Improvement of nAb assays with poor sensitivity and drug tolerance – challenges and solutions

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Outline of the presentation

- Once-Weekly semaglutide - peptide-based drug – GLP-1 analogue
- Original assays
- Post marketing commitments
- Re-development efforts (assay platform, sample pre-treatment, control antibodies)
- Revalidation of new assays
- Take home message
OW Semaglutide – a long-acting analogue of human GLP-1

**Immunogenicity**
Risk: Low

1-2% develop transient ADAs of low titres

**Endogenous counterpart with redundant function**

No impact of ADAs on efficacy and safety

**bAb measured during trial – nAb measured at follow-up only**

No nAb has been detected (in vitro or in vivo)

**HbA1c**

Cut point

Ab+ve samples (68 patients out of totally 4747)
Assay Characteristics of original assays

Ready to use cells from frozen
Same incubation time
Drug-sample
Sample and cells

Sample pre-treatment: 16% PEG 6000
Drug level at EC80
MRD: 30% sample

assay medium, 20% FBS

<table>
<thead>
<tr>
<th>Cell-based NAb assay</th>
<th>Control Ab</th>
<th>On-board drug nM</th>
<th>Assay sensitivity (ng/ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Semaglutide</td>
<td>mAb</td>
<td>0</td>
<td>3400</td>
</tr>
<tr>
<td></td>
<td></td>
<td>1</td>
<td>3400</td>
</tr>
<tr>
<td></td>
<td></td>
<td>2</td>
<td>3400</td>
</tr>
<tr>
<td>Endogenous GLP-1</td>
<td>Mix of 3 mAbs</td>
<td>0</td>
<td>6900</td>
</tr>
<tr>
<td></td>
<td></td>
<td>1</td>
<td>8800</td>
</tr>
<tr>
<td></td>
<td></td>
<td>2</td>
<td>ND</td>
</tr>
</tbody>
</table>
The two PMCs

PMC-1
• Develop and validate a sensitive assay to assess the neutralizing activity of anti-semaglutide antibodies and its cross-neutralizing effect on native GLP-1

PMC-2
• Conduct a study to assess the incidence of neutralizing antibodies to semaglutide and GLP-1 in subjects treated with semaglutide using the assays developed under PMC-1.
The samples may be obtained in pre-existing clinical studies.
Sensitivity and drug tolerance

Assay technical challenges

• Sensitivity and drug tolerance are **counteracting** parameters in **cell-based assays**

• Which parameters to change to ensure optimal sensitivity and drug tolerance?

![Graph showing sensitivity and drug tolerance balance]
## Parameters

<table>
<thead>
<tr>
<th>Assay platform</th>
<th>Pre-treatment</th>
<th>Control antibodies</th>
</tr>
</thead>
<tbody>
<tr>
<td>• Cells</td>
<td>• Acid and PEG</td>
<td>• mAb</td>
</tr>
<tr>
<td>• Seeding density</td>
<td>• PEG</td>
<td>• pAb</td>
</tr>
<tr>
<td>• Assay medium</td>
<td>• Protein A/G/L</td>
<td>• Human IgG antibodies from yeast or phage display libraries</td>
</tr>
<tr>
<td>• Incubation time</td>
<td>• Heat treatment</td>
<td></td>
</tr>
<tr>
<td>• Drug concentration</td>
<td>• Liposorb</td>
<td></td>
</tr>
<tr>
<td></td>
<td>• SPEAD/BEAD</td>
<td></td>
</tr>
</tbody>
</table>
**Assay Platform**

<table>
<thead>
<tr>
<th>Semaglutide (nM)</th>
<th>Sensitivity (ng/ml Ab)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>BHK cells</td>
</tr>
<tr>
<td>0</td>
<td>582</td>
</tr>
<tr>
<td>1.25</td>
<td>1293</td>
</tr>
<tr>
<td>10</td>
<td>8596</td>
</tr>
<tr>
<td>30</td>
<td>25000</td>
</tr>
<tr>
<td>60</td>
<td>66000</td>
</tr>
</tbody>
</table>

**Dose response and % FBS dependency, BHK-GLP-1 cells**

- **Cells**
  - BHK, DiscoverX, HEK cells
  - CLBA based on BHK cells

- **Seeding density**
  - 7000, 10000, 15000 cells/well

- **Assay medium**
  - % FBS in medium

- **Incubation time**
  - 1 hr, 3 hr, 5 hr

- **Drug concentration**
  - Reduce from EC80 to EC60

Increased FBS => increased EC50 level of semaglutide
=> Poorer sensitivity (in theory)
**Assay Platform**

- **Final assay platform**
  - Cells (BHK cells transfected with the hGLP-1R)
  - Seeding Cell density (7000 cells/well)
  - Incubation times (3 hrs)
  - Sample volume (30%)
  - Assay medium, (20% FBS)

