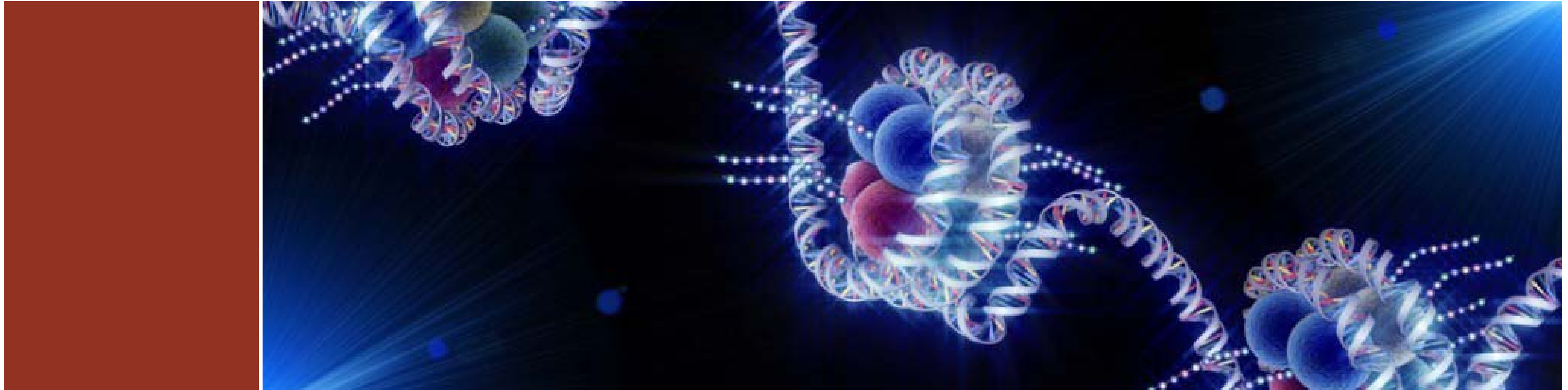

ADA assays for gene therapy projects: analytical challenges

Lydia Michaut

EBF meeting, Barcelona, 19-Nov-2015

Break-out session : "Varying Perspectives on ADAs"

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Presentation outline

- Bioanalytical support to gene therapy compounds
- Preclinical ADA assay
  - Semi-quantification of antibodies against an AAV-8 viral vector
- Clinical ADA assays
  - Semi-quantification of antibodies against an Ad5 viral vector
  - Semi-quantification of antibodies against an intracellular therapeutic protein
- Summary and discussion

