

Introduction to Experimental Design at CRUK-CI

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tinyurl.com/cruk-edesign

Agenda

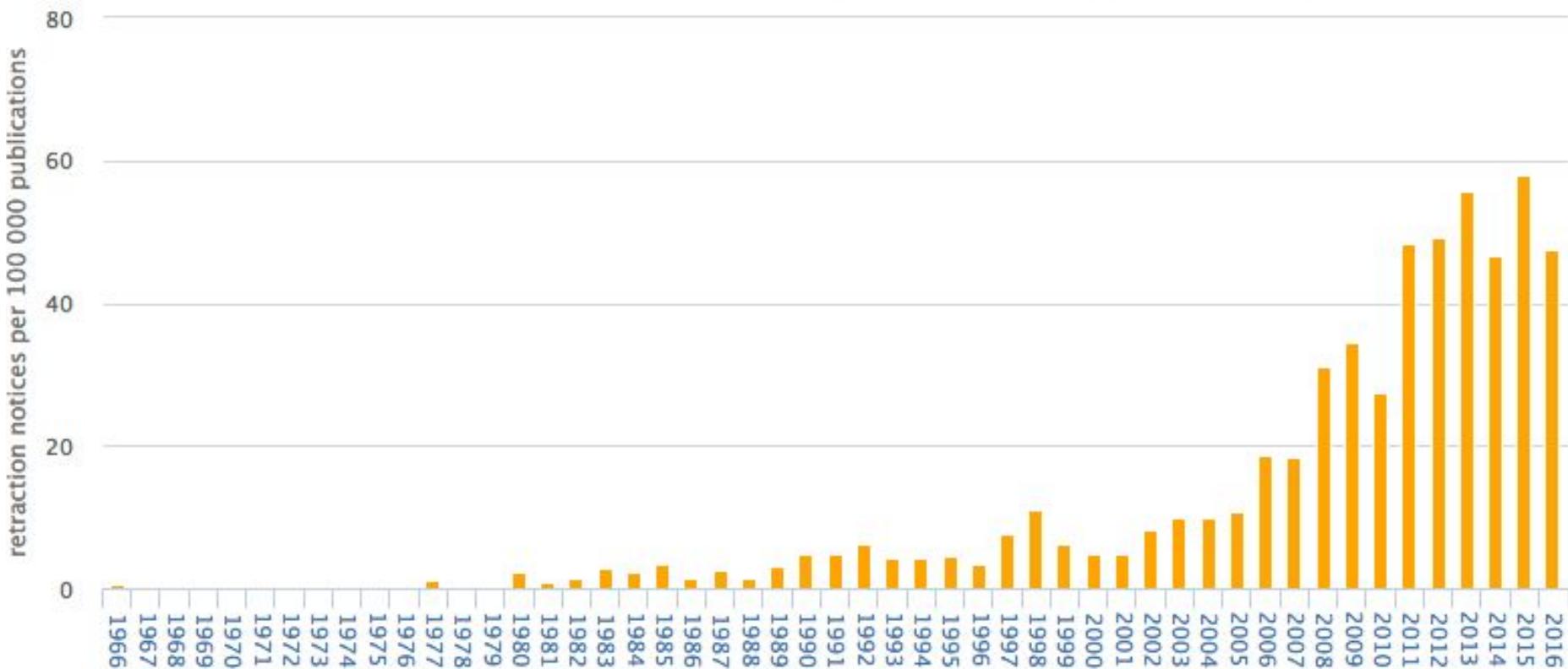
- Why perform experiments?
- Why think about experimental design?
- What makes for a well designed experiment?
- Aspects of experimental design
 - Experimental variables
 - Power: variance and replicates
 - Bias: confounding factors, randomisation, and controls
- Experimental design types
- Experimental design at CRUK-CI

Why Perform Experiments?

Why Think About Experimental Design?

Crises in Reproducible Research!!

