

Tools for comparative metagenomics

Georg Zeller

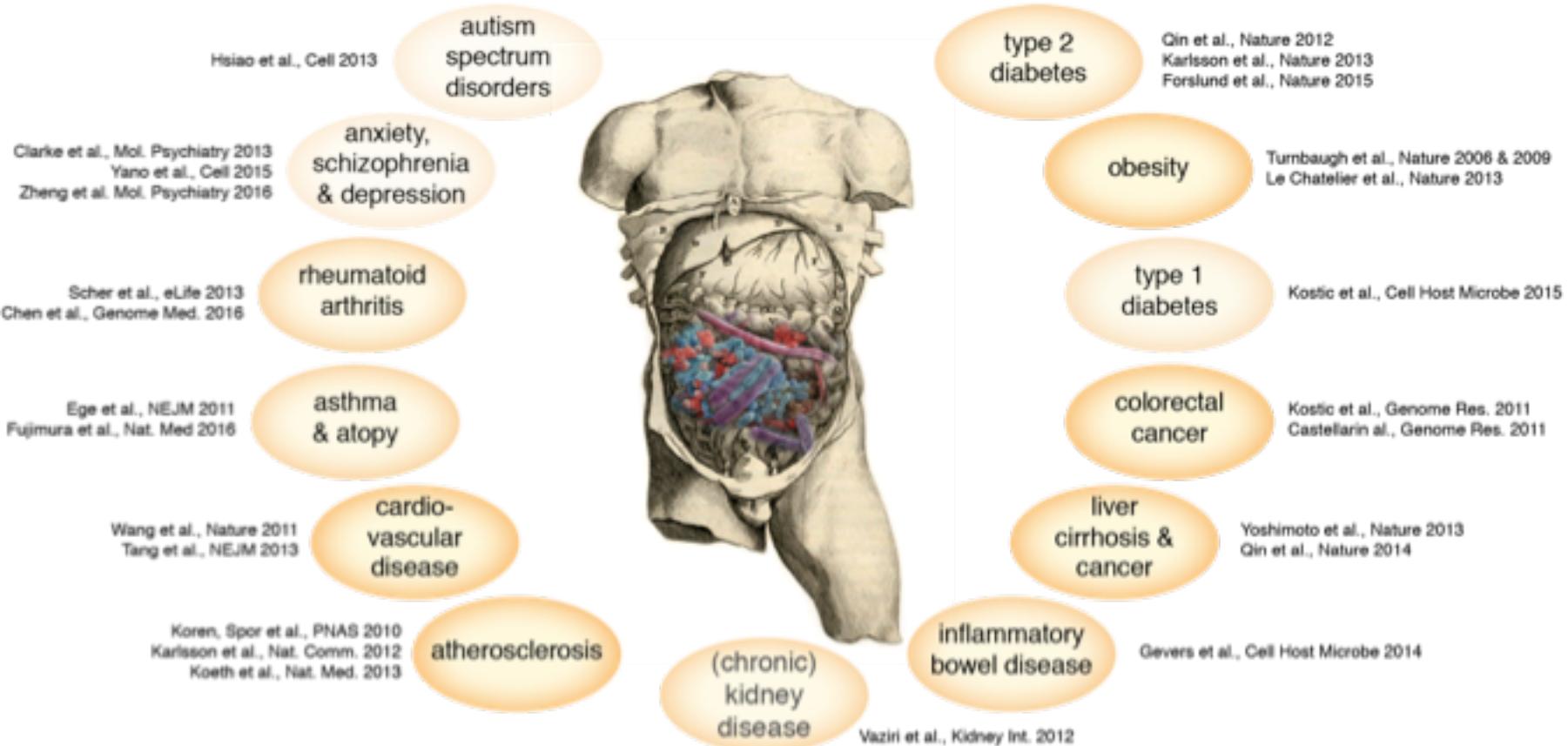
Biological diversity of microbial ecosystems



Comparative metagenomics – Exploring biological factors affecting microbiome composition

- How do microbial communities **differ between habitats?**
 - E.g.: mouse vs. human gut, farm soil vs. grassland
- What **external factors are associated** with community composition?
 - E.g. ocean temperature & depth, soil pH, human diseases
- Is a microbial community **stable over time, how does it cope with environmental fluctuations?**
 - E.g. seasonality, host age, global warming, changing diets, co-evolution with host
- How do microbial communities **respond to perturbations?**
 - E.g. antibiotics and other drug treatments, fertilization, pollution, pathogen invasion

Comparing microbiome composition in case-control studies (disease associations)



Compare the microbiome of a patients (cases) to appropriate unaffected volunteers (controls)
and identify changes in microbiome composition between groups

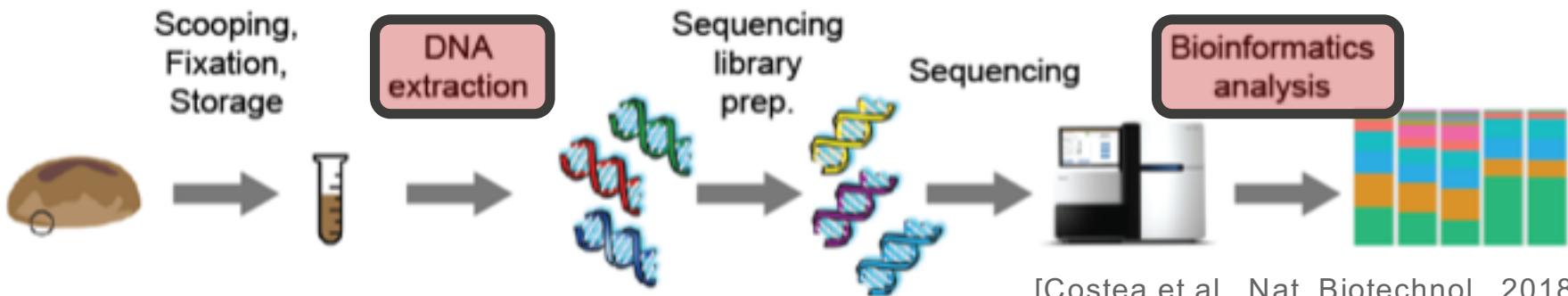


Technical and biological effects on community composition can be challenging to deconvolute

- Technical factors can strongly affect microbial community profiles (**batch effects**), e.g. DNA extraction protocols, sequencing approach (16S primers), bioinformatics profiling
- Biological factors other than that of interest can affect profiles (**confounders**): E.g Medication, lifestyle, host demographics



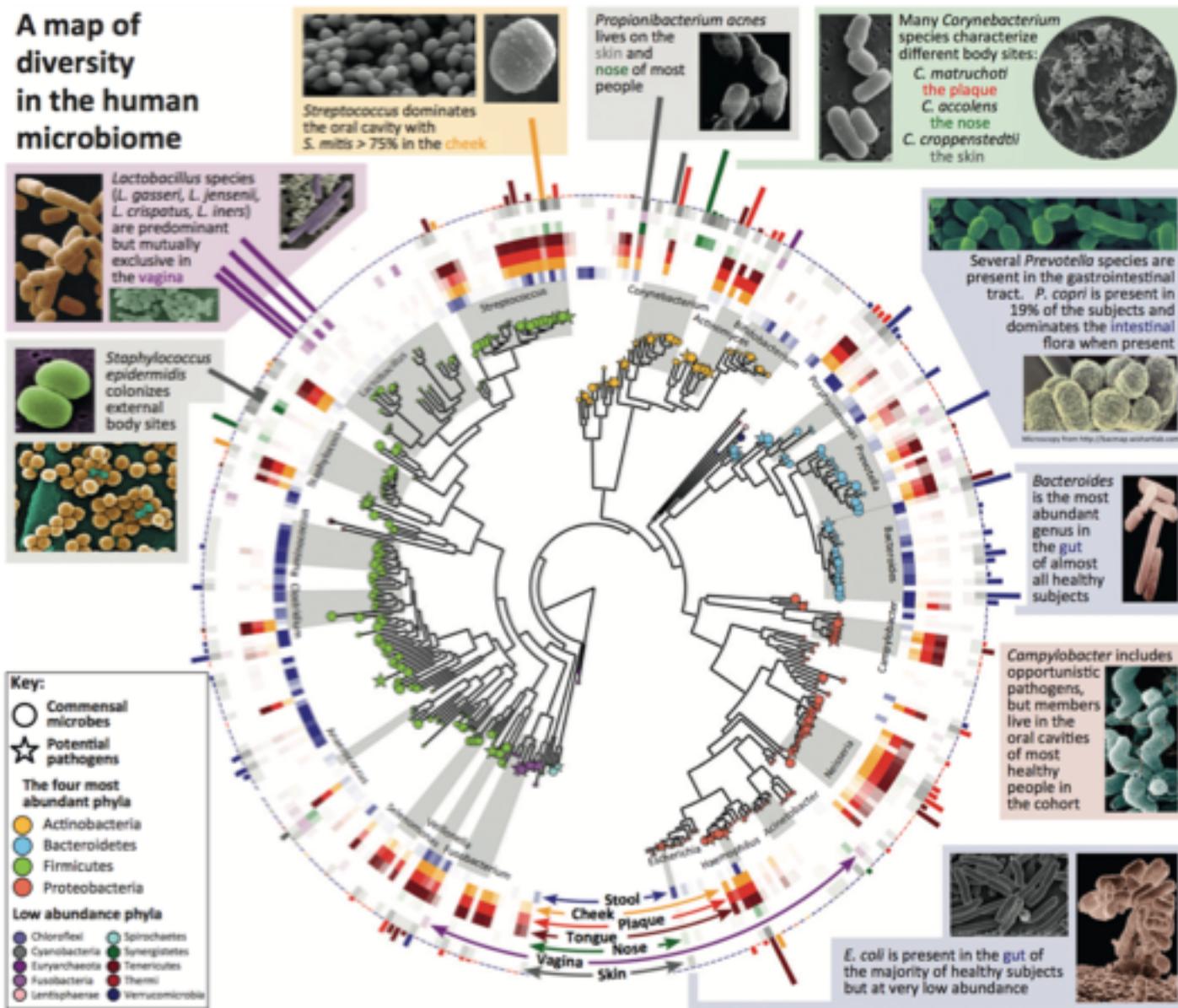
[Schmidt et al., Cell, 2018]



PART I: **EXPLORATION, VISUALIZATION & TESTING**

Let's start with visualization...

A map of diversity in the human microbiome



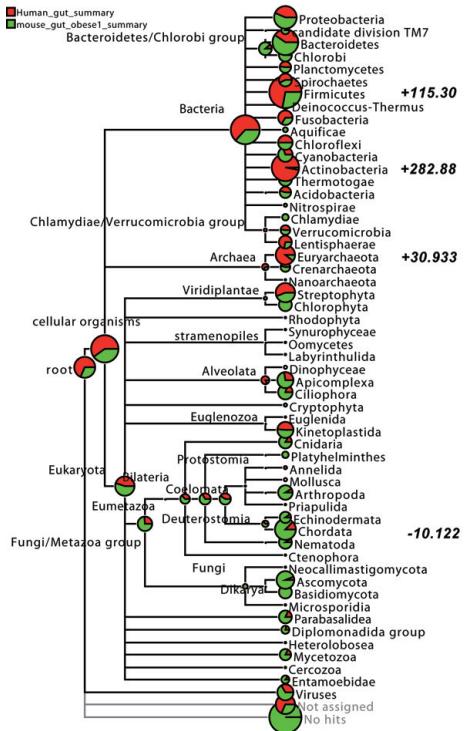
Visual community comparison

Relative abundance of taxa as

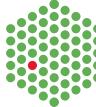
- Heatmap (taxa vs environment/conditions)
 - Bar or pie charts
 - Alongside a phylogenetic or taxonomic tree
- Important limitation:
variance between samples of the
same group is concealed by “averaging”

How to visualize sample-to-sample variance?