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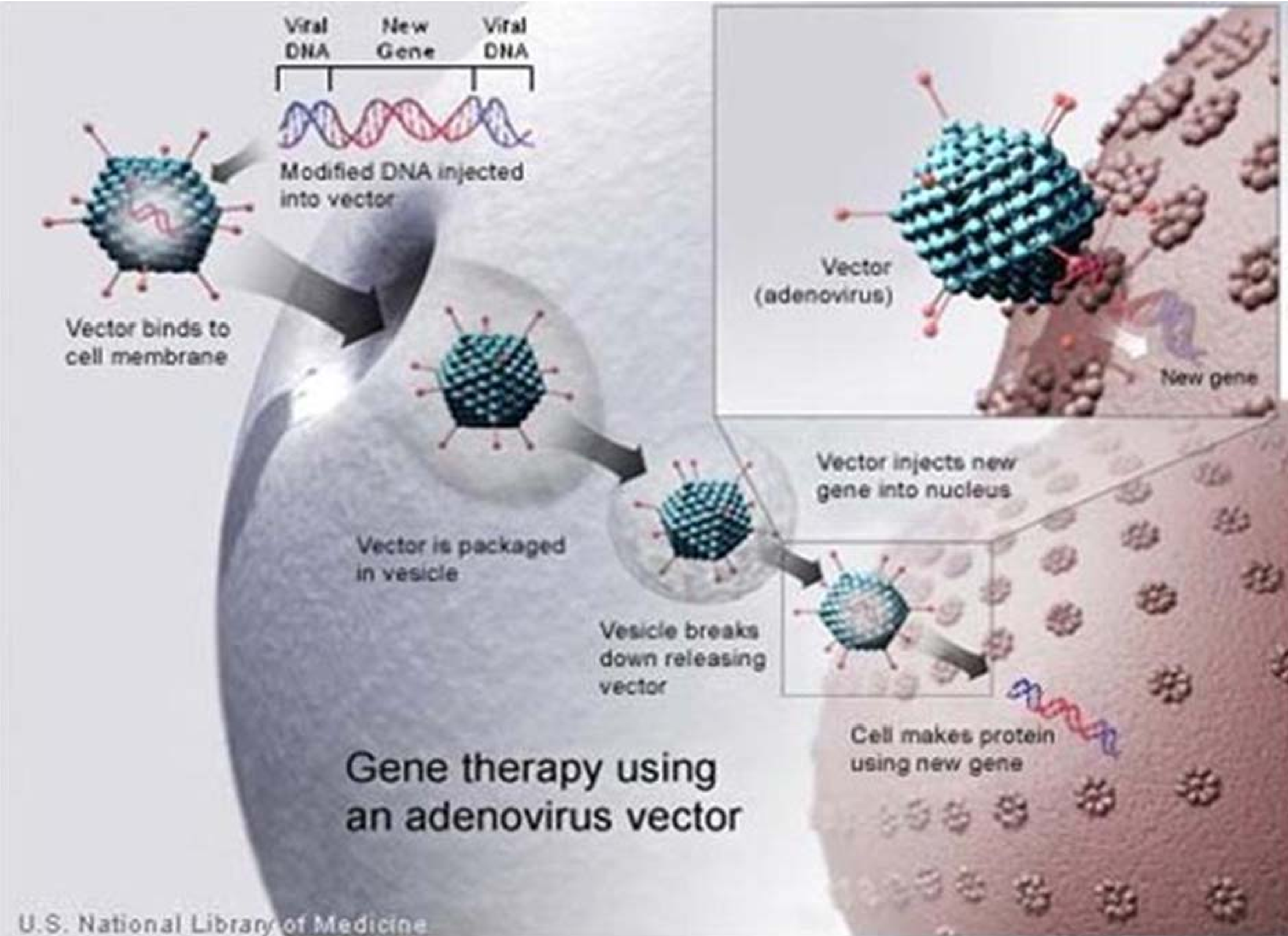
Mode of Action of Gene Therapies



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Bioanalytical support to gene therapy products

- **Vector capsid** controls cell tropism, host immune response, and influences manufacturing process and regulatory path.

- **Vector construct** controls expression levels and cell types in which the therapeutic gene is transcribed.

- **Today's examples:**
  - Adeno-associated Virus 8 (AAV-8) and Adenovirus serotype 5 (Ad5) viral vectors
  - Local administration
  - Transgenes encoding intra-cellular therapeutic proteins

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Semi-quantification of anti-AAV8 antibodies in monkey serum

- Literature reports pre-existing antibodies in up to 100% of monkeys
- The high seroprevalence of AAV serotypes in non-human primates presents an important challenge in the evaluation of vectors performance in animal models and normally an exhaustive screening of monkey colonies for pre-exisitng antibodies is required.
- One important approach to minimize the contact of the vector with the pre-existing antibodies includes targeting a tissue or organ via direct infiltration rather than via the circulation

frontiers in IMMUNOLOGY

REVIEW ARTICLE
published: 18 October 2013
doi: 10.3389/fimmu.2013.00341

Humoral immune response to AAV

*Roberto Calcedo and James M. Wilson**

Gene Therapy Program, Department of Pathology and Laboratory Medicine, University of Pennsylvania, Philadelphia, PA, USA

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Anti-AAV8 antibody assay

Development

- Evaluated and selected Protein A/G as detection reagent -> ability to bind both:
  - human IgM (A/E)
  - All IgG subclasses (human and mouse)
- Commercially-available mouse positive control (PC)
- Sandwich assay: empty viral capsid (GMP batch) coated on the ELISA plate / colorimetric detection
- Negative Control (NC):
  - Use of IgG-depleted serum: common procedure in the case of pre-existing Abs, could not be envisaged here
  - Generation of NC pool with a few sera with very low signal which was not specific for the viral capsid (*i.e* which was not decreased/reduced by addition of excess capsid)

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Anti-AAV8 antibody assay

Presence of pre-existing anti-AAV8 antibodies in > 80% of animals

- Sandwich ELISA validated for GLP-Tox sample analysis
- Samples were directly run in the confirmatory assay

  No confirmatory cut point could be established => determination of the global precision of the confirmatory assay (n=108): 24%

  √ Any sample with a %inhibition > 24% was confirmed positive for anti-AAV8 presence.

