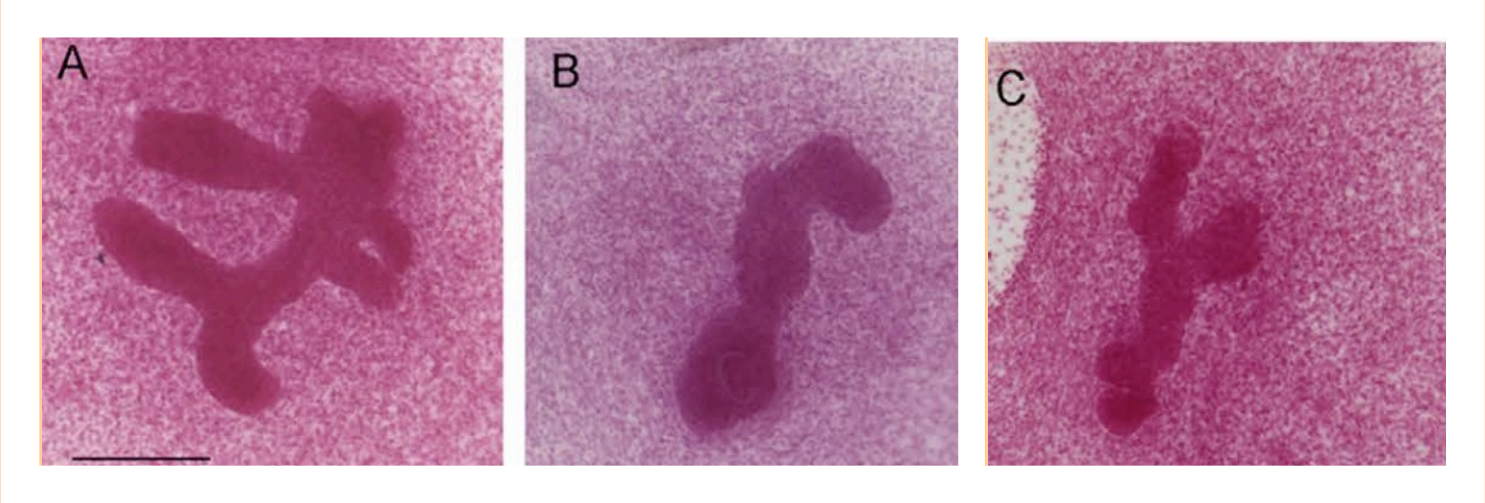
1nM BPA

1 nM BPA+

Most likely mediated by ER alpha

Speroni et al, Sci Rep 2017

Physiologically relevant doses of estradiol decrease ductal area

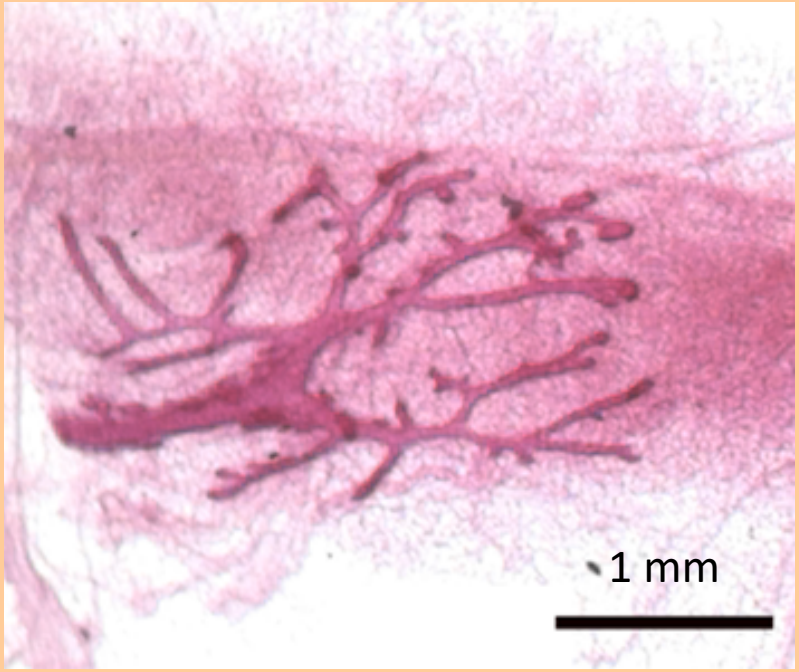


Carmine stained whole mounts of cultured mammary explants in (A) Control (CDFBS), (B) 1 nM E2, (C) 10 pM E2. Scale bar: 200 μ m.