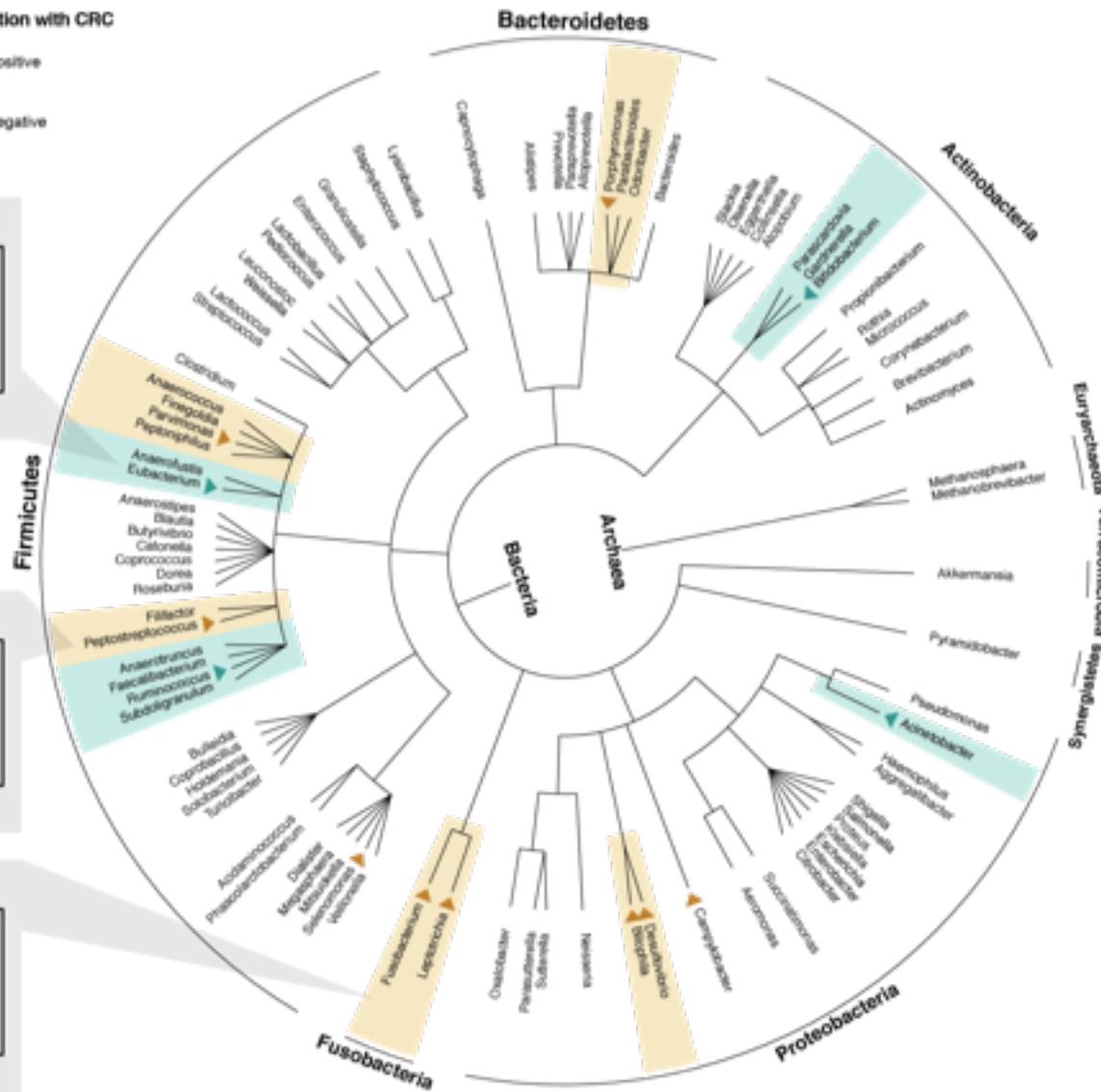
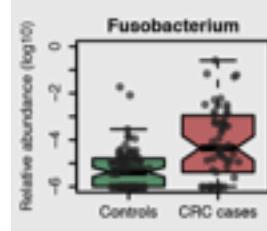
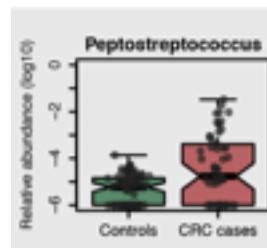
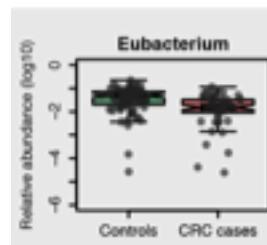
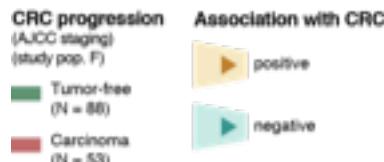
- Boxplots (visual account of variance within groups)
- Heatmaps (taxa vs samples, grouped by condition)
- Ordination (embedding high-dimensional space in 2 or 3D)



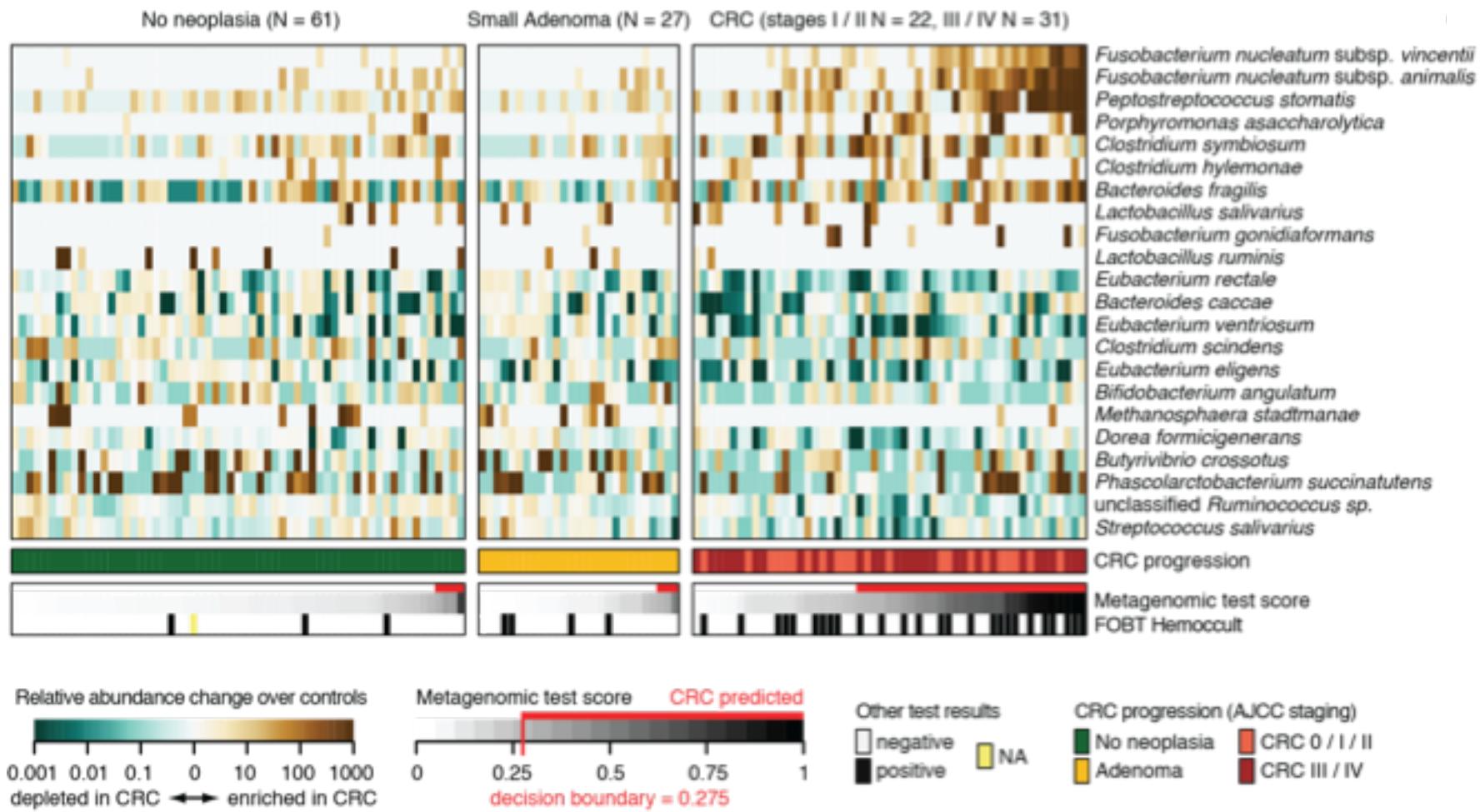
[Fig. from Huson et al. BMC Bioinform 2009; MEGAN]



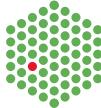
Example I: Colorectal-cancer associated gut microbiome alterations visualized on taxonomic tree



Example II: Heatmap of gut microbiome changes in colorectal cancer



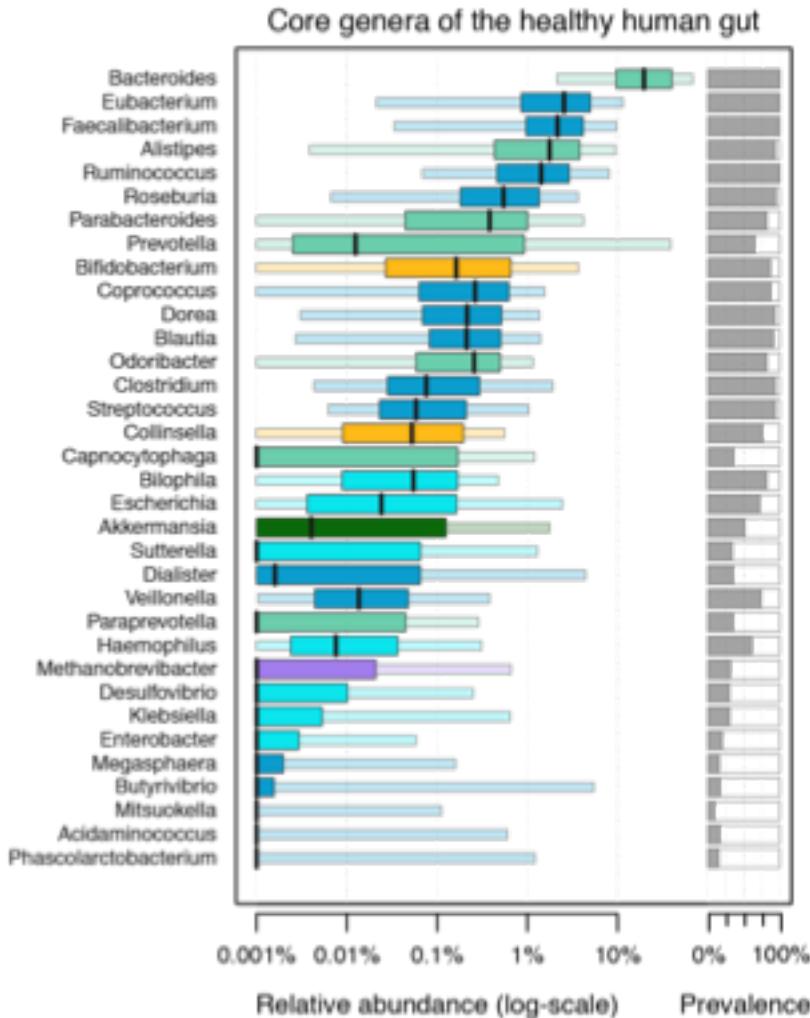
[Zeller*, Tap*, Voigt* et al., Mol. Syst. Biol. 2014; SIAMCAT]