**Increased** sample volume in combination with higher FBS percentage => improved sensitivity

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### Sensitivity in absence and presence of drug

<table>
<thead>
<tr>
<th>Sample volume</th>
<th>1% FBS and Drug at EC60 (sensitivity ng/ml)</th>
<th>20% FBS and Drug at EC60 (sensitivity ng/ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>5%</td>
<td>10%</td>
</tr>
<tr>
<td>0</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Semaglutide nM</td>
<td></td>
<td></td>
</tr>
<tr>
<td>1.25</td>
<td></td>
<td></td>
</tr>
<tr>
<td>5</td>
<td>2899</td>
<td>1560</td>
</tr>
<tr>
<td>10</td>
<td>6250</td>
<td>19655</td>
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<tr>
<td></td>
<td>25000</td>
<td>50000</td>
</tr>
<tr>
<td></td>
<td>78618</td>
<td>157237</td>
</tr>
</tbody>
</table>
Sample Pre-treatment

- Sample pre-treatment in original assays
  - 16% PEG 6000 precipitation
- Optimized pre-treatment
  - Different PEG sizes and percentages
  - 10% PEG 6000 is optimal
- Other pre-treatment regimes tested
  - Acid dissociation & PEG precipitation
    Loss of antibody, loss of sensitivity
  - Liposorp
    Lowers background, no effect on DT
  - HSA addition
    No effect
  - Heat pre-treatment
    Destroys Ab before drug
  - SPEAD
    Loss of sensitivity

Higher % PEG =>
More unspecific precipitation (drug and HSA) =>
Poorer Drug tolerance
Determination of drug concentration for stimulation

Semaglutide
EC60 = 400 pg/mL

GLP-1
EC60 = 20 pg/mL

- Same Assay platform
  - BHK cells
  - 7000 cells/well
  - 20% FBS in assay medium
  - 30% sample
- Optimised sample pre-treatment
  - 10% PEG 6000 precipitation
- Same control antibodies

Estimate theoretical sensitivity when determining the drug concentration used for stimulation -> Do I have a problem?

100 ng/mL Ab ~0.7nM binds 1.4nM drug.
Assay Characteristics of re-validated assays 1

**Ready to use cells from frozen**

**Same incubation time**
- Drug-sample
- Sample and cells

**Same platform**

**Sample pre-treatment, 10% PEG6000**

**Drug level at EC60**

**MRD: 30% sample**

**Assay medium, 20%FBS**

### Sensitivity in absence and presence of drug

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<thead>
<tr>
<th>Cell-based NAb assay</th>
<th>Control Ab</th>
<th>On-board drug nM</th>
<th>Old assay (ng/ml)</th>
<th>New assay (ng/ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Semaglutide mAb</td>
<td>0</td>
<td>3400</td>
<td>665</td>
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<tr>
<td></td>
<td>1</td>
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<td>1000</td>
<td></td>
</tr>
<tr>
<td></td>
<td>2</td>
<td>3400</td>
<td>2000</td>
<td></td>
</tr>
<tr>
<td>Endogenous GLP-1 Mix of 3 mAbs</td>
<td>0</td>
<td>6900</td>
<td>590</td>
<td></td>
</tr>
<tr>
<td></td>
<td>1</td>
<td>8800</td>
<td>460</td>
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<tr>
<td></td>
<td>2</td>
<td>ND</td>
<td>1500</td>
<td></td>
</tr>
</tbody>
</table>

*New assay: Validated by BioAgilytix EU*
**Assay Characteristics of re-validated assays**

**New assay:** Validated by BioAgilytix EU

- **Ready to use cells from frozen MRD: 30% sample**
- **Same incubation time**
- **Drug-sample**
- **Sample and cells**
- **Assay medium, 20%FBS**
- **Sample**
- **pre-treatment, 10% PEG6000**
- **Drug level at EC60**
- **Sensitivity in absence and presence of drug**
- **Cell-based NAb assay**
- **Control Ab**
- **On-board drug nM**
- **Old assay (ng/ml)**
- **New assay (ng/ml)**

<table>
<thead>
<tr>
<th>Endogenous GLP-1 Mix of 3 mAbs</th>
<th>1</th>
<th>0</th>
<th>2</th>
<th>1</th>
<th>0</th>
<th>2</th>
<th>3400</th>
<th>6900</th>
<th>3400</th>
<th>5900</th>
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<th>1500</th>
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<tr>
<td>8800</td>
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**The agency agreed that sensitivity in absence of on-board drug (1-2 nM) in follow-up samples is acceptable.**

**However, the agency acknowledged it could not be further optimised and released us from the PMCs.**

**BUT the sensitivity in presence of on-board drug (1-2 nM) in follow-up samples is not.**

**Impact on label:** The in vitro neutralizing activity of the antibodies is uncertain at this time.

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**However, the agency acknowledged it could not be further optimised and released us from the PMCs.**

