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Hormone Testing:

Comparison of Blood /Saliva/Urine Mediums

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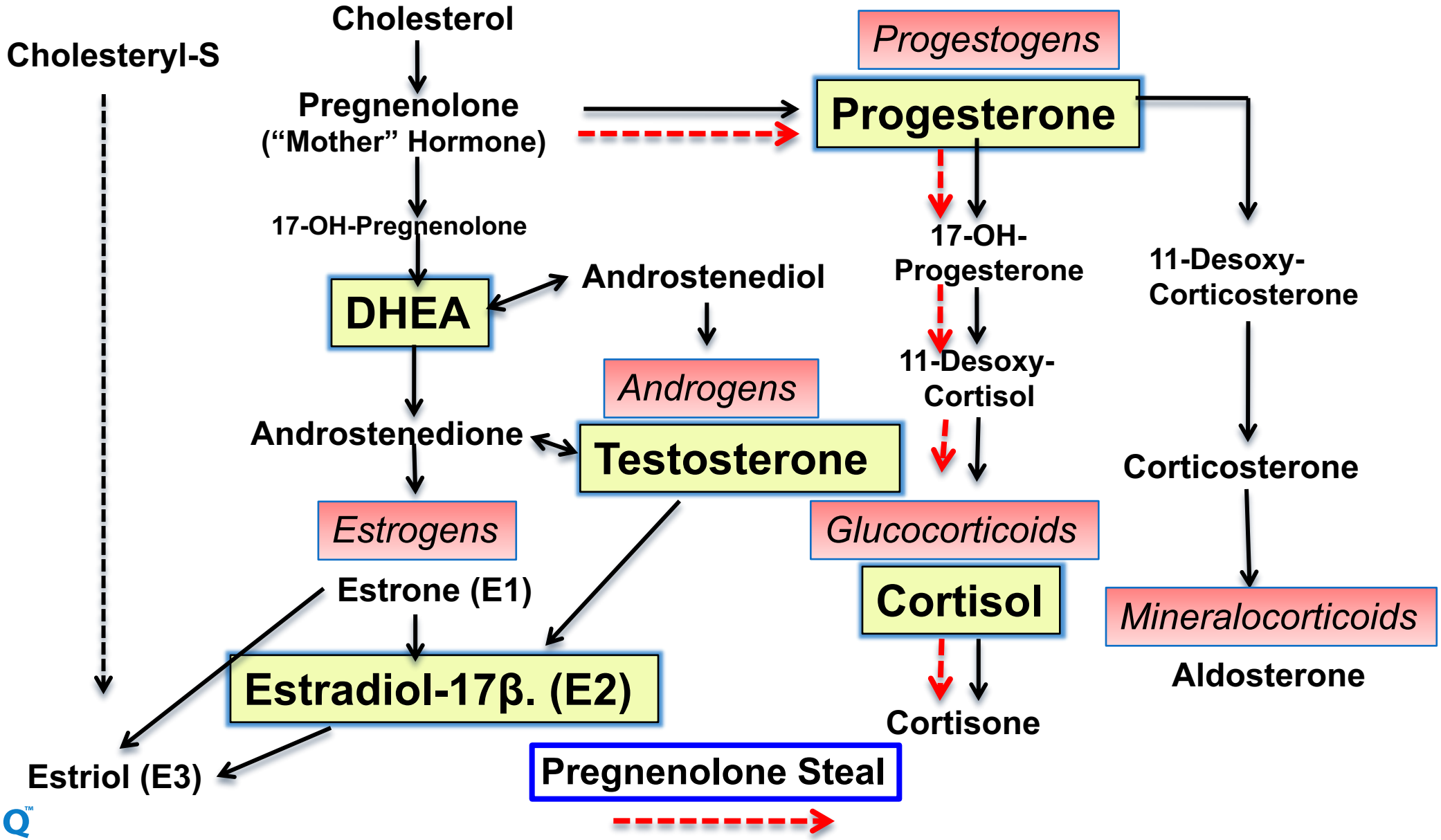
AFDNP Inc. November 3, 2021

Accurately Assessing Sex and Adrenal Hormones

- Why Test?
- Which ‘Matrix’ should be used?
  - Matrix is the sample medium, other than the analyte
- Which is the ‘Best’?
  - What are the questions that need answers?

Endogenous Steroid Hormone Pathways



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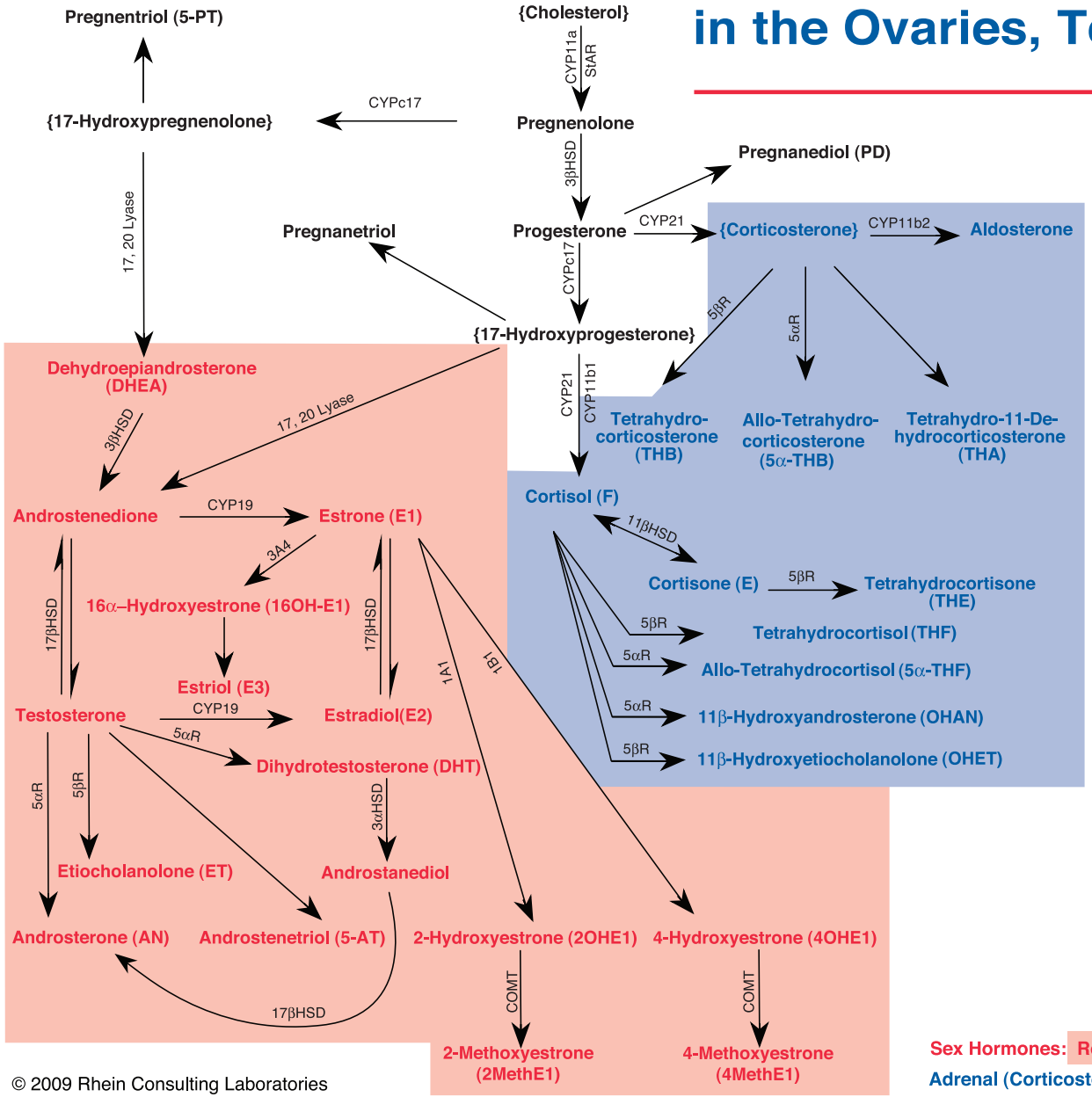
Biosynthesis and Metabolism of Steroid Hormones as Produced in the Ovaries, Testes and Adrenals



Enzymatic Steps:

3βHSD:
3 - beta - Hydroxysteroid dehydrogenase

5αR: 5 - alpha - Reductase

5βR: 5 - beta - Reductase

11βHSD:
11 - beta - Hydroxysteroid dehydrogenase

17βHSD:
17 - beta - Hydroxysteroid dehydrogenase

17,20 Lyase: 17,20 - Desmolase

CYP11a: Cholesterol side chain cleavage

StAR: Steroidogenic acute regulatory protein

CYP11b1: 11 - beta - Hydroxylase

CYP11b2: 18 - Oxidase

CYPc17: 17 - alpha - Hydroxylase

CYP19: Aromatase

CYP21: 21 - Hydroxylase

Estrogen Metabolism:

1A1: Cytochrome p450 1A1 (CYP1A1)

1B1: Cytochrome p450 1B1 (CYP1B1)

3A4: Cytochrome p450 3A4 (CYP3A4)

COMT: Catechol-O-Methyl-transferase

Sex Hormones: Red

Adrenal (Corticosteroids) Hormones: Blue

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General ways Hormone Levels Measured or Assayed

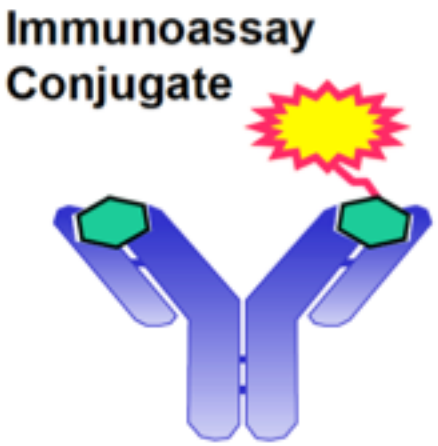
1. Immunochemical assays: RIA, ELISA (EIA), FIA,

2. Gas or liquid chromatographic (GC) technology
   in combination with mass spectrometry (MS)
   - either single or triple quadrupole MS

RIA = Radioimmunoassay; ELISA (EIA) = Enzyme-Linked ImmunoSorbent Assay; FIA = Fluorescent Immunoassays

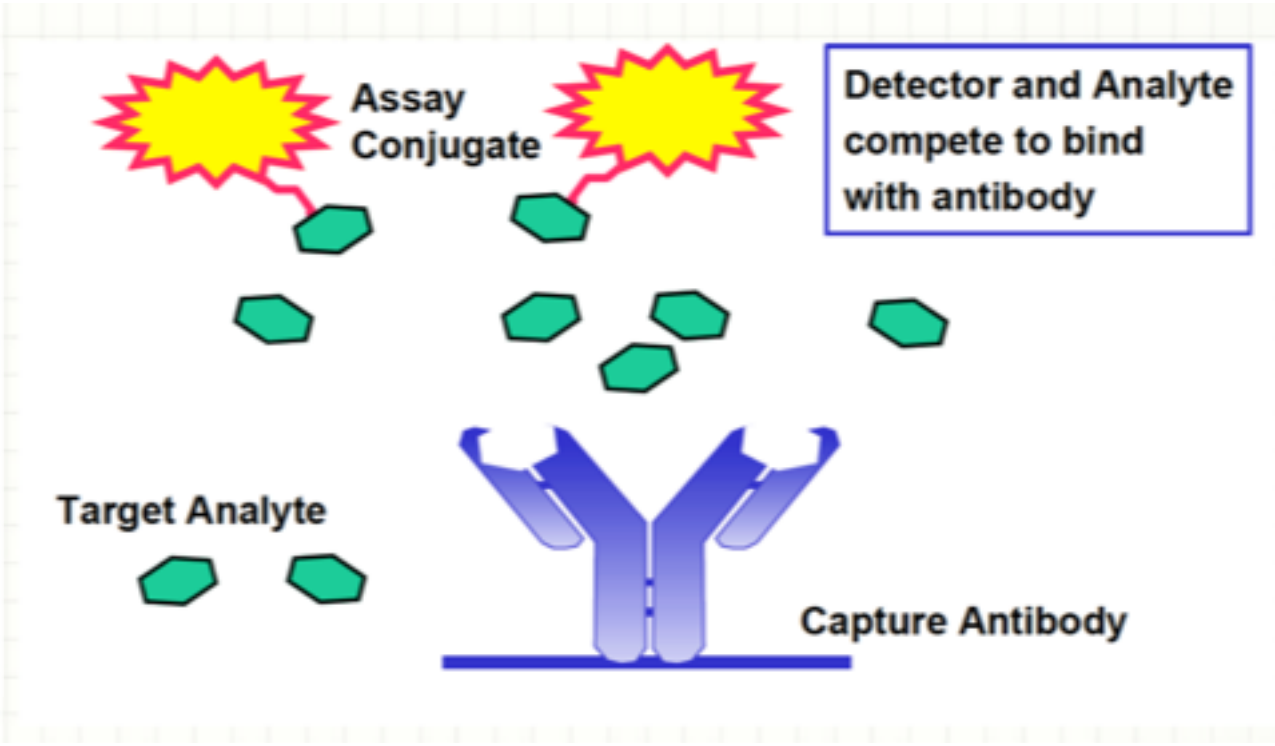
Immunoassays

- An analytical method which uses antibodies as reagents to quantitate specific analytes



Detectable Label

- Radiolabel (RIA)
- Enzyme (EIA)
- Fluorescence (FIA)
- Luminescence
- Electrochemical
- Visual
  - Colloidal gold
  - Colored latex



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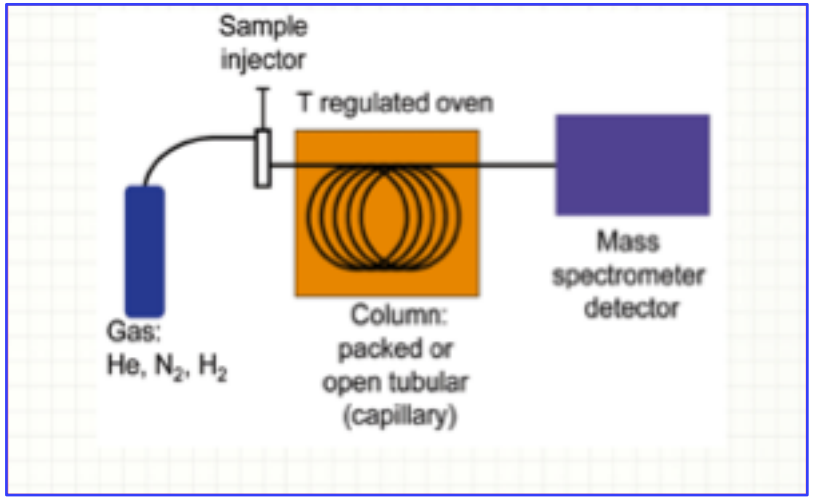
GC/MS: Basic Principles

- Principles of Gas/Liquid Chromatographic Assays coupled with
  - Mass Spectrometry or Tandem MS•MS



Molecular Fingerprinting

Complex mixtures separated in GC column
Detected by MS
Generates characteristic mass spectrum for each compound
Used as a "**fingerprint**" for identification purposes

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Steroid Testing in Different Body Fluids

- **Endogenous:**
  - All 3 main body fluids: serum/plasma/DBS, saliva, wet/dry urine can monitor endogenous primary active steroid hormones
    - Accuracy issue if steroid at very low levels, (salivary & blood Estradiol & Testo.)
    - Testing endogenous sex hormones in serum well characterized in med. lit.
      - less so in urine, saliva, and capillary whole blood (DBS)

- **Exogenous:**
  - Some of the 3 fluids are NOT appropriate for the measurement of exogenously delivered steroid hormones; specifically those delivered by oral or topical administration