Example III: Individuality of the gut microbiome

Based on 95 most abundant and prevalent gut species from 364 healthy individuals (stool samples, 3 continents)

- Bacteroidetes
- Firmicutes
- Proteobacteria
- Actinobacteria
- Verrucomicrobia
- Euryarchaeota



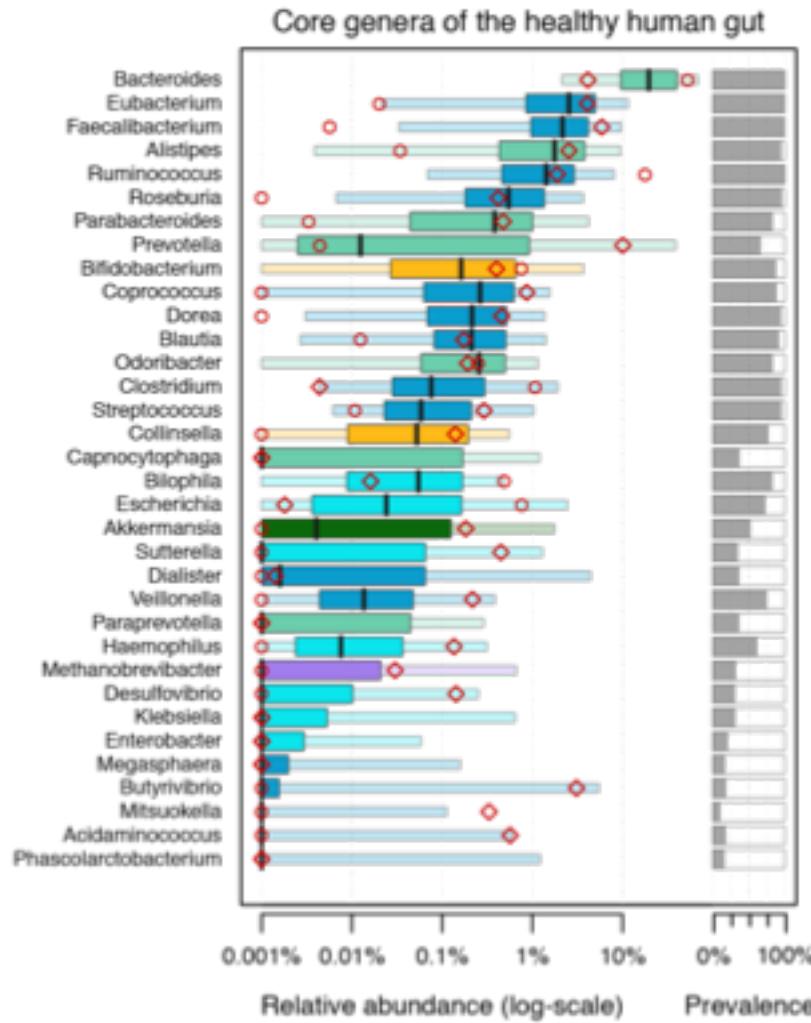
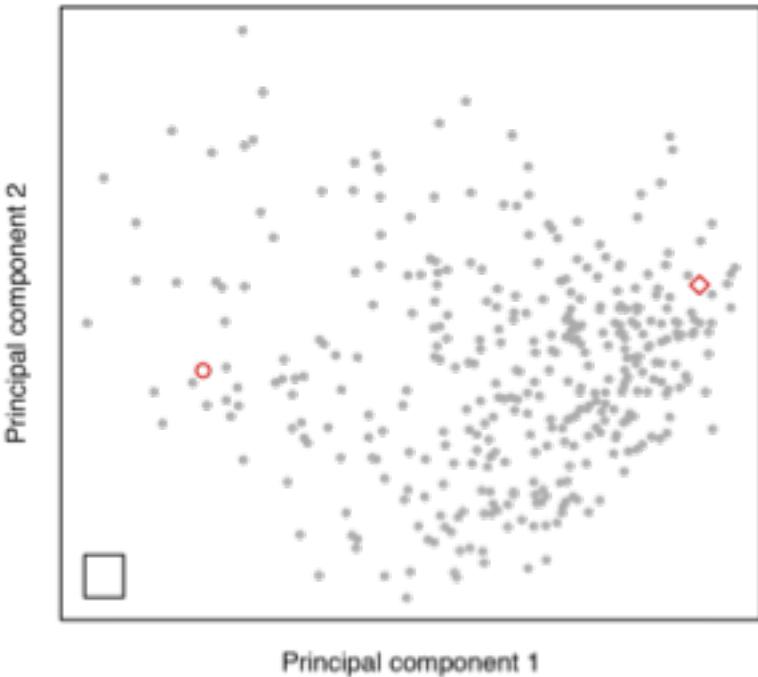


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Ordination of individual samples



Extreme within-group variance is a key characteristic of many microbial communities

Tools for microbial community comparison

Assessing differences in overall community structure

- **Community (dis-)similarity / measures:**
Summarize dissimilarity between two communities as a single number
 - Clustering
 - Comparing dissimilarity within and between groups
- **Ordination:**
Reduce high-dimensional data to 2 to 3 dimensions while preserving dissimilarity relation between samples as well as possible
 - Use with custom dissimilarity
 - Visualization by projection
 - Analyze major sources of variance

Testing for changes in individual taxa

- Apply a statistical test to each taxon to ask whether its abundance significantly differs between groups
- Robust, nonparametric tests (Wilcoxon or Kruskal Wallis tests) are recommended
- Correct for multiple testing to e.g. control the false discovery rate
 - Individual taxa affected by treatment, useful biomarkers, ecological indicator species, ...
 - Often more sensitive and interpretable than ordination!

Dissimilarity measures for microbial community comparison

Euclidean

$$d_{kl} = \sqrt{\sum_{i=1}^n (p_{ik} - p_{il})^2}$$

p_{ij} is the (relative) abundance of taxon i in sample j

Manhattan

$$d_{kl} = \sum_{i=1}^n |p_{ik} - p_{il}|$$

n equals the number of taxa

Gower

$$d_{kl} = \frac{1}{n} \sum_{i=1}^n \frac{|p_{ik} - p_{il}|}{\max(p_i) - \min(p_i)}$$

Canberra

$$d_{kl} = \frac{1}{n} \sum_{i=1}^n \frac{|p_{ik} - p_{il}|}{p_{ik} + p_{il}}$$

n equals the number of taxa with nonzero abundance in at least one sample

Bray-Curtis

$$d_{kl} = \frac{\sum_{i=1}^n |p_{ik} - p_{il}|}{\sum_{i=1}^n (p_{ik} + p_{il})}$$

Jaccard

$$d_{kl} = \frac{b_{kl}}{(1+b_{kl})}$$

b_{kl} is the Bray-Curtis dissimilarity between sample k and sample l .

unweighted Unifrac

$$\frac{\sum_{i=1}^n b_i |q_{ik} - q_{il}|}{\sum_{i=1}^n b_i \max(q_{ik}, q_{il})}$$

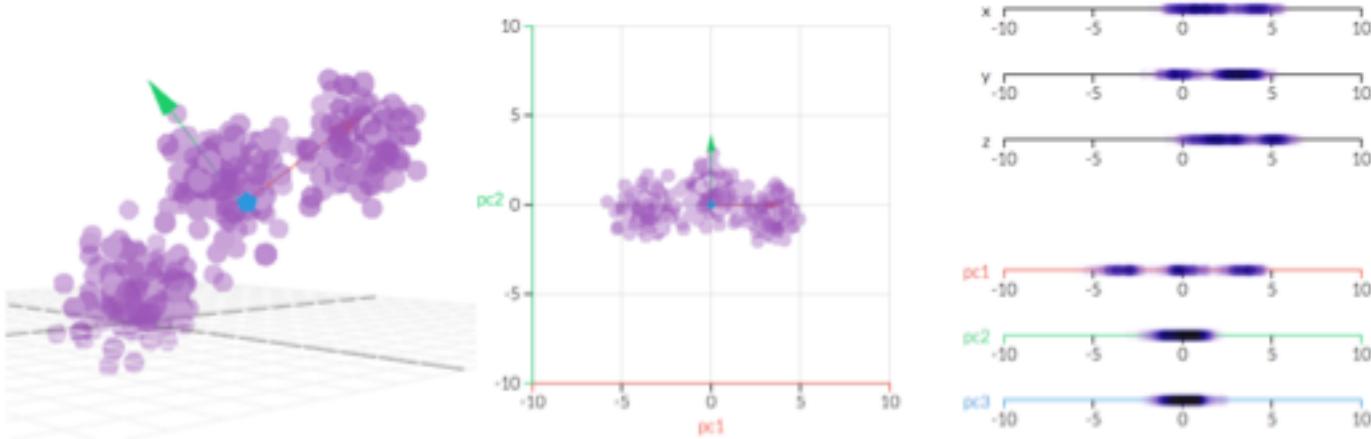
q_{ik} is 1 if taxon i is present in sample k and 0 otherwise.

weighted Unifrac

$$\sum_{i=1}^n b_i \left| \frac{p_{ik}}{\sum_{i=1}^n p_{ik}} - \frac{p_{il}}{\sum_{i=1}^n p_{il}} \right|$$

b_i refers to branch i . $\sum_{i=1}^n p_{ik}$ is equal to the sum of abundances in sample k .