**BUT the sensitivity in presence of on-board drug (1-2 nM) in follow-up samples is not.**

**Impact on label:** The in vitro neutralizing activity of the antibodies is uncertain at this time.
Control antibody

Monoclonal antibody directed against drug
Several tested
Original mAb best sensitivity

Polyclonal Ab directed against drug
Rabbits, Guinea pigs, Goats
2 pAbs approaching original mAb

Yeast display human IgG antibodies
Appr. 200 tested in bAb
Several rounds of maturation
Appr. 12 tested in nAb
3 better than mAb
2 selected for validation
**Binding kinetics of original control mAb**

- Slow association may impact sensitivity adversely
- Very slow dissociation may impact drug tolerance adversely

<table>
<thead>
<tr>
<th>Sample ID</th>
<th>KD (M)</th>
<th>kon (1/Ms)</th>
<th>kdis (1/s)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Semaglutide w/ 2 ug/ml Load</td>
<td>&lt;1.0E-12</td>
<td>8.74E+04</td>
<td>&lt;1.0E-07</td>
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<tr>
<td>Semaglutide w/ 20 ug/ml Load</td>
<td>&lt;1.0E-12</td>
<td>8.14E+04</td>
<td>&lt;1.0E-07</td>
</tr>
</tbody>
</table>
Binding kinetics of 3 yeast derived human IgG antibodies – exploratory data

Faster association may impact sensitivity positively
Faster dissociation may impact drug tolerance positively
Assay Characteristics of re-validated assays 2

- Ready to use cells from frozen
- Same incubation time
  - Drug-sample
  - Sample and cells
- Same platform
- Sample pre-treatment, 10% PEG 6000
- Drug level at EC60
- MRD: 30% sample
- Assay medium, 20% FBS

<table>
<thead>
<tr>
<th>Cell-based NAb assay</th>
<th>On-board drug</th>
<th>Old assay (ng/ml)</th>
<th>New assay 1 (ng/ml)</th>
<th>New assay 2 (ng/ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>nM</td>
<td>original control mAb</td>
<td>original control mAb</td>
<td>New Human IgG control mAb</td>
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<tr>
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<td>3400</td>
<td>665</td>
<td>98</td>
</tr>
<tr>
<td></td>
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<td>ND</td>
<td>1500</td>
<td>314</td>
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</tbody>
</table>
Assay Characteristics of re-validated assays 2

Ready to use cells from frozen
Same incubation time
Drug-sample
Sample and cells

Sample pre-treatment

Everything is the same EXCEPT the control antibody
Demonstrating better sensitivity and drug tolerance

<table>
<thead>
<tr>
<th>Assay Medium</th>
<th>Old assay (ng/ml)</th>
<th>New assay 1 (ng/ml)</th>
<th>New assay 2 (ng/ml)</th>
<th>New Human IgG control mAb</th>
</tr>
</thead>
<tbody>
<tr>
<td>Drug-sample</td>
<td>0</td>
<td>3400</td>
<td>665</td>
<td>98</td>
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<tr>
<td>Sample</td>
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<td>Sample</td>
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<td>Old control</td>
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<tr>
<td>Original</td>
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<td>8800</td>
<td>460</td>
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</tr>
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<td>2</td>
<td>ND</td>
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<td>314</td>
</tr>
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Take home messages

• **By changing the control antibody, a better sensitivity and drug tolerance of the assay could be demonstrated**
  • Sensitivity and drug tolerance dependent on relation-ship between epitope, drug-receptor affinity and drug-antibody affinity

• **Identification of control antibody**
  • Explore different platforms
    • mAb, pAb, display technology
  • Understand the binding kinetics of your control antibody

• **Know your assay**
  • Understand the theoretical sensitivity based on the concentration of drug used for stimulation of cells

• **Pro-active communication and consultation with authorities**
  • Timely and clear manner prior to phase 3
  • Request a meeting with regulators if necessary
  • Describe assay challenges, and strategies for how this will be addressed
  • Describe Immunogenicity risk strategy
  • Dependent on the Immunogenicity risk
    • Discuss/justify the suitability of the proposed strategy despite the lack of a sensitive nAb assay
    • Evaluation of clinically relevant in vivo nAb by PK, PD, ADA
Thanks to All for a lot of hard work for more than 2 years

- Novo Nordisk
  - Louise Jørgensen
  - Kristina Truelsen Jakobsen
  - Winni Andersen
  - Steffan Svejgaard Petersen
  - Mafalda dos Santos Marques Resende
  - Madeleine Dahlbäck
  - Dorte Bianca Corlin Wøldike
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  - Imke Müller
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  - Frank Höpner
  - Isabel Machens