  √ Samples with % inhibition < 24% **and** high levels of anti-AAV8 antibodies (*OD>2)* were re-analyzed diluted in the confirmatory assay:

  ➢ Dilution factor individually set for each sample on the basis of a saturated signal => *simple criterion to ensure that the re-analyzed sample was not too much diluted: signal of the unspiked diluted sample had to be high enough to allow at least 25% inhibition.*

- Samples confirmed positive were analyzed in the titration assay:

  ➢ *4 dilutions only to maximize the SA throughput ...*

  ➢ *increased risk of repeats if the choosen dilution was too low*

  ➢ Applied «classical» (two-fold) titration dilution steps => *a posteriori*, 4 or 8x dilution steps would have been more suitable for this assay.

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Anti-AAV8 antibody assay

Summary of challenges

- Low throughput assay, longer time than usual required for SA:
  - 89% of the samples were confirmed positive and analyzed in the titer assay (4 dilutions)
  - >150 titrations had to be repeated at higher dilutions
  - *a posteriori*: calibration-curve based format would have been preferable, especially when a high number of samples have to be analyzed.

- Assay very «thirsty» in reagents:
  - Viral particle used for coating and in confirmatory assay:

  => over 80 vials à 2.5×10^{12} vg/mL were required for AD, AV and SA.

  - NC pool:
    - two new pools of NC had to be prepared and qualified during SA.
    - titers > 1024 were not be reanalyzed at further dilutions but reported as > 1024 and extrapolated to 2048 for graph representation.

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Anti-AAV8 antibody assay

Lessons learnt for clinical assay development

- Directly titer the anti-vector antibodies (no screening nor confirmatory assays).

- Establish a minimum significant ratio (MSR) value to evaluate the relevance of titer changes between two time points.

- Plan for enough reagents supply (viral particle *(GMT lot from CMO)*, recombinant therapeutic protein...).

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Semi-quantification of anti-Ad5 antibodies in human serum

Implementing the lessons learnt with the first GT program

- Presence of pre-existing antibodies was confirmed in > 90% of the individuals tested during assay development and validation -> used as an advantage for the positive control pool generation.
- Negative control : IgG-depleted human serum pool
- Assay specificity confirmed through immunodepletion during development and validation => confirmatory assay won't be performed during SA
- Calibration-curve based approach first tested on the basis of the lesson learnt for the previous project: could not be implemented due to lack of parallelism.

  => Titration curve (Cut point = mean NC + 3xSD) with preliminary «screening» at two dilutions to determine the level of anti-Ad5 response.
- Minimum significant ratio = 4.1=> need to see a least a 4.1-fold difference between titers from 2 timepoints to conclude that they are statistically different

  ---> implementation of 4-fold dilution steps in the titration assay.

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Semi-quantification of antibodies against the protein encoded by the viral transgene

Whereas the immense majority of Bx drug targets are either soluble or on the cell surface, gene therapy therapeutic proteins are intracellular, even intranuclear.

-> Intranuclear protein, positively charged, complex 3D structure => difficult to produce in its native conformation

- Protein was produced in micro-organisms (*E. coli* and yeast) devoid of the chaperones allowing folding in higher eukaryotes.
- Protein solubility issues (2 providers tested) => must be resuspended in urea, which impaired further ruthenylation and biotinylation and therefore the use of the bridging format for the ADA assay

=> only possible assay format was to coat with the recombinant human protein and detect with protein A/G

