QUANTITATIVE ANALYSIS in ENDOCRINOLOGY

aim: to generate ***reliable*** *quantitative information*

ANALYTICAL PERFORMANCE
- Sensitivity
- Specificity
- Precision
- Accuracy
- …

CLINICAL EFFICACY
- DIAGNOSTIC POWER
  - Sensitivity
  - Specificity
- EFFECTIVE THERAPY
- GOOD PATIENT CARE
- …

QUANTITATIVE ANALYSIS in ENDOCRINOLOGY

PRE-ANALYTICS

WHAT? Hormones or metabolites; lipids, aminoacids, peptides, proteins

WHERE? plasma, serum, saliva, urine, faeces, tissues, hair, nails, CSF

WHEN? awake, early morning, night; menstrual phase, season

ANALYTICS

HOW?
- ✓ Ligand Binding and Immunoassay
- ✓ High Performance/Pressure Liquid Chromatography (HPLC)
- ✓ Gas Chromatografy – Mass Spectrometry (GC-MS)
- ✓ Liquid Chromatography – Tandem Mass Spectrometry (LC-MS/MS)

QUANTITATIVE ANALYSIS

ANALYTICAL METHOD (or assay) ≠ **ANALYTICAL TECHNIQUE**

- ✓ Technique
- ✓ Specific instrument/assay type
- ✓ Analytical conditions
- ✓ Calibration
- ✓ Biological fluid/sample
- ✓ Sample processing and reagents

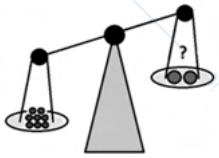
QUANTITATIVE ANALYSIS

MEASUREMENT PROCEDURE ≠ **ANALYTICAL METHOD**

- ✓ Method
- ✓ Sample storage and stabilizers
- ✓ Sample withdrawal condition
- ✓ Patient's condition at sampling
- ✓ Functional tests

QUANTITATIVE ANALYTICAL METHOD

Collect *Prepare* *Identify* *Isolate* *Detect*



Quantify *Report*

HOW to DEFINE a RELIABLE QUANTITATIVE ASSAY

ANALYTICAL PARAMETERS

PRECISION	**ACCURACY**
SENSITIVITY	**SPECIFICITY**
STABILITY	**TRACEABILITY**
RANGE	**LINEARITY**

Comparison with established methods

plus others specific for particular techniques

ASSAY VALIDATION

Definition and Guidelines

EUROPEAN MEDICINES AGENCY
SCIENCE · MEDICINES · HEALTH

CLINICAL AND LABORATORY STANDARDS INSTITUTE

FDA

Validation of methods provides documented evidences, generated by established experimental procedures, that a method is suited for its intended use and that it fulfills the necessary quality requirements.

For quantitative tests, this implies verifying method performance specifications that are clinically relevant.

Method validation is performed before it is applied to the intended use.

Method performance has to be monitored over time in the laboratory using it.

Validation has to be repeated whenever a relevant modification is introduced.

ASSAY TYPE

«In house» or laboratory-developed methods *or* *Commercial kits*

The overwhelming majority of methods employed in the clinical laboratory are commercial kits that have been validated by the manufacturer and are applied without modifications.

The user lab should 1) verify method performance, 2) carry out comparison experiments with the previously used assay, and 3) generate the reference interval for the new method (or confirm the previous).

PRECISION and ACCURACY

poor precision poor accuracy

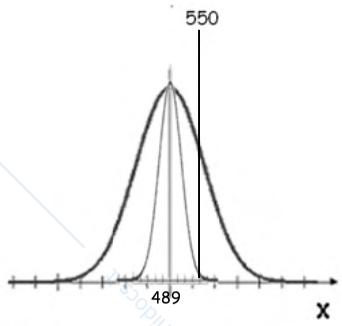
good precision poor accuracy

poor precision good accuracy

good precision good accuracy

Chose the most suitable method…

Value	Method 1	Method 2
Expected	Measures	Measures
550	498	398
	472	473
	504	521
	468	458
	501	567
	503	523
	500	603
	469	371
mean	489	489
st.dev.	16,46	79,89
c.v.%	3,4	16,3
Accuracy%	89,0	89,0



PRECISION

Agreement among repeated measurements.

Repeatability: within lab performed in the same conditions
Intra-assay, or intra-day
Inter-assay, or inter-day

Reproducibility: among different laboratories
Same conditions or not

ACCURACY

Agreement between the value obtained by the method under investigation and the target value, which can be nominal or established by a reference measurement procedure.

SPECIFICITY

What is being measured is the molecule of interest and not others.

Ability to assess unequivocally the analyte in the presence of various components in a complex matrix.

SELECTIVITY
To differentiate and quantify the analyte in the presence of other components in the sample.

IDENTIFICATION
To discriminate among compounds sharing similar structures.

SENSITIVITY

Lowest measurable concentration

Definitions	Signal / Noise ratio
LOD: limit of detection. The lowest amount in a sample which can be detected, but not quantified.	3
LOQ: limit of quantification. The lowest concentration which can be determined with adequate accuracy and precision.	10

DETECTION method

RECOVERY

Percentage of the analyte originally present in the specimen that reaches the end of the procedure, and is available for detection.

STABILITY

Change in the analyte concentration occurring between sample collection and quantitation.

Usually (but not limited to) analyte degradation.

CHEMICAL/PHYSICAL

E.g.: hydrolysis, oxidation, isomerization, induced by temperature, pH, light

BIOLOGICAL

Enzyme-mediated degradation, e.g.: GLP1 by DPPIV.
Compound release from blood cells in plasma.

PRE-ANALYTICAL

- ✓ Sample withdrawal (additives in vacuum disposables);
- ✓ Processing (e.g. centrifugation and separation);
- ✓ Transport (humudity, time, temp);
- ✓ Storage conditions (-80°C; -20°C; +4°C …);
- ✓ Freeze/thawing stability.

ANALYTICAL

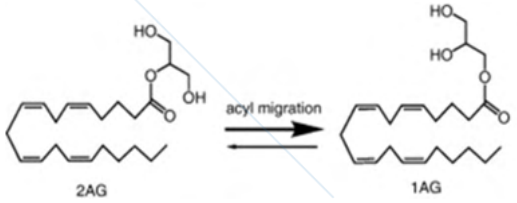
- ✓ Sample preparation (solubility, pH, buffers, solvents).
- ✓ Time between prep and analysis.
- ✓ Analytical conditions (temperature, denaturating agents,…).

STABILITY

Endocannabinoids are lipid mediators derived by phospholipids of the plasma membrane.

They are ubiquitously synthesized and rapidly released in the bloodstream on demand.

2-arachidonoyl glycerol 1-arachidonoyl glycerol



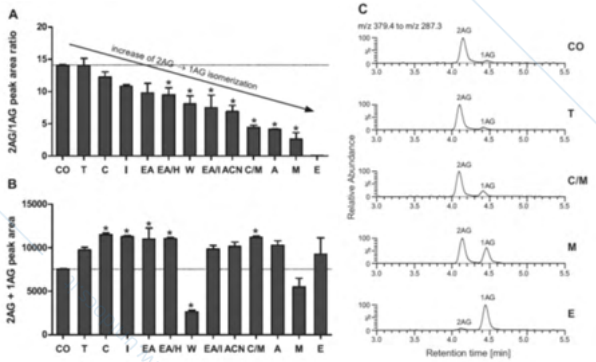
Spontaneous, non-enzymatic isomerization converting 2AG in 1AG in biological samples as well as in pure standard.

Poor chemical stability!

STABILITY

Chemical stability impacting the pre-analytical and analytical phase!

Blood sample needs to be immediately centrifuged, plasma separated and frozen. Estimation of 2AG→1AG conversion and of overall degradation during different procedures for EC extraction from plasma.

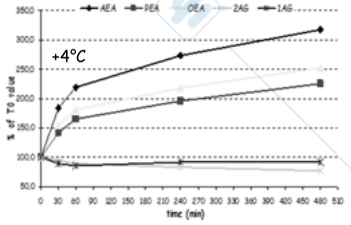


Zoerner et al., 2011

STABILITY in blood samples

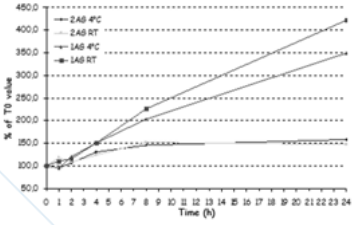
Biological stability impacting the pre-analytical phase!

WHOLE BLOOD



Release of new AEA, PEA and OEA from membranes of blood cells *ex vivo*.

PLASMA



Synthesis of new 2AG and 1AG from triglycerides breakdown *ex vivo*.

Fanelli et al., JLR 2012

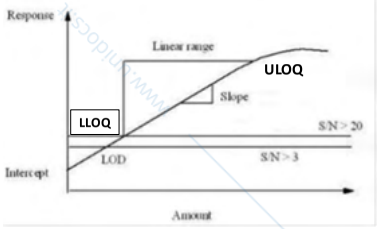
QUANTIFICATION RANGE

(DYNAMIC RANGE)

Range of concentration in which the method can quantify the analyte with adequate precision and accuracy. Comprised between:

LLOQ
Lower limit of quantification ⟸ **SENSITIVITY (S/N ratio)**

ULOQ
Upper limit of quantification ⟸ **LINEARITY**

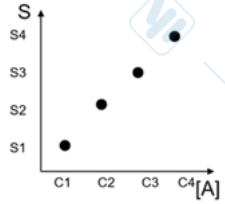


Linear relationship between assay's response and analyte concentration.
A defined increment in the concentration elicts a increment in signal with a proportion that is mantained at any concentration level within a defined range.

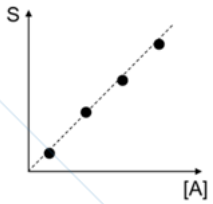
THE CALIBRATION CURVE

Absolute quantification requires the availability of the pure analyte standard.

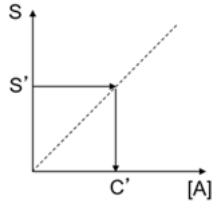
QUANTIFICATION BY EXTERNAL STANDARD:



Standard samples at known and increasing concentrations (CALIBRATORS) being analyzed.



Calculation of a calibration curve by regression analysis between signals and concentrations.



Back-calculation (INTERPOLATION) of signal in unknown samples on the curve equation to obtain the measured concentration.

THE CALIBRATION CURVE

QUANTIFICATION BY INTERNAL STANDARD (IS)

(chromatographic or mass spec methods)**:**

IS is an **analogue** or an **isotope** of the analyte: very similar (ideally identical) physical-chemical features.

The IS is added in known and constant amount to samples and calibrators.

IS and analyte within samples and calibrators have the same behavior during sample preparation and analysis. E.g.: recovery, stability, interference etc…

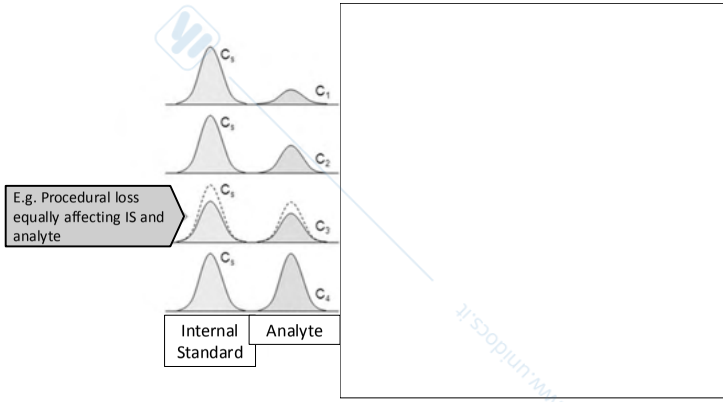
The ratio betweeen Analyte and IS is stable and unaffected throughout the prodecure.

The ratio of Analyte *vs* IS signal is therefore more precise and accurate than Analyte signal.

THE CALIBRATION CURVE

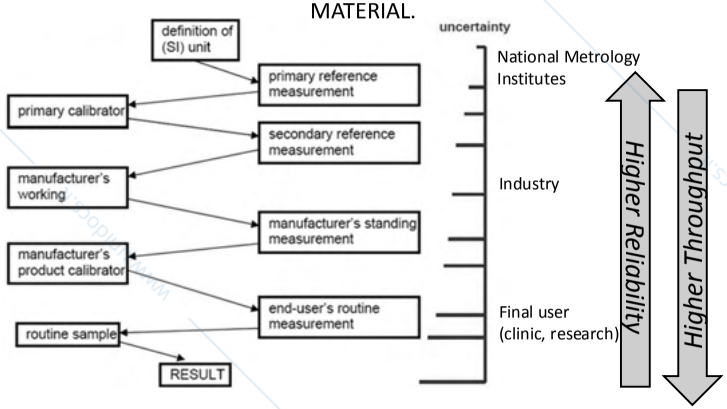
QUANTIFICATION BY INTERNAL STANDARD (IS)

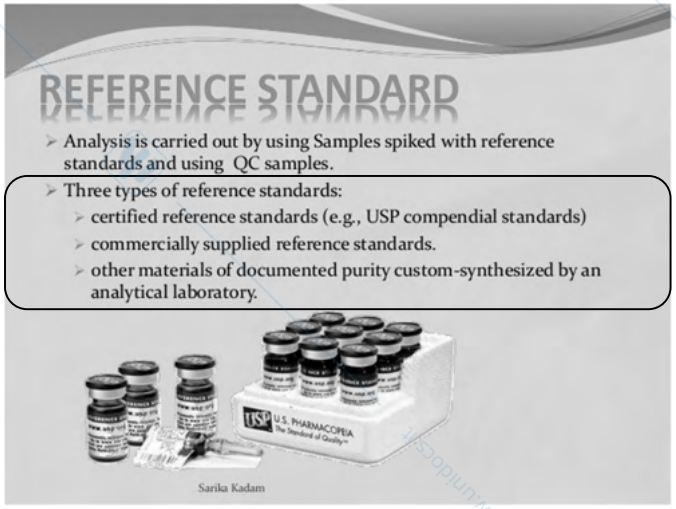
(chromatographic or mass spec methods)**:**



THE TRACEABILITY CHAIN

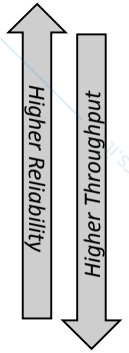
Results generated by any method need to be TRACEABLE to a high order / primary REFERENCE MEASUREMENT PROCEDURE of PRIMARY MATERIAL.





ANALYTICAL TECHNIQUES FOR QUANTITATIVE PURPOSES

- ✓ Gas Chromatography – Mass Spectrometry (GC-MS)
- ✓ High Performance/Pressure Liquid Chromatography (HPLC)
- ✓ Liquid Chromatography – Tandem Mass Spectrometry (LC-MS/MS)
- ✓ Ligand Binding and Immunoassay



IMMUNOASSAYS WERE FIRST DEVELOPED WITHIN THE ENDOCRINOLOGY FIELD!

1959: **radioimmunoassay (RIA)**
for INSULIN measurement

Hormone concentration detected in the nM / pM range

↓

Development of LABORATORIES for clinical chemistry purposes

Clinical Advancement! Professional Occupation!

IMMUNOASSAYS principle

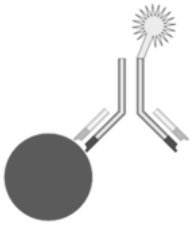
selective binding of the analyte

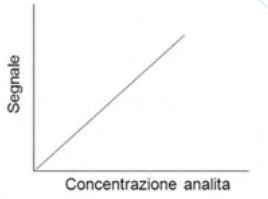
Antigen – Antibody

Estrinsic property of the molecule
"shape"

NON-COMPETITIVE IMMUNOASSAY

- Tracer: label or enzyme conjugated with the Ab
- Antibody: added in excess of concentration

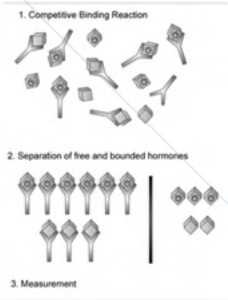




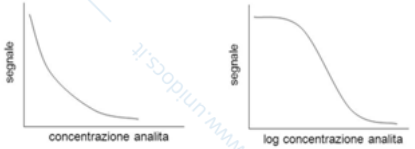
Signal increases at increasing hormone concentration

COMPETITIVE IMMUNOASSAY

Fig. 2. Basic principle of a competitive radioimmunoassay.
The assay system is set up by defined amounts of a specific antibody and of the radiolabelled steroid (tracer). The analyte present in a sample competes with the tracer for the binding sites of the antibodies. With increasing concentrations of the analyte, a higher proportion of the tracer is displaced from the antibodies. Measurement of the radioactivity either in the fraction of the free or in the fraction of the antibody bound steroids after separation allows to work out the concentration of the analyte in a sample by comparing its inhibitory effect on the binding of the tracer to specific antibody with the inhibitory effect of known standards.



- Tracer: labelled analogue of the analyte
- Antibody: added in limiting concentration



Signal decreases at increasing hormone concentration

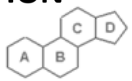
WHERE DO WE GET THE ANTIBODY? GENERATION of the POLYCLONAL ANTISERUM

IMMUNOGEN COMPOUND INOCULATION IMMUNIZATION

Able to elicit an immune reaction in the host animal.

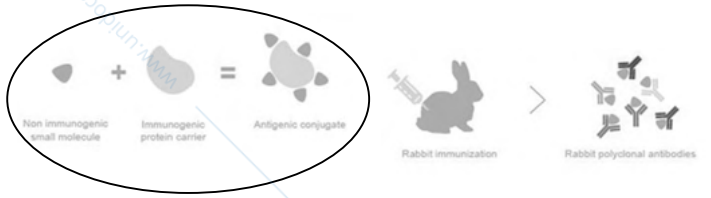
SPECIES-SPECIFIC COMPOUND

WHERE DO WE GET THE ANTIBODY? POLYCLONAL ANTISERUM GENERATION

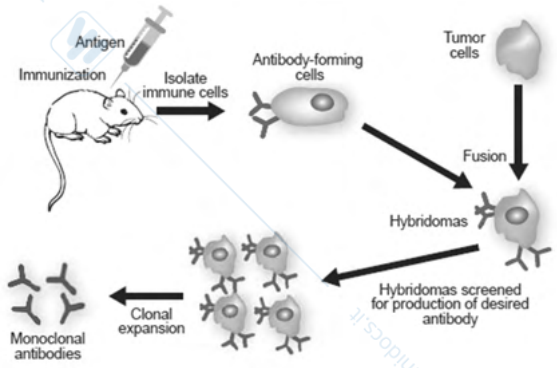


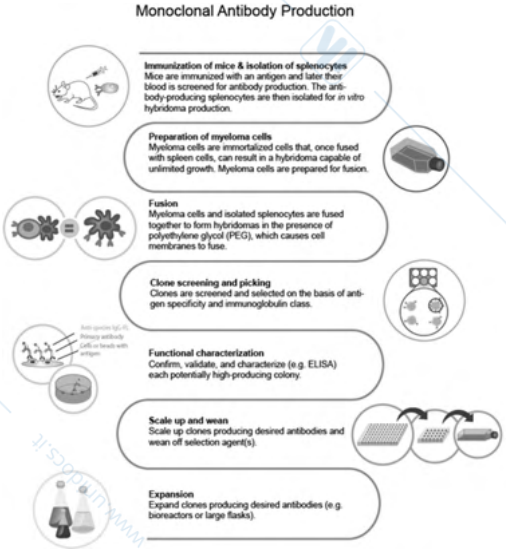
Steroids are small molecules identical across species

NON-IMMUNOGEN COMPOUND INOCULATION IMMUNIZATION

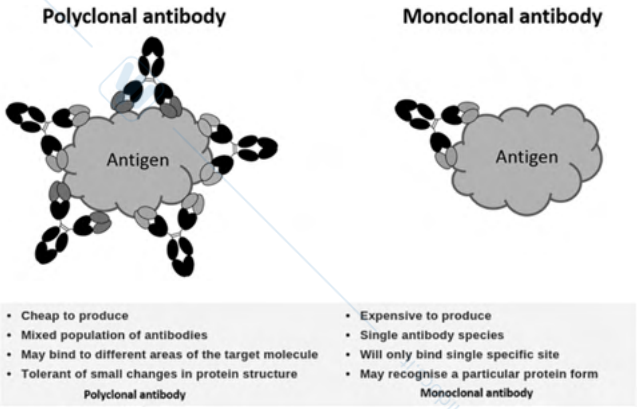


WHERE DO WE GET THE ANTIBODY? CLONE SELECTION

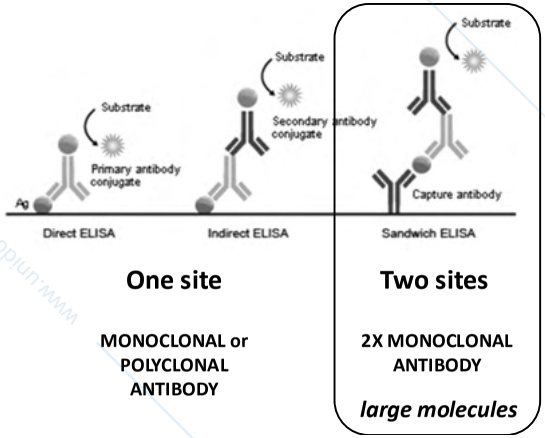




WHICH ONE IS MORE SPECIFIC?



IMMUNOASSAY DESIGN



One site

MONOCLONAL or POLYCLONAL ANTIBODY

Two sites

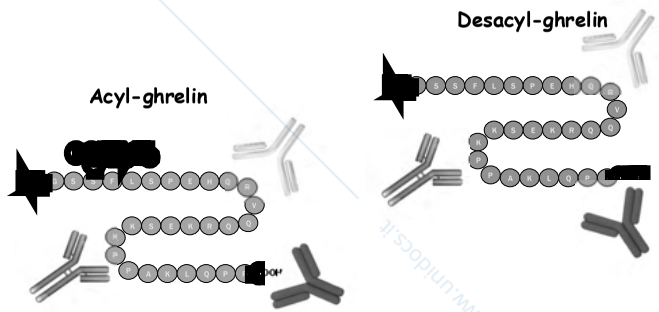
2X MONOCLONAL ANTIBODY

large molecules

IMMUNOASSAYS SPECIFICITY

If you wish to measure Total GHRELIN

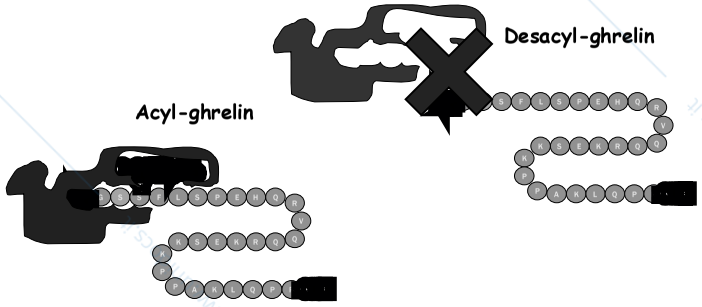
Polyclonal antibodies directed toward the C-term or the overall molecule.



IMMUNOASSAYS SPECIFICITY

If you wish to measure Acyl-GHRELIN

Monoclonal antibodies directed toward the N-terminal



IMMUNOASSAY SPECIFICITY
2X MONOCLONAL ANTIBODY

Glucagon-like peptide-1 (GLP-1)

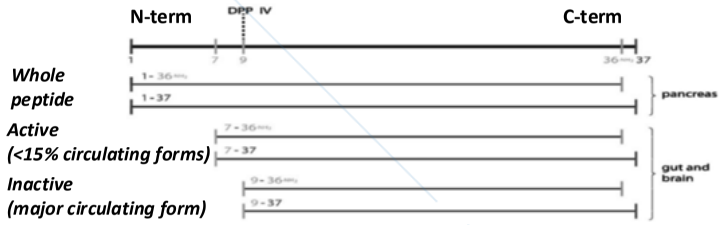


Figure 1. Diagrammatic illustration of the structures of the six molecular forms of glucagon-like peptide-1.

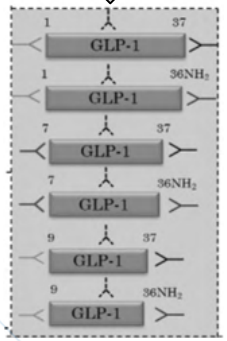
Bak et al., 2012

IMMUNOASSAY SPECIFICITY
2X MONOCLONAL ANTIBODY

1° Antibody: selects the isoform of interest
2° Antibody: selects intact peptide, avoids degradation products

GLP-1 Ab vs Side-viewing:
Prevents the detection of further proteolytic products

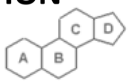
GLP-1 ACTIVE
Ab vs N-term:
7-36NH2



GLP-1 TOTAL
Ab vs C-term:

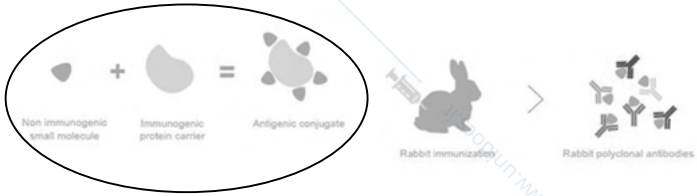
1-36NH2 +
7-36NH2 +
9-36NH2

WHERE DO WE GET THE ANTIBODY?
POLYCLONAL ANTISERUM GENERATION

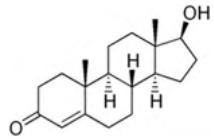


Steroids are small molecules identical across species

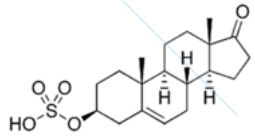
NON-IMMUNOGEN COMPOUND INOCULATION IMMUNIZATION



IMMUNOASSAY SPECIFICITY

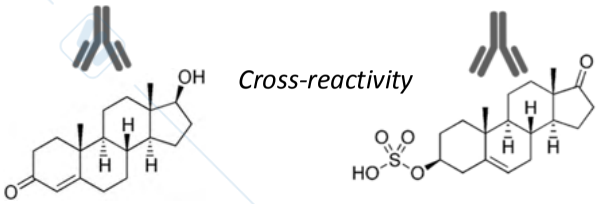


TESTOSTERONE:
pg/ml – ng/ml



DHEA-S:
µg/ml

IMMUNOASSAY SPECIFICITY

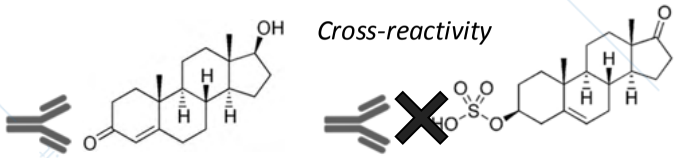


poorly specific antibody

TESTOSTERONE: **pg/ml – ng/ml**

DHEA-S: **µg/ml**

IMMUNOASSAY SPECIFICITY



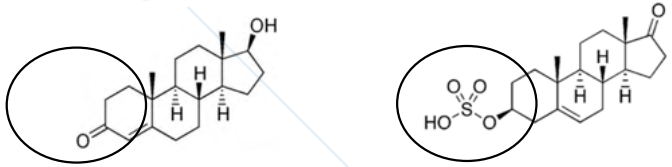
highly specific antibody

TESTOSTERONE: **pg/ml – ng/ml**

DHEA-S: **µg/ml**

IMMUNOASSAY SPECIFICITY

Reactive site used for conjugation with carriers. Usually no Ab are generated toward this epitope.



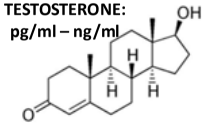
TESTOSTERONE: **pg/ml – ng/ml**

DHEA-S: **µg/ml**

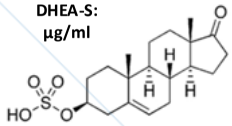
STEROID CROSS-DETECTION in IA

Structurally related compounds

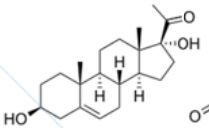
TESTOSTERONE: **pg/ml – ng/ml**



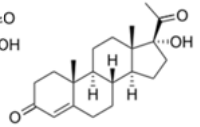
DHEA-S: **µg/ml**



17OH-PREGNENOLONE

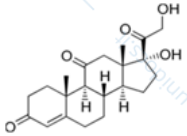


17OH-PROGESTERONE

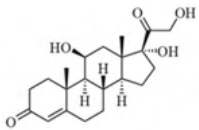


- ***Depends on the epitope.***
- ***Influenced by large differences in concentration levels.***

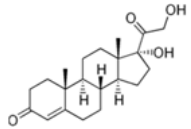
CORTISONE



CORTISOL

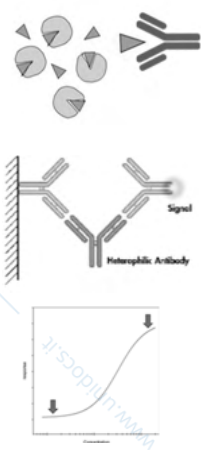


11DEOXYCORTISOL



PITFALLS in DIRECT IMMUNOASSAYS

- release from **binding protein**
- **matrix interference**
- **Anti-reagent Ab** (heterophilic Ab, rheumatoid factor)
- **Narrow dynamic range**



LIGAND-BINDING methods

Used in the early days for non-immunogen small molecules such as STEROIDS.

Binding proteins

Natural hormone carriers

Receptors

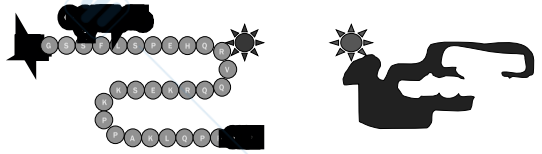
Corticosteroid binding globulin CBG.

Sex hormones binding globulin SHBG.

Steroid receptors.

IMMUNOASSAYS SENSITIVITY

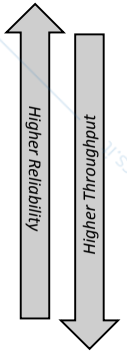
TRACERS



= Radio-isotope	^{3}H, ^{125}I
= Enzyme	peroxydase, phosphatase
= Fluorophore	fluorexine
= Cromophore	luminol

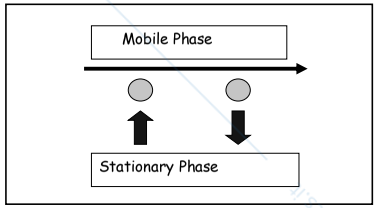
ANALYTICAL TECHNIQUES FOR QUANTITATIVE PURPOSES

- ✓ Gas Chromatography – Mass Spectrometry (GC-MS)
- ✓ High Performance/Pressure Liquid Chromatography (HPLC)
- ✓ Liquid Chromatography – Tandem Mass Spectrometry (LC-MS/MS)
- ✓ Ligand Binding and Immunoassay



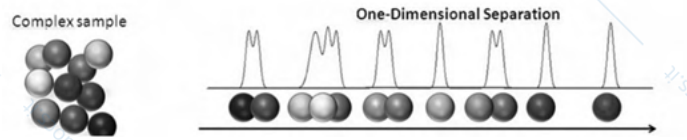
CHROMATOGRAPHY
principle

Compound's **relative affinity** for two non-miscible phases at equilibrium according to its **physical-chemical** features.



CHROMATOGRAPHY

Separation in time of compounds in a complex mixture according to physical-chemical features.



CHROMATOGRAPHY

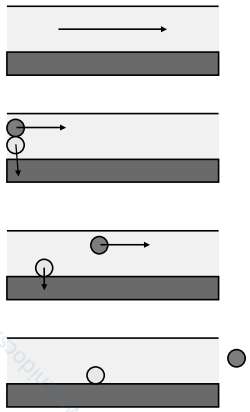
different affinity of the compounds to be analyzed for the stationary phase and for the mobile phase

The mobile phase flows in contact with the stationary phase.

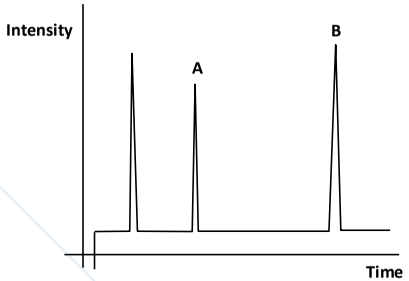
A competition is established between the stationary phase and the mobile phase for the retention of each analyte.

The migration of the analytes along the stationary phase occurs with different speeds.

Analytes are eluted at different times, and are therefore separated.



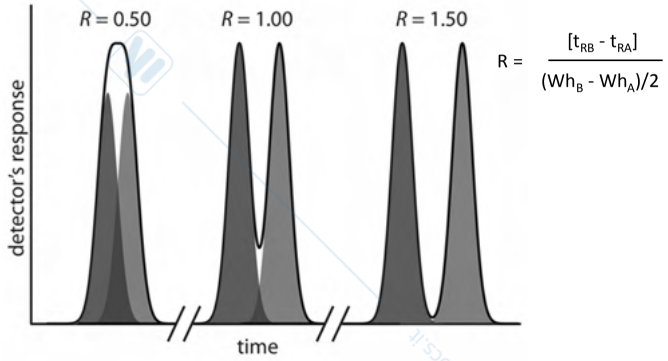
COLUMN CHROMATOGRAPHY



t_R retention time: time taken by the substance to travel through the stationary phase. This a specific feature of the molecule at defined analytical conditions. It is unvaried at constant analytical conditions.

- ✓ **CHROMATOGRAM**: diagram of signal in time.
- ✓ **CHROMATOGRAPHIC PEAK**: discrete time space in which the analyte is eluted from the system (gaussian shape).

COLUMN CHROMATOGRAPHY SPECIFICITY and RESOLUTION

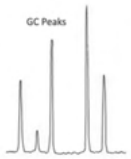


$$R = \frac{[t_{RB} - t_{RA}]}{(Wh_B - Wh_A)/2}$$

Poor resolution may lead to the indistinct measurement of two or more species within the same chromatograpic peak.

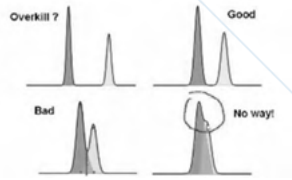
COLUMN CHROMATOGRAPHY SPECIFICITY and RESOLUTION

GAS (GC)



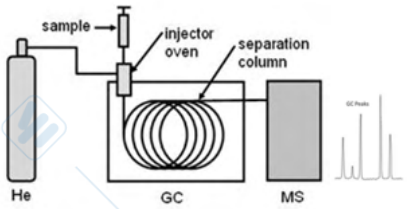
HIGH SEPARATION
HIGH SPECIFICITY
but
POOR VERSATILITY

LIQUID (LC)



GOOD SEPARATION
GOOD SPECIFICITY
and
HIGH VERSATILITY

GAS CHROMATOGRAPHY



GAS phase → **VOLATILE**

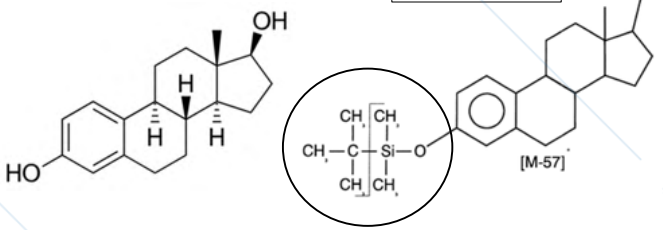
High temperature → **THERMOSTABLE**

Analytes features required for GC-MS analysis

COMPROMISE:
DERIVATIZATION

DERIVATIZATION

Covalent binding of chemical groups with high volatility and thermo-stability

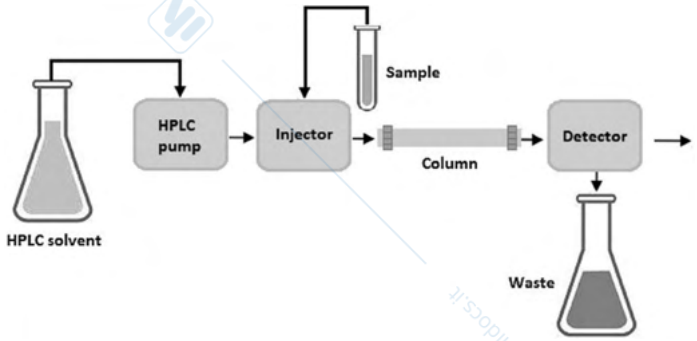


Example of ESTROGENS derivatization prior GC-MS analysis:

N-methyl-N-tert.-butyldimethylsilyl-trifluoroacetamide (MTBSTFA) + containing 1% tert.-butyldimethylchlorosilane (TBDMCS) to form tert.-butyldimethylsilyl derivatives.

Long reaction time.
Extensive sample purification.

HIGH PRESSURE / PERFORMANCE LIQUID CHROMATOGRAPHY (HPLC)



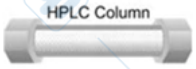
LC CLASSES

Based on the type of physical-chemical interaction between the analyte and the stationary phase, the following classes are defined:

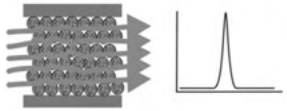
	STATIONARY PHASE	SEPARATION
ADSORPTION CHROMATOGRAPHY	Solid.	Based on the different tendency of the solutes to adsorb on the active surface of the adsorbent according to the polarity of the mobile phase.
PARTITION CHROMATOGRAPHY	Solid or liquid immiscible with the mobile phase, supported within an inert holder.	Occurs on the basis of the different solubility of the usual in the two phases.
ION EXCHANGE CHROMATOGRAPHY	Ion exchange resin.	Based on electrostatic interactions that take place between solute and stationary phase as a function of the ionic strength or the pH of the aqueous eluent.
EXCLUSION CHROMATOGRAPHY	Dimensional filter.	Based on the size of the solute and its tendency to be retained within the particles.
AFFINITY CHROMATOGRAPHY	Protein or functional groups immobilized on a solid support.	Based on a highly specific interaction between antigen and antibody, enzyme and substrate, receptor and ligand, or protein and nucleic acid.

HPLC STATIONARY PHASE: COLUMN

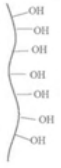
COLUMN GEOMETRY
- ✓ Lenght: 5 – 20 cm
- ✓ Diameter: 1 – 4.6 mm
- ✓ Particle size: 1.3 – 5 µm
- ✓ Porosity



CHEMISTRY of the stationary phase
- ✓ SILICA (activated)
- ✓ POLIMERS (solid or gel)

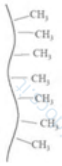


Adsorption/partition chromatography



Direct Phase
Stationary Phase:
Polar

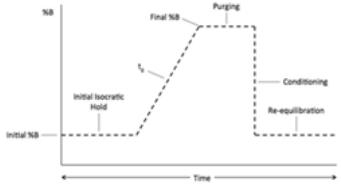
Mobile Phase:
non-polar → polar



Reversed Phase
Stationary Phase:
Non-polar (C8, C18, C6)

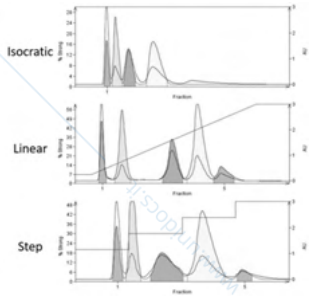
Mobile Phase:
polar → non-polar

CHROMATOGRAPHIC GRADIENT



Steady changes of the mobile phase composition during the chromatographic run.

MP A: water-based, polar
MP B: solvent-based, non polar



Gradients begin with “weak” elution conditions and end with “strong” elution conditions.

Weaker elution conditions provide adequate compound retention so that all compounds will not immediately elute from a chromatography column. Stronger conditions elute compounds retained better on the column.

A properly designed gradient will separate the target molecule from its impurities.

HIGH PRESSURE / PERFORMANCE LIQUID CHROMATOGRAPHY (HPLC)

- VERSATILE: liquid environment is comfortable to most of biologic molecules
- RAPID: 5 – 30 min
- REPRODUCIBLE
- AUTOMATABLE
- SENSITIVITY: …depends on the detector…

CHROMATOGRAPHY DETECTOR

On line "sensor" for compound eluting from the system.
It determines the sensitivity.

Most often used in clinical biochemistry:

GC → MASS SPECTROMETER

HPLC →

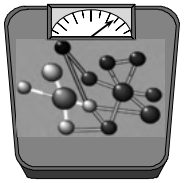
Detector Type	Sensitivity	Selectivity	Qualitative Information	Compounds
Variable-Wavelength	high, low ng range	low, one signal	low, only by RTs	vitamins, lipids, antibiotics
Diode-Array	high, low ng range	high, several signals at different wavelengths	high, RTs and spectra	colors, aspartame, lipids, flavors, antioxidants, preservatives
Fluorescence	very high, low pg range	very high, light absorbance followed by emission of light must be possible	low, only by RTs	mycotoxins, vitamins, amino acids, carbamates, glyphosate
Electrochemical	very high, low pg range	very high, oxidation or reduction must be possible	low, only by RTs	vitamins, ions, carbohydrates
Refractive Index	low, high ng range	low, all compounds influence refractive properties	low, only by RTs	carbohydrates
Conductivity	very high, low pg range	high, ionic form of compound necessary	low, only by RTs	ions, organic acids, inorganic acids
Mass Spectrometry	high, low ng range	very high, selective search for specified masses	very high molecular weight and fragmentation information	all

ANALYTICAL TECHNIQUES FOR QUANTITATIVE PURPOSES

- ✓ Gas Chromatography – Mass Spectrometry (GC-MS)
- ✓ High Performance/Pressure Liquid Chromatography (HPLC)
- ✓ Liquid Chromatography – Tandem Mass Spectrometry (LC-MS/MS)
- ✓ Ligand Binding and Immunoassay

Higher Reliability ↑ / *Higher Throughput* ↓

MASS SPECTROMETRY



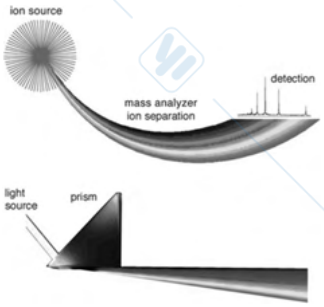
Measures the weight of ions
m/z: mass-to-charge ratio

"Mass spectrometry is the art of measuring atoms and molecules to determine their molecular weight. Such mass or weight information is sometimes sufficient, frequently necessary, and always useful in determining the identity of a species.

To practice this art one puts charge on the molecules of interest, i.e., the analyte, then measures how the trajectories of the resulting **ions respond in vacuum to various combinations of electric and magnetic fields**."

John B. Fenn, Nobel Prize 2002

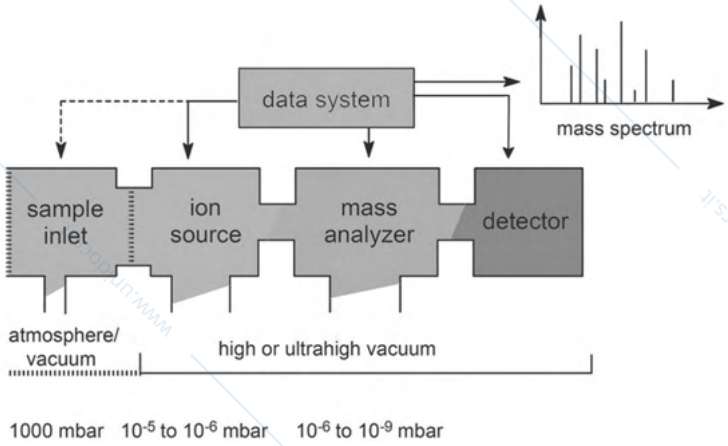
MASS SPECTROMETRY



A technique for measuring and analyzing molecules, that involves introducing enough energy into a (neutral) target molecule to cause its ionization and disintegration.

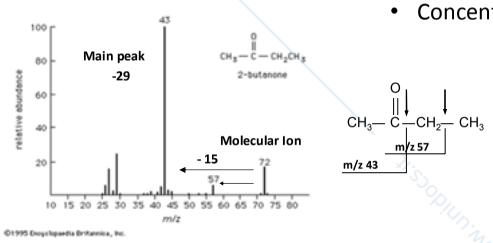
The resulting primary ions and their fragments are then analyzed, based on their mass/charge ratios, to produce a "molecular fingerprint."

MASS SPECTROMETRY
COMPONENTS



MASS SPECTRUM

- ✓ Diagram of ion abundance as related to their mass, expressed as their m/z.
- ✓ It is a specific feature of the molecule in defined and constant analytical conditions.
- ✓ Provides information about:
  - Mass
  - Isotopic ratio
  - Fragmentation pattern
  - Concentration



Structural Information by MS

Useful for characterizing the molecular structure and for identifying the compound.

Molecular Weight determination
- ➢ nominal
- ➢ accurate (elemental composition)
  - ➢Isotope pattern
  - ➢High resolution

Fragmentation
- ➢Fragmentation rules
- ➢Libraries („fitting“)
- ➢MS/MS (or MS^n)

ATOMS, ELEMENTS and ISOTOPES

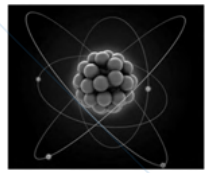
Molecules are groups of atoms.
Atoms weight is determined by **protons** and **neutrons** (electron mass is negligible).

Isotopes differ for the number of neutrons. Isotopes of the same elements are present in nature with defined ratios.

Isotope ratios provide useful information (e.g. for identification, for distinguishing natural from synthesis products).