Retraction notices per 100 000 publications by year of Entrez record creation



47 of 53 high-profile cancer studies were not reproducible!

The screenshot shows the header of the Nature journal website with the title 'nature International weekly journal of science'. Below the header is a navigation bar with links: Home, News & Comment, Research, Careers & Jobs, Current Issue, Archive, Audio & Video, and a partially visible link starting with 'F'. Below the navigation bar is a breadcrumb trail: Archive > Volume 483 > Issue 7391 > Comment > Article. The main content area features the title 'NATURE | COMMENT' followed by the article title 'Drug development: Raise standards for preclinical cancer research' by C. Glenn Begley & Lee M. Ellis. To the right of the title are three sharing icons: a person icon, an envelope icon, and a square icon.

Drug development: Raise standards for preclinical cancer research

C. Glenn Begley & Lee M. Ellis

Affiliations | Corresponding author

Nature 483, 531–533 (29 March 2012) | doi:10.1038/483531a

Published online 28 March 2012

Consequences of Poor Experimental Design...

- **Cost** of experimentation. We have a responsibility to CRUK donors!
- **Limited & Precious** material, esp. clinical samples.
- **Immortalization** of data sets in public databases and methods in the literature. Our bad science begets more bad science.
- **Ethical concerns** of experimentation: animals and clinical samples.

A Well-Designed Experiment

Should have

- Clear objectives
- Focus and simplicity
- Sufficient power
- Randomised comparisons

And be

- Precise
- Unbiased
- Amenable to statistical analysis
- Reproducible

Ronald A. Fisher(1890-1962)



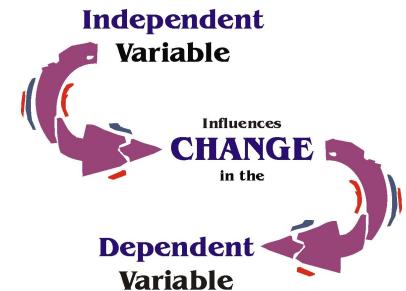
*“To consult the statistician **after** an experiment is finished is often merely to ask him to conduct a **post mortem** examination. He can perhaps say what the experiment died of.” (1938)*

Aspects of Experimental Design

- Experimental Factors
- Power
 - Sources of Variance
 - Replicates
- Minimising Bias
 - Confounding factors
 - Randomisation wherever a decision is to be made
 - Controls for both measured and unmeasured factors
 - Controls

Experimental Factors

- Factors: aspects of experiment that change and **influence the outcome** of the experiment
 - e.g. time, weight, drug, gender, ethnicity, country, plate, cage etc.
- Variable type depends on type of measurement:
 - Categorical (**nominal**), e.g. gender
 - Categorical with ordering (**ordinal**), e.g. tumour grade
 - **Discrete**, e.g. shoe size, number of cells
 - **Continuous**, e.g. body weight in kg, height in cm
- Independent and Dependent variables
 - Independent variable (IV): what you change
 - Dependent variable (DV): what changes due to IV
 - “**If (independent** variable), **then (dependent** variable)”