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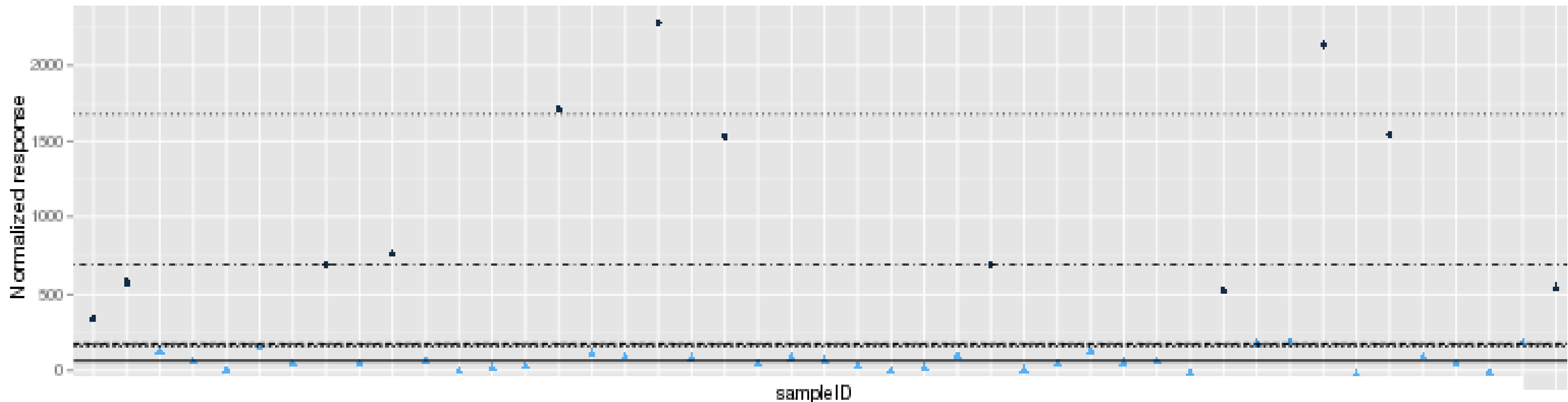
Assay development dataset

30% of confirmed «responders» in naive individual sera



Coating with rh protein
Detection with protein A/G

N=45 individual sera
66 measurements on 6 plates (=> 6 NC)
21 sera were measured twice, the others once.

=> 30% of confirmed «responders» (black squares) by the mixADA software

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Cutpoint selection by mixture models and prediction intervals: the mixADA package

Schaarschmidt F, Hofmann M, Jaki T, Grün B, Hothorn LA. (2015)

Statistical approaches for the determination of cut points in anti-drug antibody bioassays. J Immunol Methods.2015 Mar;418:84-100. doi: 10.1016/j.jim.2015.02.004. Epub 2015 Feb 27.

Cut points in immunogenicity assays are used to classify future specimens into anti-drug antibody (ADA) positive or negative. To determine a cut point during pre-study validation, drug-naive specimens are often analyzed on multiple microtiter plates taking sources of future variability into account, such as runs, days, analysts, gender, drug-spiked and the biological variability of un-spiked specimens themselves.

Five phenomena may complicate the statistical cut point estimation: i) drug-naive specimens may contain already ADA-positives or lead to signals that erroneously appear to be ADA-positive, ii) mean differences between plates may remain after normalization of observations by negative control means, iii) experimental designs may contain several factors in a crossed or hierarchical structure, iv) low sample sizes in such complex designs lead to low power for pre-tests on distribution, outliers and variance structure, and v) the choice between normal and log-normal distribution has a serious impact on the cut point.

We discuss statistical approaches to account for these complex data: **i) mixture models, which can be used to analyze sets of specimens containing an unknown, possibly larger proportion of ADA-positive specimens,** ii) random effects models, followed by the estimation of prediction intervals, which provide cut points while accounting for several factors, and iii) diagnostic plots, which allow the post hoc assessment of model assumptions. All methods discussed are available in the corresponding R add-on package mixADA.

