

Selection of ERCs and longitudinal evaluation of IRMS data

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ERC Characteristics

- Not affected by external administration of forbidden or licit substances
- Related to the metabolic pathway of the TCs
- In enough concentration to facilitate sample preparation

IRMS Markers

TCs	ERCs
Androsterone (A)	11b-hydroxy-androsterone (11OHA)
Etiocholanolone (Etio)	11b-hydroxy-etiocholanolone(11OHE)
5 α -androstane-3 α ,17 β -diol (5aAdiol)	11-keto-etiocholanolone (11KE)
5 β -androstane-3 α ,17 β -diol (5Adiol)	5 α -androst-16-en-3 α -ol (16-en)
Testosterone (T)	Pregnanediol (PD)
Epitestosterone (E)	Pregnanetriol (PT)
DHEA	
Epiandrosterone (EA)	
.....	

IRMS Markers Confounding factors

ERCs	
11b-hydroxy-androsterone (11OHA)	Adrenosterone (11-Oxo)
11b-hydroxy-etiocholanolone(11OHE)	Adrenosterone
11-keto-etiocholanolone (11KE)	Adrenosterone Cortisol
5 α -androst-16-en-3 α -ol (16-en)	Small variation may be due to Pregnenolone
Pregnanediol (PD)	Pregnenonolone Progesterone
Pregnanetriol (PT)	Not reported

WADA EQAS List – Appendix A Confounding Factors

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ii. Non-Prohibited Substances (Confounding Factors) that May Alter the Urinary Steroid Profile			
Confounding Factor	Markers of the Steroid Profile affected	Target Substances	Normative document
Ethylglucuronide (EtG)		<ul style="list-style-type: none"> • EtG 	TD EAAS
Inhibitors of 5 α -reductase: <ul style="list-style-type: none"> • Finasteride • Dutasteride 	Testosterone (T) and <i>Metabolites</i> : Androsterone (A); Etiocholanolone (Eti); 5 α -Androstane-3 α ,17 β -diol (5 α Adiol); 5 β -Androstane-3 α ,17 β -diol (5 β Adiol); and epimer Epitestosterone (E).	<ul style="list-style-type: none"> • Carboxy-finasteride • 4-hydroxy- and 6-hydroxy-dutasteride 	
Ketoconazole Fluconazole Miconazole		<ul style="list-style-type: none"> • Ketoconazole • Fluconazole • Miconazole 	
iii. Substances that May Alter Carbon isotopic Ratios of ERCs			
Confounding Factor	ERC affected	Comments / Solutions	Normative document
Pregnenolone, Progesterone	Pregnanediol (PD)	Shift of affected ERC $\delta^{13}\text{C}$ values towards exogenous range (≤ -25.0 ‰), leading to negative IRMS results. Use of alternative ERC required.	TD IRMS
Adrenosterone (11-oxo)	11 β -hydroxy-androsterone (11-OH-A) and 11-oxo-etiocholanolone (11-oxo-Eti)		
Cortisol, Cortisone (prohibited, but only <i>In-Competition</i> by systemic routes)	n/a	n/a	

Intra-individual variability of the ERCs

Statistical analysis of the ERC variation for non-sports population expressed as the SD (%) and Range (%) of the data of the single individuals.

	Female		Male		All	
	SD	Range	SD	Range	SD	Range
μ	0.23	0.58	0.28	0.73	0.26	0.67
median	0.21	0.51	0.28	0.75	0.28	0.70
SD	0.14	0.35	0.12	0.32	0.13	0.34
max	0.52	1.32	0.55	1.44	0.60	1.4
min	0.07	0.16	0.08	0.16	0.10	0.2
range	0.45	1.16	0.48	1.28	0.50	1.3
$\mu+2*SD$	0.50	1.29	0.52	1.37	0.52	1.35
Percentile						
95.0%	0.49	1.27	0.48	1.26	0.50	1.30
97.5%	0.51	1.30	0.54	1.41	0.53	1.37

We proposed a maximum **SD of 0.54 %** and **range of 1.20 %** as acceptance criteria to detect ERC outliers when several are used.

Rapid Commun Mass Spectrom. 2019;33:579–586.

