



CALUX[®] bioassays for the detection of anabolic androgenic steroid abuse in sport doping control

Corine Houtman

BioDetection Systems BV

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- **Introduction** doping abuse in sports
 anabolic androgenic steroids
- **WADA project:** application of CALUX assays in doping
 control?
- **Results I:** activity profiling pure compounds
- **Results II:** mixtures
- **Results III:** analysis of urine samples
- **Conclusions and outlook**

- **Doping: present-day issue, not new**
- 17th ct: Dutch settlers provided with tonic sauce (“doop”) by Indians in New –Amsterdam.
- 19th ct: cyclists: caffein, cocain (delay fatigue)
- 1967: Tom Simpson dies in TdF (amphetamin)
- 1970s and 1980s: systematic doping of athletes in East Germany
- 1988: Ben Johnson stripped of gold medal 100m sprint OG (stanozolol)
- 1998: Festina group disqualified in TdF (EPO)
- 2006, 2007: large doping scandals in TdF (blood transfusion, testosterone)



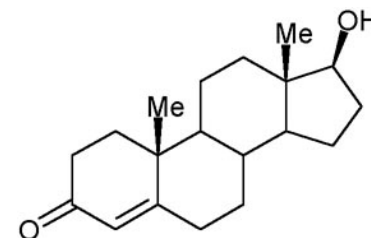
1999: Establishment **World Anti-doping Agency (WADA)** to promote and coordinate the fight against doping in sport internationally.

WADA :

- **Protect athlete's health, ensure fair play**
- **World Anti-doping code, Prohibited list:**
 - substances (anabolic agents, β 2 agonists, EPO etc.)
 - methods (blood transfusion, gene doping)
- **In- and out-of-competition testing**
- **2006 tests:**
 - 2941 urine; 265 blood; 1230 EPO samples
 - 57 adverse analytical findings, 12 anabolic agents

Anabolic androgenic steroids (AAS) - I

- **Derived from male hormone testosterone**
- **Mechanism:** activation androgen receptor



- **Effects:**
 Anabolic: muscular growth (tissues)
 Androgenic: secondary male sexual characteristics (organs)

- **Synthetic modifications:**
 delay degradation
 magnify anabolic, minimize androgenic effects
 circumvent chemical analysis





Anabolic androgenic steroids (AAS) - II

- **Current analysis for doping control:**
GC or LC separation, MS or MS/MS detection
- **Advantage:**
sensitive, specific, analysis of inactive metabolites
- **Concern:**
risk of overlooking compounds with similar activities but unknown structures
designer steroids (THG, norbolethone)

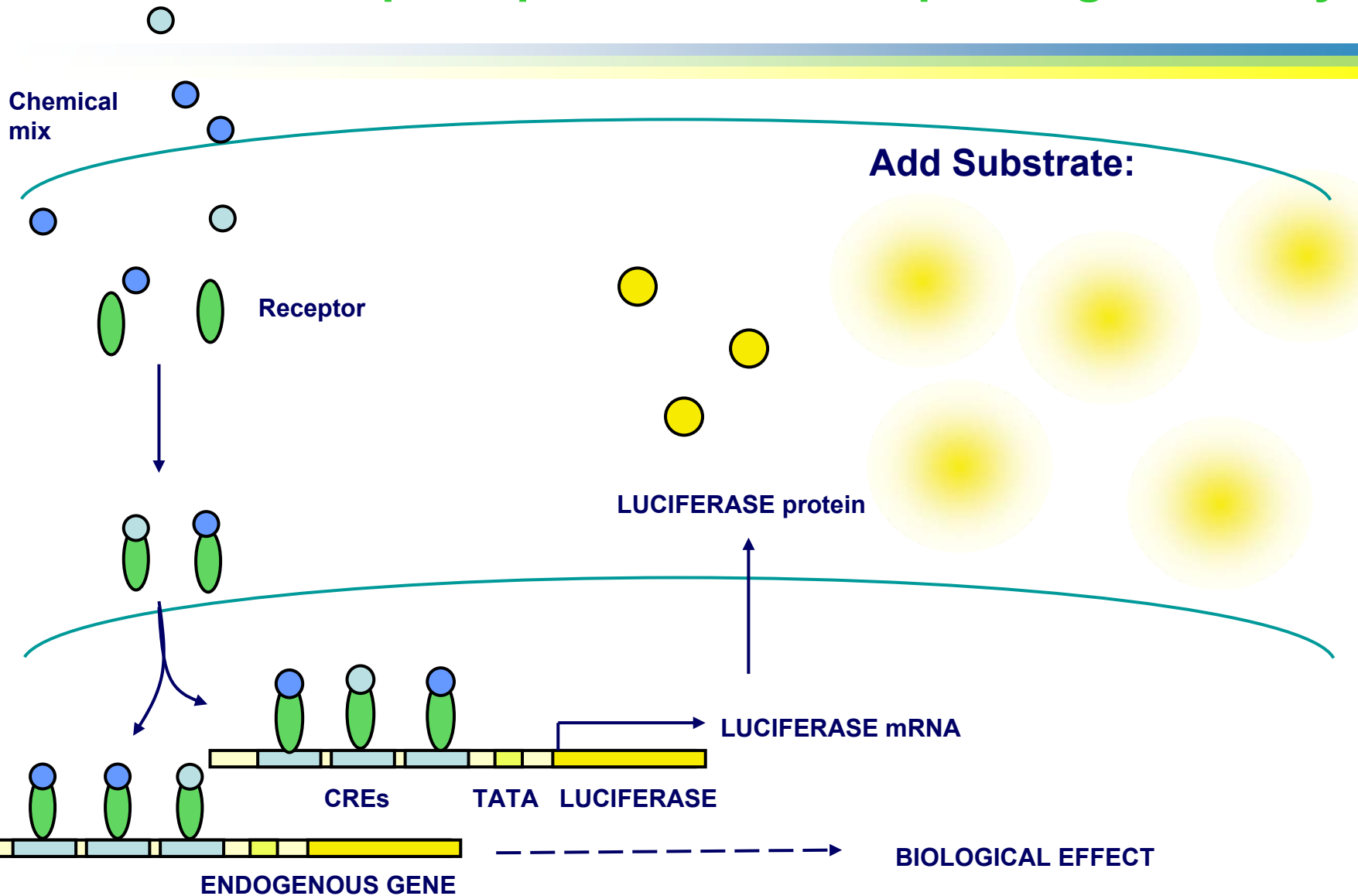


Anabolic androgenic steroids (AAS) - III

- **CALUX bioassays use receptor activation as endpoint**
- **Independent of knowledge about chemical structure**



General principal of CALUX[®] reporter gene assays



- **CALUX bioassays use receptor activation as endpoint**
- **Independent of knowledge about chemical structure**
- **Panel of CALUX bioassays for steroids available:**
 - estrogens (estrogen alpha and beta receptor – ER α , ER β)
 - androgens (androgen receptor – AR)
 - progestins (progesterone receptor – PR)
 - glucocorticoids (glucocorticosteroid receptor – GR)

=> Use CALUX assays to detect AAS with known and unknown structures?

