DIPARTIMENTO

SCIENZE MEDICHE E CHIRURGICHE – DIMEC

Centro unificato di Ricerca Biomedica applicata

La steroidologia nell'epoca della cromatografia liquida abbinata alla spettrometria di massa: successi, limiti e nuovi orizzonti

Flaminia Fanelli

Medical Biotechnologist, PhD

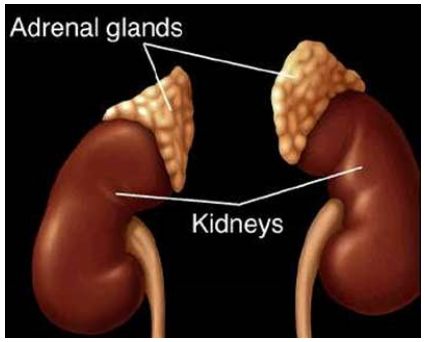
Unit of Endocrinology and Prevention and Care of Diabetes

Center for Applied Biomedical Research

Department of Medical and Surgical Sciences

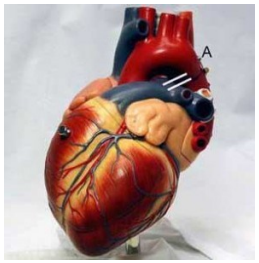
Alma Mater Studiorum University of Bologna

Bologna, Italy

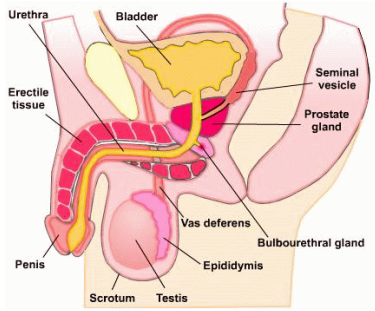


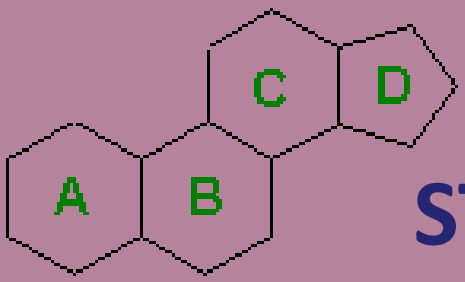
Adrenal tumors

Hypogonadism
Osteoporosis

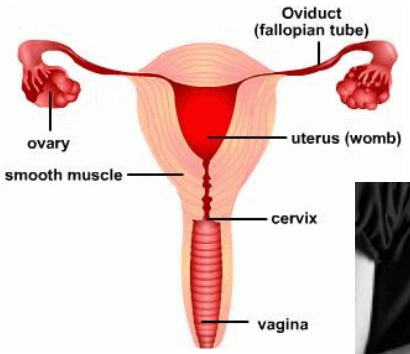


Hypertension
Cardiovascular risk
Obesity
Diabetes





STEROID HORMONES



Reproduction

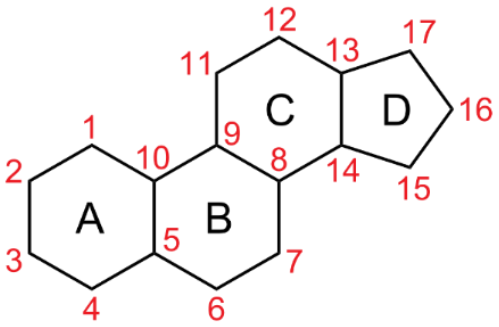
Hyperandrogenism

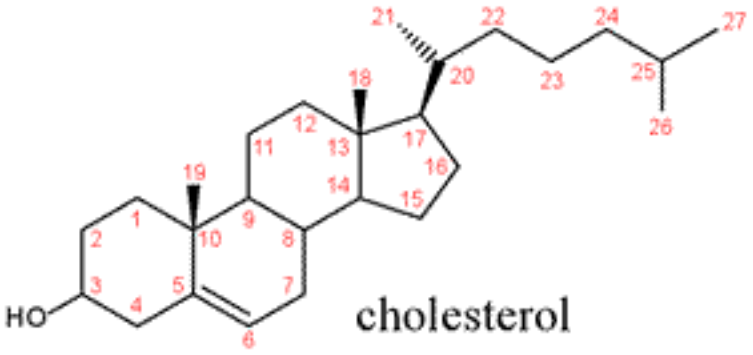
Genetic deficit steroidogenesis

Disorder of Sex Development

STEROID STRUCTURE and CLASSIFICATION

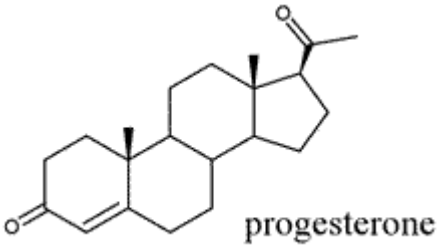
cyclopentane perhydrophenanthrene



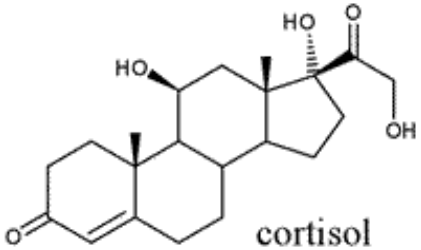


C21

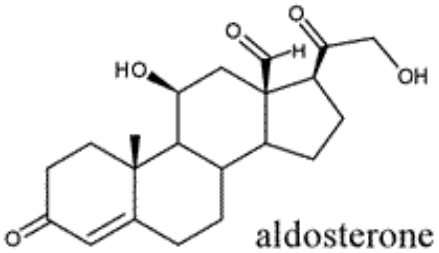
PROGESTOGENS



GLUCOCORTICOIDS

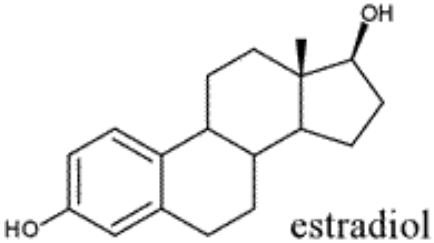


MINERALOCORTICOIDS

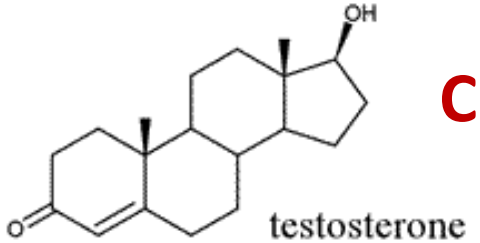


ESTROGENS

C18

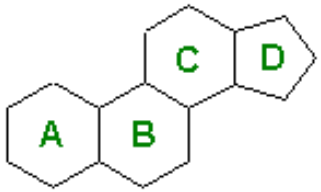


ANDROGENS



C19

PERFORMANCE REQUIREMENTS for STEROID HORMONE MEASUREMENT



SPECIFICITY *vs* hundreds of endogenous and exogenous steroids

- Structural related or unrelated isobars, isomers, epimers, conjugated metabolites
- Drugs

SENSITIVITY and **WIDE DYNAMIC RANGE**

- Gender: in females and in males
- Fertility status: Menstrual phase / Pregnancy / Menopause
- Functional tests: adrenal stimulation and suppression

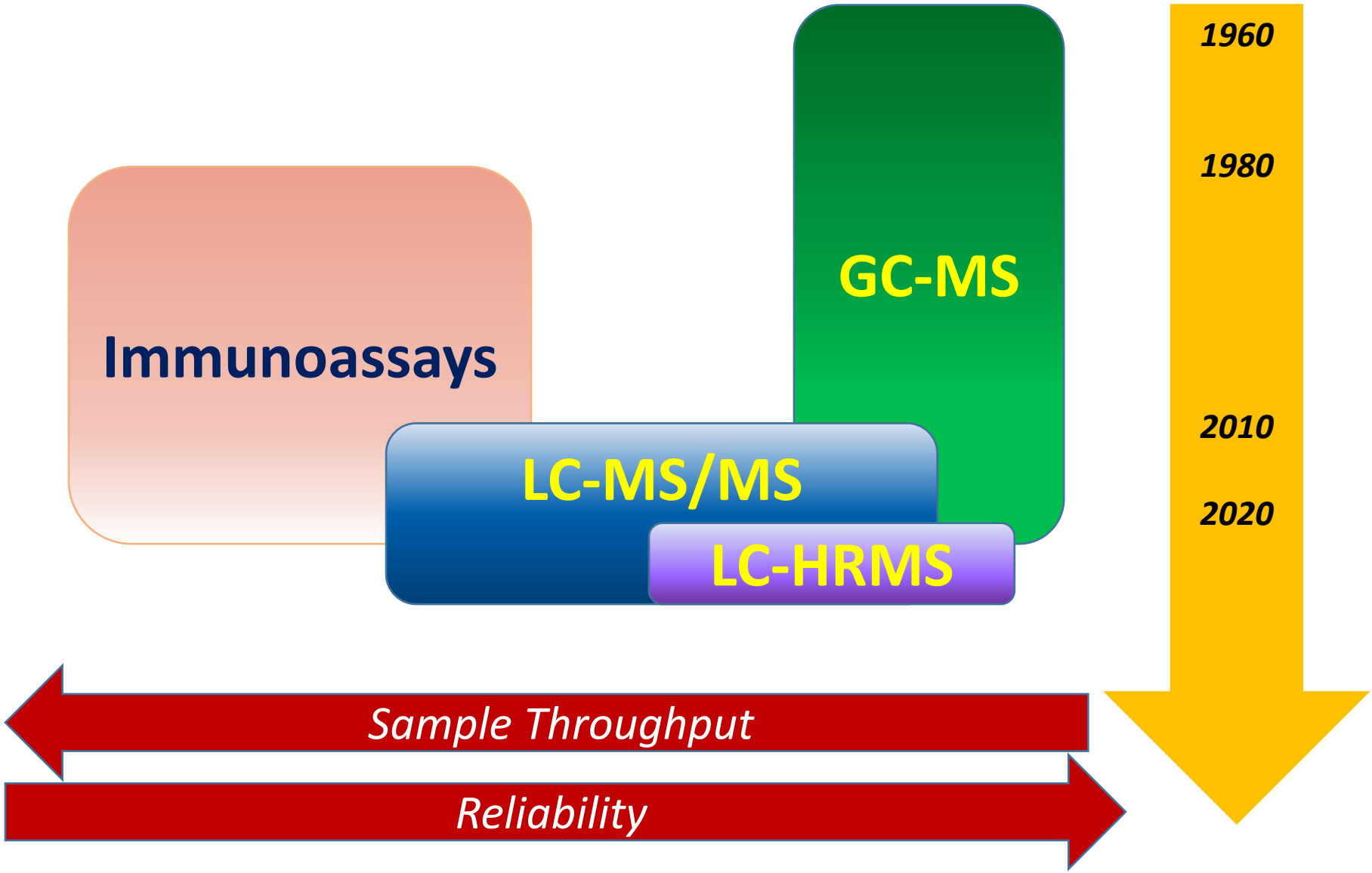
ACCURACY and **PRECISION** small but clinically relevant intra- and inter-individual variations