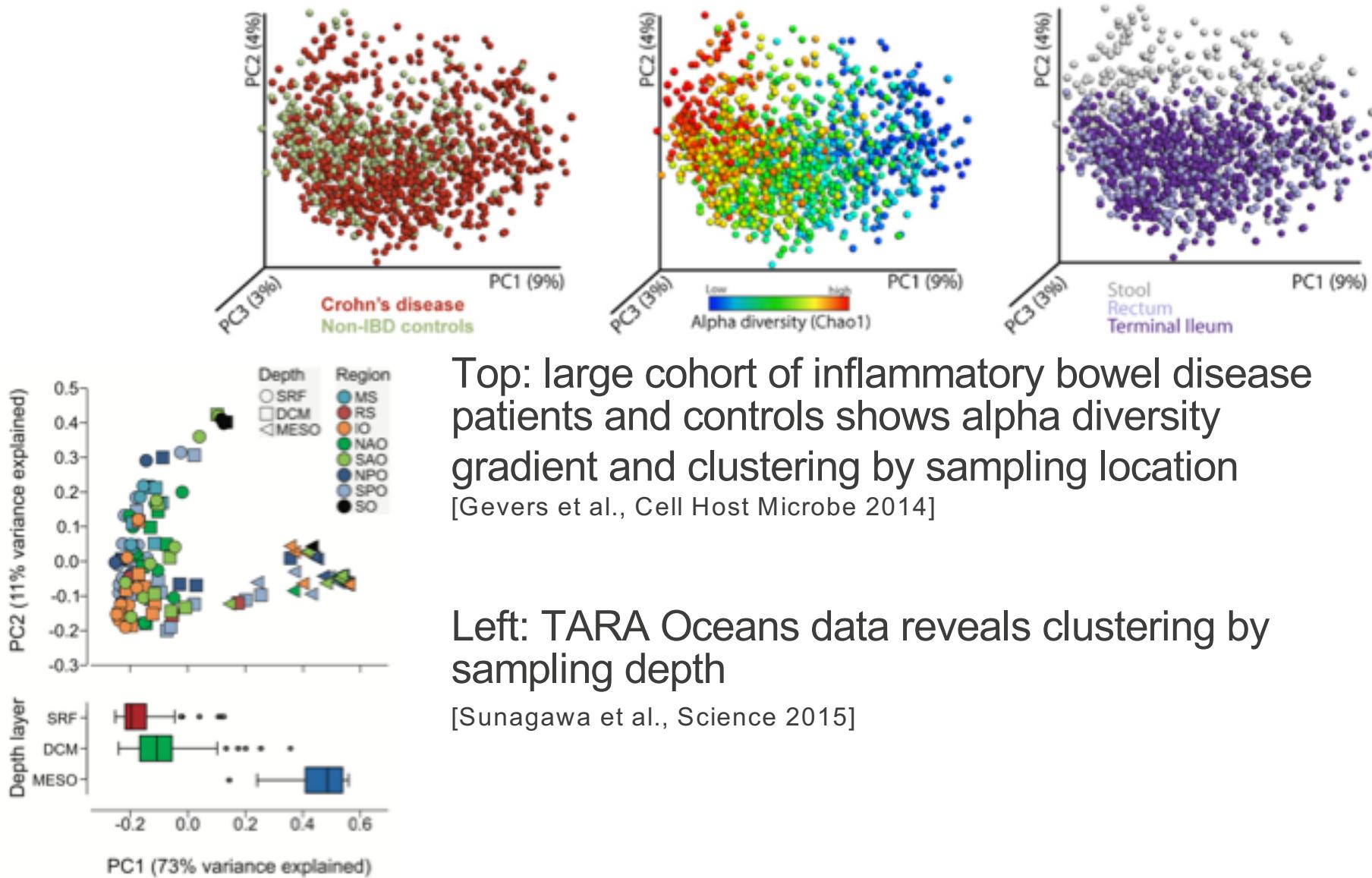
Ordination: principal component analysis and generalizations



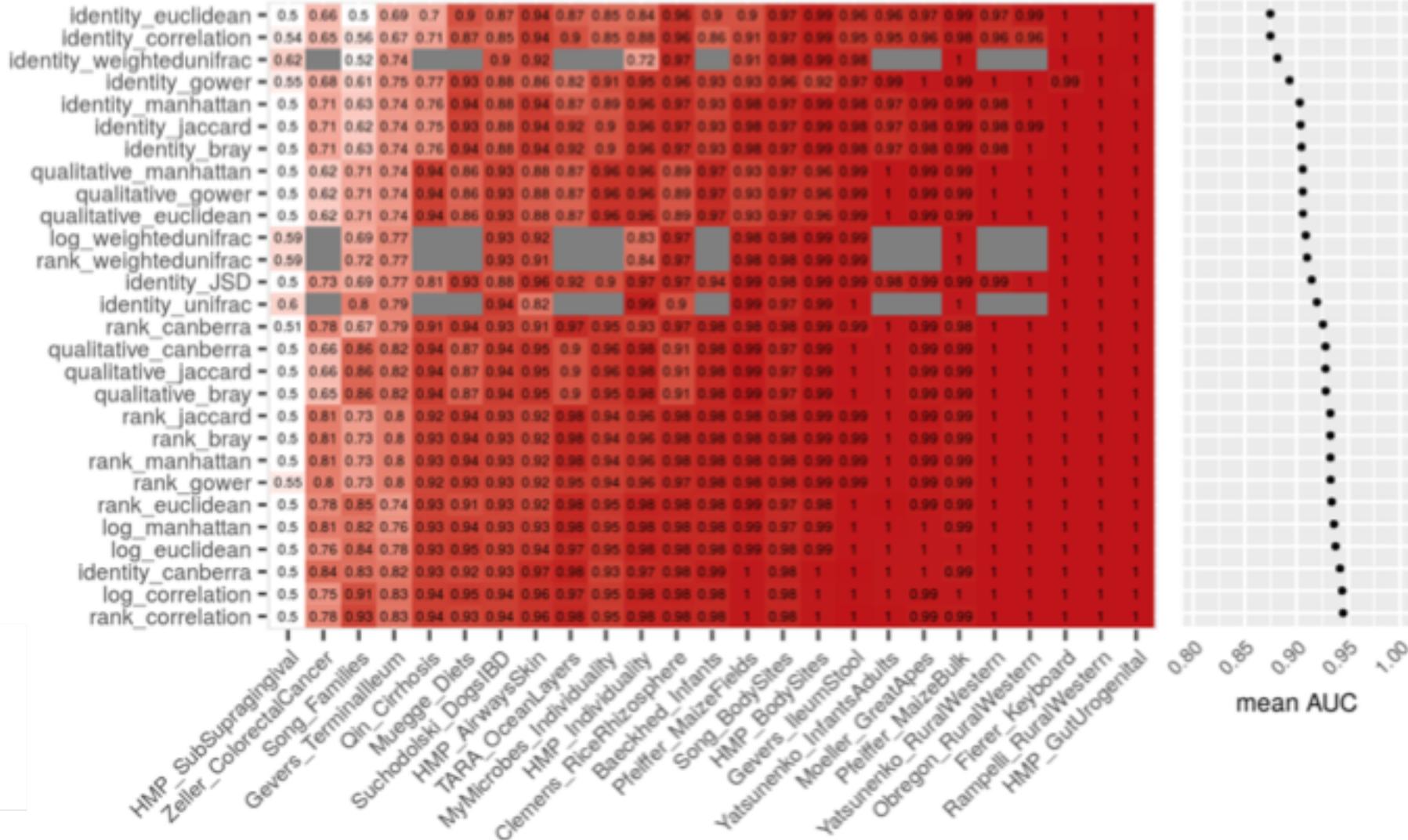
[Fig source: <http://setosa.io/ev/principal-component-analysis/>]

- PCA: transformation into an orthogonal basis such that first principal component is aligned with most variance and so on. Project to 2D (or 3D) for visual exploration
 - Implicitly works with Euclidean distance
- PCoA: Generalization that finds a low-dimensional embedding that preserves a pre-specified distance matrix as well as possible.
 - Can be used with any user-supplied dissimilarity matrix

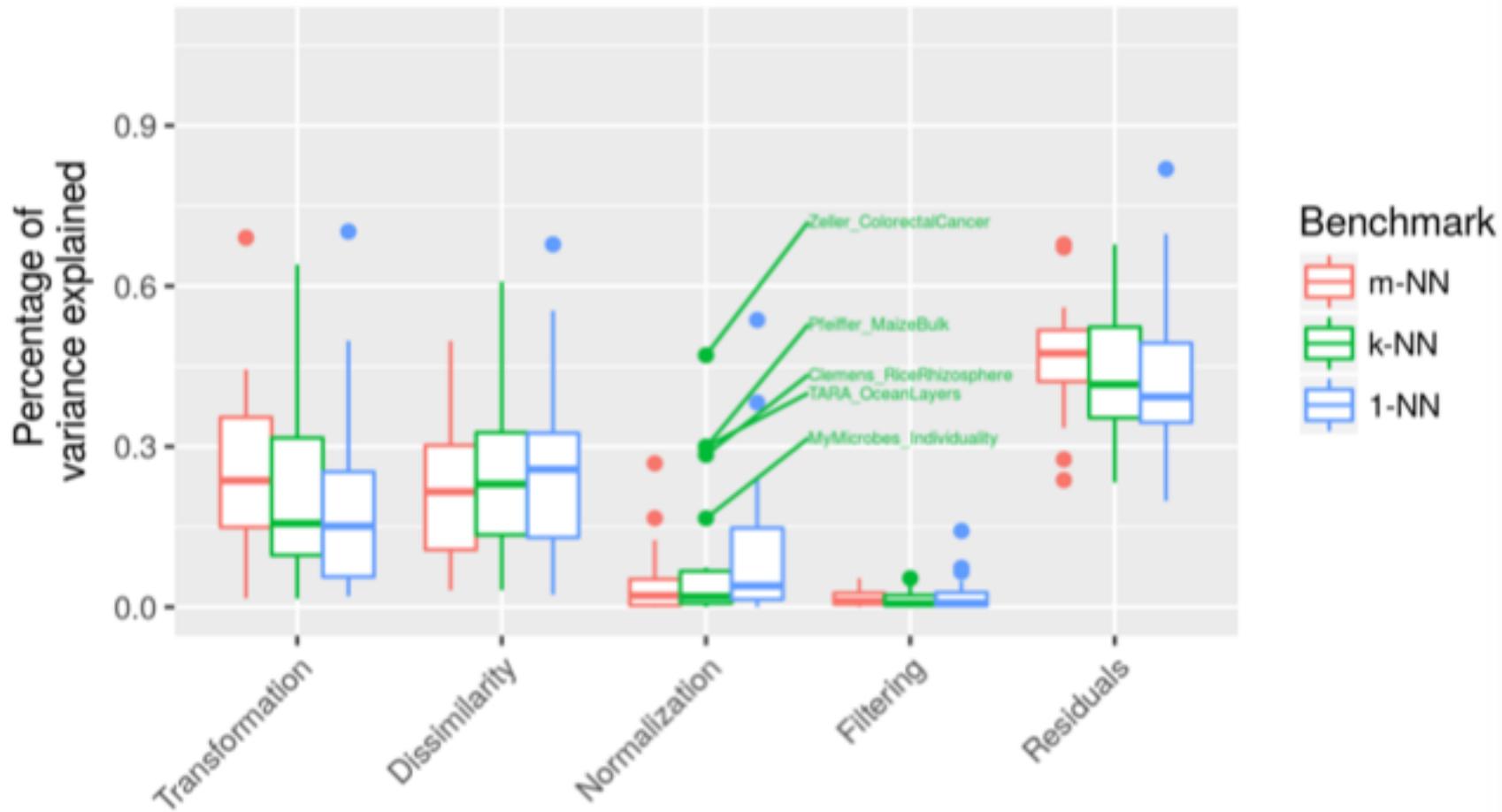
Ordination as a tool to reveal ecological differences between microbial community samples