Element	Isotope	Rel. Abund.	Isotope	Rel. Abund.	Isotope	Rel. Abund.
Carbon	^{12}C	100	^{13}C	1.11		
Hydrogen	^{1}H	100	^{2}H	.016		
Nitrogen	^{14}N	100	^{15}N	.38		
Oxygen	^{16}O	100	^{17}O	.04	^{18}O	.20
Sulfur	^{32}S	100	^{33}S	.78	^{34}S	4.40
Chlorine	^{35}Cl	100			^{37}Cl	32.5
Bromine	^{79}Br	100			^{81}Br	98.0

MASS DEFINITIONS



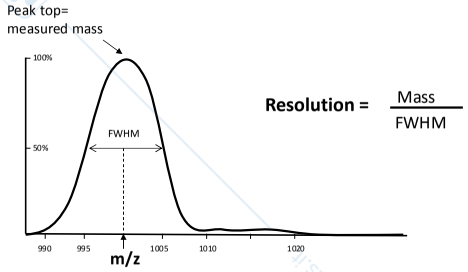
UNIT

a.m.u.: atomic mass unit (also Dalton or Thomson). Defined as 1/12 of C mass, arbitrarily set at 12.

- **Nominal Mass:**
  The integral sum of protons and neutrons in an atom (also called the atomic mass number), e.g. C=12, H=1, O=16.
- **Average Mass**
  The sum of the average of the isotopic masses of the atoms in a molecule, e.g. C=12.01115, H=1.00797, O=15.9994.
- **Monoisotopic Mass**
  The sum of the exact or accurate masses of the lightest stable isotope of the atoms in a molecule, e.g. C=12.000000, H=1.007825, O=15.994915.

MASS SPECTROMETRY ACCURACY and RESOLUTION

Mass Accuracy (ppm) $= \dfrac{\text{Measured mass} - \text{Theoretical mass}}{\text{Theoretical mass}} \times 10^6$

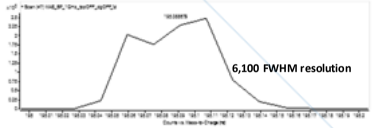


FWHM:
Full width at half maximum

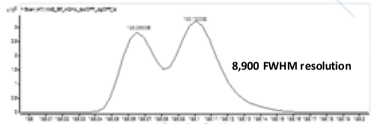
Resolution $= \dfrac{\text{Mass}}{\text{FWHM}}$

MASS SPECTROMETRY RESOLUTION

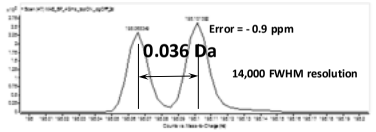
Different types of MS analyzers have different resolution.



Methyl 5-acetylsalicylate
$[M+H]^+$ 195.065185 *m/z*



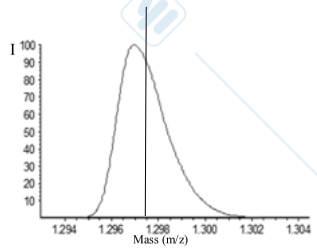
Butyl paraben
$[M+H]^+$ 195.101571 *m/z*



MASS and SPECTRUM

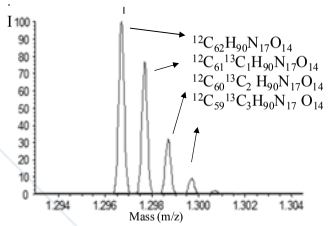
Mass spectra of Angiotensin I

Average mass 1297.50248



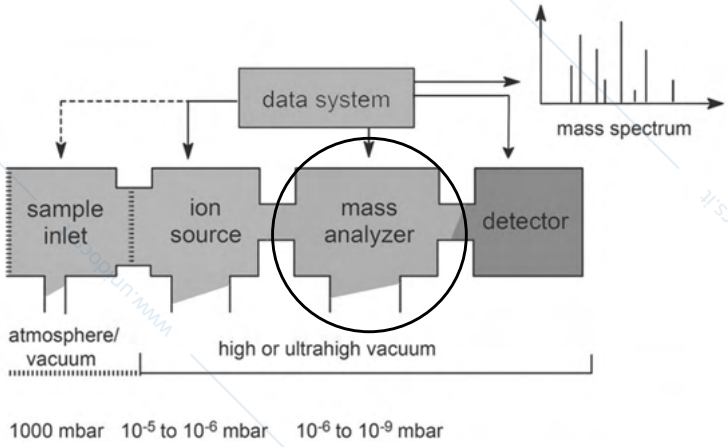
R = 1000

Monoisotopic mass 1296.68518



R = 5000

MASS SPECTROMETER COMPONENTS

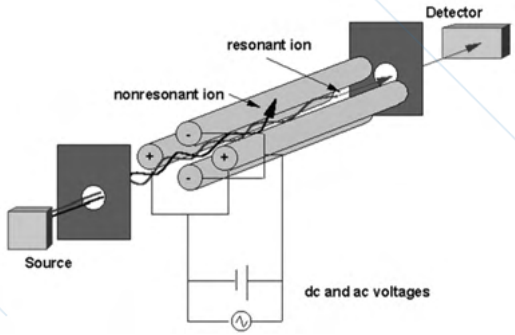


DIFFERENT TYPES OF MASS ANALYZERS

Compromise between
RESOLUTION and **LINEARITY**

- Quadrupole and Triple Quadrupole (Tandem MS/MS):
  - Quantitative analyses
    High sensitivity, linearity, reproducibility. Low resolution.
- Ion Trap :
  - Qualitative analyses
    High resolution, multiple fragmentation cycles, mass accuracy. Low linearity.
- Time of Flight (TOF):
  - Qualitative analyses
    High resolution, exact mass. Low linearty.

QUADRUPOLE - MS



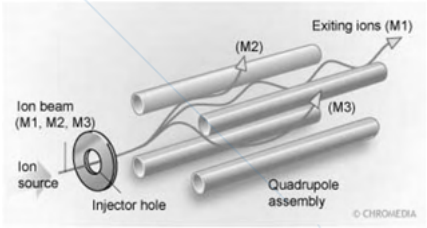
Combined effect of **electric field** and **radio frequency** to stabilize the path of certain ions, but not others.

QUADRUPOLE - MS

ION FILTER

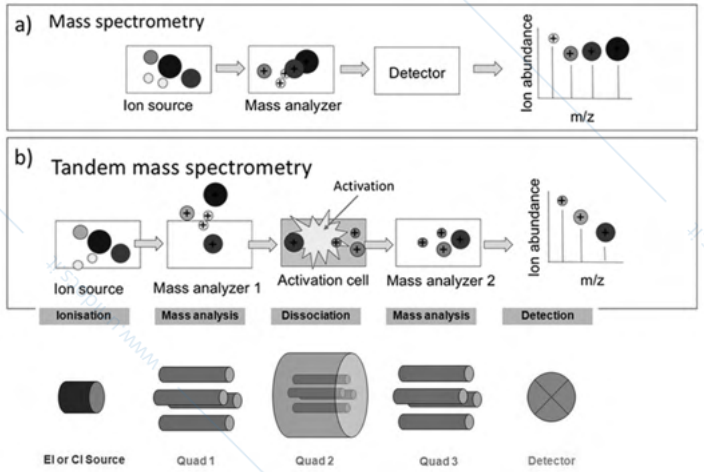
Trajectories of ions with unwanted m/z (e.g. M2 and M3) are unstable in the set DC + RF combination.

Only ions displaying the desired m/z will assume a stable trajectory and pass through the quadrupole (e.g. M1).

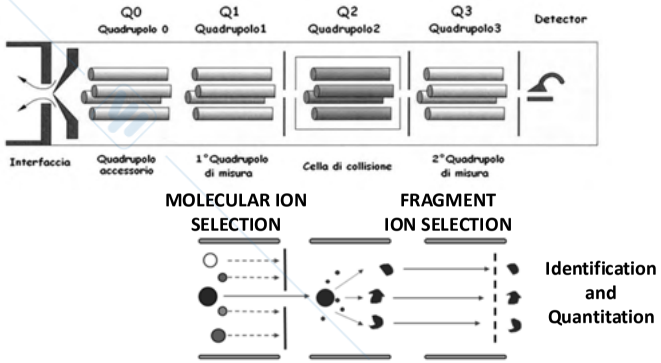


A huge number of compounds in a biological sample will share the same m/z…how can we improve the specificity?

TANDEM MS in TRIPLE QUADRUPOLE



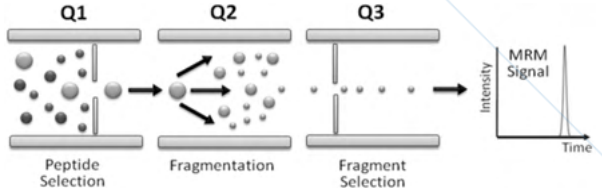
TRIPLE QUADRUPOLE – TANDEM MS/MS



Specificity is enhanced by the selection of fragment ions (Q3 m/z) specifically generated by the molecular ion (Q1 m/z) of the compound of interest.

TRIPLE QUADRUPOLE – TANDEM MS

MS/MS TRANSITION

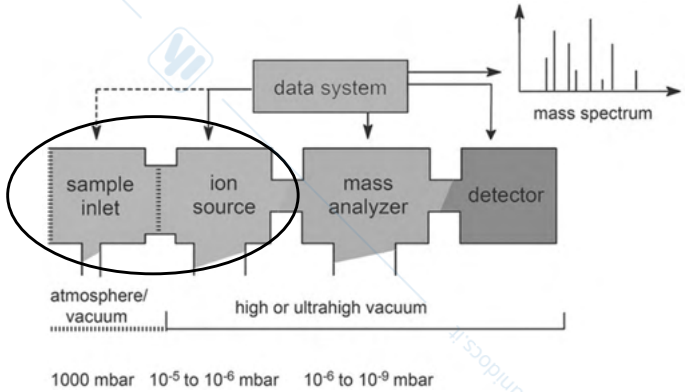


Filter on a fix m/z

Filter on a fix m/z

DC/RF combinations can be switched in msec.
Multlipe Q1/Q3 pairs (**transitions**) can be monitored in the same experiment. This is called **MULTIPLE REACTION MONITORING**.

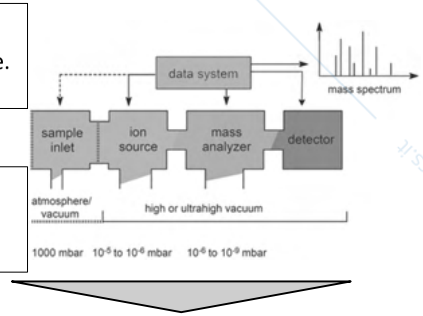
MASS SPECTROMETER COMPONENTS



MASS SPECTROMETER COUPLING CHROMATOGRAPHY WITH MS

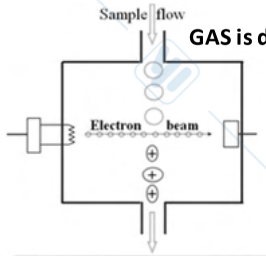
GC
Gas flow at high temperature.
Easily removed.

LC
Liquid flow at high pressure.
Hard to be removed.



VACUUM

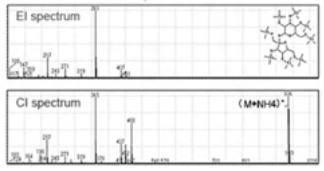
ION SOURCE
GC – MS INTERFACE



GAS is discarded

Hard ionization: the molecule is fragmented during the ionization.

Unsuitable for TANDEM MS experiments.



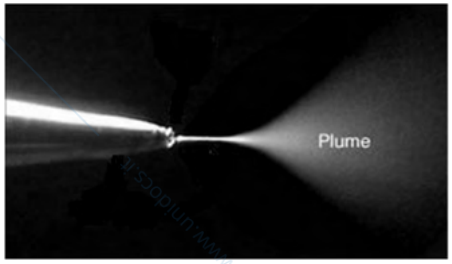
Electronic Impact

Chemical Ionization

ION SOURCE
LC – MS INTERFACE

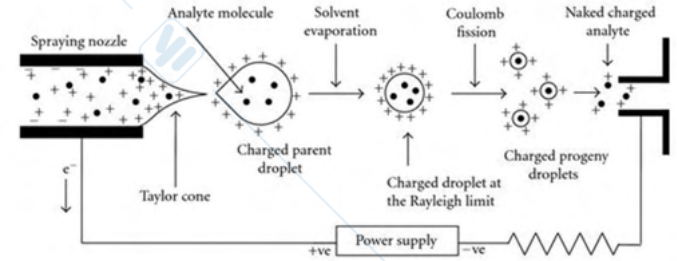
Atmospheric pressure ionization source for LC-MS coupling

"Electrospray ionization for mass spectrometry of large biomolecules" (Science, 1989)



John B. Fenn, Nobel Prize 2002

ION SOURCE
LC – MS INTERFACE

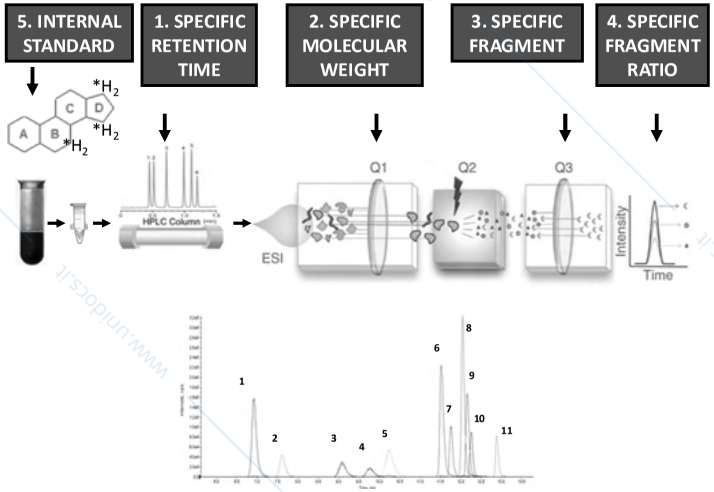


Liquid mobile phase is nebulized, droplets are desolvated until the ion is expelled.

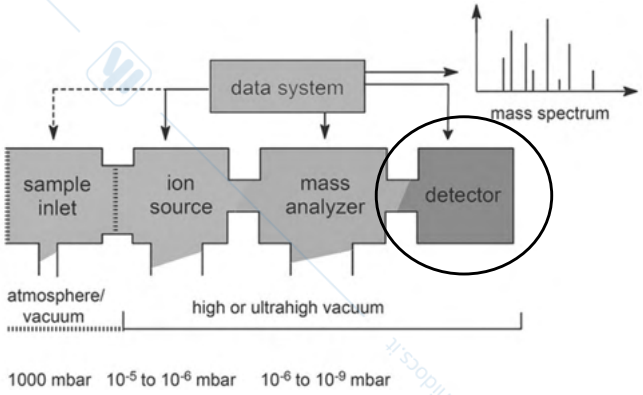
Soft ionization: single charged molecular ion $[M+H]^+$
Ideal for Tandem MS experiments.

E.g.: m.w.= 288 → m/z= 289

LC-MS/MS SPECIFICITY

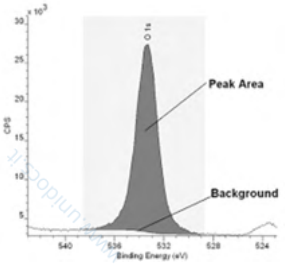


MASS SPECTROMETER COMPONENTS



DETECTOR and QUANTITATION

Electromultiplier or photomultiplier
Ion current traveling across the 3Q undergoes a direct impact on the surface of a electromultiplier plate. Ion current is converted in an electric current which is amplified by a photomultiplier tube. The electric current is registered by a computer and converted into signals.



The peak area is calculated and used for deriving molecule abundance.

The Area ratio (Analyte/IS) is used for quantitation.

LC-MS/MS on 3Q platforms

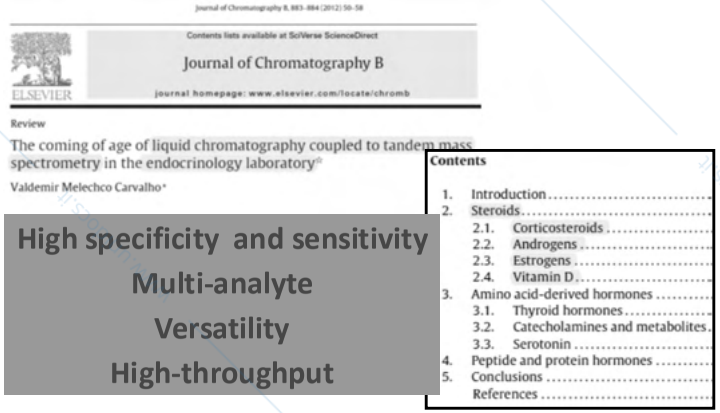
Ideal for small molecules
Quantitation of large multi-analyte panel
High practicability and throughput (compared to GC)

- ✓ *High specificity*
- ✓ *Good – optimal sensitivity*
- ✓ *Internal Standard - High Accuracy*
- ✓ *Wide linear range (high scan speed)*

Limited m/z range: <3000 amu
Low resolution
Low mass accuracy

LC-MS/MS is the technology of choice for small molecule quantitation in biological fluids.

Endocrinology is a major field of application.


Journal of Chromatography B, 883–884 (2012) 50–58

Contents lists available at SciVerse ScienceDirect

Journal of Chromatography B

journal homepage: www.elsevier.com/locate/chromb

Review

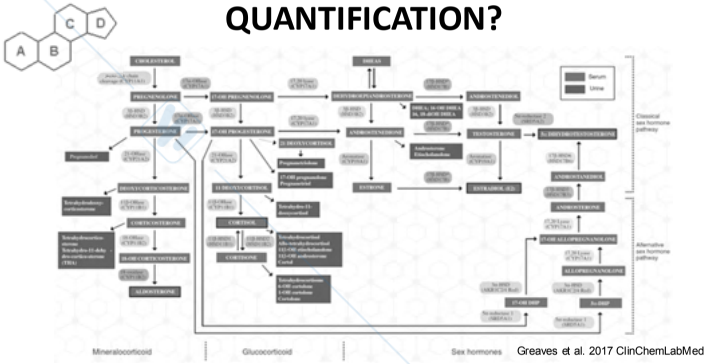
The coming of age of liquid chromatography coupled to tandem mass spectrometry in the endocrinology laboratory☆

Valdemir Melechco Carvalho*

Contents

High specificity and sensitivity
Multi-analyte
Versatility
High-throughput

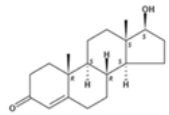
Is LC-MS/MS suitable for STEROID QUANTIFICATION?



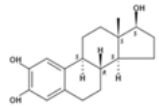
Large family including >800 compounds.
Similar structure.
ISOMERS: same molecular weight, same atomic composition.
ISOBARS: same molecular weight, different atomic composition.

STEROID CROSS-DETECTION in LC-MS/MS

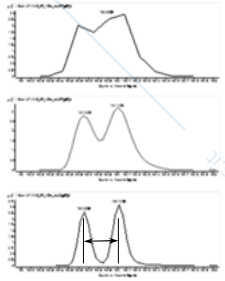
ISOBARS: same molecular weight, different atomic composition, DIFFERENT exact mass.



Name: Testosterone
Formula: C19H28O2
Nominal Mass: 288
Exact Mass: 288.2089301



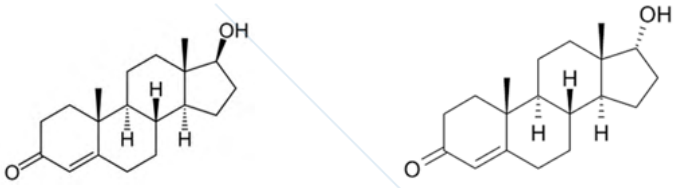
Name: 2-Hydroxyestradiol
Formula: C18H24O3
Nominal Mass: 288
Exact Mass: 288.1725446



High Resolution MS could distinguish among ISOBARS…but unfortunately 3Q instruments cannot.

STEROID CROSS-DETECTION in LC-MS/MS

ISOMERS: same molecular weight, same atomic composition, SAME exact mass



TESTOSTERONE

EPITESTOSTERONE

$C_{19}H_{28}O_2$

288.208923

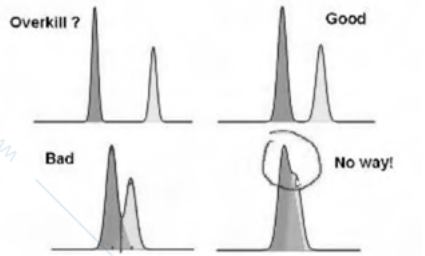
Cannot be distinguished by any MS analyzers!

STEROID CROSS-DETECTION in LC-MS/MS

CHROMATOGRAPHIC SEPARATION is of utmost importance for ensuring high specificity.

extended run time to separate isomers and isobars

GC > UPLC > HPLC



STEROID MEASUREMENT IN THE CLINICAL LAB

	GC-MS	LC-MS/MS	Direct IAs
ANALYTICAL QUALITY			
Specificity	HIGHEST	HIGH	Variable/POOR
Matrix Influence	None	Manageable	Major issue
Accuracy guarantee	Isotopic internal standard	Isotopic internal standard	None
Application for REFERENCE METHODS	Gold Standard	Incoming	No way
Multi-analyte potential	HIGHEST	HIGH	SINGLE analyte
PRACTICABILITY			
Experience requirement	HIGH	HIGHEST	Almost none
Bench work	Extensive	Variable	None
Run time	Elevated 30 min	Medium 5 – 25 min	Low <5 min
Throughput	Low	Good	Optimal
Ready to use Kits	None	Incoming	Many
Costs	Instrumental	Instrumental	Reagents

LC-MS/MS for PEPTIDE/PROTEIN QUANTIFICATION

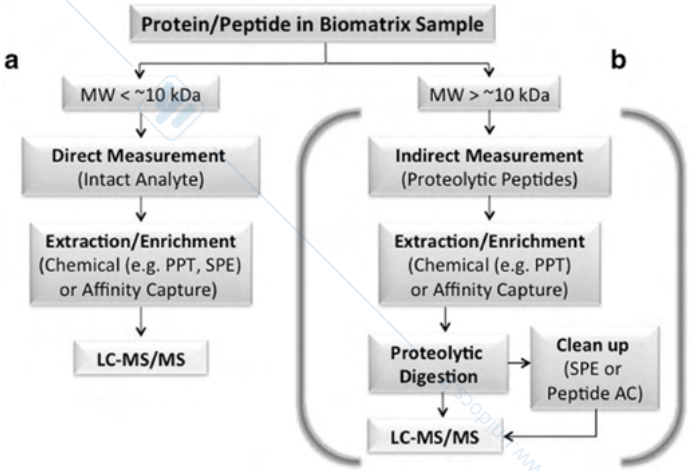
Medium-large molecules.

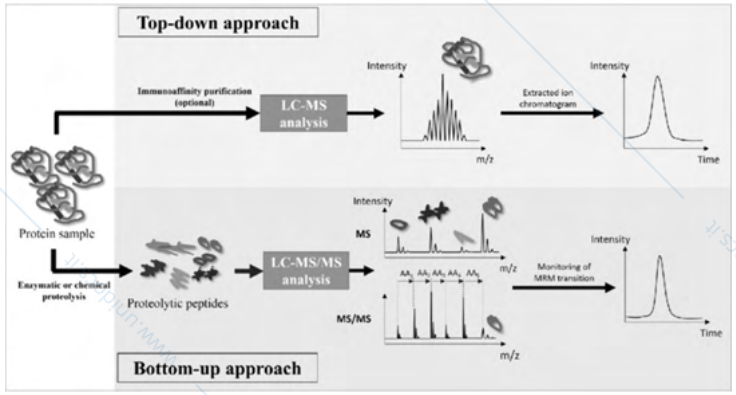
Way more challenging than small molecules.

- High mass accuracy is required
- Large m/z range
- Nano LC flow (nano-HPLC)
- Difficult chromatography
- Poor ESI sensitivity (multicharge ions)
- Multiple isoformes/PTM (intact proteins)
- Complex sample prep (peptides obtained by trypsin digestion)
- Lack of certified standards
- Lack of internal standards
- …

QTof are ideal for protein analysis…however quantitation is poor.
3Q has low resolution, poor accuracy, and is limited to 3000amu.

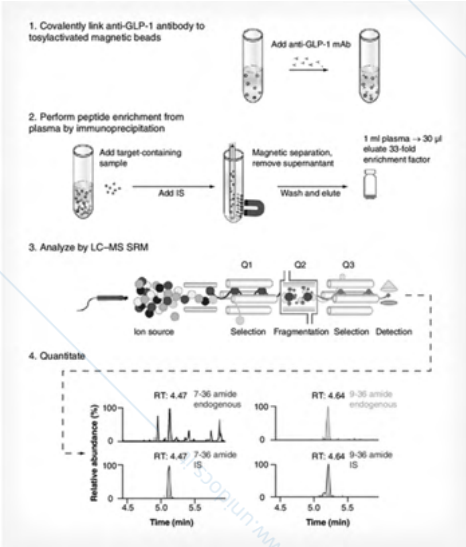
LC-MS/MS for PEPTIDE/PROTEIN QUANTIFICATION





LC-MS/MS for GLP-1

30 aa
3297.7 Dalton



Chappell et al., 2014

LC-MS/MS for IGF-1

70 aa
7,649 Daltons

Clinical Chemistry and Laboratory Medicine (CCLM) | Volume 56: Issue 11

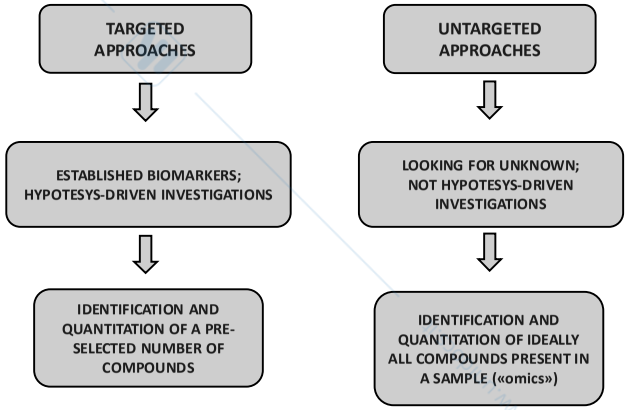
A quantitative LC-MS/MS method for insulin-like growth factor 1 in human plasma

Kees J. Bronsema, Frank Klont, Frank B. Schalk, Rainer Bischoff, Ido P. Kema and Nico C. van de Merbel

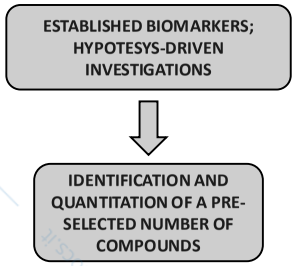
DOI: https://doi.org/10.1515/cclm-2017-1042 | Published online: 01 May 2018

A liquid chromatography-tandem mass spectrometry (LC-MS/MS) method was developed that employs **^{15}N-IGF1** as an internal standard. The method features **urea-based IGF1/IGFBP-complex dissociation** which is directly followed by **tryptic digestion**. Following solid-phase extraction (SPE) sample clean-up of the digest, IGF1 is detected by means of two **signature peptides** that enable quantification of total IGF1 as well as discrimination between IGF1 proteoforms with 'native' and modified or extended N-terminal sequences.

MASS SPECTROMETRY-BASED APPROACHES TO PROTEIN AND METABOLYTE ANALYSIS

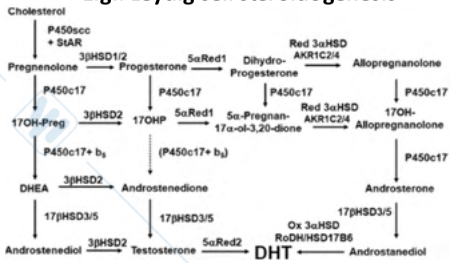


DEVELOPMENT OF TARGETED ASSAYS



- Get the pure standard and IS
- Instruct the MS to detect it
- Optimize best LC conditions
- Optimize sample preparation
- Optimize collection/storage
- Define the calibration range
- Validate the assay
- Measure unknown samples

FIRST…DEFINE TARGETS!
E.g.: Leydig cell steroidogenesis

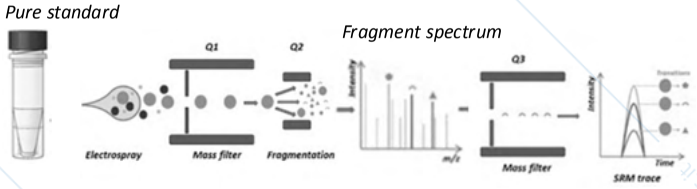


PURCHASE PURE STANDARD

Available?
Purity?
Cost?

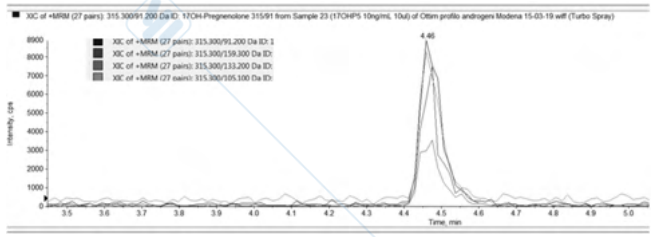
NECESSARY for targeted approaches
Method development
Method calibration

CREATE a MS/MS detection method for each compound



Analyze the pure standard to identify the molecular ion.
Fragment the molecular ion to explore the fragment spectrum.
Select the most abundant and specific fragments.
Define the Q1 → Q3 MRM transition (AKA MS/MS).

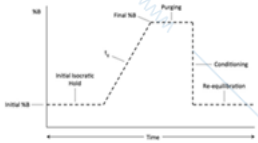
17OHP5: BEST MRM SELECTION; Molecular Weight: 332.5 g/mol



CREATE a LC method

C8, C18, C6-phenyl, BEH etc…

H2O, MeOH, ACN, formic acid, ammonium formiate etc…



Inject the pure standard.

Use MRM as detector.

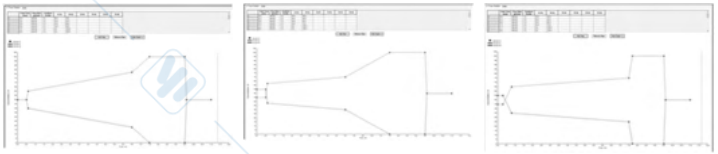
Test multiple columns, mobile phases, flow, additives, gradient conditions…

Find the best compromise between run time, peak shape, resolution, separation etc…

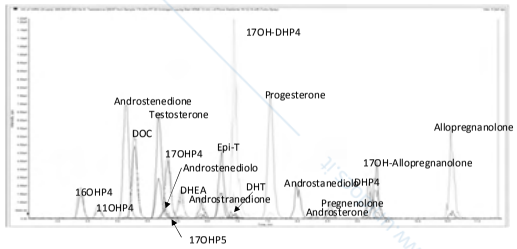
Define retention time for each compound.

Test various chromatographic gradients

Changes in time in the relative abundance between mobile phases, aiming at increasing the eluotropic power.



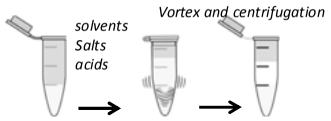
PANEL including 20 androgens and proandrogens



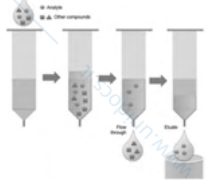
TECHNIQUES FOR SAMPLE PREPARATION

- ✓ *Remove interfering components.*
- ✓ *Increase analyte concentration.*
- ✓ *Improve the performance and the reliability of the measurement, but time consuming.*

PROTEIN PRECIPITATION

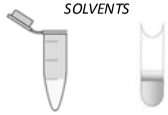


SOLID PHASE EXTRACTION



LIQUID – LIQUID EXTRACTION

NON-miscible SOLVENTS



OPTIMIZE A SAMPLE PREP PROTOCOL

Use LC-MS/MS as detector.

Test different options (SPE, LLE…).

For each option, optimize conditions (solvents, volumes, temperature, disposables etc…).

Find the best compromise between recovery and selectivity. Reduce matrix effect as much as possible.

Fanelli et al., 2012

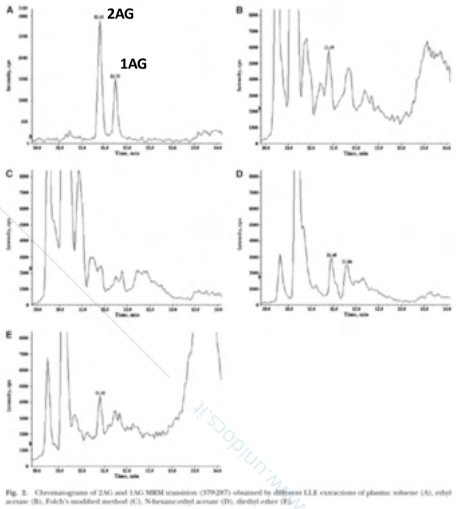


Fig. 2. Chromatograms of 2AG and 1AG MRM transition (379/287) obtained by different LLE extractions of plasma: toluene (A), ethyl acetate (B), Folch's modified method (C), N-hexane:ethyl acetate (D), diethyl ether (E).

DEFINE the CALIBRATION RANGE

What is the expected level of the analyte in the specimens?

Known from literature?

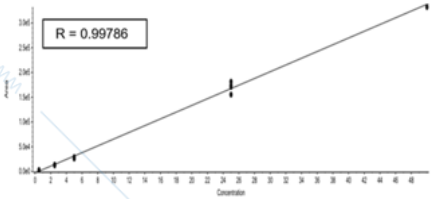
To be hypotesized based on analyte response in the sample, then refined?

Both physiological and pathological levels covered?

Minimum 5 calibrators plus 0 required.

Define IS concentration.

For each compound!



VALIDATION

ANALYTICAL PARAMETERS

PRECISION	**ACCURACY**
SENSITIVITY	**SPECIFICITY**
STABILITY	**TRACEABILITY**
RANGE	**LINEARITY**

APPLICATION TO CLINICAL PURPOSES

Comparison with established methods

Harmonization and Standardization

Generation of Reference Intervals

LC-MS/MS method development and validation: The International Guidelines

Typical method development includes assessing:

- Selectivity
- Carry-over
- Recovery
- Matrix effect
- Sensitivity
- Accuracy
- Precision
- Stability

TABLE 1. Commonly used criteria for the performance characteristics of LC–MS/MS methods intended for use in clinical diagnostics

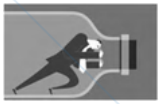
	Imprecision, %	Accuracy, %	Qualitative identification	Comments
Precision	≤10	na	yes	na
LOQ	≤15	≥80	yes	At least ⅓ of the concentration corresponding to the clinical decision level, s/n ≥ 5
LOD	na	na	yes	Quantitation may not be accurate, s/n ≥ 3
ULOL	≤10	≥85	yes	Highest concentration at which accuracy and imprecision are acceptable
AMR	≤10	≥85	yes	Should overlap clinically relevant range of concentrations
Carry-over	na	na	na	Less than LOD

LOQ, limit of quantitation; LOD, limit of detection; ULOL, upper limit of linearity; AMR, analytical measurement range.

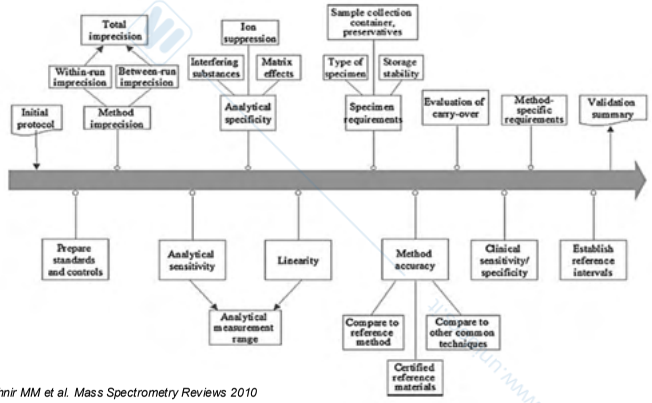
FDA U.S. FOOD & DRUG ADMINISTRATION

EUROPEAN MEDICINES AGENCY SCIENCE MEDICINES HEALTH

Kushnir MM et al. Mass Spectrometry Reviews 2010



LC-MS/MS method development and validation: A Long Way to Go



Kushnir MM et al. Mass Spectrometry Reviews 2010

UNTARGETED APPROACHES

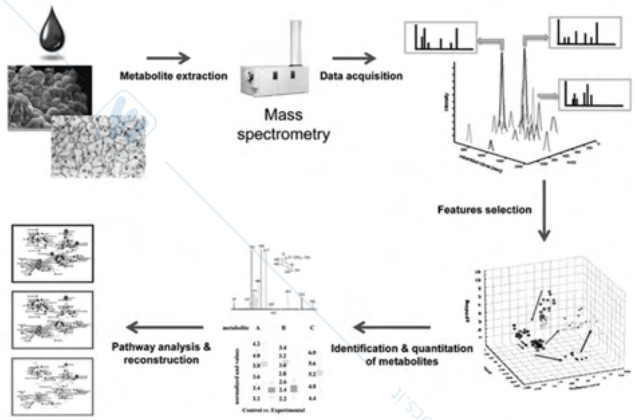
LOOKING FOR UNKNOWN; NOT HYPOTESYS-DRIVEN INVESTIGATIONS

⇩

IDENTIFICATION AND QUANTITATION OF IDEALLY ALL COMPOUNDS PRESENT IN A SAMPLE («omics»)

- Get samples from classes to be compared
- Minimal, unselective sample-prep
- Long and simple LC separation
- MS full scan acquisition
- Filter data to remove noise and artifacts
- Compare «features» among classes via multidimensional statistic analysis
- Select informative features
- Identify compounds via databases
- Confirm identity by pure standard

UNTARGETED APPROACHES



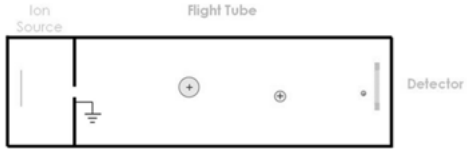
UNTARGETED APPROACHES

Require High Resolution and Accurate Mass MS to distinguish and to register the maximum possible information from thousands of compounds in a biological sample.

Coupling with UPLC or GC.

Time Of Flight TOF

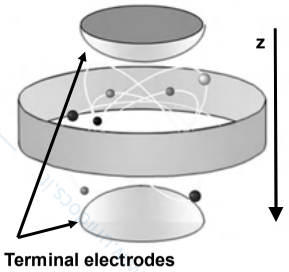
Ions are accelerated before entering a chamber without field. Ions travel the defined distance within the flight tube taking a time inversely proportional to its m/z.



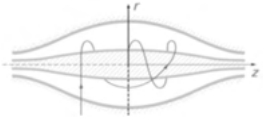
Ion Trap

Ions are trapped in a three-dimensional quadrupolar field, where the terminal electrodes control the stability of the trajectories along the z axis

To obtain a mass spectrum, the radiofrequency of the terminal electrodes is gradually increased, causing a progressive destabilization of the heavier ions that are expelled along the z axis.



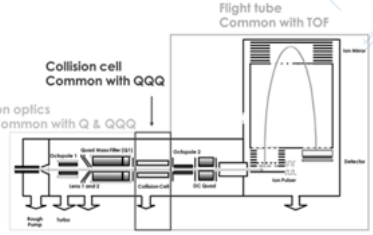
ORBITRAP



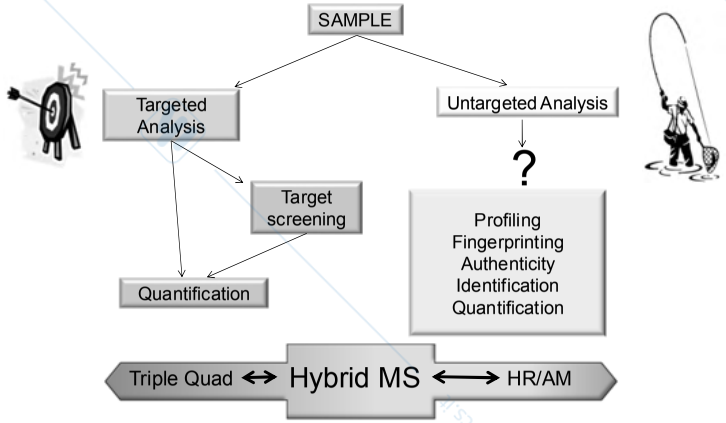
Ion trap in which moving ions are trapped around an electrode.
Electrostatic attraction is compensated by centrifugal force arising from the initial tangential velocity.
Potential barriers created by end-electrodes confine the ions axially.
One can control the frequencies of oscillations (especially the axial ones) by shaping the electrodes appropriately.
Axial frequency can be used for mass analysis.

Hybrid MS analyzers
E.g.: qqTOF

Combining the best features of each MS analyzer.