- **WADA project (2004-2007):** *Development of a tight bioassay-based control system for steroids and other prohibited substances in sport doping*
- **Workpackages:**
 1. Establishment of suitable CALUX bioassays for the detection of steroids
=> pure compounds and mixtures
 2. Method development for CALUX analysis of urine and plasma
 3. Determination of endogenous steroid activities in urine samples
 4. Design of an integrated system for sport doping control using a combination of CALUX bioassays and chemical-analytical methods



I. Steroidal activities of pure compounds



Results I: concentration response curves of pure compounds in AR CALUX

pure solutions of >120 steroids:

- endogenous AAS
- exogenous AAS
- metabolites
- potential designer AAS

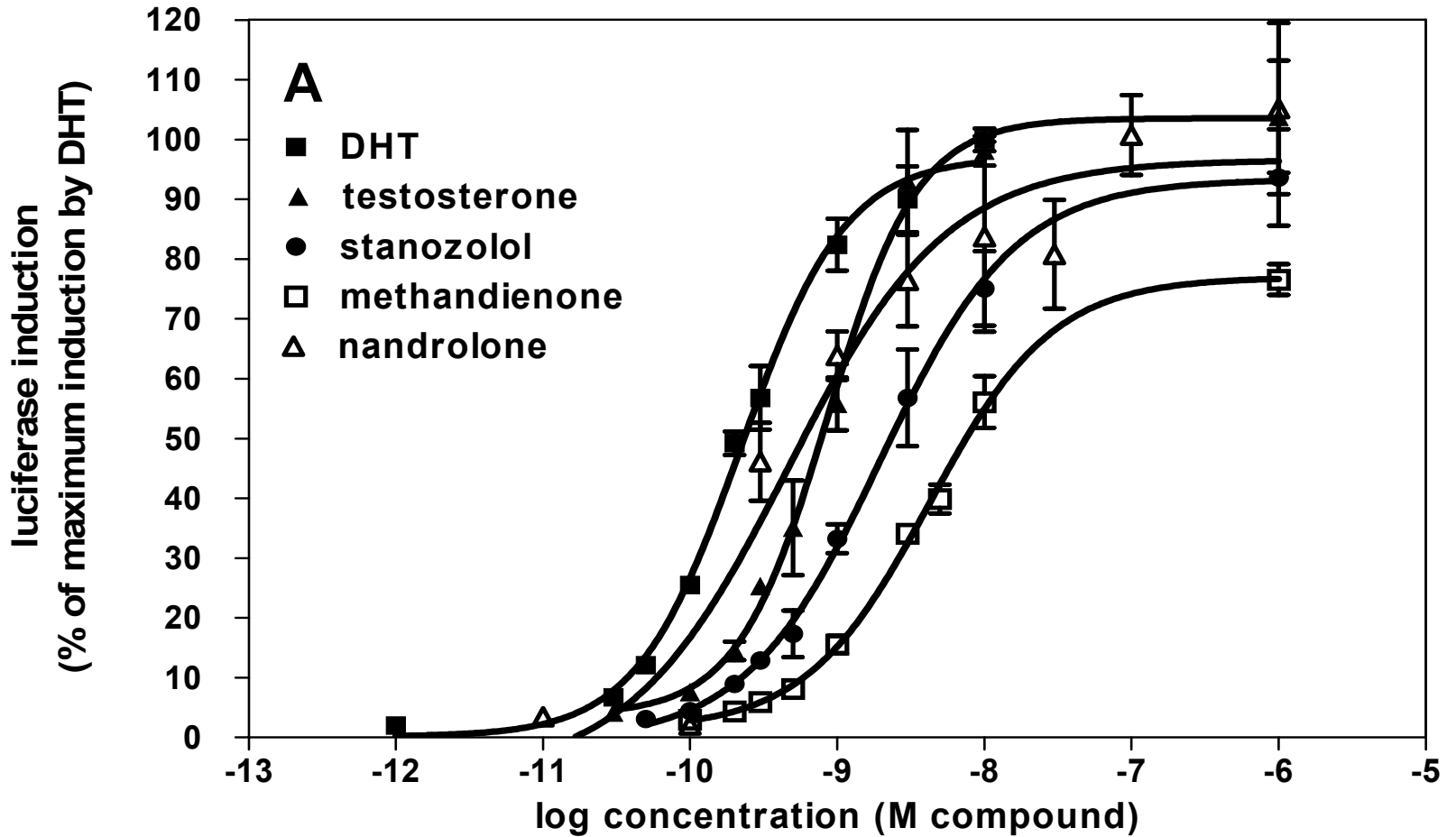
Tested in panel of 5 CALUX bioassays:

- AR, ER α , ER β , GR, PR
- concentration response curves

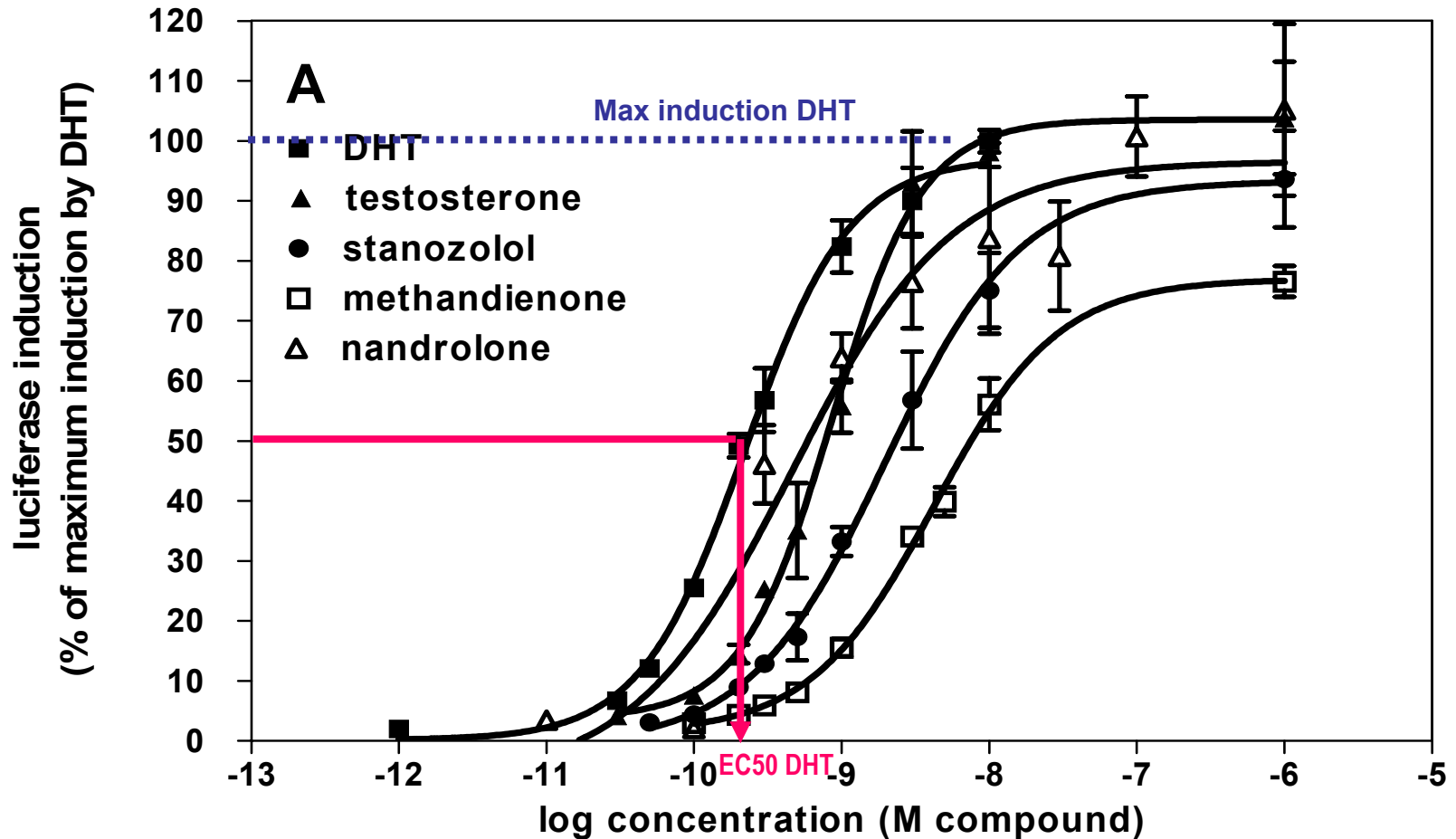
Compare activity with that of reference compounds:

- DHT, 17 β -E2, dexamethasone, org2058

Results I: concentration response curves of pure compounds in AR CALUX



Results I: concentration response curves of pure compounds in AR CALUX



- Maximum induction: % of maximum induction by DHT

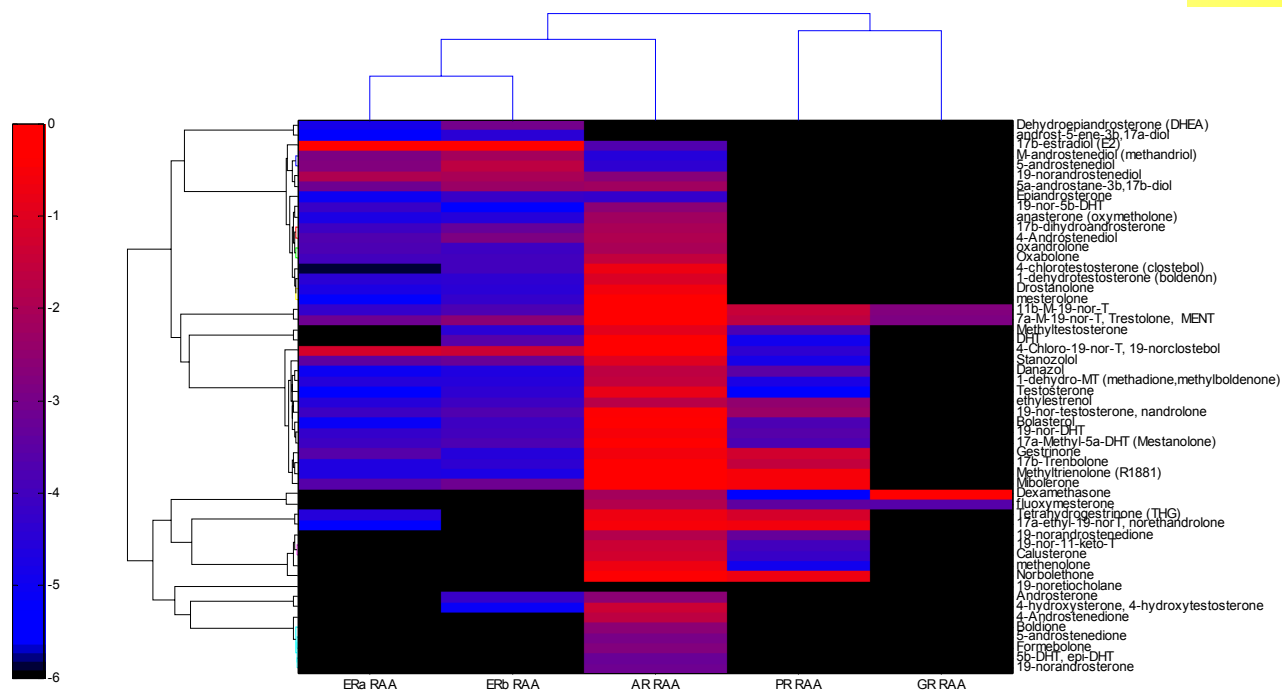
- Relative Agonistic Activity: $RAA = EC_{50} \text{ DHT} / EC_{50} \text{ compound}$