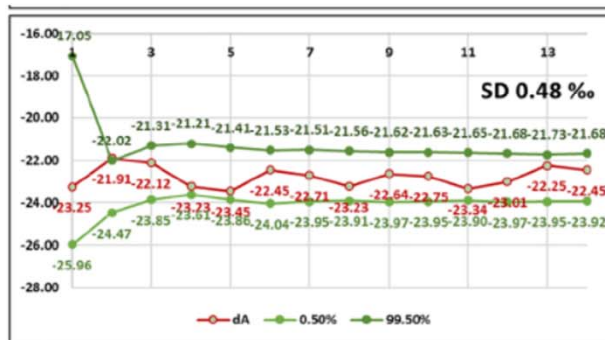
How many ERCs we can get?

Distribution of the number of ERCs measured by sample

#ERC	2016		2017		2018	
	N	%	N	%	N	%
4	404	84.3%	333	92.8%	234	91.4%
3	66	13.8%	22	6.1%	21	8.2%
2	9	1.9%	4	1.1%	1	0.4%
Total samples	479		359		256	

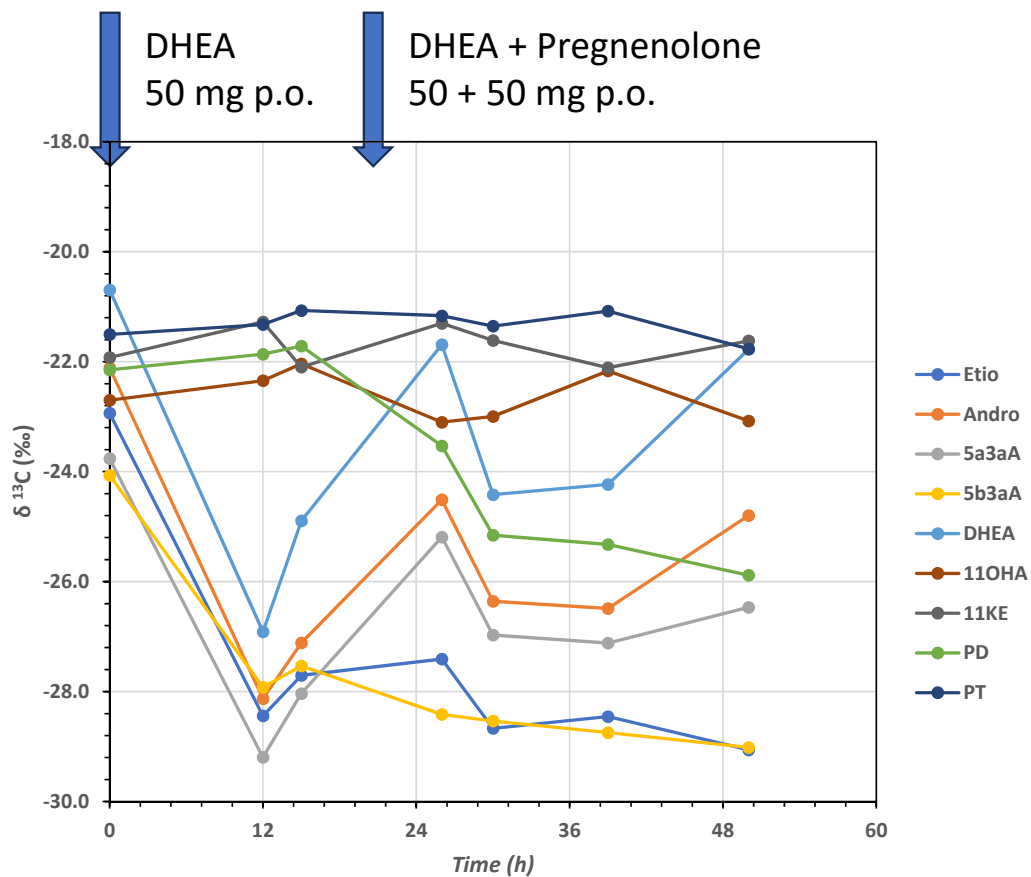
Rapid Commun Mass Spectrom. 2019;33:579–586.