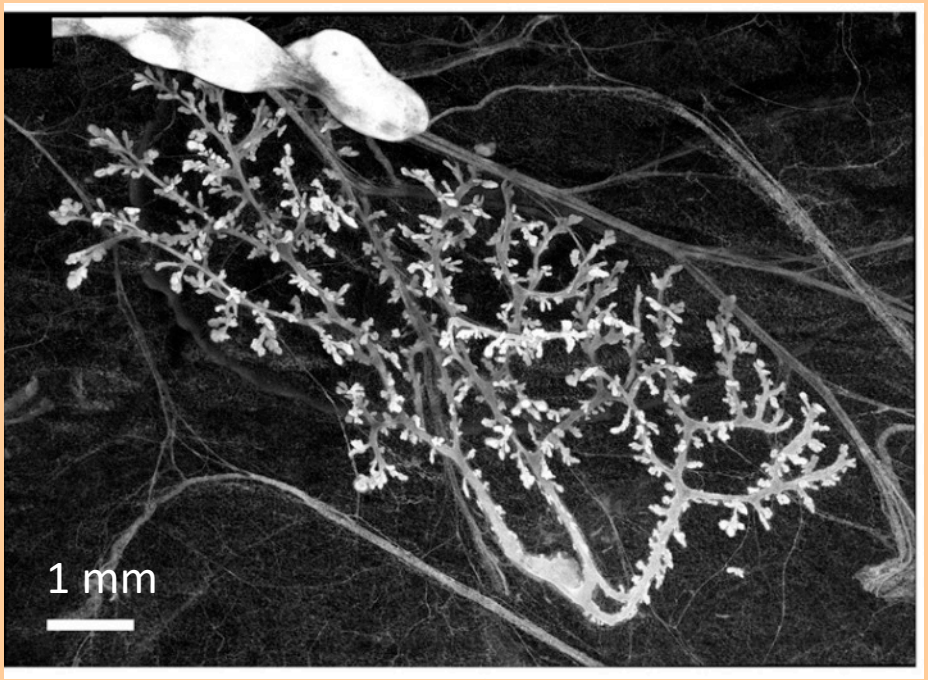
Speroni et al, Sci Rep 2017

PND21 mammary glands

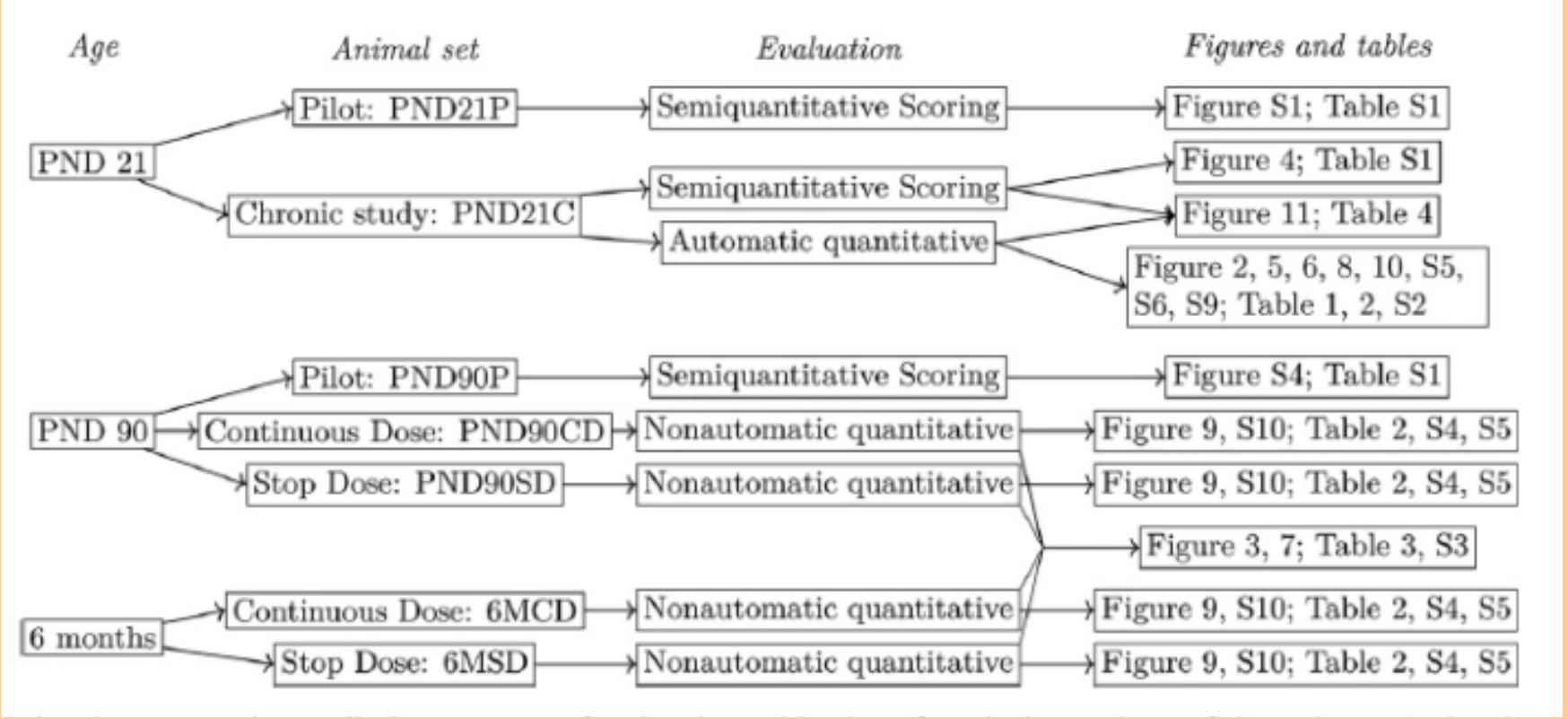
CD1 MOUSE



SD RAT



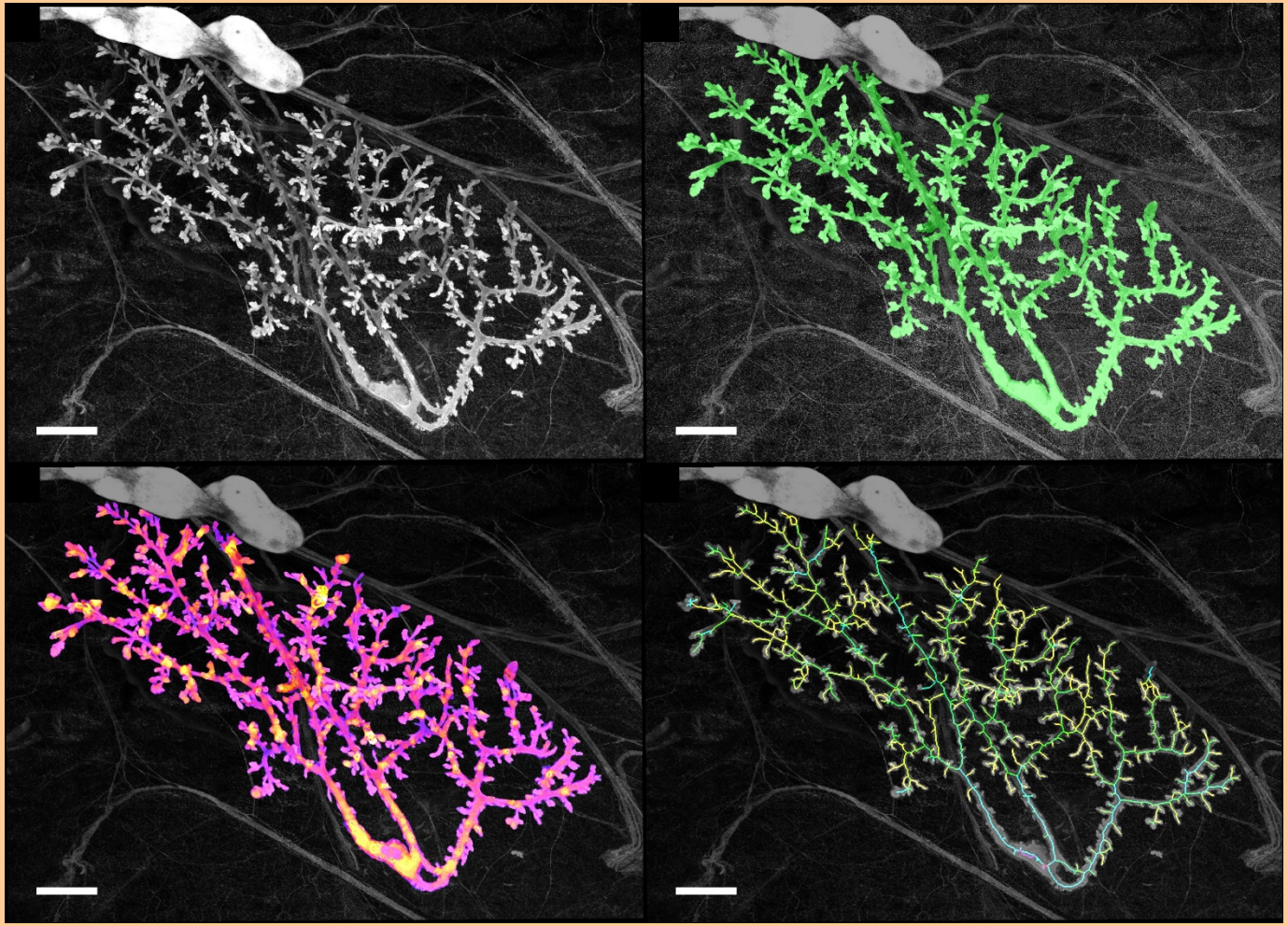
Experimental design

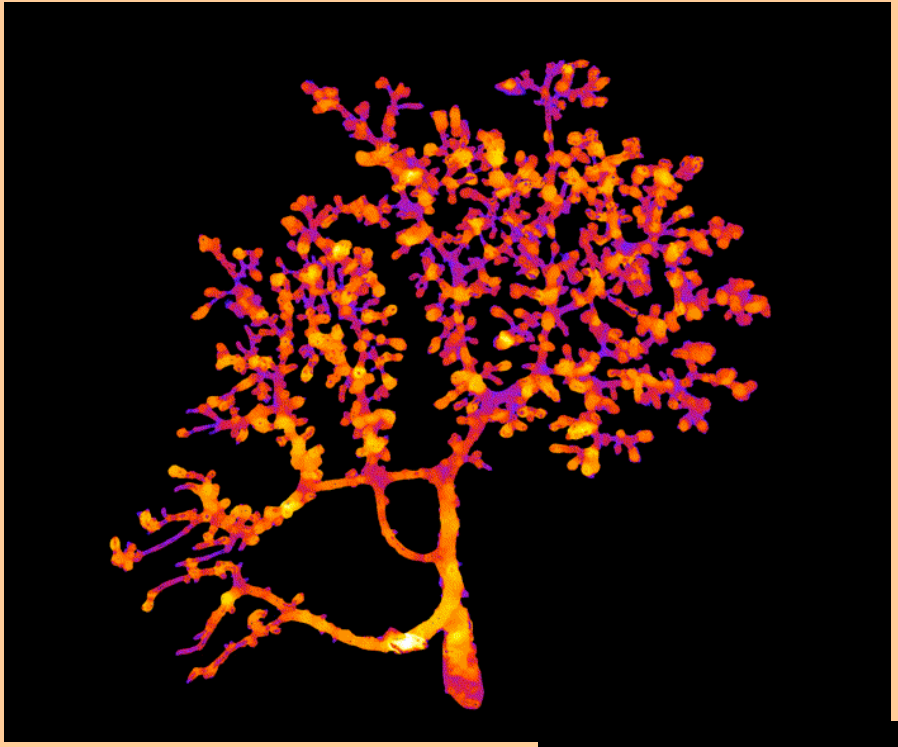


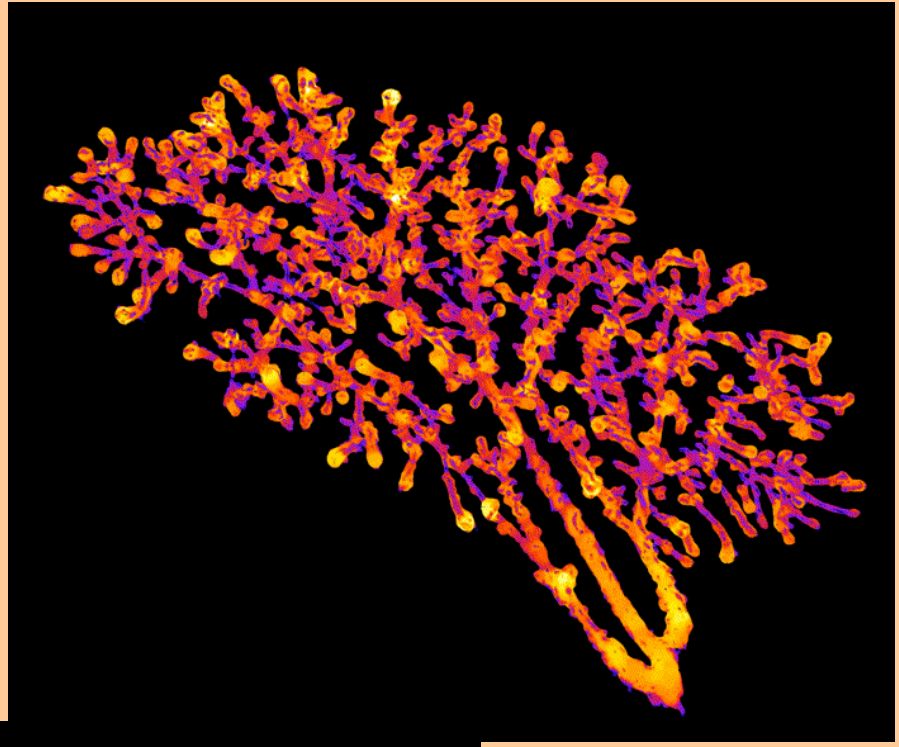