Results I: AAS screening using a panel of assays

Compound	AR CALUX			PR CALUX			ERa CALUX			ERb CALUX			GR CALUX		
	logEC50 (M)	RTA (%)	RAA 1=DHT	logEC50 (M)	RTA (%)	RAA 1=org2058	logEC50 (M)	RTA (%)	RAA 1=E2	logEC50 (M)	RTA (%)	RAA 1=E2	logEC50 (M)	RTA (%)	RAA 1=dex
WADA 2005 compounds															
Mibolerone	-10.11	92	2.0479	-9.78	98	0.3069	-7.17	89	0.0002	-6.77	89	0.0008	>-5	<5	0.0000
5a-dihydro-testosterone (DHT)	-9.94	100	1.0000	-5.38	76	0.0000	>-5	<5	0.0000	-6.24	106	0.0002	>-6	<5	0.0000
Methyltrienolone (R1881)	-9.92	69	1.0625	-9.80	88	0.3281	-6.16	61	0.0000	-5.15	53	0.0000	>-6	<5	0.0000
4-chloro-19-nor-T, 19-norclostebol	-9.86	101	1.1398	-5.91	45	0.0000	-9.63	95	0.0362	-8.44	95	0.0401	>-5	<5	0.0000
17b-trenbolone	-9.83	104	0.9434	-8.61	96	0.0261	-6.16	105	0.0000	-5.50	109	0.0000	>-5	<5	0.0000
Bolasterone	-9.79	106	0.9665	-6.38	77	0.0002	-5.68	160	0.0000	-5.82	110	0.0001	>-5	<5	0.0000
Mesterolone	-9.57	108	0.5894	>-5	45	0.0000	-5.60	151	0.0000	-5.60	87	0.0001	>-5	<5	0.0000
Norbolethone	-9.56	99	0.5654	-9.52	90	0.2124	-4.90	173	0.0000	>-5	8	0.0000	>-5	<5	0.0000
19-nor-T (Nandrolone)	-9.52	92	0.4857	-7.98	61	0.0050	-6.76	94	0.0001	-6.18	80	0.0002	>-5	<5	0.0000
17a-ethyl-19-nor-T (Norethandrolone)	-9.47	89	0.4611	-9.67	95	0.2359	-5.30	120	0.0000	>-5	40	0.0000	>-5	<5	0.0000
Gestrinone	-9.33	60	0.3321	-8.91	79	0.0525	-7.19	87	0.0001	-5.30	75	0.0000	>-5	<5	0.0000
Drostanolone	-9.33	115	0.3320	>-5	16	0.0000	-6.08	91	0.0000	-5.43	62	0.0000	>-5	<5	0.0000
Methenolone	-9.26	104	0.2875	-5.35	92	0.0000	>-5	48	0.0000	>-5	38	0.0000	>-6	23	0.0000
4-chlorotestosterone (Clostebol)	-9.21	108	0.2589	>-5	25	0.0000	-5.90	114	0.0000	-5.72	78	0.0001	>-5	<5	0.0000
17a-methyl-DHT (Mestanolone)	-9.21	105	0.2545	-6.26	86	0.0001	-6.65	123	0.0001	-5.78	93	0.0001	>-5	<5	0.0000
Tetrahydrogestrinone (THG)	-9.20	70	0.2459	-9.02	104	0.0673	-6.26	121	0.0000	>-5	18	0.0000	>-5	<5	0.0000
Testosterone (T)	-9.18	94	0.1460	>-5	78	0.0000	-5.34	127	0.0000	-5.56	96	0.0000	>-5	<5	0.0000
17a-methyl-T (MT)	-9.08	108	0.1965	-6.17	70	0.0002	>-5	100	0.0000	-5.17	100	0.0000	>-5	<5	0.0000
Stanozolol	-8.95	73	0.1397	-5.39	64	0.0000	-7.36	145	0.0002	-6.64	101	0.0006	>-5	<5	0.0000
1-dehydrotestosterone (Boldenon)	-8.83	87	0.1072	>-5	30	0.0000	-6.35	111	0.0000	-5.49	87	0.0000	>-5	<5	0.0000
Calusterone	-8.72	72	0.0816	-6.04	85	0.0001	>-5	<5	0.0000	>-5	9	0.0000	>-5	<5	0.0000
4-hydroxytestosterone (4-hydroxy-T)	-8.57	111	0.0579	>-5	20	0.0000	>-5	75	0.0000	>-5	70	0.0000	>-5	<5	0.0000
1-dehydro-MT (Methandienone, Methylboldenone)	-8.40	95	0.0401	-5.60	61	0.0000	-6.29	137	0.0000	-5.30	57	0.0000	>-5	<5	0.0000
Oxabolone	-8.37	92	0.0366	>-5	25	0.0000	-6.85	99	0.0001	-5.89	89	0.0001	>-5	<5	0.0000
4-androstenedione	-8.34	82	0.0572	>-5	<5	0.0000	>-5	<5	0.0000	>-5.0	<5	0.0000	>-5	<5	0.0000
Danazol	-8.27	56	0.0289	-6.65	52	0.0003	-5.76	124	0.0000	-5.19	49	0.0000	>-5	<5	0.0000
Ethylestrenol	-8.17	63	0.0231	-7.83	85	0.0034	-6.25	107	0.0000	-5.84	93	0.0001	>-5	<5	0.0000
Fluoxymesterone	-8.14	78	0.0217	-6.92	72	0.0004	>-5	<5	0.0000	>-5.0	8	0.0000	-5.72	48	0.0002
19-nor-androstenedione	-8.13	101	0.0213	-6.97	17	0.0005	-5.24	98	0.0000	>-5	30	0.0000	>-5	<5	0.0000
4-androstenediol	-7.99	55	0.0153	>-5	<5	0.0000	-7.09	130	0.0001	-6.96	124	0.0013	>-5	<5	0.0000
Oxandrolone	-7.90	83	0.0124	>-5	<5	0.0000	-7.03	96	0.0001	-5.79	80	0.0001	>-5	<5	0.0000
5a-androstane-3a,17b-diol	-7.88	80	0.0121	>-5	<5	0.0000	-6.70	108	0.0000	-6.50	90	0.0005	>-5	<5	0.0000
Anasterone (Oxymetholone)	-7.77	95	0.0093	>-5	12	0.0000	-6.05	136	0.0000	-5.31	90	0.0000	>-5	<5	0.0000
5a-androstane-3b,17b-diol	-7.71	69	0.0082	>-5	<5	0.0000	-7.66	120	0.0004	-7.57	98	0.0054	>-5	<5	0.0000
Boldione	-7.41	75	0.0029	>-5	<5	0.0000	>-5	<5	0.0000	>-5	<5	0.0000	>-5	<5	0.0000
3a-hydroxy-5a-androstan-17-one (Androsterone)	-7.39	13	0.0038	>-5	<5	0.0000	>-5	74	0.0000	-5.70	51	0.0001	>-5	<5	0.0000
19-nor-androstenediol	-7.31	56	0.0032	>-5	15	0.0000	-8.85	128	0.0060	-7.85	100	0.0105	>-5	<5	0.0000
Formebolone	-7.16	115	0.0016	>-5	<5	0.0000	>-5	<5	0.0000	>-5	<5	0.0000	>-5	<5	0.0000
5-androstenedione	-6.95	94	0.0014	>-5	<5	0.0000	>-5	45	0.0000	>-5	40	0.0000	>-5	<5	0.0000
19-norandrosterone	-6.77	22	0.0009	>-5	<5	0.0000	-5.20	105	0.0000	>-5	35	0.0000	>-5	<5	0.0000
5b-DHT (epi-DHT)	-6.70	51	0.0006	>-5	<5	0.0000	>-5	17	0.0000	>-5	8	0.0000	>-5	<5	0.0000

Results I: Steroid activity profiling using hierarchical cluster analysis



- >120 prohibited and potential designer AAS tested for androgenic, estrogenic, progestagenic and glucocorticoid activity
- 98% of compounds active in 1 or more assays (conc 10^{-12} tot 10^{-5} M)
- 88% androgenic, some estrogenic, progestagenic => AR CALUX

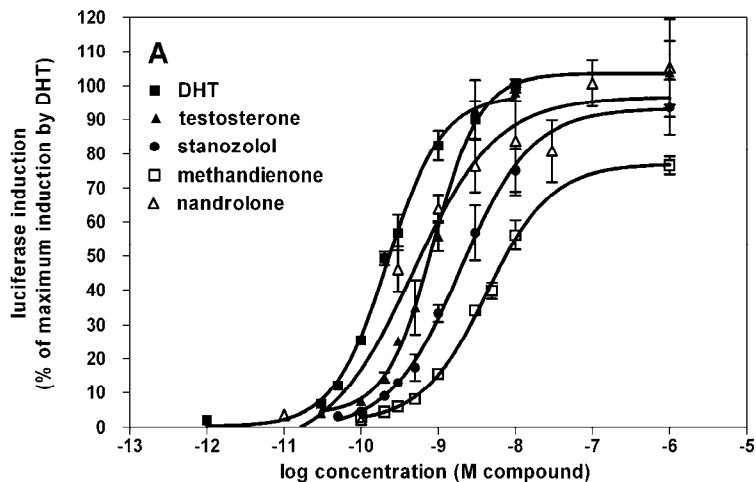
II. Description of mixture behaviour of AAS in the AR CALUX bioassay

How do mixtures of AAS behave in the AR CALUX assay?