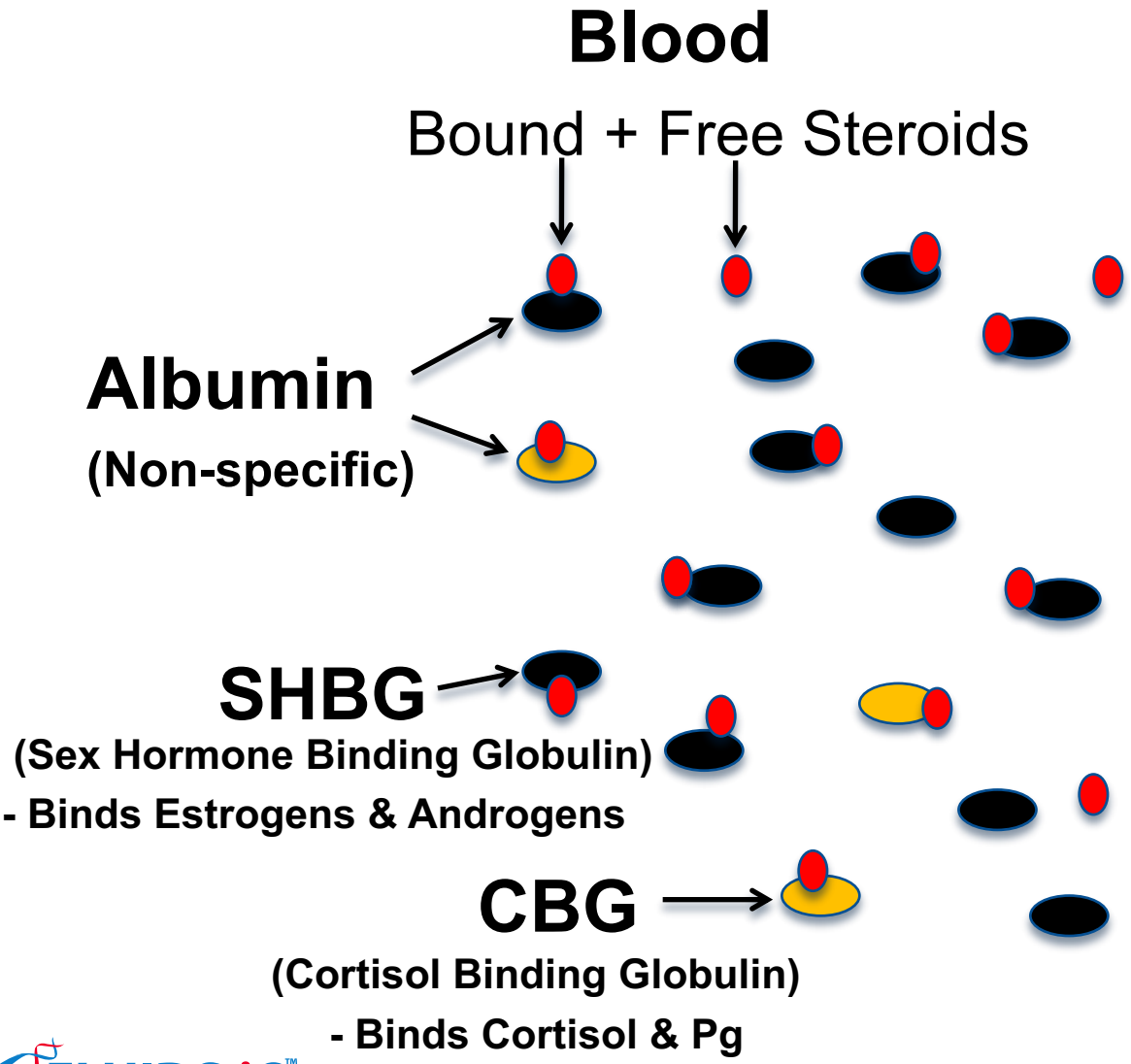
Consideration of the Relative Merits of the Three Primary Analytical Matrices Used to Test Hormones

Blood – Saliva – Urine

Consideration of the Relative Merits of the Three Primary Analytical Matrices Used to Test Hormones

Blood (Serum)

Steroid Hormones in Blood



Serum Sampling

- **Advantages:**
  - Readily available
  - Cost-effective
  - Well-established reference ranges
  - Widely utilized and accepted
    - Standard of measurement by conventional medical community
    - Certain hormones not readily assayed in fluid matrices other than serum

Serum Sampling

- **Advantages:**
- Measures Sex Hormone Binding Globulin (SHBG) &

  Cortisol Binding Globulin (CBG)
- Ideal for testing Peptide Hormones:
  - Thyroid hormones including Reverse T3, as well as thyroid antibodies
  - FSH, LH, prolactin, fasting insulin:

    - These hormones, because of their molecular weight alone, do not appear in filtrates, such as saliva or urine.

Serum Sampling

- **Disadvantages:**
- Hormone concentrations are time-dependent
  - ultradian (hr to hr), diurnal or circadian periodicity
- Rapid clearance: Short half-life
- Pulsatile secretion leads to major fluctuations, especially sex hormones
- Limited sensitivity and specificity for many hormones

Serum Sampling

- **Disadvantages:**
- Invasive collection process
- Specificity often poor in immunologically based assays,
  - e.g., RIA & ELISA (cross-reactivity)
- Recent advances using LC/MS•MS: Vastly improved specificity

  but, often at very significant additional expense
- Comprehensive hormone profiling in serum can be cost-prohibitive

Serum Sampling

- **Disadvantages:**
- Serum provides only a snapshot in time – **Spot Test**
- Rapid fluctuations in serum levels of certain sex hormones makes repeat testing necessary for meaningful data: Inconvenient and cost-prohibitive
- Separate Tests for Total and Free hormone are often required

Oral Hormone Delivery and Testing

Challenges of Using Serum

- Bio-identical estrogens (estradiol, estriol, estrone), progestogens (progesterone), & androgens (testosterone & DHEA), are all used **orally** as a form of **HRT**
  - **10x physiological dosing is required to achieve a physiological level of the active hormone** in whatever body fluid used for testing

Causes **false high hormone levels in serum (eg; Pg)**

- Most commercial serum immunoassays that rely on polyclonal Abs overestimate true levels (eg; Pg), especially women using oral therapy (Pg)

HRT = Hormone Restoration (Replacement) Therapy

Serum Testing for Hormones

- Limited utility for sex hormones, because:
  - No distinction made between **bound & free** hormone.
  - Estradiol, estrone, estriol and Pg reported as **total** hormones
  - Free hormones assays not common, leading to misleading results
    - Hormone levels appear to be normal, or even high normal, because of an abundance of **bound** hormone
    - **However, if the free hormone level is low, patient can be functionally deficient even with a normal total hormone level**

Serum Testing for Hormones

Estrogens: Estrone (E1), Estradiol (E2), Estriol (E3)

- E2 is the most common female hormone measured in serum
- E1 available from many labs, but not tested as often
- E3 testing not routinely performed, but is an important estrogen
  - Protective. Binds to Estrogen Receptor Beta (ERb), which inhibits cell proliferation and is a potent tumor suppressor

Progesterone:

- Monitoring its supplementation in serum poses a problem
- Transdermal progesterone does raise serum Pg levels in a statistically significant manner, but the magnitude of change is quite small
  - Can lead to excess Pg dosing as practitioners strive to achieve therapeutic levels

Serum Testing for Hormones

Testosterone:

- Serum T testing is an exception. Commonly available as both total and free, and therefore can be useful in assessing hormone balance

- Serum hormone testing **does not** measure estrogen, androgen, & adrenal **metabolites**
  - Assist in understanding a patient's condition and help to guide treatment options