Not only ERCs but also the TCs



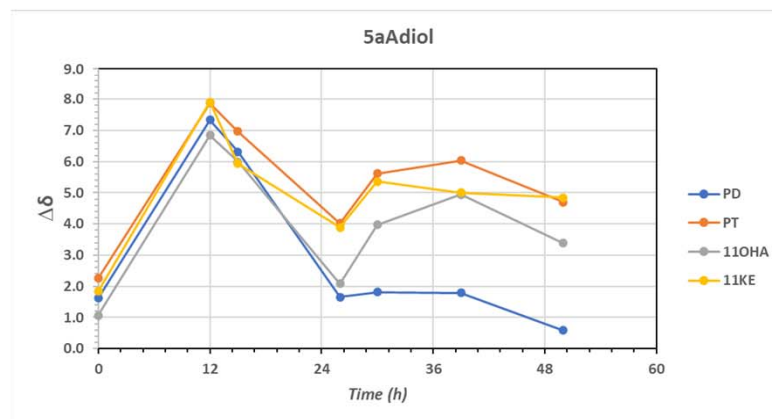
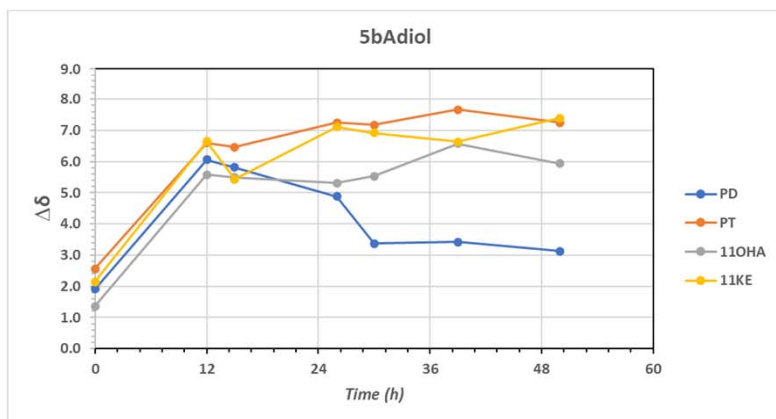
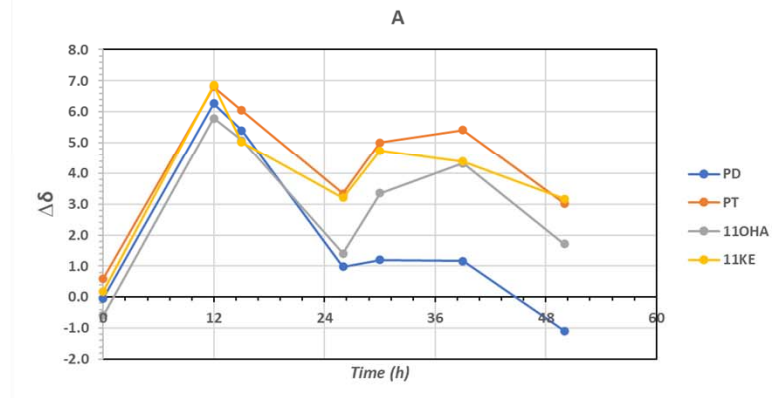
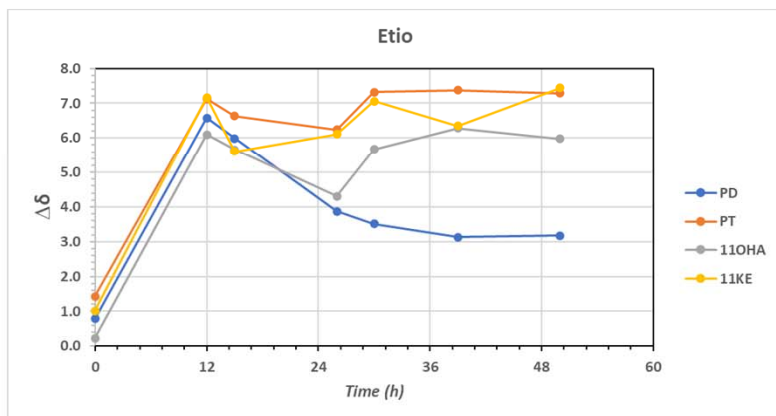
Drug Test Anal. 2022;14:1877–1890