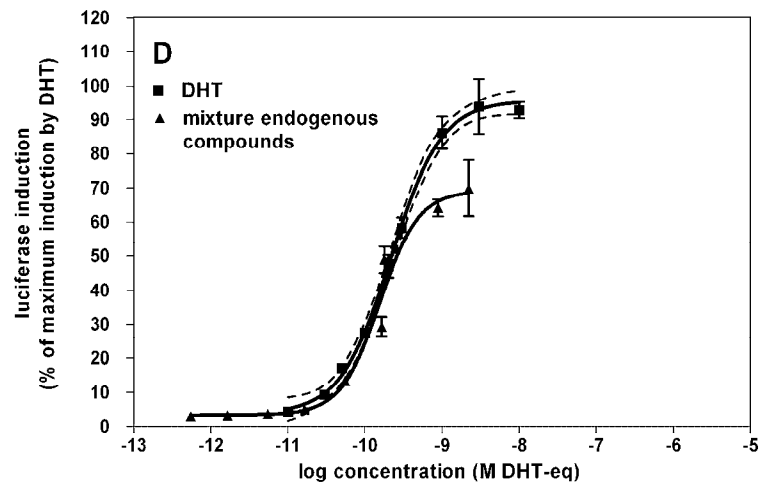
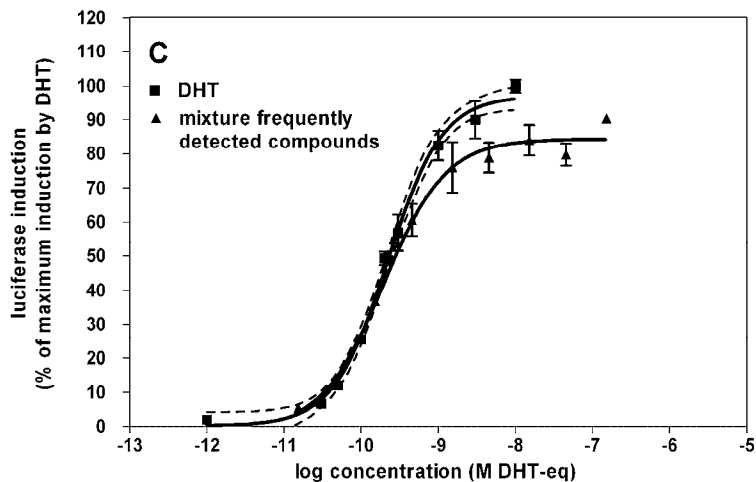
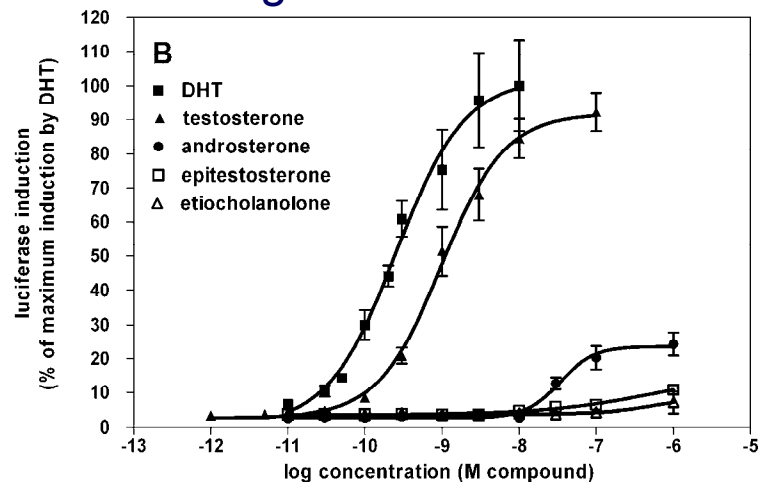
- urine / plasma: mixtures of steroids (+ doping?)
- AR CALUX measures combined androgenic activity
- independent similar action => concentration addition ?
- $\sum_{i=1}^n C_i * RAA_i = c_{sum}$ (DHT-equiv.) => overlap mixture and DHT curve