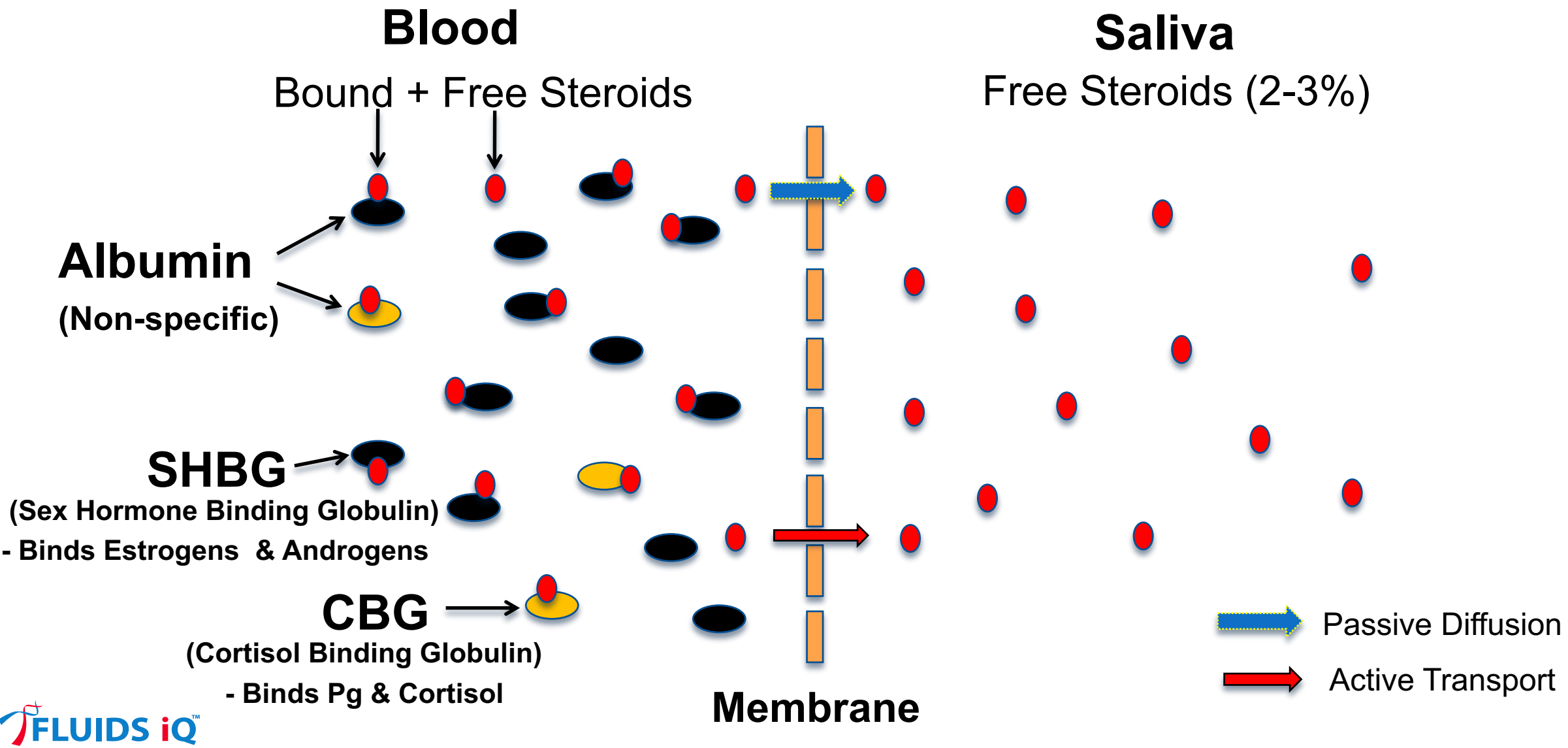
Blood Testing for Hormones

- Almost always use the term ‘blood’ synonomously with ‘Serum’
- Can use ‘**Dried Blood Spot**’ as a modality
- Advantages:
  - Less invasive than serum
  - Ease and reliability of transport
  - Allows for home testing by patient
  - Allows for testing in offices without phlebotomists, etc
- Disadvantages:
  - Tissue level test: Must compare to serum levels
  - Using whole blood. Must account for Hg effect and coagulants

Consideration of the Relative Merits of the Three Primary Analytical Matrices Used to Test Hormones

Saliva

Bound vs Free Steroid Fractions



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Oral Hormone Delivery and Testing
Challenges of Saliva Sampling

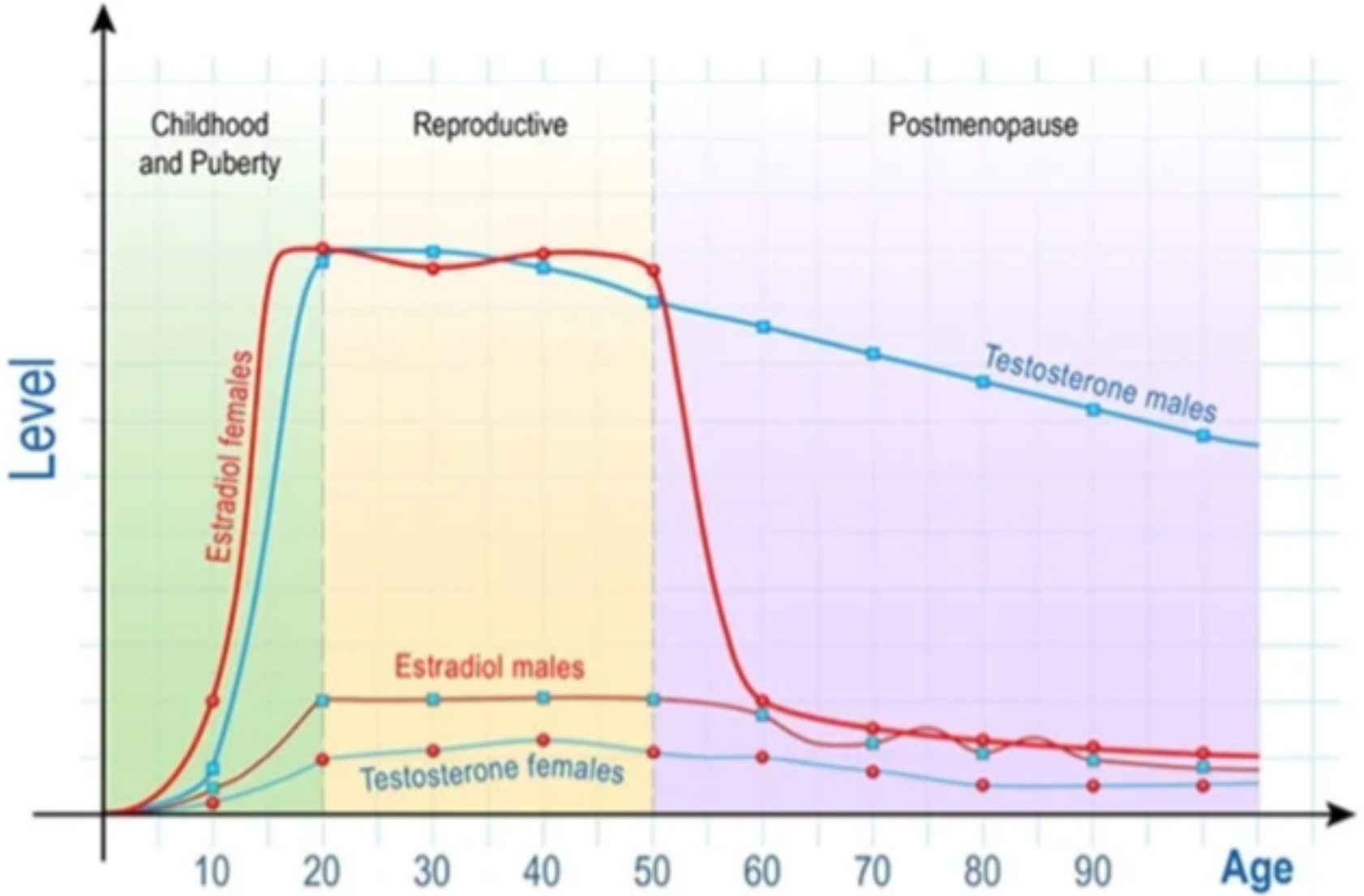
- Endogenously or exogenously-produced hormones in bloodstream:
  - 97–98% of the active hormone bound up by specific proteins
  - 2%–3% released in capillary beds and into interstitial space & tissues

- In saliva - most metabolites from oral hormone therapy filtered out by salivary gland. Only **active** hormones enter saliva
  - ***Salivary hormones more representative of the amount of bioactive steroid present in the bloodstream, and its bioavailability to target tissues***

Saliva Sampling

- **Advantages:**
  - Excellent Medium for ***Rapid Testing***
  - Accurate measure of clinically validated biomarkers
  - Non-invasive, with low infection rate in sample collection
  - Measures **free hormone fraction**
  - Ideal for **multiple or serial sampling** per day or month
    - More versatility than serum for evaluating un-supplemented hormone status
  - Accessible to practitioners, without a license, to order blood tests