- Differential diagnosis,
- Monitoring therapy / hormone replacement

Requests by clinicians include **PANEL** rather than single steroids

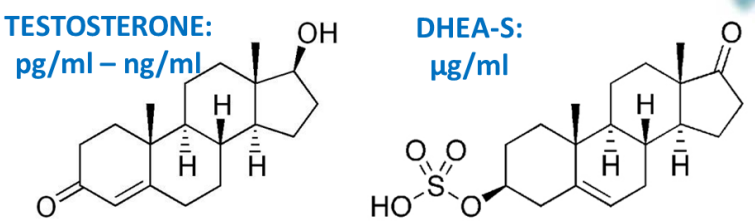
Ideally **NO INFLUENCE** from different MATRIX

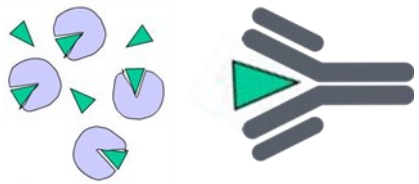
QUANTITATIVE ANALYTICAL TECHNIQUES FOR STEROID MEASUREMENT

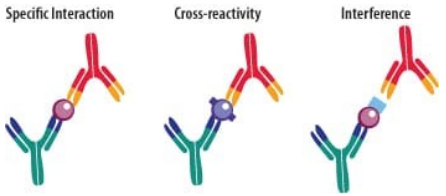


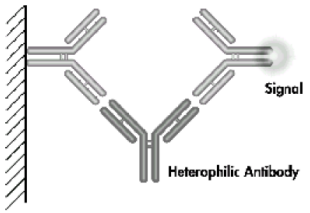
PITFALLS in DIRECT IMMUNOASSAYS

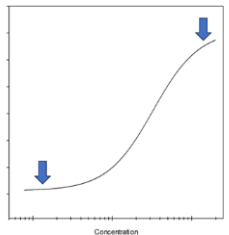
- **Cross reactivity**
- release from **binding protein**
- **matrix interference**
- **Anti-reagent Ab** (heterophilic Ab)
- **Biotine** supplementation
- **Narrow dynamic range**



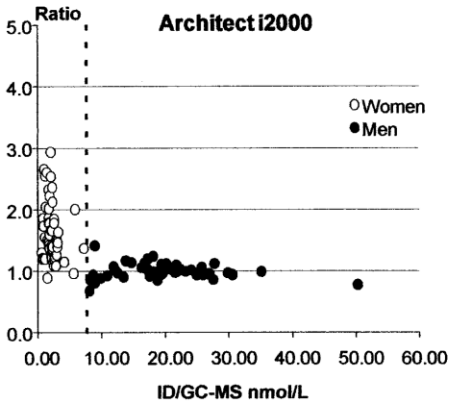


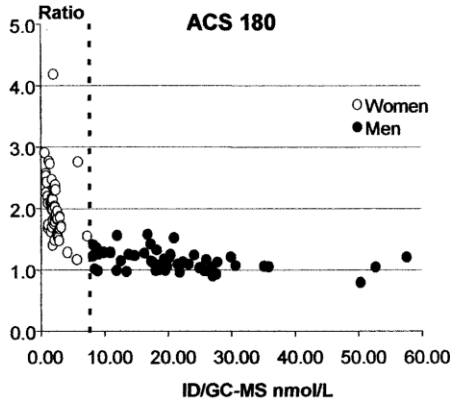


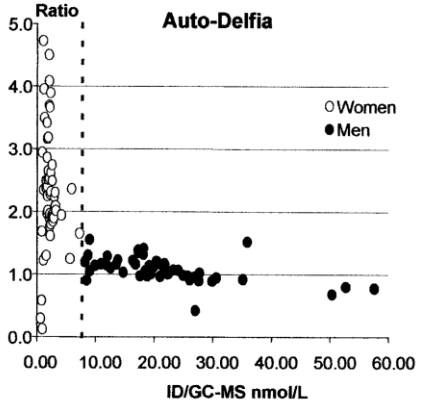


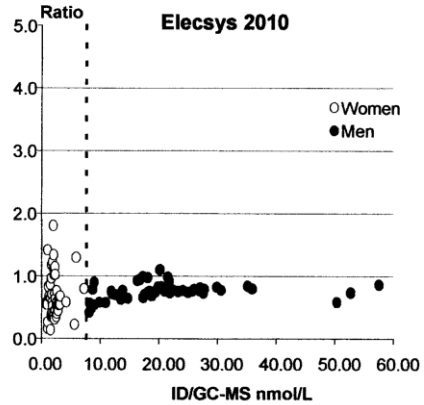


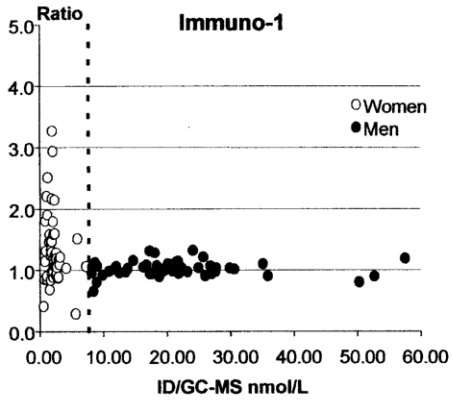
TESTOSTERONE IN THE IMMUNOASSAY AGE

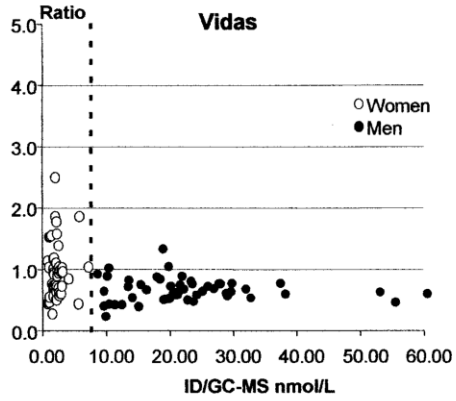


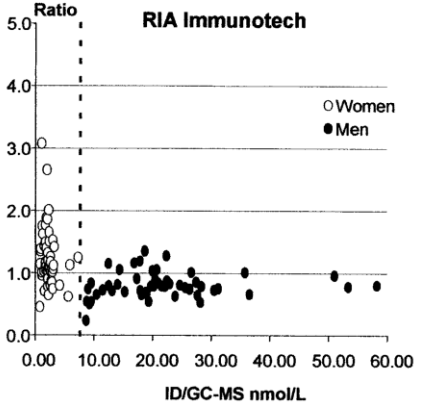


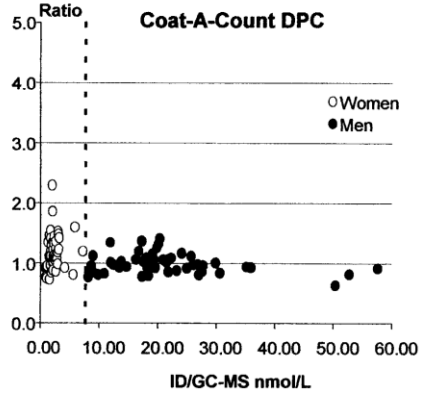


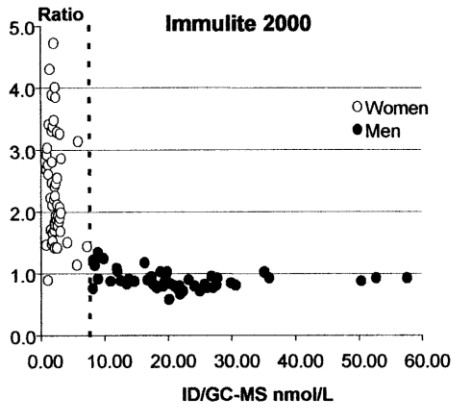


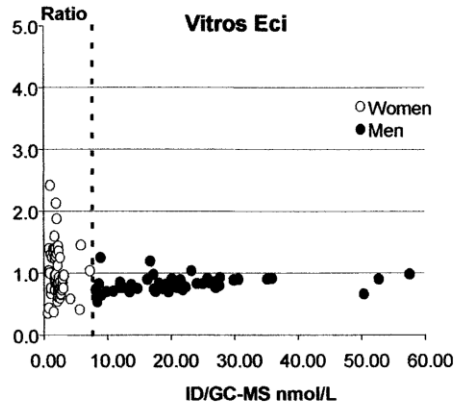












Immunoassays

vs

GC-MS reference method

Taieb et al., Clin Chem 2003

DIRECT IMMUNOASSAY in the ROUTINE LAB

HIGH COSTS

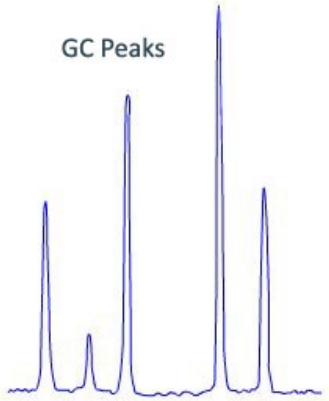
SERIOUS UNCERTAINITIES

Diagnosis

Therapy

COLUMN CHROMATOGRAPHY – MASS SPECTROMETRY

GAS (GC)

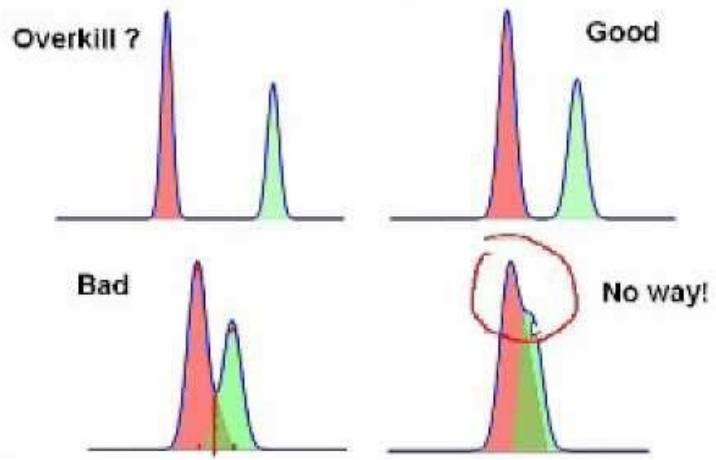


HIGH SEPARATION
HIGH SPECIFICITY

but

POOR VERSATILITY

LIQUID (LC)