Results II: Mixtures of androgens behave additively in AR CALUX

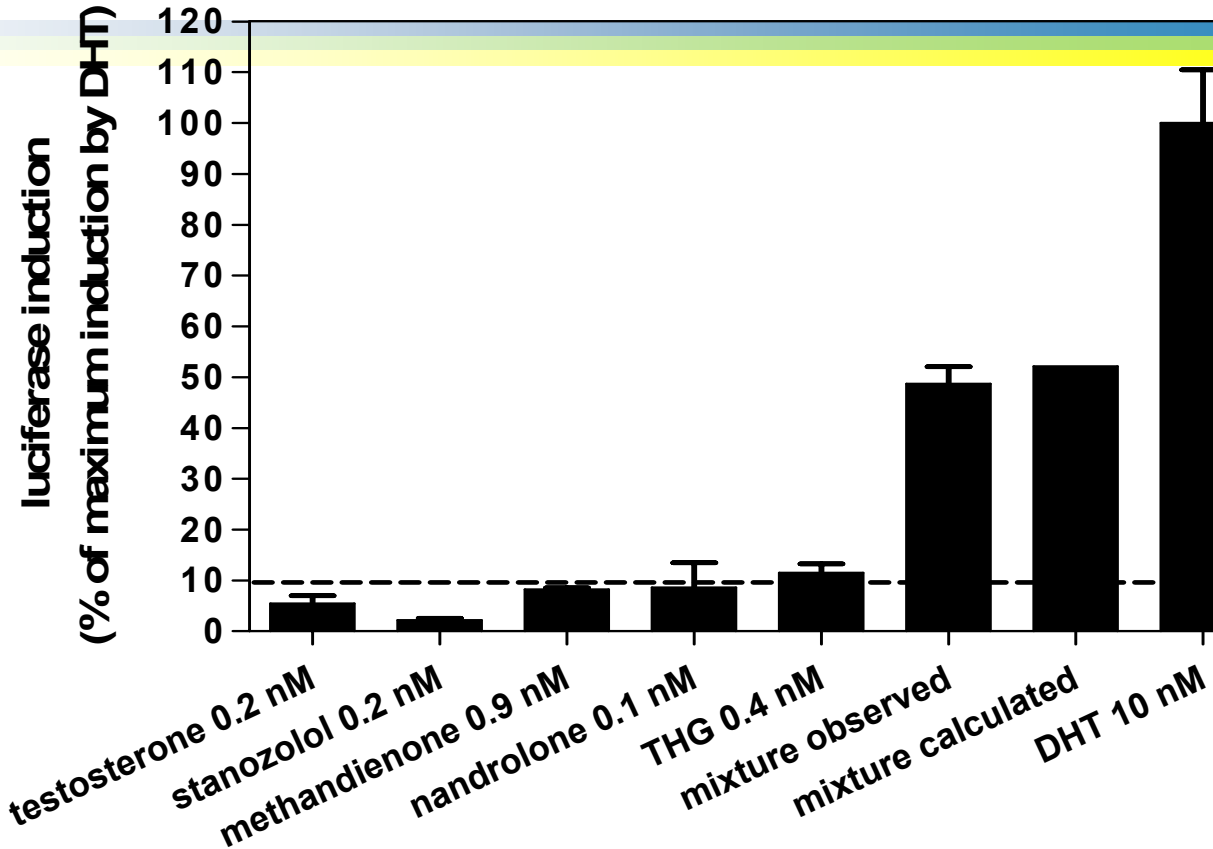
4 frequently detected AAS



Endogenous steroids



Results II: Mixtures of androgens behave additively in AR CALUX



Low individual concentrations, significant mixture effects

Mixture behaviour predictable with CA

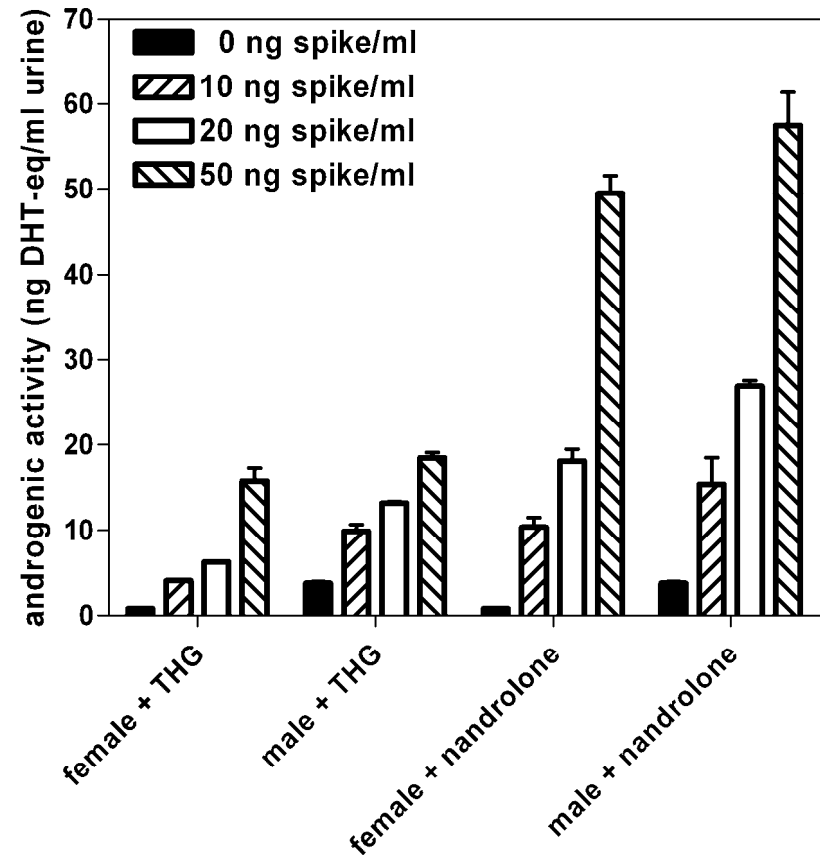
=> use of RAA allowed

=> compare bioassay activity with chemically determined concentrations

III. Analysis of steroidal activities in urine

Can AR CALUX discriminate between doping positive and negative samples?

- Analysis of spiked samples
- conc. dep. activity increase
- compare with background levels!



Determination of background steroidal activities

- 102 urine samples athletes
- gender, race, vegetarian diet, contraception use
- all samples negative in doping analysis

Aims:

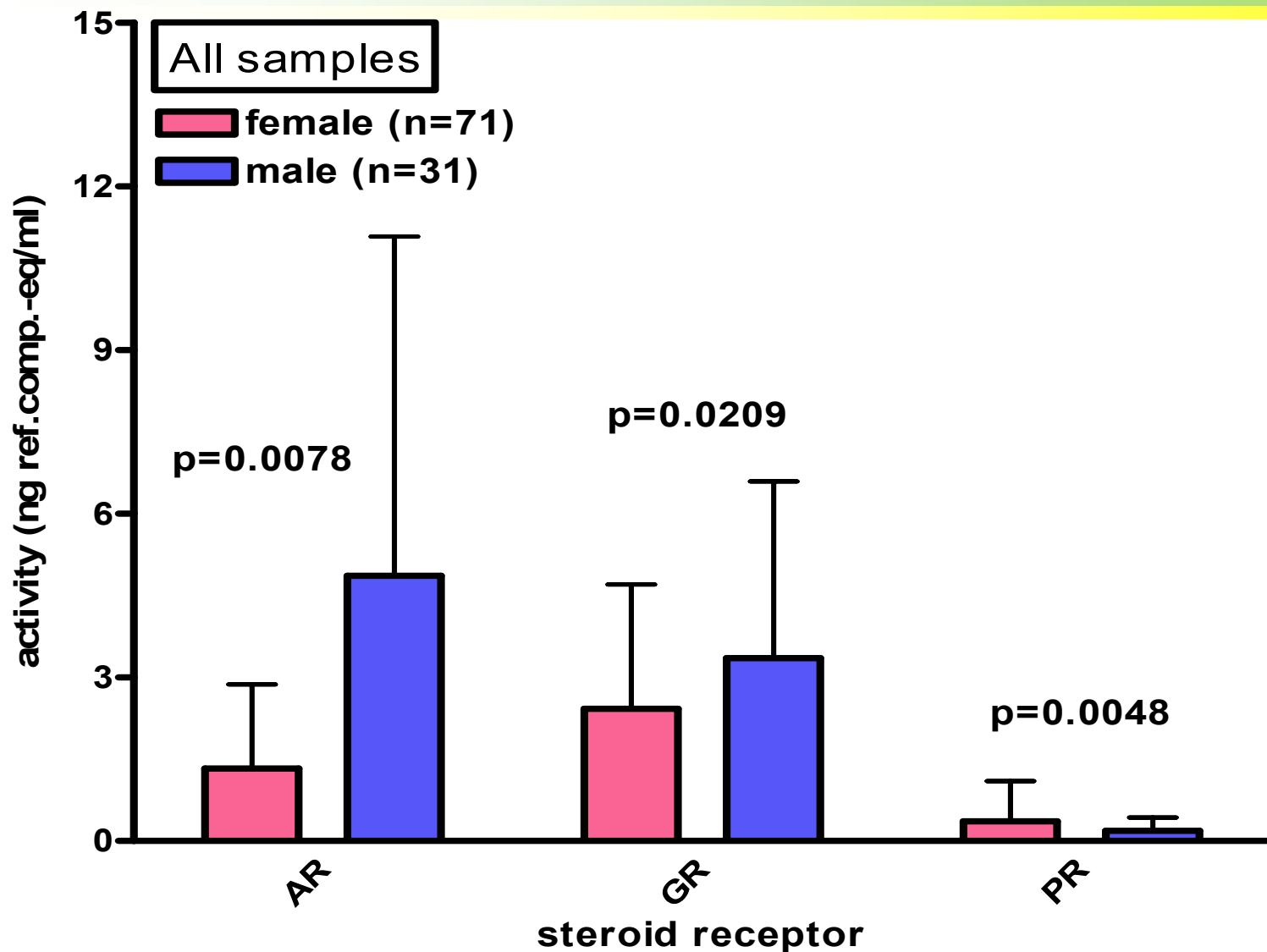
- estimation endogenous steroidal activity levels
- inter-individual variations
- correlations between activities and gender, race etc