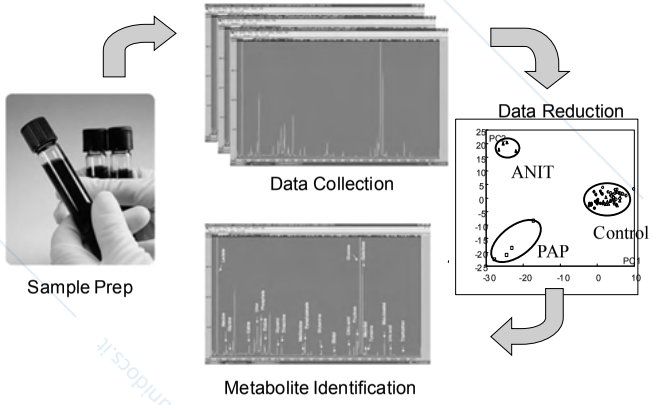


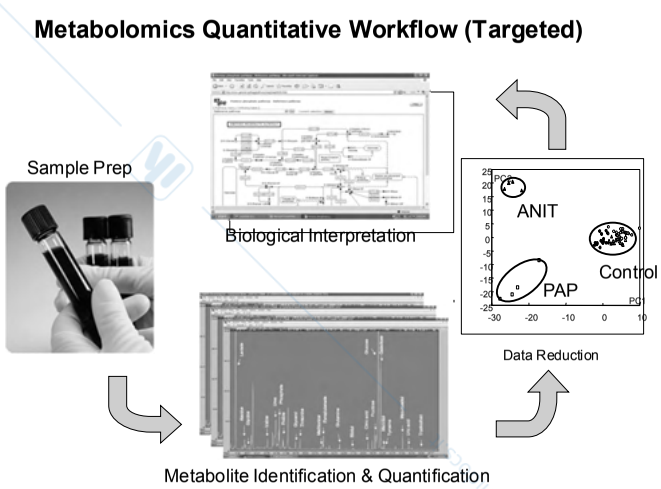
Targeted vs. Untargeted analysis workflow

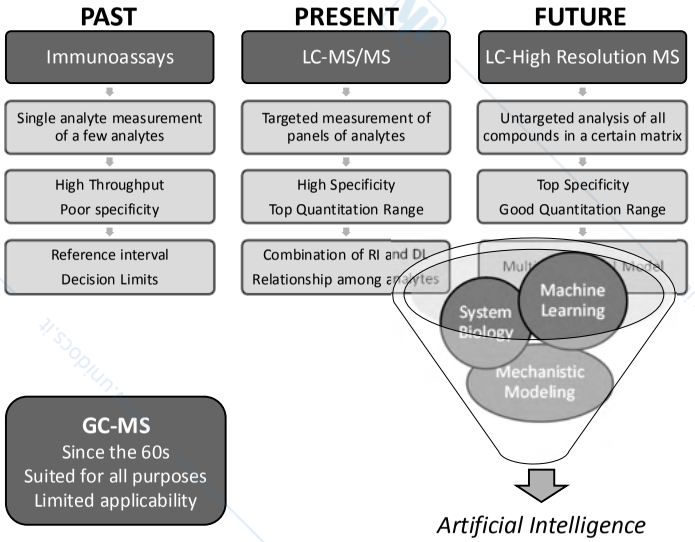


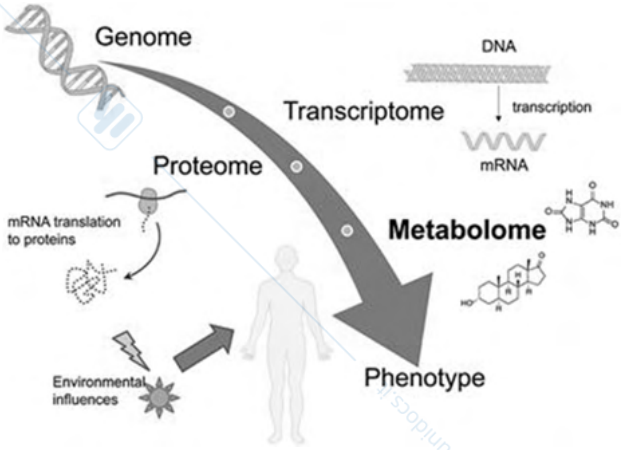
Untargeted and targeted approaches can be complementary

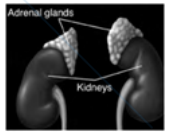
Metabolomics Profiling Workflow (Untargeted)







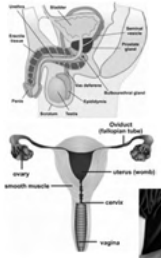




Adrenal tumors

Hypogonadism
Osteoporosis

Hypertension
Cardiovascular risk
Obesity
Diabetes



STEROID HORMONES

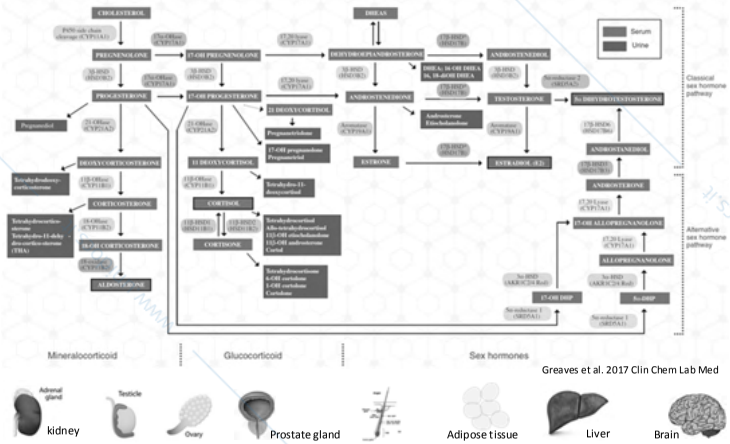
Reproduction

Hyperandrogenism

Genetic deficit steroidogenesis
Disorder of Sex Development

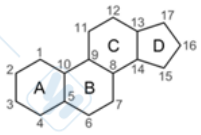
STEROIDOME

The totality of steroid compounds pertaining to all possible pathways
> 800 steroid compounds listed in Human Metabolome Database

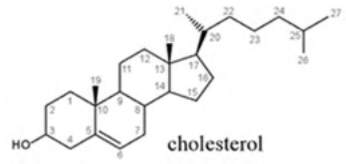


Greaves et al. 2017 Clin Chem Lab Med

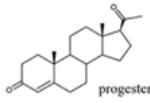
STEROID STRUCTURE

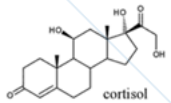


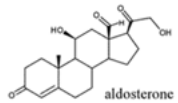
Cyclopentane-perhydro-phenanthrene



C21







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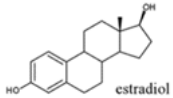
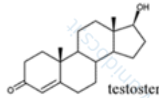
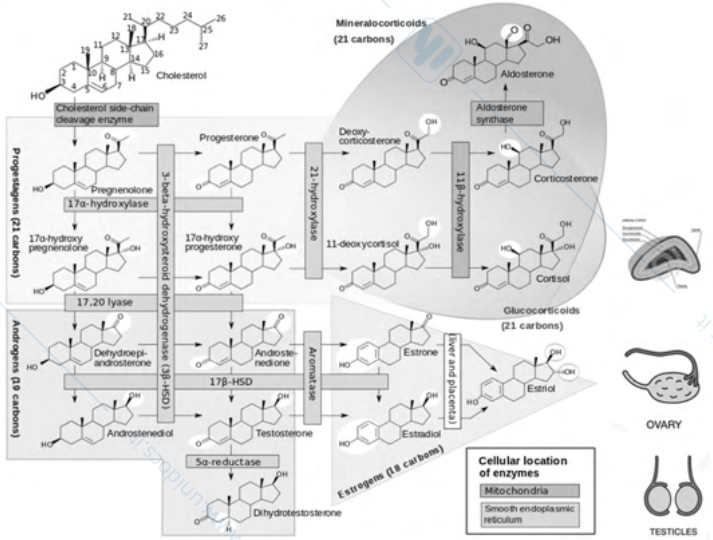
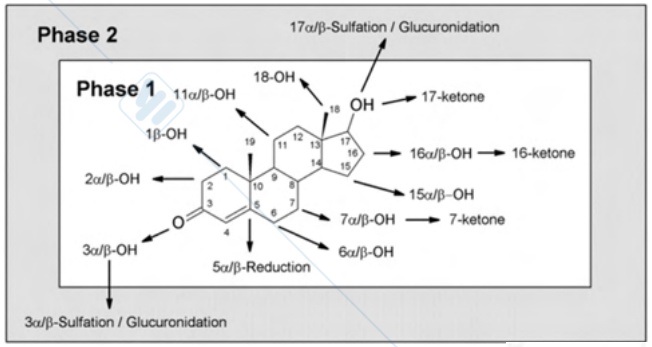


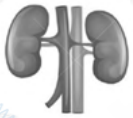


C19



STEROID EXCRETION





STEROID INVESTIGATION IN HUMAN FLUIDS

Early morning

- ✓ Secretion
- ✓ Complex matrix

24 hour

- ✓ Daily exposure
- ✓ Steroid conjugated metabolites

Multiple time points

- ✓ Free – active fraction
- ✓ Very low levels

PRE-ANALYTICAL considerations for quantitative testing

- What tube for sample collection

Blood:
Serum: gel separator, beads clot activator
Plasma: EDTA, Li-Heparin, Na-Heparin

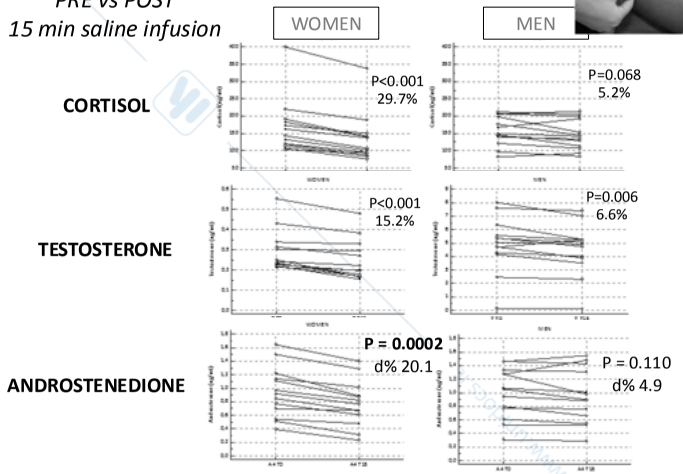
SALIVA:
Whole: Passive drool/Direct spitting
Swab for mucin removal: organic/sinthetic polymer; citric acid for saliva stimulation

PRE-ANALYTICAL considerations for STEROID quantitative testing

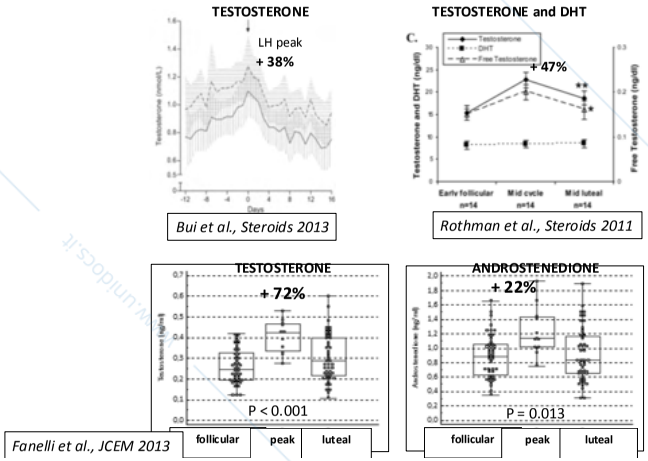
- Patient conditions
  - Time of the day: early morning 07:30 – 09:30
  - Nutritional status: fasting
  - Drug suspension and how long: e.g.: glucocorticoid, estroprogestins therapies; 1 day, 1 week, months…
  - Needle stress: 10-15 min saline infusion
  - Menstrual phase: usually follicular phase, except progesterone in luteal phase
  - Special pathologies: es: enzyme deficits: cross-detection from supra-physiologic levels of unusual steroids
- Withdrawal conditions
  - HPA or HPG stimulation test: ACTH or hCG, resp.
  - HPA suppression test : DEXAMETHASONE test
  - Body orientation: aldosterone/renin CLINO- (supine) or ORTHO- (standing) STATISM

SAMPLING CONDITION
VENIPUNCTURE STRESS

PRE vs POST
15 min saline infusion



IMPACT OF THE MENSTRUAL CYCLE ON ANDROGEN LEVELS
Healthy Adult and Young Adult Women



PERFORMANCE REQUIREMENTS for STEROID HORMONE MEASUREMENT

SENSITIVITY and **WIDE DYNAMIC RANGE**
- Gender: in females and in males
- Fertility status: Menstrual phase / Pregnancy / Menopause
- Functional tests: adrenal stimulation and suppression

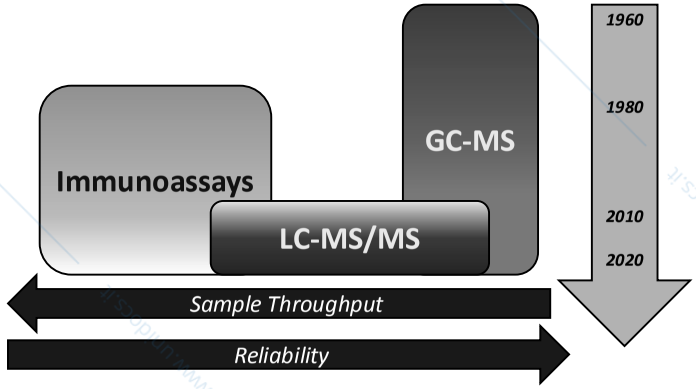
ACCURACY small but clinically relevant intra- and inter-individual variations
- Differential diagnosis,
- Monitoring therapy / hormone replacement

SPECIFICITY *vs* thousands of endogenous and exogenous steroids
- Structural related or unrelated isobars, isomers, epimers, conjugated metabolites
- Drugs

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

Ideally **NO INFLUENCE** from different MATRIX

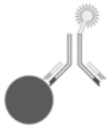
QUANTITATIVE ANALYTICAL TECHNIQUES FOR STEROID MEASUREMENT

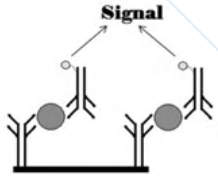


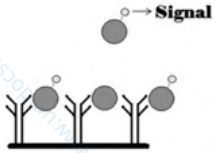
IMMUNOASSAYS

selective binding of the measurand
Antigen – Antibody

indirect property of the molecule
"shape"







Once upon a time…

ELSEVIER

Clinica Chimica Acta 231 (1994) 107–113

Short communication

Measurement of steroid hormones in plasma by isocratic high performance liquid chromatography coupled to radioimmunoassay

Stefano Boschi*[a], Rosaria De Iasio[a], Paolo Mesini[a], Gian Franco Bolelli[b], Raffaella Sciajno[c], Renato Pasquali[d], Maurizio Capelli[e]

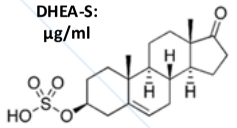
[a]Servizio di Farmacologia Clinica, Policlinico S.Orsola, Via Massarenti, 9, 40138 Bologna, Italy
[b]Istituto di Citomorfologia Normale e Patologica, Sez. di Bologna, Bologna, Italy
[c]Servizio di Fisiopatologia della Riproduzione, University of Bologna, Bologna, Italy
[d]Clinica Medica I, Endocrine Unit, University of Bologna, Bologna, Italy
[e]Laboratorio Centralizzato, Policlinico S.Orsola, Bologna, Bologna, Italy

STEROID CROSS-DETECTION in IA

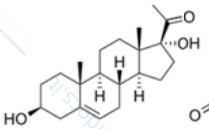
Structurally related compounds

TESTOSTERONE: pg/ml – ng/ml

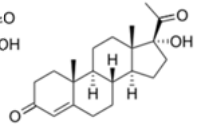
DHEA-S: µg/ml



17OH-PREGNENOLONE

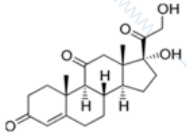


17OH-PROGESTERONE

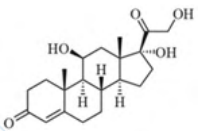


- *Depends on the epitope*
- *Different concentration range*
- *Enzymatic deficit/blockage*

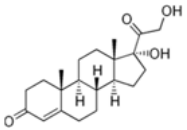
CORTISONE



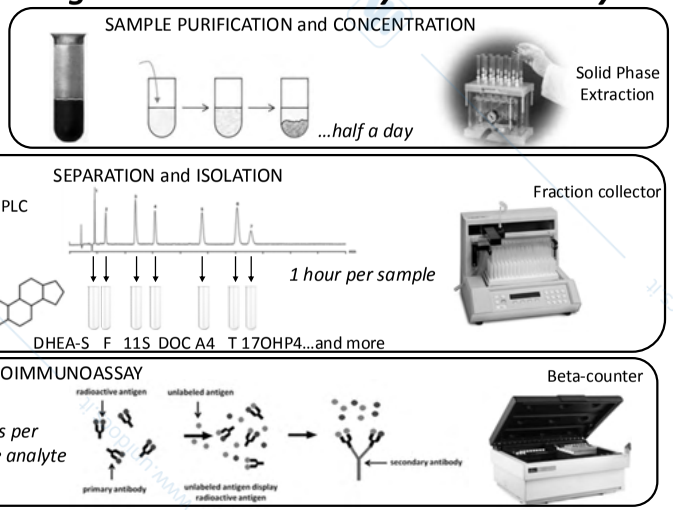
CORTISOL



11DEOXYCORTISOL



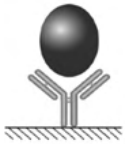
..the gold standard in early immunoassays…



PURIFICATION + HPLC + RIA
for steroid measurement

SENSITIVITY SPECIFICITY THROUGHPUT

DIRECT IMMUNOASSAY in the ROUTINE LAB



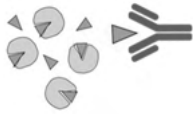
- *commercial KIT + automation*
- *no sample preparation*
- *no costs for personnell*

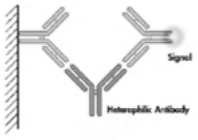
SENSITIVITY SPECIFICITY THROUGHPUT

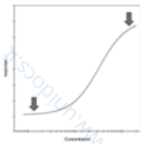
CENTRAL LABORATORY S.ORSOLA-MALPIGHI HOSPITAL
~ 45000 measurements / year (2014)

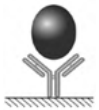
PITFALLS in DIRECT IMMUNOASSAYS

- release from **binding protein**
- **matrix interference**
- **Anti-reagent Ab** (heterophilic Ab, rheumatoid factor)
- **Biotine** supplementation
- **Narrow dynamic range**

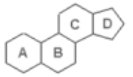


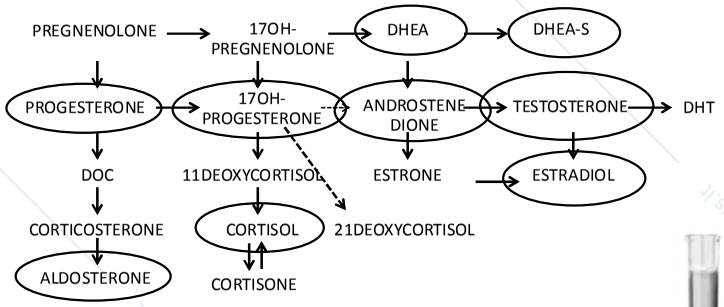






DIRECT IMMUNOASSAY in the ROUTINE LAB

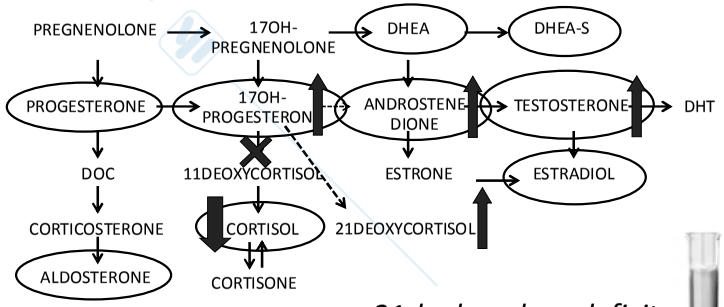




poor performance

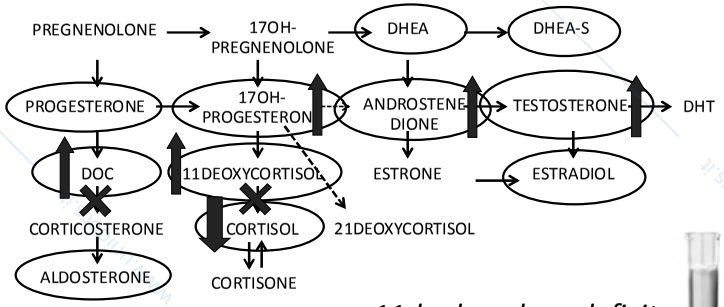
is this panel enough?

DIRECT IMMUNOASSAY in the ROUTINE LAB



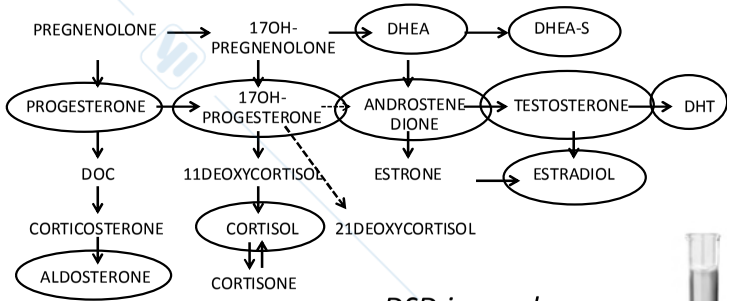
21-hydroxylase deficit
CAH and DSD in newborns
NCCAH in adults

DIRECT IMMUNOASSAY in the ROUTINE LAB



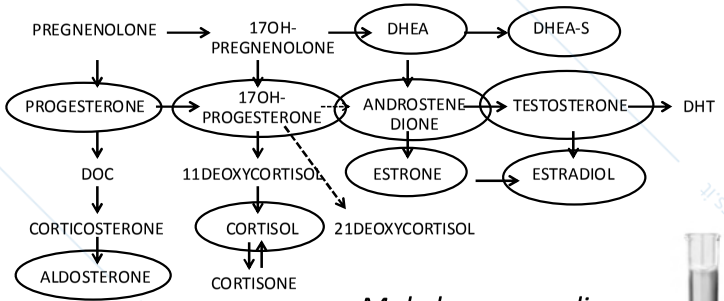
11-hydroxylase deficit
CAH and DSD in newborns
NCAH in adults

DIRECT IMMUNOASSAY in the ROUTINE LAB

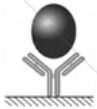


DSD in newborns
Male hypogonadism
Female hyperandrogenism

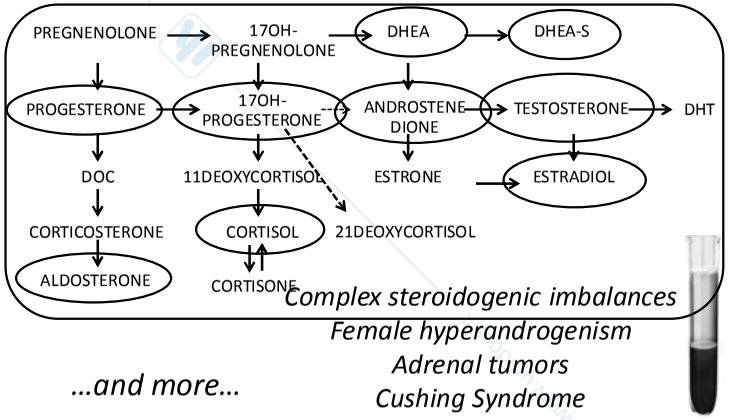
DIRECT IMMUNOASSAY in the ROUTINE LAB



Male hypogonadism
Male gynecomastia
Female hyperandrogenism



DIRECT IMMUNOASSAY in the ROUTINE LAB



Complex steroidogenic imbalances
Female hyperandrogenism
Adrenal tumors
Cushing Syndrome

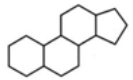
…and more…

DIRECT IMMUNOASSAY in the ROUTINE LAB

HIGH COSTS

SERIOUS UNCERTAINITIES
Diagnosis
Therapy

LIMITED PANEL OF STEROIDS



CLINICAL PRACTICE UPDATE

Unusual results from immunoassays and the role of the clinical endocrinologist

Alison M. Jones and John W. Honour

Clinical Chemistry 49, No. 8, 2003

Editorial

Herold DA, Fitzgerald RL

Immunoassays for Testosterone in Women: Better than a Guess?

Clinical Chemistry 52, No. 9, 2006

Editorials

Stowasser M, Gordon RD

Aldosterone Assays: An Urgent Need for Improvement

POSITION STATEMENT: Utility, Limitations, and Pitfalls in Measuring Testosterone: An Endocrine Society Position Statement

William Rosner, Richard J. Auchus, Ricu

jcem.endojournals.org J Clin Endocrinol Metab, April 2013, 98(4):1376–1387

Challenges to the Measurement of Estradiol: An Endocrine Society Position Statement

William Rosner, Susan E. Hankinson, Patrick M. Sluss, Hubert W. Vesper, and Margaret E. Wierman

JCEM THE JOURNAL OF CLINICAL ENDOCRINOLOGY & METABOLISM

SPECIAL FEATURE

Editorial

Requirement for Mass Spectrometry Sex Steroid Assays in the *Journal of Clinical Endocrinology and Metabolism*

D. J. Handelsman and L. Wartofsky

ANZAC Research Institute (D.J.H.), Concord Hospital, University of Sydney, Sydney, NSW 2139, Australia;

SPECIAL FEATURE

Editorial

***Editorial:* The New Instructions to Authors for the Reporting of Steroid Hormone Measurements**

SPECIAL FEATURE

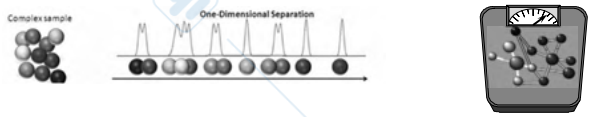
Commentary

Case for the Wider Adoption of Mass Spectrometry-Based Adrenal Steroid Testing, and Beyond

Phillip J. Monaghan, Brian G. Keevil, Paul M. Stewart, and Peter J. Trainer

«HYPHENATED» TECHNIQUES

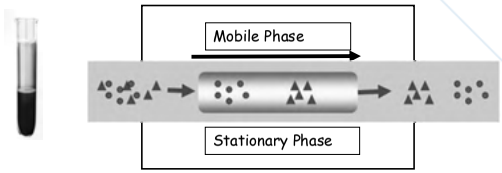
CHROMATOGRAPHY - MASS SPECTROMETRY



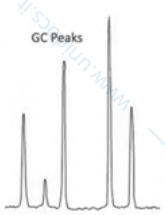
Separation and Identification of compounds in a complex mixture according to physical-chemical features

CHROMATOGRAPHY

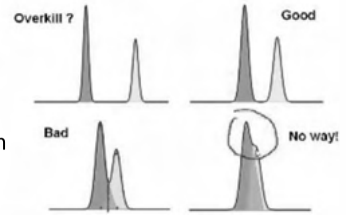
differential affinity for mobile and stationary phase



GAS (GC) **LIQUID (LC)**



different resolution power

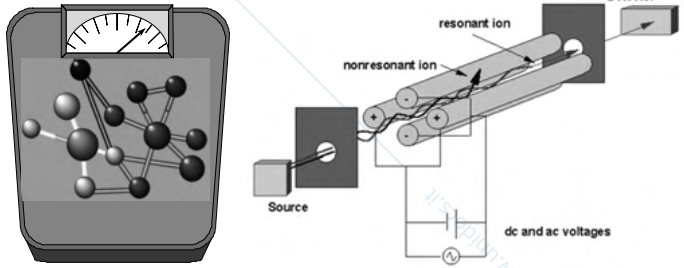


MASS SPECTROMETRY

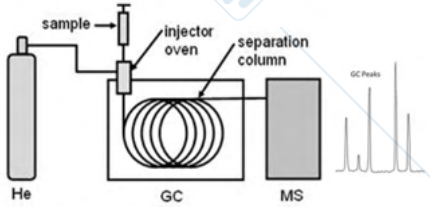
QUADRUPOLE / TRIPLE QUADRUPOLE

low mass resolution
ideal for quantitative / TARGETED analysis
multi-analyte

m/z ionized compounds



GC-MS REQUIREMENTS



GAS phase → **VOLATILITY**

High temperature → **THERMAL STABILITY**

COMPROMISE for STEROID MEASUREMENT:

DERIVATIZATION
Analyte covalent modification
Long reaction time + extensive purification

GC-MS

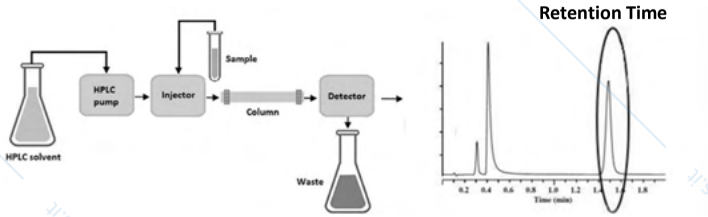
for steroid measurement

SENSITIVITY SPECIFICITY THROUGHPUT

STILL:

- Technique used for **Reference Measurement Procedure** in Reference Laboratories
- Characterization of URINE steroids (**untargeted** approaches)

HIGH PRESSURE LIQUID CHROMATOGRAPHY (HPLC)



VERSATILE (liquid environment suited for biological molecules)

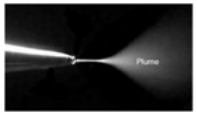
SHORTER RUN TIMES

EASIER SAMPLE PREPARATION

COUPLING LC and MS: a recent achievement

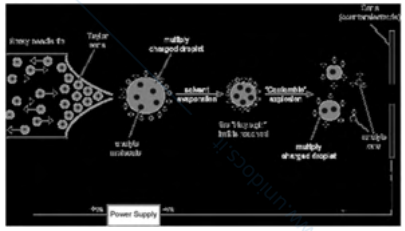
THE INTERFACE

HPLC	ATMOSPHERIC PRESSURE IONIZATION	MS
Large volumes of liquids High pressure	«SOFT»	Ions High vacuum



Pos Ion $[MW+1]^+$
Neg Ion $[MW-1]^-$

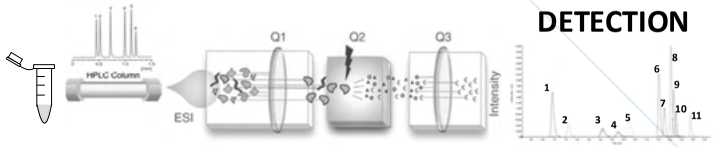
Nobel Prize
John B. Fenn
(2002)



THE POWER OF LC-MS/MS

3 SPECIFICITY STEPS

MULTIANALYTE DETECTION



1. **HPLC**
RETENTION TIME
Affinity for analytical column

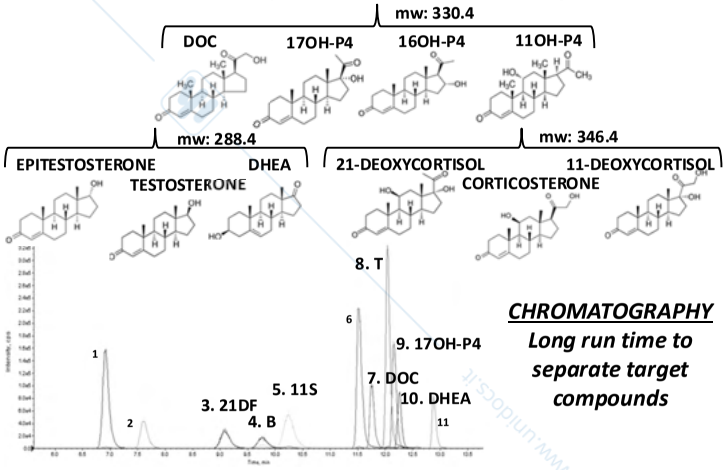
2. **MS1**
MOLECULAR WEIGHT
Selection of the Precursor Ion

3. **MS2**
STRUCTURE
Selection of Fragment Ions

SENSITIVITY SPECIFICITY THROUGHPUT

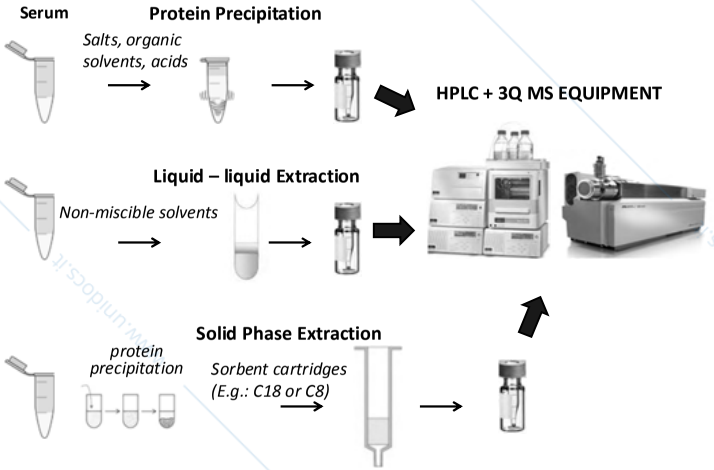
STEROID CROSS-DETECTION in MS/MS

Isobar/Isomer compounds: same molecular weight (mw)



CHROMATOGRAPHY
Long run time to separate target compounds

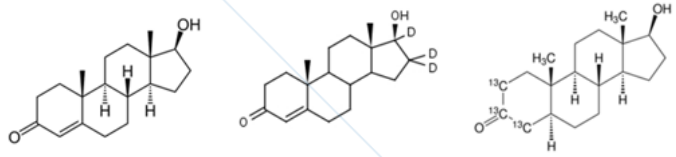
Sample preparation for LC-MS/MS analysis



QUANTITATION BY ISOTOPIC DILUTION

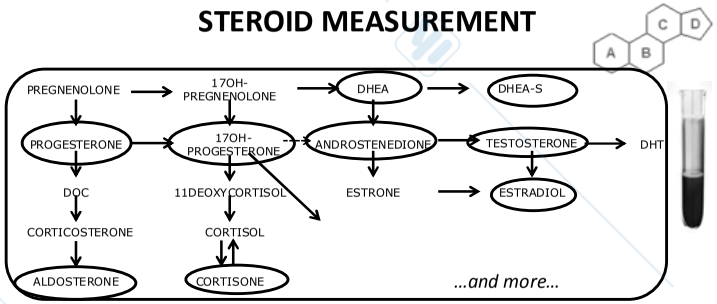
Internal Standard:

- Stable isotopes (^{2}H, ^{13}C) of the same analyte
- Same physical-chemical behaviour
- **Different m/z**



- Analyte / IS ratio is unchanged across the pre-analytical and analytical procedure
- **Analyte / IS signal ratio** is used for quantitation

STEROID MEASUREMENT



DIRECT IMMUNOASSAYS	LC-MS/MS
➢ Limited panel of steroids	➢ Wide dynamic range
➢ Poor specificity	➢ Multi-analytic steroid panels
➢ Poor sensitivity	➢ High specificity
➢ Poor inter-IA and inter-lab comparability	➢ High sensitivity

LC-MS/MS for STEROID PROFILING

making the difference in modern endocrinology

BETTERS
The measurement of known steroids

ENLARGES
The spectrum of measurable steroids

- ✓ Bettering current diagnostic tools
- ✓ Enlarging disease characterization
- ✓ Define novel diagnostic/predictive criteria

LC-MS/MS for STEROID PROFILING

making the difference in modern endocrinology

BETTERS
The measurement of known steroids

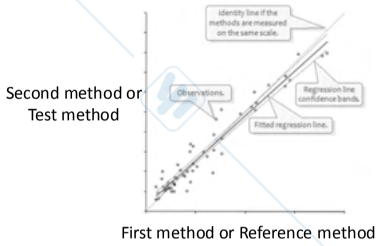
- ✓ Bettering current diagnostic tools

ImmunoAssays flaw:

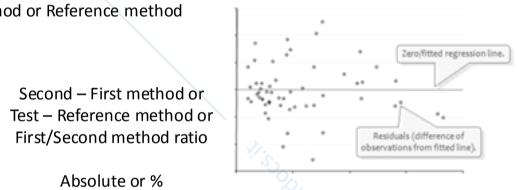
Comparison vs MS methods

REPORTING METHOD'S COMPARISON

REGRESSION

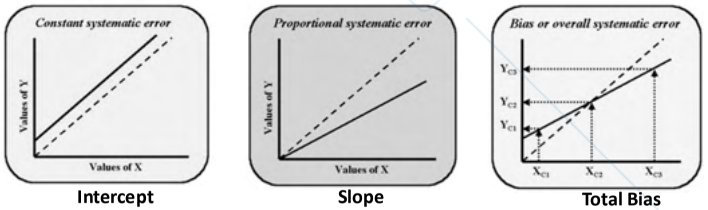


BLAND - ALTMAN



REPORTING METHOD'S COMPARISON

Regression Plot

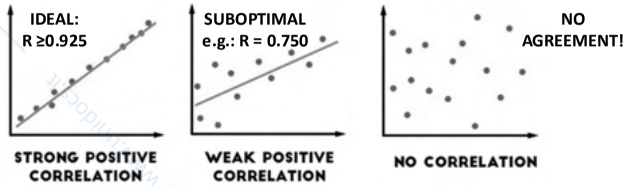


Intercept
Equality line: intercept=0
Fitting line: coeff (90CI)

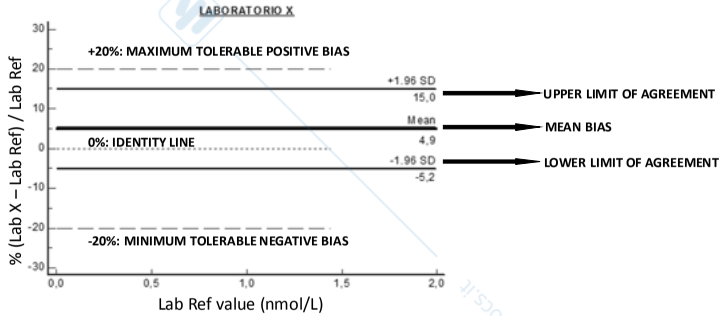
Slope
Equality line: slope=1
Fitting line: coeff (90CI)

Total Bias

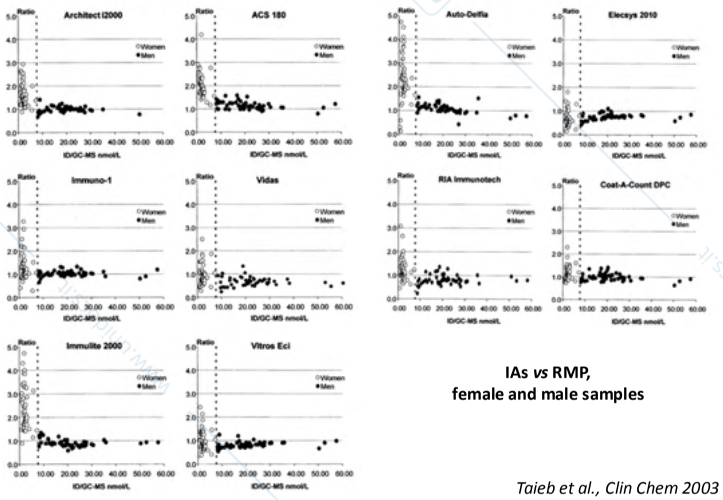
RANDOM ERROR: correlation coefficient R



REPORTING METHOD'S COMPARISON
Bland and Altman's Plot



SERUM TESTOSTERONE

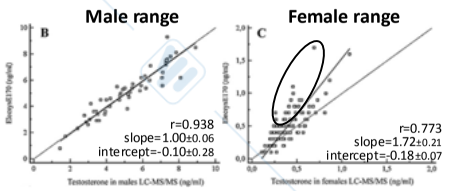


IAs *vs* RMP,
female and male samples

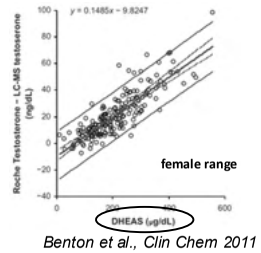
Taieb et al., Clin Chem 2003

SERUM TESTOSTERONE and SEX STEROIDS in Bologna

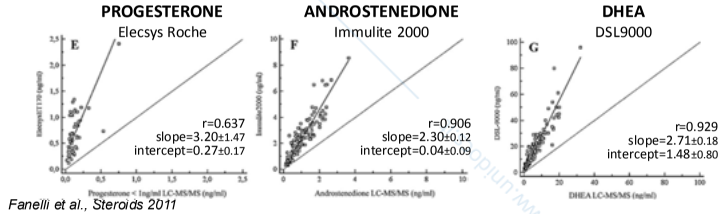
LC-MS/MS *vs* Elecsys Roche



% bias Elecsys Roche *vs* LC-MS/MS



Benton et al., Clin Chem 2011

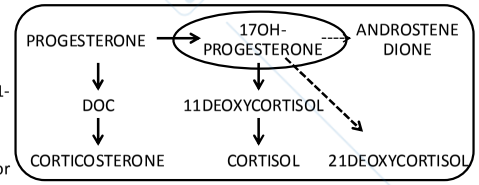


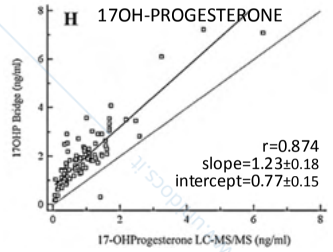
Fanelli et al., Steroids 2011

CONGENITAL ADRENAL HYPERPLASIA

Routine Lab:

- ✓ **17OHprogesterone only**
- ✓ No discrimination between 21-hydroxylase and other forms
- ✓ ACTH-stimulation test
- ✓ Genetic analysis mandatory for confirmation



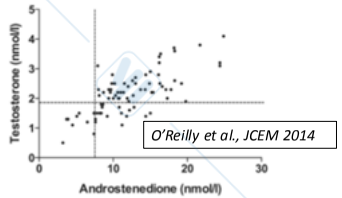


Fanelli et al., Steroids 2011

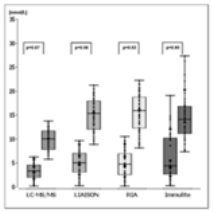
LC-MS/MS:
17OHprogesterone +
21Deoxycortisol +
Corticosterone
Help in differentiating Non Classical Adrenal Hyperplasia from PCOS.

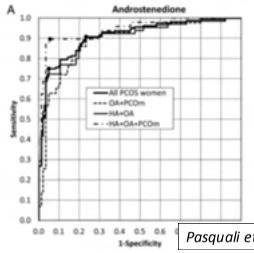
ANDROSTENEDIONE

MS-restricted biomarker of PCOS

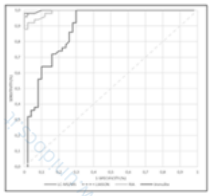


O'Reilly et al., JCEM 2014





Pasquali et al., JCEM 2016



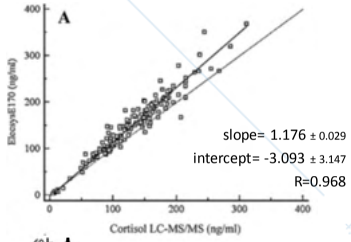
Van Helden et al, Clin Biochem 2018

CORTISOL

ROUTINE IMMUNOASSAYS *vs* LC-MS/MS

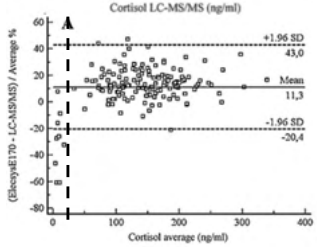
159 de-identified samples collected among sera from S.Orsola-Malpighi Hospital routine laboratory

good comparability at basal levels (12% bias)



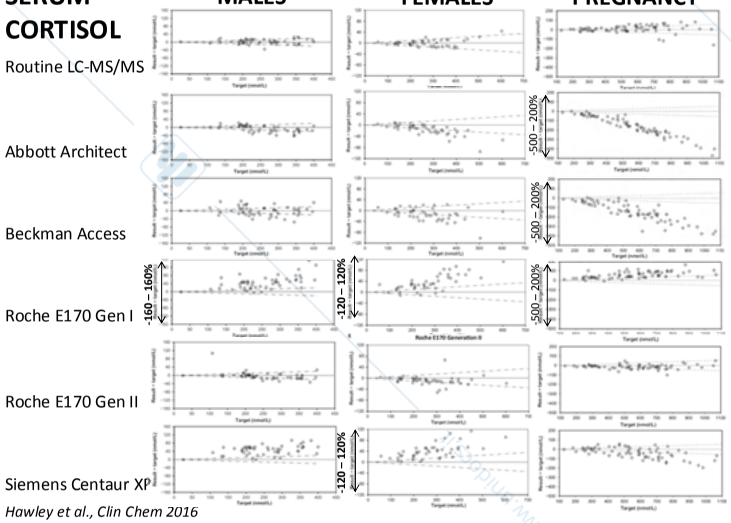
Cortisol levels post dexamethasone suppression test:

False negatives!



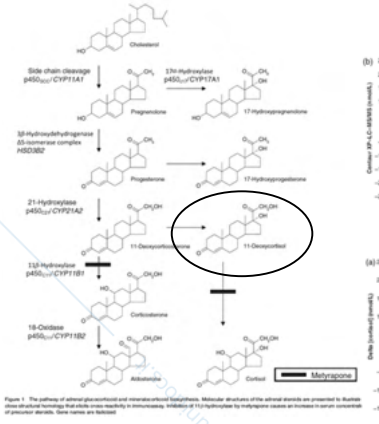
Fanelli et al., Steroids 2011

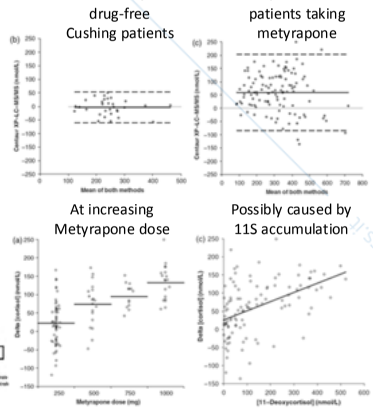
SERUM CORTISOL



Hawley et al., Clin Chem 2016

CORTISOL MEASUREMENT DURING METYRAPONE therapy

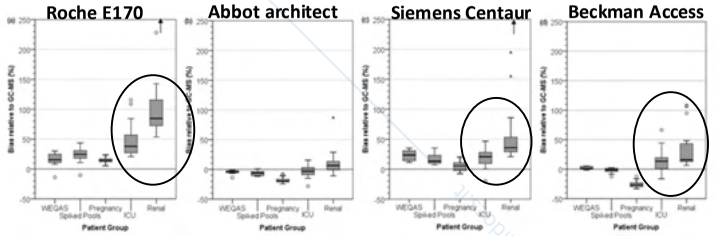




Monaghan et al., Ann Clin Biochem 2011

SERUM CORTISOL

HPA hyperactivation or reduced clearance.
Possible accumulation of glucocorticoid intermediates interfering with Ias.



Dodd et al., 2016

Post-ACTH test SERUM CORTISOL

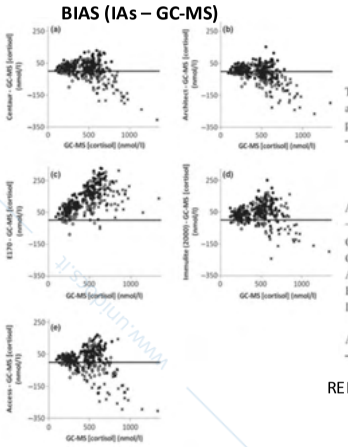


Table 4. Assay-specific estimated lower reference limits for post-adrenocorticotrophin cortisol according to gender and oral contraceptive pill (OCP)-status

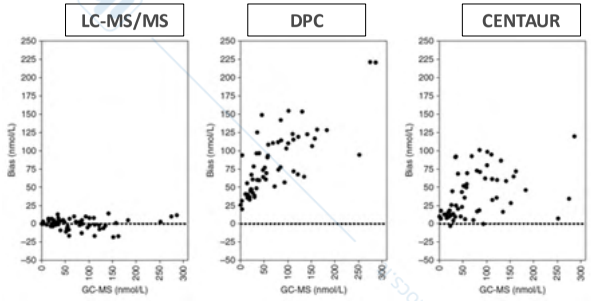
Assay	Males	Non-OCP females	Combined male and Non-OCP female subjects*	OCP females
GC-MS	418	421	420	643
Centaur	448	446	446	619
Architect	430	416	NA	577
E170	574	524	NA	791
Immulite (2000)	469	478	474	688
Access	459	455	NA	604

REFERENCE VALUES VARY ACCORDING TO THE ASSAY

El Farhan et al., Clinical Endocrinology 2013

URINARY FREE CORTISOL

Bias *vs* GC-MS



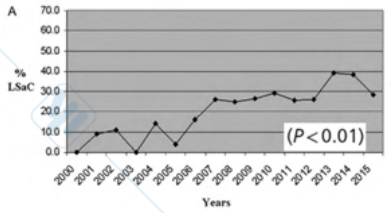
Wood et al., Ann Clin Biochem 2008

SALIVA as a biological fluid

1. **Painless, non stressful, non invasive.**
2. **No need for assistance from health care providers and structures. Self performed!**
3. **Allows for multiple sampling within the same day or in the night.**
4. **Provides information on the FREE or BIOAVAILABLE fraction.**

…but…high SENSITIVITY is required to detect the low steroid levels.

LATE NIGHT SALIVARY CORTISOL TESTING



European Registry on Cushing's syndrome (ERCUSYN)

Valassi et al., EJE 2017

Characteristics of reported midnight salivary cortisol assays.