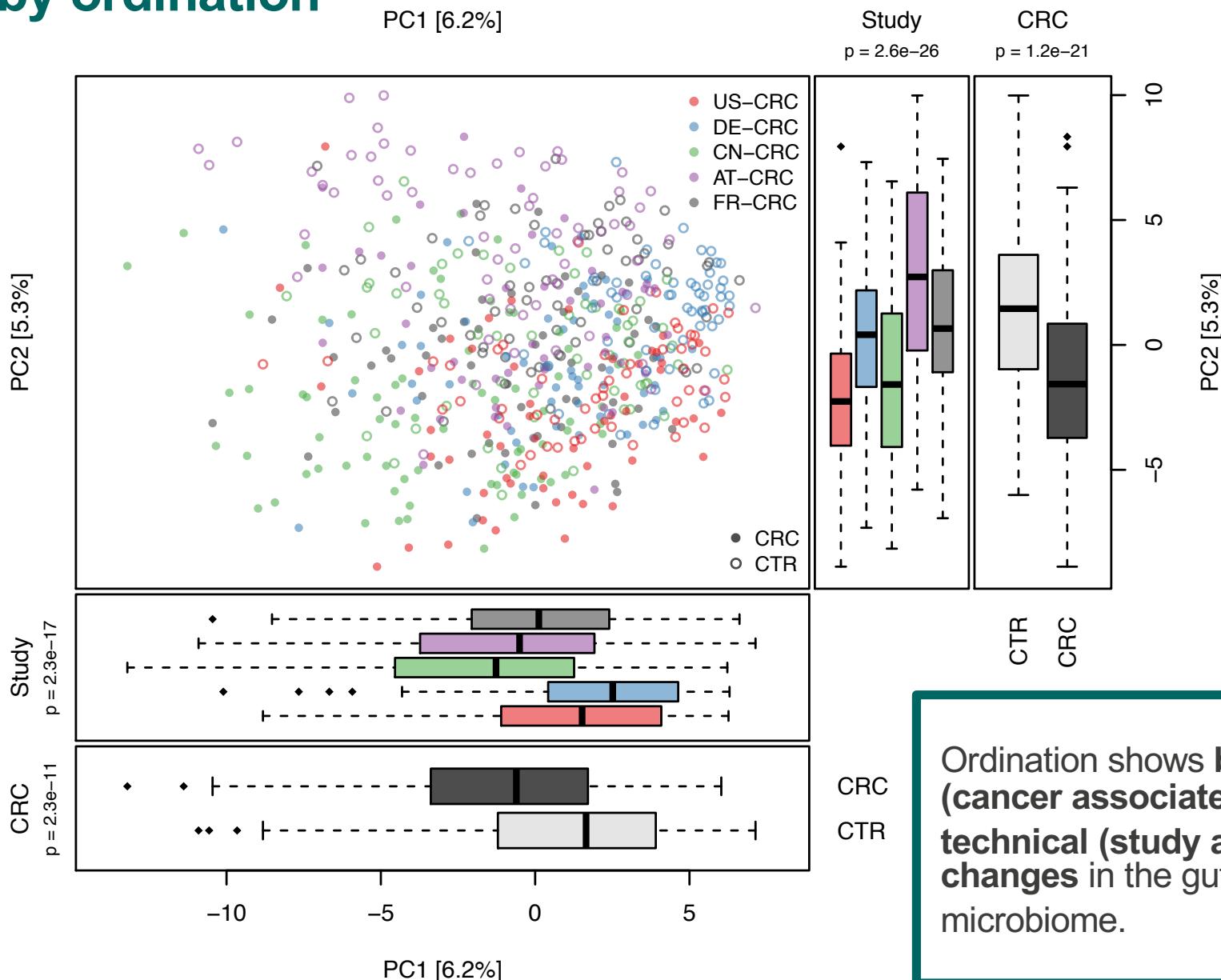
Comparison of dissimilarity measures



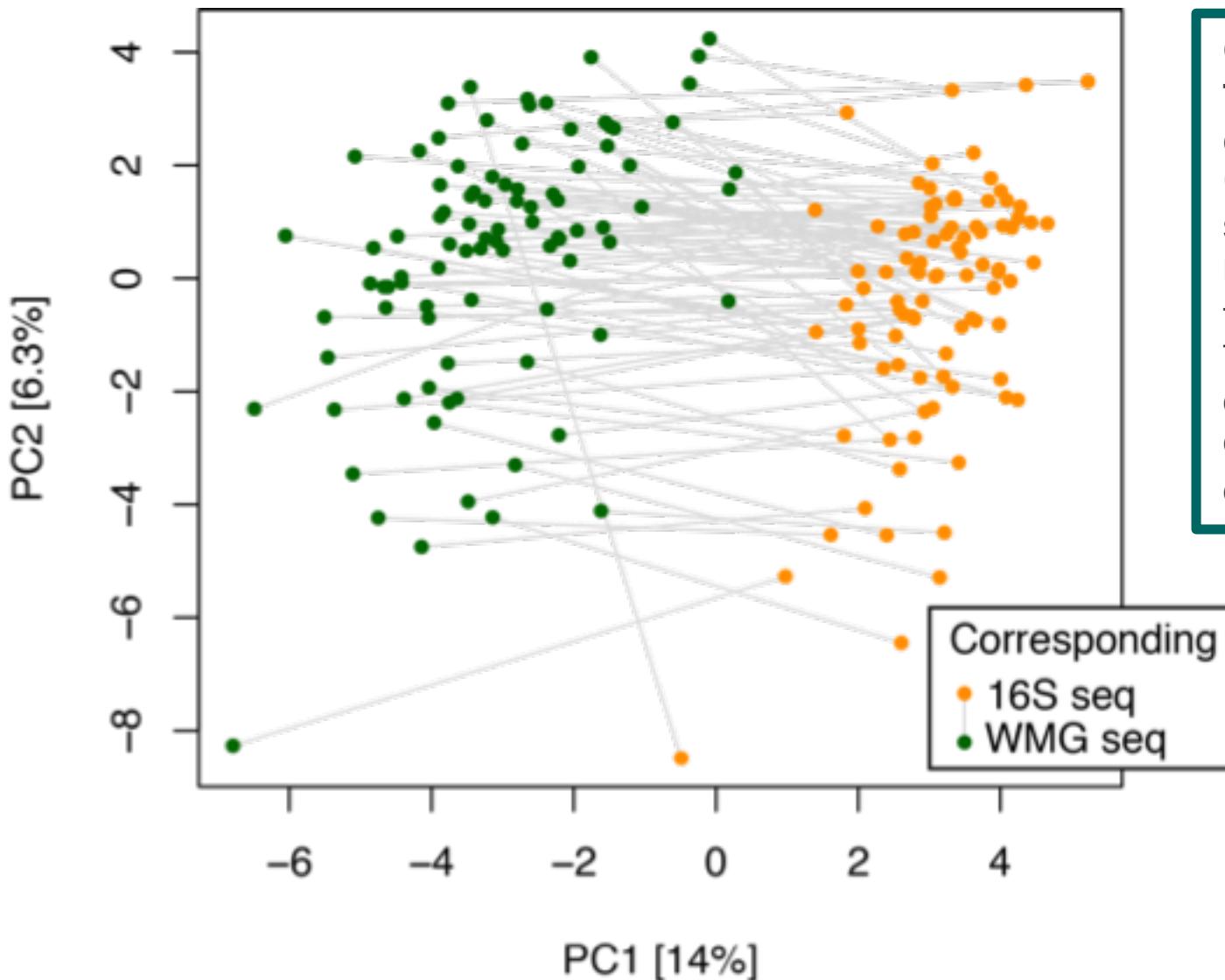
Factors influencing dissimilarity analysis



Joint visualization of multiple influences by ordination



Example of technical differences due to sequencing approach



Ordination shows **technical differences** (16S versus shotgun metagenomics of the same samples) to be more dramatic than any expected biological difference.

Tools for microbial community comparison

Assessing differences in overall community structure

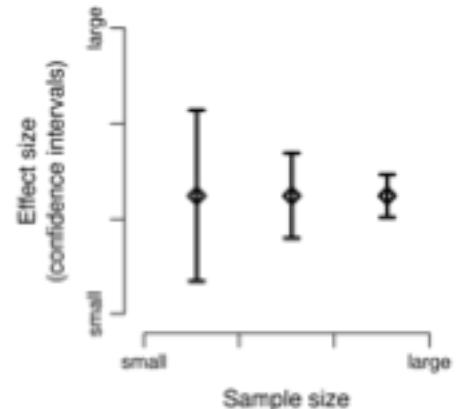
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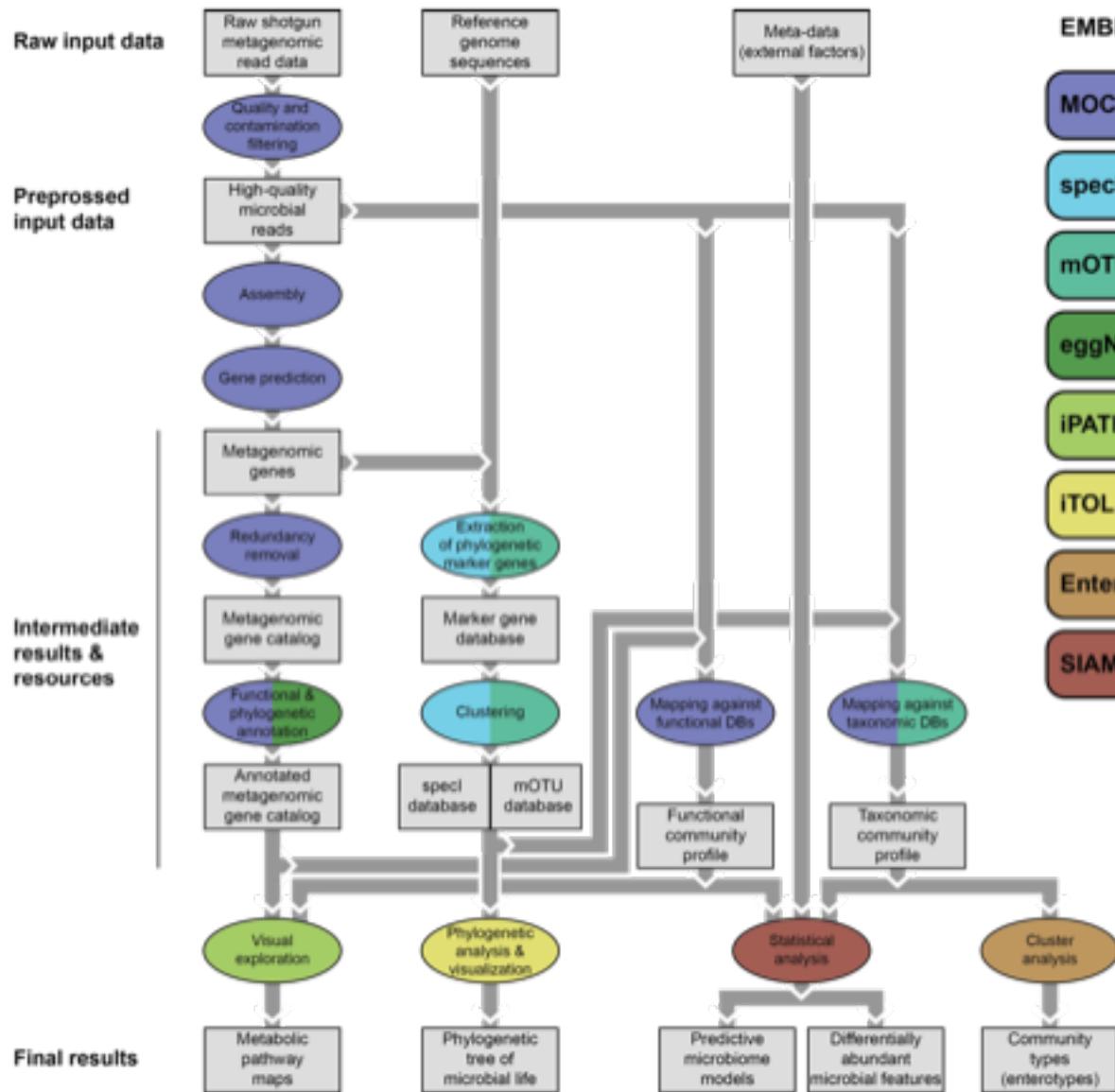
Summary of caveats