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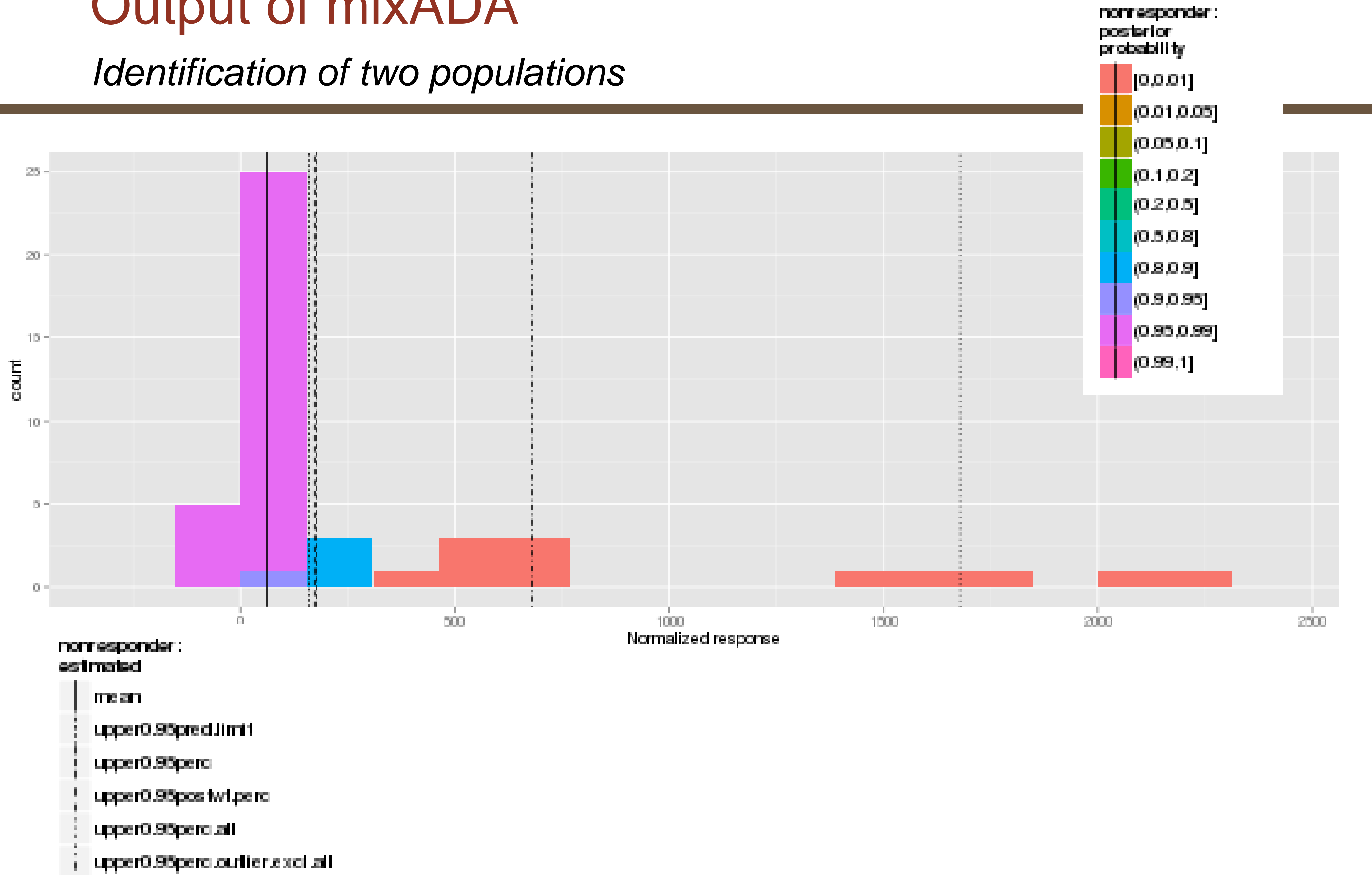
Output of mixADA

Identification of two populations



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The various approaches for SCP value calculation

*Normal distribution of data assessed and demonstrated**

SCP value	group	estimated	Description
62.8636	nonresponder	mean	N=33
160.3973	nonresponder	upper0.95pred.limit	Prediction limit can be used since we have here a normal distribution
172.3512	nonresponder	upper0.95postwt.perc	Percentile approach introduced a posteriori to deal with special cases
177.4000	nonresponder	upper0.95perc	Percentile approach
1677.8000	all	upper0.95perc.all	N=45
680.5750	all	upper0.95perc.outlier.excl.all	Shankar approach with 1.5 interquartile range rule for outlier exclusion

**Normal Box-Cox-Lambda and LRT for normality and log-normality for sample ID means (non-responder)*

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Discussion

Coating with an equimolar mix of peptides spanning the whole protein sequence instead of the «misfolded» recombinant full-length protein.