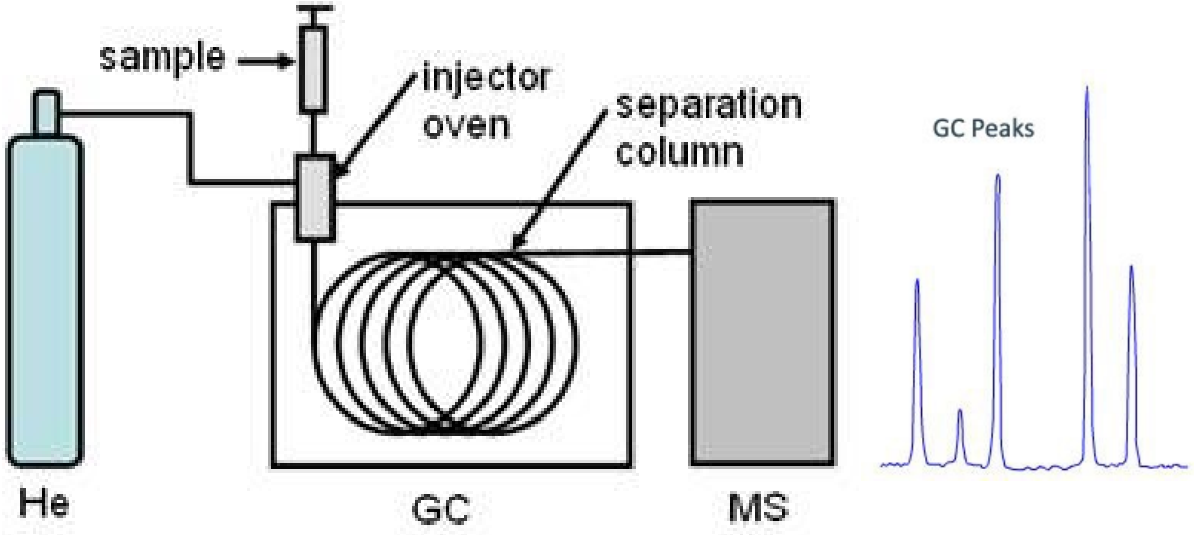


GOOD SEPARATION
GOOD SPECIFICITY

and

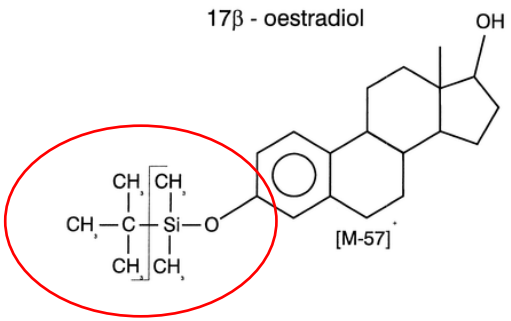
HIGH VERSATILITY

GAS CHROMATOGRAPHY



GAS phase High temperature

Analytes need to be volatile and thermostable



COMPROMISE:

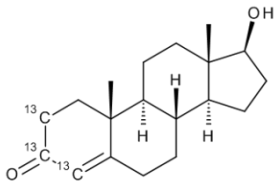
DERIVATIZATION

Long reaction time.

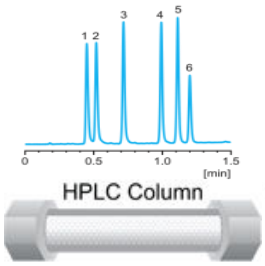
Extensive sample purification.

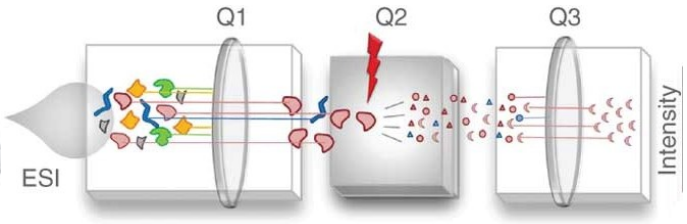
THE POWER OF LC-MS/MS

ISOTOPIC DILUTION

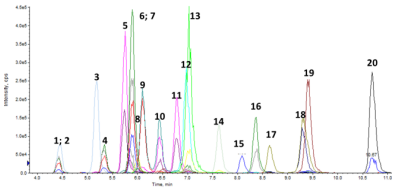


3 SPECIFICITY STEPS





MULTIANALYTE DETECTION



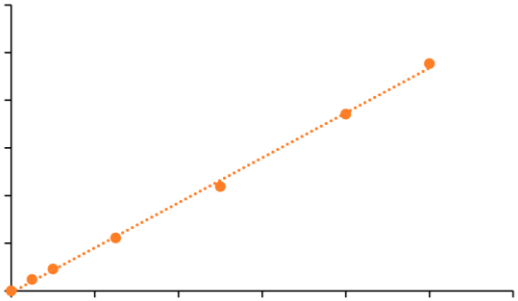
1. HPLC RETENTION TIME

2. MS1 MOLECULAR WEIGHT

3. MS2 STRUCTURE

LARGE DYNAMIC RANGE

5 orders of magnitude



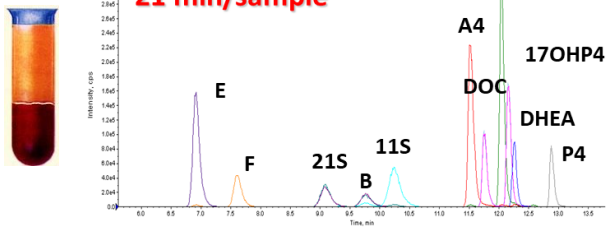
SENSITIVITY

SPECIFICITY

UNIBO/CRBA IN HOUSE METHODS FOR STEROID PROFILING

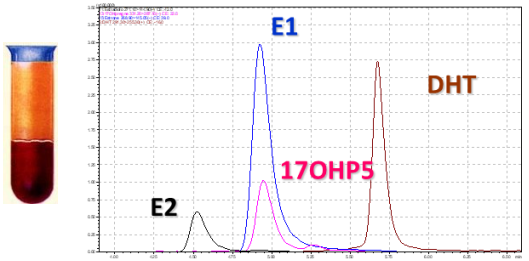
API 4000 QTrap

Panel 1: 11 steroids in serum

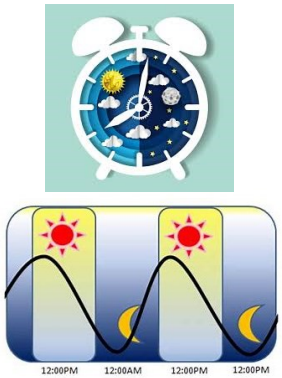


Fanelli et al., Steroids 2011

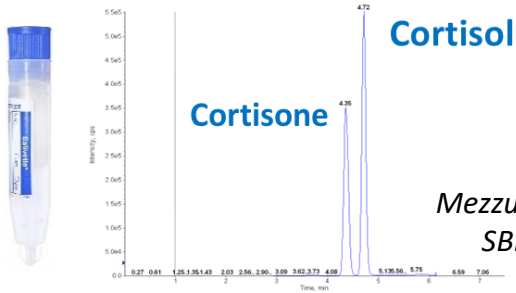
Panel 2: 4 steroids in serum



Mezzullo et al., SBMB 2020

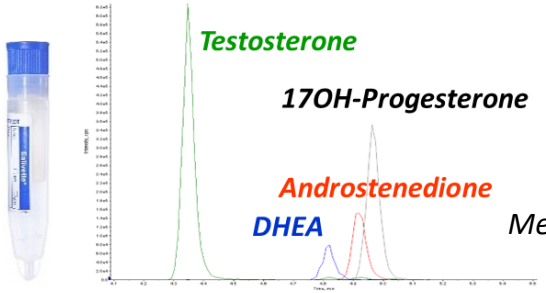


Panel 3: Salivary glucocorticoids



Mezzullo et al., SBMB 2016

Panel 4: Salivary androgens

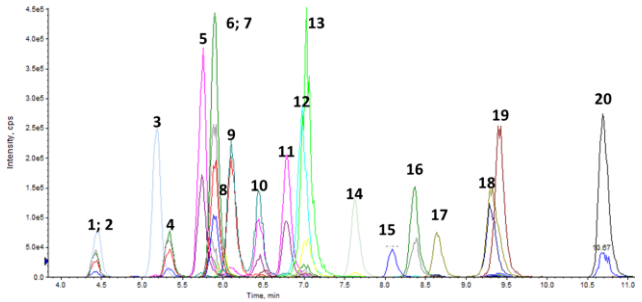


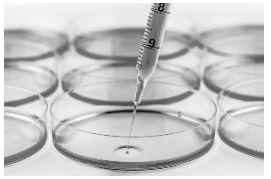
Mezzullo et al., CCLM 2017

Panel 5: 21 steroids from Leydig cells

(Canonical and backdoor androgen pathways)

Fanelli et al., SBMB 2023





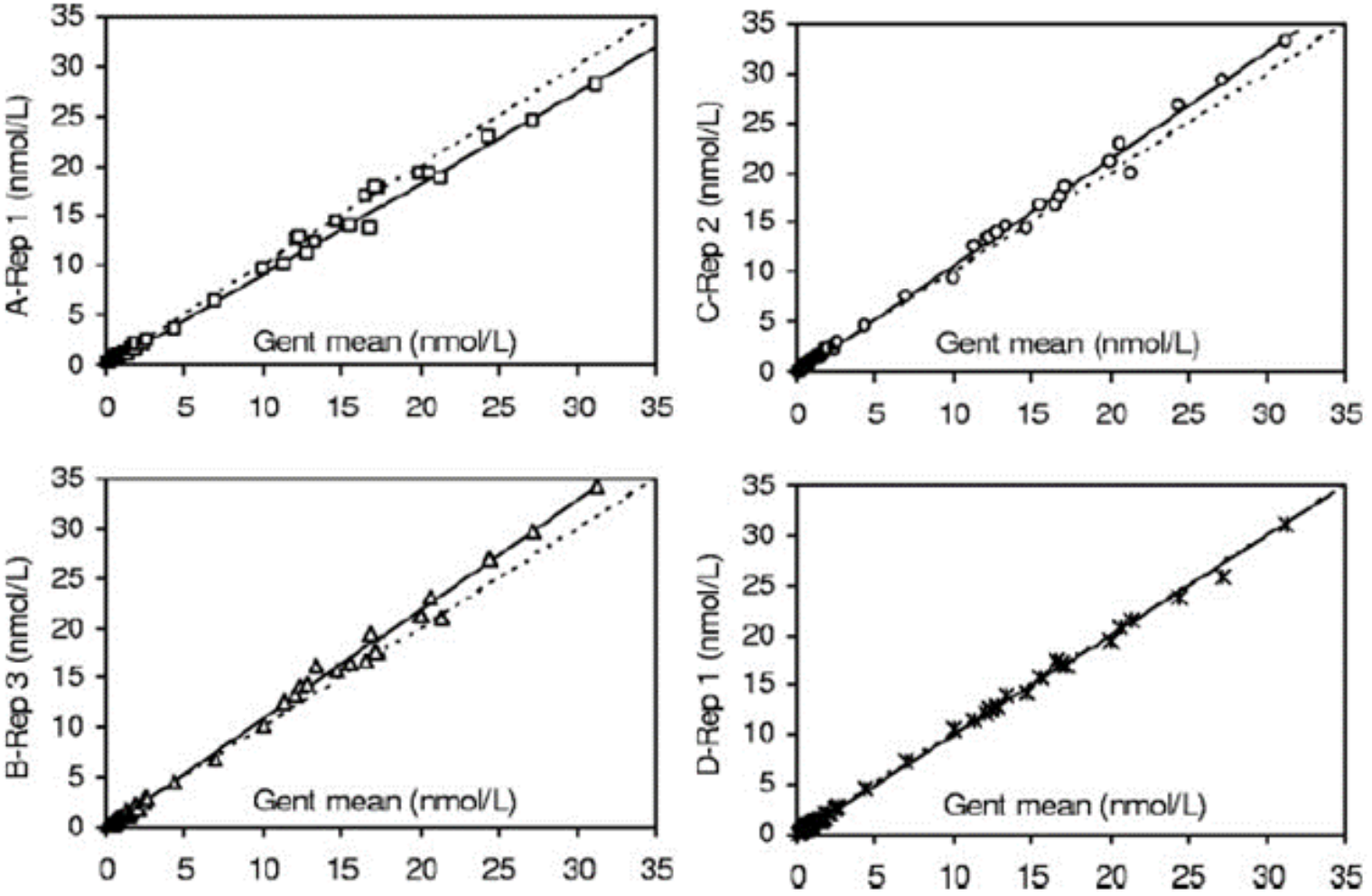
THE SUCCESS
of LC-MS/MS in the last 20y

- **Improving the measurement of traditional diagnostic steroids**
- Allowing the measurement of steroid panels
- Easing the path toward harmonization of measurements