3D image analysis

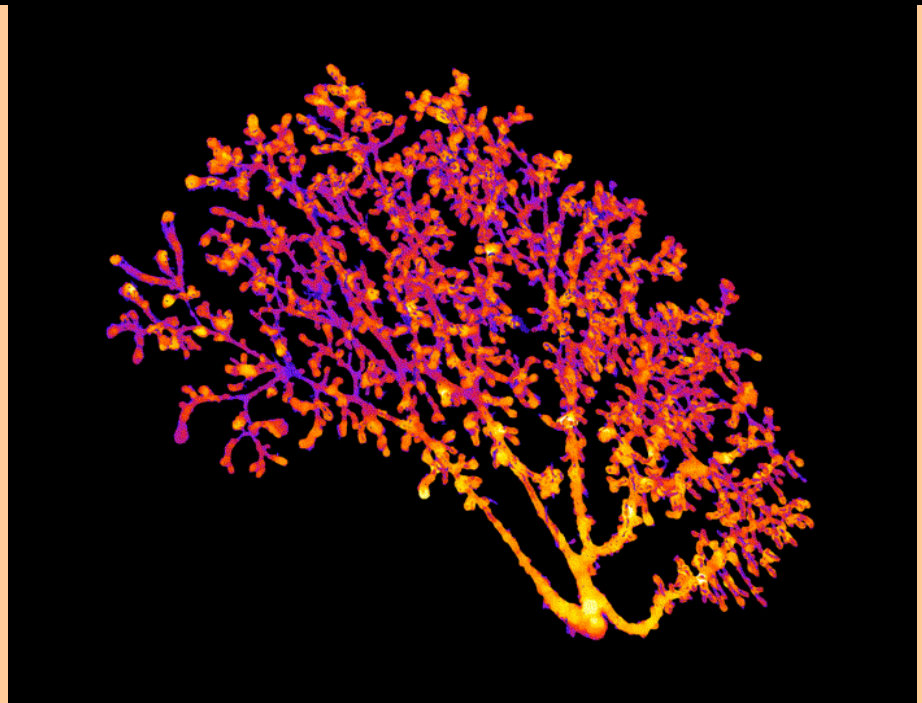
- Image acquisition (confocal microscope)
- Segmentation
- Analysis

  ->~90 features

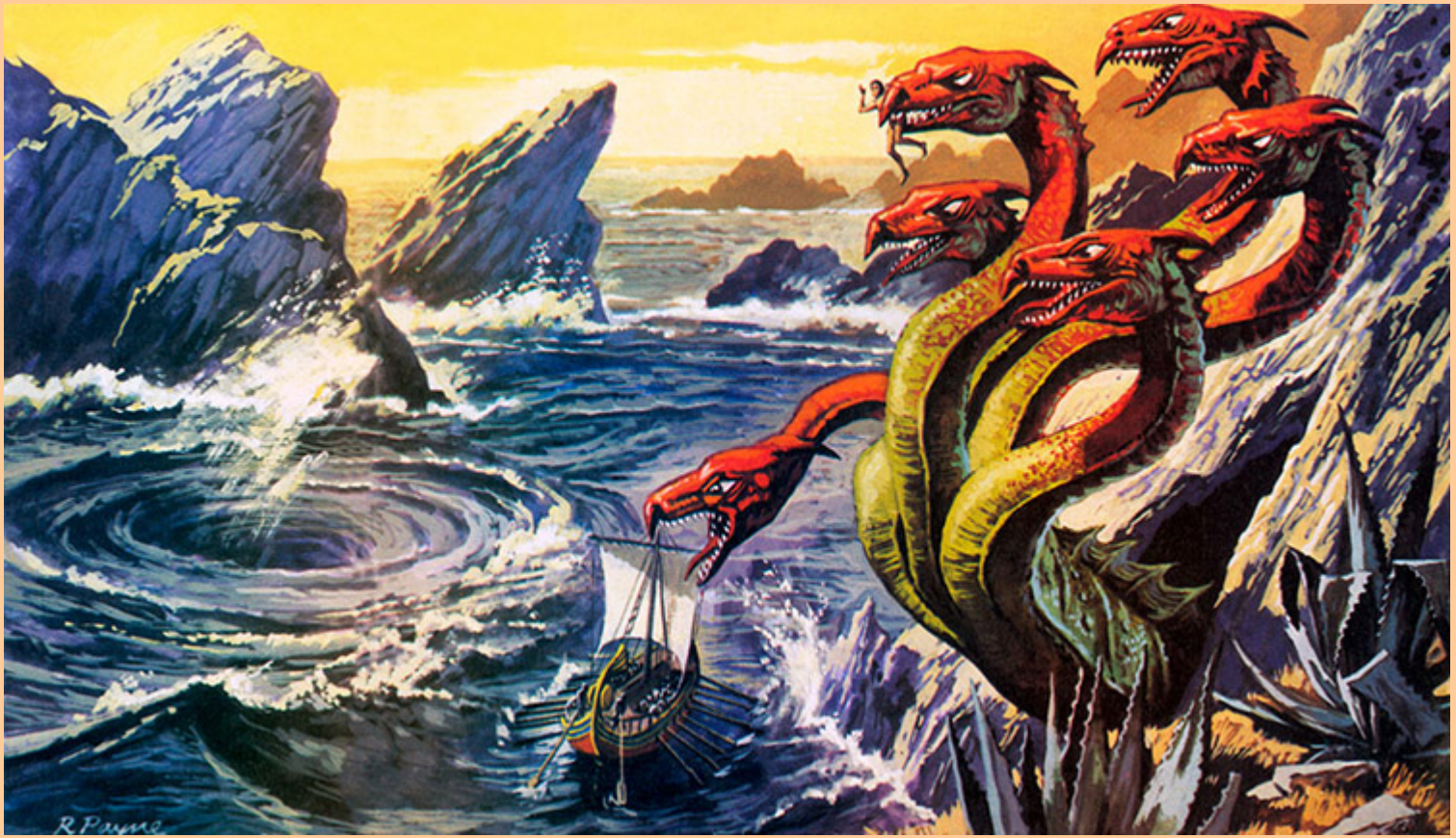








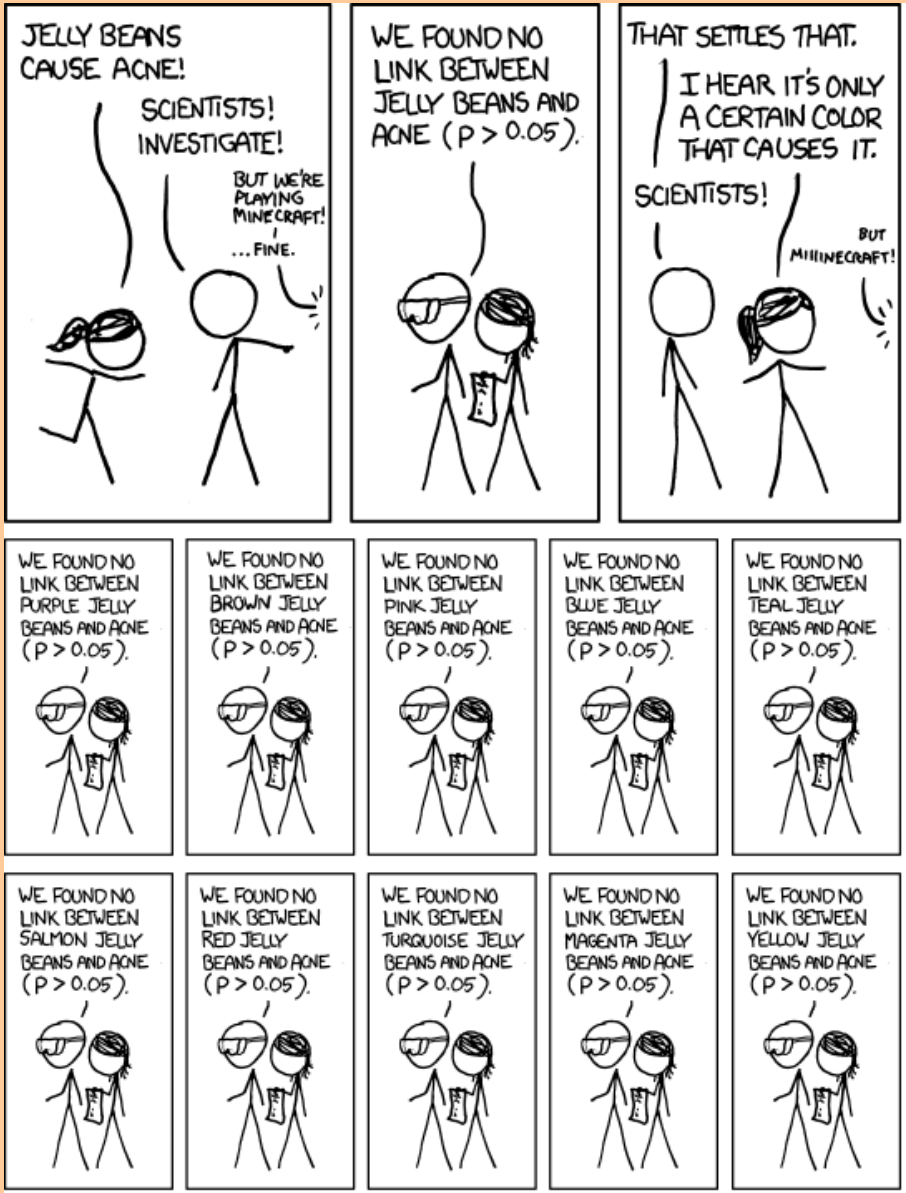
Statistical analysis between Scylla and Charybdis