Effect of pregnenolone on PD

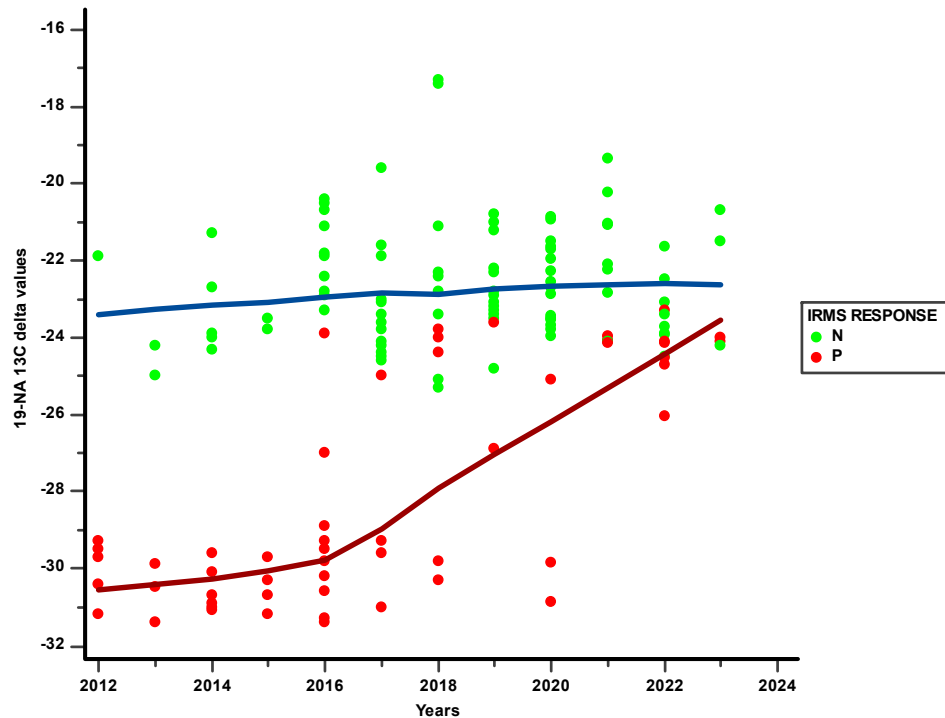


PT not affected by Pregnenolone administration

Effect of pregnenolone on $\Delta\delta$



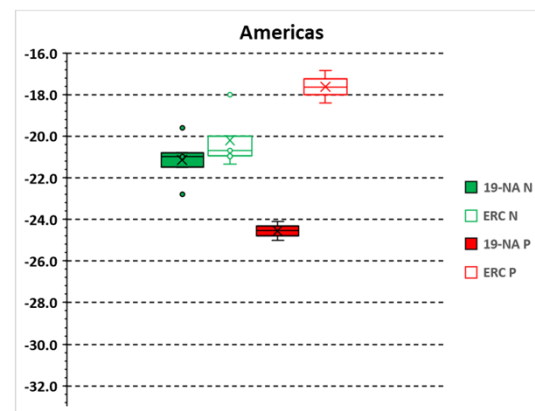
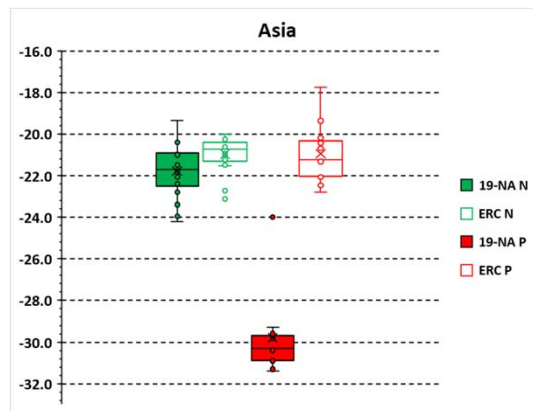
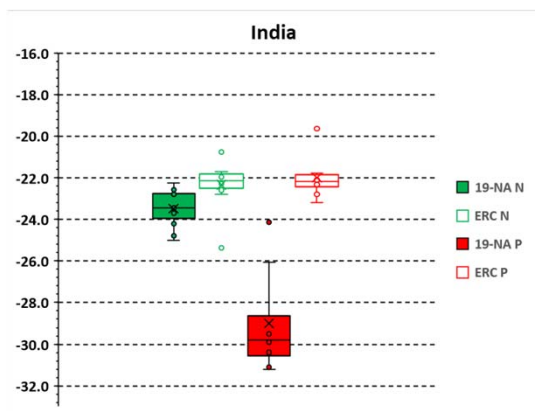
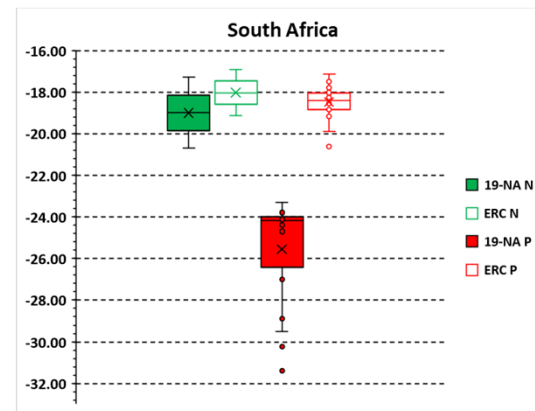
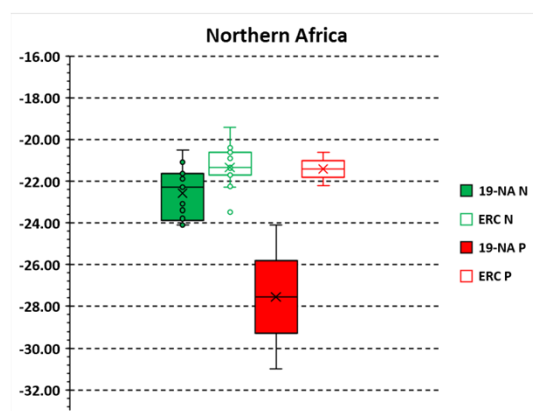
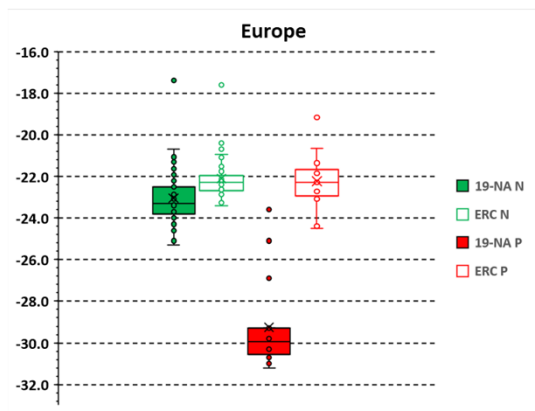
19-NA IRMS data vs Time