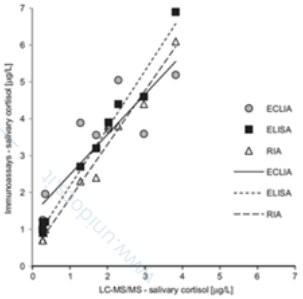
Assay	Mean value in patients with Cushing's syndrome (μg/dl)	Reference value (μg/dl)	Cutoff value (μg/dl)	Sensitivity of the assay %	Specificity of the assay %	Reference
RIA	0.87 ± 0.16	0.043 ± 0.003	0.13	92	100	Raff et al.
RIA	0.97 ± 0.13	0.18 ± 0.02	0.35	92.7	93.1	Putignano et al.
RIA	1.23 ± 2.06	0.18 ± 0.02	0.2	100	96	Yaneva et al.
RIA	0.72 (0.26–1.94)	0.07 (0.01–0.16)	0.22	100	100	Viardot et al.
RIA	0.97 ± 1.56	0.12 ± 0.031	0.21	93	100	Doi et al.
FPIA	1.6 ± 0.3	0.26 ± 0.02	0.55	93	100	Papanicolau et al.
RIA	0.9 ± 0.9	0.09 ± 0.008	0.28	100	87	Castro et al.
ELISA	0.58 ± 0.1	0.0025 ± 0.001	0.08	88	82	Restituto et al.
RIA	0.88 ± 0.24	0.16 ± 0.01	0.40	100	100	Nunes et al.
RIA	0.92 ± 0.38	0.28 ± 0.03	0.29	100	71	Present study
ECLIA	1.1 ± 0.41	0.2 ± 0.02	0.35	100	89	Present study

FPIA, fluorescence polarization immunoassay; ELISA, enzyme-linked immunosorbent assay; ECLIA, electrochemiluminescent immunoassay.

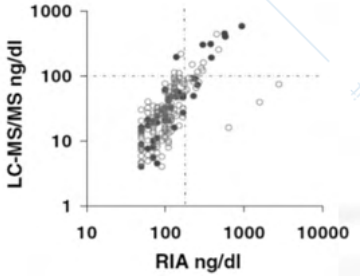
Beko et al., Chimica Clinica Acta, 2010

SALIVARY CORTISOL

IAs *vs* LC-MS/MS

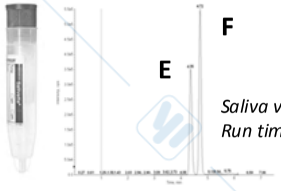


Baecher et al., J Lab Med 2013

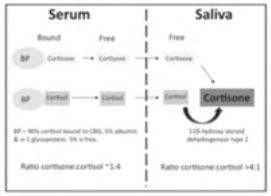


Baid et al., JCEM 2007

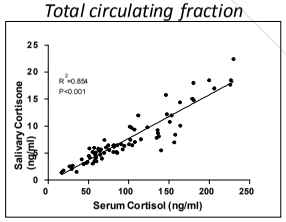
SALIVARY STEROID PROFILING by LC-MS/MS



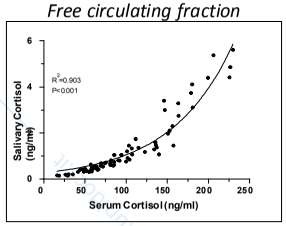
Saliva volume: 50 µl
Run time: 7.5 min



Salivary Cortisone
Total circulating fraction

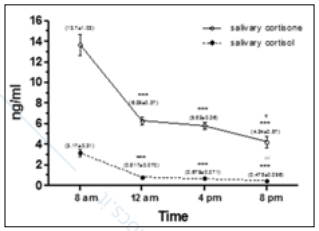


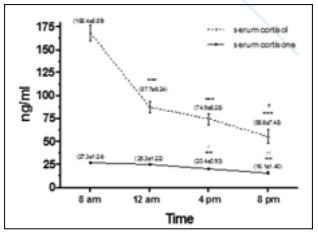
Salivary Cortisol
Free circulating fraction



Mezzullo et al., J Ster Bioch Mol Biol 2016

SALIVARY GLUCOCORTICOIDS BY LC-MS/MS AS A TOOL FOR ANALYZING THE CIRCADIAN RHYTHM





Mezzullo M. et al. JSBMB 2016

SALIVARY ANDROGENS

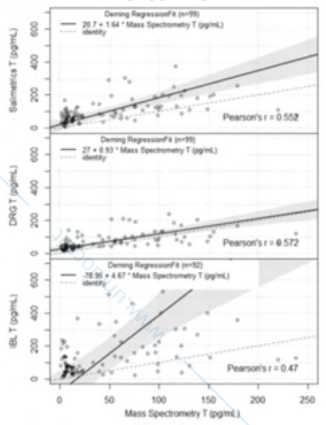
"Salivary testosterone has also been shown to be a **reliable substitute** for **free testosterone** measurements but cannot be recommended for general use at this time, since the methodology has **not been standardized** and adult male **ranges are not available** in most hospital or reference laboratories**»**

Wang et al., Consensus Statement EJE 2008

SALIVARY ANDROGENS

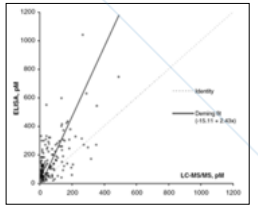
IA vs MS

TESTOSTERONE

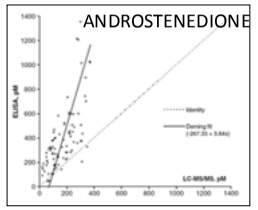


Welker et al., Psychoneuroendocrinology 2016

TESTOSTERONE

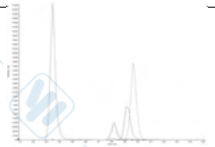


ANDROSTENEDIONE

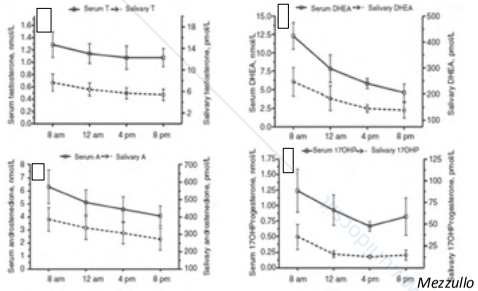


Turpeinen U. et al. Clinica Chimica Acta 2012

SALIVARY ANDROGENS BY LC-MS/MS AS A TOOL FOR ANALYZING THE CIRCADIAN RHYTHM

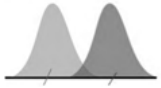


1. **Testosterone**
2. **DHEA**
3. **Androstenedione**
4. **17OHProgesterone**

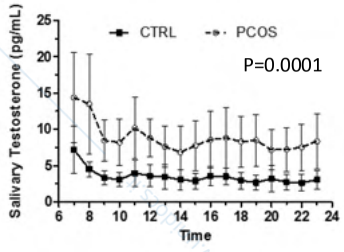


Mezzullo et al., JSBMB 2017

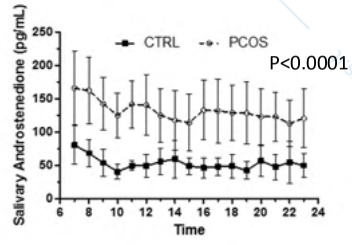
SALIVARY ANDROGENS IN PCOS



TESTOSTERONE



ANDROSTENEDIONE



CIRCADIAN RHYTHM

Mezzullo et al., ECE 2018

LC-MS/MS for STEROID PROFILING

making the difference in modern endocrinology

BETTERS
The measurement of known steroids

ENLARGES
The spectrum of measurable steroids

- ✓ Bettering current diagnostic tools
- ✓ Enlarging disease characterization
- ✓ Define novel diagnostic/predictive criteria

LC-MS/MS for STEROID PROFILING

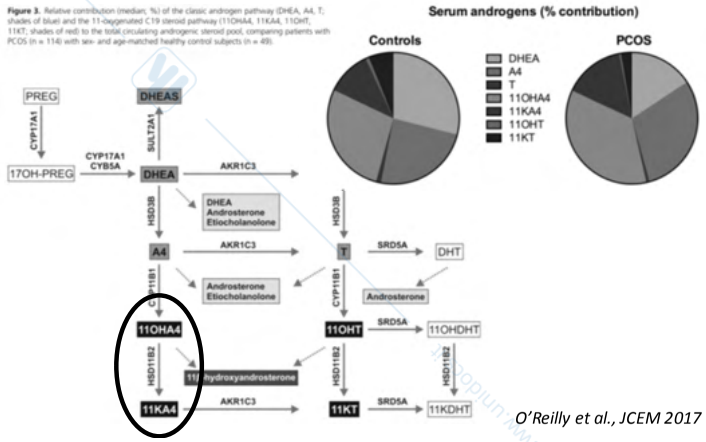
making the difference in modern endocrinology

PAST IA[illegible] few steroids
PRESENT LC-MS/MS[illegible]arge targeted panels of steroids
FUTURE HR-MS[illegible]ntargeted steroidomics

ENLARGES
The spectrum of measurable steroids

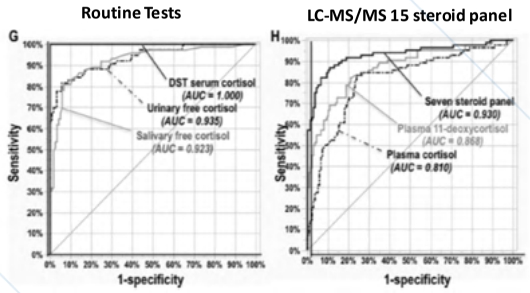
- ✓ Enlarging disease characterization
- ✓ Define novel diagnostic/predictive criteria

FORGOTTEN STEROIDS
11 OXO ADRENAL ANDROGENS



O'Reilly et al., JCEM 2017

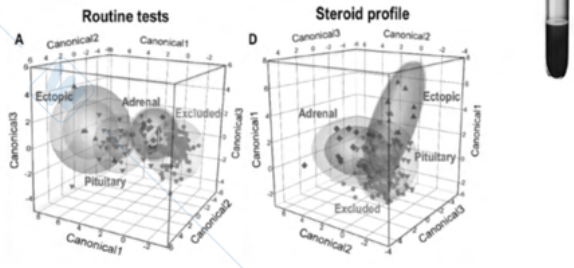
LC-MS/MS PROFILINF FOR DIAGNOSING AND SUBTYPING CUSHING SYNDROME



15 steroid panel by lc-ms/ms
similar diagnostic efficacy
Compared to 3 routine tests

Eisenhofer et al., Clin Chem 2018

LC-MS/MS PROFILINF FOR DIAGNOSING AND SUBTYPING CUSHING SYNDROME



Actual Grouping B	Predicted 1. Adrenal	2. Pituitary	3. Ectopic	4. Excluded	
1. Adrenal	13	0	0	0	100%
2. Pituitary	1	32	4	4	80%
3. Ectopic	0	1	4	0	80%
4. Excluded	0	0	0	114	100%

Actual Grouping B	Predicted 1. Adrenal	2. Pituitary	3. Ectopic	4. Excluded	
1. Adrenal	20	0	0	1	95%
2. Pituitary	2	45	0	4	88%
3. Ectopic	0	0	12	0	100%
4. Excluded	3	10	1	124	90%

Fig. 3. Results of discriminant analyses for use of routine diagnostic tests (A, B, C) compared to 10 steroids of the steroid panel (D, E, F) that provided optimal discrimination of the 4 patient groups (ectopic CS ▲, pituitary CS ▼, adrenal CS ◆, CS excluded ●).

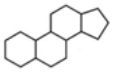
Eisenhofer et al., Clin Chem 2018

LC-MS/MS for STEROID PROFILING

making the difference in modern endocrinology

BETTERS
The measurement of known steroids

ENLARGES
The spectrum of measurable steroids



- ✓ Bettering current diagnostic tools
- ✓ Enlarging disease characterization
- ✓ Define novel diagnostic/predictive criteria

What's the next challenge?

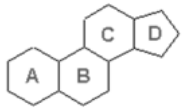
Each LAB is still establishing its own Reference Intervals, Diagnostic Cut-Off, Decision Limits
NOT Transferable to other LABS or METHODS

Table 27. Cortisol (F) reference intervals according to age, menstrual phase, and fertility status in the female population

Age/ Tanner stage	Menstrual phase/ fertility status	LRL–URL, percentile	LC-MS/MS		Immunoassay		
			LRL–URL, nmol/L	Reference [geographic group[a]]	LRL–URL, nmol/L	Direct or routine assay	Reference [geographic group[a]]; population study
17 y		2.5–97.5			240–665[b,c,d]	CLIA, Immulite, DPC	Elmlinger et al., 2002 [8]
16–40 y		2.5–97.5	138–810[b,c,e,i]	Kulle et al., 2013 [8]			
17–19 y		2.5–97.5			242–648[b,c,d]	CLIA, Immulite, DPC	Elmlinger et al., 2002 [8]
18–19 y		2.5–97.5			245–617[b,c,d]	CLIA, Immulite, DPC	Elmlinger et al., 2002 [8]
18–49 y	luteal	0–100	150–822[b,c]	Eisenhofer et al., 2017 [8]			
18–54 y	premeno-pausal	2.5–97.5	131–551[f]	Fanelli et al., 2011 [9]			
18–54 y	follicular	2.5–97.5	112–551[f]	Fanelli et al., 2011 [9]			
19–49 y	follicular	0–100	97–979[b,c]	Eisenhofer et al., 2017 [8]			
45–77 y	postmeno-pausal	0–100	124–698[b,c]	Eisenhofer et al., 2017 [8]			
45–86 y	postmeno-pausal	2.5–97.5	157–498[f]	Fanelli et al., 2011 [9]			

Fanelli et al., 2018

What can we still ask to LC-MS/MS??



Harmonization of steroid hormone measurement

HARMONIZATION of MEASURES

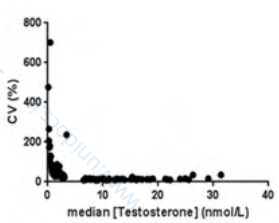
Lab results are «comparable irresepective of the measurement procedure used and where and when a measurement was made.» Miller at al., 2014

Hormonal values have «the same meaning», no matter the lab and the assay that generated them.

Mandatory for establishing univoque, lab- and assay- independent REFERENCE VALUES and CLINICAL DECISION LIMITS to be applied worldwide.

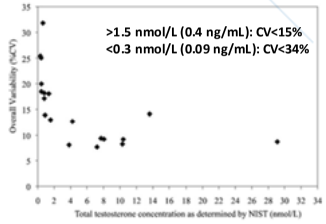
REPRODUCIBILITY OF SERUM TESTOSTERONE

7 labs using IAs



Groenestege et al., Clin Chem 2012

9 LC-MS/MS assays



Vesper et al., Steroids 2009

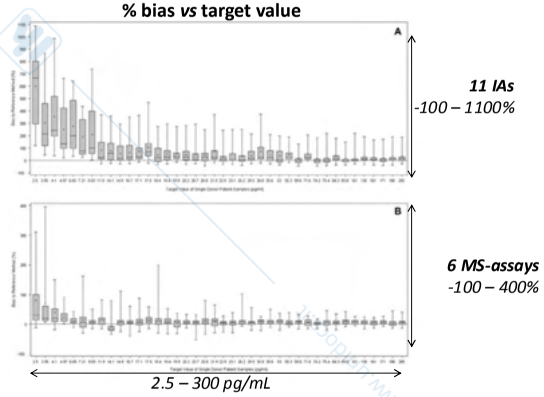
REPRODUCIBILITY OF SERUM ESTRADIOL

Steroids. 2014 April ; 82: 7–13.

High variability in serum estradiol measurements in men and women

Hubert W. Vesper[a,*], Julianne C. Botelho[a], Meghan L. Vidal[a], Yasamin Rahmani[a], Linda M. Thienpont[b], and Samuel P. Caudill[a]

% bias *vs* target value

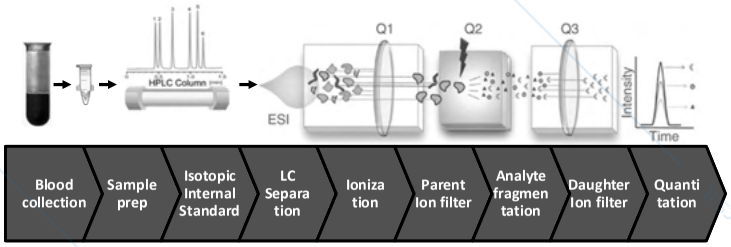


11 IAs
-100 – 1100%

6 MS-assays
-100 – 400%

2.5 – 300 pg/mL

LC-MS/MS is a COMPLEX MULTISTEP PROCEDURE



multiple options available at each step
several differences among LC-MS/MS assays

➢ What is the Impact on the final result?

STANDARDIZATION is obtained when results are harmonized and also referable to the top of the traceability chain by means of certified materials, or **ANCHOR POINTS**.

		Certified Reference Material	Reference Measurement Procedure	Reference Lab	EQA programs	Matrix-based Calibrators
ROUTINE LAB	Cortisol	Yes	yes	yes	yes	x
	Progesterone	yes	yes	yes	yes	x
	Testosterone	yes	yes	yes	yes	x
	Estradiol	yes	yes	yes	yes	x
	Aldosterone	x	yes	yes	yes	x
	17OH-Progesterone	x	yes	yes	yes	x
	DHEAS	x	x	x	yes	x
	DHEA	x	x	x	yes	x
	Androstenedione	x	x	x	yes	x
NOT IN ROUTINE	DHT	x	x	x	incoming	x
	Estrone	x	x	x	x	x
	11Deoxycorticosterone	x	x	x	x	x
	Corticosterone	x	x	x	x	x
	11Deoxycortisol	x	x	x	x	x
	21Deoxycortisol	x	x	x	x	X
	Cortisone	x	x	x	x	x

NOT YET AVAILABLE

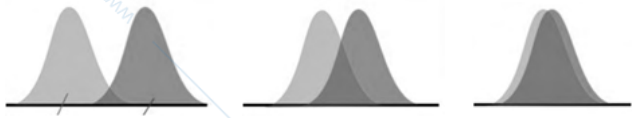
INTERPRETING THE LABORATORY RESULT: TURNING NUMBERS INTO SOMETHING MEANINGFUL

REFERENCE INTERVALS «normality»

CUT-OFF or THRESHOLD VALUES
E.g.: between different diseases; associated with signs and symptoms Appearance.

Variable effectiveness depending on the analyte/disease/condition

HEALTH
DISEASE



DEFINITION of REFERENCE INTERVALS

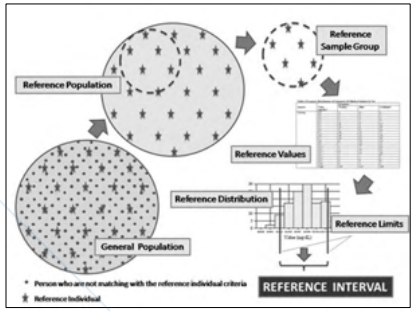
before '60s (and still today commonly speaking)

«normal values» or «normal ranges»

«NORMAL» is ambiguous, subjective, may have different meanings
OUT OF NORMALITY is not necessarily ABNORMAL

DEFINITION of REFERENCE INTERVALS

The term "reference interval" (RI) was defined in 1986 by the International Federation of Clinical Chemistry (IFCC)



REFERENCE INDIVIDUALS
comprise a
REFERENCE POPULATION
from which is selected a
REFERENCE SAMPLE GROUP
on which are determined
REFERENCE VALUES
on which is observed a
REFERENCE DISTRIBUTION
from which is calculated
REFERENCE LIMITS
that define a
REFERENCE INTERVAL

OBSERVED VALUE
(patient test result)
can be compared with

Solberg H (on behalf of IFCC) Clin Chim Acta 1987; 167: 111-18.

CONSIDERATIONS on RI GENERATION

ISSUE	ACTION
Health definition	Health criteria Specify physiologic condition (pregnancy) Specify population (athletes)
Intra- and Inter-individual biological variability	Partition Number of cases
Calculation	Define limits Proper statistics
Pre-analyticals (sampling condition, subject condition, timing)	Standardized procedures
Analyticals	Traceability to high order methods/materials Harmonization of measures

DEFINING HEALTH

✓ *absence of any disease?*

✓ *reporting general wellbeing?*

✓ *no drug assumption?*

✓ *define health criteria according to physiopathologic conditions known to impact the hormone and the scope of the RI*

Multiple levels

Anamnestic Questionnaire/Interview

Physical Examination

Lab / Imaging testing

SPECIFICATIONS within the "Health" concept

INTER-INDIVIDUAL DIFFERENCES
GENDER
AGE
GENOME
ENVIRONMENT
LIFESTYLE
ETHNICITY

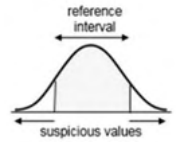
DEFINE SPECIFIC POPULATIONS

INTRA-INDIVIDUAL CHANGES
Circadian Rhythm
Fast/feeding
Female menstrual/reproductive/menopausal status
Ageing
Season
Environment
Stress

STANDARDIZE SPECIFIC CONDITIONS

WHERE DO WE SET THE REFERENCE LIMITS?

...in medio stat virtus...



100%
99%
95%
90%
75%

LOWER REFERENCE LIMIT LRL

UPPER REFERENCE LIMIT URL

WORKFLOW FOR RI GENERATION …IN REAL LIFE

✓ ***Relying on overall laboratory results***

Thousands of numbers.
Assuming that «the majority» are «normal»… though people doing blood testing are not-healthy by definition!
No info on subjects and on preanalytics to make selections. Only statistical elimination of outliers…

✓ ***Relying on the kit manufacturer***

REFERENCE POPULATION
Diagnostic Kit Manufacturers

ARCHITECT «The expected ranges for the ARCHITECT Testosterone assay were obtained by testing specimens drawn from 77 premenopausal females, 20 postmenopausal females, and 82 males. For this study, specimens from premenopausal females were categorized as ovulating, and **using oral contraceptives**." "It is recommended that **each laboratory establish its own expected ranges**"

Population	n	Testosterone Value (ng/mL) Minimum	2.5 Percentile	97.5 Percentile	Maximum
Females	97	0.09	0.13	1.08	1.30
Premenopausal	77	0.09			1.09
Ovulating	52	0.09			1.09
Using Oral Contraceptives	25	0.13			0.83
Postmenopausal	20	0.12			1.30
Males	82	1.56	1.66	8.11	8.77
<50 years old	70	1.66			8.77
50 years and older	12	1.56			5.63

Testosterone 2017-11, V 9.0 English

Test subjects	N	Percentiles Median	5-95th	Median	5-95th
		ng/mL		nmol/L	
Males 20-49 years	136	5.36	2.49-8.36	18.6	8.64-29.0
Males ≥ 50 years	78	4.76	1.93-7.40	16.5	6.69-25.7
Females 20-49 years	89	0.271	0.084-0.481	0.941	0.290-1.67
Females ≥ 50 years	71	0.162	0.029-0.408	0.563	0.101-1.42

ELECSYS "The following table shows the results obtained with the Elecsys Testosterone II assay in an **apparently healthy** group of 214 males and 160 females without intake of contraceptive and prescription drugs (study number CIM 000669). Blood samples were taken between **6.30 am and 1.00 pm**."

ACCESS "…Testosterone levels were measured in human serum and **heparinized plasma** from **apparently healthy** male and female patients using the Access Testosterone assay."…"The data is representative; **each laboratory should establish its own reference ranges** to assure proper representation of specific populations and sample types."

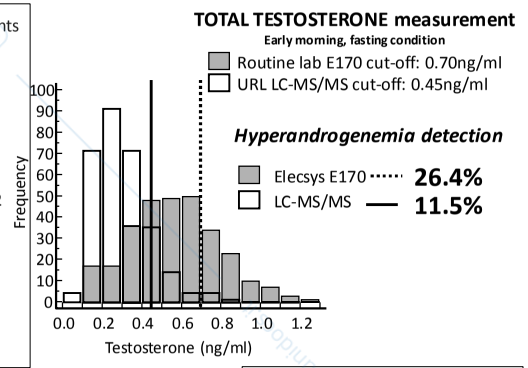
Reference Group & Age Range	Sample Type	n	Median Concentration ng/mL (nmol/L)	95% Reference Interval ng/mL (nmol/L)
Males (18 – 66 years)	Serum	240	3.84 (13.32)	1.75 – 7.81 (6.07 – 27.10)
	Plasma (heparinized)	240	3.84 (13.32)	1.68 – 7.58 (5.83 – 26.30)
Females (21 – 73 years)	Serum	240	0.39 (1.01)	< 0.1 – 0.75 (< 0.35 – 2.60)
	Plasma (heparinized)	240	0.41 (1.42)	< 0.1 – 0.90 (< 0.35 – 3.12)

MEASURING TESTOSTERONE IN FEMALE PATIENTS IA vs LC-MS/MS

N=295 consecutive patients
Referring for:
Hyperandrogenism
Menstrual irregularities
Infertility

Age 18 – 54y
BMI 29.6±8.0kg/m²
N°menses in last 6m: 4±2
mF-G score: 8.0±4.9

Other causes excluded:
Hypercortisolism
Congenital adrenal hyperplasia
Androgen-secreting tumor
Hyperprolactinemia
Ovarian failure

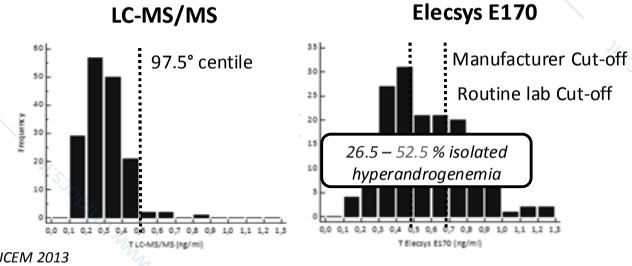


Fanelli et al.. Unpublished data

MEASURING TESTOSTERONE IN YOUNG HEALTHY WOMEN IA vs LC-MS/MS

Reference Population

N=162; Age: 16 – 19y
BMI: 18.5 – 24.9 kg/m²
mF-G<8; 10-13 menses/year
Normal Biochemical/Hormonal assessment
Drug and Disease free



Fanelli et al., JCEM 2013

TAKE HOME MESSAGES

LC-MS/MS is generally better than IAs
but it is NOT FOOLPROOF by definition

- proper **ANALYTICAL VALIDATION** and maintenance
- QCs, survey program, standardization

LC-MS/MS is not as "routinable" as Immunoassays yet

- Sample preparation
- Long runtime
- Experienced staff
- Ready to Use kits are now available

LC-MS/MS has the potential for achieving universal harmonization but…

Interpretation of Results is <u>still</u> ASSAY and LAB dependent

- Reference Intervals
- Diagnostic Threshold

LC-MS/MS :

a number of applications, in the clinical field and byond!

THE DIFFUSED SYSTEMS OF LIVING ORGANISMS

- Nervous System
- Immune System
- Endocrine System
- Genome

Why «diffused»:

They modulate and integrate in a unified way all the body's functions

They regulate metabolic functions

They regulate vital homeostatic functions

The overall control network is highly integrated and operates to keep constant the inner medium of higher organisms –HOMEOSTASIS- living in the external medium –ENVIRONMENT- featured by variable phisical-chemical features –ADAPTATION- in order to guarantee the survival and the reproduction of the individuals.

ENDOCRINE SYSTEM

Communication machinery acting through special signaling molecules -HORMONES- released by specific secreting cells -ENDOCRINE CELLS- to control the functional status of other cells -TARGET CELLS- able to sense the signal by means of specific molecules -RECEPTORS-.

Principal functions of the endocrine system

- Maintenance of the internal environment in the body (maintaining the optimum biochemical environment).
- Integration and regulation of growth and development.
- Control, maintenance and instigation of sexual reproduction, including gametogenesis, coitus, fertilization, fetal growth and development and nourishment of the newborn.

ENDOCRINOLOGY PILLARS

- Endocrine glands
- Hormone classification
- Hormone transport in the bloodstream
- Target cells and endocrine systems

boundaries of general definitions and classificantions are very fleeting

THE ENDOCRINE SYSTEM

ENDOCRINE CELLS

Express sensors for signals activating or inhibiting hormone synthesis or secretion.

Express the enzymatic and structural machinery for synthesizing and secreting hormones. Each endocrine cell is specific for a single hormone (rarely two hormones).

TARGET CELLS

Express the enzymatic machinery for synthesizing HORMONE RECEPTORS: molecules recognising and specifically binding the signaling compound. Same target cell may express receptors for multiple hormones.

HORMONES

Signaling compounds are able to activate or inhibit certain functions in other cells.

EFFECT

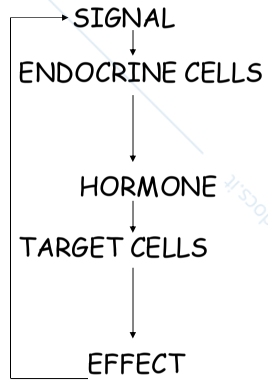
the HORMONE-RECEPTOR bond elicits a response in the target cell able to activate or inhibits complex signaling pathways, finally resulting into the hormone-specific BIOLOGIC EFFECT (e.g.: electric, metabolic, mechanic, secretory, transport of molecules)

ENDOCRINE SYSTEM SELF-REGULATION

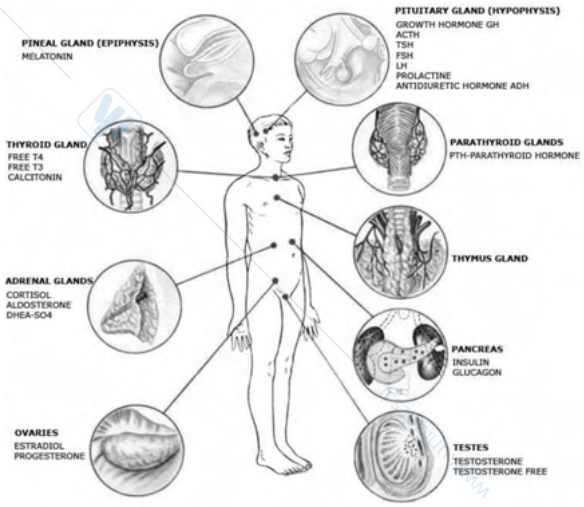
The ENDOCRINE SYSTEM is never static. Its components vary in function of physiologic stimuli, able to influence the hormone circulating level, which, in turn, influences the magnitude of the initial signal.

FEED-BACK turn off the communication line, to allow it to sense new stimuli

FEED-FORWARD



THE ENDOCRINE SYSTEM



ENDOCRINE CELLS AND GLANDS

- Endocrine cells able to sense stimuli, and to synthetize and secrete hormones.
- Endocrine glands are complex structures or organs secreting hormones in the bloodstream

•Endocrine cells are either organized in glands (localized endocrine system), or in simple groups of cells with variable ditribution in other organs (diffused endocrine system, neuroendocrine cells, APUD, in the gastric-intestinal tract)

what we call «HORMONES»

From the greek: ορμοσ,
Meaning "to excite" or «to arouse» or «setting in motion».

Defined in early XX century
by Ernest Henry Starling,
physiologist at the University College, London.

Famous experiments performed in 1902
by Starling and Bayliss.
Discovery of Secretin, the messenger between gastric-intestinal tract and pancreas.

Lecture at the Royal College of Physicians in London,
June 20th 1905.

HORMONES

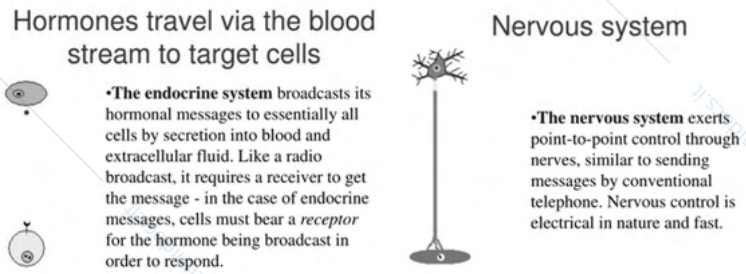
Professor
Ernest Henry Starling

«These chemical messengers (…) have to be carried from the organ where they are produced to the organs which they affect, by means of the bloodstream, and the continually recurring physiological needs of the organism must determine their repeated production and circulation through the body.»

HORMONE keynotes

- Hormones are synthesized and released <u>following biochemical signals</u> generated by various and different modulation systems
- Hormones are synthesized and secreted according to specific, dynamic and variable <u>chronobiologic rules</u>
- Hormones are usually sinthesized by <u>precursors</u>
- Hormones secreted in the bloodstream may be active or may require <u>activation by specific cells</u> in order to perform their action
- Some hormones stimulates the synthesis and the secretion of other hormones –<u>TROPIC</u>-
- Hormone action needs to be limited in time. Homeostasis is mantained by <u>feedback loops.</u>

THE BODY'S COMMUNICATION SYSTEMS



ENDOCRINE vs SYNAPTIC SIGNALING
a matter of time

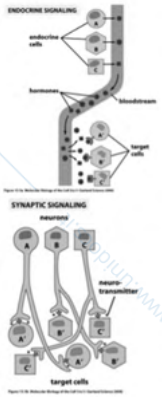
Unlike the nervous system, where information is transmitted very quickly, the endocrine system acts slowly.

Hormones released into the bloodstream need 5 – 10 seconds to elicit the first effect.

More often, hormones work within 30 minutes up to 3 hours.

Some, like growth hormone, give effects that are only visible after a few months.

ENDOCRINE vs SYNAPTIC SIGNALING
a matter of space



The contrast between endocrine and synaptic signaling. In complex animals, endocrine cells and nerve cells work together to coordinate the diverse activities of the billions of cells. Whereas different endocrine cells must use different hormones to communicate specifically with their target cells, different nerve cells can use the same neurotransmitter and still communicate in a highly specific manner.

(A) Endocrine cells secrete hormones into the blood, which signal only the specific target cells that recognize them. These target cells have receptors for binding a specific hormone, which the cells "pull" from the extracellular fluid.

(B) In synaptic signaling, by contrast, specificity arises from the synaptic contacts between a nerve cell and the specific target cells it signals. Usually, only a target cell that is in synaptic communication with a nerve cell is exposed to the neurotransmitter released from the nerve terminal (although some neurotransmitters act in a paracrine mode, serving as local mediators that influence multiple target cells in the area).

Long distance
Low concentration
(10^{-6} – 10^{-9} M)
Target specificity:
Hormone variety
Hormone Receptor

Short distance
High concentration
(10^{-4} M)
Target Specificity:
Synaptic Contact
Same neuro-transmitter, multiple functions

HORMONE CLASSIFICATION

Multiple criteria:

1. According to Chemical Nature
2. Production site
3. According to Function
4. On the basis of Mechanism of Action
5. According to Nature of Action
6. On the basis of Stimulation of Endocrine Glands
7. On the basis of Functional Interconnections

HORMONE CLASSIFICATION

ACCORDING TO THEIR CHEMICAL NATURE

- Peptides / Proteins /Glycoproteins
- Amine
- Steroids
- Eicosanoids

HORMONE CLASSIFICATION

ACCORDING TO THE PRODUCTION SITE

- Hypothalamus/Pituitary gland
- Thyroid/Parathyiroid
- Gastric-intestinal-pancreatic system
- Adrenal gland cortex and medulla
- Testicle/Ovary/Placenta

HORMONE CLASSIFICATION

ACCORDING TO FUNCTION

- Hypothalamic Neuro-hormones
- Pituitary Tropic Hormones
- Sex Hormones
- Metabolic and Trophic Hormones
- Vasoactive Hormones
- Hormones for water, saline and mineral homeostasis

HORMONE CLASSIFICATION

ON THE BASIS OF MECHANISM OF ACTION

- Group I hormones: binding to intracellular receptors (usually lipophilic) (steroids and thyroid hormones)

- Group II hormones: binding to cell surface receptors (usually water-soluble). Further divided according to the second messenger
  - cAMP
  - phospholipid, inositol, Ca^{2+}

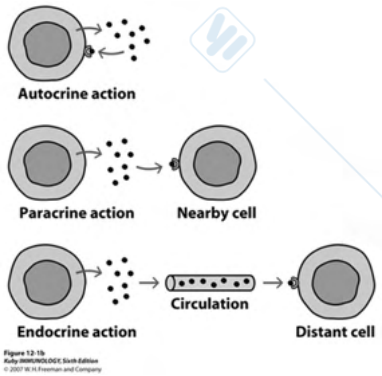
HORMONE CLASSIFICATION

ACCORDING TO THE NATURE OF ACTION

- Endocrine: distant effect
- Paracrine: local effect
- Autocrine: self effect

HORMONE CLASSIFICATION

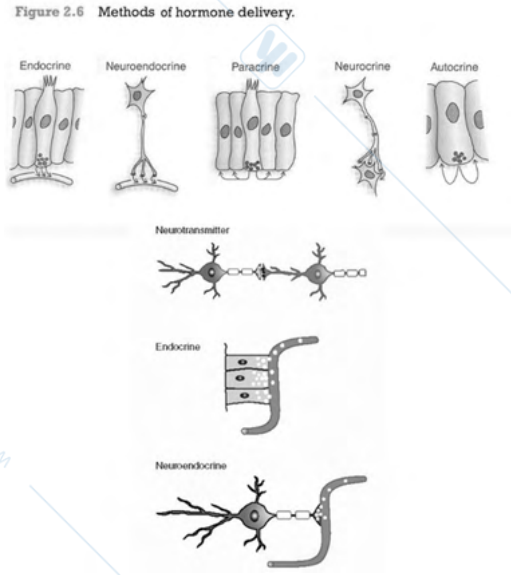
ACCORDING TO THE NATURE OF ACTION



Released in the interstitial fluid to act on the releasing cell
E.g.: cytokines, growth factors

Released in the interstitial fluid to act on adjacent cells
E.g.: eicosanoids

Traveling through the bloodstream to distant targets
E.g.: thyroid hormones



HORMONE CLASSIFICATION

ON THE BASIS OF STIMULATION OF ENDOCRINE GLANDS

- Tropic hormones: stimulate other endocrine cells/glands
- Non-tropic hormones: non-endocrine target tissues

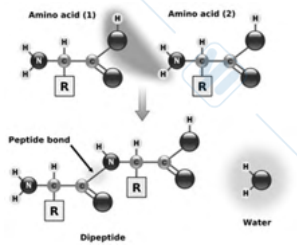
HORMONE CLASSIFICATION

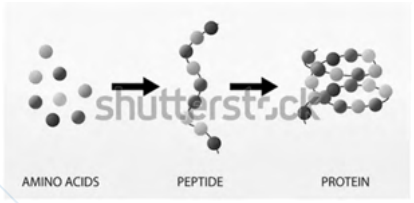
ON THE BASIS OF FUNCTIONAL INTERCONNECTIONS

The AXES

- Hypothalamus – Pituitary - Thyroid
- Hypothalamus – Pituitary - Adrenal
- Hypothalamus – Pituitary - Gonads
- Renin – Angiotensin - Aldosterone

PEPTIDE/PROTEIN/GLYCOPROTEIN HORMONES





<50 aa <5000 Da

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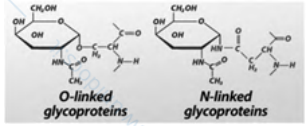
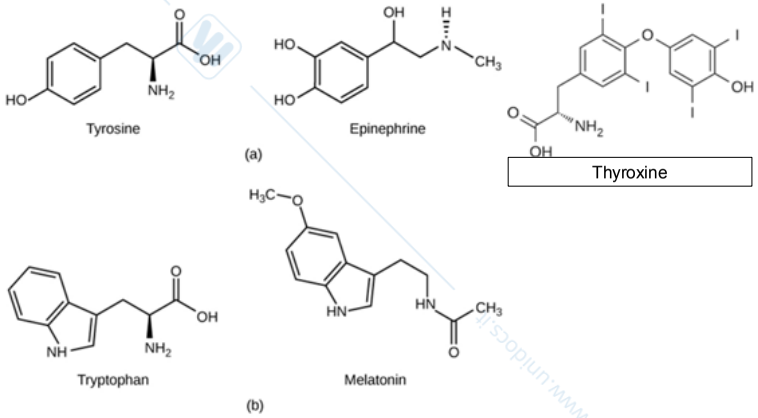


MAJOR PEPTIDE/PROTEIN/GLYCOPROTEIN HORMONES

Hormone	N° of aa	Production Site	Main biological function
Peptides and polypeptides			
Adrenocorticotropic hormone (ACTH, corticotropin)	39	Adenohypophysis	Regulates adrenal gland secretion
Angiotensin (I and II)	10 and 8	Kidney	Regulates arterial blood pressure
Antidiuretic Hormone (ADH, vasopressin)	9	Neurohypophysis	Regulates water absorption at the kidney level
Calcitonin	32	Thyroid	Regulates Ca^{2+} metabolism
Cholecystokinin (CCK)	8 - 59	Intestine	Regulates intestine mobility and secretions; neurohormone
Endothelin	21	Vascular endothelium	Vasoconstriction, hypertensive
Gastrin	14-34	Gut	Regulates acidity in the gut lumen
Glucagon	29	Pancreas	Increases glycaemia
Insulin	51	Pancreas	Regulates glucose utilization
Corticotropin-Releasing Hormone (CRH)	41	Hypothalamus	Regulates ACTH production
Gonadotropin Releasing Hormone (GnRH)	10	Hypothalamus	Regulates LH and FSH production
Growth Hormone Releasing Hormone	29	Hypothalamus	Regulates GH production
Thyrotropin Releasing Hormone	3	Hypothalamus	Regulates TSH production
Atrial natriuretic peptide (ANP)	28	Heart	Regulates the diuresis
Oxytocin	9	Neurohypophysis	Regulates lactation and uterus motility in pregnancy
Parathyroid hormone (PTH)	34	Parathyroids	Regulates Ca^{2+} metabolism
Vasoactive intestinal polypeptide (VIP)	28	Intestine; pancreas	Regulates intestine mobility and secretions; neurohormone
Pancreatic polypeptide (PP)	36	Intestine	Regulates intestine mobility and secretions
Prolactin (PRK)	199	Adenohypophysis	Regulates milk production
Renin	340	Kidney	Regulates arterial blood pressure
Somatomedin-C or Insulin like Growth Factor (IGF-1)	70	Liver	Mediates the effects of GH
Somatotropin or Growth Hormone (GH)	191	Adenohypophysis	Regulates growth
Glycoproteins			
Follicle-stimulating Hormone (FSH)	92+111	Adenohypophysis	Regulates gonad hormone production
Lutenizing Hormone (LH)	92+120	Adenohypophysis	Regulates gonad hormone production
Thyroid-stimulating Hormone (TSH)	92+118	Adenohypophysis	Regulates thyroid hormone production
Chorionic Gonadotropin (hCG)	237	Placenta	Regulates pregnancy progression
Erythropoietin	165	Kidney	Regulates red cell production at the bone marrow

MAJOR AMINO ACIDS DERIVED HORMONES

Mw: 200 – 800 Da

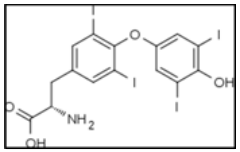


MAJOR AMINO ACIDS DERIVED HORMONES

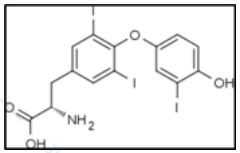
Hormone	Production site	Main biological function
Tryptophan-derived		
Melatonin	Pineal gland	Regulates wake-sleep cycle
Serotonin	Hypothalamus and gastric-intestinal tract	Neuromodulator
Tyrosine-derived		
Epinephrine and Norepinephrine	Adrenal	Regulates stress response and arterial blood pressure
Dopamine	Hypothalamus	Neuromodulator
Thyroxine and	Thyroid	Regulates energy metabolism
Triiodothyronine	Thyroid and peripheral tissues	Regulates energy metabolism

IODOTHYRONINE
THYROID HORMONES

THYROXINE – T4



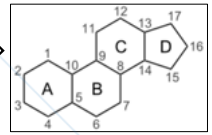
TRIIODOTHYRONINE – T3



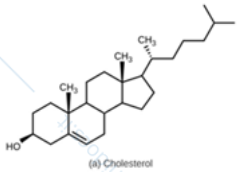
In 2016 levothyroxine, a manufactured form of thyroxine, was the most prescribed medication in the United States with more than 114 million prescriptions

STEROID HORMONES

cyclopentane perhydrophenantrene ⇨



cholesterol



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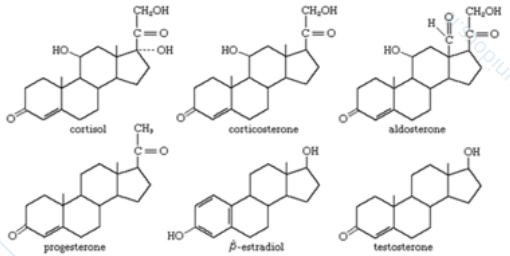
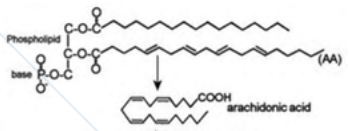


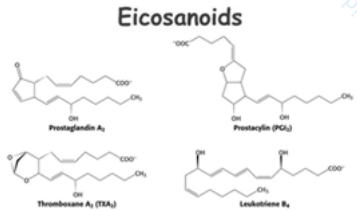
MAJOR STEROID HORMONES

Hormone	Production site	Main biological function
Aldosterone	Adrenal	Regulates arterial blood pressure
Corticosterone	Adrenal	Regulates arterial blood pressure
Cortisol	Adrenal	Regulates glucose and protein metabolism; tissue reactivity etc…
Dihydrotestosterone (DHT)	Skin; prostate, seminal vescicles etc…	Androgenic action, masculinization
Testosterone	Testicle	Androgenic action, anabolic
Estradiol (E2)	Ovary	Regulates menstrual cycle; feminization
Progesterone	Ovary	Regulates menstrual cycle
25OH-colecalciferol (25OH-VitD)	Liver	Regulates Ca^{2+} metabolism (intestinal absorption)
1,25(OH)2-colecalciferol (1,25(OH)2-VitD)	Kidney	Regulates Ca^{2+} metabolism (intestinal absorption)

EICOSANOIDS
HORMONES DERIVED BY POLY-UNSATURATED FATTY ACIDS

Mw: 200 – 500 Da





MAJOR EICOSANOID HORMONES

Hormone	Production site	Main biological function
Prostaglandin	Prostate and others	Regulates inflammatory response and capillary circulation
Prostacyclin	Blood vessel wall	Regulates capillary circulation and coagulation
Thromboxane	Platelets and others	Regulates inflammatory response and coagulation
Leukotriene	Various	Regulates inflammatory response

HORMONE TRANSPORT IN THE BLOODSTREAM

- Steroid hormones, unlike protein hormones, are sinthesized on demand and rapidly secreted in the bloodstream. Due to their lipophilic nature, cannot be stored.
- Carriers acts as a hormone *reservoir*, ensuring hormone distribution throughout the body, and protecting them from inactivation and excretion (bile, urine).
- Hormone-specific carriers are: sex hormone binding globulin -SHBG-, corticosteroid binding globulin -CBG- (globuline leganti i corticosteroidi), thyroxine-binding globulin -TBG-
- Albumin is a non-specific carrier, but in large abundance in blood.