- Beware of technicalities such as **inappropriate data preprocessing, strange dissimilarity measures and projection artefacts!**
 [see e.g. Morton et al. mSystems 2017]
- **Disentangling biological and technical effects** can be difficult!
 [e.g. geography and DNA extraction protocol effects, as these are rarely uncorrelated]
- **Confounding** with biological effects from uncontrolled sources is difficult to detect and correct for!
 [see e.g. Forslund et al. Nature, 2015, Qin et al. Nature 2012, Karlsson et al. Nature 2013]
- Correlation does not imply **causation!**
 [see e.g. <http://www.tylervigen.com/spurious-correlations>]
- Statistical significance should not be confused with **effect size**, nor with **biological significance!**

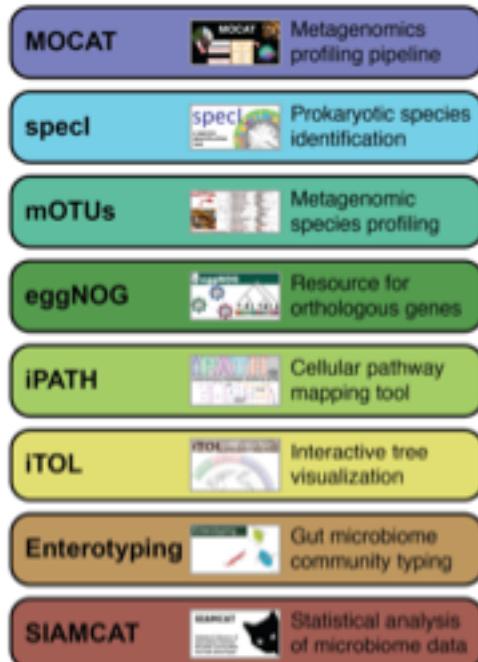


EMBL microbiome analysis toolkit

www.microbiome-tools.embl.de



EMBL Microbiome Tools



git.embl.de/zeller/microbiome_analysis_notebooks/

PART II: STATISTICAL MODELLING

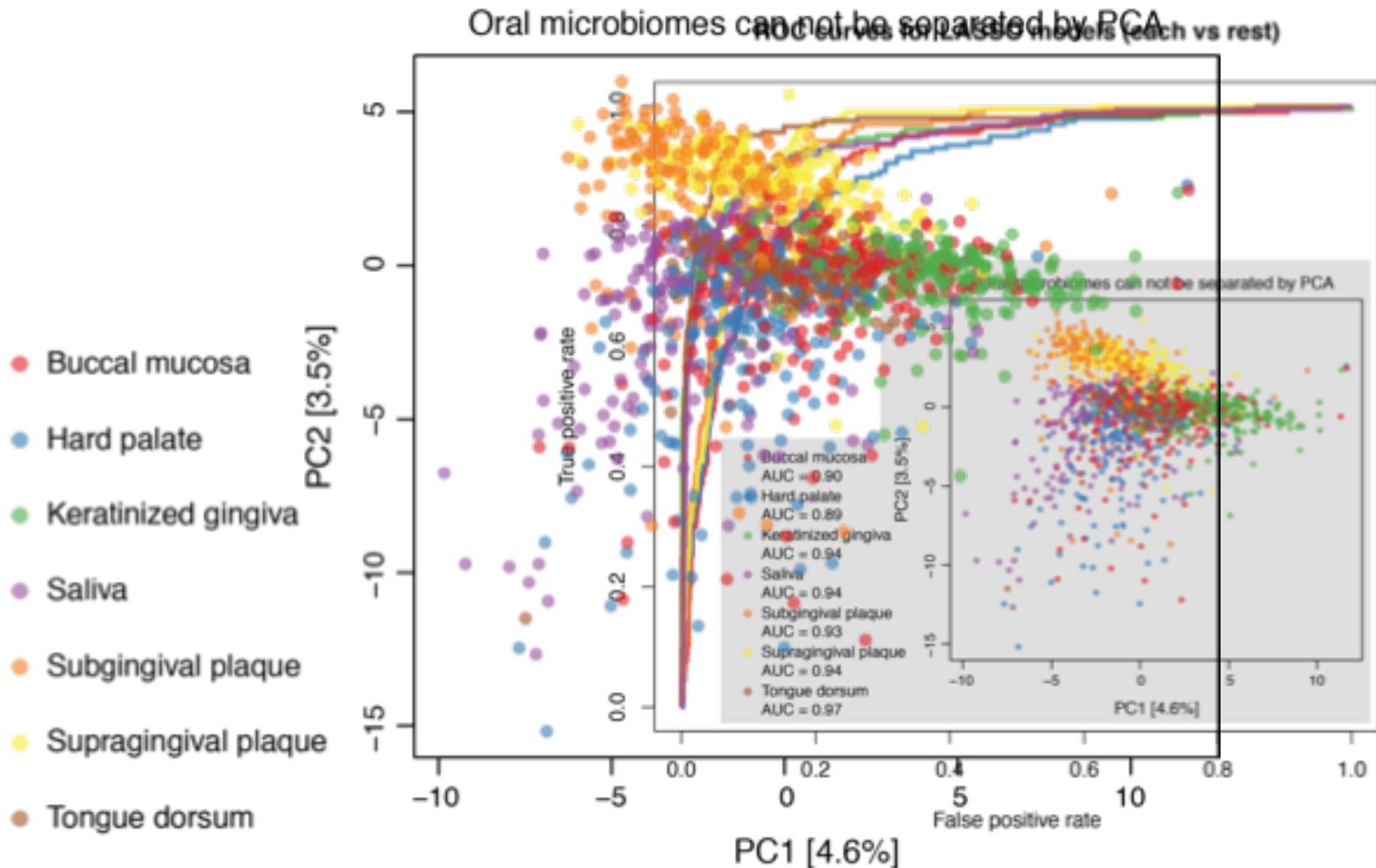
Why statistical modeling / machine learning?

- Modeling ideally **extracts the essence** of a biological phenomenon
- Good models make **accurate predictions on new data** (necessary e.g. for microbiome-based diagnostics)
- **Prediction accuracy** is often a more **meaningful measure of association** than statistical significance of differences
- **Sparse statistical models** are „in between“ univariate and multivariate testing, e.g. based on „a few“ taxa (**biomarkers**)
- Suitable methods can **select predictive taxa** (and ignore others)

$$y_i = f(\mathbf{x}_i) + \epsilon$$

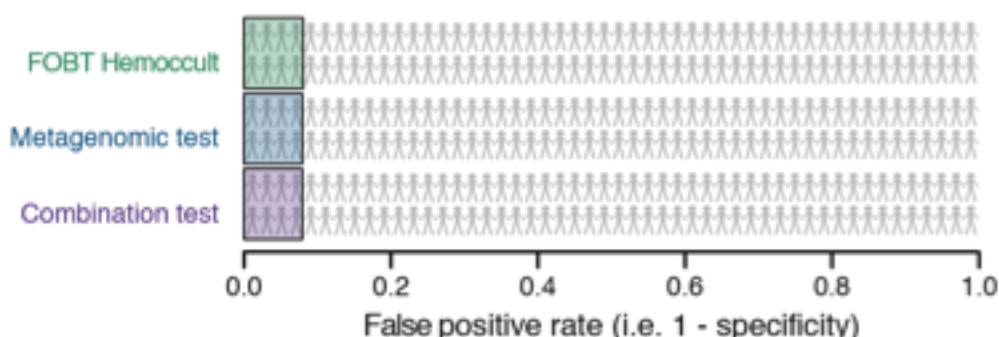
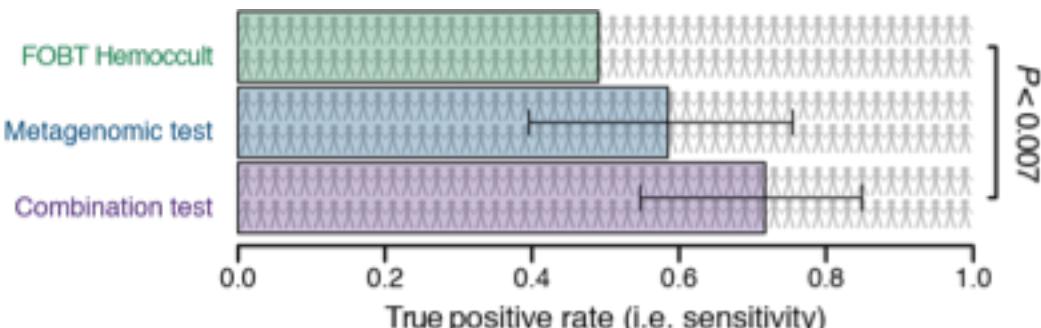
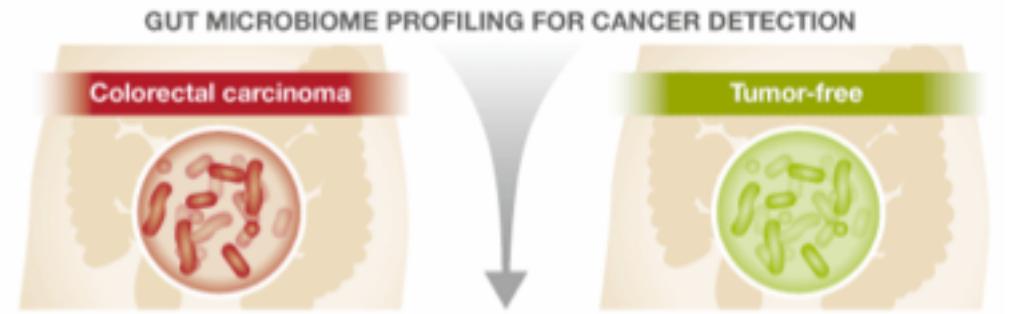
For i samples / patients
 y_i – label (e.g. disease or control)
 x_i – features (e.g. species abundance profile, a vector)
 f – our model
 ϵ – modeling error

PCoA vs modeling



While it can be difficult to resolve for each oral microbiome sample the precise sampling site using PCoA (with various dissimilarity measures), one can train statistical models that can very accurately recognize sample origin.

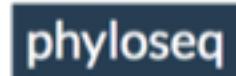
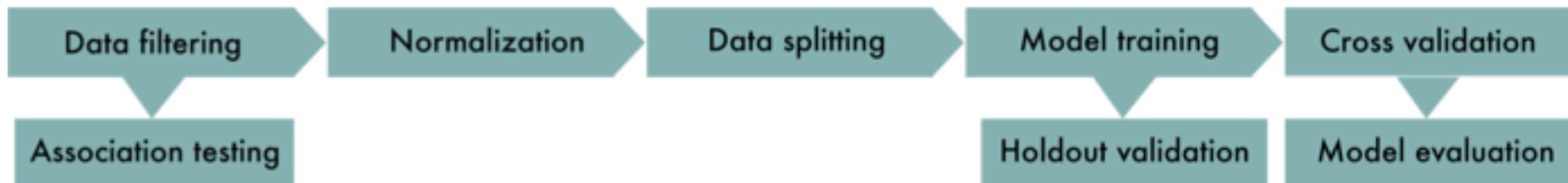
Detecting colorectal cancer (CRC) from fecal microbiome profiles



- Collected stool samples from 53 colorectal cancer (CRC) patients and 88 healthy controls
- Used metagenomic sequencing and profiled gut bacterial species
- Can microbiome differences be used for non-invasive detection of cancer?
- How does metagenomic detection compare to standard clinical tests (FOBT)?