Results III: background steroidal activities in urine of athletes

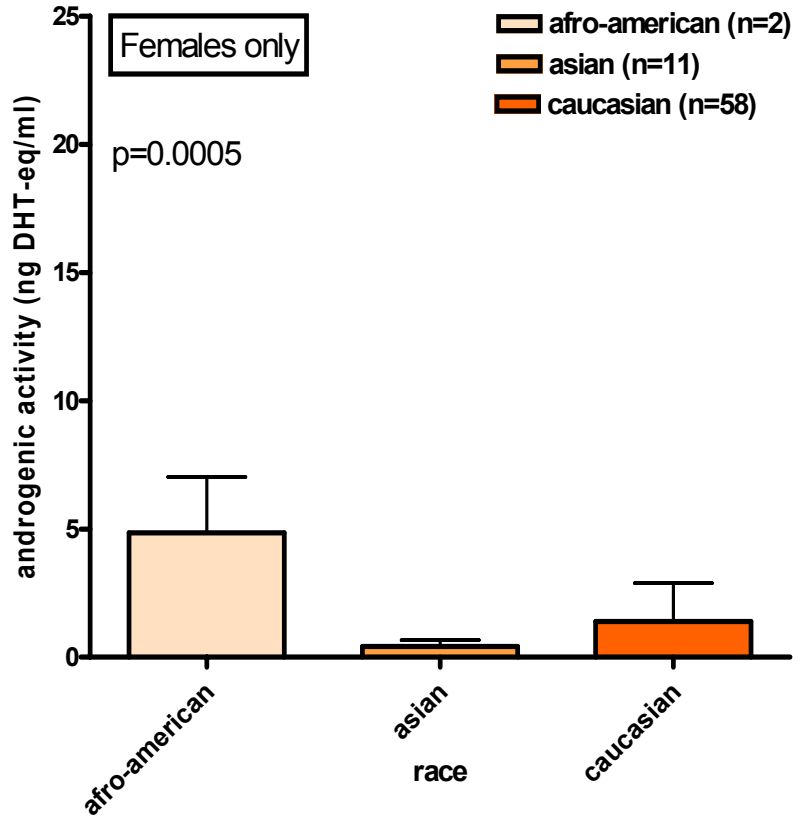
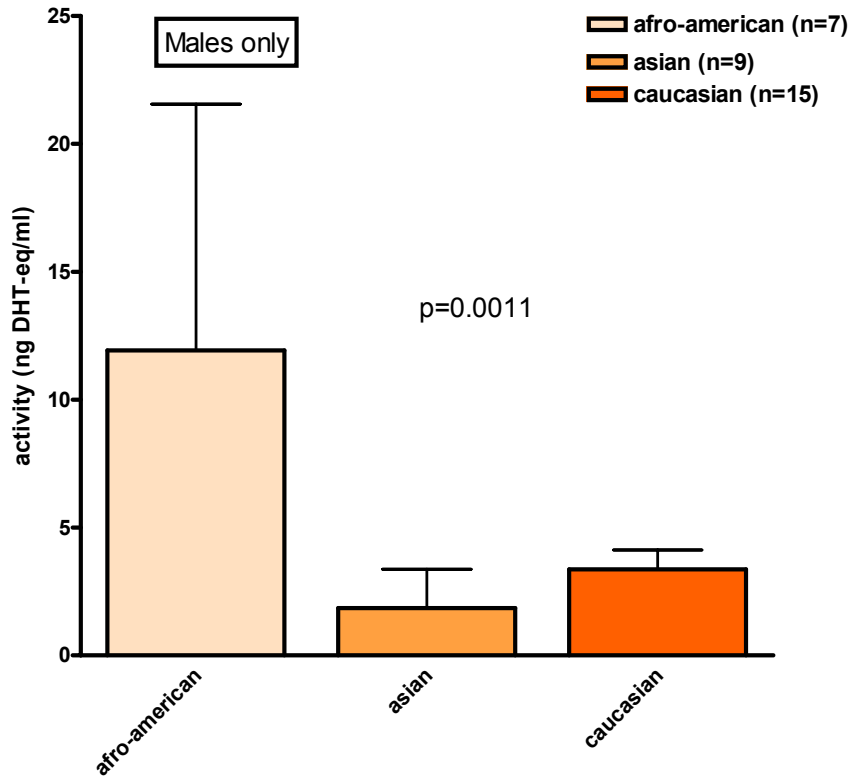
ref. comp. (ng/ml)	AR		ER α			ER β		GR		PR	
	DHT-eq		E2-eq			E2-eq		Dex-eq		Org2058-eq	
	♀	♂	♀	♀*	♂	♀	♂	♀	♂	♀	♂
Mean	1.33	4.86	5.46	2.24	2.15	12.24	11.66	2.42	3.35	0.36	0.19
sem	0.18	1.12	2.60	0.27	0.50	3.29	6.26	0.27	0.58	0.09	0.04
median	0.85	2.99	1.73	1.66	1.45	3.73	2.47	1.91	2.43	0.10	0.11
stddev	1.54	6.22	21.92	2.25	2.77	27.43	34.86	2.28	3.24	0.74	0.24
minimum	<lod	0.26	0.01	0.01	0.13	0.25	0.24	<lod	<lod	<lod	<lod
maximum	8.12	25.42	179.3	9.56	15.32	167.3	191.9	10.60	15.78	4.60	1.22
95% value	3.9	15.4	42.0	6.0	6.9	58.5	70.8	6.2	8.9	1.6	0.6
sample size	71	31	71	69	31	71	31	71	31	71	31