=> Miss the antibodies directed against conformational epitopes.

However, the aspecific responders suggests that the folding of the full-length protein produced in *E.coli* may not be correct either => likely missing the real conformational epitopes with the current strategy too.

Checks: confirmatory assay run with the full lenght protein.

Several positive controls commercially available.

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T-cell mediated (cellular) immune response to intra-cellular antigens

- From Chuah et al. (2013) Gene therapy for hemophilia (DOI: 10.1111/jth.12215)

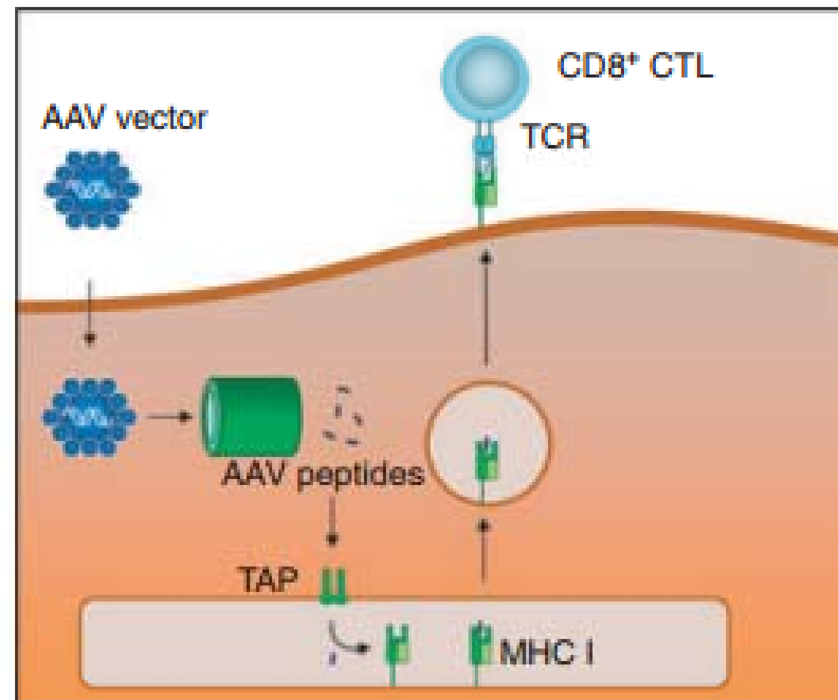

Fig. 3. Hypothetical model of adeno-associated virus (AAV)-specific T-cell immune responses. Following AAV transduction, AAV vector proteins (blue) are degraded by proteasomes, after which they are presented to cytotoxic T cells by major histocompatibility complex (MHC) class I molecules. At high vector doses, this results in the killing of the transfected cell by the cytotoxic T cells. CTL, cytotoxic T lymphocyte; TAP, transporter associated with antigen processing; TCR, T-cell receptor.

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Covance Harrogate:

Heather Wells

John Castle

James Munday

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Glossary

- ADA: anti-drug antibody(ies)
- NC: negative control
- rh: recombinant human
- vg: vector genomes
- Ig: immunoglobulin

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Varying Perspectives on ADAs

Day 2 break-out (Jupiter room) 16:20 17:40

Moderator: Matthew Barfield <Matthew.Barfield@gsk.com>

- 16:20 16:40 Nicolas White, MedImmune : CBA, LBA or NA – Regulatory Sense on Non-Sense
- 16:40 17:00 Gregor Jordan, F. Hoffmann-La Roche: Development of a bioanalytical method for the characterization of immune complexes
- 17:00 17:20 Lydia Michaut, Novartis: Anti-Vector antibody assays for gene therapy projects: analytical challenges
- 17:20 17:40 Ludovicus Staelens, UCB BioPharma: Approach to simultaneous detection, (semi-)quantification and isotyping of ADA in plasma samples by LC-MS/MS