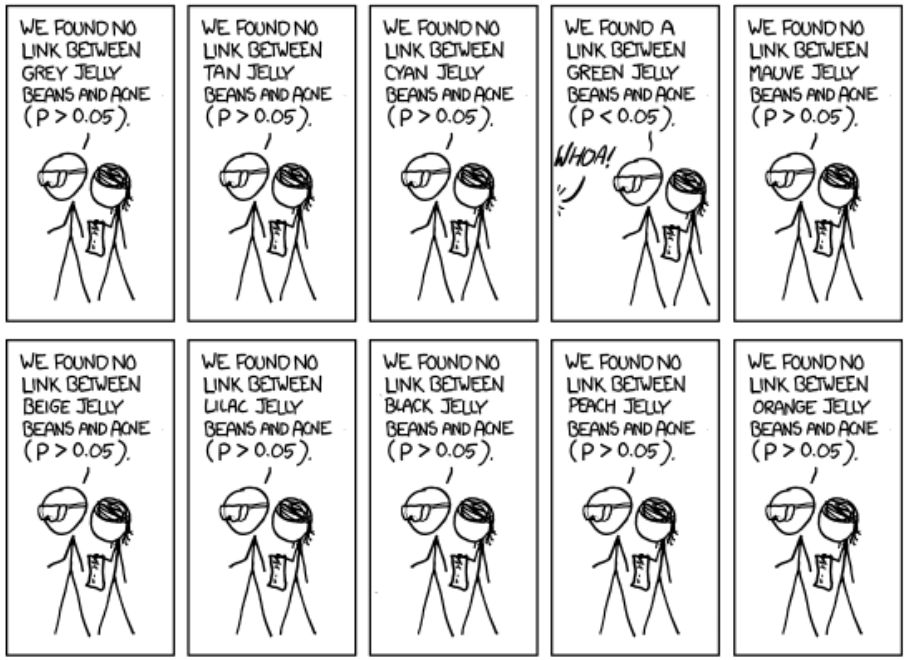


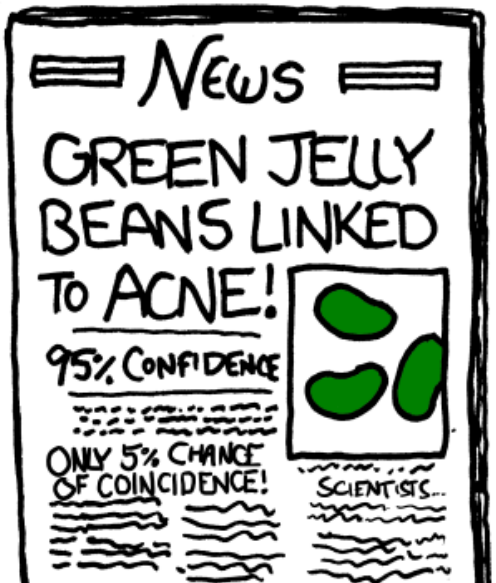
Charybdis

Scylla

Statistical analysis: Charybdis







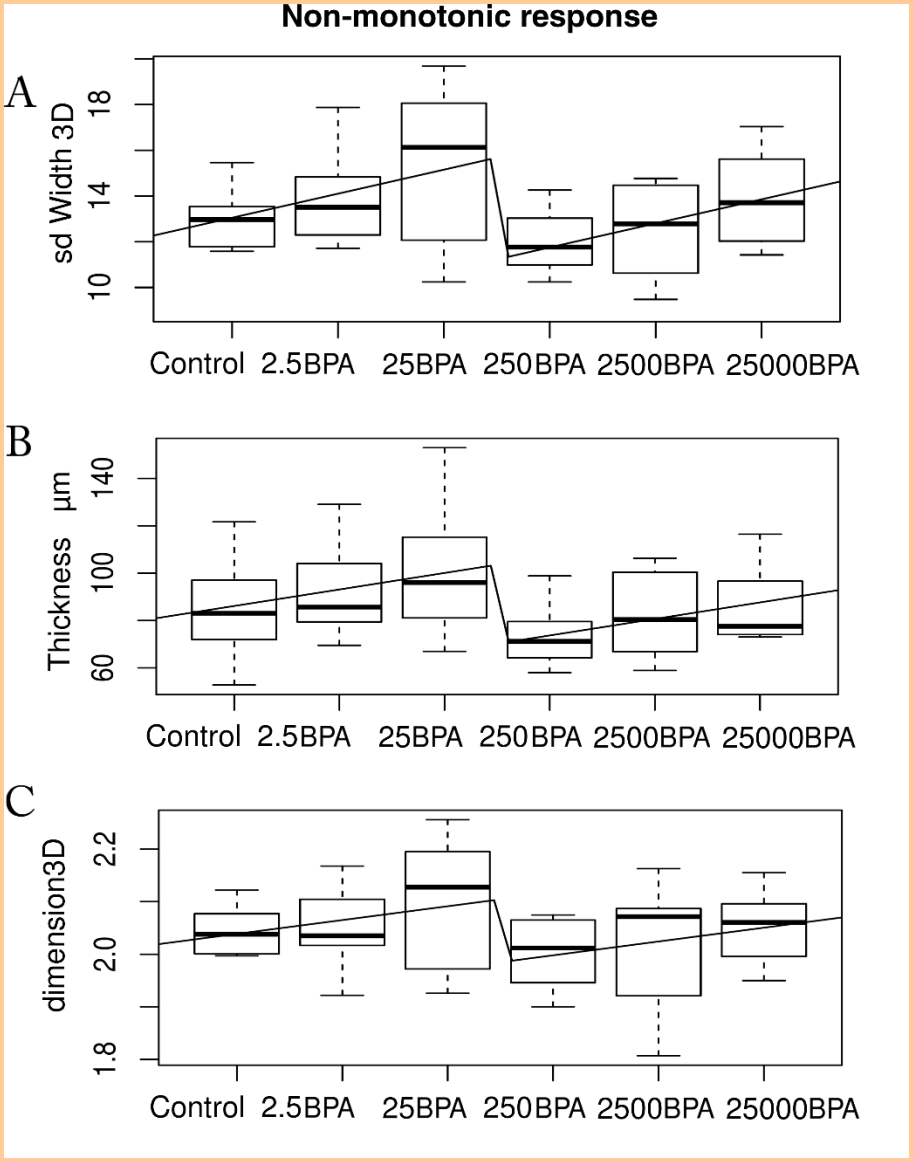
Source https://xkcd.com/882/

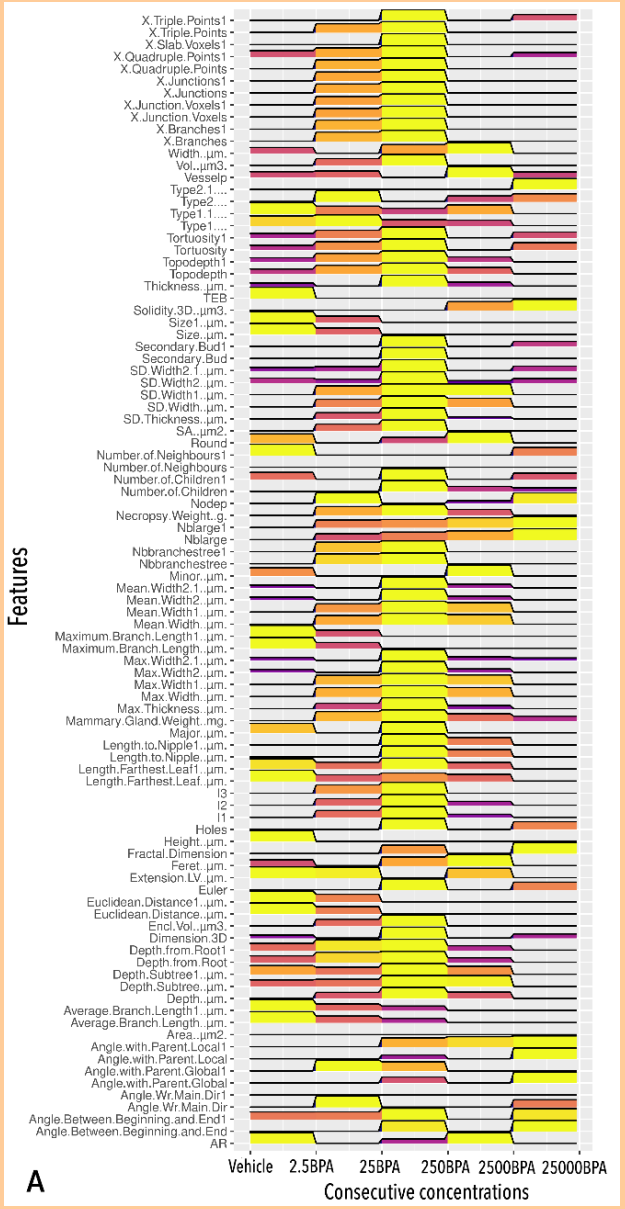
Statistical analysis: Scylla

- **Charybdis**: the more we observe, the more we need to correct for multiple comparisons, the stronger the effects need to be

- **Scylla**: there is a clear method to analyze monotonic response. For