Variation in Sex Hormone levels with Age



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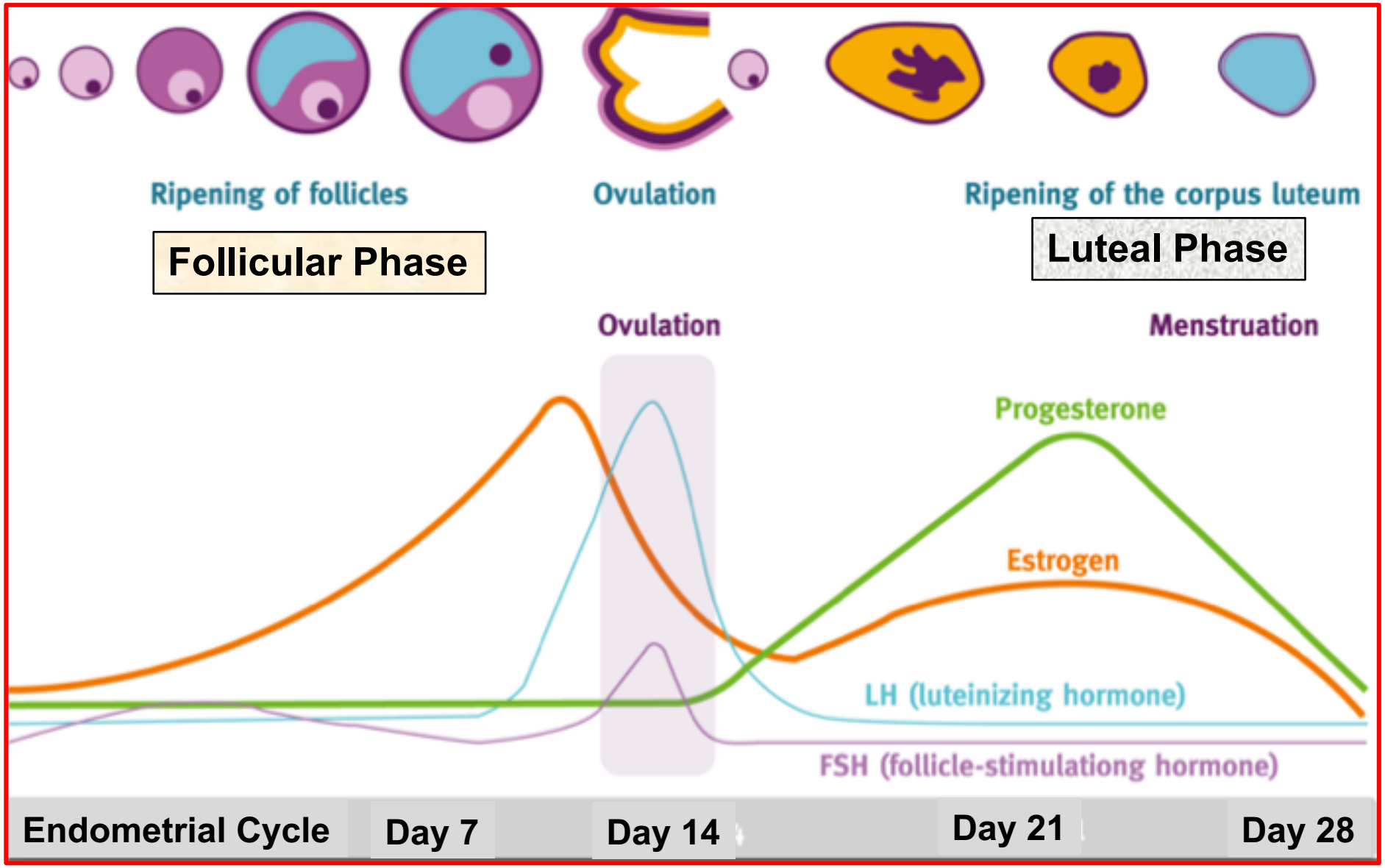
Ratan, NM-2019

Female Sex Hormones

Follicular Development



Pituitary & Ovarian Hormone Levels

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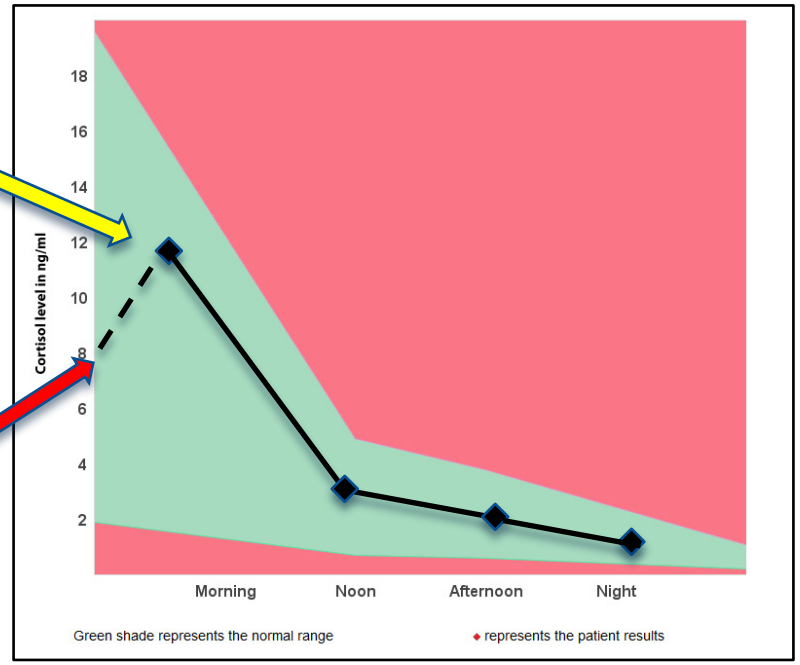
Cortisol

Normal Cortisol: Diurnal (daily) rhythm is apparent

Morning Cortisol

- Key to HPA axis evaluation
- Sample > 30 min after awakening
  - Allows time for peak serum CAR to reach saliva

Cortisol Awakening Response (CAR)



Total Cortisol: Sum of the 4 Cs

Elevated levels: Hypercortisolism or exogenous cortisol source.

Depressed levels: Hypoadrenal function

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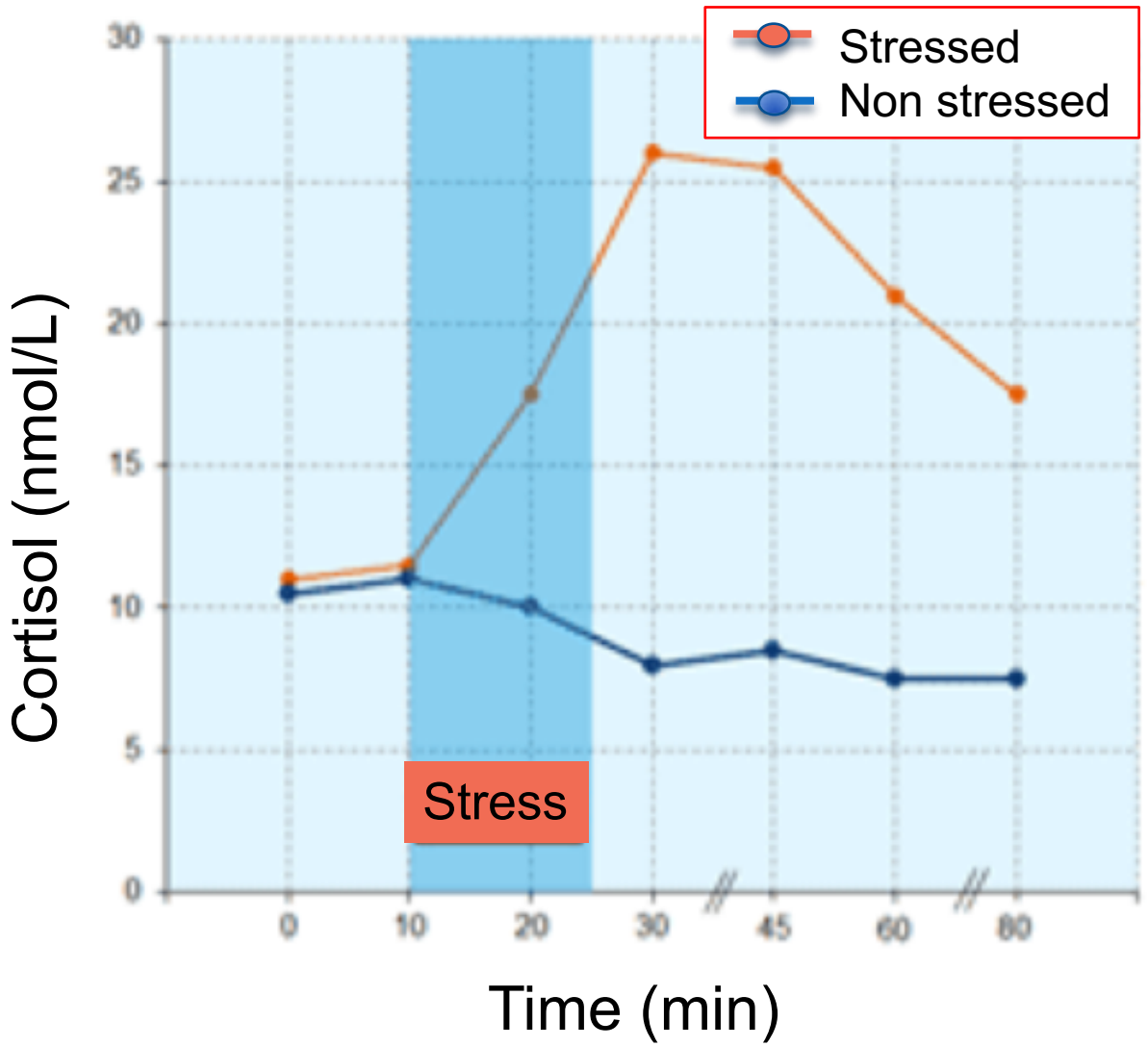
Cortisol and Stress Measurement