HORMONE TRANSPORT IN THE BLOODSTREAM

• Water soluble hormones, like protein and small polar catecholamines, can easily travel in the bloodstream in free form, and are rapidly degraded.

• Small lipid hormones such as steroids or thyroid hormones are water-insoluble. To be vehiculated in the bloodstream they bind to carrier plasma proteins: large part is trasported by spefic glycoproteins, whereas a minor part is bound to albumin.

• Carrier proteins play important role of in physiologic and pathologic conditions, by influencing hormone reserve, availability, activity, catabolism, plus direct functions of carrier-hormone complex

HORMONE RECEPTORS

• Protein hormones and catecholamines act through plasma membrane receptors (group II).

• Steroids and iodothyronines enter the cell and bind citosolic or nuclear receptors (group I).

• The transport across the cell membrane may be passive (steroids) or mediated by specific transporter (iodothyronines).

• Some hormones enter the cell bound to their specific carrier (estrogens – SHBG).

Principal functions of the endocrine system

- Maintenance of the internal environment in the body (maintaining the optimum biochemical environment).
- Integration and regulation of growth and development.
- Control, maintenance and instigation of sexual reproduction, including gametogenesis, coitus, fertilization, fetal growth and development and nourishment of the newborn.

FUNCTIONS OF THE ENDOCRINE SYSTEM

HOMEOSTASIS

Many hormones are involved in the manteinance of internal physical-chemical physiologic conditions.

- Temperature: iodothyronine

- Hydrosaline equilibrium (water volume and electrolites): ADH, ANP, Aldosterone, PTH, Calcitriol

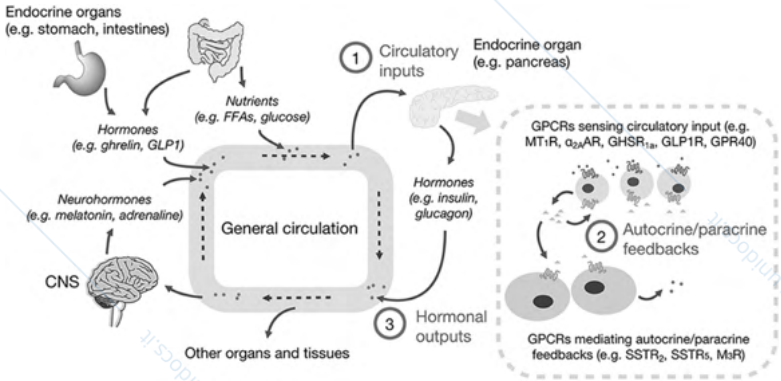
- Arterial Blood Pressure and cardiac activity: noradrenalin, adrenalin, angiotensin II, Endothelin, ANP, prostaglandin

- Response to stress: ACTH-Cortisol, Adrenalin

FUNCTIONS OF THE ENDOCRINE SYSTEM

ENERGY METABOLISM

Many hormones are involved in regulating food absorption (Gastrin, Cholecystokinin, Secretin,…) and metabolization (Insulin, Glucagon, Adrenalin, Glucocorticoids, GH, Iodothyronines) for ensuring the synthesis of new substances and the production of energy that can be used for biological processes or stored in depots (fat; glycogen).



FUNCTIONS OF THE ENDOCRINE SYSTEM

GROWTH AND DEVELOPMENT

Many hormones have essential importance for ensuring growth and development in the right direction, timing and entity, at the

neuropsychologic level:
Thyroid Hormones

somatic level:
Thyroid Hormones, GH-IGF-1, Insulin, sex steroids, PTH-Calcitriol

FUNCTIONS OF THE ENDOCRINE SYSTEM

REPRODUCTION

- Gametogenesis: Gonadotropins
- Libido and erectile function: Testosterone
- Fertilization, Nesting, Pregnancy: Estrogens, Progesteron, Chorionic Gonadothropin
- Delivery: Oxytocin
- Breast-feeding: Oxytocin and Prolactin

FUNCTIONS OF THE ENDOCRINE SYSTEM

REPRODUCTION

Many hormones are crucial regulators of all the processes inolved in reproduction. These key players are regulated by different mechanism and act with a specific timing and in proper amount.

- Embryo sexual differentiation: requires production or suppression of Testosterone and its conversion to DHT.
- Pubertal development of secondary sexual features (external and internal genitalia, breast, hair, fat distribution, bone and muscle development) and differentiation of sexual behaviour: sex steroids – estrogens and androgens are present in both sexes but in different proportion

HORMONE TYPE OF ACTION

Biological effects of a given hormone can be:

- SINGLE: selectively acting on one type of target cell.
  E.g.: TSH only acts on thyroid follicular cells
- MULTIPLE: acts on various type of target cells.
  E.g.: Iodothyronines act on almost all cells in the body

HORMONE TYPE OF ACTION

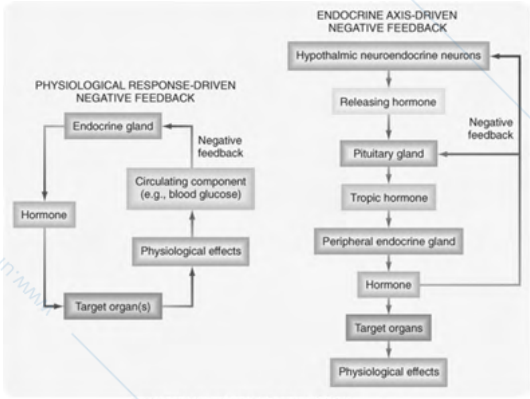
Biological effects of multiple hormones can INTERACT:

- SYNERGISTIC: two or more hormones have the same effect on target cells. E.g.: Glucagone, Epinephrin, Cortisol, GH, iodothyronine: all contribute to increase glycaemia.
- ANTAGONIST: the effect of one hormone is counteracted by the effect of a second hormone. E.g.: Insulin lowers glycaemia, in contrast with Glucagone, Epinephrin, Cortisol, GH, iodothyronine.
- PERMESSIVE: the effect of one hormone is only realized in presence of the effect of a second hormone. E.g.: iodothyronines stimulate the expression of epinephrine receptors.
- MODULATOR: the effect of the hormone is diminished or enhanced by the effect of another hormone. E.g.: angiotensin II vasoconstriction effect at the kidney is diminished by vasodilator prostaglandines (induced by AgII).

REGULATION OF ENDOCRINE RESPONSES

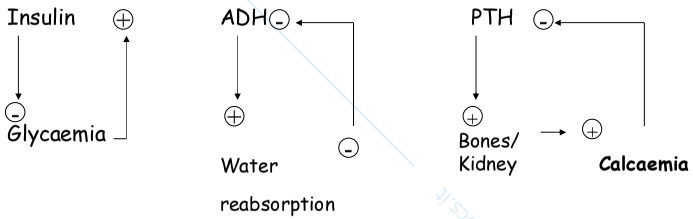
FUNCTIONAL FEEDBACK vs HORMONAL FEEDBACK

Guaranteeing system responsiveness



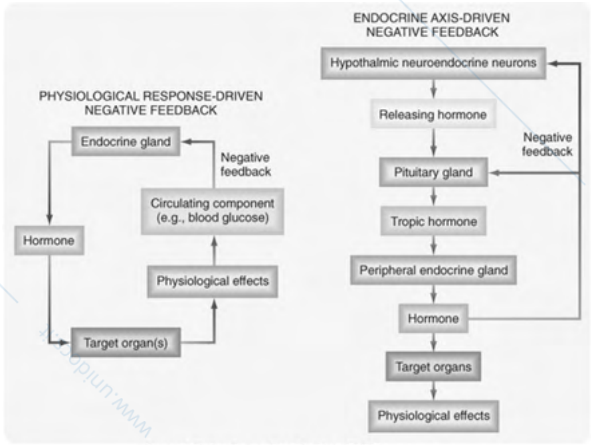
FUNCTIONAL FEEDBACK

Hormone secretion is regulated by the functional effects induced in the target cells.

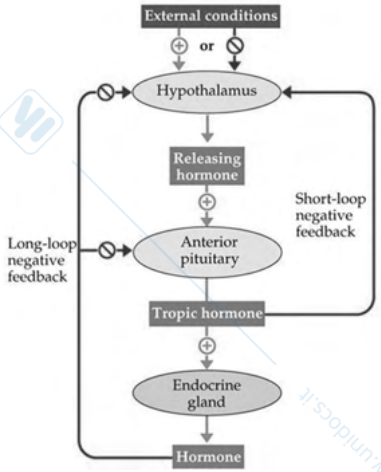


REGULATION OF ENDOCRINE RESPONSES

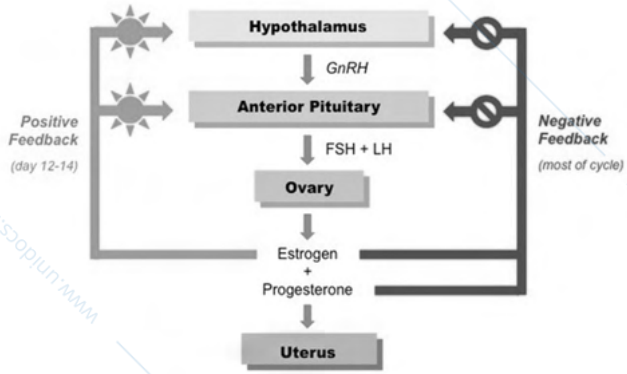
FUNCTIONAL FEEDBACK vs HORMONAL FEEDBACK



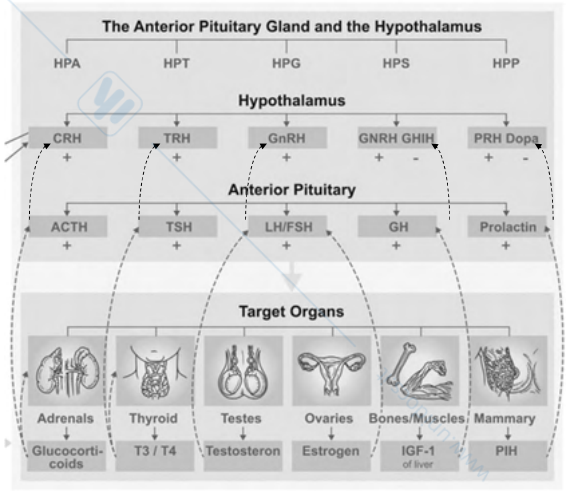
HORMONAL FEEDBACK REGULATION MULTIPLE LEVELS



NEGATIVE OR POSITIVE (OR BOTH) FEEDBACK REGULATION



HORMONE FEEDBACK



SECRETION RHYTHMS AND PULSATILITY

Secretion of many hormones is not continue but PULSATILE:

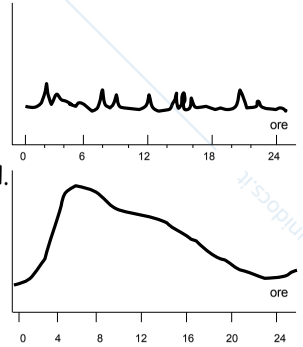
Basal hormone concentration in blood is low, but increases in sudden and transitory peaks called SECRETORY BURSTS.

Bursts may vary in frequency and amplitude, thereby influencing blood level.

Message is not only represented by hormone level in the blood

Changes in frequency and amplitude pattern represent a communicating code

The hormone secreting time pattern determines regular fluctuation of blood levels, known as RHYTHMS.

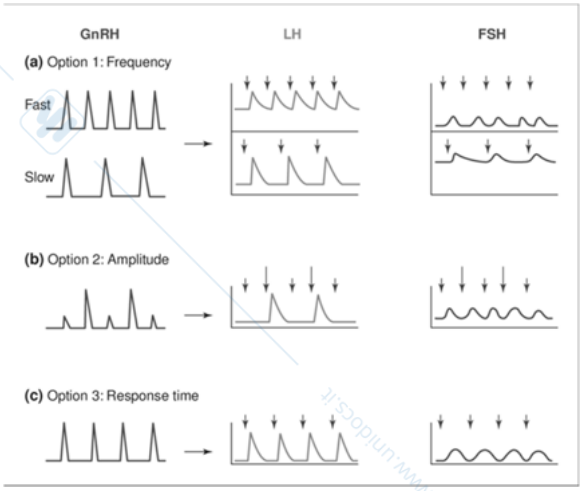


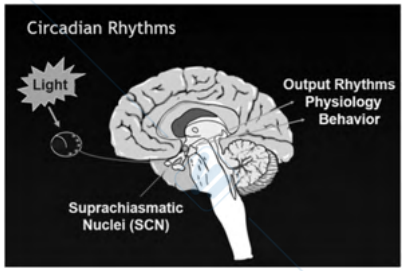
SECRETION RHYTHMS AND PULSATILITY

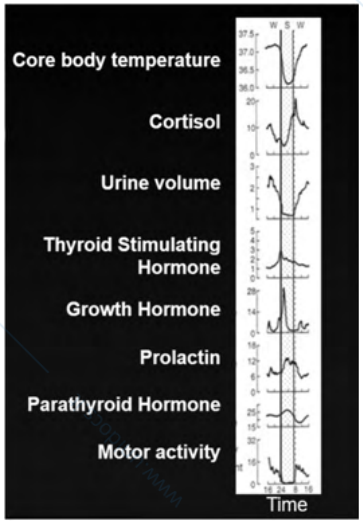
high **frequency** GnRH pulses favours the LH over FSH concentration; low frequency GnRH favouring FSH over LH

low **amplitude** GnRH selectively releasing FSH over LH

changes in **response time**, with FSH taking longer to respond than LH



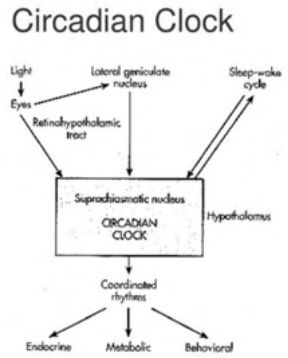




MEDIATORS OF HORMONE RHYTHM

Complex neural circuits integrated at the hypothalamic level – Suprachiasmatic Nucleus

Sensing day/night and wake/sleep alternations.



Episodic secretion of hormones

- Response-stimulus coupling enables the endocrine system to remain responsive to physiological demands
- Secretory episodes occur with different periodicity
- Pulses can be as frequent as every 5-10 minutes

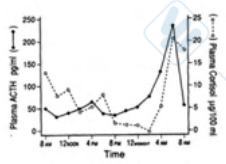
Rhythm according to periodicity:

- ~ 1 hour: circhoral
- < 24 hours: ultradian
- ~24 hours: circadian
- > 24 hours: infradian

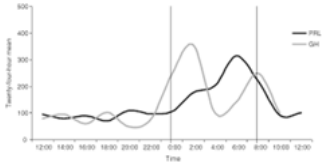
CIRCADIAN RHYTHM

Most of the hormones are CIRCADIAN: blood level fluctuates, varying from maximum (AZIMUT) and minimal (NADIR) levels, to rise again at the following cycle.

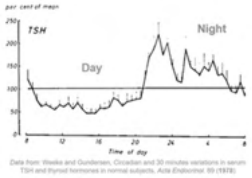
CORTISOL and ACTH: azimut around 4 am, followed by a slow decrease ending in the nadir around midnight



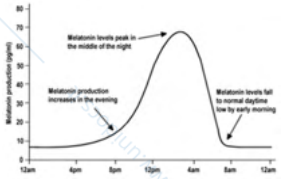
GH and PRL: azimut in the night, fllowed by a steep decrease, then keeping constant until the next sleep



TSH: azimut around 20-24 am, nadir 9-12 am



Melatonin: increases in the evening, azimut around 2 am, then steep decrease. Flat low levels in the morning hours



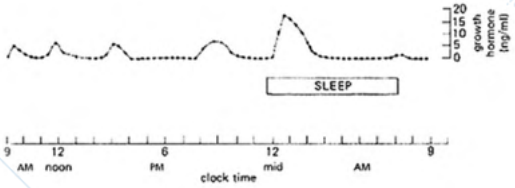
ULTRADIAN RHYTHM

recurrent periods (bursts) throughout the 24h

e.g.:

TSH: bursts every 90-120 min

GH: variable pattern



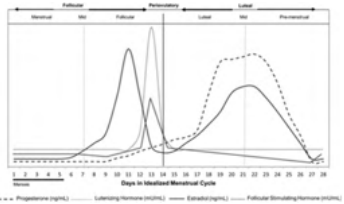
INFRADIAN RHYTHM

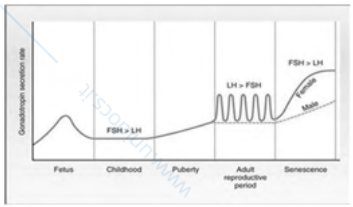
Long term fluctuation:

Gonadotropines and Sex hormones

FSH-LH, estrogens and progesterone

- Menstrual fluctuation over 28days
- Lifetime fluctuation
  - pre-puberale
  - Reproductive
  - Menopausal/Senescence





Real life consequences of the knowledge about hormone secretion modes and feedback systems:

Hormone laboratory testing in research and clinical endocrinology

1) The concentration of a certain hormone is to be determined together with other hormones and parameters influencing its level - SIMULTANEOUSLY

iodothyronines	+	**TSH**
sex steroids	+	**FSH-LH**
cortisol	+	**ACTH**
Aldosterone	+	**Renin, Na, K**
Insulin	+	**Glucose**
PTH	+	**Calcium-Phosphate**
GH	+	**IGF-1**

2) Taking into account the cycle trend of hormone with a secretion rhythm:
- **ACTH and cortisol are higher in the morning and lower in the evening (circadian rhythm).**
- **FSH/LH, estradiol and progesterone have different fluctuation across the follicular, ovulatory and the luteal phase (infradian rhythm).**

3) Taking into account that for plusatile hormones a single evaluation may be not exaustive, as it may have been randomly performed during a secretory burst (high) or far from it (low).

4) Considering the impact of stress (cortisol, ACTH, prolactin), of training (GH, prolactin) or of drugs on some hormone levels.

5) Meaningful hormone levels may be obtained in conditions that simulate a physiological increase or suppression of its secreton (functional tests of stimulation / inhibition).

HORMONE MECHANISMS OF ACTION ON TARGET CELLS

In order to provide a response to the hormone signal, the target cell needs to be equipped with

1. A device to recognize the hormone: specific receptors

RECEPTORS: Highly specific proteins displaying a selective binding for a certain compound (ligand) and for structural analogues (agonists)

2. A machinery for transforming the signal represented by the hormone-receptor bound into a cascade of reactions ending up in the biologic response: receptor down-stream events.

The Ligand-Receptor complex induces the allosteric modification of the receptor, altering (usually activating) its biological activity, and triggering a series of reactions.

HORMONE RECEPTORS ACT AS TRANSDUCTOR OF HORMONE SIGNAL

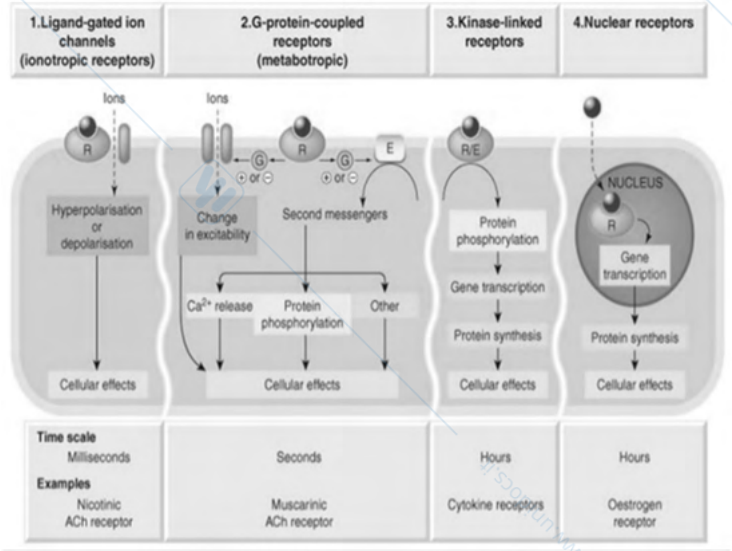
HORMONE MECHANISMS OF ACTION ON TARGET CELLS

Major feature of a RECEPTOR:

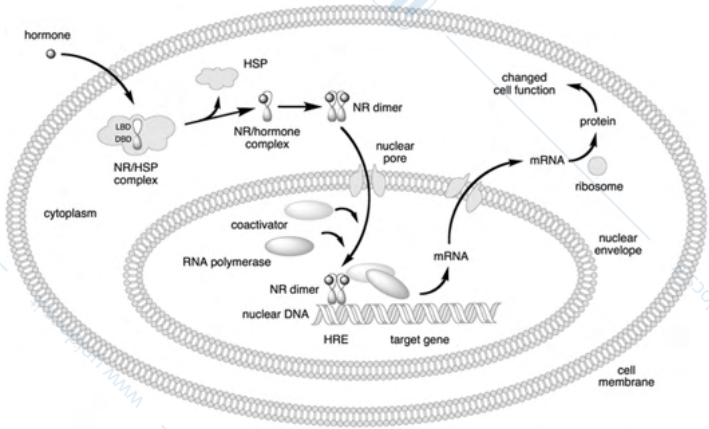
affinity, specificity, saturability

Classification of Receptors based on cell localization:

- Membrane receptors:
  catecholamine, peptide hormones, prostanoids
- Intracellular (cytosol, nucleus) receptors:
  iodothyronine, steroid hormones

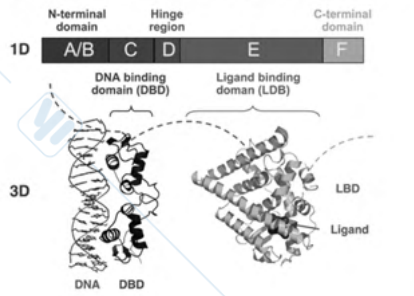


HORMONES ACTING AT THE NUCLEAR LEVEL



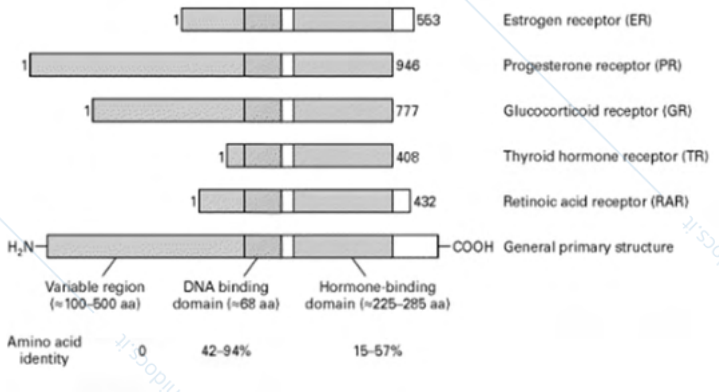
Changing gene expression.
«Genomic function»

Structural Organization of Nuclear Receptors

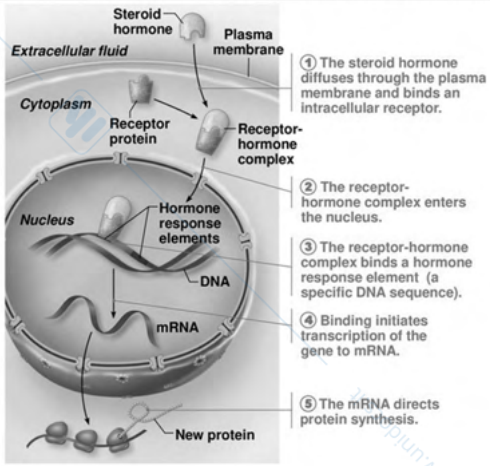


A/B Domain: regulatory domain at the N-term. Variable.
C Domain: DNA-binding domain. It is composed of the zinc-finger structure and binds the hormone response elements (HRE) on target genes.
D Domain: central flexible structure.
E Domain: Ligand binding domain.
F Domain: C-term.

NUCLEAR RECEPTOR SUPERFAMILY

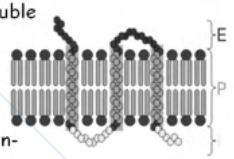


Mechanisms of Steroid Action



PLASMA MEMBRANE RECEPTORS

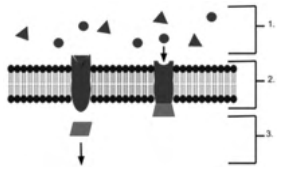
Integral membrane protein permanently included in the double phospholipid layer.



- Extracellular domain exposing the ligand binding site
- Transmembrane domain
- Intracellular domain exposing the activation site for down-stream post-receptor reactions.

Converts the external signal carried by the hormone in a within-cell signal triggering a cascade of down-stream events ending up in a biological response.

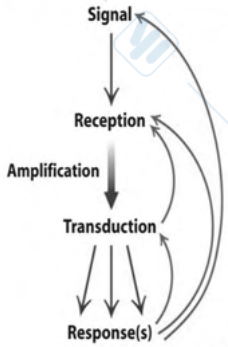
Classification based on the mechanism of signal transduction:



- G-protein coupled receptors
- Tyrosin-kinases receptors
- Citokine receptor
- Ion channel-coupled receptors

SIGNAL TRANSDUCTION PATHWAYS ASSOCIATED WITH MEMBRANE RECEPTORS

Signal transduction depends on molecular circuits



1. Release of the primary messenger. - ligand
2. Reception of the primary messenger.
3. Delivery of the message inside the cell by the second messenger(cAMP, cGMP, Ca^{2+}, IP_3, DAG...).
4. Activation of effectors that directly alter the physiological response.
5. Termination of the signal.

HORMONES CLASSIFICATION ACCORDING TO THE RECEPTOR TYPE

Tab. 1-III – Classificazione dei recettori di membrana.

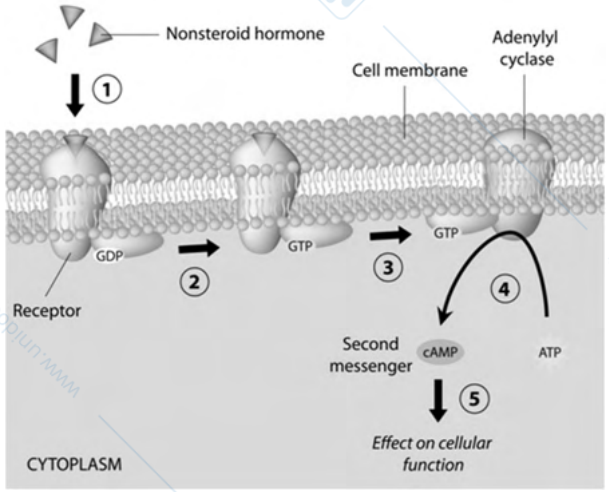
Recettore	Segnale
Recettori accoppiati alle G-protein [G protein-coupled seven transmembrane receptor (GPCR)]	
TSH, FSH, LH, ACTH, MSH, PTH, GHRH, CRH, glucagone	Canali del calcio
TRH, GnRH	G_q, G_{11}
α-adrenergico	Giα
β-adrenergico	Gsα, adenilato-ciclasi
Recettori tirosino-chinasici	
IGF-1, insulina	Tirosino-chinasi, IRS
Nerve growth factor, epidermal growth factor	Tirosino-chinasi, Ras
Recettori delle citochine	
GH, PRL	Janus kinase, tirosino-chinasi
Recettori serino-chinasici	
Transforming growth factor β, anti-mullerian hormone, activine	Serino-chinasi

UNIENDO cap. 1 pag.9

HORMONES CLASSIFICATION ACCORDING TO THE RECEPTOR TYPE

Adenylyl Cyclase Mechanism (cAMP)	Phospholipase C Mechanism (IP_3/Ca^{2+})	Steroid Hormone Mechanism	Tyrosine Kinase Mechanism	Guanylate Cyclase Mechanism (cGMP)
ACTH	GnRH	Glucocorticoids	Insulin	Atrial natriuretic peptide (ANP)
LH	TRH	Estrogen	IGF-1	Nitric oxide (NO)
FSH	GHRH	Progesterone	Growth hormone	
TSH	Angiotensin II	Testosterone	Prolactin	
ADH (V_2 receptor)	ADH (V_1 receptor)	Aldosterone		
HCG	Oxytocin	1,25-Dihydroxycholecalciferol		
MSH	α_1 Receptors	Thyroid hormones		
CRH				
Calcitonin				
PTH				
Glucagon				
β_1 and β_2 receptors				

G-PROTEIN COUPLED RECEPTORS



a) G PROTEIN (TRANSDUCTOR)

-Transmits the signal from the Receptor to the Effector.

- Bound to Guanosinic nucleotides (GDP-GTP).

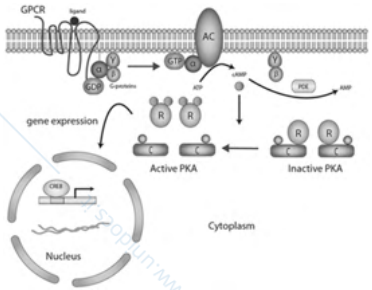
- Structure: heterotrimers of α, β, γ subunits. β and γ subunits are common to many G-protein type. α subunits is different in each G-protein type.

- α subunit shows intrinsic GTP-ase potential which is inhibited in the $\alpha+\beta+\gamma$ complex. GTP-ase activity starts when α is released by the complex, following the Hormone-Receptor binding.

- Different G Proteins act on different Effettors.

b) MEMBRANE EFFECTOR

Membrane proteins activated by the signal transmitted by the G-protein. Once activated, they increase or reduce the cytosol level of second messengers.



Effectors can be

- enzimes

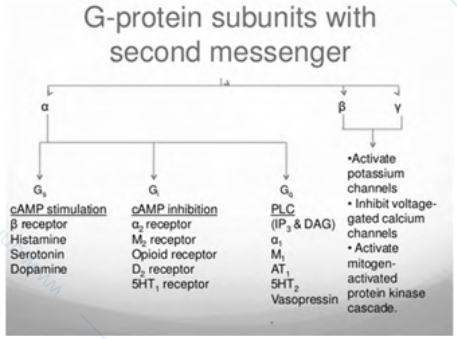
ADENYLYL CYCLASE: AC
GUANYLYL CYCLASE: GC
PHOSPHOLIPASE C: PLC
PHOSPHOLIPASE A2: PLA2

- ion channels (Ca^{2+}, K^+)

c) SECOND MESSENGERS

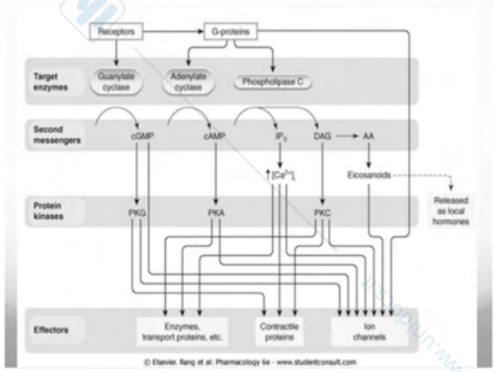
Cytosol small molecules whose level is regulated by Membrane Effectors. Able to influence the activity of downstream Kinase-Protein in the cytoplasm.

- Cyclic nucleotides : **cAMP** and **cGMP**
- Phospholipid derivative: **DAG** and **phosphatidyl-inositol (IP3)**
- Arachidonic acid-derivative: **Prostanoids**
- **Ca++**



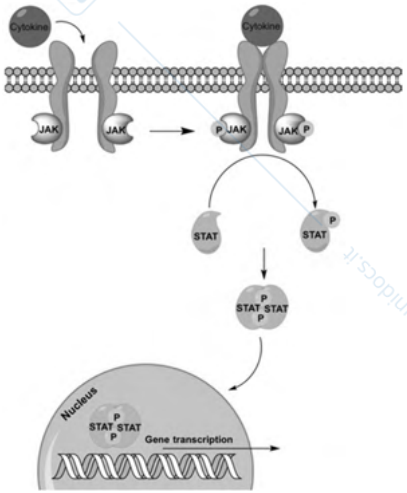
d) PROTEIN-KINASE (PK)
Cytoplasm protein with phosphorilating potential.
Regulated by second messengers

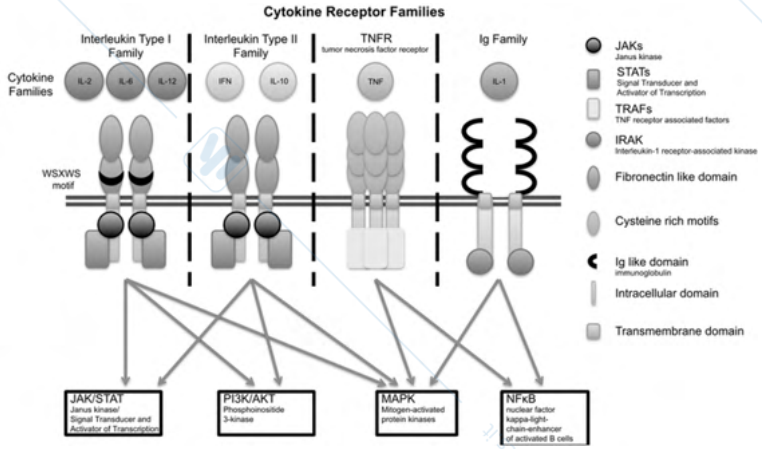
PKA:	regulated by	cAMP
PKG:	regulated by	cGMP
PKC:	regulated by	DAG
PK-Calcium:	regulated by	Ca++



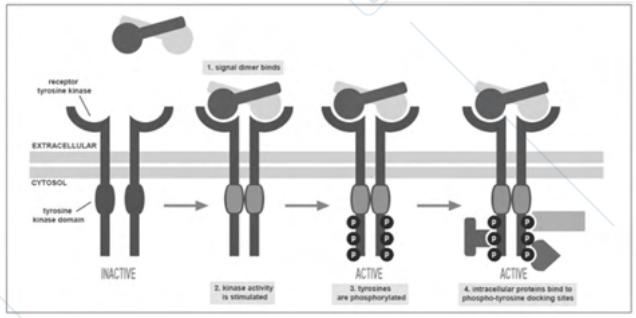
CYTOKINE RECEPTOR

Cytokine receptors are transmembrane glycoproteins commonly composed of several subunits.
There are different types of cytokine receptors regarding the composition of subunits. We have monomeric and multimeric receptors, and the latter can be homo- or heteromultimeric.
Binding of extracellular ligands to cell-surface receptors can recruit additional molecules in the ligand-receptor complex within the cell membrane and in the cytosol.



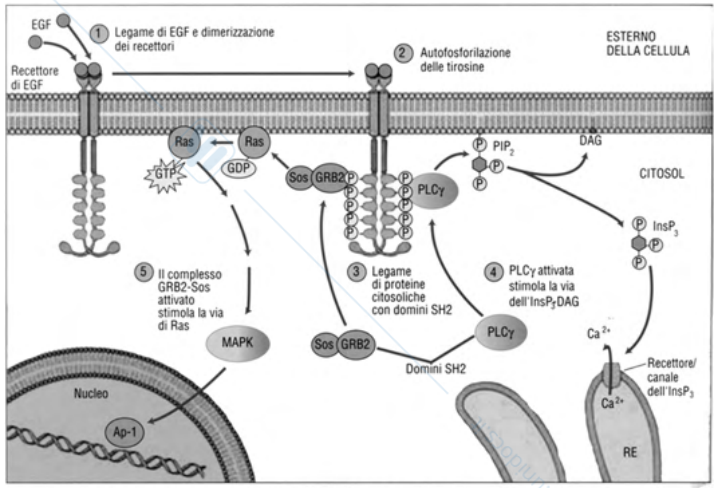


TYROSIN-KINASE RECEPTORS



TKr are characterized by specific domains within the extracellular portion that interact with the ligand, a single transmembrane domain and a tyrosine kinase domain in part exposed to the cell interior. The tyrosine kinase activity is tightly regulated by multiple autoinhibitory mechanisms including an inhibitory conformation of the extracellular domain, the transmembrane domain, the juxtamembrane domain, and the activation loop. Ligand binding to the extracellular domain causes a conformational switch that leads to the activation of the tyrosine kinase domain.

TYROSIN-KINASE RECEPTORS



Esempio delle vie di trasduzione associate a recettori per fattori di crescita.

TYROSIN-KINASE RECEPTORS

Membrane receptors with INTRINSIC kinase activity.

In the non ligand bound form the activity is inhibited.

Activates following dimerization induced by ligand-receptor binding.

STRUCTURE: membrane integral protein in monomeric or tetrameric form.

E.g.: Insulin-R, IGF-1R, growth factors (PDGF-R, EGF-R)

INSULIN-R and IGF-1R are HETEROTETRAMERS:

The single heterodimer is composed by α and β subunits holded together by S-S bridges.

Two heterodimers are holded together by S-S bridges on the α subunits.

- α subunits contain the insulin binding-site
- β subunits contain the extracellular, transmembrane and intracellular domains including the catalitic domain.

HUMAN ENDOCRINE DISEASES

1. Hormone over-production
2. Hormone under-production
3. Altered hormone response at the target tissue
4. Tumours of the endocrine glands or other tissues impacting on hormone secretion.

Next lessons

Biotechnologist role in Endocrinology

Like in other biomedical disciplines….

- Basic research
- Biomarker discovery
- Drug development

Plus…

Endocrinologists are required to assess patients and diagnose endocrine disorders through extensive use of <u>laboratory tests</u> for

HORMONE MEASUREMENT

HOW ARE HORMONES MEASURED?

Next lessons

LABORATORY MEDICINE and ENDOCRINOLOGY

*Aim of a quantitative assay: to generate **reliable** quantitative information*

ANALYTICAL REQUIREMENTS

- Sensitivity
- Specificity
- Precision
- Accuracy

CLINICAL REQUIREMENTS

- DIAGNOSTIC POWER
  - Sensitivity
  - Specificity
- EFFECTIVE THERAPY
- GOOD PATIENT CARE

QUANTITATIVE ANALYSIS in ENDOCRINOLOGY

PRE-ANALYTICS

WHAT? hormones, lipidids, peptides, metabolites

WHERE? plasma, serum, saliva, urine, faeces, tissues, hair, nails, CSF...

WHEN? awake, early morning, night; menstrual phase, season

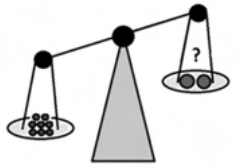
ANALYTICS

HOW?

- ✓ Ligand Binding and Immunoassay
- ✓ High Performance/Pressure Liquid Chromatography (HPLC)
- ✓ Gas Chromatografy – Mass Spectrometry (GC-MS)
- ✓ Liquid Chromatography – Tandem Mass Spectrometry (LC-MS/MS)

QUANTITATIVE ANALYTICAL METHOD

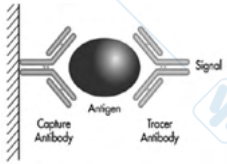
Collect *Prepare* *Identify* *Isolate* *Detect*



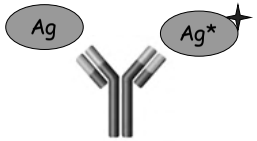
Report

IMMUNOASSAY …based on an indirect feature of the molecule

Non competitive



Competitive…



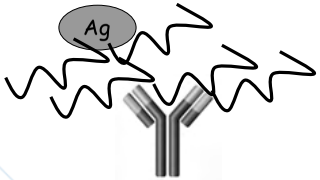
RIA, FIA, IRMA, ELISA, ECLIA, CLIA…

HIGH THROUGHPUT:

- REAGENTS SUPPLIED AS KIT
- AUTOMATIZATION
- POOR SAMPLE PURIFICATION

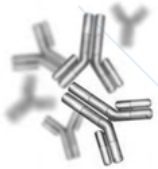
THE SPECIFICITY PROBLEM

…underestimation

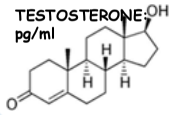


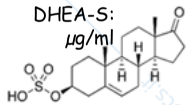
MATRIX INTERFERENCE

overestimation…

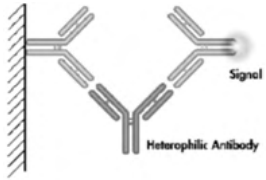


STRUCTURALLY RELATED MOLECULES

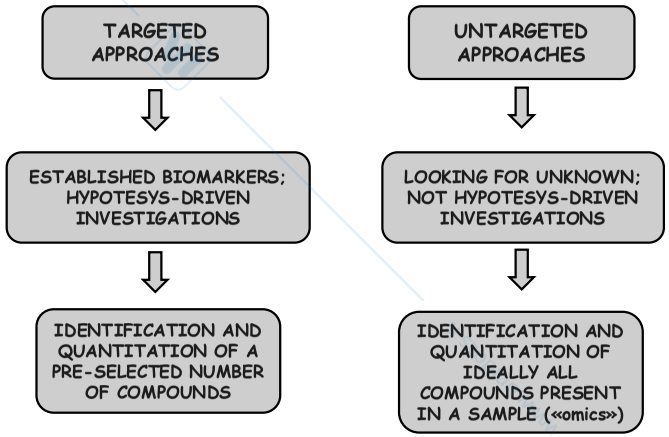




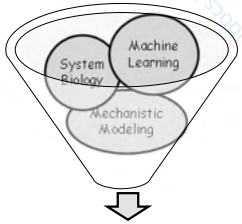
HETEROPHILIC INTERFERENCE



MASS SPECTROMETRY-BASED APPROACHES TO PROTEIN AND METABOLYTE ANALYSIS



PAST	PRESENT	FUTURE
Immunoassays	LC-MS/MS	LC-High Resolution MS
Single analyte measurement of a few hormones	Targeted measurement of panels of hormones	Untargeted analysis of all hormones in a certain matrix
High Throughput Poor specificity	High Specificity Top Quantitation Range	Top Specificity Good Quantitation Range
Reference interval Decision Limits	Combination of RI and DL, Relationships among targeted hormones	Multidimensional Model

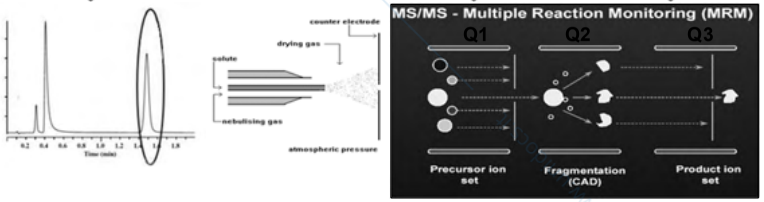


Metabolomics / Proteomics

LC-MS/MS: based on intrinsic feature of the molecule

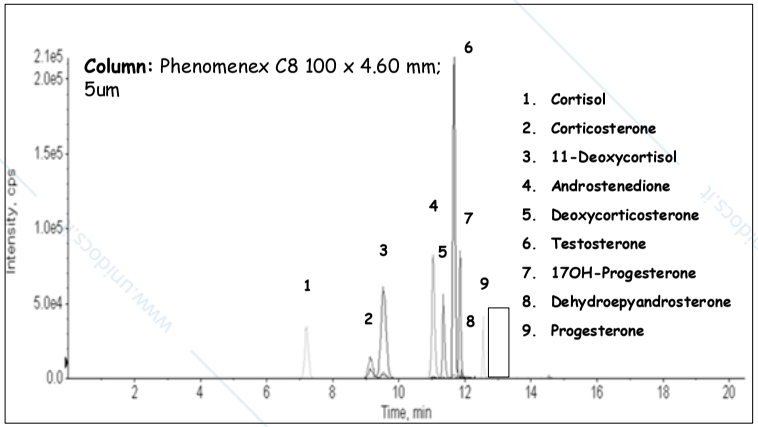
3 SPECIFICITY STEPS

Liquid Chromatography	Atmospheric Pressure Ionization "soft" sources	Mass Spec 1	Mass Spec 2
RETENTION TIME	ESI	MOLECULAR WEIGHT	STRUCTURE
Interaction with a solid phase	APCI	Selection of the Precursor Ion	Selection of Fragments
	APPI		
	+ -		

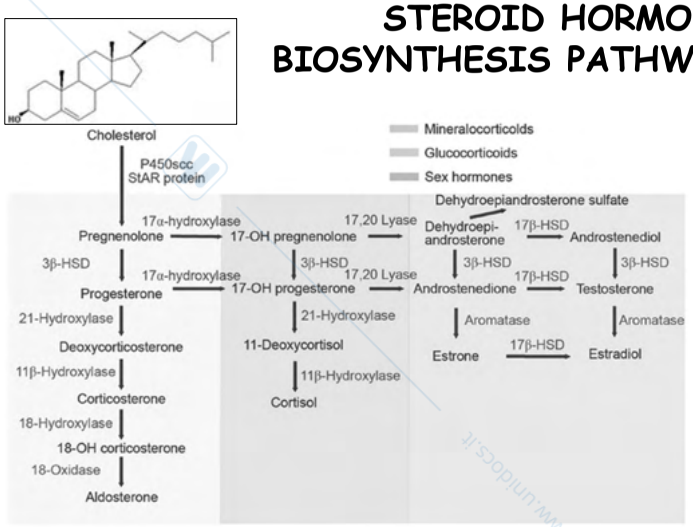


Fanelli F et al Steroids 2011

SIMULTANEOUS PROFILING OF NINE SERUM STEROIDS



STEROID HORMONE BIOSYNTHESIS PATHWAY



OBESITY

ADIPOSE TISSUE AS AN EVOLUTION STRATEGY

Wells JCK Dis Model Mech 2012

The pan-human profile of adiposity was shaped over our evolutionary past, reflecting ecological pressures that favored a number of unusual traits that are characteristic of our species. These traits include large brains and fully bipedal locomotion, but also key social and behavioral capacities, and they have been widely assumed to have been favored by the emerging 'savannah' environment in east and southern Africa.