Results III: steroid activities in urine of athletes correlation with gender



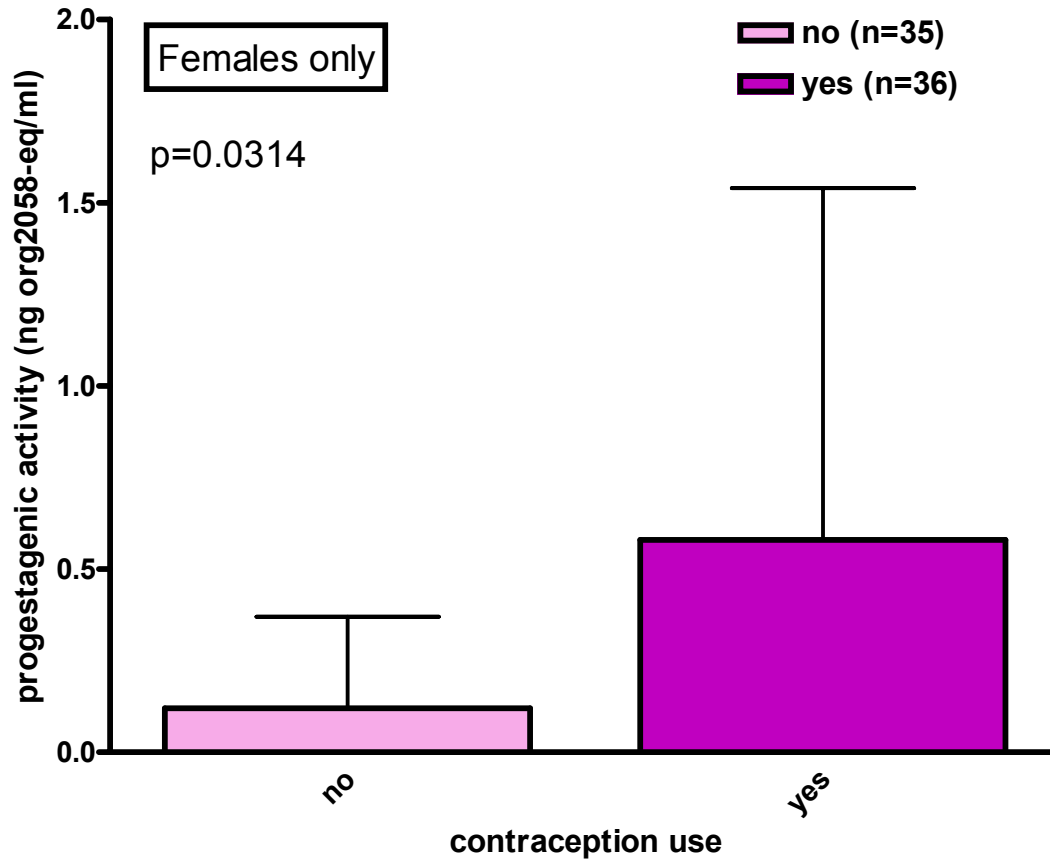


Results III: steroid activities in urine of athletes correlation androgenic activity with race

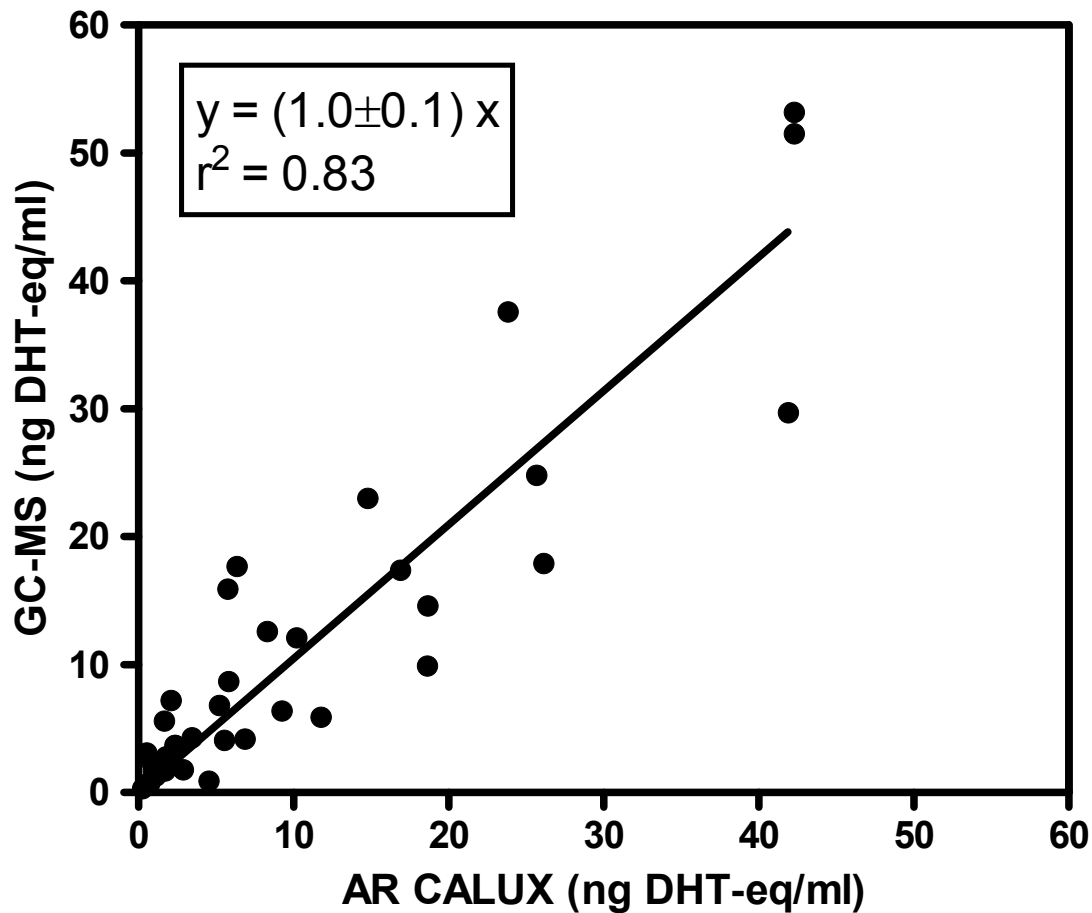




Results III: steroid activities in urine of athletes correlation progestagenic activity and contraception



Σ DHT and Testosterone



$$[DHT] * RAA_{DHT} + [T] * RAA_T$$

Pure compounds and mixtures

- CALUX bioassays are able to detect the activities of AAS on the WADA prohibited list and designer anabolic steroids
- Most AAS show androgenic activity, some also estrogenic, progestagenic
- Androgenic mixture activity of AAS correctly predicted with Concentration Addition

Analysis of urine samples

- Sensitive analysis steroidal activities in urine (and plasma) samples
- Concentration dependent increase of activity by AAS doping
- Considerable inter-individual variations in background levels

- Background steroidal levels correlated with gender, race, contraception use.
- Background androgenic activity largely explained by endogenous DHT and T.

Future challenges:

- Discrimination between doping positive and negative samples
 - analysis positive samples
 - group / individual specific normal background levels?
 - separation endogenous and exogenous AAS?
- Combination of CALUX + chemical analysis could provide a tight system for doping detection

• BDS

Bram Brouwer
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Monique van de Heijning
Bart Pieterse
Edwin Sonneveld
Marije Westra

• RIVM (ARO)

Henk Herbold
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• WADA

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