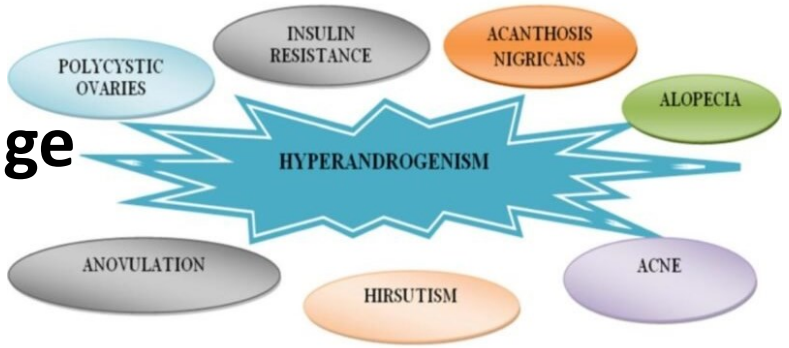
Clinical Chemistry 54:8
1290–1297 (2008)

State-of-the-Art of Serum Testosterone Measurement by Isotope Dilution–Liquid Chromatography–Tandem Mass Spectrometry

Linda M. Thienpont,[1*] Katleen Van Uytfanghe,[1] Stuart Blincko,[2] Carol S. Ramsay,[3] Hui Xie,[3] Robert C. Doss,[3] Brian G. Keevil,[4] Laura J. Owen,[4] Alan L. Rockwood,[5,6] Mark M. Kushnir,[5] Kelly Y. Chun,[7] Donald W. Chandler,[7] Helen P. Field,[8] and Patrick M. Sluss[9]

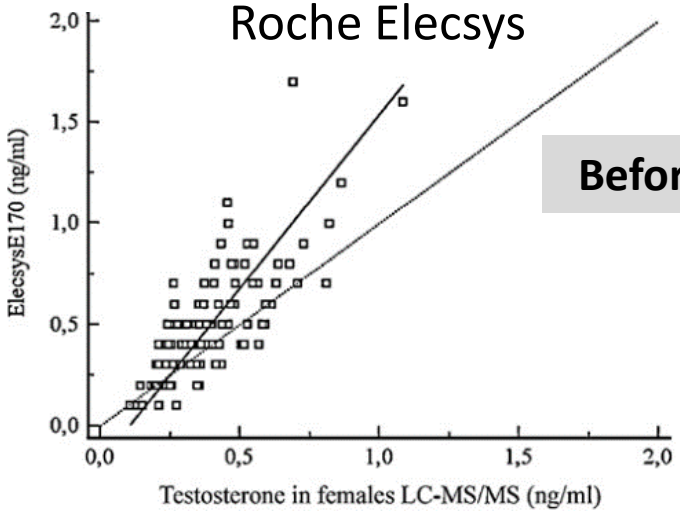


FEMALE HYPERANDROGENISM
5-10% of women in reproductive age



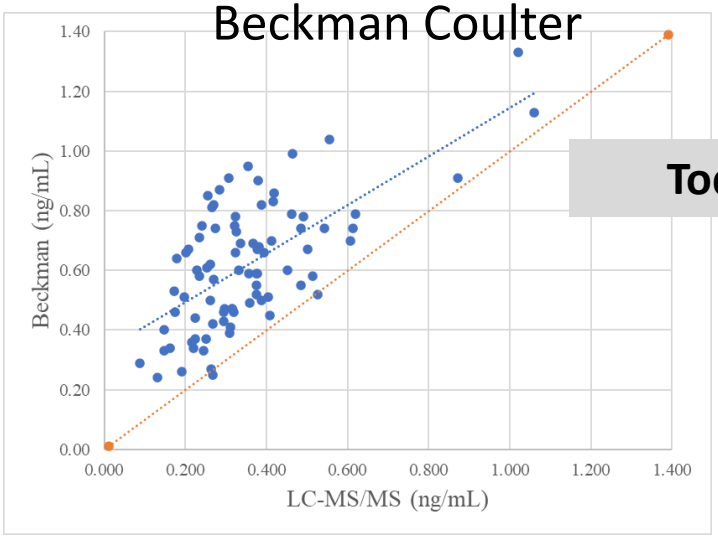
Serum TESTOSTERONE

Roche Elecsys



Before 2015

Beckman Coulter

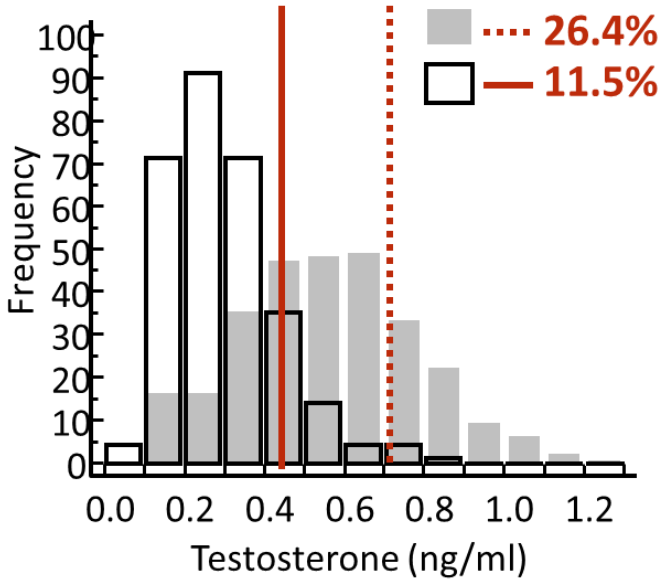


Today

Routine lab - IA cut-off: 0.70 ng/mL

LC-MS/MS cut-off: 0.45 and 0.50 ng/mL

N=295 patients (18 – 54y) referring for: Hirsutism, Menstrual irregularities, Infertility or Obesity



Fanelli et al., unpublished data

ADRENAL INCIDENTALOMA

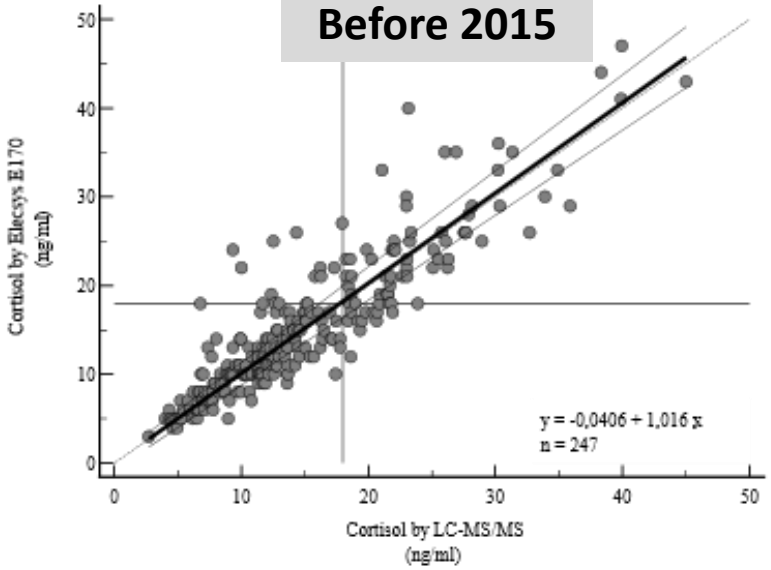
4-10% of the population

DEXAMETHASONE SUPPRESSION TEST FOR THE DIAGNOSIS OF MILD AUTONOMOUS CORTISOL SECRETION

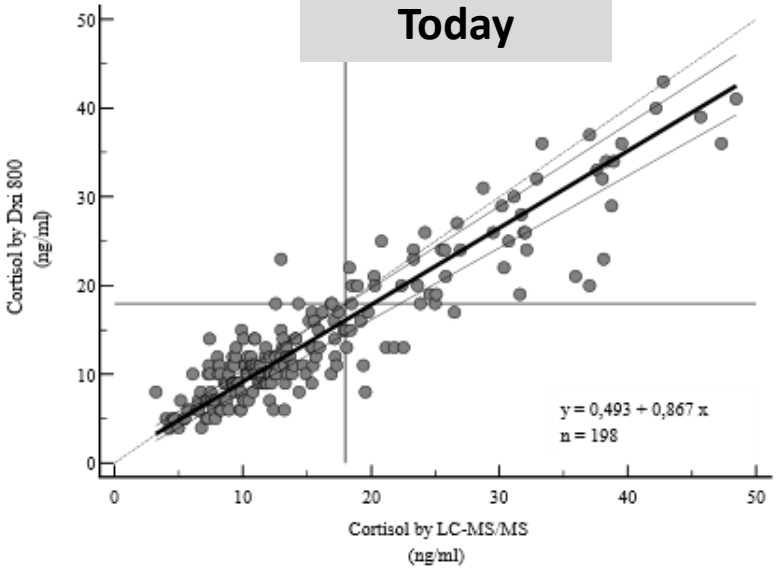
ELECSYS E170
5% possible false positives.
6.5% possible false negatives.

DXI 800
0.9% possible false positives.
4.6% possible false negatives.

Before 2015



Today



N° of DST at our Unit in Bologna: 40 per month.

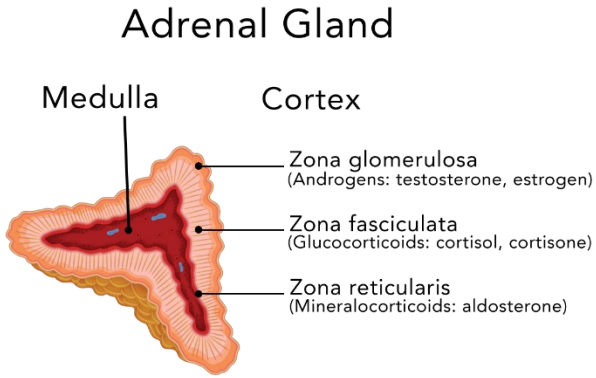
2 patients per month potentially wrongly discharged….