WE WERE MADE FOR SAVING ENERGY, NOT FOR WASTING IT

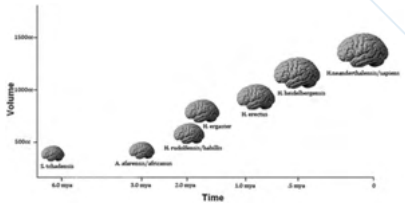
Thrifty genotype hypothesys

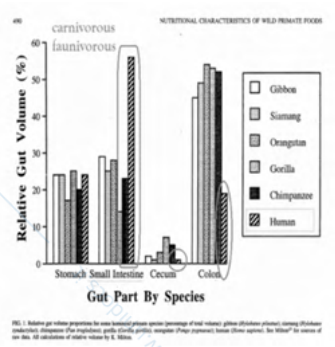
Phylogenesis of the fat mass

About 2 millions years ago, our ancestors stood up for the first time. Probably at that time, the uniform layer of subcutaneous fat in primates of both sexes became differentiated to produce eventually the specific human and sexual characteristics seen today.
Fat in women developed in the lower part of the body (HIP) in relation to the upright posture, the mechanical condition of pregnancy and the need to have reserves for the newborn.
In men, fat is less useful and was reduced by half. It was depositated predominantely in the upper body (TRUNK), where it was less likely to hinder mobility and aptitude for wrestling.
It is possible that natural selection increased this differentation.

To maintain an adequate and stable metabolic rate, the massive brain expansion was associated with corresponding adjustments in gut-size. Increasing availability of easily digestible energy and nutrient-rich food sources might have been the key prerequisite for this GUT-BRAIN COEVOLUTION

the high neuron number in the human brain generates a particularly high energy requirement, equivalent to 20% of basal metabolism even in adult life, and 80% in newborns.





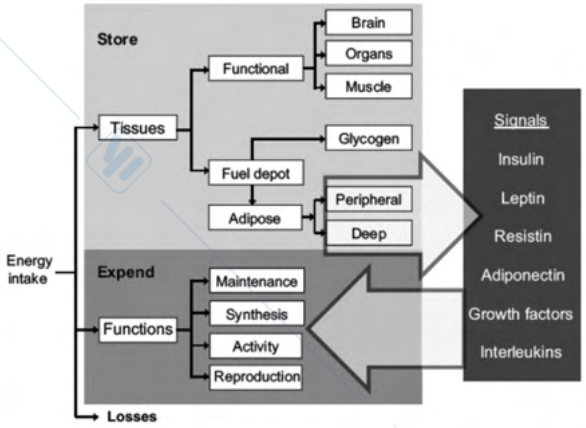


Fig. 5. Energy metabolism as an 'allocation game', with incoming energy allocated to ongoing biological functions (energy expenditure) or a range of energy stores. The allocation 'decisions' are assumed to be orchestrated by various signaling molecules. Adapted with permission (Wells, 2009a).

For species with energy stores, the allocation of energy between competing functions is potentially subject to greater control, allowing more complex life-history strategies. Indeed, a recent study found that adipose tissue is negatively associated with brain size across mammals, highlighting that storing energy and processing information are alternative ways of dealing with ecological uncertainty. Humans, however, have both large brains and high adiposity, making both strategies possible.

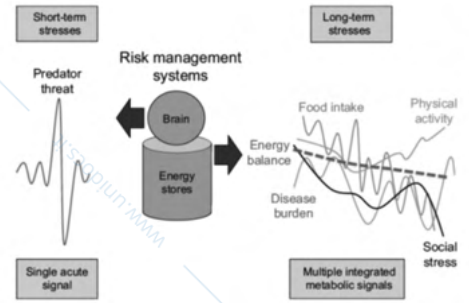


Fig. 1. Adiposity as risk management. This model treats adipose tissue as an endocrine regulatory system integrating multiple signals of energy supply and demand in order to optimize the allocation of energy across competing functions. This slower-response endocrine system contrasts with the faster-response regulation of behavior by the neural system (the brain), in response to acute signals. The example shows an individual responding to negative energy balance caused by increasing energy demand and decreasing supply.

ADIPOSE TISSUE PLASTICITY Adipose tissue accounts for 10% of the body mass in a normal newborn, for 15-20% in adult men and for 25-30% in adult women.

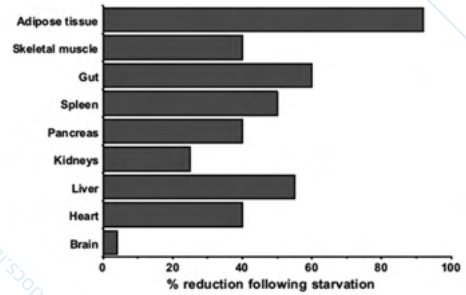


Fig. 3. Percentage reduction in the mass of various organs and tissues during starvation. During starvation, the amount of adipose tissue decreases substantially, but most other tissues also decline. Based on published data (Rivers, 1988).

OBESITY

- Obesity is a clinical condition featured by the excess of adipose tissue either for an increase in fat volume (hyperthrofic component) or in the number of adipocyte (hyperplastic component) or both.

- Major risk factor for life-threatening diseases (diabetes, cardiovascular diseases, cancer, etc…)

BODY MASS INDEX: BMI

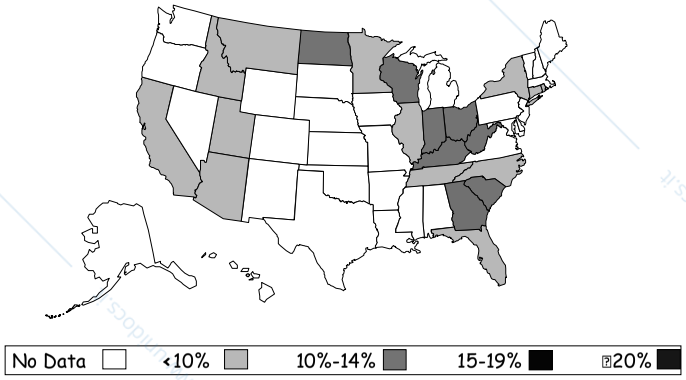
Ratio between Body Weight (Kg) and squared Height (m)

$$BMI = Kg/m^2$$

Classification based on BMI

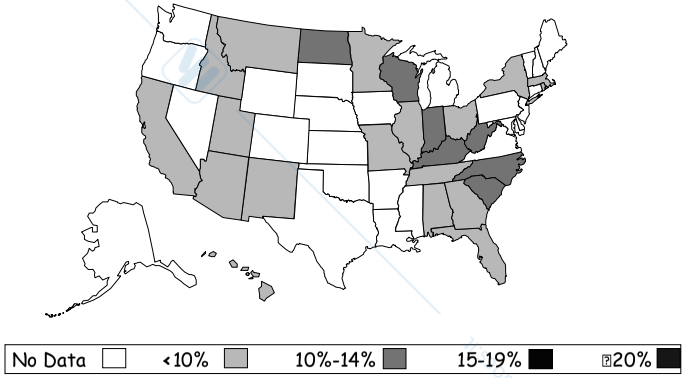
Class	BMI (kg/m2)
Underweight	< 18,5
Normalweight	18,5-24,9
Overweight	25-29,9
Obesity I	30-34,9
Obesity II	35-39,9
Obesity III	> 40

1985



1986

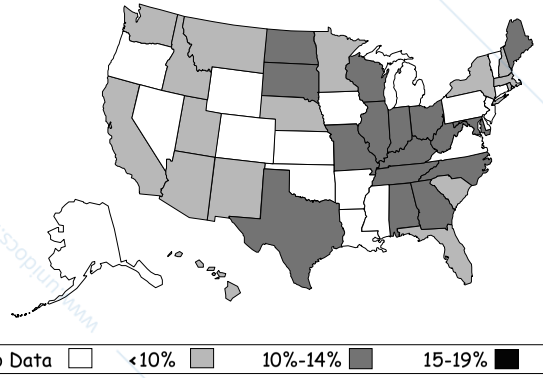
(*BMI ≥ 30, or ~ 30 lbs overweight for 5'4" person)



No Data | <10% | 10%-14% | 15-19% | ≥20%

1987

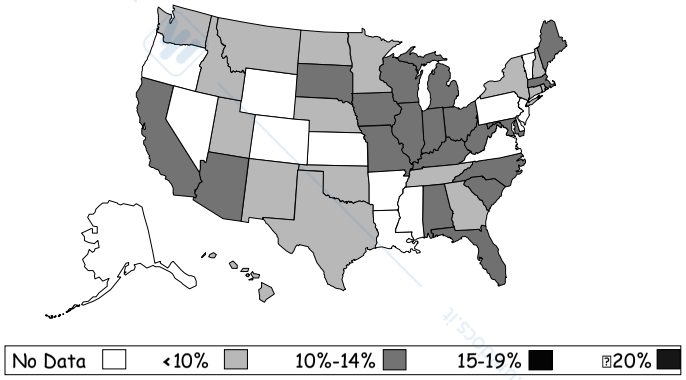
(*BMI ≥ 30, or ~ 30 lbs overweight for 5'4" person)



No Data | <10% | 10%-14% | 15-19% | ≥20%

1988

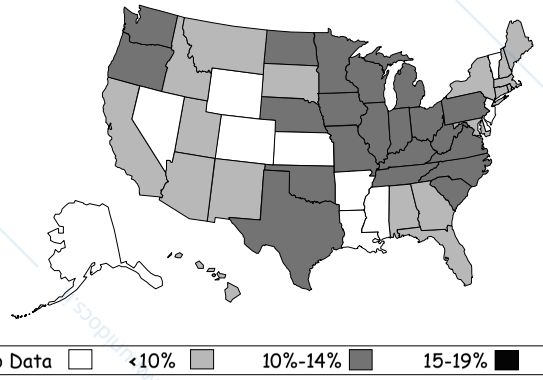
(*BMI ≥ 30, or ~ 30 lbs overweight for 5'4" person)



No Data | <10% | 10%-14% | 15-19% | ≥20%

1989

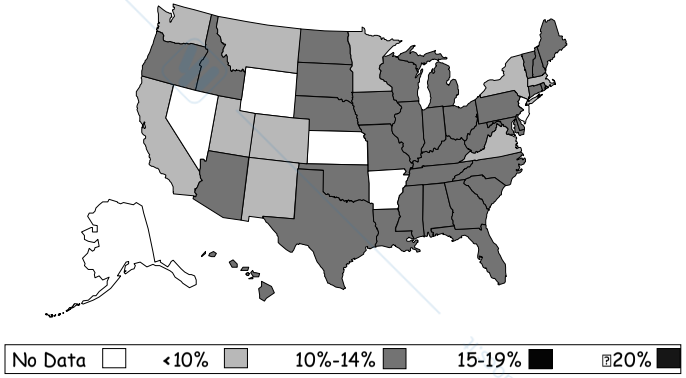
(*BMI ≥ 30, or ~ 30 lbs overweight for 5'4" person)



No Data | <10% | 10%-14% | 15-19% | ≥20%

1990

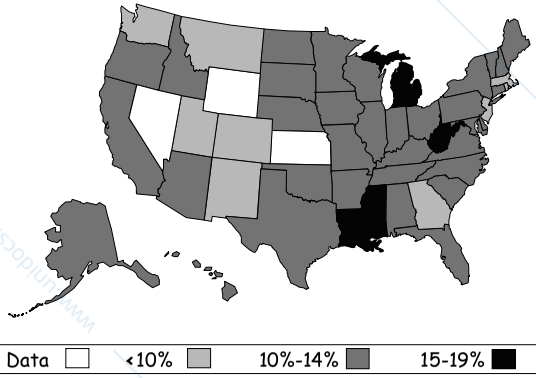
(*BMI ≥ 30, or ~ 30 lbs overweight for 5'4" person)



No Data | <10% | 10%-14% | 15-19% | ≥20%

1991

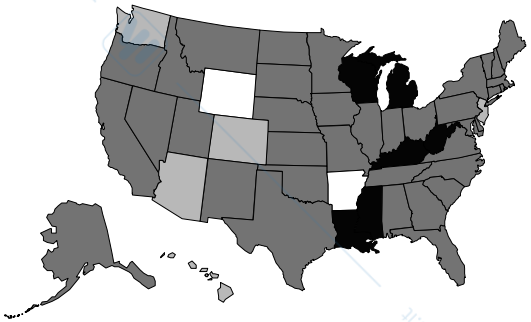
(*BMI ≥ 30, or ~ 30 lbs overweight for 5'4" person)



No Data | <10% | 10%-14% | 15-19% | ≥20%

1992

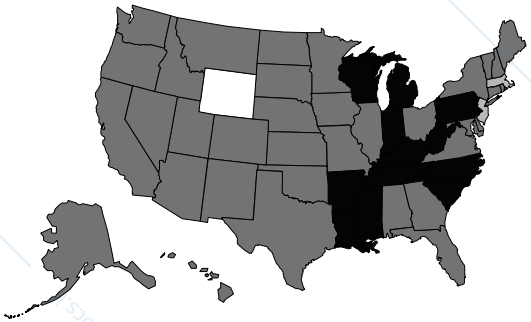
(*BMI ≥ 30, or ~ 30 lbs overweight for 5'4" person)



No Data | <10% | 10%-14% | 15-19% | ≥20%

1993

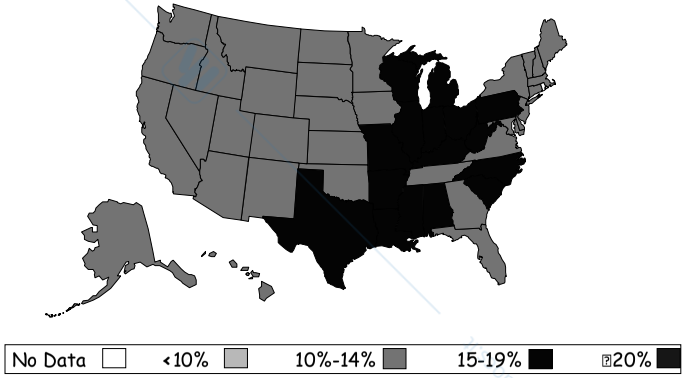
(*BMI ≥ 30, or ~ 30 lbs overweight for 5'4" person)



No Data | <10% | 10%-14% | 15-19% | ≥20%

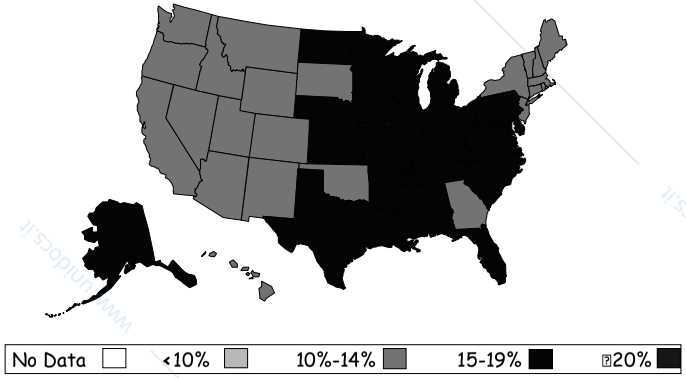
1994

(*BMI ≥ 30, or ~ 30 lbs overweight for 5'4" person)



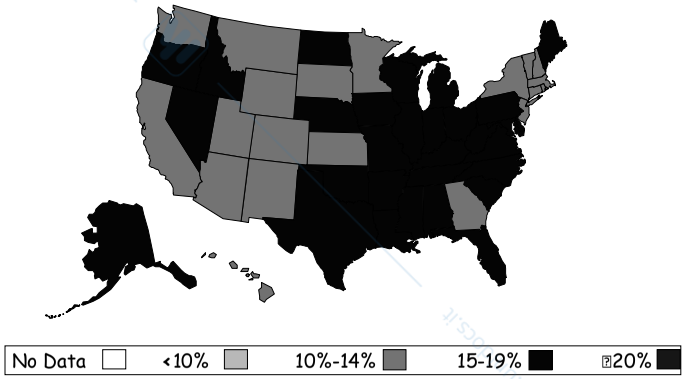
1995

(*BMI ≥ 30, or ~ 30 lbs overweight for 5'4" person)



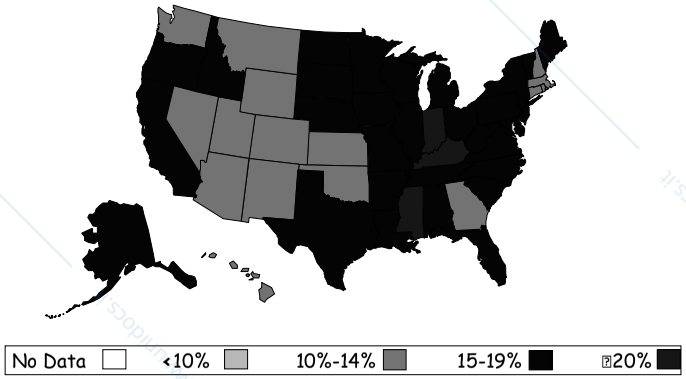
1996

(*BMI ≥ 30, or ~ 30 lbs overweight for 5'4" person)



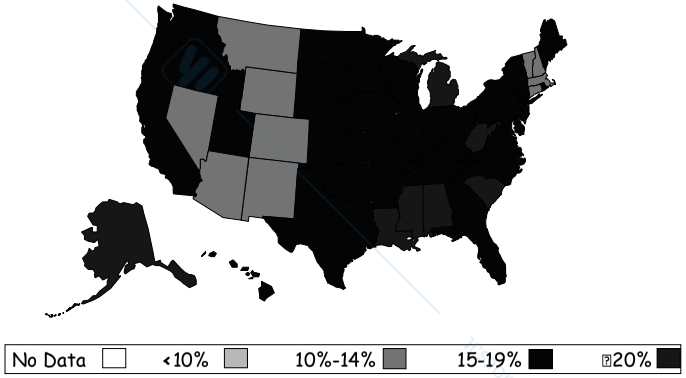
1997

(*BMI ≥ 30, or ~ 30 lbs overweight for 5'4" person)



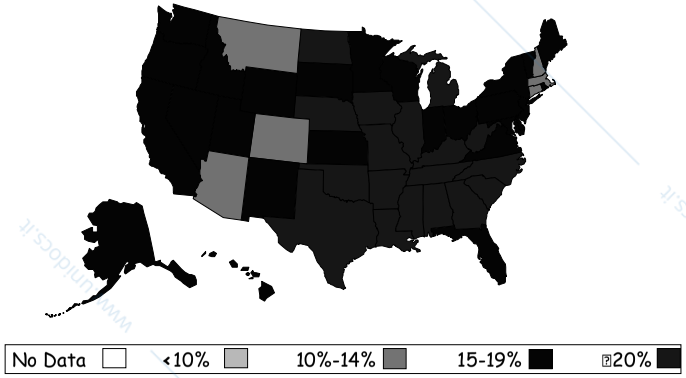
1998

(*BMI ≥ 30, or ~ 30 lbs overweight for 5'4" person)



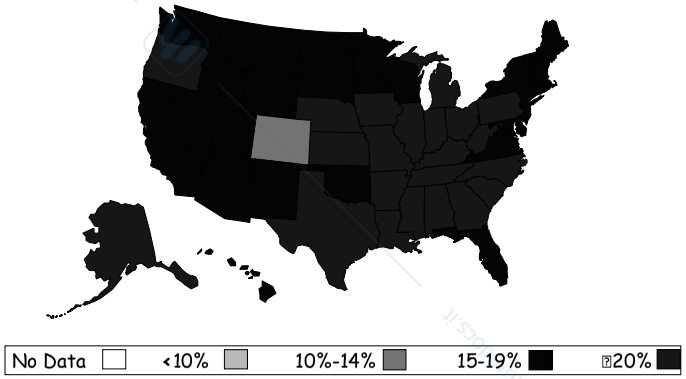
1999

(*BMI ≥ 30, or ~ 30 lbs overweight for 5'4" person)

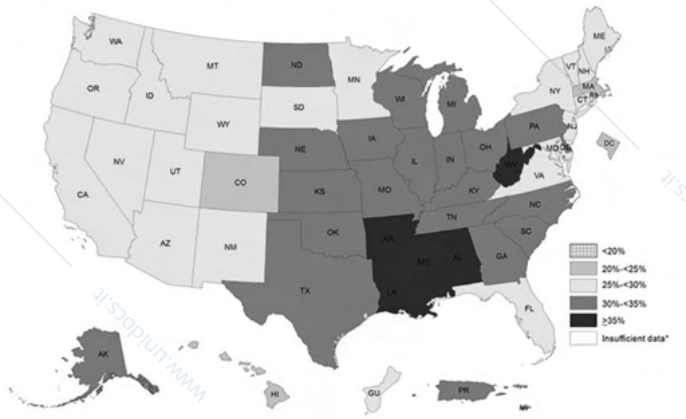


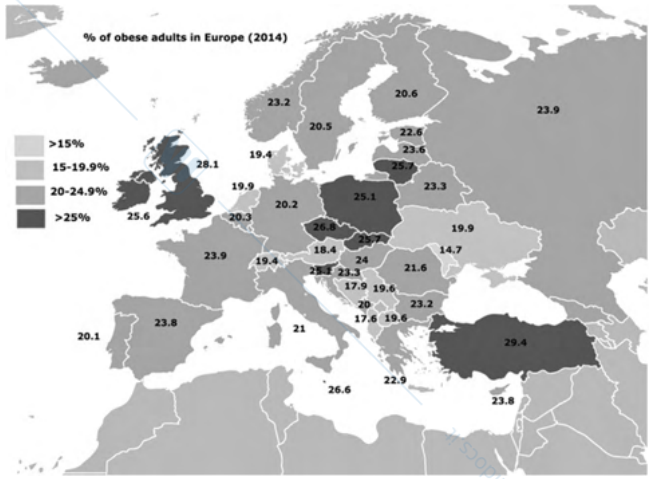
2000

(*BMI ≥ 30, or ~ 30 lbs overweight for 5'4" person)

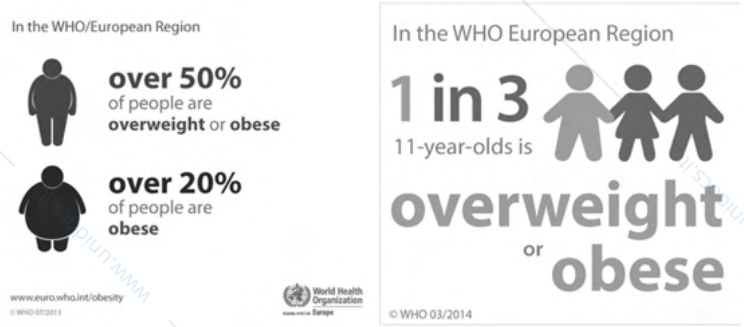


USA 2016



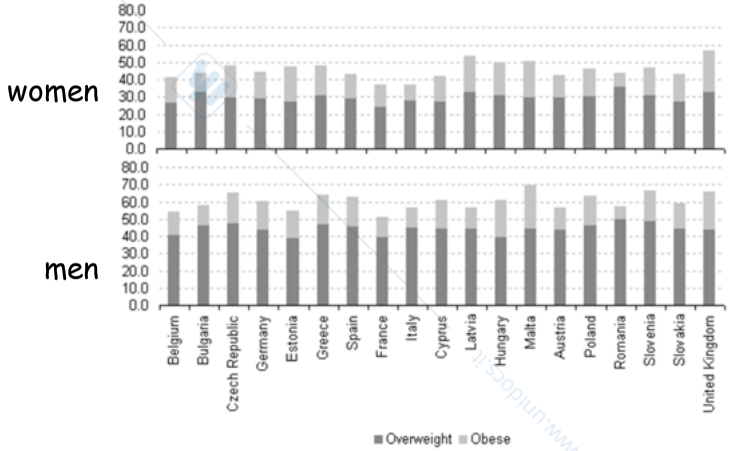


OBESITY IN EUROPE



Residents 507.4 M

OVERWEIGHT AND OBESITY IN EUROPE (2014)



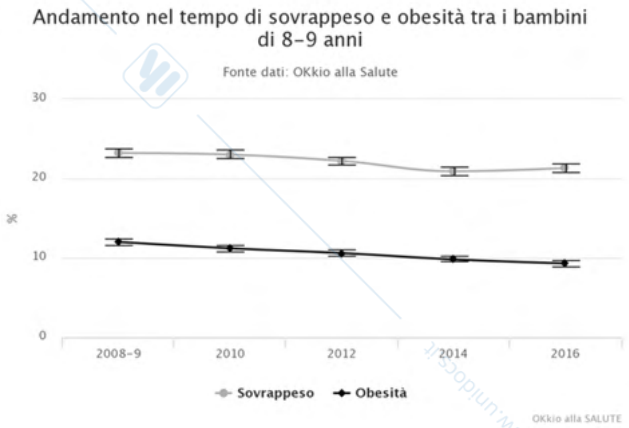
http://ec.europa.eu/eurostat/statistics-explained/index.php/Overweight_and_obesity_-_BMI_statistics

According to the report «Osserva-salute 2016», based on the ISTAT Multipurpose Invesigation "Aspetti della vita quotidiana"

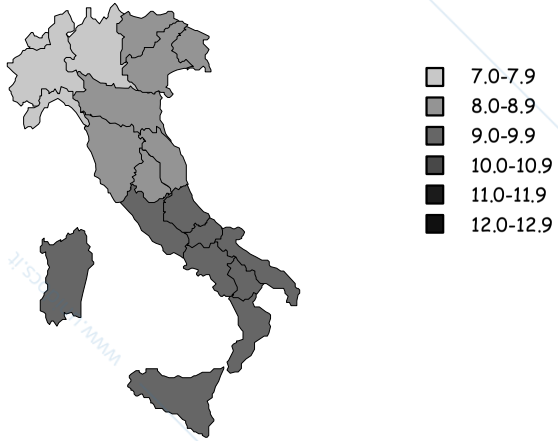
in Italian adult population in 2015,
One in three people is overweight (35.3%) and
1 in 10 people is obese (9.8%)

Overall, 45.1% of the subjects aged ≥18 y.o. has an excess weight.

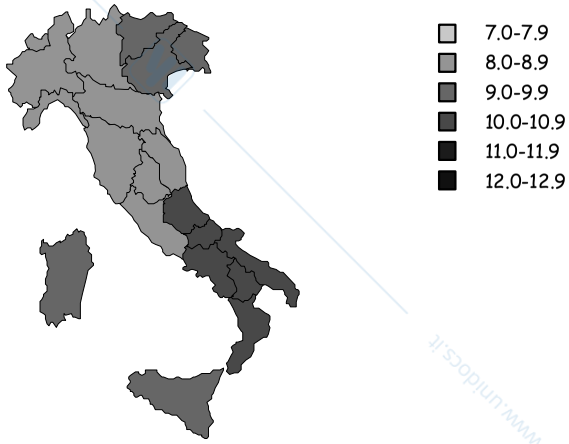
CHILDHOOD OBESITY



OBESITY PREVALENCE IN ADULTS
ISTAT 2001



OBESITY PREVALENCE IN ADULTS
ISTAT 2003



OBESITY PREVALENCE IN ADULTS
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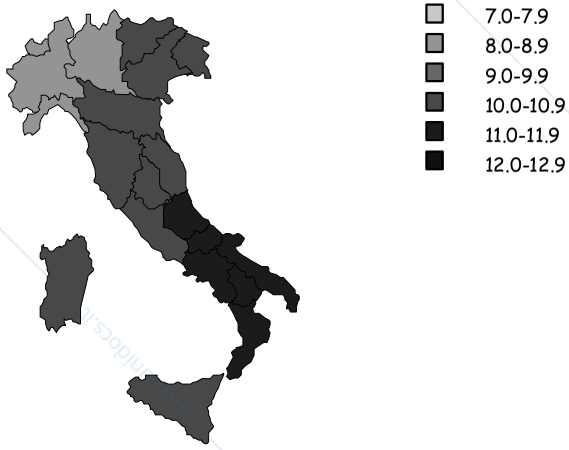
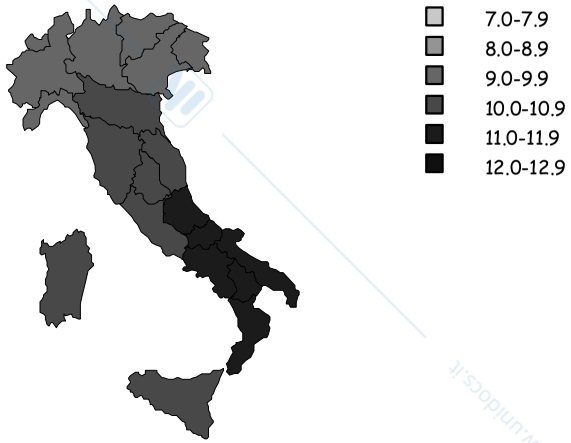
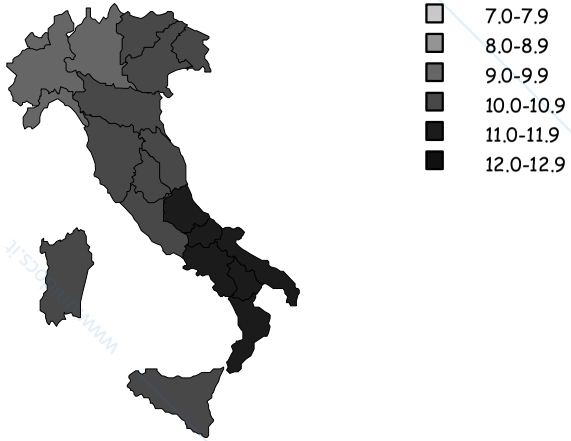


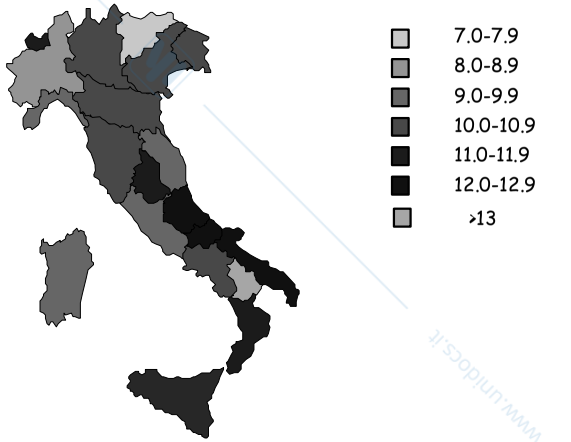
OBESITY PREVALENCE IN ADULTS
ISTAT 2009



OBESITY PREVALENCE IN ADULTS
ISTAT 2012

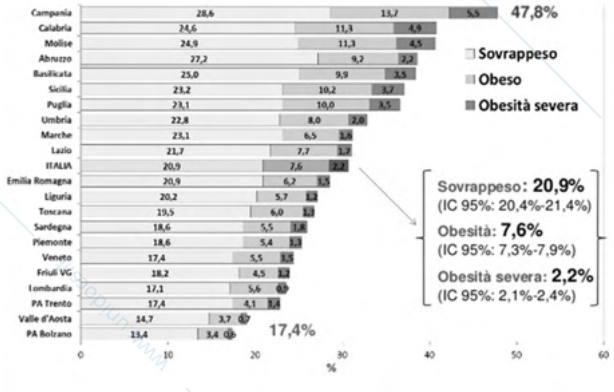


OBESITY PREVALENCE IN ADULTS
ISTAT 2013

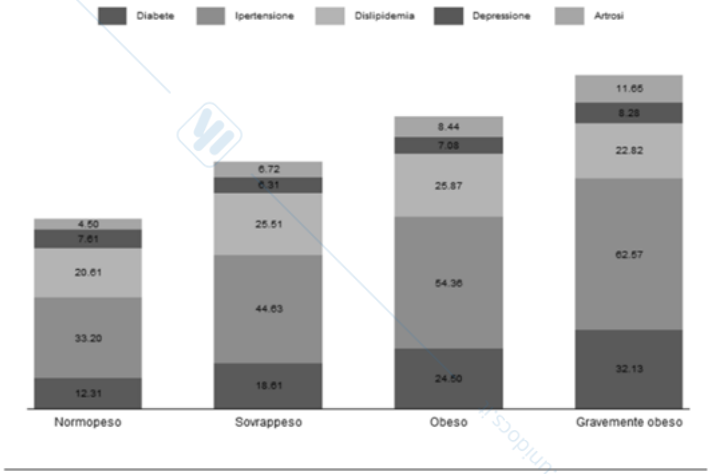


CHILDHOOD OBESITY

Bambini di 8-9 anni: sovrappeso e obesità per Regione, OKkio alla SALUTE 2014



DISEASE OCCURRENCE PER BMI CLASS



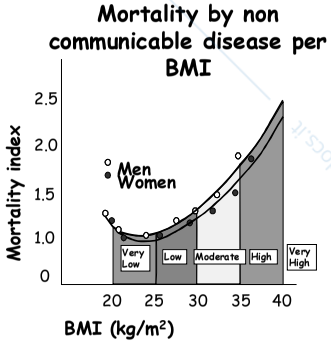
http://www.fondazionetorvergataeconomia.it/wp-content/uploads/2012/11/Rapporto_Spesa_sanitaria_BMI_ISS_4-2014.pdf

gallbladder
gallbladder

OBESITY IS A RISK FACTOR FOR A NUMBER OF SEVERE DISEASES

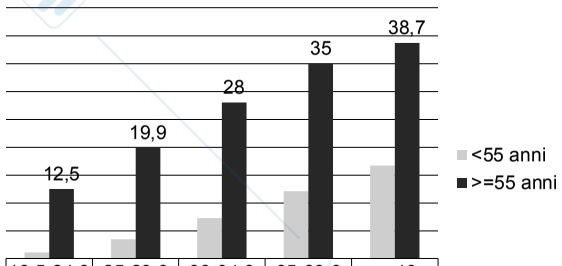
NON COMMUNICABLE DISEASES

1. Type II Diabetes
2. Cardiovascular Diseases
3. Cancer
4. Gallbladder
5. Obstructive Sleep Apnea
6. Musculoskeletal disorders (osteoarthritis)
7. Hypogonadism
8. Infertility



PREVALENCE OF DIABETES ACCORDING TO BMI CLASS

(SISSI PROJECT; N=2,705,211)



	18.5-24.9	25-29.9	30-34.9	35-39.9	>=40
<55 anni	1,1	3,5	7,3	12,1	16,7
>=55 anni	12,5	19,9	28	35	38,7

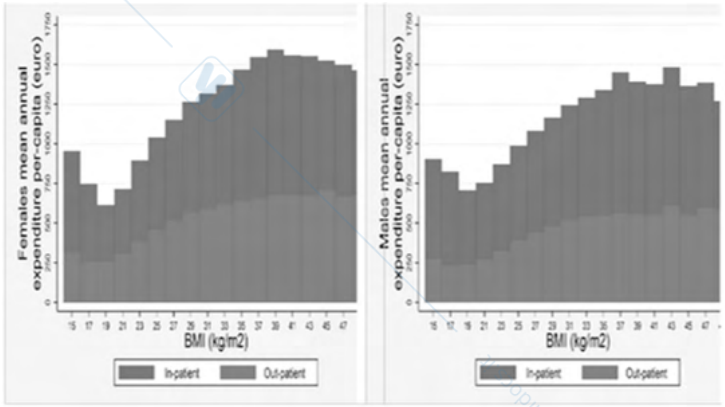
COVID-19 and Italy: what next?

Andrea Remuzzi, Giuseppe Remuzzi

"The mean age of those who died in Italy was 81 years and more than two-thirds of these patients had diabetes, cardiovascular diseases, or cancer, or were former smokers."

The Lancet.
Published Online March 12, 2020
https://doi.org/10.1016/ S0140-6736(20)30627-9

In Italy, obesity accounts for **2.5 billions/year €**
Hospital-costs excluded



Atella et. 2014

WORLD HEALTH ORGANIZATION FACT SHEETS
KEY FACTS

- Worldwide obesity has nearly **tripled** since 1975.
- In 2016, **39%** of adults aged ≥ 18 y.o. (1.9 billion people) were overweight, and 13% (650 million people) were obese.
- Most of the world's population live in countries where overweight and **obesity kills more people than underweight**.
- In 2016 41 million children ≤5 y.o. were overweight or obese.
- Over 340 million children and adolescents aged 5-19 were overweight or obese in 2016.
- **Obesity is preventable.**

https://www.who.int/en/news-room/fact-sheets/detail/obesity-and-overweight

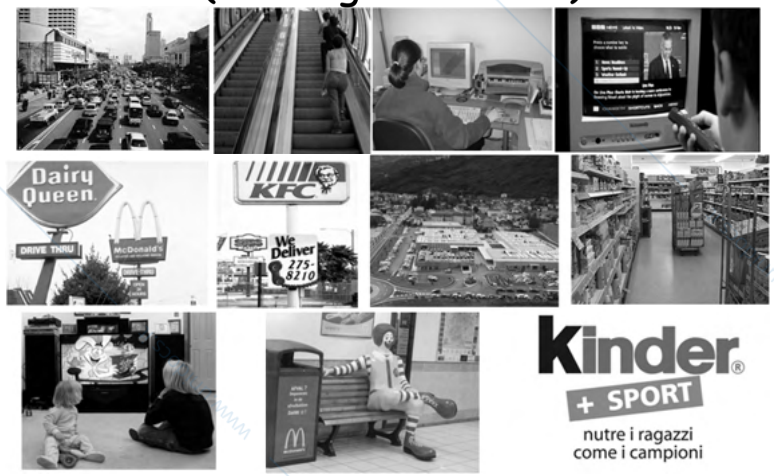
WHY OBESITY IS RISING

In popular wisdom, obesity is the product of too much food and too little activity, seemingly implicating personal responsibility.
This perspective increasingly seems to be over-simplistic.
Attention has begun to turn to a wider range of possible environmental or demographic risk factors, such as central heating, shifts in sleep patterns, chronic psychosocial stress, exposure to television screens, later age at first birth, environmental pollutants, etc.

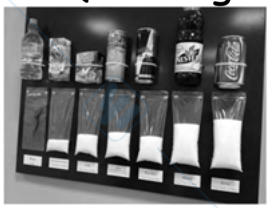
Obesity can be considered a disease 'outside' the body, deriving from an inappropriate food supply and marketing system, producing a niche to which individuals then vary in their susceptibility.

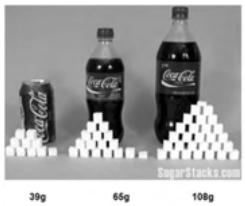
If the disease component of obesity lies not in adipose tissue itself, but in the interaction between adipose tissue biology and our modern industrialized environment, efforts to combat obesity would be much more effective if they prioritized 'external' environmental change rather than attempting to manipulate 'internal' biology through pharmaceutical or behavioral means.

«OBESOGENIC» ENVIRONMENT
('obesogenic niche')



«OBESOGENIC» ENVIRONMENT
('obesogenic niche')





OBESITY HERITABILITY

Studies in twins comparing monozygous and dizygous twins report 48 – 80%.
Only performed in industrialized countries, where favorable environmental conditions might maximize the contribution of genetic factors to phenotypic variability.
Under greater ecological constraint, heritability might well be lower.

Table 1. Heritability coefficients from a selection of large twin studies for anthropometric indices of adiposity

Population	Age	n	Outcome	Heritability (%)	Reference
China	Adults	1260 twin pairs	BMI	61	Lee et al., 2010
			Waist	75	
Finland	Adults	~5278 twin pairs	BMI	80	Hjelmborg et al., 2008
United Kingdom	4 years	3582 twin pairs	BMI	48	Haworth et al., 2008
	11 years	4251 twin pairs	BMI	78	
United States	20 years	4071 twin pairs	BMI	77	Stunkard et al., 1986a
	Adults	1224 twin pairs	BMI	76	Watson et al., 2010

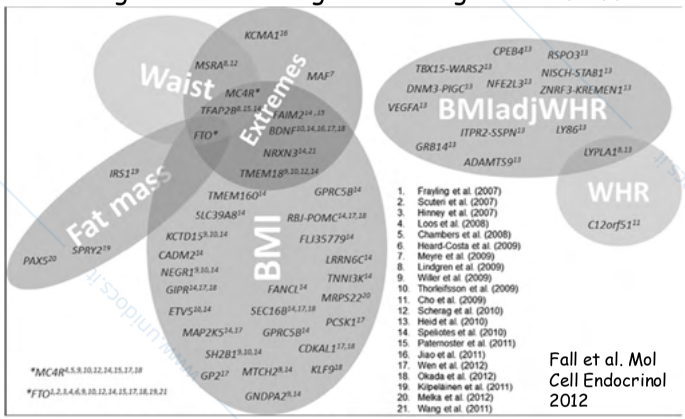
OBESITY does not only affect rich countries…

Facing a double burden of disease
Many low- and middle-income countries are now facing a "double burden" of disease.
•While these countries continue to deal with the problems of infectious diseases and undernutrition, they are also experiencing a rapid upsurge in noncommunicable disease risk factors such as obesity and overweight, particularly in urban settings.
•It is not uncommon to find undernutrition and obesity co-existing within the same country, the same community and the same household.

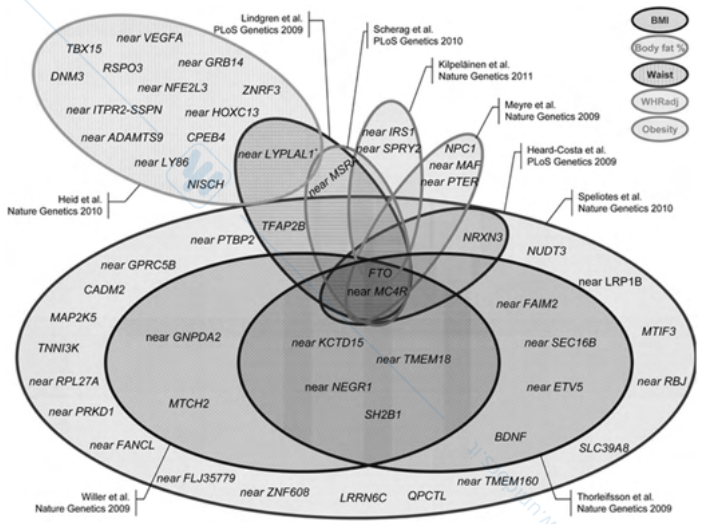
Children in low- and middle-income countries are more vulnerable to inadequate pre-natal, infant, and young child nutrition. At the same time, these children are exposed to high-fat, high-sugar, high-salt, energy-dense, and micronutrient-poor foods, which tend to be lower in cost but also lower in nutrient quality. These dietary patterns, in conjunction with lower levels of physical activity, result in sharp increases in childhood obesity while undernutrition issues remain unsolved.

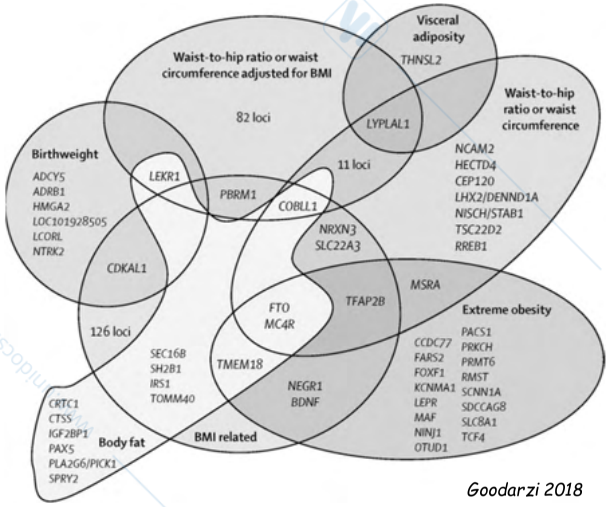
GENOME-WIDE ASSOCIATION STUDIES OF OBESITY

Continuous traits having a polygenic architecture, with each associated gene contributing a small magnitude of effect



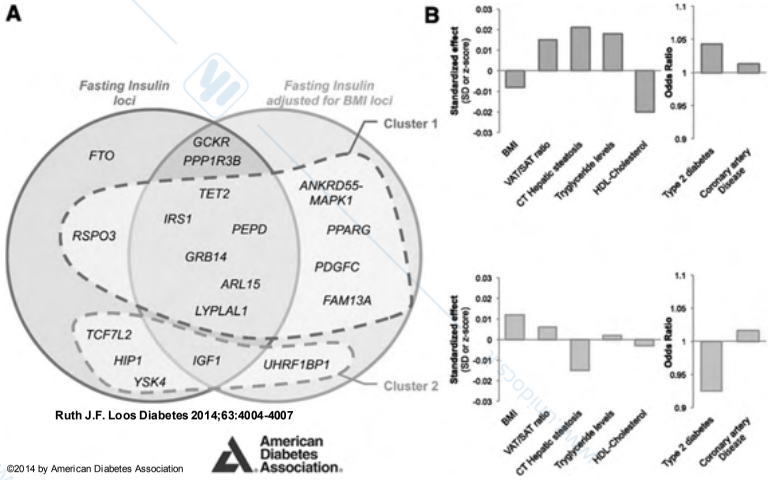
Fall et al. Mol Cell Endocrinol 2012





Goodarzi 2018

Insulin-associated loci - Adiposity – Cardiometabolic diseases



Ruth J.F. Loos Diabetes 2014;63:4004-4007

American Diabetes Association.

