hGH (and IGF-I) doping in sports: New methods of detection

Martin Bidlingmaier
Endocrine Laboratories
Medizinische Klinik – Innenstadt
Ludwig-Maximilians University
Munich / Germany
Martin Bidlingmaier holds shares in CMZ Assay GmbH
Identification of the growth-hormone-releasing peptide-2 (GHRP-2) in a nutritional supplement

Andreas Thomas, Maxie Kohler, Joachim Mester, Hans Geyer, Wilhelm Schänzer, Michael Petrou and Mario Thevis

obtained as „nutritional supplement“ OTC in Cyprus 2009
Black market product bought in Germany; box contained vials with both, rhGH and cadaveric hGH!

August 2007: Trainer of a German junior amateur cycling team finds „Jintropin“
and „Igtropin“ (recombinant IGF-I)
Problems in detecting GH / IGF-I abuse

Physicochemical properties

- Recombinant and endogenous hormones have same amino acid sequence
- GH/IGF-I have no N-linked glycosylation sites (unlike other peptide hormones e.g. EPO)

Physiological properties of GH

- Secretion pulsatile, short half-life (<15 min.)
- Secretion stimulated by sleep, exercise and stress
  - concentration per se does not prove exogenous administration!
Problems in detecting GH abuse

CORRIERE DELLA SERA
14 ottobre 2000
Sangue fuori norma per cinque ori di Sydney
Rosolino, Agostino Abbagnale, Idem, Bellutti e Trillini: valori alti nei test sull' ormone della crescita……..

“Growth hormone outside normal range in 5 Italian gold medal winners in Sydney”
Problems in detecting GH abuse

"Normal range" for GH???

<table>
<thead>
<tr>
<th>Time (min)</th>
<th>Glucose (mmol/l)</th>
<th>Insulin (pmol/l)</th>
<th>GH (µg/l)</th>
<th>IGF-I (µg/l)</th>
<th>IGFBP (µg/l)</th>
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<tbody>
<tr>
<td>A</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
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<tr>
<td>0</td>
<td>4.4</td>
<td>21.5</td>
<td>16.9</td>
<td>315</td>
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<td>20</td>
<td>4.7</td>
<td>21.6</td>
<td>18.6</td>
<td>305</td>
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<td>40</td>
<td>4.6</td>
<td>21.6</td>
<td>14.5</td>
<td>298</td>
<td></td>
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<tr>
<td>60</td>
<td>4.6</td>
<td>21.5</td>
<td>11.2</td>
<td>284</td>
<td></td>
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<tr>
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<td></td>
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<td></td>
<td></td>
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<tr>
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<td>4.5</td>
<td>50.2</td>
<td>9.9</td>
<td>262</td>
<td>3881</td>
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<td>30</td>
<td>4.6</td>
<td>28.6</td>
<td>7.8</td>
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<td>3647</td>
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<tr>
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<td>4.6</td>
<td>21.5</td>
<td>5.7</td>
<td>235</td>
<td>3942</td>
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<td>90</td>
<td>4.9</td>
<td>14.3</td>
<td>3.3</td>
<td>244</td>
<td>3782</td>
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<tr>
<td>120</td>
<td>5.2</td>
<td>28.6</td>
<td>1.9</td>
<td>239</td>
<td>3895</td>
</tr>
<tr>
<td>Normal range</td>
<td>3.8–6.6</td>
<td>43–186</td>
<td>0.06–5</td>
<td>100–400</td>
<td>1700–4000</td>
</tr>
</tbody>
</table>

Armanini et al., Br J Sports Med 2002
Strategies to discriminate endogenous from exogenous GH

1. the “marker approach” - pharmacodynamic endpoints of GH action
2. the “isoform approach” - molecular composition of GH in circulation
3. Potential new methods:
   - Ways to detect IGF-I abuse
   - Measurement of GH by LC-MS/MS?
Thanks to...