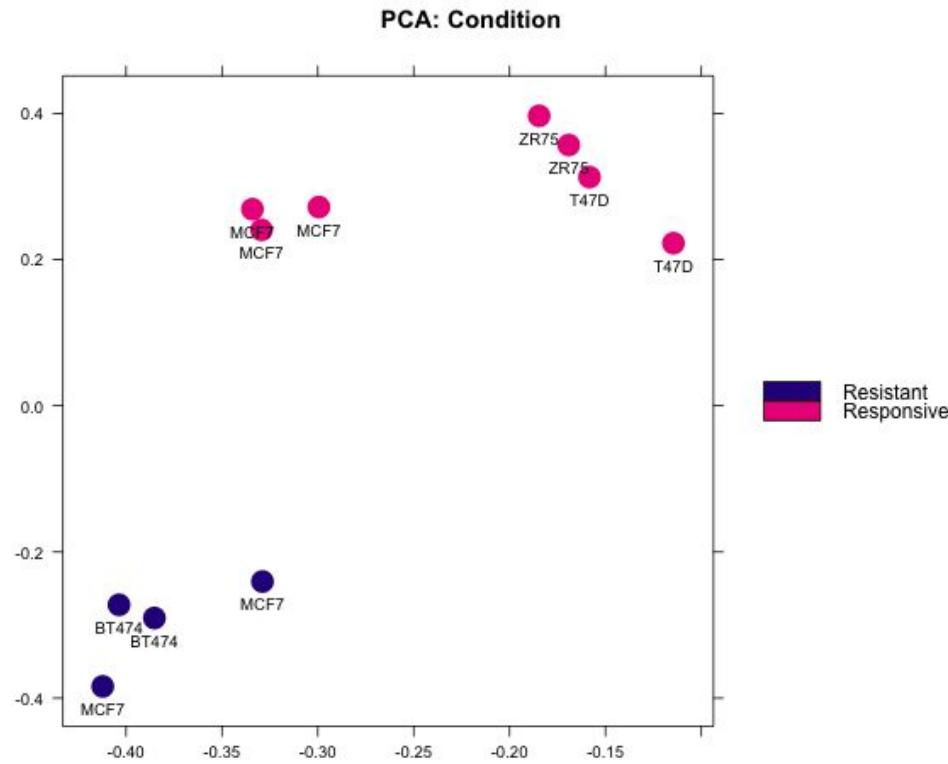


Sources of Variation

- Biological “noise”
 - Biological processes are inherently stochastic
 - Single cells, cell populations, individuals, organs, species....
 - Timepoints, cell cycle, synchronized vs. unsynchronized
- Technical noise
 - Reagents, antibodies, temperatures, pollution
 - Platforms, runs, operators
- Consider in advance and control

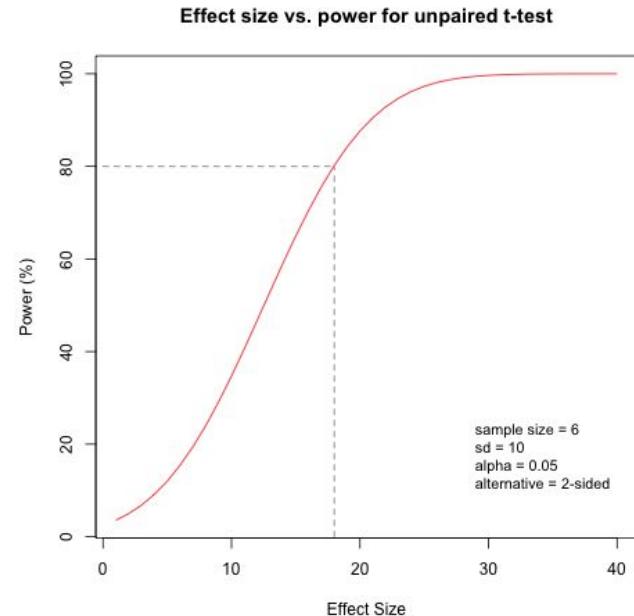
Types of Replication

- Biological replication:
 - *In vivo*:
 - Patients
 - Mice
 - *In vitro*:
 - Different cell lines
 - Re-growing cells (passages)
- Technical replication:
 - Experimental protocol
 - Measurement platform (i.e. sequencer)



How many samples?

- Why do you need replicates?
- Calculating appropriate sample sizes
 - Power calculations
 - Planning for precision
 - Resource equation
- Power: the **probability** of detecting an **effect** of a specified size if present.
 - Identify and control the **sources of variability**
 - Biological variability
 - Technical variability
 - Using **appropriate numbers** of samples (sample size/replicates)
 - Power calculations estimate sample size required to detect an effect *if degree of variability is known*
 - Depends on δ , n, sd, α , H_A
 - If adding samples increases variability, that alone won't add power!