Proposed classes of "thrifty gene" (promoting energy-saving) associated with:

- appetite
- metabolism or thermogenesis
- predisposition for physical activity
- adipocyte lipid storage capacity
- lipid oxidation
- insulin metabolism
- infant growth
- pubertal timing

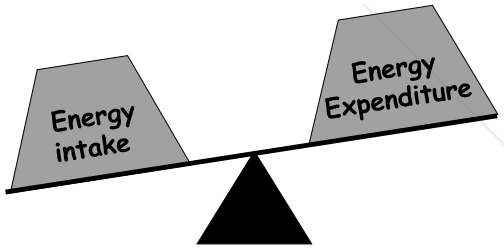
TACKLING OBESITY

Research into the molecular basis of body weight regulation has increased exponentially in recent years, following the discovery of an increasing number of relevant hormones, peptides and other signaling molecules, allowing numerous regulatory pathways to be elucidated.

Although a number of pharmaceutical products have been developed, few have been classified safe for human use. The complexity and redundancy of molecular pathways of weight regulation seem to hinder the identification of safe 'entry points' that are still capable of generating substantial effects. There is no obvious rate-limiting step in energy metabolism and, because adiposity is a continuous trait, no individual gene generates a large magnitude of impact on phenotype.

However, humans can deliberately fatten themselves. Obesity can therefore be said to have a specific behavioral component, and behavior might therefore seem the obvious target for both treatment and prevention. The lack of success in behavioral prevention programs is indicated by upward trends in obesity prevalence in many industrialized countries.

OBESITY CAUSES



nutritional, physical activity, endocrine, genetic, iatrogenic factors

BODY MASS INDEX: BMI

BMI defines the SEVERITY of obesity

Ratio between Body Weight (Kg) and squared Height (m)

$$BMI = Kg/m^2$$

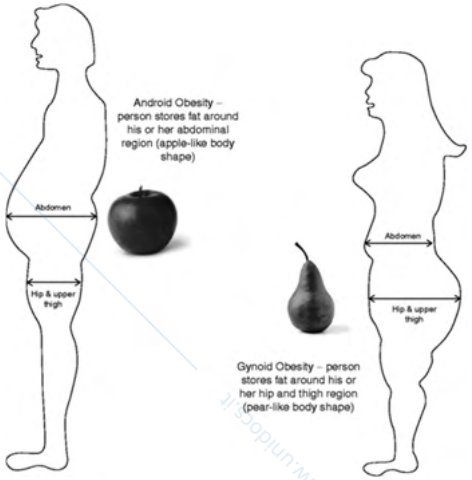
Classification based on BMI

Class	BMI (kg/m2)
Underweight	< 18,5
Normalweight	18,5-24,9
Overweight	25-29,9
Obesity I	30-34,9
Obesity II	35-39,9
Obesity III	> 40

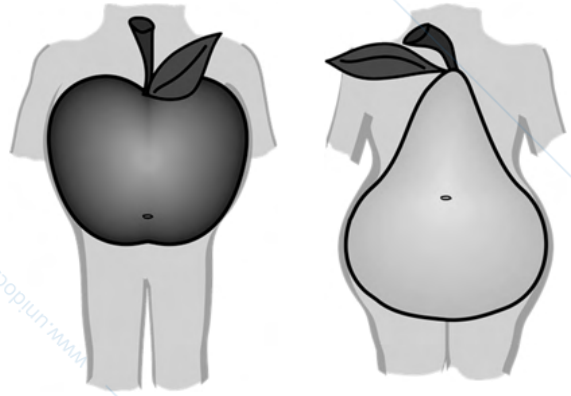
ABDOMINAL OBESITY

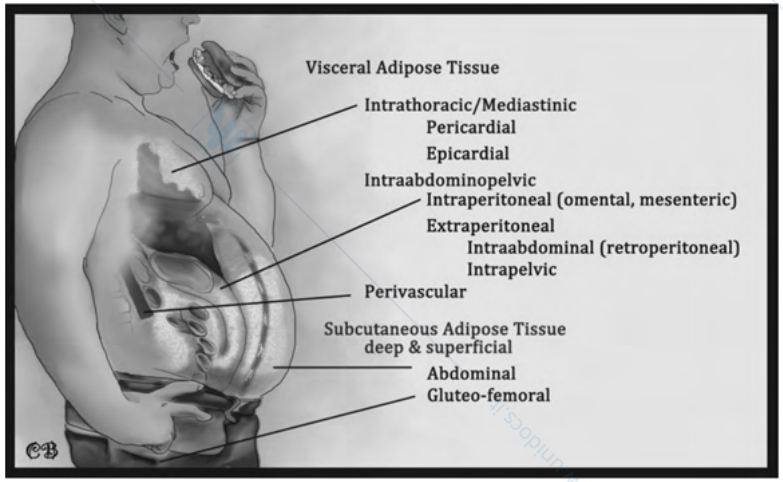
Overweight/obese subjects show relevant differences in the distribution of excess fat.

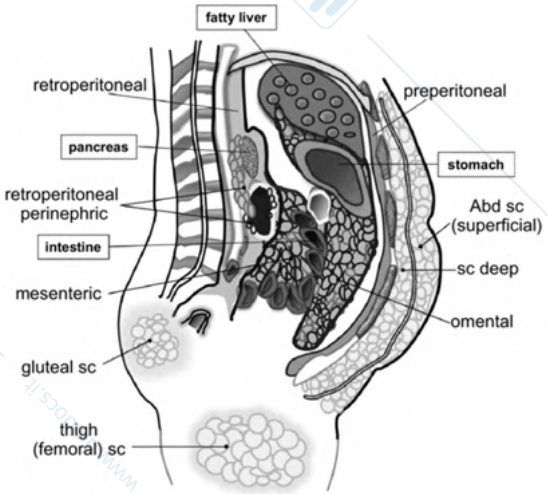
Risks for health are higher when abdominal fat predominates in comparison with gluteal-femoral fat.



Android (apple) vs gynoid (pear) obesity



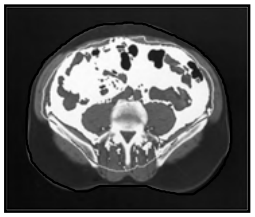




TOTAL and VISCERAL adipose tissues

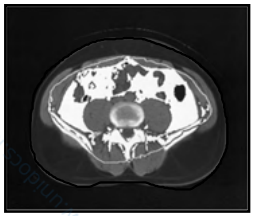
Fat mass: 19.8 kg
VAT: 155 cm²

- Atherogenic dyslipidemia
- Hyperglycemia
- Insulin resistance
- Elevated blood pressure
- Proinflammatory profile
- Prothrombotic profile
- Endothelial dysfunction



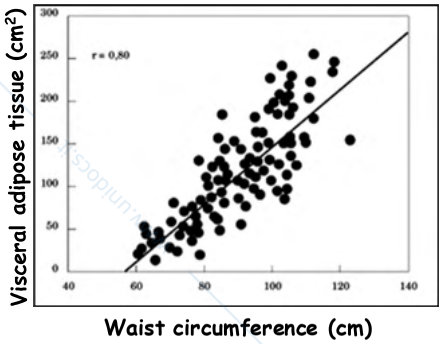
Fat mass: 19.8 kg
VAT: 96 cm²

- Normal lipid profile
- Normal glycemia
- Insulin sensitive
- Normal blood pressure
- Low inflammatory profile
- Normal fibrinolytic profile
- Normal endothelial function



Relationship between WAIST CIRCUMFERENCE and VISCERAL ADIPOSE TISSUE

Evaluation of the amount of abdominal visceral fat per mean values of waist circumference



Cardiovascular risk is influenced by BMI and by fat distribution:
Risk is directly associated with waist circumference, which, in turn, is associated with the abundance of peri-visceral fat.

WAIST CIRCUMFERENCE IS A MARKER OF INTRA-ABDOMINAL OBESITY

- Easily measured by a tape measure
- Waist circumference identifies patients at high cardiovascular risk
- In association with biochemical parameters such as Triglycerides, waist circumference is highly predictive of cardiovascular events.

Després 2001; Lemieux 2000; Empana et al 2004

Abdominal obesity and waist circumference thresholds

Abdominal Obesity	Waist Circumference
Men	> 102 cm
Women	> 88 cm

Adult Treatment Panel guidelines (ATP III) CRITERIA

ATP III: The Metabolic Syndrome*

*Diagnosis is established when ≥ 3 of these risk factors are present.

Risk Factor	Defining Level
Abdominal obesity (Waist circumference)	
Men	> 102 cm (> 40 in)
Women	> 88 cm (> 35 in)
TG	≥ 150 mg/dL
HDL-C	
Men	< 40 mg/dL
Women	< 50 mg/dL
Blood pressure	≥ 130/≥ 85 mm Hg
Fasting glucose	≥ 110 (≥ 100)† mg/dL

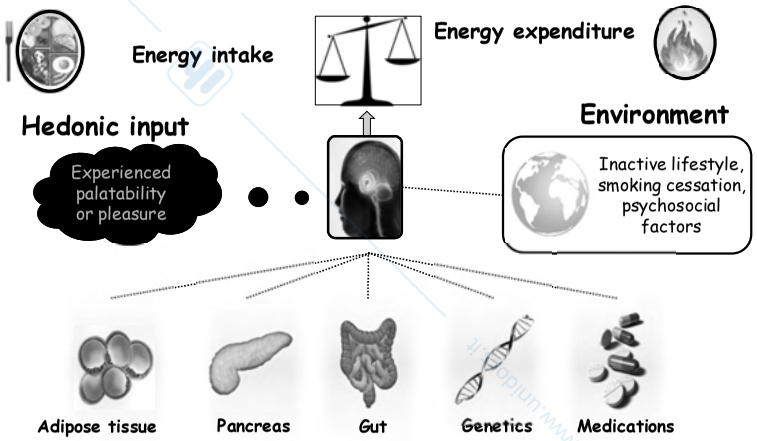
†2003 New ADA IFG criteria

HDL-C, high-density lipoprotein cholesterol.
Expert Panel on Detection, Evaluation, and Treatment of High Blood Cholesterol in Adults. *JAMA*. 2001;285:2486-2497.

OBESITY

PATHOPHYSIOLOGY

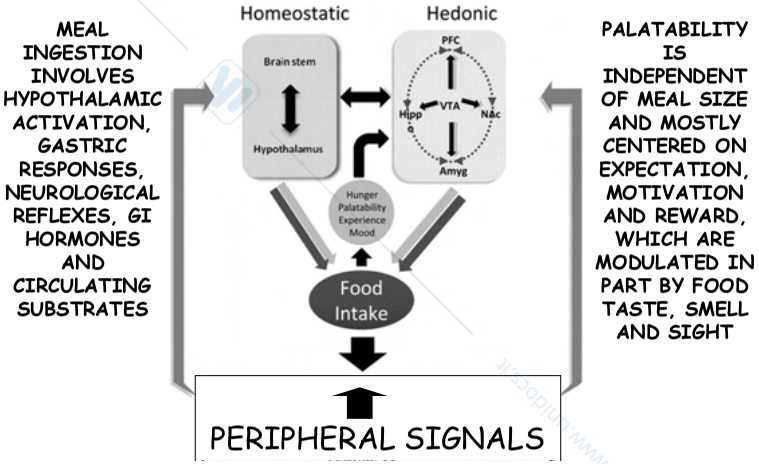
OBESITY IS A COMPLEX AND MULTIFACTORIAL DISEASE



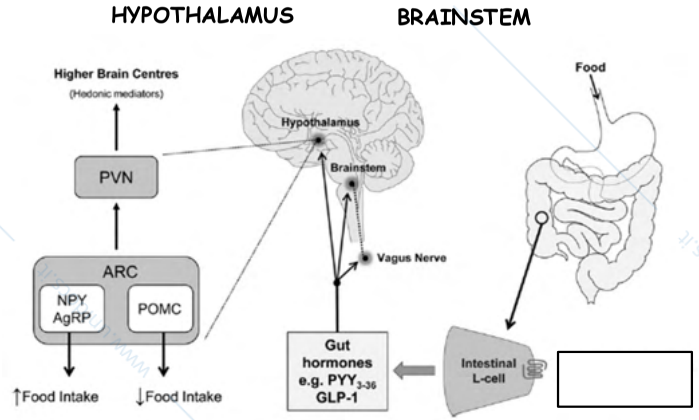
Control of Appetite

Homeostatic Hedonic

MEAL CONSUMPTION, INCLUDING QUANTITY AND QUALITY OF FOOD,
IS REGULATED BY INTEGRATION OF
THE HUNGER/SATIETY AND PALATABILITY SENSATIONS



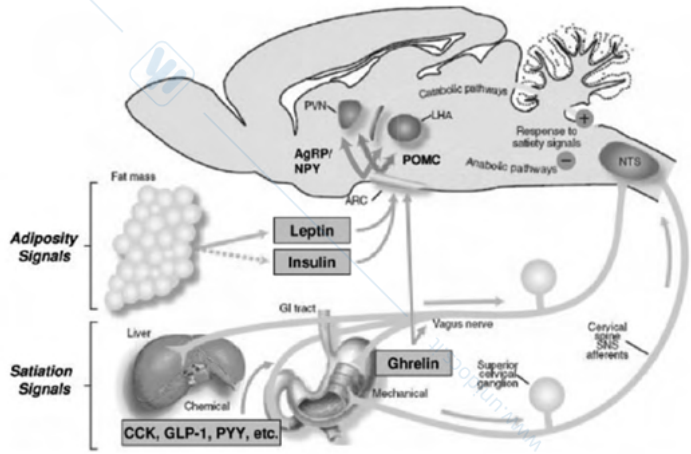
HOMEOSTATIC CONTROL OF FOOD INTAKE: TWO HUBS



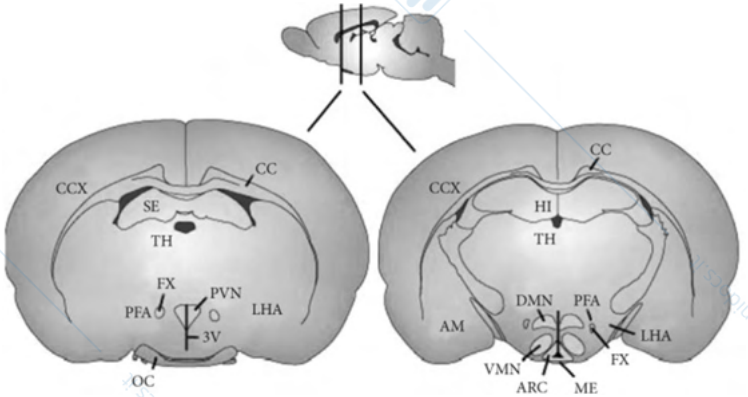
Hypothalamus. 95% of genes involved in obesity are expressed at the hypothalamic level.

Sam AH et al. Neuropharmacology 2012

ENERGY AVAILABILITY SIGNALS

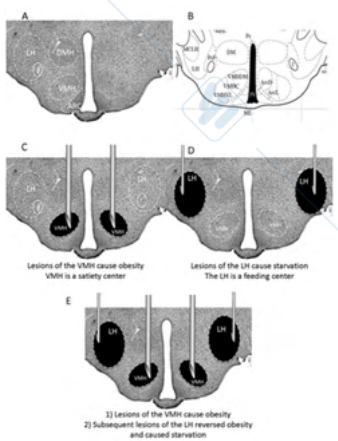


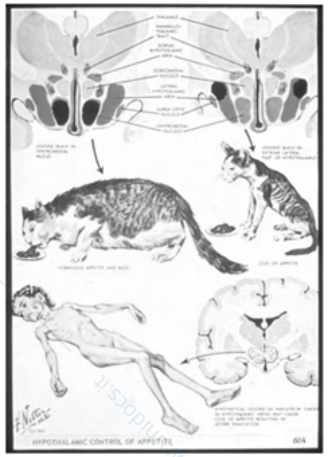
Hypothalamic nuclei involved in appetite regulation



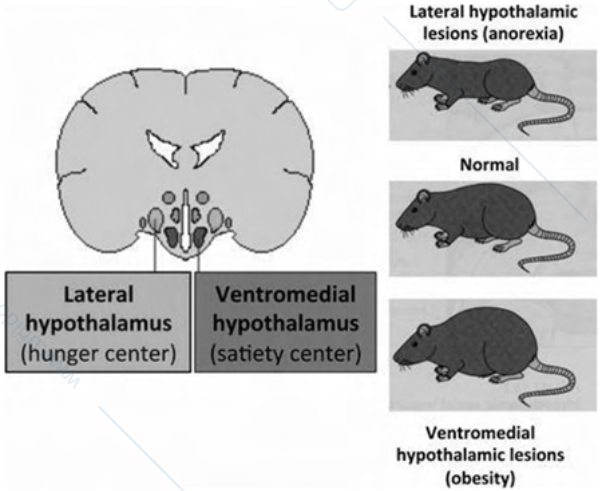
ARC, arcuate nucleus; AM, amygdala; CC, corpus callosum; CCX, cerebral cortex; DMN, dorsomedial nucleus; FX, fornix; HI, hippocampus; LHA, lateral hypothalamic area; ME, median eminence; OC, optic chiasm; PFA, perifornical area; PVN, paraventricular nucleus; SE, septum; 3V, third ventricle; TH, thalamus; VMN, ventromedial nucleus.

HISTORICAL VIEW ON BRAIN CONTROL OF ENERGY METABOLISM



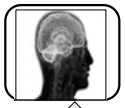


Lesion in the lateral hypothalamus → loss of the hunger center → anorexia
Lesion in the central hypothalamus → loss of the satiety center → obesity

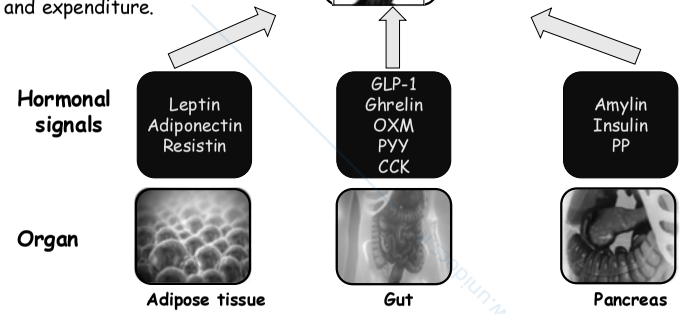


MULTIPLE HORMONAL SIGNALS INFLUENCE APPETITE

Signals reflecting energy stores, recent nutritional state, and other parameters are integrated in the central nervous system, particularly in the hypothalamus, to coordinate energy intake and expenditure.

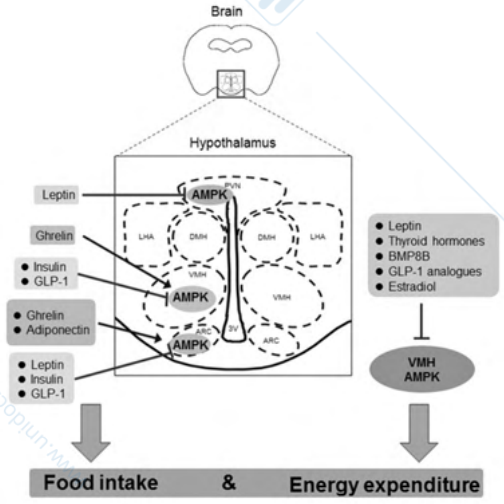


Key neuronal systems integrate and translate the information into efferent responses

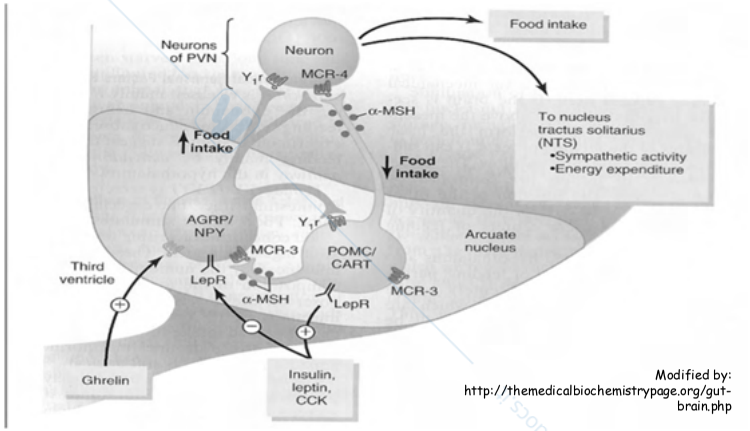


New targets for drug development in the treatment of obesity!
Targeting only one site is likely to be less effective than targeting two or more sites.

AMP-activated protein kinase (AMPK)

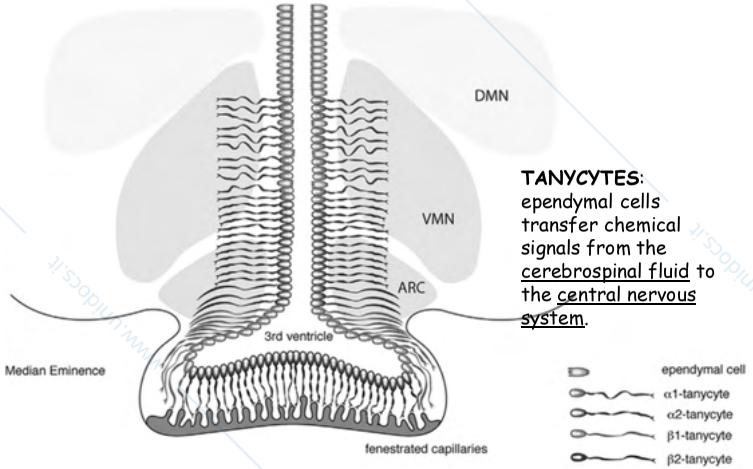


HOMEOSTATIC REGULATION OF FOOD INTAKE



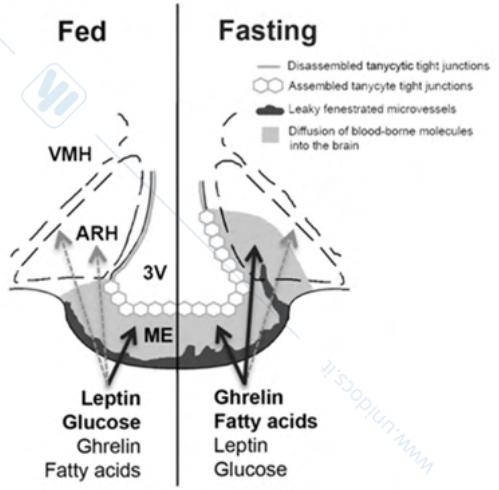
Leptin receptors in AgRP neurons seem to have a more pronounced role on body weight, whereas those in POMC on glucose/insulin profile

THE NUCLEUS ARCUATUS IS ADJACENT TO THE MEDIAN EMINENCE, A CIRCUMVENTRICULAR ORGAN WITH FENESTRATED CAPILLARIES AND HENCE A PERMEABLE BLOOD BRAIN BARRIER



TANYCYTES: ependymal cells transfer chemical signals from the cerebrospinal fluid to the central nervous system.

The focal plasticity of the dual-faced blood-hypothalamus barrier



PARABIOSIS EXPERIMENTS: A CIRCULATING FACTOR IN THE BLOOD SUPPRESSES FOOD INTAKE

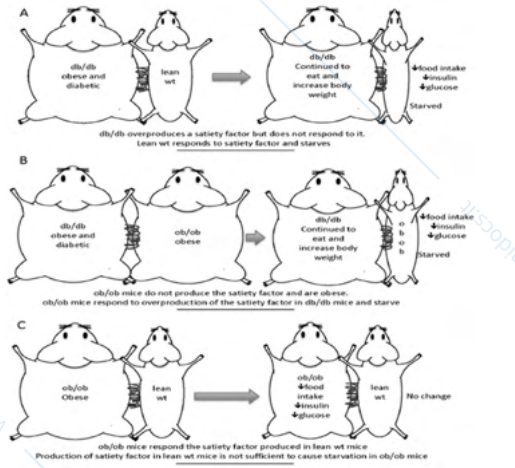
Coleman DL Diabetologia 1973

two mice are joined together surgically and develop single, shared circulatory system.

db/db mouse: model for type II diabetes

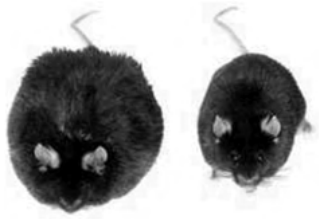
db/ob mouse: model for obesity

The key circulating factor will be identified in 1994 and is now known as LEPTIN



Leptin

- Endocrine messenger conveying an anorectic signal
- Protein encoded by the *ob* gene, with a cytokine-like structure;
- Synthesized mainly by the adipose tissue and released in the bloodstream;



ob/ob : deficit in leptin hormone

db/db : deficit in leptin receptor

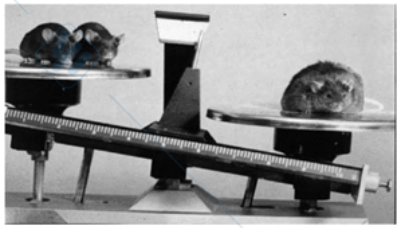
↓

Hyperphagia
Decreased energy expenditure
Increased fat accumulation

↓

Obesity,
Insulin-Resistance

ob/ob mouse

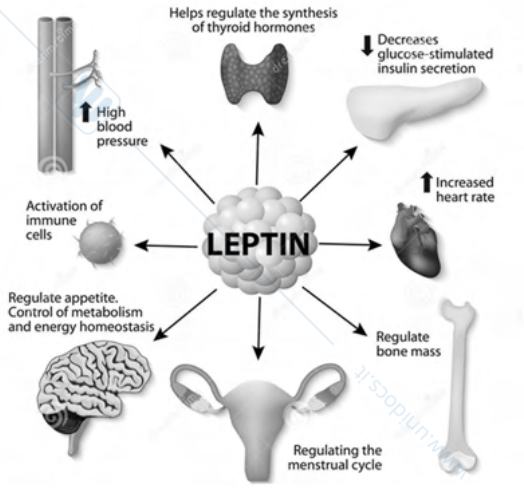


Genotype **mutation in leptin gene**

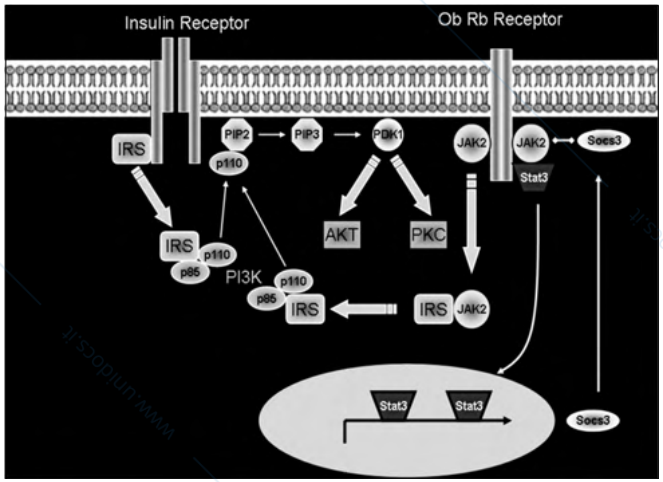
Phenotype **Absence of leptin in the bloodstream**
Obesity
Hyperphagia
Infertility

Phenotype is reverted by Leptin administration

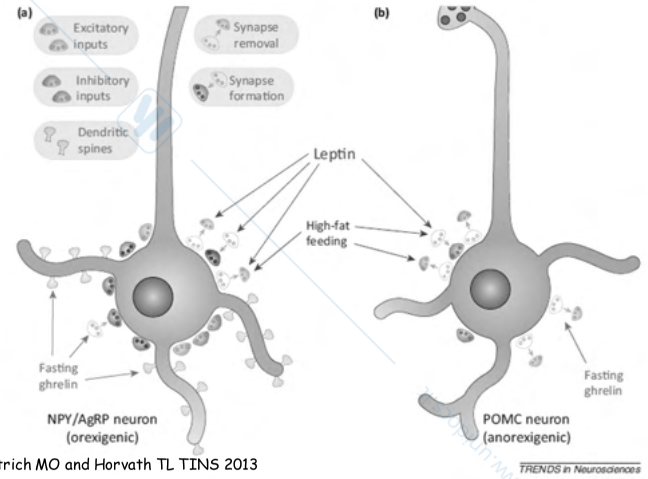
LEPTIN EFFECTS



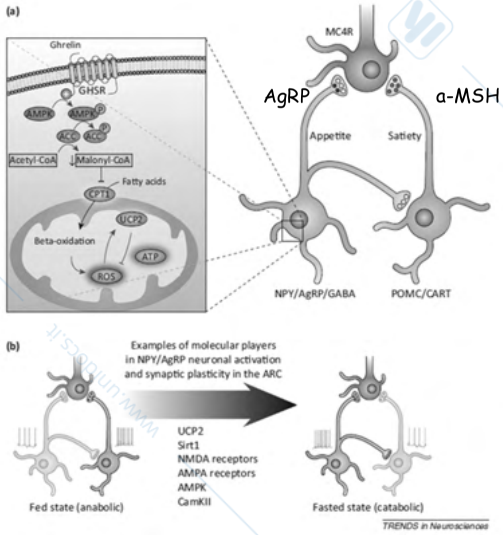
Leptin and Insulin are secreted in proportion to the amount of fat and glucose in the body. These "adiposity" hormones enter the brain by transport through the blood-brain barrier and interact with specific neuronal receptors, primarily in the hypothalamus, to affect energy balance.



SYNAPTIC PLASTICITY IN THE ARCUATE NUCLEUS



Dietrich MO and Horvath TL TINS 2013



Orexigenic and Anorexigenic neurons project at the PARAVENTRICULAR NUCLEUS expressing the Melanocortin 4 Receptor (MC4R)

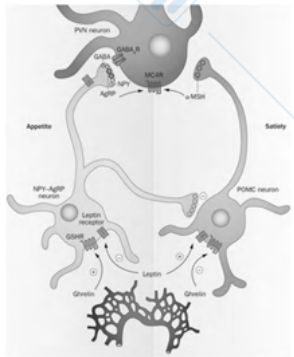
Agouti-related Peptide (AgRP)

Pro-opiomelanocortin (POMC) cleavage generates a-Melanocyte-stimulating hormone (a-MSH)

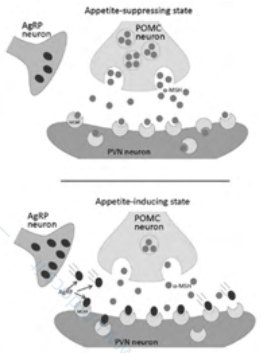
INTERACTION BETWEEN AgRP AND a-MSH AT MC4R SITES IN THE PVN

Orexigenic and Anorexigenic neurons project at the PARAVENTRICULAR NUCLEUS expressing the Melanocortin 4 Receptor (MC4R)

a-MSH: (POMC cleavage product) MC4R ligand
AgRP: MC4R antagonist



Nasrallah CM and Horvath TL Nature Rev Endo 2014

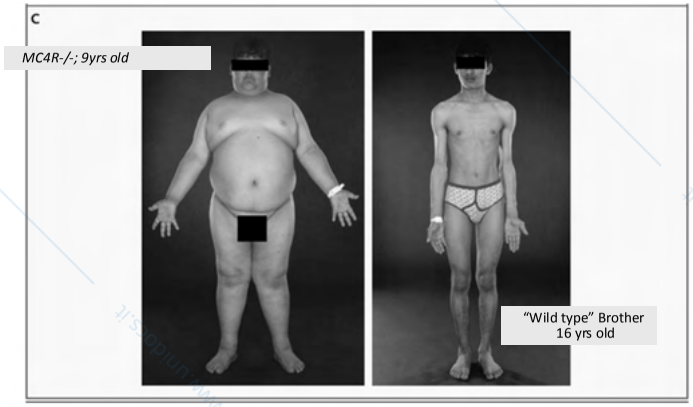


Reichenbach A et al 2012

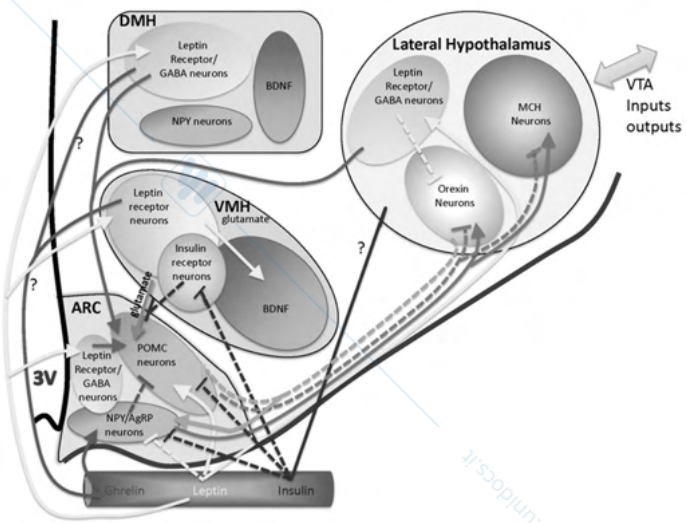
Gene mutations inactivating MC4R induce a permanent orexigenic stimulus

Clinical Spectrum of Obesity and Mutations in the Melanocortin 4 Receptor Gene

I. Sadaf Farooqi, M.D., Ph.D., Julia M. Keogh, B.Sc., Giles S.H. Yeo, Ph.D., Emma J. Lank, B.Sc., Tim Cheetham, M.D., and Stephen O'Rahilly, M.D.

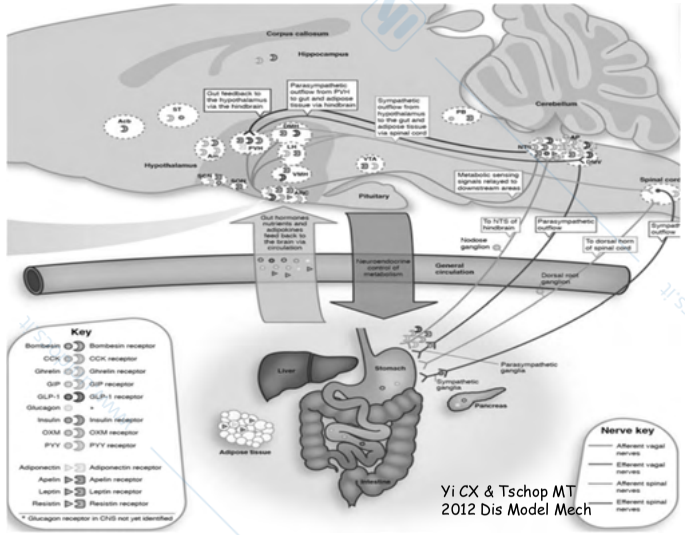


N Engl J Med 2003;348:1085-95.



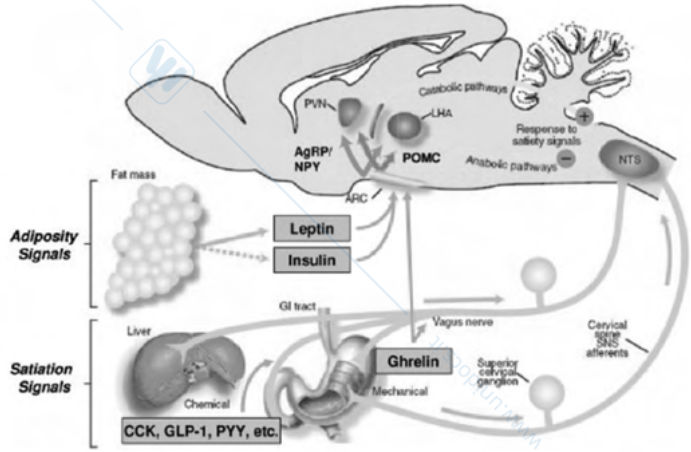
Reichenbach A et al. 2012

HYPOTHALAMUS – BRAINSTEM INTERPLAY

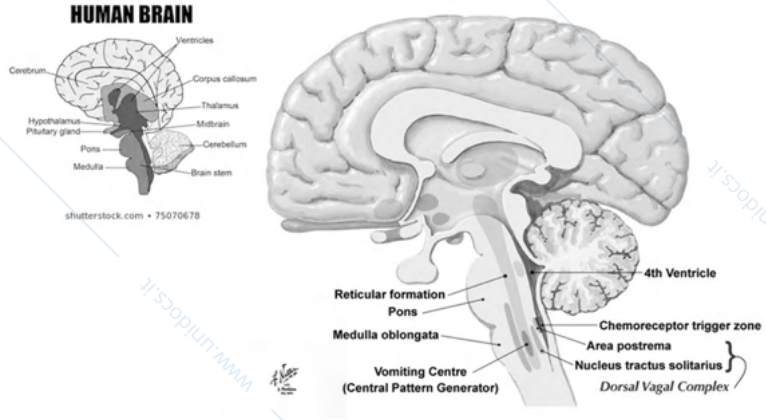


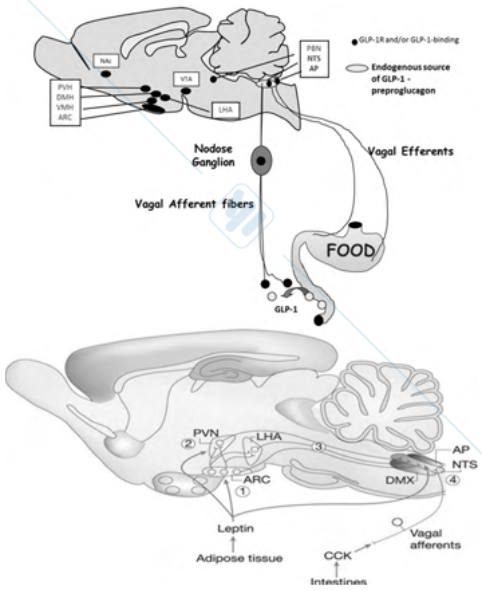
Yi CX & Tschop MT
2012 Dis Model Mech

ENERGY AVAILABILITY SIGNALS



ANOTHER MAJOR INTEGRATIVE GUT-BRAIN HUB
AREA POSTREMA and
NUCLEUS OF TRACTUS SOLITARIUS
in the BRAINSTEM





GI – BRAINSTEM INTERPLAY

1) Gastrointestinal vagal afferents are activated by mechanoreceptors and chemoreceptors, and converge in the nucleus of the tractus solitarius of the brainstem. Neuronal projections from the NTS, in turn, carry signals to the hypothalamus.

2) Gut hormones also alter the activity of the ascending vagal pathways from the gut to the brainstem.

3) Circulating gut hormones may influence the NTS through the adjacent circumventricular organ, the area postrema.

HOMEOSTATIC GI SIGNALS

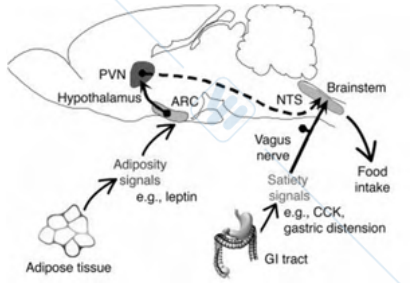
SATIATION
feelings of fullness that contribute to the decision to stop eating

SATIETY
prolongation of the interval until reappearing of hunger or of a drive to eat

GI hormones that affect satiation	
Gut-derived peptide	**Effect on food intake**
CCK	Decrease
GLP-1	Decrease
PYY	Decrease
Apo A-IV	Decrease
Enterostatin	Decrease
Bombesin-family peptides	Decrease
Oxyntomodulin	Decrease
Amylin	Decrease
Ghrelin	Increase

Woods SC and Alessio DA. J Clin Endocrinol Metab 2008; 93:11: S37–S50.

CHOLECYSTOKININ (CCK)



The prototypical satiation signal is the duodenal peptide cholecystokinin (CCK), which is secreted in response to dietary lipids or proteins and which activates receptors on local sensory nerves in the duodenum, sending a message to the brain via the vagus nerve that contributes to satiation.

Principal site of release	Factors stimulating or inhibiting secretion	Receptor	Site of action	Actions
PROXIMAL SMALL INTESTINE	FAT & PROTEINS↑	CCKAR or CCK-1	VAGUS nerve Area Postrema Nucleus tractus Solitarius	Increasing SATIATION

GHRELIN

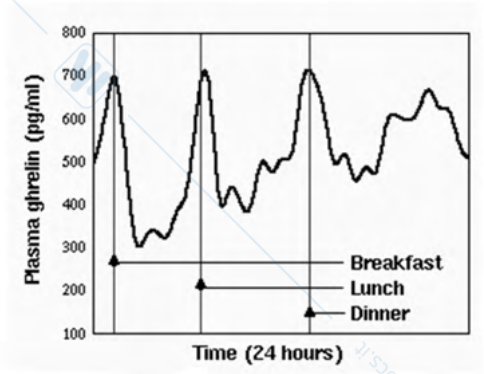
Ghrelin is a 28-amino acid acylated peptide secreted by the stomach.

In normal subjects, ghrelin levels are highest in the fasted state. Ghrelin levels are chronically elevated in people with weight loss due to anorexia nervosa or dietary restriction.

In contrast to other gut hormones, plasma ghrelin levels decrease after meals and are low in obese subjects.

Principal site of release	Factors stimulating or inhibiting secretion	Receptor	Site of action	Actions
GASTRIC MUCOSA (X/A-like cells)	Caloric restriction ↑ Macronutrients ↓	GHS-R1A-1R	VAGUS nerve BRAINSTEM HYPOTHALAMUS	Meal initiator

GHRELIN BLOOD CONCENTRATION FLUCTUATES THROUGHOUT THE DAY, RISING BEFORE A MEAL AND THEN DECREASING UPON CONSUMPTION

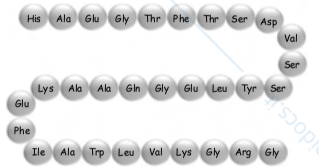


Cummings DE et al. Diabetes 2001

GLUCAGON-LIKE PEPTIDE-1 (GLP-1)

- GLP-1 is a 31 amino acid peptide
- Member of incretin family
- Secreted predominantly by L-cells in the gut, but also at the brainstem level (NTS)

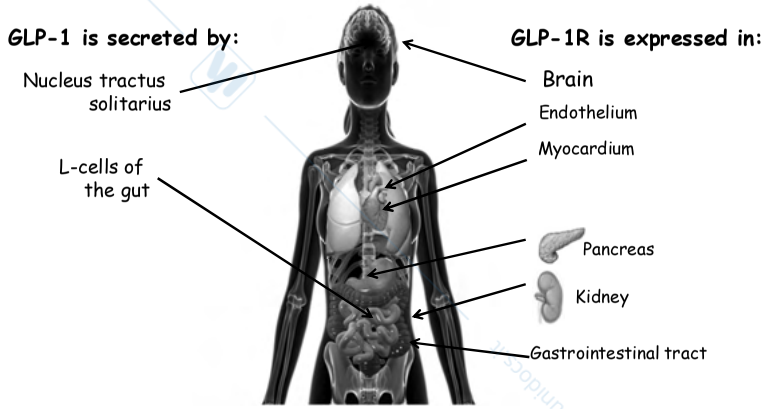
Human endogenous GLP-1



Enzymatic degradation by DPP-4
Half-life =1.5–2 min

Merchenthaler *et al. J Comp Neurol* 1999; Baggio & Drucker. *Gastroenterology* 2007; Ducker & Nauck. *Lancet* 2006

GLP-1 SECRETION and RECEPTOR EXPRESSION

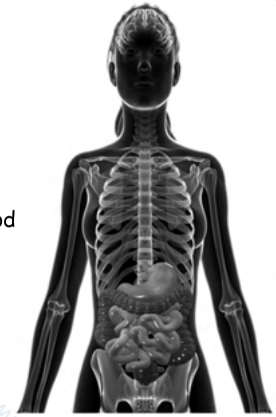


Merchenthaler *et al. J Comp Neurol.* 1999; Baggio & Drucker. *Gastroenterology* 2007; Ban *et al. Circulation* 2008; Vrang *et al. Prog Neurobiol* 2010; Pyke *et al. Endocrinology* 2014.