non-monotonic response, the fallback method is to perform multiple comparisons with the negative control.
    * We lose the ability to asses the response curve as a whole
    * We lose statistical power by multiple testing

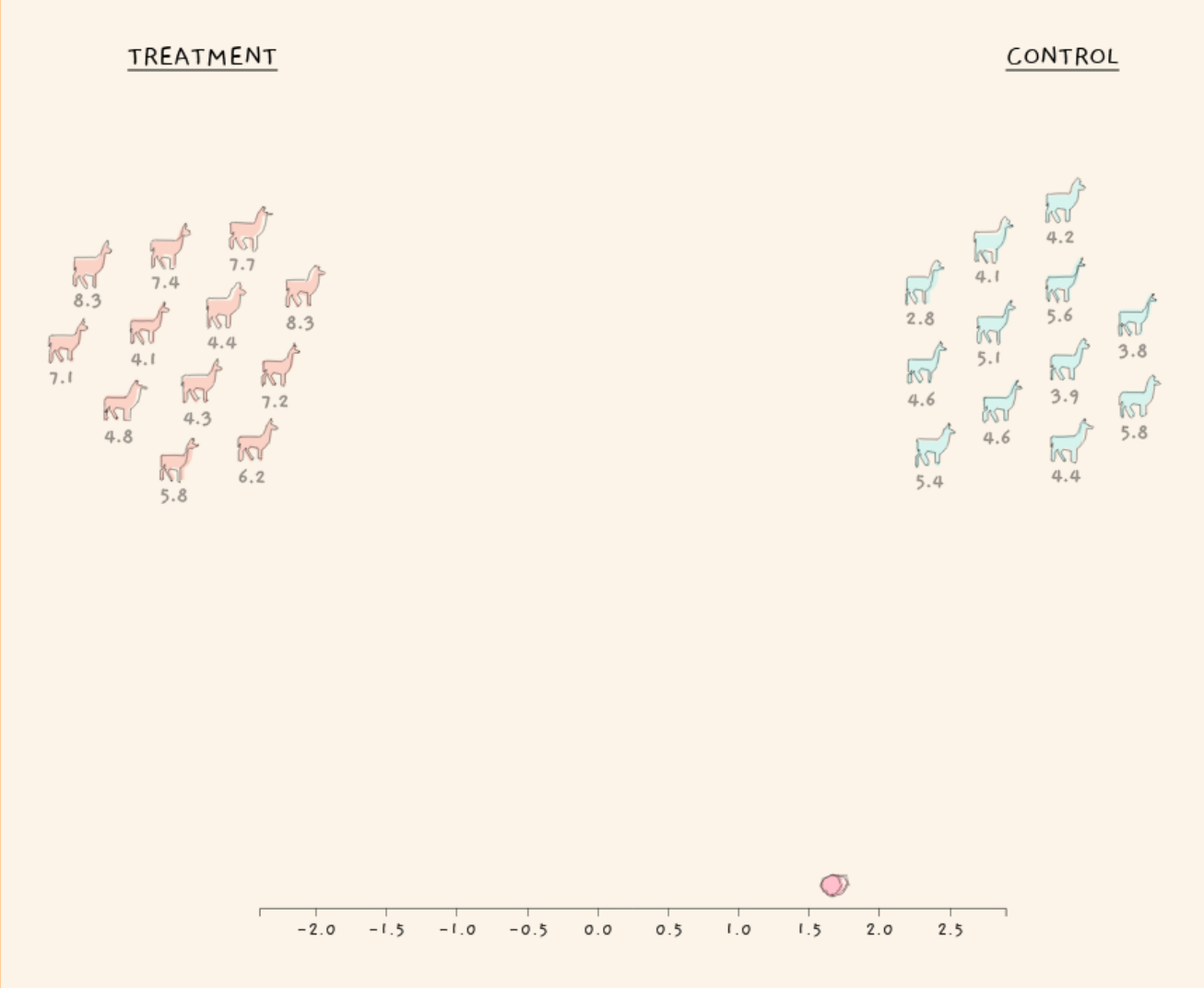
Observations





Method: permutation test

- Define the random variable of interest X
- Compute its value for the experimental data



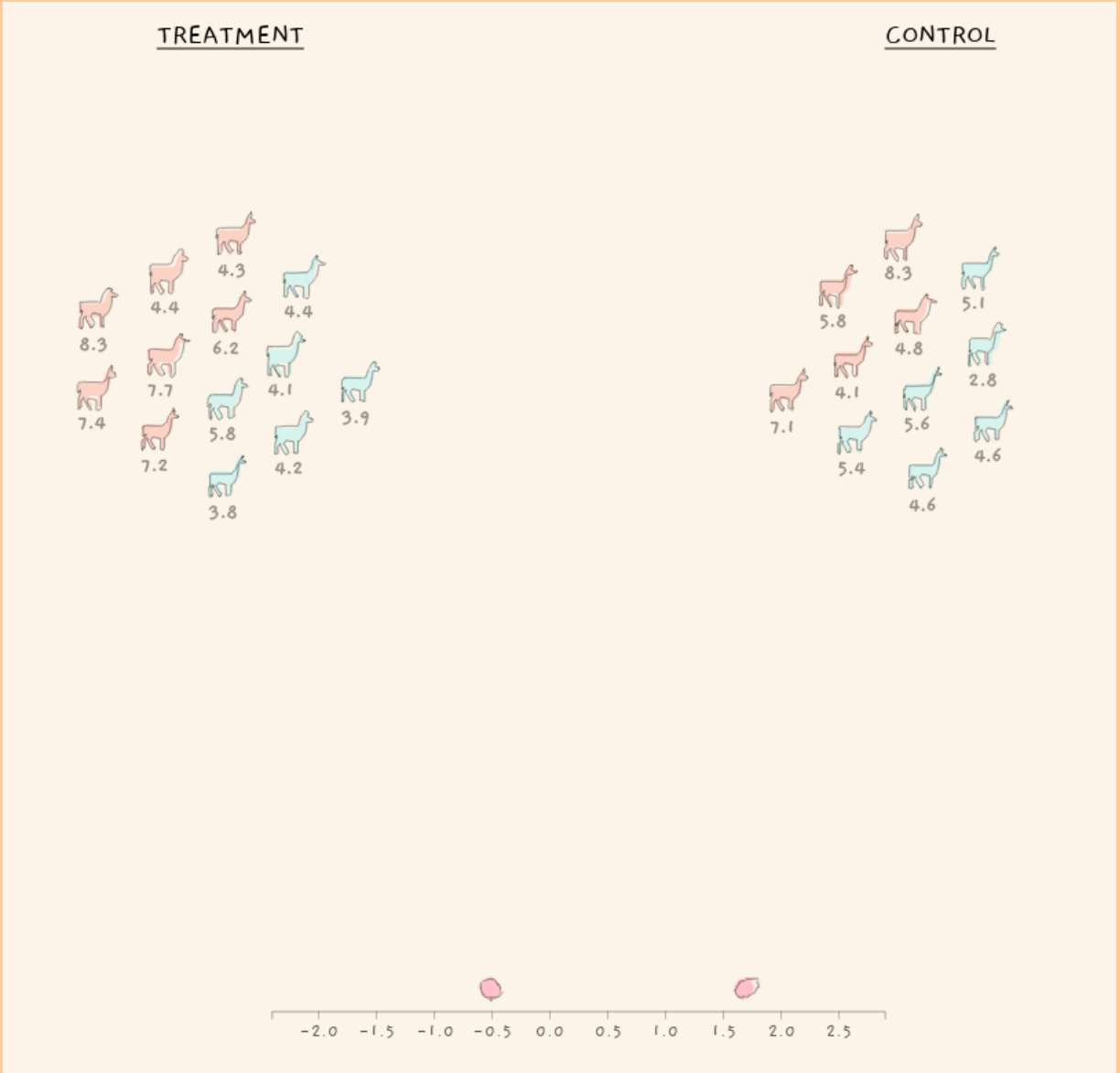
Source https://www.jwilber.me/permutationtest/