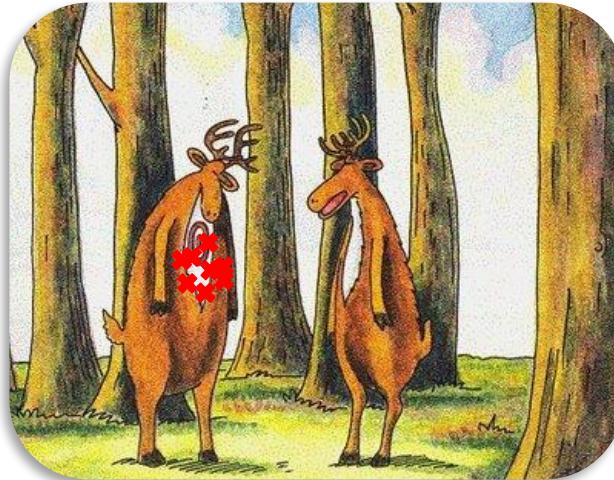
Forms of Bias

Type of Bias	Description
Selection bias	Systematic differences between baseline characteristics or treatment groups that are being compared.
Performance bias	Systematic differences between groups in exposure to factors other than the interventions of interest (aka confounding or extraneous factors).
Attrition bias	Systematic differences between groups due to samples being withdrawn from the study or excluded from the analyses.
Detection or Measurement bias	Systematic differences between groups in how outcomes are assessed or determined, e.g. measurement errors and inefficient use of data.
Reporting bias	Systematic differences between reported and unreported findings due to manipulation in the reporting of findings such as selective or distorted reporting , e.g. papers with more 'interesting results' are more likely to be submitted and accepted for publication.

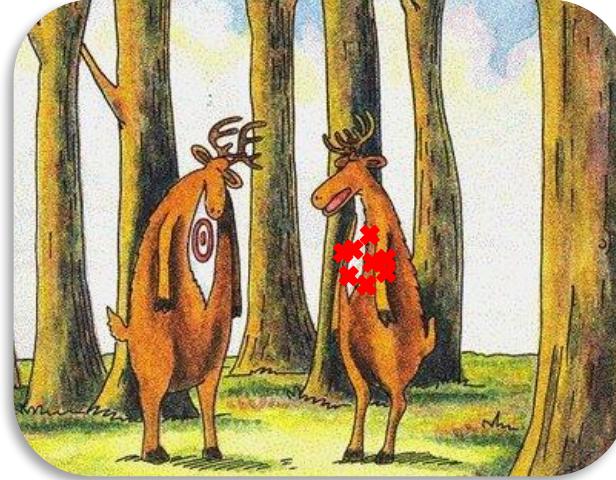
Precision, Accuracy & Bias

Precise

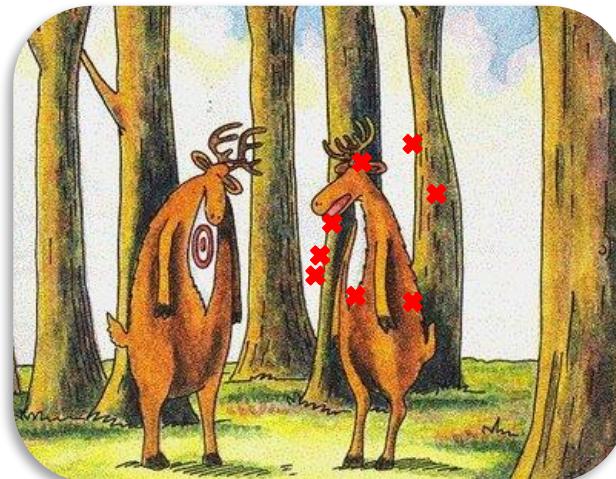
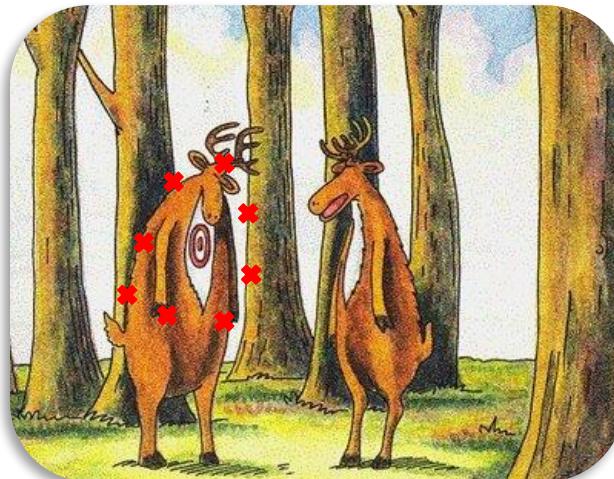
Accurate