A large number of CP are subcontracted from other Laboratories and increased along the years

Time dependent or sample origin dependent?

Delta values vs samples' origin



19-NA 2023 cases

Sample	4131	4337	4836	5419	6230	6647	7010	7012	7396	7507	7506	7508	7773	8227	8228
Gender	F	F	M	F	F	F	M	M	F	M	F	M	F	M	M
Sport Discipline	Athletic Marathon	Athletics Mid distance	Para-Athletics Standing Throws	Triathlon Triathlon	Athletics Middle distance	Athletics Sprint	Fencing Sabre	Weightlifting	Triathlon Triathlon	Cycling Mountain Bike	Wrestling	Powerlifting Bench Press	Athletic Long Distance	Boxing	Boxing
Origin	SA	Qatar	SA	World Triathlon	Qatar	SA	India	Cuba	Italy	SA	SA	SA	Qatar	India	India
19-NA	5.5	12.9	7.2	2.4	7.4	13.2	11	20.5	6.3	9.5	17.8	1.5	3.3	3.1	3
19-NE	3.3	5.6		1.2	4	7.5	2.2	5.9			10.3				5.7
19-NA/19-NE	1.7	2.3		2.0	1.9	1.8	5.0	3.5			1.7				0.5
19-NA	-25.03	-23.70	-24.37	-20.38	-24.06	-24.09	-24.76	-24.07	-23.59	-24.07	-24.08	-23.91	-23.85	-24.08	-21.01
19-NE	-24.84	-24.04			-23.66	-24.07		-24.05			-24.26				-21.89
A	-19.27	-19.19	-20.27	-19.03	-19.57	-19.73	-21.52	-18.27	-22.29	-18.33	-29.31	-21.95	-20.09	-20.23	-20.13
PD	-19.41	-19.24	-20.56	-19.18	-19.55	-19.62	-21.67	-18.53	-22.62	-16.62	-23.88	-19.40	-20.12	-20.30	-19.79
PT	-19.20	-18.92	-20.57	-18.56	-19.62	-19.40	-21.60	-18.00	-22.38	-17.38	-23.77	-22.18	-19.80	-20.15	-20.44
A-19-NA	5.76	4.51	4.10	1.35	4.49	4.37	3.24	5.80	1.30	5.73	-5.22	1.96	3.76	3.85	0.88
PD-19NA	5.62	4.47	3.81	1.20	4.51	4.47	3.09	5.54	0.97	7.45	0.20	4.51	3.73	3.78	1.23
PT-19-NA	5.83	4.79	3.80	1.82	4.44	4.69	3.16	6.07	1.21	6.68	0.31	1.73	4.05	3.94	0.57
Mean	-19.29	-19.11	-20.47	-18.92	-19.58	-19.58	-21.60	-18.27	-22.43	-17.44	-25.65	-21.18	-20.00	-20.23	-20.12
SD	0.11	0.17	0.17	0.32	0.03	0.17	0.07	0.26	0.17	0.86	3.16	1.54	0.18	0.08	0.33
Amplitude	0.21	0.32	0.30	0.62	0.06	0.33	0.15	0.53	0.33	1.72	5.54	2.78	0.32	0.16	0.66
	AAF	AAF	AAF	NEG	AAF	AAF	AAF	AAF	NEG	AAF	NEG	NEG	AAF	AAF	NEG