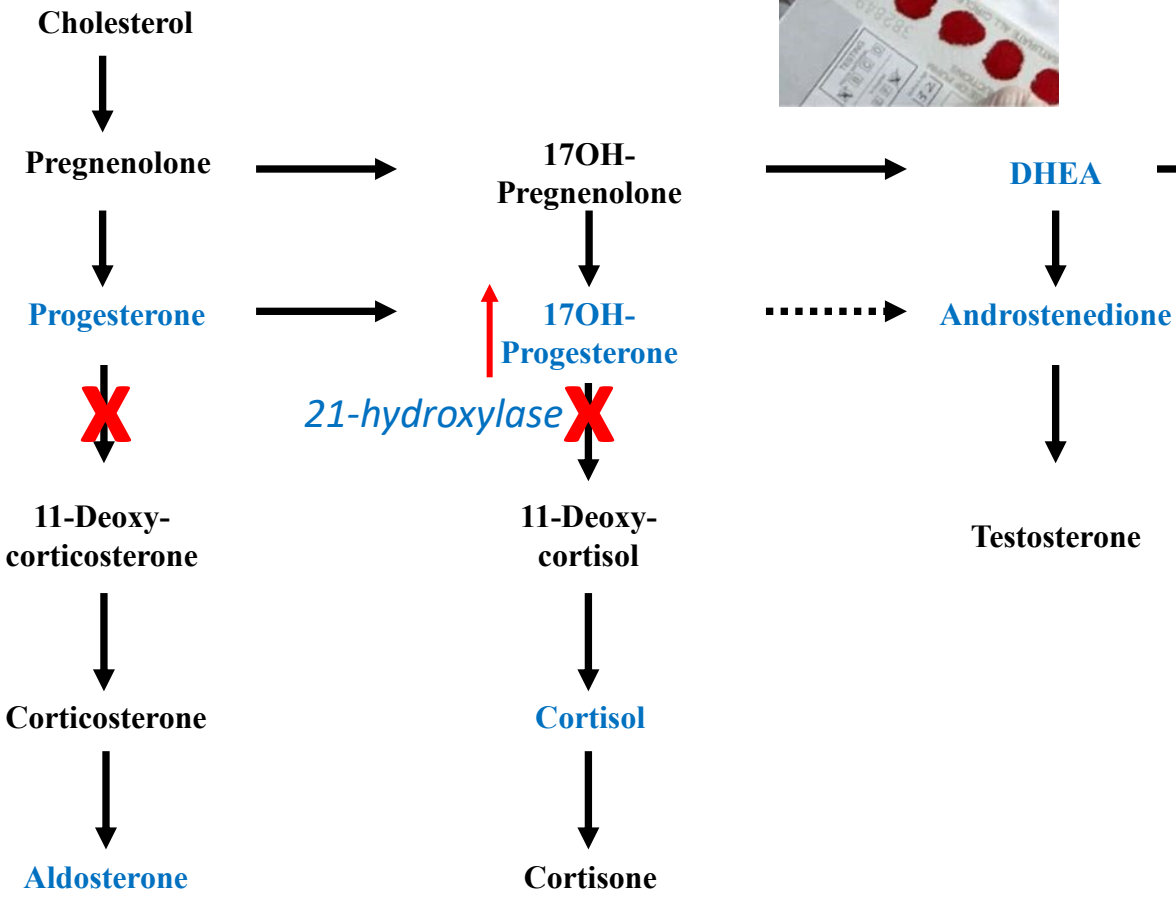
Rotolo et al., manuscript in preparation

THE SUCCESS
of LC-MS/MS in the last 20y

- Improving the measurement of traditional diagnostic steroids
- **Allowing the measurement of steroid panels**
- Easing the path toward harmonization of measurements

CONGENITAL ADRENAL HYPERPLASIA



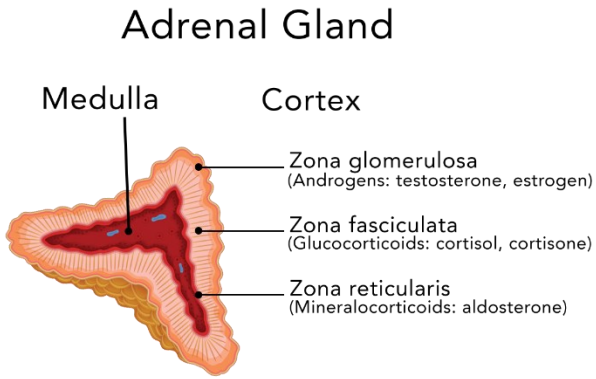


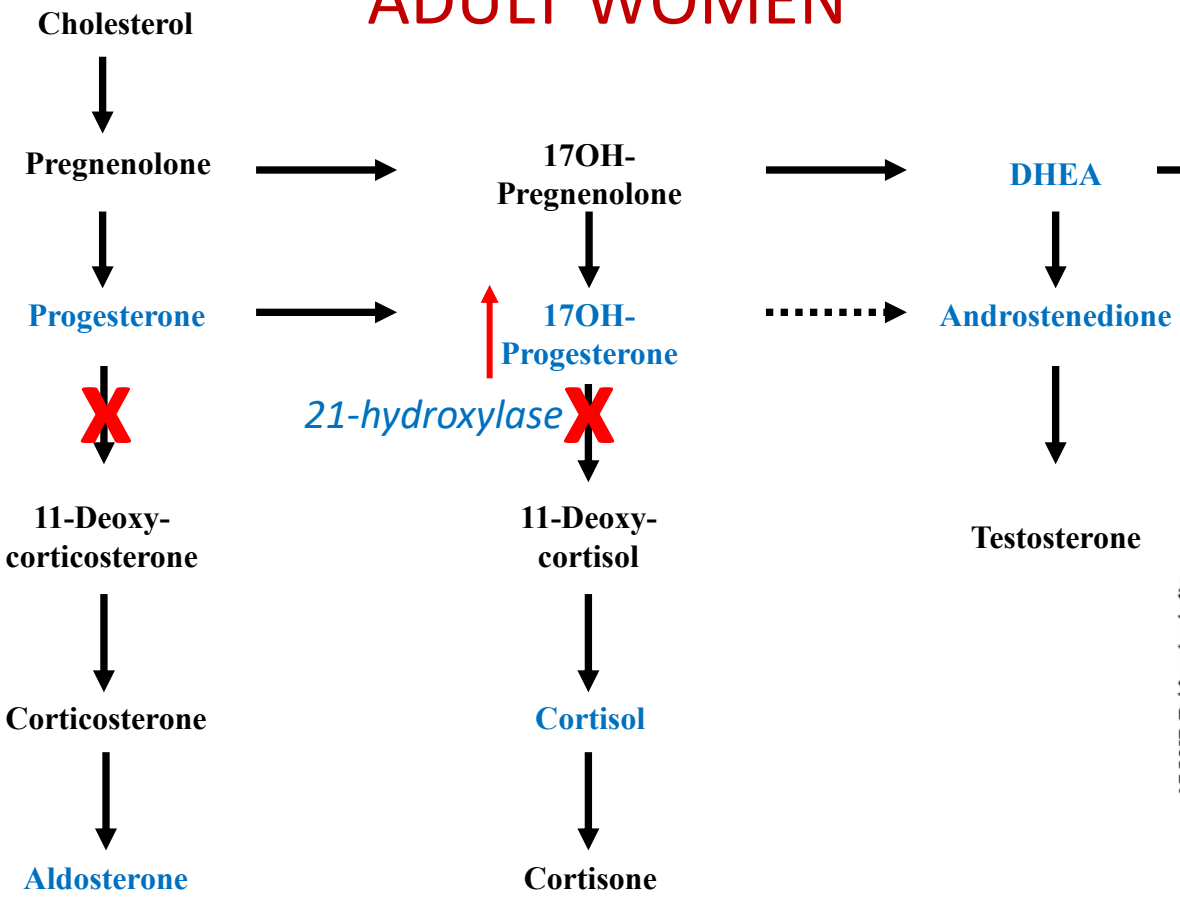
Hormones measured in the routine lab

Hormones NOT measured in the routine lab

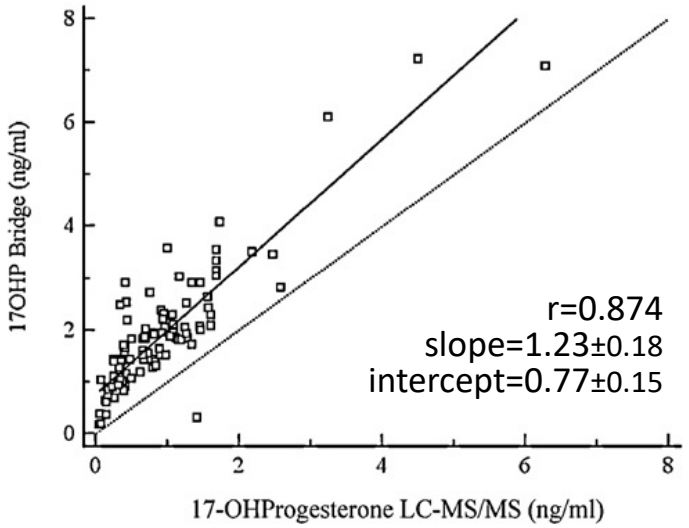
CONGENITAL ADRENAL HYPERPLASIA

DIFFERENTIATION **NON CLASSIC CAH** FORM **PCOS** IN ADOLESCENT AND ADULT WOMEN





17OH-PROGESTERONE RIA vs LCMSMS



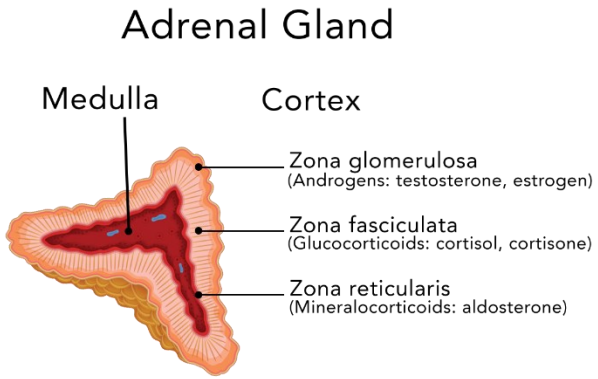
Hormones measured in the routine lab

Hormones NOT measured in the routine lab

Fanelli et al., Steroids 2011

CONGENITAL ADRENAL HYPERPLASIA

DIFFERENTIATION **NON CLASSIC CAH** FORM **PCOS** IN ADOLESCENT AND ADULT WOMEN



Cholesterol

Pregnenolone → 17OH-Pregnenolone → DHEA → DHEAS

Progesterone → 17OH-Progesterone → Androstenedione → 11βOH-Androstenedione → 11-keto-Androstenedione

21-hydroxylase

11-Deoxy-corticosterone → Corticosterone → Aldosterone

11OH-Progesterone → 11-keto-Progesterone

11-Deoxy-cortisol → Cortisol → Cortisone

21-Deoxycortisol → 21-Deoxycortisone

Testosterone → 11βOH-Testosterone → 11-keto-Testosterone

Hormones measured in the routine lab

Hormones NOT measured in the routine lab

Unconventional RE-DISCOVERED steroids

DIAGNOSING 21-NCAH by LC-MS/MS STEROID PROFILING

Table 2 Comparison of steroid concentrations between patients with nonclassic 21-hydroxylase deficiency (N21OHD) and controls. Data are expressed as median (interquartile range).