Biased

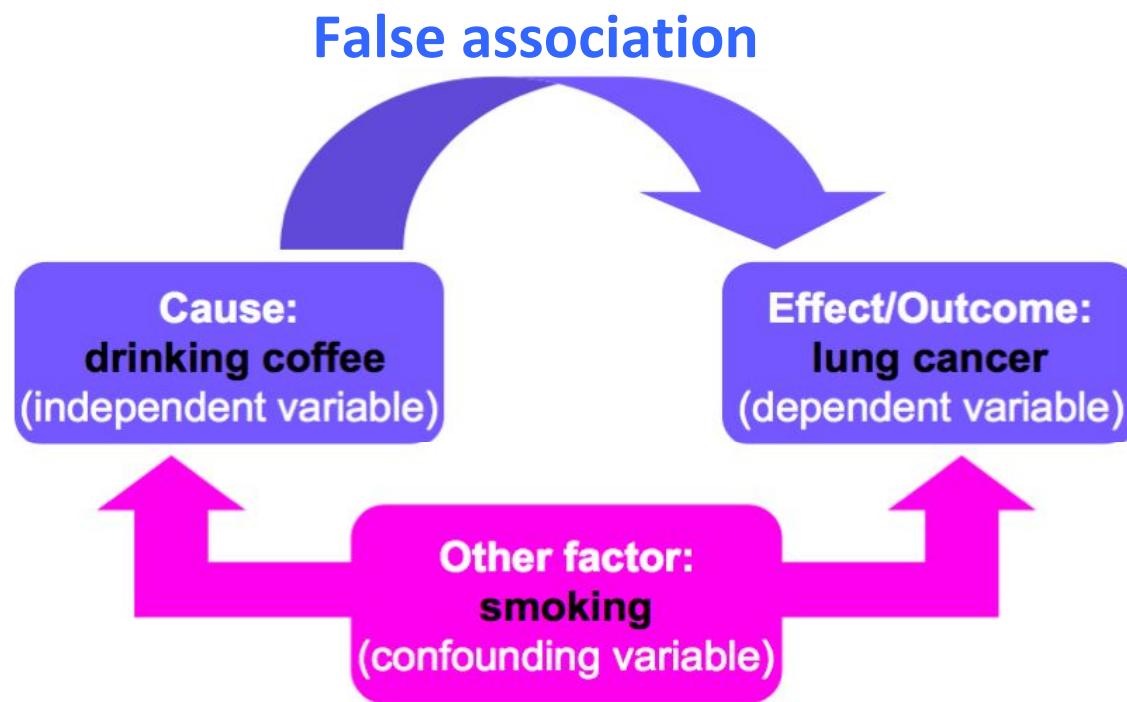


Imprecise



Confounding Factors

- Also known as **extraneous, hidden, lurking or masking factors**, or the **third variable or mediator variable**.
- May mask an actual association or **falsely** demonstrate an apparent association between the independent & dependent variables.
- Hypothetical Example would be a study of coffee drinking and lung cancer.