Zida Wu
Christian J. Strasburger

GH2000/2004 team:
R. Holt, E. Basset, P. Sonksen
R. Dall
J. Wallace

Anti-Doping laboratories:
London (D. Cowan, Ch. Bartlett)
Lausanne (M. Saugy, S. Giraud)
Sydney (Ch. Howe)
Kreischa (A. Kniess)
Rome, Athens, Cologne

WADA / IOC
BiSp (German Institute for Sport Sciences)

Australian consortium:
A. Nelson, K. Ho
Marker approach

Pharmacodynamic endpoints of GH: the GH 2000/2004 project

P-III-P

PINP

Osteo

ICTP

IGF-I

IGFBP-3

ALS

IGFBP-2

ICTP

cartilage

bone

pituitary derived

recombinant

hGH

liver
Marker approach

GH effects on IGF and Bone/Collagen Turnover
double blind, placebo controlled study
n= 40 placebo, 29 GH 0.1 IU/kg, 30 GH 0.2 IU/kg

IGF-I

P-III-P

IGF: Dall et al., J Clin Endocrinol Metab 2000
Bone: Longobardi et al., J Clin Endocrinol Metab 2000
Marker approach

Combination of markers allows discrimination

“Score” from discriminant functions

Men = - 6.586 + 2.905 * p3p + 2.100 * igf - 101.737 / age

Women = 8.459 + 2.454 * p3p + 2.195 * igf – 73.666 / age

Powrie et al. GH & IGF Research 2007
Marker approach

Discrimination between “normal” and “doped” (cut off for 1:10,000 risk of false positive)

GH2000 study (n=28 males)

Kreischa study (n=10 males)

Erotokritou-Mulligan et al. GH & IGF Research 2007
Effect of musculo-skeletal or soft-tissue injury
(Amateur and elite athletes, within 10 days after injury)

- no change in IGF-I
- increase in P-III-P (41.1 ± 16.6%)
- peak after 14 days
- no false positives

Impact on score
(n=127 amateur/ 16 elite athletes)

Erotokritou-Mulligan et al., J Clin Endocrinol Metab 2008
Marker approach

Influence of Demographic Factors and Sport Type
(n = 995 athletes, cross sectional)

Minor impact of

Ethnicity

Sporting type

Nelson AE et al., J Clin Endocrinol Metab 2006
Marker approach

Influence of Demographic Factors and Sport Type (n = 995 athletes)

Major impact of age

Nelson et al., J Clin Endocrinol Metab 2006
Marker approach

Influence of age: New studies in adolescent athletes

Cut off suggesting doping

- Increase in score with advancing puberty
- No false positives, but caution recommended in this age group

**FIG. 3.** Calculated GH-2000 scores for 85 elite male and 72 elite female adolescent athletes. Scores were calculated using the results from DSL IGF-I assay and CIS P-III-P assay. The *horizontal dotted line* shows GH-2000 score of 3.7 (the proposed cutoff that suggests doping with GH).

Guha N et al., J Clin Endocrinol Metab 2010
Marker approach

Influence of assays used: Comparability?

- IGF-I assay initially used (Nichols) discontinued
- P-III-P assays: mono vs. polyclonal ABs, different standard preparations

Guha N et al., J Clin Endocrinol Metab 2010
Summary: Marker approach

- IGF and collagen/bone markers increase with GH application

- Score from combination of IGF-I and P-III-P allows detection of GH application

- Sensitivity >80% on treatment, progressively decreasing within days after stop, but some cases positive for 2 weeks

- Little impact of ethnicity, sporting type and injury

- Crucial points:
  - Assay specific normative data, allowing score adjustment for age and gender
  - Standardization of analytical methods (assays)
Isoform approach

Differences in the molecular composition of GH in circulation

pituitary derived

hGH

22 kD hGH
20 kD hGH
multimeric hGH fragments etc.

recombinant

monomeric 22 kD-hGH only
### Background: hGH-Isoforms in circulation

<table>
<thead>
<tr>
<th>Isoform Description</th>
<th>Percentage (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>22 kDa monomer total</td>
<td>48</td>
</tr>
<tr>
<td>20 kDa monomer total</td>
<td>9</td>
</tr>
<tr>
<td>Acidic GH (desamido- &amp; acyl-GH)</td>
<td>5</td>
</tr>
<tr>
<td>22 kDa non-covalent dimers</td>
<td>14</td>
</tr>
<tr>
<td>22 kDa disulphide dimers</td>
<td>6</td>
</tr>
<tr>
<td>20 kDa non-covalent dimers</td>
<td>5</td>
</tr>
<tr>
<td>22 kDa non-covalent oligomer</td>
<td>7</td>
</tr>
<tr>
<td>22 kDa disulphide oligomer</td>
<td>3</td>
</tr>
<tr>
<td>Others</td>
<td>3</td>
</tr>
</tbody>
</table>