Steroid (nmol/L)	**N21OHD** ($n = 32$)	**Controls** ($n = 54$)	**Ratio of medians N21OHD/controls**	***P***
17OHP4	18.1 (8.7–33.0)	2.8 (0.9–4.3)	6.5	<0.0001
16OHP4	2.4 (1.4–4.9)	0.6 (0.4–0.9)	4.1	<0.0001
21dF	1.8 (1.3–5.3)	0.0 (0.0–0.3)	N/A	<0.0001
Progesterone	0.8 (0.5–1.4)	0.4 (0.3–0.8)	1.8	0.0018
Cortisol	202.5 (125.9–442.8)	249.1 (208.9–373.7)	0.8	0.197
Cortisone	53.7 (30.5–72.3)	51.5 (40.3–60.8)	1.0	0.855
Corticosterone	1.6 (0.8–3.5)	5.9 (3.5–13.6)	0.3	<0.0001
11dF	0.8 (0.5–1.4)	0.9 (0.4–1.6)	0.8	0.626
DOC	0.3 (0.3–0.4)	0.5 (0.4–0.9)	0.6	0.113
A4	4.3 (2.0–8.1)	3.9 (2.2–8.5)	1.1	0.798
Testosterone	1.1 (0.5–2.7)	1.6 (0.8–4.3)	0.7	0.195
11OHA4	9.6 (5.1–25.9)	4.5 (2.9–7.1)	2.1	<0.0001
11KA4	1.1 (0.7–1.7)	0.6 (0.4–0.8)	1.7	0.0001
11OHT	1.0 (0.3–1.7)	0.4 (0.3–0.6)	2.2	0.0019
11KT	1.8 (1.2–3.3)	0.9 (0.6–1.3)	2.0	<0.0001
11KT/Testosterone	1.8 (1.2–3.0)	0.6 (0.2–0.9)	3.3	<0.0001
11OHA4/A4	2.6 (2.1–4.6)	1.2 (0.7–2.1)	2.0	<0.0001
A4/testosterone	3.8 (2.8–4.5)	3.5 (2.4–4.2)	1.3	0.274

A4, androstenedione; DOC, 11-deoxycorticosterone; 17OHP4, 17α-hydroxyprogesterone; 16OHP4, 16α-hydroxyprogesterone; 21dF, 21-deoxycortisol; 11dF, 11-deoxycortisol; 11OHA4, 11β-hydroxyandrostenedione; 11KA4, 11-ketoandrostenedione; 11OHT 11β-hydroxytestosterone; 11KT, 11-ketotestosterone.

Turcu et al., EJE 2020

21-NCAH vs PCOS

High 21-deoxycortisol 100% sensitivity

≥0.087 ng/mL 76.9% specificity

High 17OHProgesterone ≥1.79 ng/mL 72.7% sensitivity

plus 100% specificity

Low Corticosterone ≤8.76 ng/mL

41.9%
true negative
PCOS women not needing further genetic analysis or ACTH testing

Oriolo et al., JEI 2020

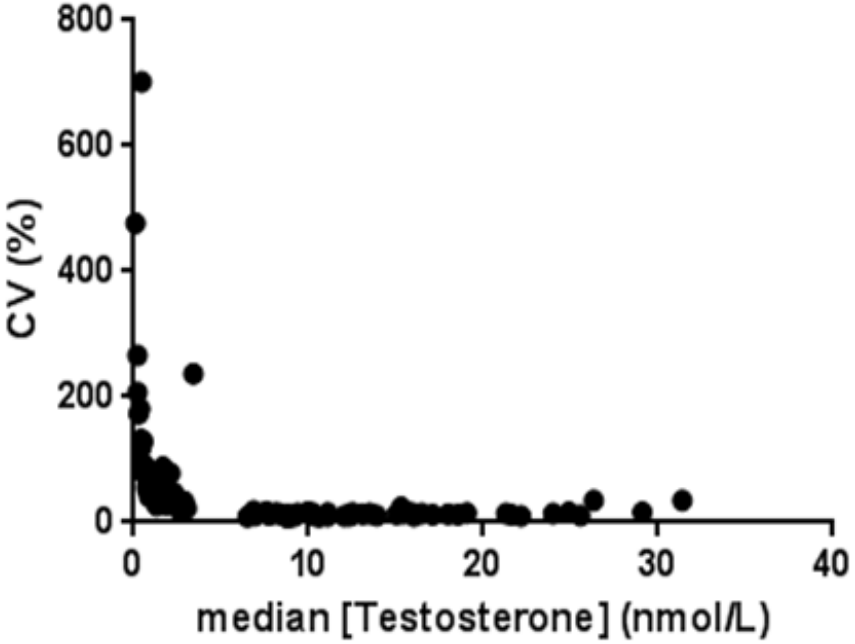
THE SUCCESS
of LC-MS/MS in the last 20y

- Improving the measurement of traditional diagnostic steroids
- Allowing the measurement of steroid panels
- **Easing the path toward harmonization of measurements**

REPRODUCIBILITY OF STEROID MEASUREMENTS

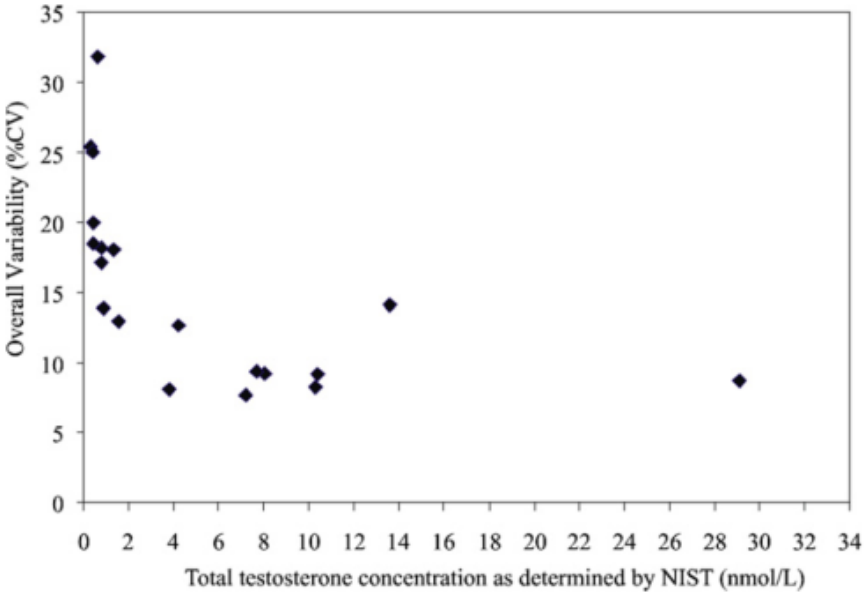
TESTOSTERONE

7 labs using IAs



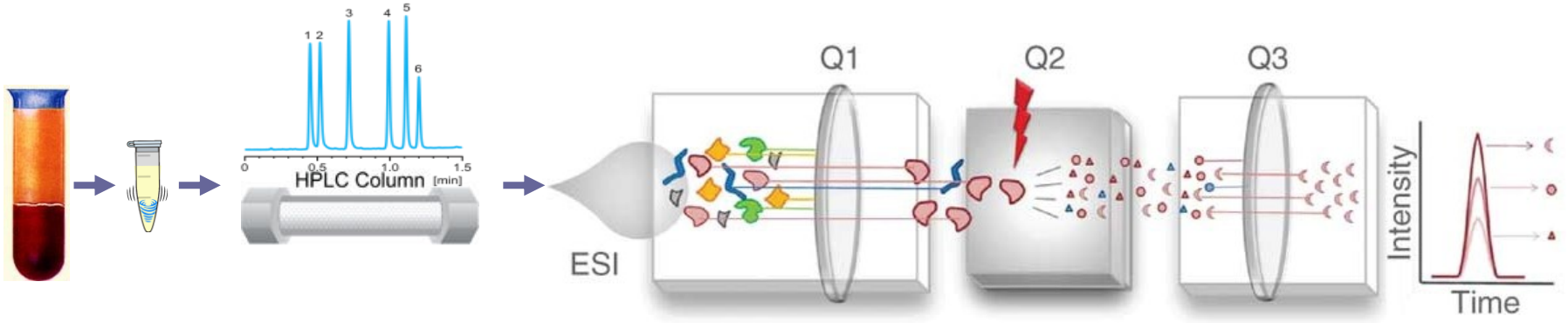
Groenestege et al., Clin Chem 2012

9 LC-MS/MS assays



Vesper et al., Steroids 2009

LC-MS/MS is a COMPLEX MULTISTEP PROCEDURE



Blood collection → Sample prep → Isotopic Internal Standard → LC Separation → Ionization → Parent Ion filter → Analyte fragmentation → Daughter Ion filter → Quantitation

Multiple options available for each step.

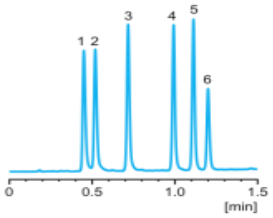
Several technical differences among LC-MS/MS assays.

➢ What is the Impact on the final result?

HarmoSter CONSORTIUM

9 Measuring Centers all over Europe

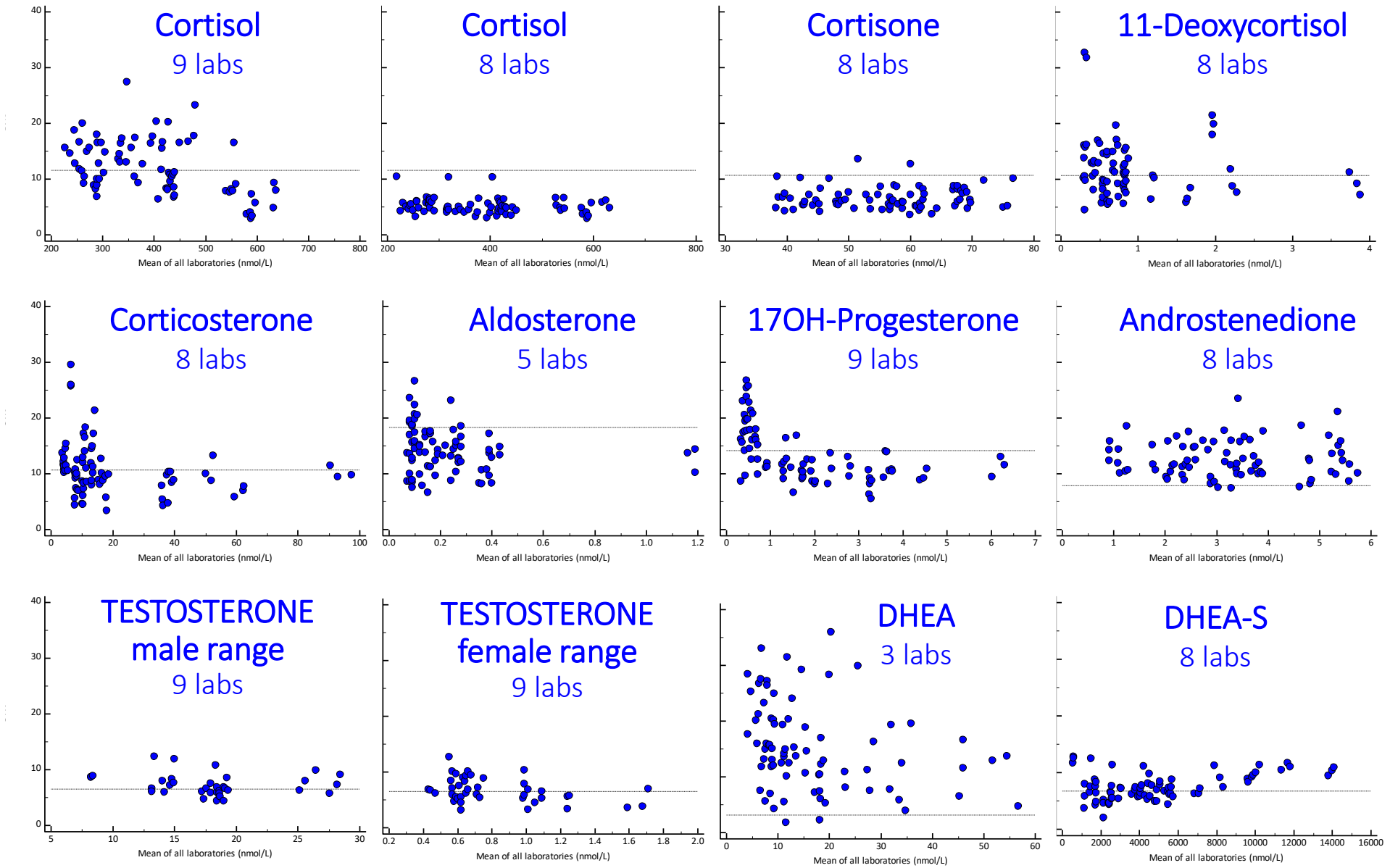
9 different LC-MS/MS assays



- University of **Bologna**, IT
- **Manchester** NHS Foundation Trust, UK
- **Amsterdam** University Medical Center, NL
- Canisius-Wilhelmina Hospital, **Nijmegen**, NL
- Technische Universität **Dresden**, DE
- University Hospital, **Erlangen**, DE
- Hospital of the University of **Munich**, DE
- University Hospital of **Lausanne**, CH
- Ente Ospedaliero Cantonale, **Bellinzona**, CH

INTER-LABORATORY VARIABILITY

Maximum allowable imprecision



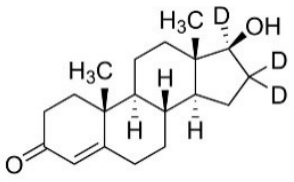
Fanelli et al., CCLM 2022a, 2022b and 2024

THE LIMITS

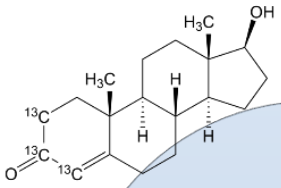
SOURCES OF VARIABILITY IN RESULTS BY DIFFERENT LC-MS/MS METHODS

Interaction among analytical factors and individual patient sample within each methods.