Confounding Factors

- Other examples:
 - Democrats were less satisfied with their sex lives than Republicans.
(ABC poll report).
 - Slightly overweight people live longer than thin people
(US Centre for Disease Control).
- **Inadequate management and monitoring** of confounding factors
 - one of the most common causes of researchers wrongly assuming that a correlation leads to a causality.
- If a study does not consider confounding factors,
don't believe it!

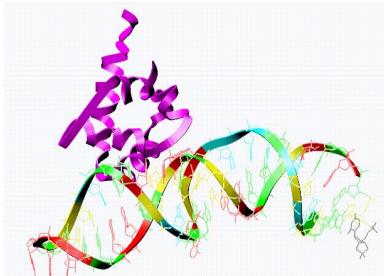
Genetic Signatures of Exceptional Longevity in Humans

Paola Sebastiani,^{1,*} Nadia Solovieff,¹ Annibale Puca,² Stephen W. Hartley,¹ Efthymia Melista,³ Stacy Andersen,⁴ Daniel A. Dworkis,³ Jemma B. Wilk,⁵ Richard H. Myers,⁵ Martin H. Steinberg,⁶ Monty Montano,³ Clinton T. Baldwin,^{6,7} Thomas T. Perls^{4,*}

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- GWAS study: 800 centenarians vs. controls
- Found 150 SNPs predicting centenarians with 77 % accuracy
- Problem: they used **different SNP chips** for centenarians and controls
- Retracted in 2011 following independent review and QC of data

Technical Confounding Factors: Batch Effects



RNA Extraction

Day1, Plate 1

	1	2	3	4	5	6	7	8	9	10	11	12
A	○											
B												
C												
D												
E												
F												
G												
H												

Control

Day2, Plate 2

	1	2	3	4	5	6	7	8	9	10	11	12
A	○											
B												
C												
D												
E												
F												
G												
H												

Treatment 1

Day3, Plate 3

	1	2	3	4	5	6	7	8	9	10	11	12
A	○											
B												
C												
D												
E												
F												
G												
H												

Treatment 2

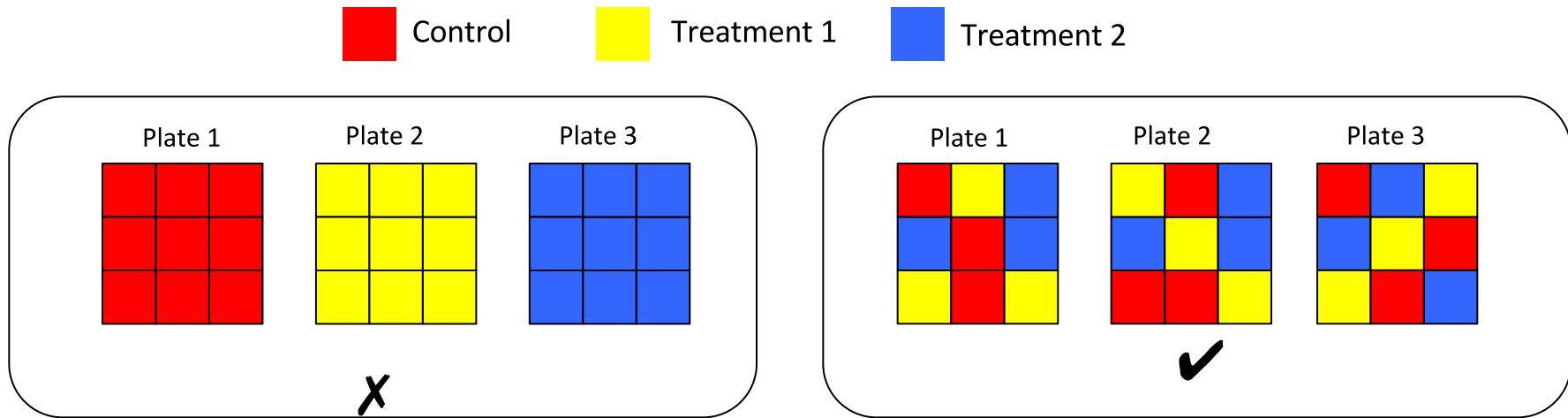
The difference between Control, Treatment 1
and Treatment 2 is confounded by **day** and **plate**.