19-NA Cases evaluation (1)

Sample	5419	7396	7506	7508	8228
Gender	F	F	F	M	M
Sport Discipline	Triathlon Triathlon	Triathlon Triathlon	Wrestling	Powerlifting Bench Press	Boxing
Origin	World Triathlon	Italy	SA	SA	India
19-NA	2.4	6.3	17.8	1.5	3
19-NE	1.2		10.3		5.7
19-NA/19-NE	2.0		1.7		0.5
19-NA	-20.38	-23.59	-24.08	-23.91	-21.01
19-NE	-19.88		-24.26		-21.89
A	-19.88	-22.29	-29.31	-21.95	-20.28
PD	-19.18	-22.62	-23.88	-19.40	-19.79
PT	-18.56	-22.38	-23.77	-22.18	-20.44
A-19-NA	1.35	1.30	-5.22	1.96	0.88
PD-19NA	1.20	0.97	0.20	4.51	1.23
PT-19-NA	1.82	1.21	0.31	1.73	0.57
Mean	-18.92	-22.43	-25.65	-21.18	-20.12
SD	0.32	0.17	3.16	1.54	0.33
Amplitude	0.62	0.33	5.54	2.78	0.66
	NEG	NEG	NEG	NEG	NEG

Average δ (‰) of the AAFs

Mean (19-NA)	-24.21
SD	0.40
n	10

Clear negative cases

19-NA Cases evaluation (2)

Sample	7396	7506	7508
Gender	F	F	M
Sport Discipline	Triathlon Triathlon	Wrestling	Powerlifting Bench Press
Origin	Italy	SA	SA
19-NA	6.3	17.8	1.5
19-NE		10.3	
19-NA/19-NE		1.7	
19-NA	-23.59	-24.08	-23.91
19-NE		24.26	
A	-22.29	-29.31	-21.95
PD	-22.62	23.88	-19.40
PT	-22.38	-23.77	-22.18
A-19-NA	1.30	-5.22	1.96
PD-19NA	0.97	0.20	4.51
PT-19-NA	1.21	0.31	1.73
Mean	-22.43	-25.65	-21.18
SD	0.17	3.16	1.54
Amplitude	0.33	5.54	2.78
	NEG	NEG	NEG

The absolute delta values are in the endogenous region
-23.6 is not abnormal for the geographical area
The ERCs are homogeneous (consistent)

Seems to be a real Neg

19-NA Cases evaluation (3)

Sample	7506	7508
Gender	F	M
Sport Discipline	Wrestling	Powerlifting Bench Press
Origin	SA	SA
19-NA	17.8	1.5
19-NE	10.3	
19-NA/19-NE	1.7	
19-NA	-24.08	-23.91
19-NE	-24.26	
A	-29.31	-21.95
PD	-23.88	-19.40
PT	-23.77	-22.18
A-19-NA	-5.22	1.96
PD-19NA	0.20	4.51
PT-19-NA	0.31	1.73
Mean	-25.65	-21.18
SD	3.16	1.54
Amplitude	5.54	2.78
	NEG	NEG

Androsterone can be affected by an exogenous administration

PD is abnormally enriched
But should be used as primary ERC (> -25‰)
If PD is accepted, this is an ATF, if discarded is Neg

Abnormal variability among the ERCs

19-NA Cases evaluation (4)

	Marker	δ (‰)	
TCs	19-NA	-24.08	
	19-NE	-24.26	
	A	-29.31	-29.47
	Etio	-30.27	
	5aAdiol	-30.18	
	5bAdiol	-30.12	
	T	-29.10	
ERCs	11KE	-23.35	
	11OHA	-24.46	
	PD	-23.88	-23.57
	PT	-23.77	
	Mean	-23.87	
	SD	0.46	
	Max-Min	1.11	