DEUTERIUM



CARBON 13



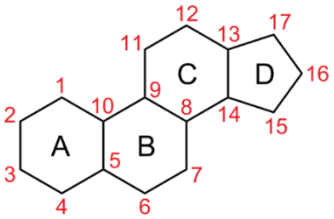
Internal Standard:
Isotope type, mol weight, ret time

LC:
Matrix effect, Isomers, Isobars

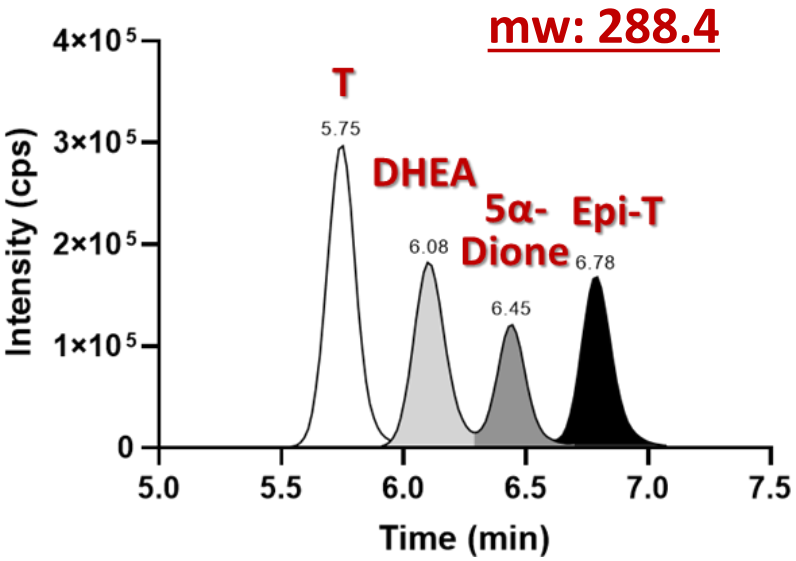
MRM:
Q1/Q3 specificit y

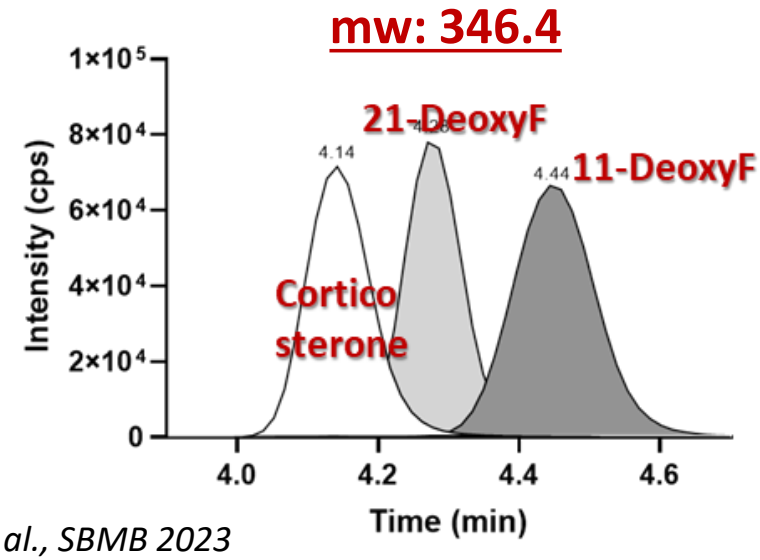
Calibration:
Surrogate matrix
Standard

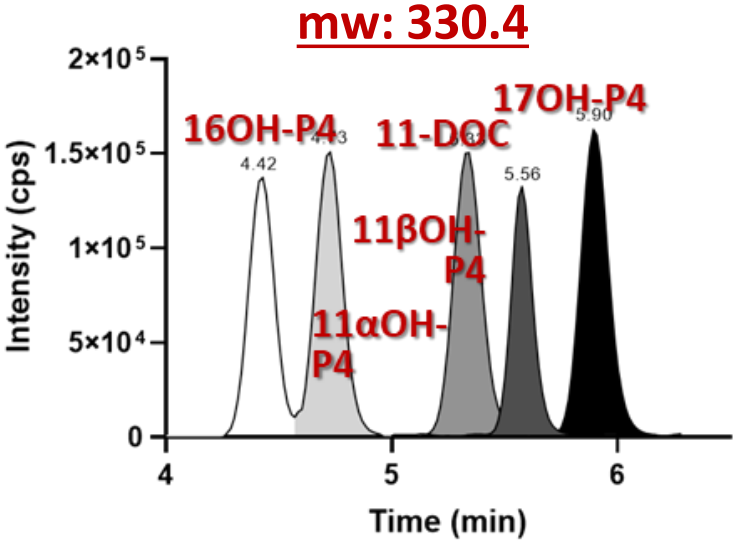
THE LIMITS
STEROID CROSS-DETECTION in LC-MS/MS



Extended run-times to separate steroids with the same molecular weight and similar MS/MS spectrum