Solutions

- Randomisation
 - Statistical analysis assume randomised comparisons
 - May not see issued caused by non-randomised comparisons
 - Make every decision random not arbitrary
- Blinding
 - Especially important where subjective measurements are taken
 - Every experiment should reach its potential degree of blinding

Randomised Block Design

- **Blocking** is the arranging of *experimental units* in groups (blocks) that are similar to one another.



- RBD across plates so that each plate contains spatially randomised **equal proportions** of:
 - Control
 - Treatment 1
 - Treatment 2controlling plate effects.

Randomised Block Design

Good design example: Alzheimer's study from GlaxoSmithKline

Plate effects by *plate*

Left PCA plot show *large plate effects*.
Each colour corresponds to a different plate

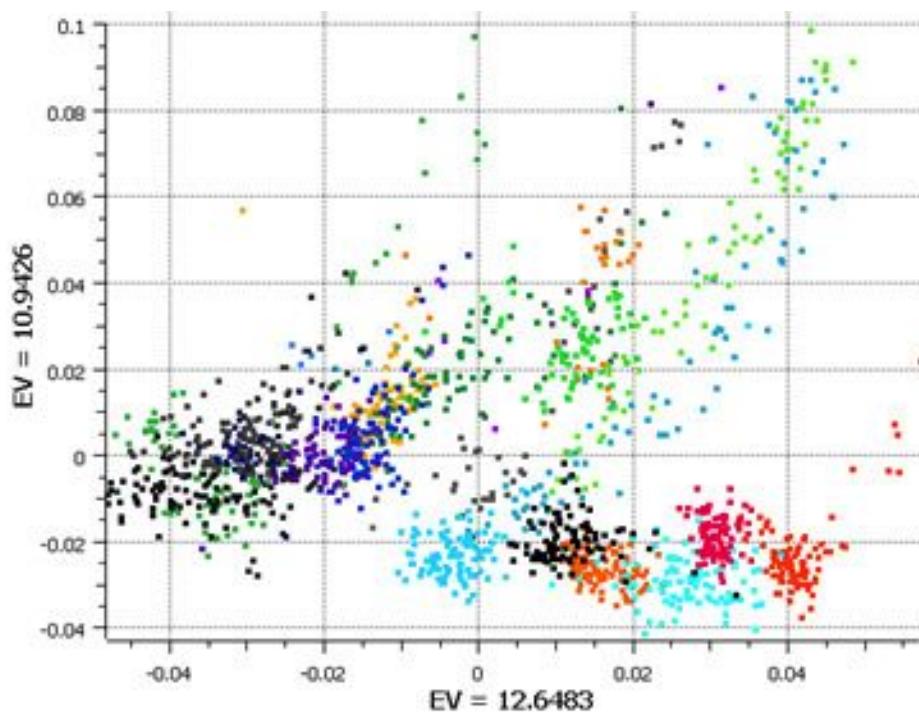
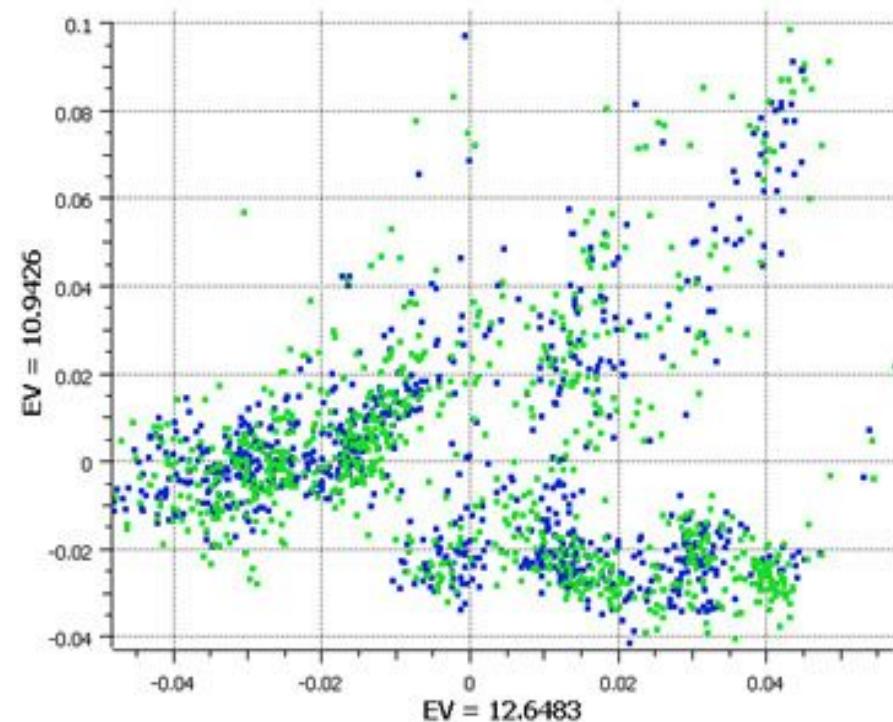