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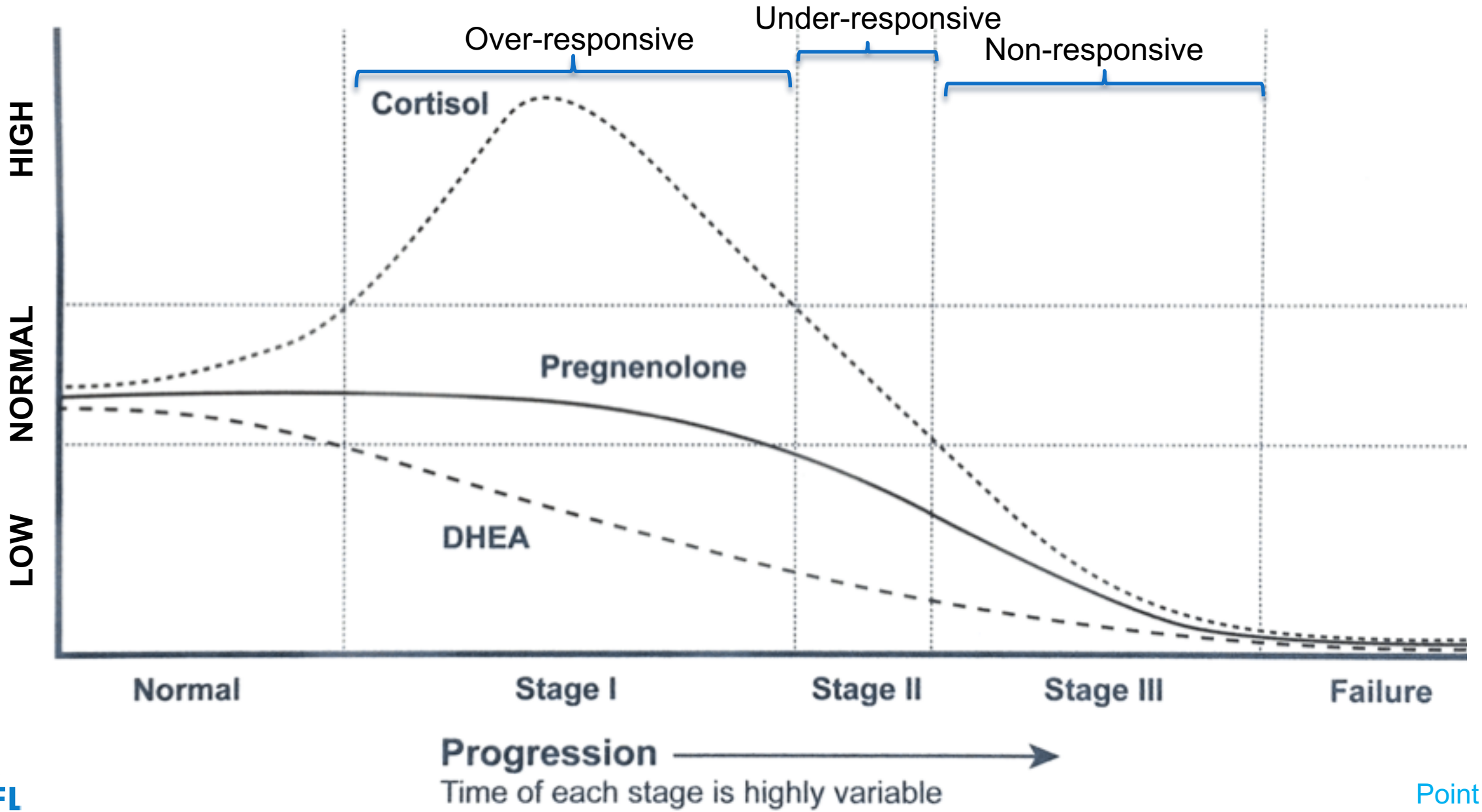
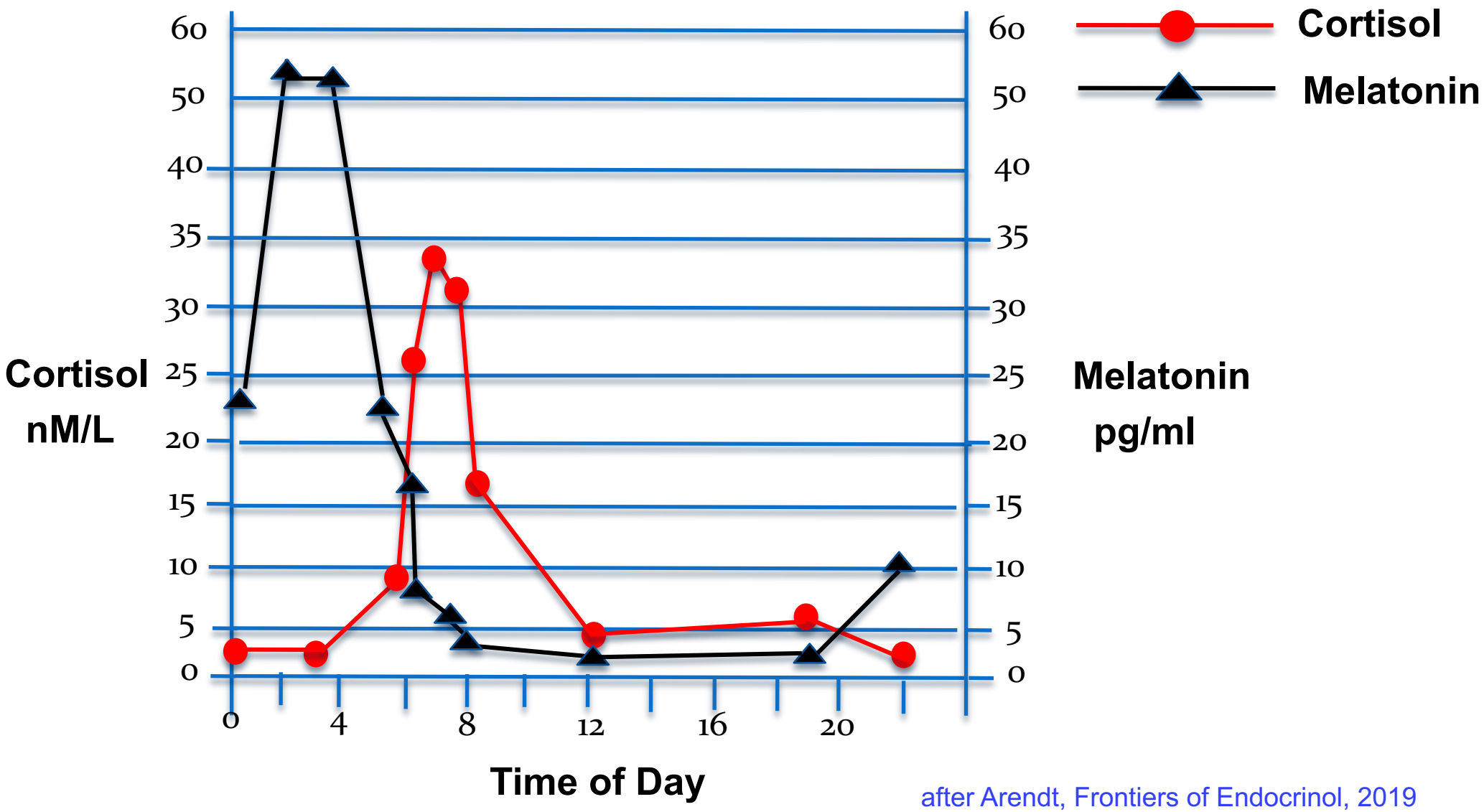
Chronic Stress & Adrenal Hormone Output



Point Inst. 2010

Daily Profiles Cycles for Cortisol & Melatonin



after Arendt, Frontiers of Endocrinol, 2019

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Patient: JOHN DOE
Sex: Male **Age:** 33 yr **Date of Birth:** 1988-10-22
Height: 6 ft 5 in **Weight:** 155 lbs **Waist size:** 44 in

Hormones: No
Health Care Professional: John Smith

Accession #:	**100035502**
Sample received:	2021-09-27
Report issued:	2021-09-27
Sample collection:	
2021-09-22	06:45 AM
2021-09-22	12:30 PM
2021-09-22	18:30 PM
2021-09-22	22:45 PM

FEMALE WELLNESS DAILY CYCLE + MELATONIN (Daytime)

17-β ESTRADIOL (E2) pg/ml: **4**

		Reference range
Female		
21-50 years	Follicular phase	1.3 - 7.8 pg/ml
	Mid cycle	3.8 - 16.0 pg/ml
	Luteal phase	1.2 - 8.4 pg/ml
51-75 years	Post Menopausal	0.6 - 4.4 pg/ml
Male		1.0 - 4.7 pg/ml

PROGESTERONE (Pg) pg/ml: **11.2**

		Reference range
Female		
	Follicular phase	19.6 - 86.5 pg/ml
	Luteal phase	99.1 - 332.6 pg/ml
	Post Menopausal	6.0 - 56.4 pg/ml
Male		12.7 - 65.1 pg/ml

Pg:E2 RATIO: **2.8:1**

Optimal (Luteal): 100 - 300:1 when E2 1.2-3.3 pg/ml

Melatonin (Daytime\Noon) pg/ml: **4.1**

Reference range 0 - 5 pg/ml

The reference range is derived from a normal distribution of results that encompass 95% of randomly selected individuals in a population.