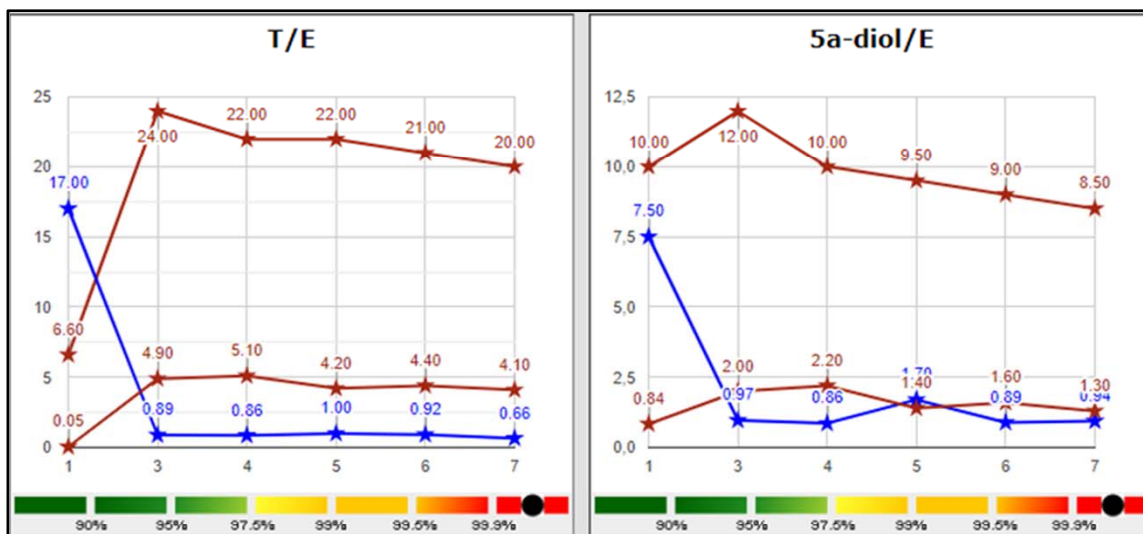
PD-TC	PT-TC	11OHA-TC	11KE-TC	Mean ERC-TC
0.31	0.20	-0.38	0.73	0.22
0.49	0.37	-0.20	0.91	0.39
5.54	5.42	4.85	5.96	5.44
6.50	6.38	5.81	6.92	6.40
6.42	6.30	5.73	6.83	6.32
6.36	6.24	5.67	6.77	6.26
5.34	5.22	4.65	5.75	5.24

Neg for 19-Norsteroids

(Is a real Neg???)

AAF for testosterone and metabolites
(Criteria i., ii., v., and vi. TD2022IRMS Art. 2.3.2)

Steroid profile case



- Female
- No confounding factors reported
- Correct assignment of the sample to the Passport Checked with PC
- Sample 1 very suspicious and IRMS requested

Sample #	Event	Date	T/E	T (ng/mL)	E (ng/mL)	5aAdiol/E	5a/5bAdiol	A/Etio	SG
1	IC	24/02/2020	17.0	110	7	7.5	0.4	1.4	1.019
2	OOC	10/12/2020	0.7	27	39	1.3	0.3	1.1	1.021
3	IC	14/02/2021	0.9	21	24	1.0	0.4	1.4	1.018
4	IC	19/06/2022	0.9	61	72	0.9	0.5	1.1	1.020
5	IC	10/10/2022	1.0	12	12	1.7	0.3	0.9	1.024
6	IC	21/10/2022	0.9	32	35	0.9	0.3	0.9	1.020
7	IC	13/12/2022	0.7	15	23	0.9	0.4	1.1	1.021

Steroid profile case (2)

Sample #	Event	Date	T/E	T (ng/mL)	E (ng/mL)	5aAdiol/E	5a/5bAdiol	A/Etio	SG
1	IC	24/02/2020	17.0	110	7	7.5	0.4	1.4	1.019
2	OOC	10/12/2020	0.7	27	39	1.3	0.3	1.1	1.021
3	IC	14/02/2021	0.9	21	24	1.0	0.4	1.4	1.018
4	IC	19/06/2022	0.9	61	72	0.9	0.5	1.1	1.020
5	IC	10/10/2022	1.0	12	12	1.7	0.3	0.9	1.024
6	IC	21/10/2022	0.9	32	35	0.9	0.3	0.9	1.020
7	IC	13/12/2022	0.7	15	23	0.9	0.4	1.1	1.021

Sample #	IRMS Results (‰)					
	T	5aAdiol	5bAdiol	A	Etio	PD (ERC)
1	-23.9	-23.7	-23.7	-22.8	-22.4	-21.4

IRMS Results $\Delta\delta_{ERC-TC}$ (‰)				
T	5aAdiol	5bAdiol	A	Etio
2.5	2.3	2.3	1.4	1