Plate effects by *case/control*

Right PCA plot shows each plate cluster contains *equal proportions* of cases (blue) and controls (green).



Experimental Controls

- Controlling errors
 - Type I: FP
 - Negative controls: should have minimal or no effect
 - Type II: FN
 - Positive controls: known effect
- Technical controls
 - Detect/correct technical biases
 - Normalise measurements (quantification)

Examples of Experimental Controls

- Wild-type organism (knockouts)
- Inactive siRNA (silencing)
- Vehicle (treatments)
- Input: fragmented chromatin (ChIP)
- Spike-ins (quantification/normalisation)
- “Gold standard” datapoints
- Multi-level controls
 - e.g. contrast Vehicle/Input vs. Treatment/Input

Design Issues: Sequencing Experiments

- Platforms (MiSeq, HiSeq, etc.)
- Library preps
- Multiplexing and pooling strategies
- Single-end vs paired end
- Sequencing depth
 - Coverage
 - Lanes
- Validation

Types of Experimental Designs

- Full random: compute associations after
- Block designs: randomisation
- Matched: tumour/normal
- Factorial/multifactorial designs: GLM
- Time series
- Hierarchical designs

CRI Experimental Design Meetings

- **Tuesday** 30 min slots (2:00-3:00pm) with Bioinformatics & Genomics Cores
- **Requirements:**
 - Email CRIExperimentalDesign@cruk.cam.ac.uk to request meeting
 - Fill in Experimental Design Form and return 1 week prior to meeting
 - **Your attendance**
 - Provide **project background** (a few slides from you)
- **Discussion:**
 - Planning, time-scale, cost, aims, scope, questions
 - Choosing the correct technology
 - Technical issues e.g. what sequencing depth?
 - Sample collection and processing methods
 - Sample information (meta-data) collection
 - Randomisation, Blocking and Replication issues
 - Analyst?
 - Pilot study?
 - Effect size & Sample-size calculation?

Experimental Design Guide

- <https://rawgit.com/bioinformatics-core-share-d-training/experimental-design/master/ExperimentalDesignManual.pdf>

tinyurl.com/cruk-edesign

Practicals

1. Clinical: Identification of prognostic biomarkers in human prostate cancer patients (Rory)
2. Animal: Effects of mutant vs wildtype HHEX in liver and brain development (Jing)
3. RNA-seq: effect of silencing a transcription factor on gene expression in a breast cancer cell line (James)
4. ChIP-seq: Transcription factor binding divergence in mice (Chandu)