DHEA-S (DS) ng/ml: **2.7**

	Reference range
Female	0.2 - 2.5 ng/ml
Male	0.2 - 2.7 ng/ml

CORTISOL (C) ng/ml

		Reference ranges
Morning	**7.2**	1.6 - 12.6 ng/ml
Noon	**4**	0.7 - 4.9 ng/ml
Afternoon	**2.5**	0.6 - 3.8 ng/ml
Night	**1.2**	0.3 - 2.9 ng/ml
TOTAL	**14.9**	3.2 - 24.2 ng/ml

TOTAL C:DS RATIO: **6:1**

Reference range 5:1 to 6:1

TESTOSTERONE (T) pg/ml: **49.2**

Age (years)	Male	Female
Less than 20	Range not applicable	
20 - 29	41.4 - 142.5	5.5 - 49.0 pg/ml
30 - 39	31.8 - 100.4	5.2 - 49.0 pg/ml
40 - 49	30.1 - 97.8	4.5 - 49.0 pg/ml
50 - 59	30.0 - 92.0	3.6 - 49.0 pg/ml
60 - 69	23.2 - 86.9	2.9 - 38.8 pg/ml
Greater than 69	Range not applicable	

Saliva Sampling

- **Disadvantages:**
  - Saliva **production** difficult for some pts. Multiple restrictions:
    - Eating, drinking, gum-chewing, make-up use
    - Difficult to collect adequate volume in people > 40, Sjogren's Syndrome, etc
    - Micro-damage from tooth brushing (~1hr), even with no bleeding signs
  - Only used to evaluate **steroid hormones**.
    - Peptide hormones (eg; growth hormone & thyroid) are not available
  - **Steroid hormone metabolites not measured in saliva**

Saliva Sampling

- **Disadvantages: Sensitivity and Specificity**
- Limited analytical sensitivity for any non Ab-based assays
- Decrease in specificity, due to cross-reactivity
- Collection can itself significantly alter hormone concentrations

Consideration of the Relative Merits of the Three Primary Analytical Matrices Used to Test Hormones

Urine

Steroid Metabolism in the Liver

Blood

Steroid

(*Fat Soluble*)

Reactions

- Oxidation
- Reduction
- Hydrolysis
- Hydration
- Dehalogenation

Phase I

(*Breakdown*)

Enzymes & Cofactors

- Vits A, C, D3, E
- Vits B2, 3, 6, 12
- Folic Acid (B9), Ca
- Glutathione/NAC
- Flavonoids

Metabolites (Intermediate)

(More *Water Soluble*)

Reactions

- Glucuronidation, Sulfation
- Glutathione conjugation
- Acetylation, Methylation
- Amino Acid conjugation

Phase II

(*Neutralization:*

Conjunction Pathway)

Excretory Derivatives

(*Water Soluble*)

Serum

Kidney

Urine

Phase III

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NAC = N-acetyl L-cysteine

Urine Sampling of Steroid Hormones

- Less common in clinical practice than either serum or saliva
- **24-hr urine collection** the preferred method for testing hormones secreted at night & during deep sleep (eg; Growth Hormone)
- Most economical & reliable way to evaluate hormone ***metabolites***
- **Must differentiate 24 hr from serial single point collection, especially in Dried Urine sampling**

Urine Sampling

- **Advantages: 24 Hour Urine**
  - Urine assays measure unbound (bio-available) hormone fraction
  - No cross-reactivity: “Gold Standard” for accuracy and reproducibility,
  - Use of 24-hr urine hormone profiles in clinical practice:
    - Correlate well with symptoms reported by pts on hormone questionnaires
  - Allows evaluation of adequacy & safety of exogenous estrogen supplementation and in assessing adrenal function
    - Certain estrogen metabolites are “good” estrogens, with protective effect on estrogen-sensitive tissues. Other metabolites have more carcinogenic effects

Urine Sampling

Advantages: 24 Hour Urine

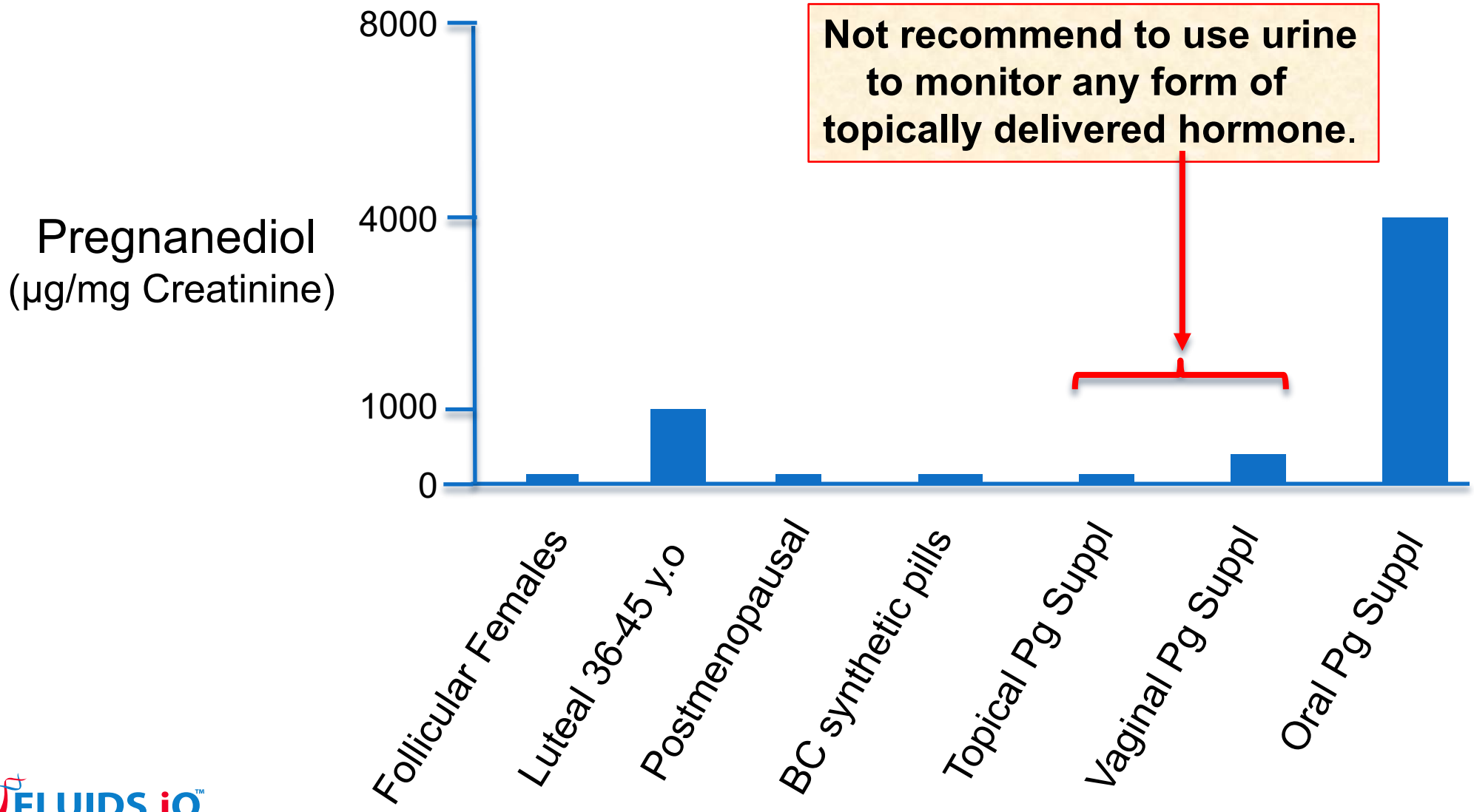
- 24 hr urine hormone panels excellent for evaluating adrenal health and f[n]
  - Virtually 100% specificity with GC-MS•MS
- Measures DHEA & cortisol, as well as cortisone (cortisol storage form)
- Measures Metabolites: A) cortisol & cortisone, B) aldosterone (& other mineralocorticoids)
  - A) Importance of metabolites seen in stressed patient with normal or high-normal cortisol.
  - B) Mineralocorticoids: Regulate salt/water balances. Low levels are a clear indicator of chronic adrenal fatigue. An excellent marker to monitor adrenal recovery with treatment