Method: permutation test

- Define the random variable of interest X
- Compute its value for the experimental data
- Assign randomly animals to “treatments” groups and compute X

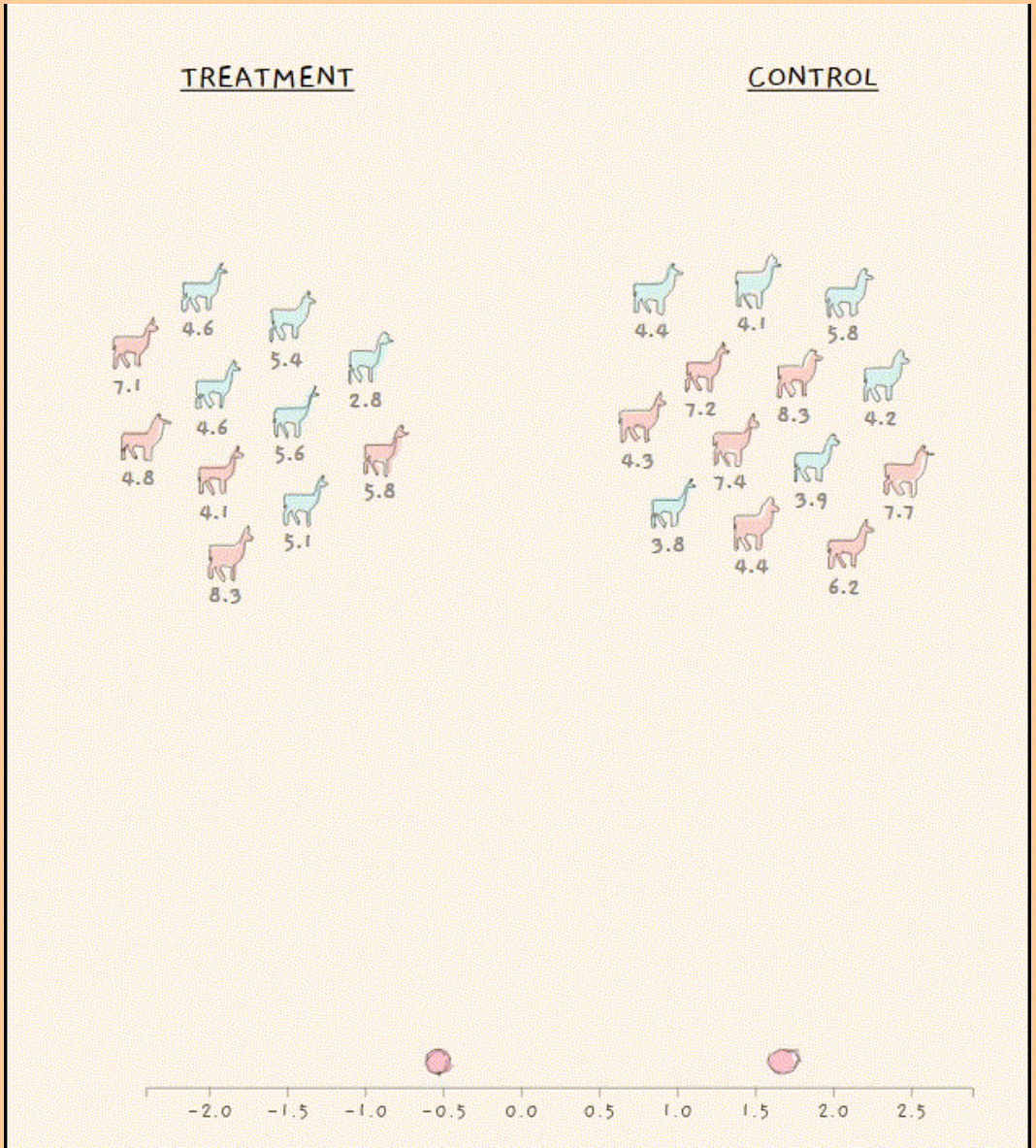
Under the null hypothesis that treatment does nothing, this is a equally probable result.

Other aspects of individuals are unchanged.



Method: permutation test

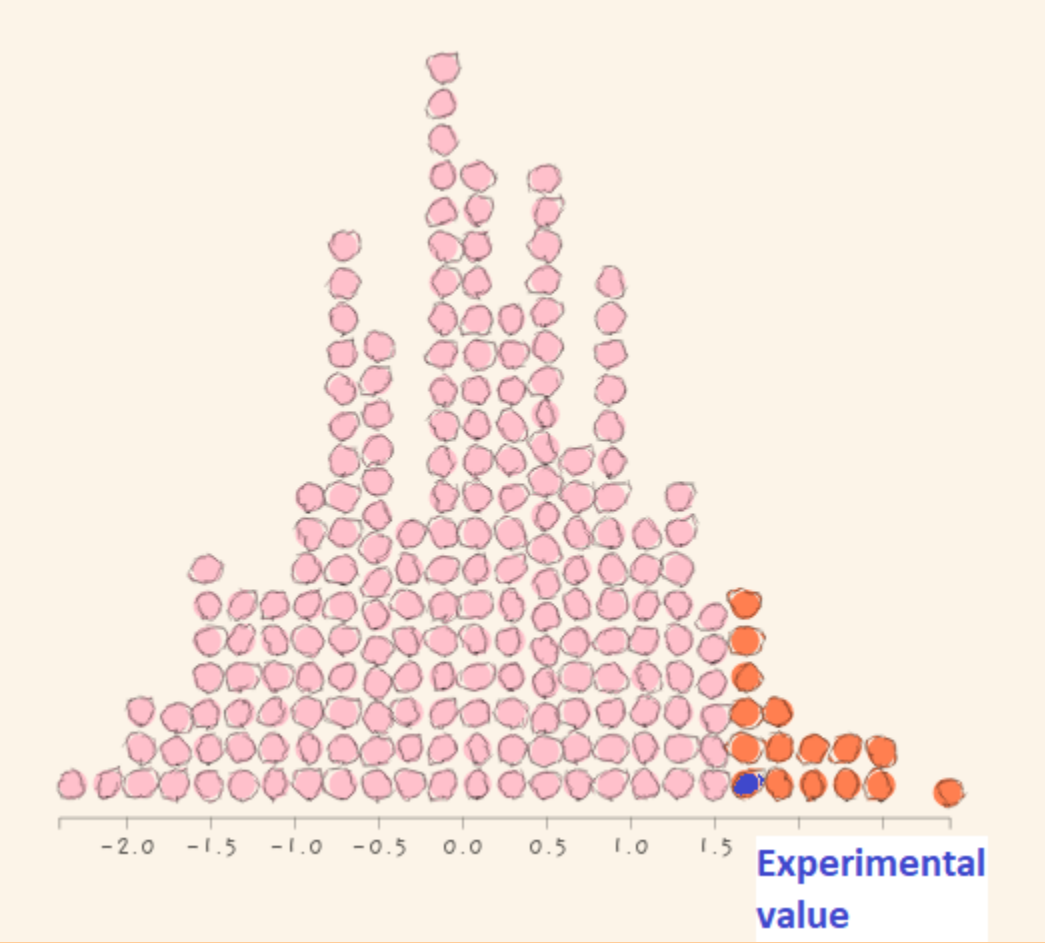
- Define the random variable of interest X
- Compute its value for the experimental data
- Assign randomly animals to treatments groups and compute X
- Iterate many times to generate the statistic of X



Method: permutation test

- How often do we get the experimental value of X by random permutations?