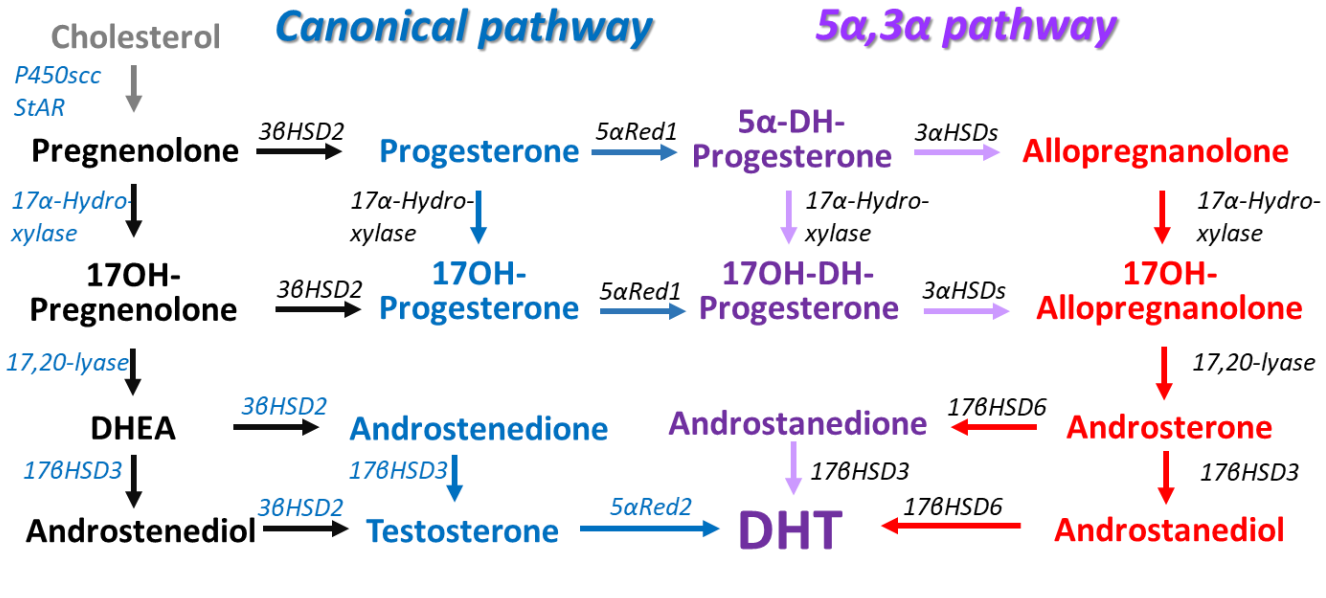


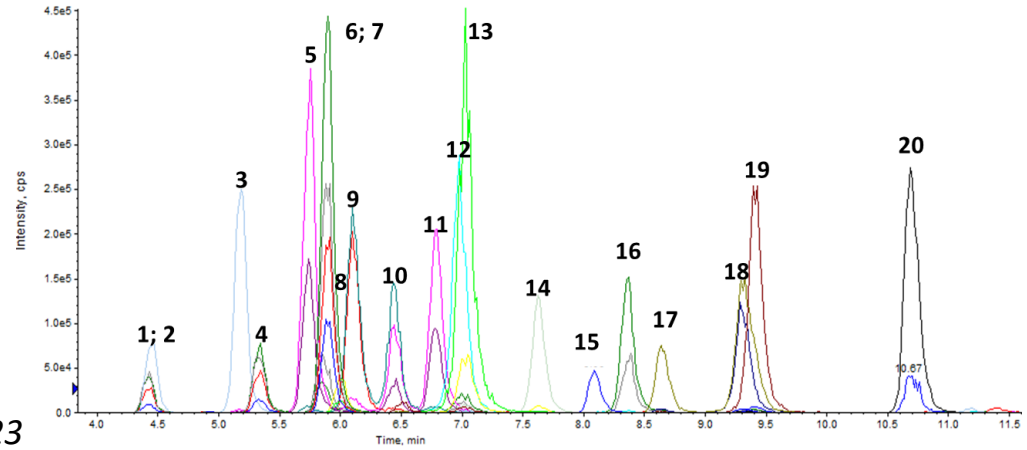


Fanelli et al., SBMB 2023

EXPLORING THE STEROIDOME

THE BACKDOOR PATHWAY FOR ANDROGEN BIOSYNTHESIS





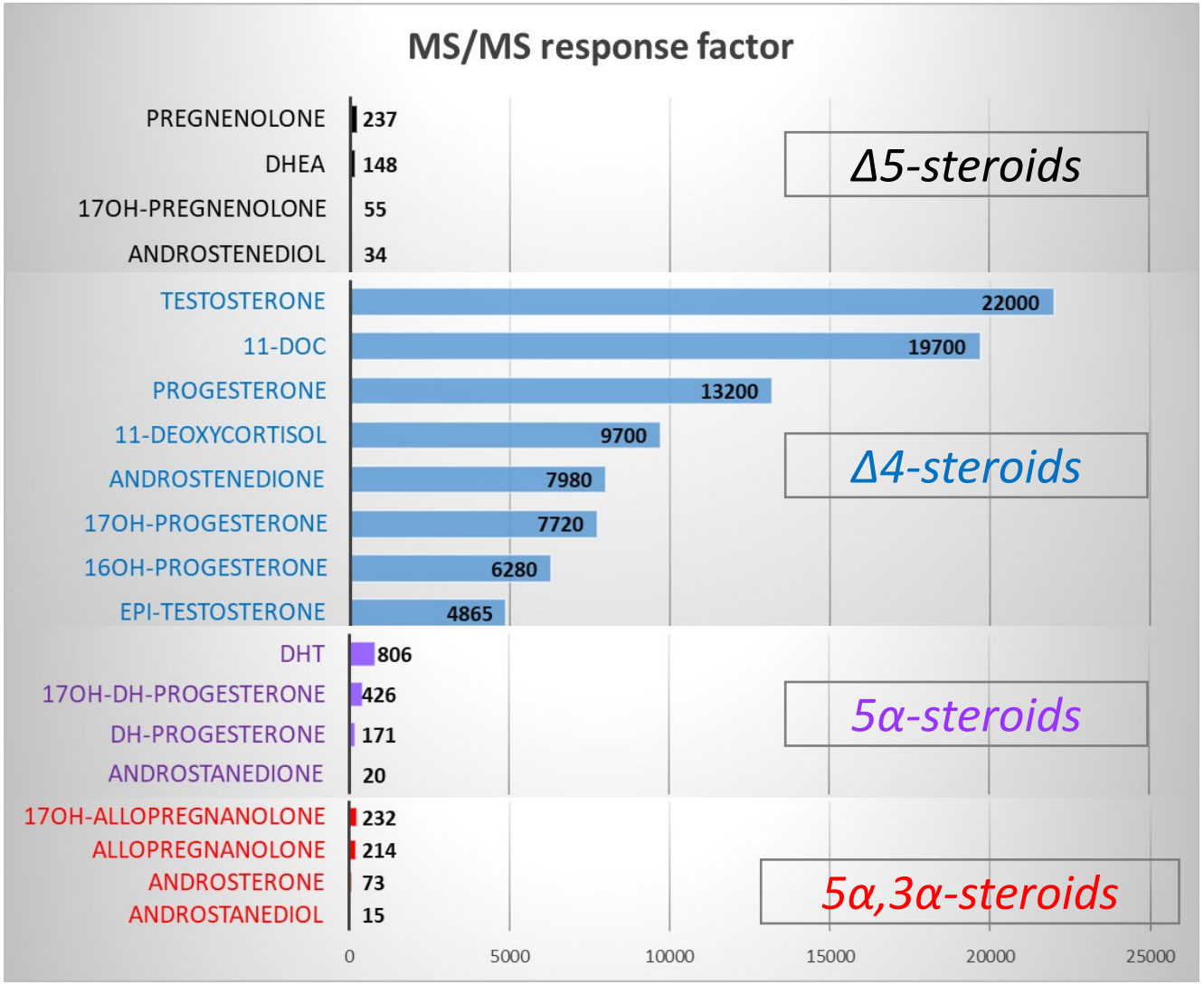
1 16OH-Progesterone (16OH-P4)
2 11-Deoxycortisol (11-S)
3 Androstenedione (A4)
4 11-Deoxycorticosterone (11-DOC)
5 Testosterone (T)
6 Androstenediol (A5)
7 17OH-Progesterone (17OH-P4)
8 17OH-Pregnenolone (17OH-P5)
9 Dehydroepiandrosterone (DHEA)
10 Androstanedione (5α-dione)
11 Epitestosterone (Epi-T)
12 Dihydrotestosterone (DHT)
13 17OH-Dihydroprogesterone (17OH-DHP4)
14 Progesterone (P4)
15 Androstanediol (3α-diol)
16 Androsterone (AN)
17 Pregnenolone (P5)
18 Dihydroprogesterone (5α-DHP4)
19 17OH-Allopregnanolone (17OH-Allo)
20 Allopregnanolone (Allo)

Fanelli et al., SBMB 2023

THE LIMITS

VARIABLE SENSITIVITY FOR DIFFERENT STEROIDS

Peak area per 1 pg on column



Fanelli et al., SBMB 2023

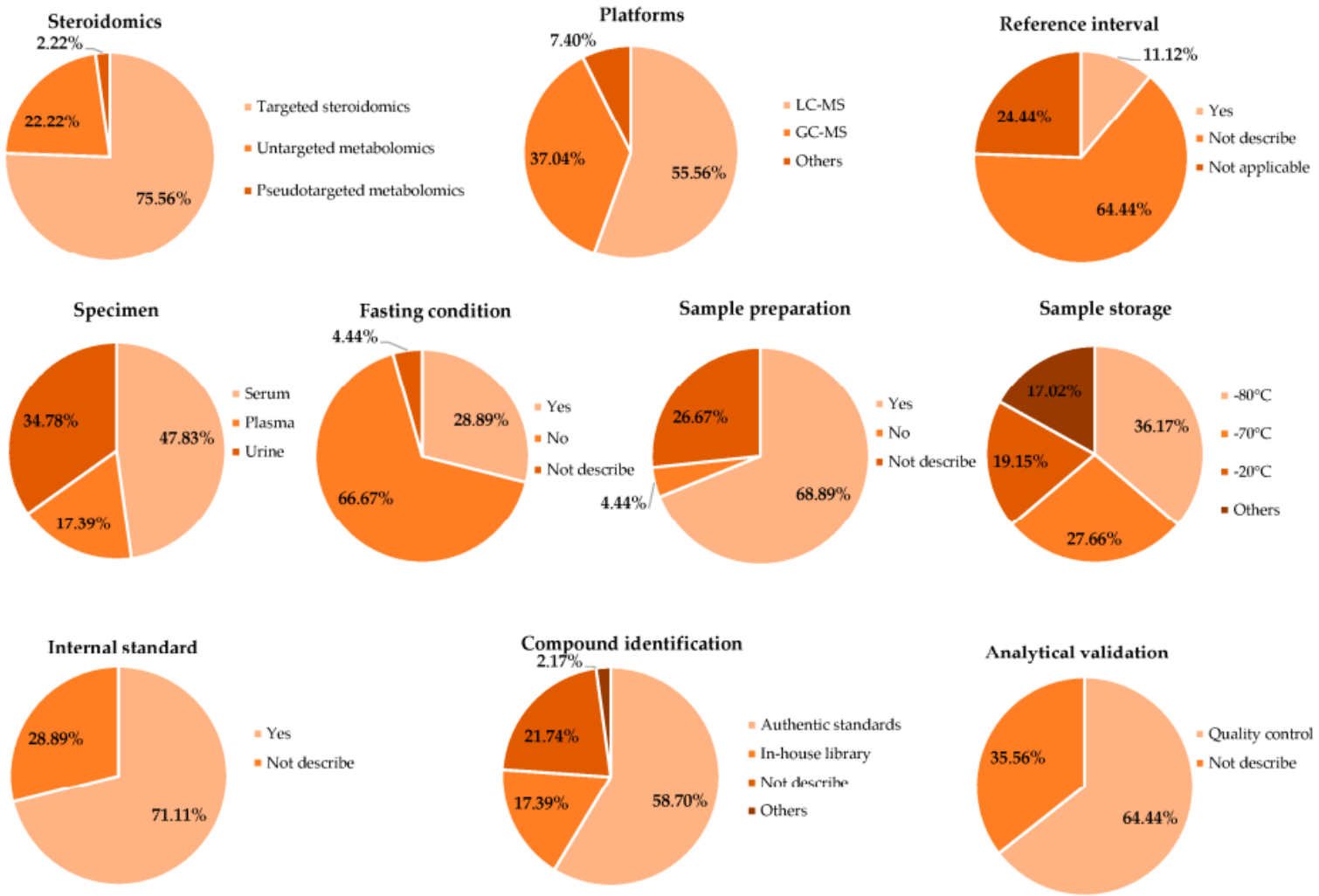
FUTURE PERSPECTIVES
FOR CLINICAL LABORATORIES

- Ready to use kits including SPE plates, calibrators, internal standard, reagents, etc… CE-IVD

- Fully automated solutions combining sample prep + LC-MS/MS

FUTURE PERSPECTIVES
«STEROIDOMICS»

HETEROGENEITY IN STUDY DESIGN AND ANALYTICAL APPROACH

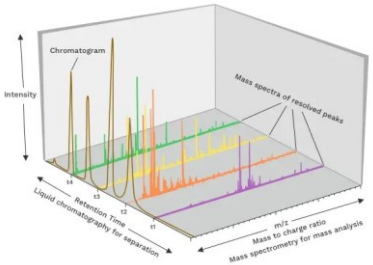


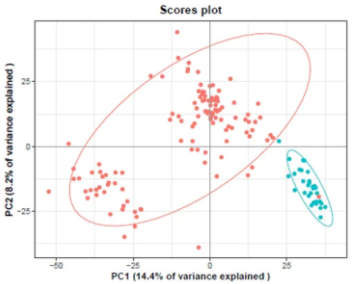
Anh et al., 2019

UNTARGETED WORKFLOW FOR UNTARGETED METABOLOMICS

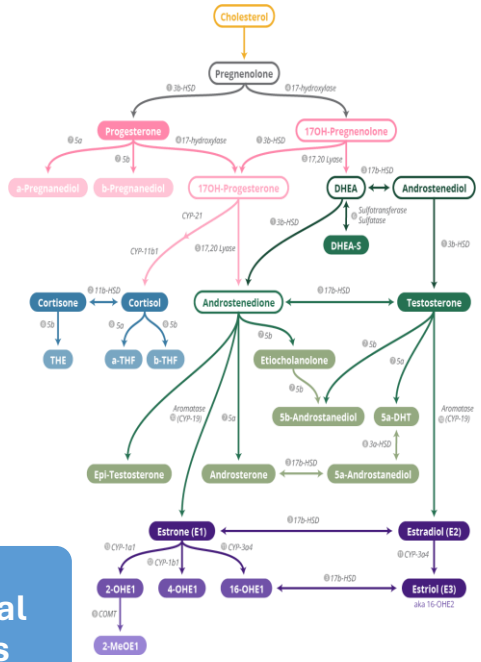
WORK IN PROGRESS

Vanquish Flex
Orbitrap Exploris 240





Pathways analysis



Sample Collection and Preparation → Data Acquisition UHPLC-HRMS → Data processing and Normalization

Statistical analysis

Machine Learning

Compound Identification

Interpretation

Biomarker Identification

STEROID ANALYTICALS
THE ON GOING (R)EVOLUTION

PAST	PRESENT	FUTURE
Immunoassays	LC-MS/MS	LC-High Resolution MS
Single analyte measurement of a few steroids	**Targeted** measurement of panels of steroids	**Untargeted** analysis of all steroids in a certain matrix
High Throughput Poor specificity	High Specificity and Sensitivity Good Throughput	Top Specificity Good Throughput
Reference interval Decision Limits	Combination of multiple RI and DL	Multidimensional Model

Bologna Team

Division of Endocrinology and Prevention and Care of Diabetes,
Dept. of Medical and Surgical Sciences, University of Bologna

Valentina Bissi
Greta Galante
Thomas Senard
Lorenzo Tucci
Kimberly Coscia
Laura Rotolo
Guido Di Dalmazi
Valentina Vicennati
Alessandra Gambineri
Uberto Pagotto