Urine Sampling

- **Disadvantages: 24 hour Urine Collection (UC)**
  - Inconvenience of 24 hr UC. Compliance often questionable
  - Results altered in pts with significant liver or renal impairment
  - Dehydration or excessive fluid intake can affect results (Creatinine)
  - Subject to misinterpretation d/o hepatic ‘1st-pass’ effect if pt on oral HRT
  - Does not elucidate the ***diurnal cortisol*** pattern
    - Clinically: Elevated cortisol/cortisone may relate to night-time cortisol spikes

Progesterone Metabolite - Pregnanediol (median)

Indirect estimate of progesterone levels in urine



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Urine Testing & Hormone Supplementation

- Reveals approximately total hormone **consumed & eliminated,** but NOT how much of the **bioactive and available** hormone is present in the systemic circulation, or has entered target tissues
- **Conclusion**: Urine testing an excellent way to evaluate endogenous production of the sex hormones, but:

Not clinically useful as a diagnostic fluid for exogenous oral or topical hormone delivery

Dried Urine Spot Tests

- Like all spot tests: Fundamental flaw & compromise - a time snapshot, rather than full representation of the 24-hr production cycle

- Because results cannot be expressed in classic µg/24 hrs, which eliminates urine volume as a variable, results are normalized to Creatinine (Cr), which creates problems for pulsatile analytes
  - Cr levels vary with subject age, diet, exercise, kidney function, as well as genetically
  - Result: Pt. ends up as own control.

    Cannot be adequately classified with typical reference range

Dried Urine Spot Tests

- Urine composition differs, based on what **precedes** the “point in time” collection
  - A first AM urine will differ, depending on whether/when person urinated during night

- Assumes Creatinine production parallels hormone output
  Never addressed in literature

- Almost no labs using this paradigm have published their methodology, nor have results been validated independently
  - Results from split samples (single sample divided & sent to various labs): may differ by > 3 orders of magnitude in case of 2 labs utilizing DU spot tests, or DU vs 24 hr
  - Derivitization of ketones issue

Dried Urine Spot Tests

In absence of published methods/data & given the small, limited urine volume in a dried urine sample for this type of analysis:

Unclear how quantitation of certain analytes can be credibly accomplished, and reproduced with confidence

The Dried Urine paradigm is arguably outside acceptable scientific standards

Requirements for Accurate Urine Steroid Profiling

"A perfect quantitative profiling technique would have the following attributes:

- 1. Accurate collection of 24-hr samples
- 2. Quantitative extraction of all steroid conjugates from urine
- 3. Complete hydrolysis of all steroid conjugates
- 4. Quantitative recovery of free steroids
- 5. Quantitative conversion of all steroids into volatile derivatives
- 6. Reproducibility of inter and intra-assays of individual steroids
- 7. Absence of impurities….."

after Shackelton, 1985

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Analysis of urinary estrogens, their oxidized metabolites, and other endogenous steroids by benchtop orbitrap LCMS versus traditional quadrupole GCMS

Adrian A. Franke · Laurie J. Custer · Yukiko Morimoto · Frank J. Nordt · Gertraud Maskarinec

Received: 15 April 2011 / Revised: 27 May 2011 / Accepted: 2 June 2011

- The literature has peer reviewed articles that compare GCMS to LCMS for 24 hr Urine samples. The 'Gold Standard'
- ***There are no studies that independently compare and contrast 24 hr to dried urine spot sampling***

benchtop orbitrap LCMS and single quadrupole GCMS. Sixteen steroidal estrogens including oxidized metabolites could be analyzed by LCMS. LCMS–GCMS Spearman rank

Introduction

Most Complete Assessment of Hormone Function

Ideal combination of tests:

- Urine: 24-hour hormone profile
- Saliva: Adrenal Function, such as 4-point cortisol and DHEA
- Serum: Thyroid panel. FSH & LH not absolutely necessary.
  - Additional appropriate work-up: CBC, serum Fe, TIBC, ferritin, comprehensive metabolic panel, Hgb A1C, fasting insulin, etc

- Each has a set of clinical strengths and limitations.
- Combination of testing methods may occasionally be appropriate.

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TIBC = total iron-binding capacity)

Hormones in Blood, Saliva & Urine

Blood	Saliva	Urine
Total steroid level	Free steroid fraction	Measures what body discards
Modified by binding proteins	Independent of binding proteins	
97 - 98% of the steroids biologically inactive	**Fraction of biologically active hormones only (2 – 3%)**	Shows level of total hormone & metabolites

Appropriate Body Fluids for Testing Exogenously Delivered Hormones

Type of Fluid	Non Endogenous	Oral Steroids	Topical Steroids	Vaginal Steroids	Troche Steroids	Pellet/IM Steroids
Serum	Yes	Yes[1]	No[2]	No[2]	Yes	Yes
Saliva	Yes	Yes	Yes[3]	Yes	No[4]	Yes
Urine	Yes	Yes[1]	No[2]	No[5]	Yes	Yes
DBS	Yes	Yes[1]	Yes[6]	Yes	Yes	Yes

1. Overestimation: Possible metabolite interference with immunoassays (GC/MS - OK)
2. Underestimation: Not reflective of tissue levels
3. Overestimation: Unless reference ranges reset higher for supplementation
4. Overestimation: Direct contamination of oral mucosa/saliva
5. Overestimation: Direct contamination of urine
6. Overestimation: If hands/fingertips used to apply hormones within 24 hrs of testing

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Zava, 2015

Appropriate Body Fluids for Testing Exogenously Delivered Hormones

Type of Fluid	Non Endogenous	Oral Steroids	Topical Steroids	Vaginal Steroids	Troche Steroids	Pellet/IM Steroids
Serum	Yes	Yes[1]	No[2]	No[2]	Yes	Yes
Saliva	Yes	Yes	Yes[3]	Yes	No[4]	Yes
Urine	Yes	Yes[1]	No[2]	No[5]	Yes	Yes
DBS	Yes	Yes[1]	Yes[6]	Yes	Yes	Yes

Saliva & DBS, but not serum/plasma or urine, are the only way to accurately monitor exogenous topical steroid hormone therapies

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Zava, 2015

Comparison of Fluid Mediums For Hormone Testing

For Endogenous Hormones

Conclusions:

All 3 body fluids are good mediums when hormones produced

Endogenously

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Conclusions: Fluid Mediums For Hormone Testing

For Exogenous Hormones

- **Serum**: For accurate oral administration of sex hormones, needs extraction & separation of metabolites
- **Urine**: Not recommended unless range re-established for dosing (eg,100–300 mg oral Pg) & range readjusted up to reflect the expected level
- **Serum/plasma & Urine**: NOT recommended for testing steroid hormones delivered by topical route of administration.

Best with Saliva

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References

- Du JY, Sanchez P, Kim L, Azen CG, Zava DT, Stanczyk FZ. Percutaneous progesterone delivery via cream or gel application in postmenopausal women: a randomized cross-over study of progesterone levels in serum, whole blood, saliva, and capillary blood. *Menopause*. 2013;20:1169–1175.
- Stanczyk FZ, Cho MM, Endres DB, Morrison JL, Patel S, Paulson RJ. Limitations of direct estradiol and testosterone immunoassay kits. *Steroids*. 2003;68:1173–1178.
- Yang DT, Owen WE, Ramsay CS, Xie H, Roberts WL. Performance characteristics of eight estradiol immunoassays. *Am J Clin Pathol*. 2004;122:332–337.
- Wang C, Catlin DH, Demers LM, Starcevic B, Swerdloff RS. Measurement of total serum testosterone in adult men: comparison of current laboratory methods versus liquid chromatography-tandem mass spectrometry. *J Clin Endocrinol Metab*. 2004;89:534–543.
- Edelman A, Stouffer R, Zava DT, Jensen JT. A comparison of blood spot vs. plasma analysis of gonadotropin and ovarian steroid hormone levels in reproductive-age women. *Fertil Steril*. 2007;88:1404–1407.
- Glaser R L, Zava DT, Wurtzbacher D. Pilot study: absorption and efficacy of multiple hormones delivered in a single cream applied to the mucous membranes of the labia and vagina. *Gynecol Obstet Invest*. 2008;66:111–118.
- Shirtcliff EA, Reavis R, Overman WH, Granger DA. Measurement of gonadal hormones in dried blood spots versus serum: verification of menstrual cycle phase. *Horm Behav*. 2001;39:258–266;

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Thank You

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