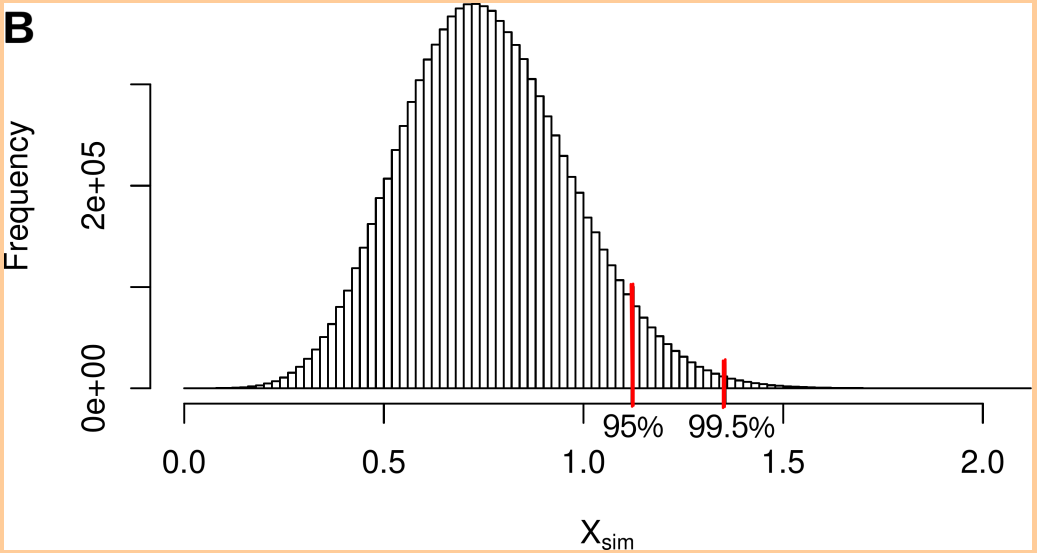
This estimates the p -value under the null hypothesis



Method: permutation test

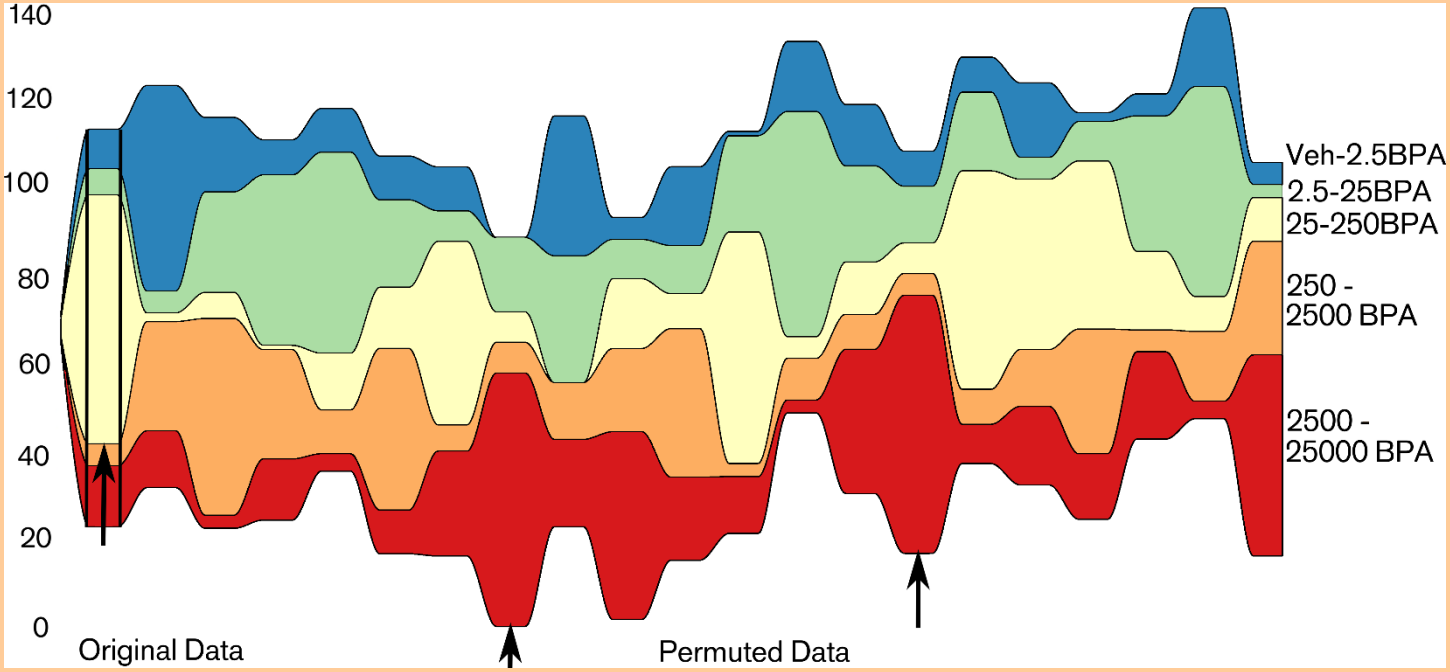
- How often do we get the observed, experimental value of X by random permutations?