[Zeller*, Tap*, Voigt* et al., Mol. Syst. Biol. 2014]

A statistical modeling workflow



COMMON
WORKFLOW
LANGUAGE

Starting with SIAMCAT

```
> source("https://bioconductor.org/biocLite.R")
> biocLite("SIAMCAT")
> browseVignettes("SIAMCAT")
```

File formats supported:

- phyloseq
- BIOM
- LEfSe
- MaAsLin
- metagenomeSeq



microbiome-tools.embl.de

The above workflow is specifically implemented in the SIAMCAT Bioconductor package, but still fairly general for any statistical modeling task.

Feature preparation

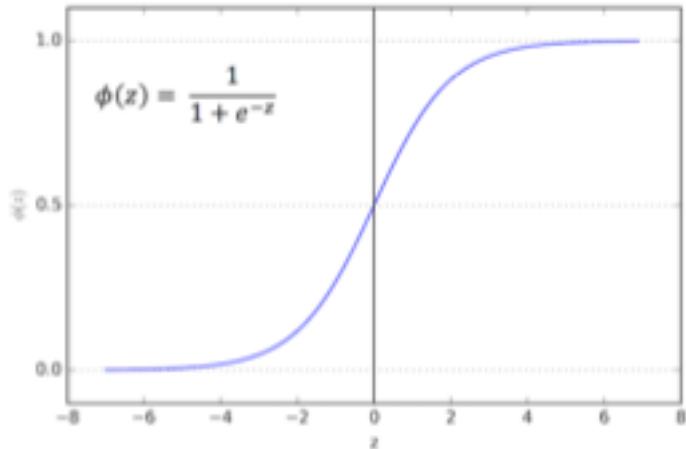
- Use your **domain expertise** to engineer features that are likely predictive of the phenomenon of interest – microbiome examples:
 - Species abundances (or higher/lower resolution profiles)
 - Metabolic pathway abundance (e.g. KEGG / CAZy maps)
 - Functional gene annotations (GO terms, domains, ...)
 - Orthologous gene families (COGs, eggNOG families, ...)
 - Toxins, virulence factors, ABX resistance genes, ...
- Consider **interpretability** –
predictive species/metabolic pathways may be better than k-mers
- Importantly, do **NOT use the label** information for feature filtering / preprocessing!

Training and prediction (with the LASSO)

The (LASSO logistic regression) model:

$$f(\mathbf{x}_i) = \phi(\mathbf{w}^T \mathbf{x}_i) = \phi\left(\sum_{j=1}^p w_j x_{ji}\right)$$

The logit function ϕ :



Fitting the model (for the simple LASSO without logit):

$$\min_{\mathbf{w}} \sum_i^n (y_i - \mathbf{w}^T \mathbf{x}_i)^2 + \lambda \sum_j^p |w_j|$$

Applying the trained model to new data:

$$\hat{y}_i = f(\hat{\mathbf{x}}_i) = \phi(\mathbf{w}^T \hat{\mathbf{x}}_i)$$

Model evaluation I – classification errors

In many applications, classes aren't equal – and neither are errors!

		True condition	
		positive ("cancer")	negative ("healthy")
Predicted condition	positive ("predicted to have cancer")	True positives TP	False positives FP (Type I errors)
	negative ("predicted not to have cancer")	False negatives FN (Type II errors)	True negatives TN

True positive rate (TPR, **sensitivity, recall**)

$$= \text{TP} / (\text{TP} + \text{FN})$$

True negative rate (TNR, **specificity**)

$$= \text{TN} / (\text{TN} + \text{FP})$$

False positive rate (FPR, $1 - \text{specificity}$)

$$= \text{FP} / (\text{TN} + \text{FP})$$

- are all **independent on prevalence** (of positives in the total population)

Precision (positive pred. value, PPV)

$$= \text{TP} / (\text{TP} + \text{FP})$$

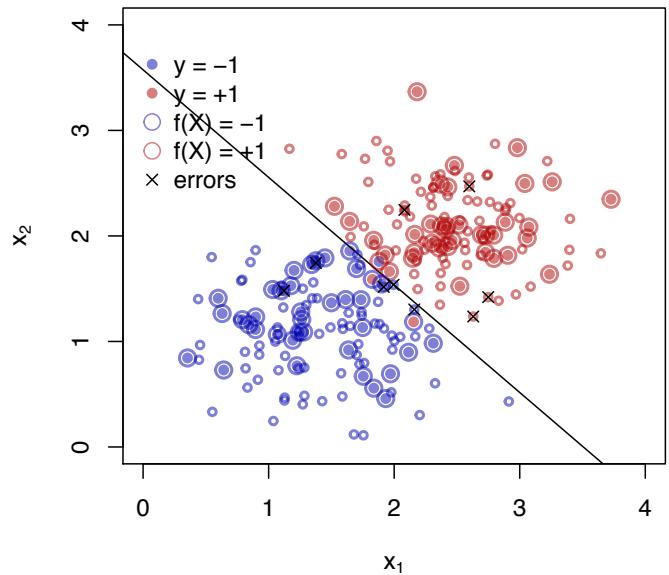
False discovery rate (FDR, $1 - \text{precision}$)

$$= \text{FP} / (\text{FP} + \text{TP})$$

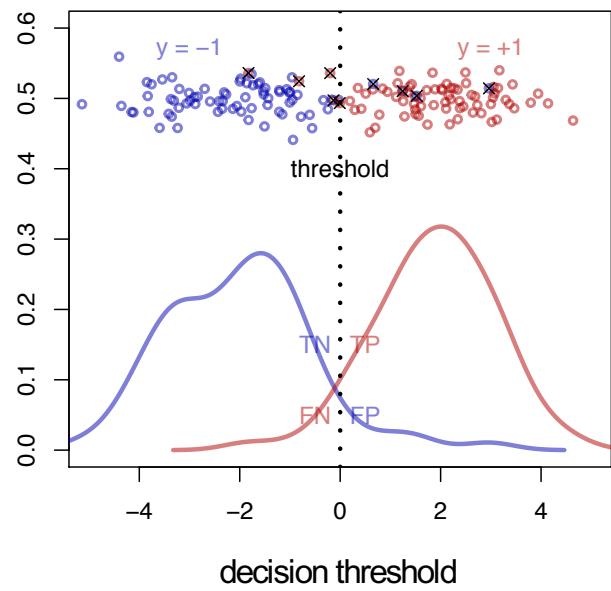
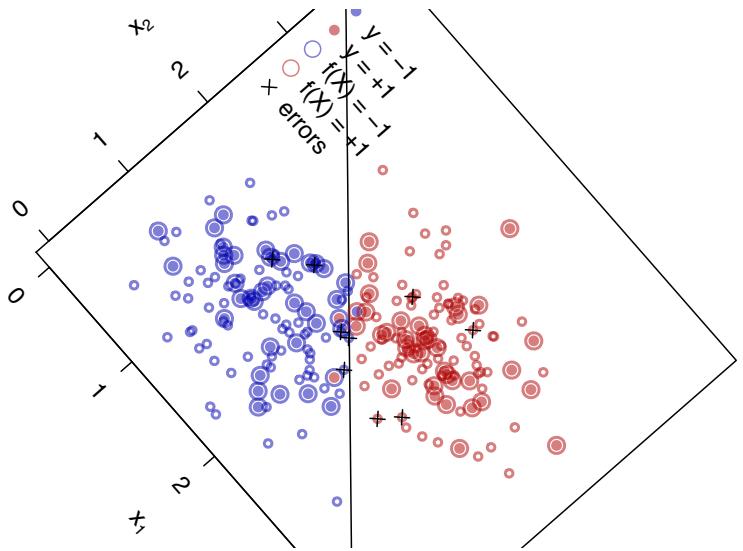
- are both **dependent on prevalence** (of positives in the total population)

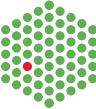
[these and more measures on en.wikipedia.org/wiki/Evaluation_of_binary_classifiers]

Model evaluation II – ROC curves

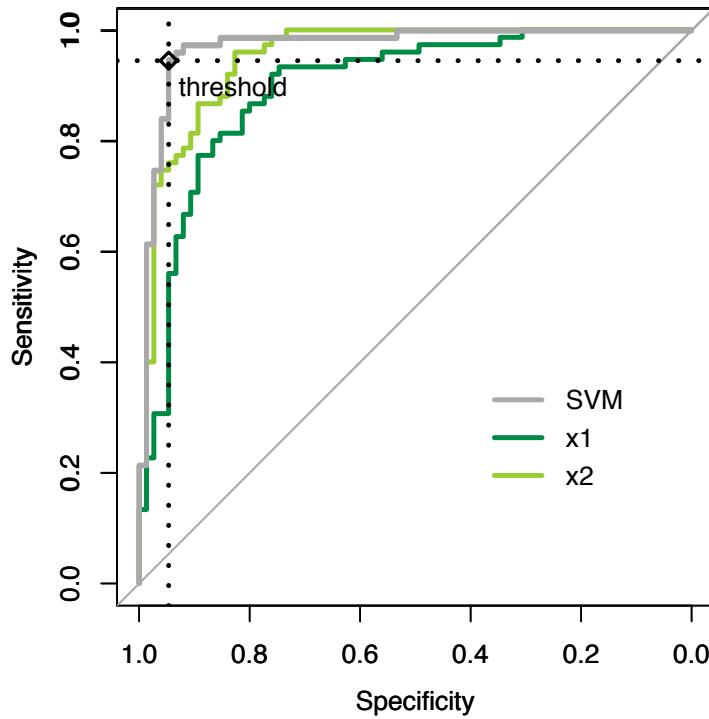
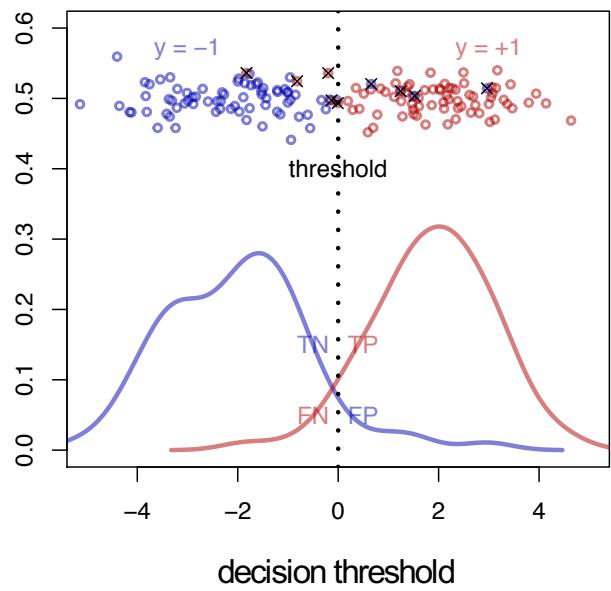
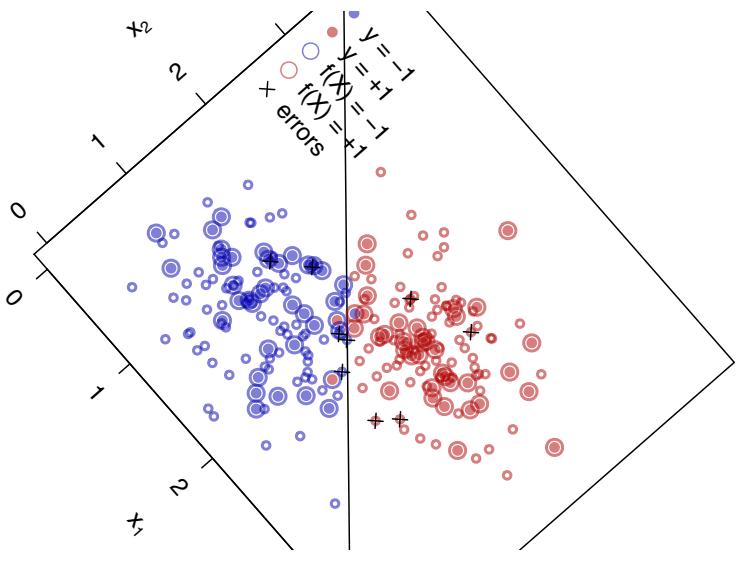