Highly suspicious of an exogenous administration of preparation in the endogenous delta region

Steroid profile case (3)

Sample #	Event	Date	T/E	T (ng/mL)	E (ng/mL)	5aAdiol/E	5a/5bAdiol	A/Etio	SG
1	IC	24/02/2020	17.0	110	7	7.5	0.4	1.4	1.019
2	OOC	10/12/2020	0.7	27	39	1.3	0.3	1.1	1.021
3	IC	14/02/2021	0.9	21	24	1.0	0.4	1.4	1.018
4	IC	19/06/2022	0.9	61	72	0.9	0.5	1.1	1.020
5	IC	10/10/2022	1.0	12	12	1.7	0.3	0.9	1.024
6	IC	21/10/2022	0.9	32	35	0.9	0.3	0.9	1.020
7	IC	13/12/2022	0.7	15	23	0.9	0.4	1.1	1.021

Sample #	IRMS Results (‰)					
	T	5aAdiol	5bAdiol	A	Etio	PD (ERC)
1	-23.9	-23.7	-23.7	-22.8	-22.4	-21.4
2	-22.4	-22.5	-21.2	-	-	-21.5
3	-21.6	-22.3	-23	-	-22.8	-21.1

IRMS Results $\Delta\delta_{ERC-TC}$ (‰)				
T	5aAdiol	5bAdiol	A	Etio
2.5	2.3	2.3	1.4	1
0.9	1	-0.3		
0.5	1.2	1.9		1.7

Highly suspicious of an exogenous administration of preparation in the endogenous delta region

Conclusions

- For some populations 19-NA detection origin is becoming very challenging.
- Fix delta values to evaluate PD ($< -25\text{‰}$) should be reviewed
- The evaluation of the isotopic status of the athlete could be a better option to discriminate which ERCs may be affected (unfortunately 3 values are needed)
- The longitudinal evaluation of the delta values may open a new way of evaluating the cases. In these cases, the consistency of the ERCs along time is useful to evaluate the basal status of the athlete

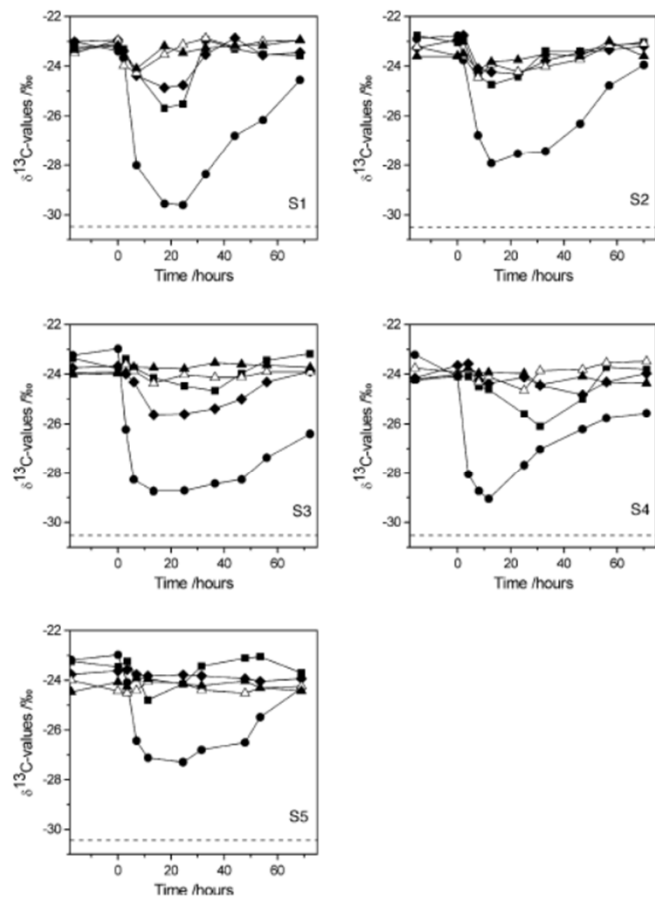


Fig. 2. Urinary $\delta^{13}\text{C}$ -values of androsterone (\blacktriangle), etiocholanolone (\triangle), pregnanediol (\bullet), 5β -androstanediol (\blacksquare) and androstenol (\blacklozenge) of five subjects (S1-S5) before and after oral administration of 50 mg PREG at $t=0$. The dash lines with $\delta^{13}\text{C}$ of -30.5‰ represent the value of the isotope ratio of PREG in the