This estimates the p -value under the null hypothesis



Results : exploratory analysis

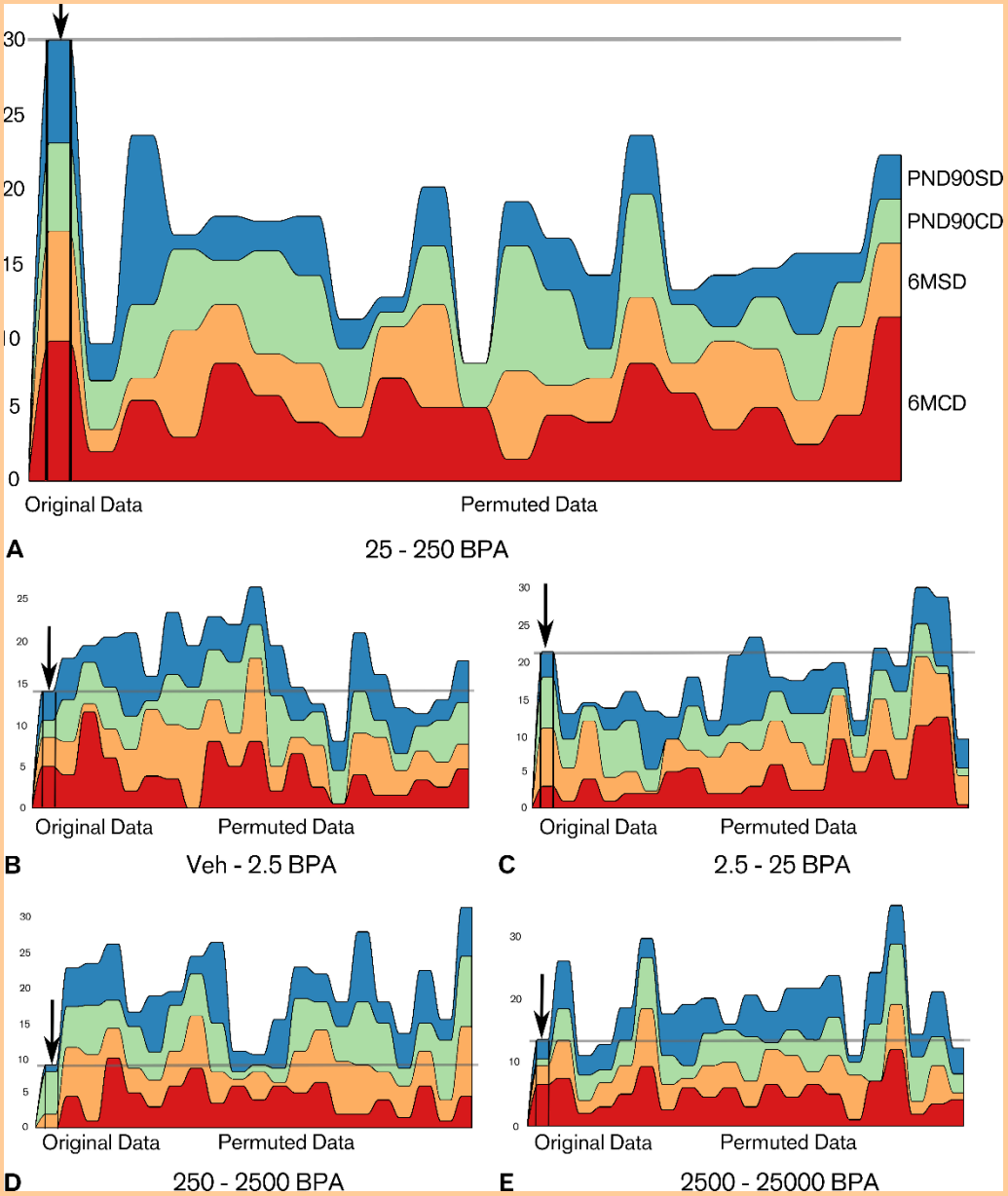
PND21: p = 0.0094



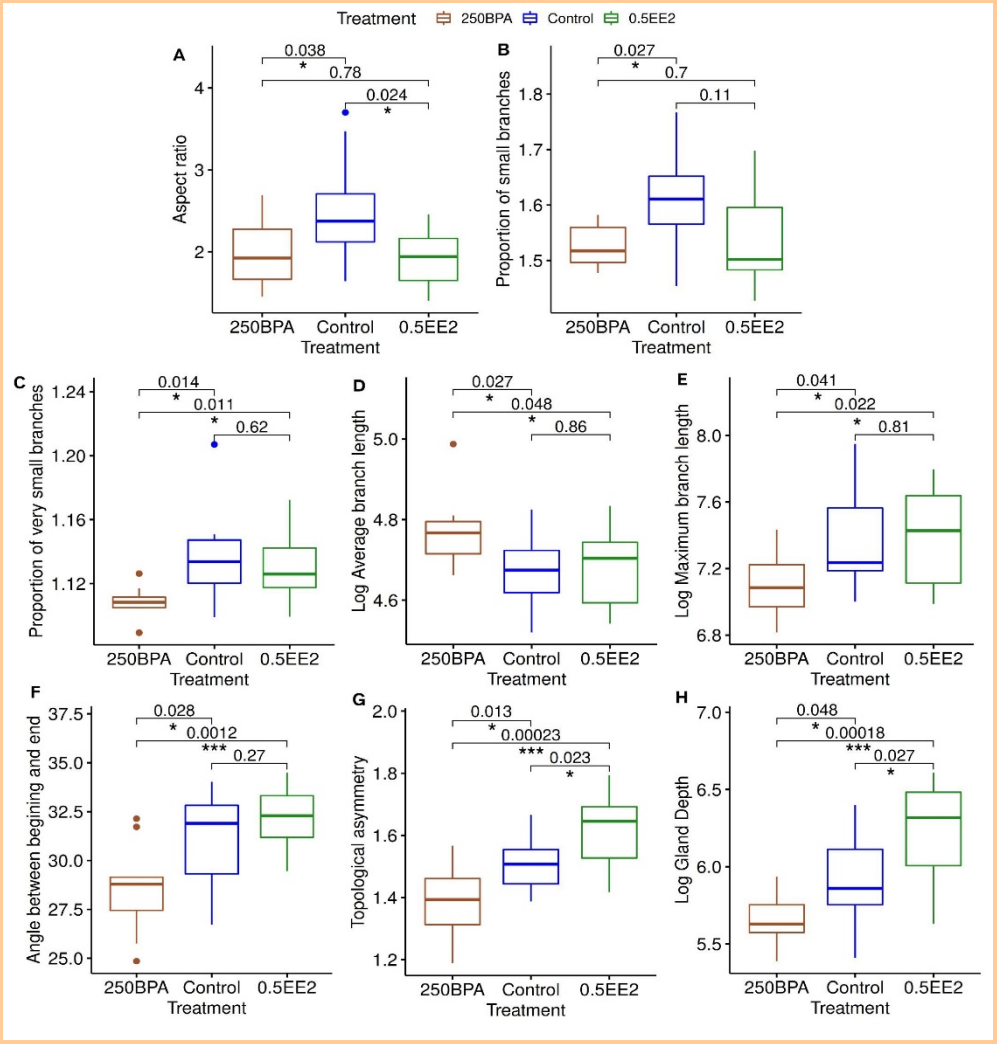
Results : confirmatory analysis

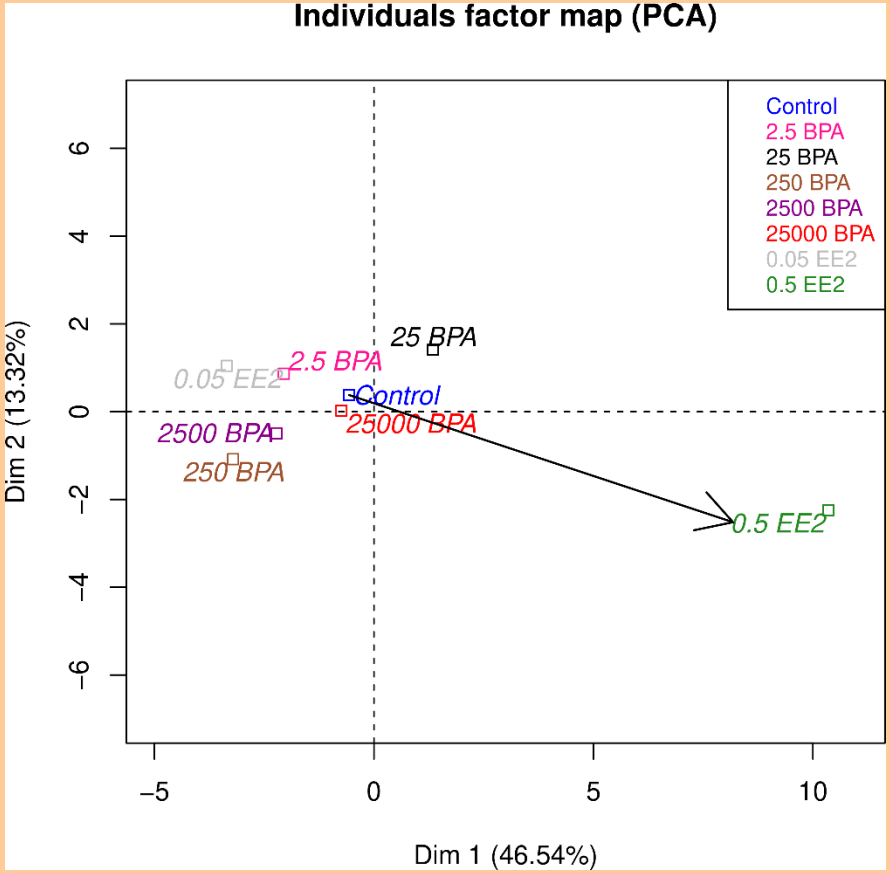
PND90 and 6 months

p = 0.0038



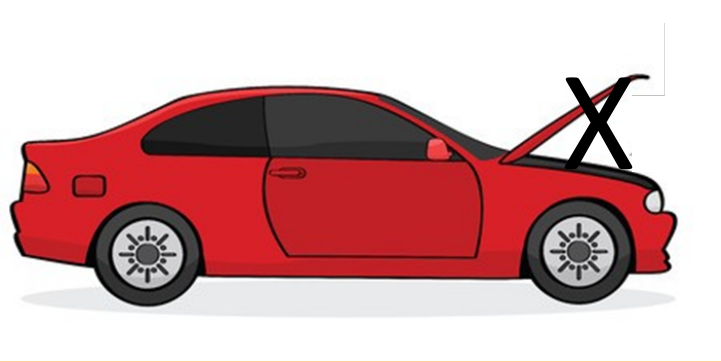
Effects of EE2 versus BPA





Mathematics and mechanisms-I

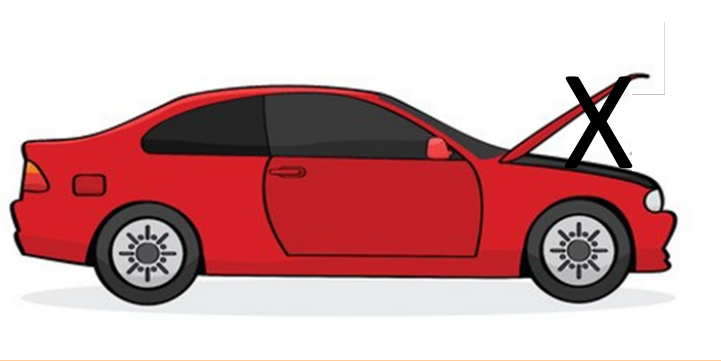
- Linear models are a powerful tool to provide evidence of a causal relationship because they quantitatively relate the changes of a putative cause with the one of the effects.
- Moreover, linear responses to small causes are a common mathematical property albeit not universal.
- Therefore, exhibiting a linear response is a powerful method to provide empirical evidence of a causal relationship in a given context.



No need to look under the hood !!!

Mathematics and mechanisms-II

- NMDRC are common in endocrinology because the putative causes are involved in multilevel, complex regulations due to the evolutionary history of hormones and their functions (Soto et al, J Med Food 1999, Geck et al, PNAS 2000, Villar-Pazos et al Sci Rep 2018).
- In this context, a more appropriate way to show the presence of causation is to show the prevalence of a specific non-monotonic pattern (here a breaking point between 25BPA and 250BPA) using pertinent and robust statistical tools.



No need to look under the hood !!!

Conclusions

This study shows:

- A clear statistical evidence of non-monotonic dose response curves of developmental exposure to BPA for multiple measurements.
- A break point in the dose-response curves between doses of 25 and 250 ug BPA/kg body weight/day
- The occurrence of non-monotonic dose response curves **at all ages** of the animals studied, with the same breaking point.
- That so-called "mechanistic" studies are NOT needed to accept that a NMDRC reflects a causal link to the exposure when the statistical methods of analysis are pertinent and rigorous (ie, similar to MDRC, good stats reveal underlying causal link).

Conclusions

- Using the same set of animals as the core CLARITY-BPA study, this analysis provides a statistical demonstration that the low-dose effects of BPA (e.g. mammary cancer already at 2.5 ug/kg/day) observed in the CLARITY-BPA core study are due to a causal relationship between the dose of BPA administered and its effect.
- This provides a counterpoint to the earlier statements made by the US FDA and National Center for Toxicological Research that the low-dose effects observed were due to random events.
- Our study shows clear statistical evidence that different estrogens can produce either similar or very different effects, depending on the endpoints being measured. This contradicts the hypothesis that BPA and ethinyl estradiol would always have similar effects.