Model evaluation II – ROC curves





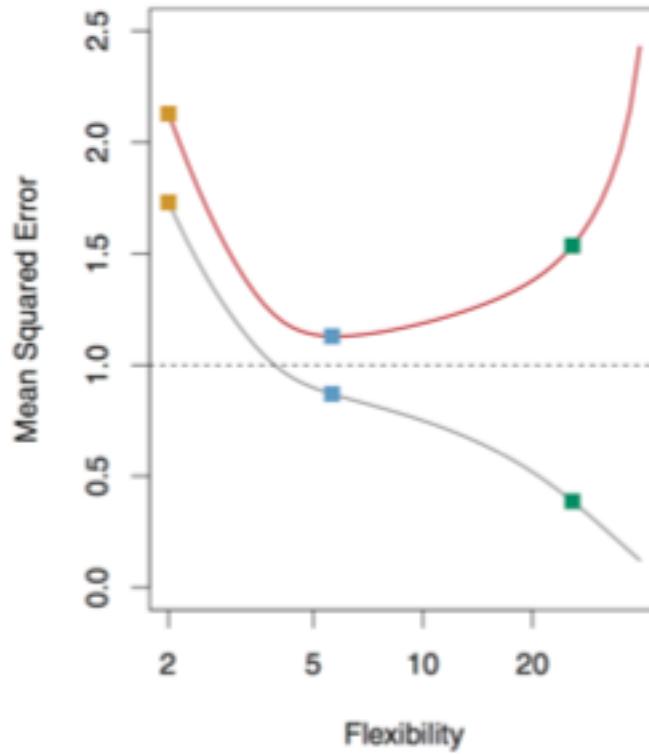
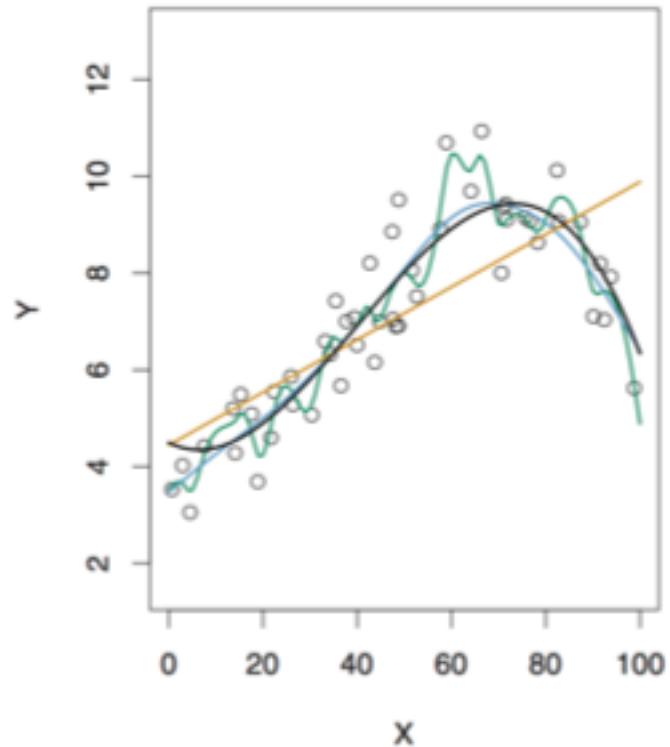
Model evaluation II – ROC curves



- Change decision threshold to obtain other trade-offs between sensitivity and specificity
- Receiver operating characteristic (ROC) curve plots all of them
- Area under the ROC curve as a summary

Model evaluation III – assessing generalization

- Minimizing the training error
With increasing flexibility, models will fit the training data better and better
- Generalization to new data sets
Overfitting the training data will result in poor generalization



Here smoothing splines are used; model flexibility / complexity increases with the degree of the polynomials.

[James, Witten, Hastie & Tibshirani, Springer 2013]

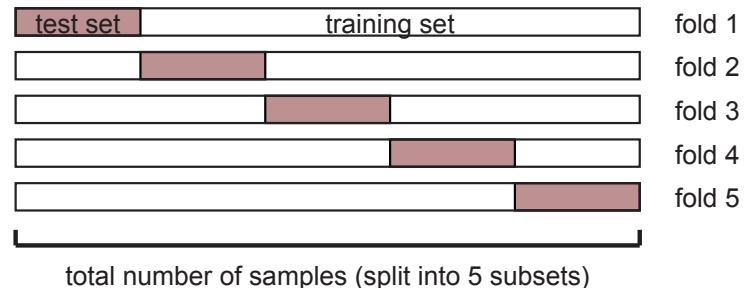
Splitting data for cross validation or external validation

Some data needs to be reserved for model evaluation....

- Validation on external data
- Cross-validation (CV)



- Train model on training set
- Test on test set
- Assess error on test predictions

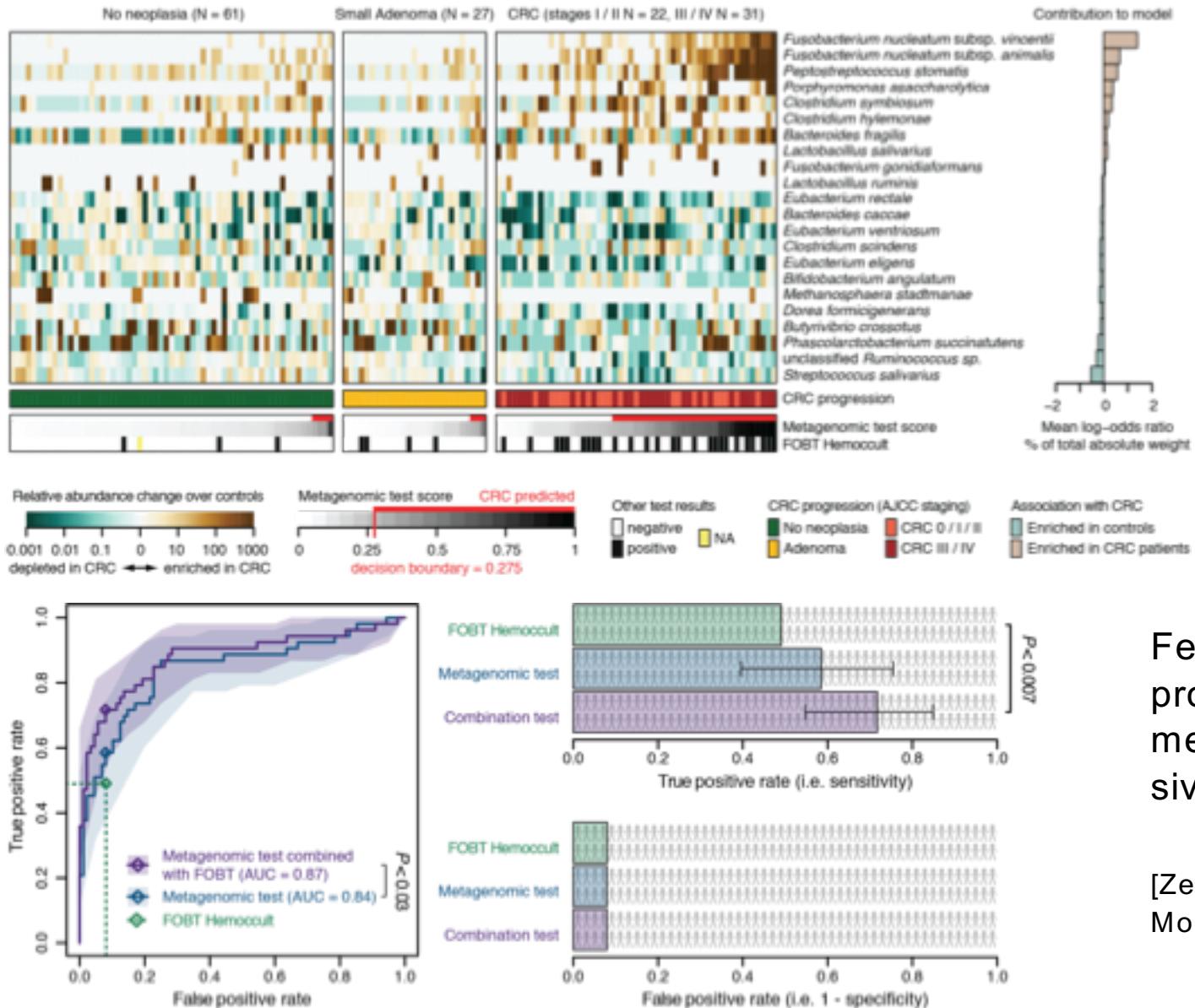


- For each CV fold:
 - Train a model on training set
 - Predict on the test set
- Either concatenate or average predictions from (all) test sets to estimate error
- More efficient use of (training) data

Cross-validation pitfalls

- **Cross validation works under the i.i.d. assumption** (observations have the same probability distribution and are mutually independent)
 - E.g. a series of (fair or unfair) coin flips is i.i.d. as the next flip doesn't depend on the previous ones.
- However, biological samples are often **not independent**:
 - Multiple time-point measurements from the same subject or related subjects
 - Spatial structure / dependencies between measurements
- Data (sets) are **not always identically distributed**
 - Batch effects: e.g. experiments or diagnostic tests performed in different labs (by different technicians, at different times, using different reagent lots, ...) may exhibit (subtle) distributional shifts

Model interpretation



Fecal metagenomic profiling as a novel method for non-invasive CRC detection.

[Zeller*, Tap*, Voigt* et al.,
Mol. Syst. Biol. 2014]

Summary of caveats

- **Model fitting is easy, model evaluation is not at all!**
Understand the generalization assessed – consult experts!
- Beware of **overfitting** – especially on small data sets – especially with complex algorithms!
[Typically $N > 50$ per group is a requirement; start with simple algorithms first]
- **Trade off interpretability** (white-box models) **and maximal prediction accuracy** wisely!
- **Models can be confounded too!**
[see e.g. Forslund et al. Nature, 2015, Qin et al. Nature 2012, Karlsson et al. Nature 2013]
- Diagnostic application is relatively straightforward, but underlying **mechanisms are generally difficult to glean** from models
(predictability does NOT imply causality!)

git.embl.de/zeller/microbiome_analysis_notebooks/