Isoform approach

Negative feedback of GH administration on pituitary GH secretion

Injection of rhGH

22 kD

HGH isoforms

Supression of pituitary hGH

Consequence: more 22kD, less other isoforms
Isolateform approach

Measurement of hGH isoforms by specific immunoassays

- **monomeric 22 kD GH**
- **20 kD GH**
- **dimeric GH**
- **multi-meric GH**
- **modif. GH**

**Assay 1** (22kD-hGH specific or “rec assay”)

**Assay 2** (permissive hGH assay or “pit assay”)

Calculate rec/pit ratio
Blinded analysis of 40 samples by rec/pit ratio

Rec/pit ratio

rhGH treated (n = 20)

endogenous GH (n = 20)

Wu et al., Lancet 1999;353(9156):895
Suppression of 20 kD hGH after injection of 22 kD hGH

Injection of rhGH

Suppression of 20 kD hGH secretion in females for 18 h (low dose) to 30 h (high dose) after rhGH

Suppression of 20 kD hGH after injection of 22 kD hGH

Assay sensitivity crucial issue for “doping test”: Something disappears which is already very low in normal males!

Epitope mapping for the mAbs used

20 kD hGH not involved!

strategy tests „monomeric 22kD“ vs. „other forms of GH“

GH isoforms during exercise

During acute exercise: 22 kD hGH peak

Immediately after exercise: non-22 kD hGH peak:

If any consequence: False negative test result

Wallace et al., J Clin Endocrinol Metab. 2001
Rec/pit ratios after injection of low and high dose of rhGH (% baseline)

A: initial rec/pit assay combination

C: second rec/pit assay combination for confirmation

Window of Opportunity?

Rec/pit ratios in 2 individuals after rhGH injection:

A: female

C: male

WoO ~ 20-30 h

Status of test implementation

• Final test version (ISO certified kits) used since 2008, meanwhile implementation in >30 WADA labs

• Independent EQAS performed regularly to ensure uniformity of results

• approximately 800 in competition (Euro2008, Beijing Olympics) and 400 out of competition samples (mainly UK) analysed

• Some countries less “active” (eg. USA 23 samples tested in 2009)

• WADA statistics for 2009: 175 ooC tests (e.g. 15 tests in biathlon)

• Februar 22, 2010: First positive case in an elite athlete (London Antidoping laboratory), 2 years ban accepted
Status of test implementation

(n=1803)

ratios obtained so far (mostly „in competition“)

„cut-offs“
(red: males / grey: females)

athlete with TUE (true GHD)

first positive case (London lab)
Summary: Isoform approach

- The relative increase in 22 kD hGH vs. other hGH isoforms after rhGH injection is detectable.
- Detection of rec. hGH use for ~ 20 – 30 hours after single injection.
- No impact of age, sex, acute exercise, ethnic background and sports discipline.
- Cut off values from normal subjects and athletes defined (WADA coordinated studies).
- Test implemented in >30 WADA accredited labs.
Potential new methods

- Ways to detect IGF-I abuse
  
  • Measurement of IGF-I by LC-MS/MS
Measurement of IGF-I by LC-MS/MS

measurement of IGF-1 and analogues is possible in human urine...
detection of synthetic IGF-1 analogues like longR3-IGF-1 is definite proof of illicit use...
...but recombinant IGF-I can not be distinguished from endogenous IGF-I!

Thomas A et al., Analyst, 2011, 136, 1003–1012
Potential new methods

- Ways to detect IGF-I abuse
  
  • Measurement of IGF-I by LC-MS/MS
  • Adaptation of the “marker approach”? 
Potential new methods

- Ways to detect IGF-I abuse
  - Measurement of IGF-I by LC-MS/MS
  - Adaptation of the “marker approach”?

- Measurement of GH by LC-MS/MS?
Current limitation of MS methods for GH:

- LoQ >3 ng/ml for 22 kD GH
- Turnaround time 1 sample per week
- Discrimination endogenous and exogenous?
Conclusion

- The marker method would provide a longer WoO but requires assay standardization.
- The isoform method is validated and in use.
- Despite short WoO the first positive case shows that intelligent testing strategies work.
- ...the main problem is that only very few samples are collected!

No sampling, no test, no cases!
SAVE THE DATE!

6th International Congress of the GRS and IGF Society

October 16 - 20, 2012
Munich · Germany

www.grs-igf2012.org