METABOLIC EFFECTS OF GLP-1

Appetite

- ⇧ Satiety
- ⇧ Fullness
- ⇩ Hunger
- ⇩ Prospective food consumption
- ⇩ Energy intake



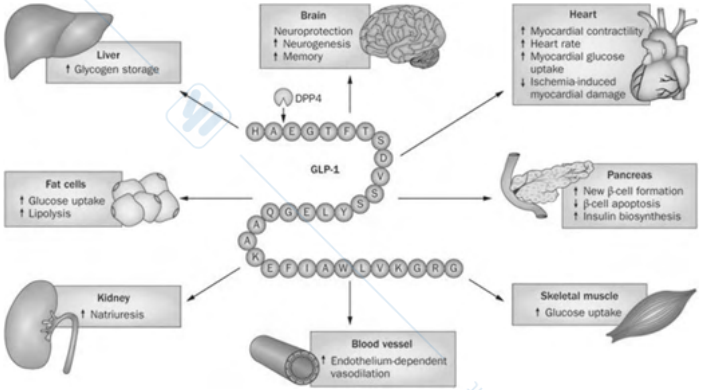
Glucose regulation

- ⇧ (Glucose-dependent) Insulin secretion
- ⇩ Glucagon secretion

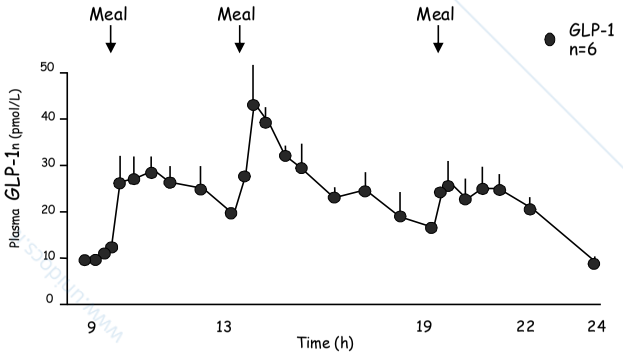
Mild gastric effects

- ⇩ Gastric acid
- ⇩ Gastric emptying

Flint *et al. J Clin Invest* 1998; Nauck *et al. Diabetologia* 1993; O'Halloran *et al. J Endocrinol* 1990; Nauck *et al. Am J Physiol* 1997



GLP-1 IS RELEASED IN RESPONSE TO FOOD INTAKE



Adapted from: Orskov *et al. Scand J Gastroenterol* 1996

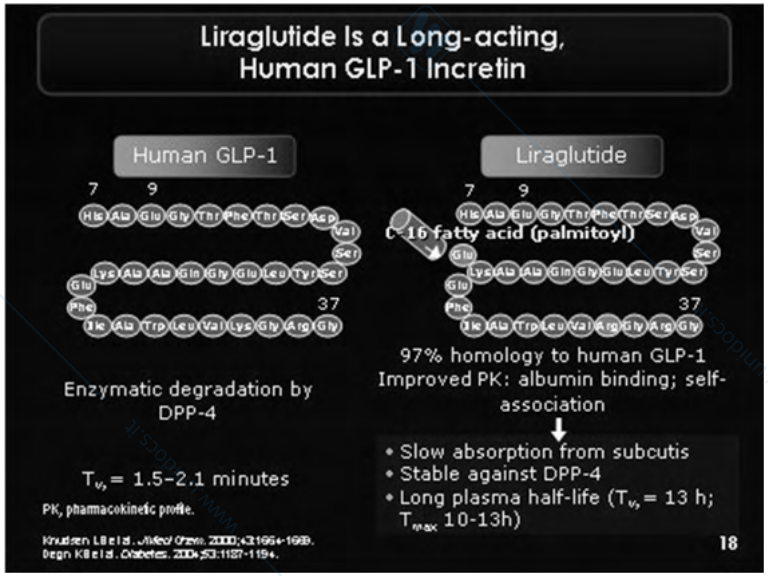
LIRAGLUTIDE

GLP-1 analog (97% homology)

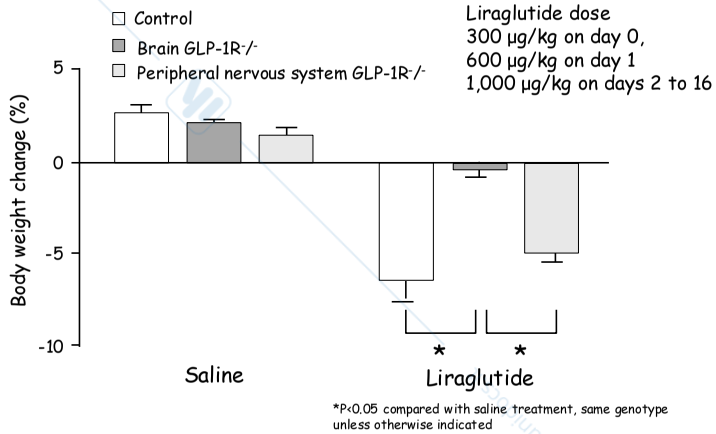
GLP-1R agonists

Glucose-lowering effect by stimulating insulin secretion by pancreas b-cells

Weight lowering effect by acting at brain level



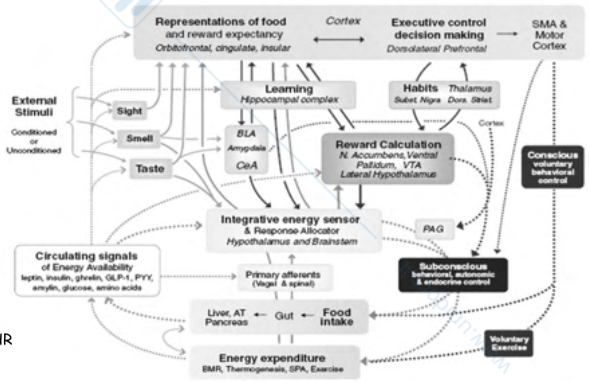
BRAIN GLP-1 RECEPTORS MEDIATE THE BODY WEIGHT LOWERING EFFECT OF LIRAGLUTIDE



Adapted from: Sisley *et al. J Clin Invest* 2014

EXTRA-HYPOTHALAMIC AREAS

A new view is to include other brain areas such as basal ganglia and cortico-limbic systems in the greater circuitry of the homeostatic regulator. This view is supported by observations of lasting effects on food intake and energy balance by manipulating such extra-hypothalamic areas. It would also be much better to explain how obesity can develop in a rapidly changing environment that primarily interacts with the cognitive and emotional brain.



Berthoud HR
PNS 2012

THE HEDONIC MECHANISM

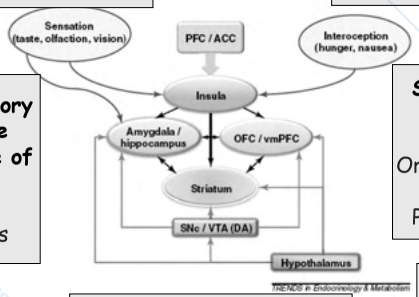
If feeding was controlled solely by homeostatic mechanisms, most of us would be at our ideal body weight, and feeding would be considered like breathing….However, humans will pay large sums of money for an excellent meal and will eat beyond their homeostatic needs if presented with highly palatable food. **Saper et al., Neuron, 2002**

Cognitive control/Attention Decision-making
Anterior cingulate cortex
Dorsolateral-
Prefrontal Cortex

Integration of autonomic responses with behavior and emotion
Insula "ingestive cortex"

Emotion/Memory Encoding the incentive value of food cues
Amygdala,
hippocampus

Signal computing and decision-making
Orbitofrontal cortex
Ventromedial-
Prefrontal Cortex

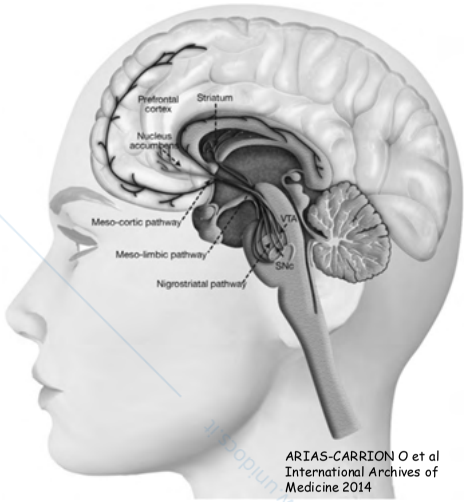


Reward, learning and motivational signal via dopamine
Nucleus accumbens/
Striatum

Origin of dopaminergic neurons
VTA/
Substantia nigra

Adapted from Dagher A.
TEM 2012

Dopaminergic neurons are located in the midbrain structures substantia nigra and the ventral tegmental area.
Their axons project to the striatum (caudate nucleus, putamen and ventral striatum including nucleus accumbens), the dorsal and ventral prefrontal cortex.



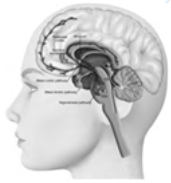
ARIAS-CARRION O et al International Archives of Medicine 2014

The mesolimbic dopamine pathway mediates the psychopharmacology of REWARD

Reward or reinforcement is an objective way to describe the positive value that an individual ascribes to an object, behavioral act or an internal physical state.

The pathway is referred as the pleasure center of the brain, with dopamine as the pleasure neurotransmitter.

When activated, this system reinforces behaviour.

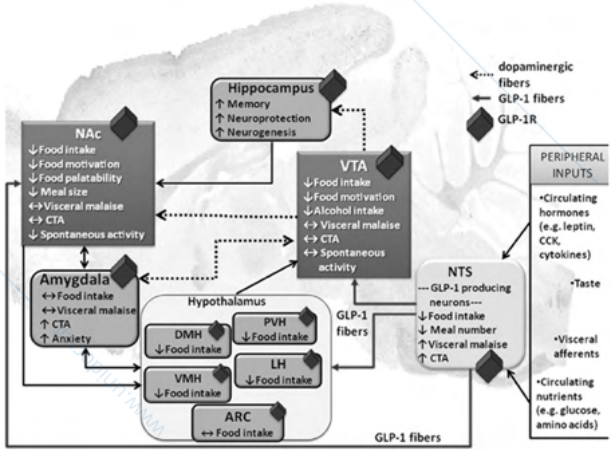


ARIAS-CARRION O et al International Archives of Medicine 2014

HORMONES PRODUCED BY THE GUT
IMPACT ON THE HEDONIC MECHANISMS

promoting the intake of the most palatable foods

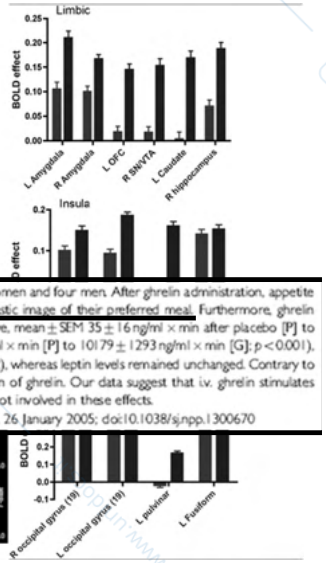
EFFECT OF GLP-1 ON FOOD INTAKE AND ASSOCIATED BEHAVIORS IS NEUROANATOMICALLY DISTRIBUTED



Skibicka KP *Front. Neurosci* 2013

Ghrelin Modulates Brain Activity in Areas that Control Appetitive Behavior

Malik et al. Cell Metab 2008

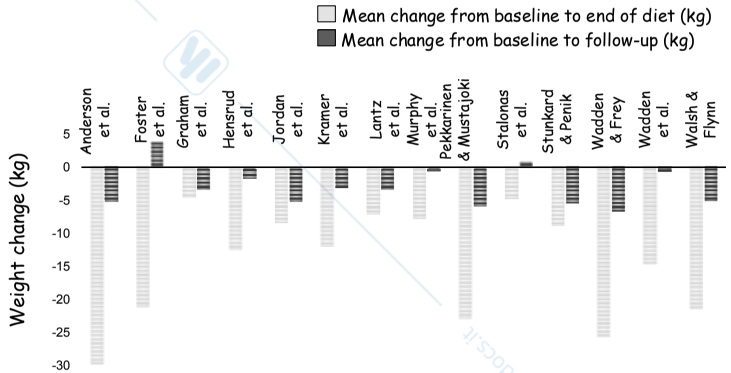


young control subjects. In order to test gender differences, we included five women and four men. After ghrelin administration, appetite was enhanced in eight of nine subjects. Seven probands reported a vivid, plastic image of their preferred meal. Furthermore, ghrelin stimulated an immediate increase in plasma levels of GH (area under the curve, mean ± SEM 35 ± 16 ng/ml × min after placebo [P] to 2808 ± 533 ng/ml × min after ghrelin [G]; $p < 0.001$), cortisol (5908 ± 984 ng/ml × min [P] to 10179 ± 1293 ng/ml × min [G]; $p < 0.001$), and ACTH (922 ± 103 pg/ml × min [P] to 3030 ± 763 pg/ml × min [G]; $p < 0.02$), whereas leptin levels remained unchanged. Contrary to placebo, glucose concentration did not decrease markedly after administration of ghrelin. Our data suggest that i.v. ghrelin stimulates appetite and images of food in young women and men. Obviously, leptin is not involved in these effects.

Neuropsychopharmacology (2005) **30,** 1187–1192, advance online publication, 26 January 2005; doi:10.1038/sj.npp.1300670

COUNTER-REGULATORY "HOMEOSTATIC" MECHANISMS DRIVING WEIGHT REGAIN

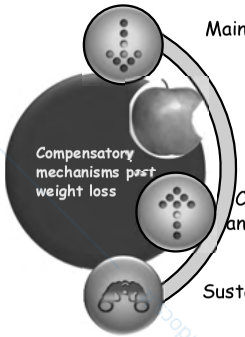
MAINTENANCE OF WEIGHT LOSS IS CHALLENGING



Follow up range from 4 to 7 years

Mann *et al. Am Psychol* 2007

PHYSIOLOGY OF WEIGHT REGAIN



Maintaining body weight at ≤10% or less decreases resting, non-resting and total energy expenditure

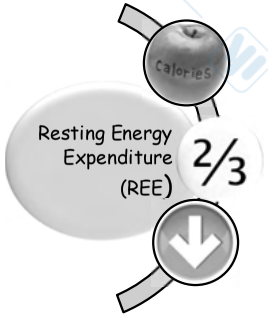
Weight loss is accompanied by an increase of fasting desire to eat, hunger and prospective food consumption

Changes in digestive hormones, suppression of thyroid axis and amplification of cortisol levels, can promote weight gain

Sustained behaviour change (DIET, PHYSICAL ACTIVITY) is necessary for long-term weight loss maintenance

Leibel *et al. N Engl J Med* 1995; Doucet *et al. Int J Obes Relat Metab Disord* 2000; Blomain *et al. ISRN Obes* 2013; Thomas *et al. Am J Prev Med* 2014

RESTING ENERGY EXPENDITURE IS REDUCED IN RESPONSE TO WEIGHT LOSS



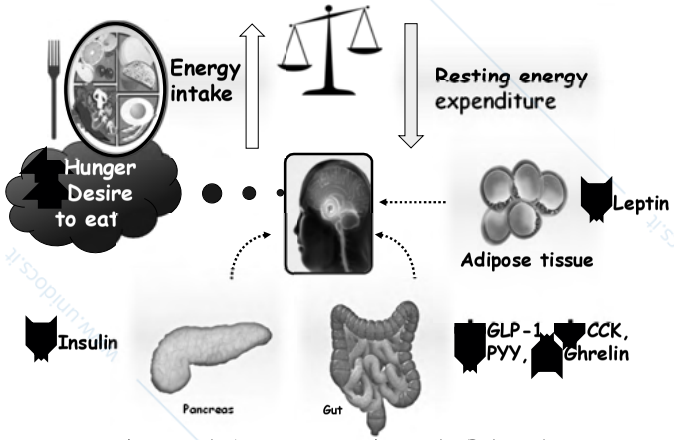
Refers to the amount of energy required to fuel the body at rest

Two-thirds of an individual's total daily energy needs

Decreased REE has been found to be a determinant of weight regain

Hill *et al. Circulation* 2012; Pasman *et al. Obes Res* 1999; Schwartz *et al. Obes Rev* 2010

PHYSIOLOGICAL RESPONSES TO WEIGHT LOSS FAVOR WEIGHT REGAIN



Schwartz *et al. Obes Rev* 2010. Sumithran *et al. N Engl J Med* 2011

ETIOLOGIC CLASSIFICATION OF OBESITY

- Genetic syndromes
- Monogenic obesity
- Endocrine/Iatrogenic
- Essential
- Psychiatric
  - *Psycogenic obesity*
  - *Bulimia nervosa*

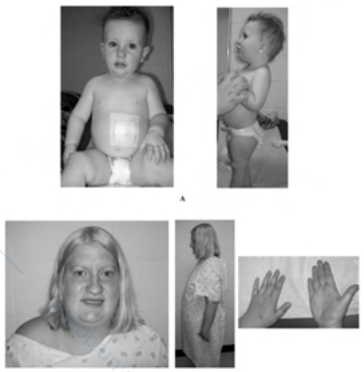
Obesity associated with genetic syndromes

- Prader-Willi Syndrome:compex inheritance pattern, central distribution of adipose tissue, short stature, hypogonadotropic hypogonadism, type II diabetes, moderate mental retardation, narrow forehead, almond-shaped eyes, thin upper lip and downturned mouth, small hands and feet, hypotonic muscles. It represents the most frequent genetic syndrome associated with obesity (1/25000). Most frequent exitus is caused by cardiorespiratory failure.
- Alström Syndrome:autosomal recessive inheritance, central obesity within age 5, no mental retardation, hypogonadotropic hypogonadism in males, type II diabetes, retinitis pigmentosa, sensorineural hear loss.
- Laurence-Moon Syndrome:autosomal recessive inheritance, central obesity within age 1, gynoide adipose tissue distribution in both sexes, moderate mental retardation, gliosis of hypothalamic nuclei, short stature, retinic dystrophy, spastic paraparesis.

Obesità associata a s. genetiche II

- Bardet-Biedl Syndrome similar to Laurence S., though neurologic complications are rare. Renal disturbes and polydactyly are typical.:
- Albright Syndrome:X-linked, typical facies and face obesity, short stature and limbs, variable mental retardation, dental retardation, calcification of soft tissues.
- Down Syndrome (21 trisomy):general obesity within age 3, short stature, variable mental retardation, typical facies tipica, clinodactyly, hypotinic muscles, cardiopathies.

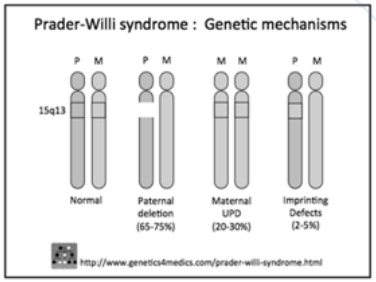
The Prader-Willi syndrome



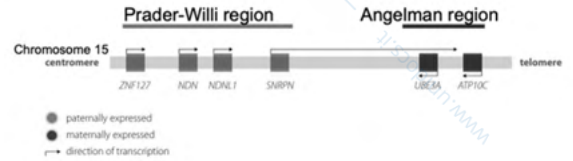
Frontal and profile views of a 16 month old female with PWS due to maternal disomy 15 and not treated with GH.
Facial, profile and hand views on a 18y old female with PWS due to the typical 15q11-q13 deletion.

PWS is a complex neurodevelopmental disorder due to errors in genomic imprinting with loss of imprinted genes that are paternally expressed from the chromosome 15q11-q13 region. Approximately 70% of individuals with PWS have a *de novo* deletion of the paternally derived 15q11-q13 region; maternal disomy 15 (both 15s from the mother) in about 25% of cases, and the remaining subjects have either defects in the imprinting center controlling the activity of imprinted genes or due to other chromosome 15 rearrangements.
Hyperphagia leading to early childhood obesity. Obesity is a significant health problem, if uncontrolled. PWS is considered the most common known genetic cause of morbid obesity in children.

In the PWS the maternal gene is silenced by imprinting, while the paternal gene is deleted. This loss causies the absence of the encoded protein/s. The causative gene is on region 15q11-q13.



The same region is involved in the *Angelman Syndrome*, however, this is casued by the imprinting of the paternal gene and deletion of the maternal gene. Symptoms are very different.



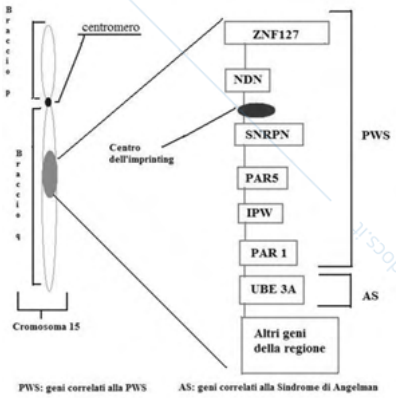
NECDIN

NECDIN is a nuclear protein expressed by some neurons.
It regulates the permanent arrest of cell growth after the embrional mitosis period, during the neuronal stabilization.

NDN gene is also expressed at the placenta level.

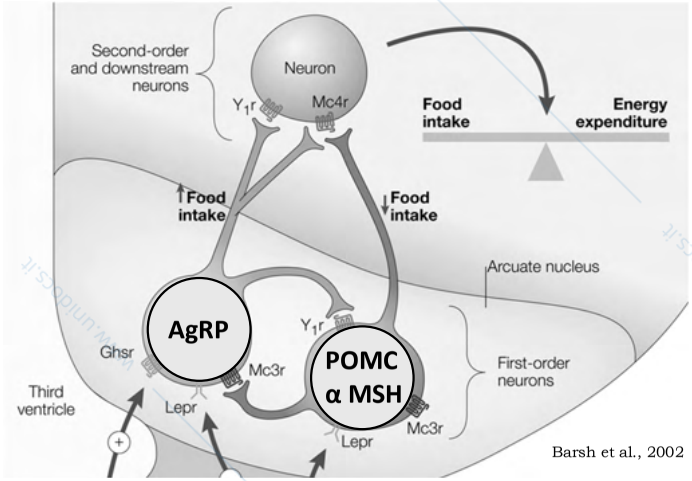
In human fybroblast the paternal NDN gene is exclusively expressed.

These findings clarify how the loss of paternal allele causes the neurologial disorders in PWS patients.



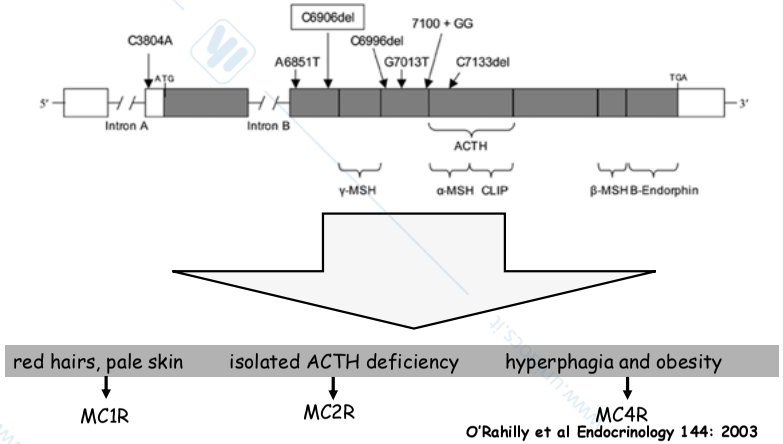
MONOGENIC OBESITY

THE HYPOTHALAMUS IS THE ENERGY BALANCE CONTROL CENTER

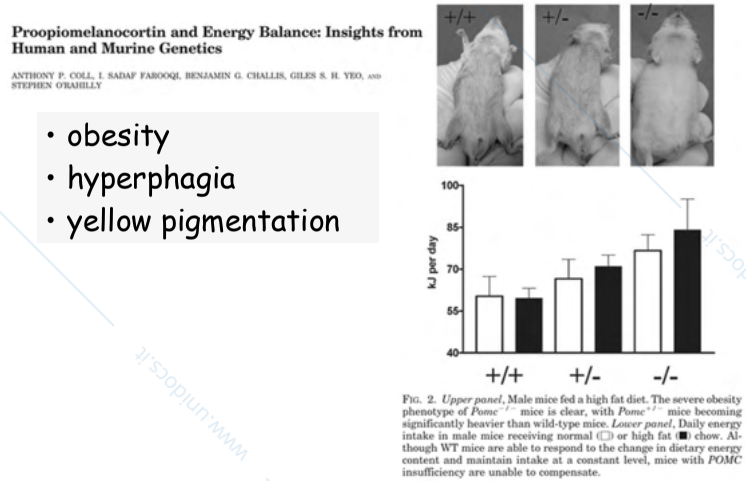


Proopiomelanocortin / a-MSH

241 aa propeptide expressed in hypothalamus and pituitary



POMC mutations



- obesity
- hyperphagia
- yellow pigmentation

POMC mutations

Severe early-onset obesity, adrenal insufficiency and red hair pigmentation caused by *POMC* mutations in humans

Heiko Krude[1], Heike Biebermann[1], Werner Luck[2], Rüdiger Horn[2], Georg Brabant[2] & Annette Grüters[1]

Red hairs
Adrenal insufficiency
Hyperphagia
Early obesity

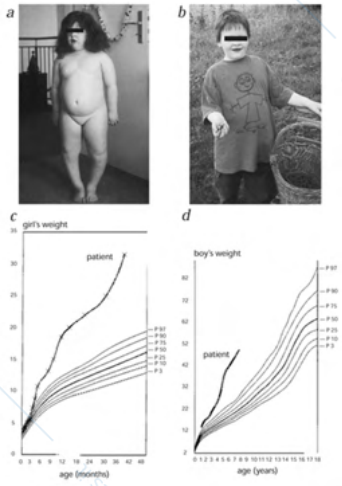


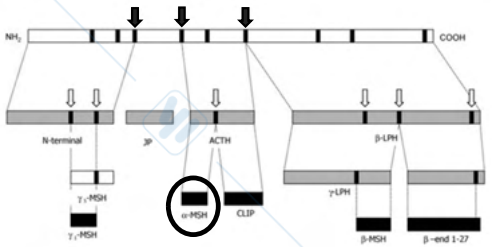
Fig. 1 Phenotype and weight curves of patient 1 and 2. a, Patient 1 is shown at three years of age, demonstrating the red-hair pigmentation and obesity. She had normal weight at birth and developed cholestasis at three weeks. Due to the history of adrenal hypoplasia in her first-born brother, the ACTH deficiency was diagnosed at 23 days and hydrocortisone substitution led to subsequent resolution of cholestasis. Since she was four months of age, the parents reported an increased appetite which led to severe early-onset obesity interfering with the ability to walk until she was two years. Mental development so far has been normal.
b, Patient 2 is shown at an age of five years. The perinatal history was complicated by transient hypoglycaemia. His birth weight was normal and obesity was first noticed at five months . After a febrile seizure attack at 12 months, blood-glucose measurement revealed hypoglycaemia and hyponatriaemia leading to an endocrine work-up which resulted in the diagnosis of adrenal insufficiency due to complete ACTH deficiency. With hydrocortisone substitution, his subsequent development was uneventful apart from abnormal eating behaviour causing progredient obesity. His intellectual and emotional assessments (HAWIK test at five years) were normal. In both children, MRT imaging revealed normal pituitary morphology. **c,d, Auxology of patients 1 (c) and 2 (d) demonstrating progredient** obesity in both cases. The weight curves of the patients are indicated in red. The photographs are reproduced with the written informed consent of the parents

Nat Genet. 1998;19:155-7.

POMC / αMSH key role in energy balance in human is also confirmed by:

- Increased risk of obesity with environmental interaction in individuals carrying non-inactivating mutations of POMC
- Hyperphagia in subjects carrying selective mutations of MSH
- Obesity in carriers of Prohormone Convertase gene (PC)-1

PROHORMONE CONVERTASE (PC)-1 MUTATIONS



PC-1 cleaves POMC, proinsulin, proglucagone (GLP-1) and other neuropeptides

Carriers of homozygous and combined heterozygous mutations are affected by :

- High POMC levels
- **Obesity**
- Hypogonadotropic Hypogonadism
- Postprandial hypoglycaemia
- Secondary hypoadrenocorticism
- Impaired intestinal motility

mice affected display short length but not obesity

GENETIC DEFICIT OF α-MSH RECEPTOR in mice

MC4-R -/- obesity, hyperglycaemia, hyperphagia, hyperinsulinaemia, increased length

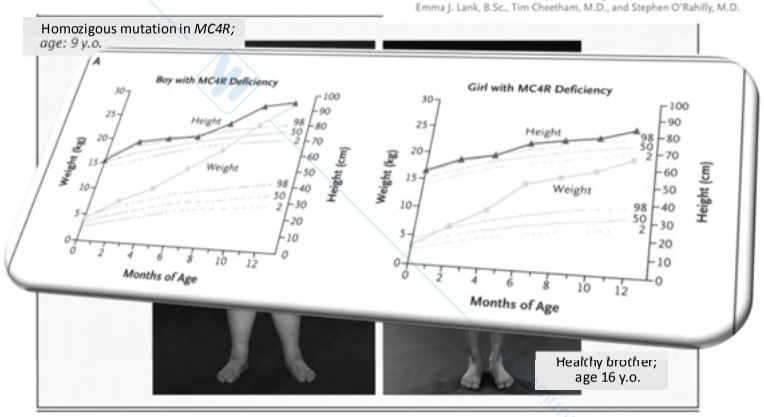
MC3-R -/- increased fat mass, decreased lean mass, reduced physical activity, prone to diet induced obesity

MC3-R -/- MC4-R -/- significantly heavier than MC4-R -/-

GENETIC DEFICIT OF α-MSH RECEPTOR in humans

Clinical Spectrum of Obesity and Mutations in the Melanocortin 4 Receptor Gene

I. Sadaf Farooqi, M.D., Ph.D., Julia M. Keogh, B.Sc., Giles S.H. Yeo, Ph.D., Emma J. Lank, B.Sc., Tim Cheetham, M.D., and Stephen O'Rahilly, M.D.



N Engl J Med 2003;348:1085-95.

Genetics of Obesity in Humans

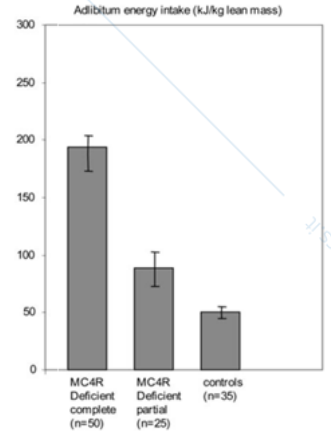
I. Sadaf Farooqi and Stephen O'Rahilly

Genotype - phenotype correlation in human MC4R deficiency.

Ad libitum food intake at test meal (30 min) for patients with complete and partial loss of function MC4R mutations.

Partial MC4R deficiency causes 2-folds higher food intake.
Complete MC4R deficiency causes 4-folds higher food intake.

Gene dosage effect.



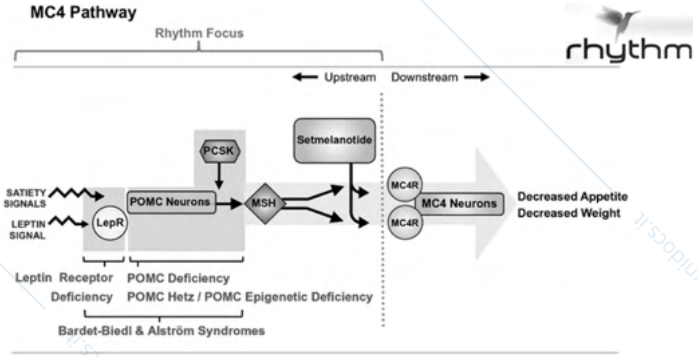
Endocrine Reviews 27: 710–718, 2006

What is the epidemiologic impact of MC4R mutations ?

- 6% of patients with pediatric obesity
- 1-2.5% of patients with BMI > 30

It is the most frequent known monogenic cause of human obesity

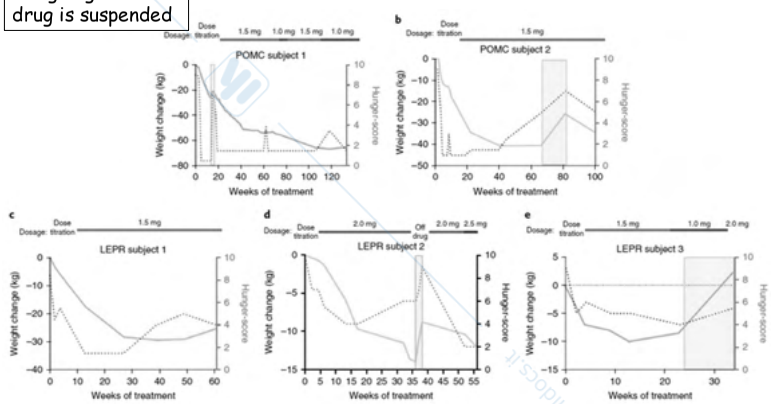
SETMELANOTIDE in MONOGENIC AND POLYGENIC OBESITY



Selective agonist of MC4R in the paraventricular nucleus (PVN) of the hypothalamus and in the lateral hypothalamic area (LHA).
Reduces appetite.
Increases resting energy expenditure.

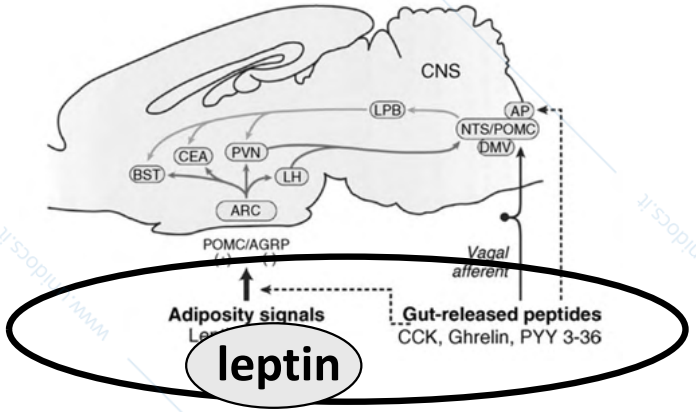
SETMELANOTIDE in MONOGENIC AND POLYGENIC OBESITY

Weight gain when drug is suspended



Clement K et al Nature Med 2018

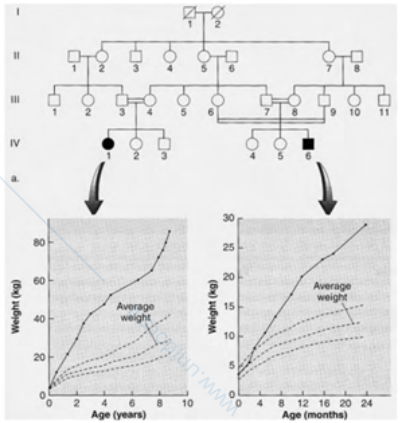
NEUROENDOCRINE CONTROL OF ENERGY METABOLISM: INTEGRATION OF CENTRAL AND PERIPHERAL SIGNALS



Peptides 27 (2006): 340-349

GENETIC DEFICIT OF LEPTIN

A few families in the world.
One Pakistan familiy carries a frameshift mutation (ΔG133) in a hotspot of a run of six guanines.
ΔG133 results in a truncated form of leptin that is misfolded and not secreted.
Heterozygote members of the ΔG133 families show leptin levels lower than ethnically matched control subjects.
This partial leptin deficiency is associated with a mean fat mass 23% greater than predicted by their height and weight.



The clinical phenotype of human congenital leptin deficiency is very similar to that seen in the *ob/ob* mouse, though with some differences

TABLE 1
Phenotypes Associated with Leptin Deficiency in Rodents (ob/ob) and Humans

Phenotype	*ob/ob*	Human leptin deficiency
Total body weight	3X Normal	Mean BMI sds = 6.2
Body composition		
Fat mass	> 50%	Mean 57% of body weight
Lean mass	Decreased	Normal for age
Bone mineral content	Decreased	Normal for age
Food intake	Increased meal size	Increased meal size and frequency
Energy expenditure		
Body temperature	Decreased in response to cold	Normal in basal state
Basal metabolic rate	Decreased oxygen consumption	Appropriate for body composition
Physical activity	Reduced	Reduced
SNS activation	Basal decreased and refractory to cold exposure	Reduced in response to cold
Metabolic responses		
Diabetes	Fasting hyperglycaemia	Normoglycaemia
Hyperinsulinaemia	Severe; resistance to exogenous insulin	Appropriate for degree of obesity
T cell-mediated immunity	Decreased CD4 cells, reduced T-cell proliferation	Decreased CD4 cells, reduced T-cell proliferation
Neuroendocrine function		
Reproductive	Hypogonadotropic hypogonadism	Hypogonadotropic hypogonadism
Thyroid	Hypothalamic and ?peripheral effects	Mild hypothalamic hypothyroidism
Growth	Stunted	Normal linear growth and IGF-1 levels
Adrenal	Corticosterone excess	Normal cortisol and ACTH levels

[Abbreviations: BMI, body mass index; SNS, sympathetic nervous system; IGF-1, insulin-like growth factor-1; ACTH, corticotropin.]

Minireview: Human Obesity—Lessons from Monogenic Disorders

STEPHEN O'RAHILLY, I. SADAF FAROOQI, GILES S. H. YEO, AND BENJAMIN G. CHALLIS

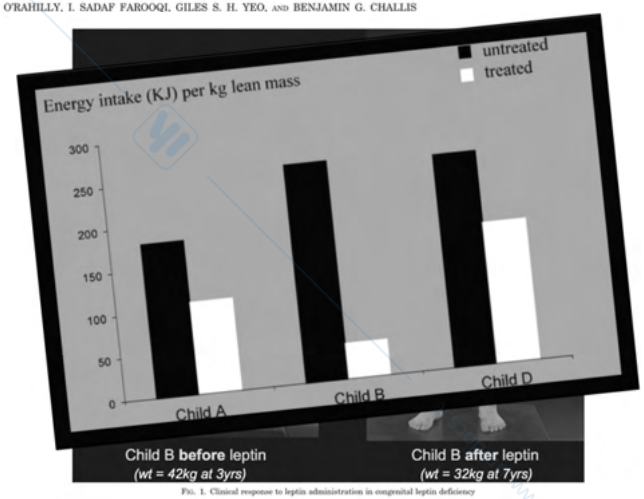


FIG. 1. Clinical response to leptin administration in congenital leptin deficiency

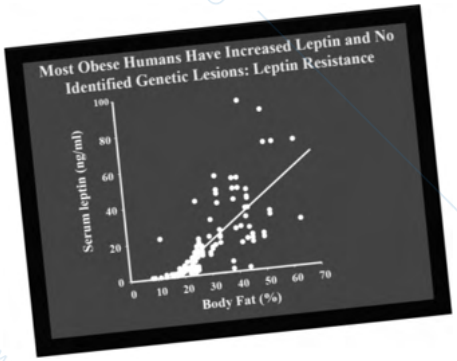
Endocrinology 144: 3757–3764, 2003

ESSENTIAL OBESITY

- The most frequent form of obesity.
- The alteration of the energy balance is caused by genetic, environmental, ethnic and socio-cultural factors.
- The genetic component is now considered to be 25-40%.
- Food intake quantitatively excessive or qualitatively unbalanced.
- Eating disorders.

LEPTIN and ESSENTIAL OBESITY

Possible mechanisms of Leptin-Resistance



- Impaired leptin transport across the blood-brain barrier
- Reduced leptin uptake in the brain
- Expression of a "Leptin-inhibitory protein"

The adipocyte

THE ADIPOSE TISSUE

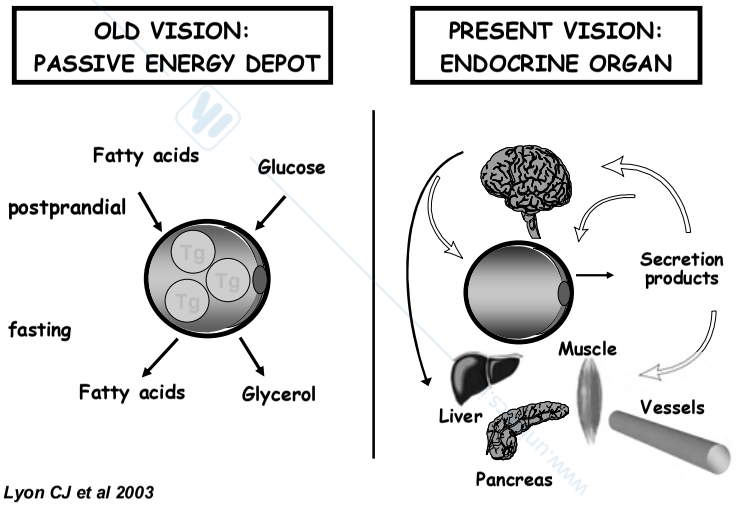
- **in the past:**
  - **a passive tissue for energy storage in the form of lipids**
  - **the only tissue capable of growing in an almost unlimited way, without losing the cell phenotype, but probably contributing to the pathogenesis of some anomalies described in obesity**
- **today?**
  - **an organ, involved in the regulation of energy balance by means of a complex network of endocrine, paracrine and autocrine mediators: the ADIPOKINES.**

THE ADIPOSE TISSUE
a number of different biological functions

- Buffering starvation
- Buffering stochasticity
- Buffering the brain
- Adaptation to cold
- Growth
- Reproduction
- Sexual selection
- Immune function
- Psychosocial stress

- Rather than being supplied by large arteries, adipose tissue depots are maintained by many smaller blood vessels from adjacent tissues, which generate close connections with these tissues
- involvement in multiple signaling pathways through which energy needs and energy availability interact
- interaction with fundamental feedback systems that proactively allocate energy between multiple competing functions

The evolution of the adipose tissue vision:

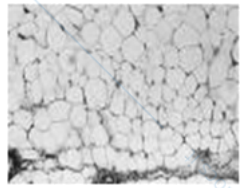


Lyon CJ et al 2003

WHITE ADIPOSE TISSUE (WAT)

The WAT has a central role in the energy metabolism. Deputed to triglycerides storage.

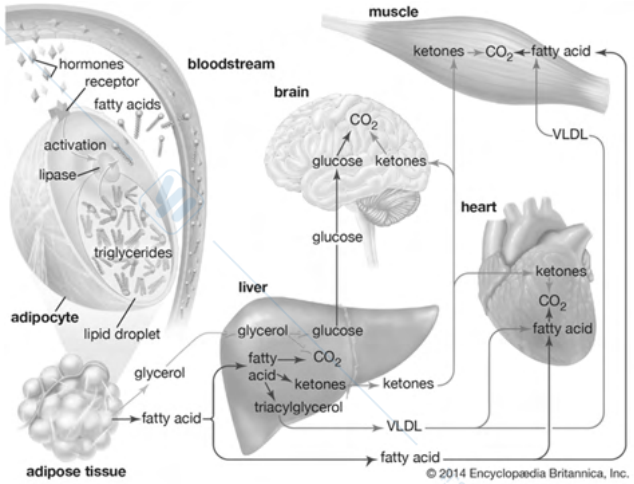
White adipocytes are UNILOCULAR: large (<100um), with central lipid vacuoles that take up much of the space within the cell. Nuclei are peripherally located, and the cytoplasm forms a thin peripheral ring around the central vacuole



The lipoprotein lipase in the adipocytes hydrolizes triglycerides in chylomicrons and in VLDL: fatty acids entering the adipocytes are then esterified.

In fasting condition, triglycerides are hydrolized to provide energy fuel.

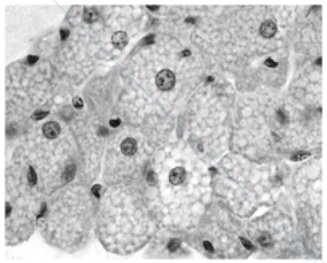
Triglycerides mainly contain:
Oleic acid (45%),
Palmitic acid (20%),
Linoleyc acid (10%),
Stearic acid (6%).



When hormones signal the need for energy, fatty acids and glycerol are released from triglycerides stored in fat cells (adipocytes) and are delivered to organs and tissues in the body.

BROWN ADIPOSE TISSUE (BAT)

Brown adipocytes are MULTILOCULAR, containing many small lipid droplets, and a high number of iron-containing mitochondria, giving them the dark color. High oxygen consumption. More capillaries. High symphatetic innervation.

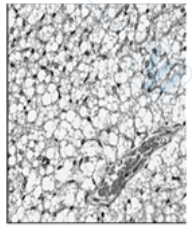


The BAT operates thermogenesis. In humans, this is mainly occurring in newborns. Not clear its impact in adults.

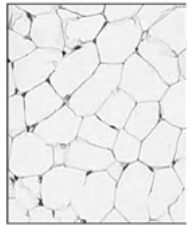
Thermogenesis is stimulated by adrenalin, which activates lipolysis. Free fatty acids act as uncoupling factors, dispelling the proton gradient of the respiratory chain, thereby turning energy into heat.

BROWN ADIPOSE TISSUE

BAT

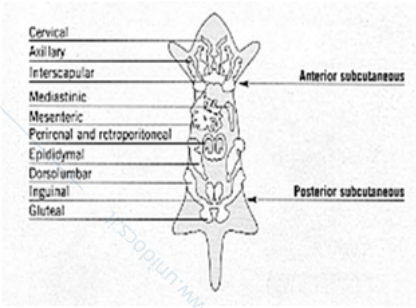


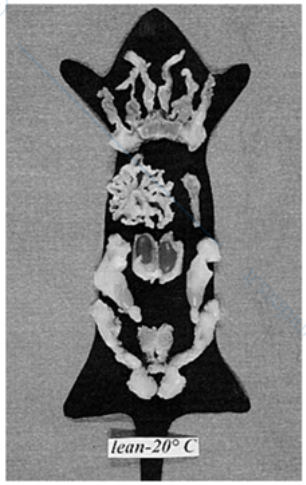
WAT



The BAT is thus defined for its brown coloring given by the high presence of iron associated with the cytochromes present in the mitochondria.
It is mainly found in animals that go into hibernation.
It is scarcely present in adult humans, but it is found in infants.

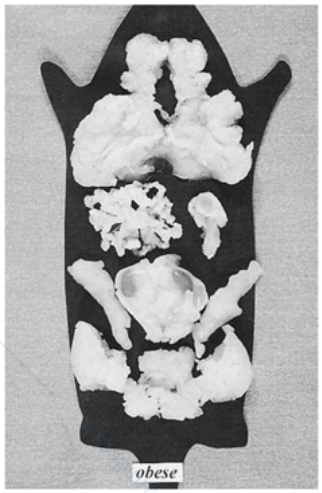
ADIPOSE ORGAN





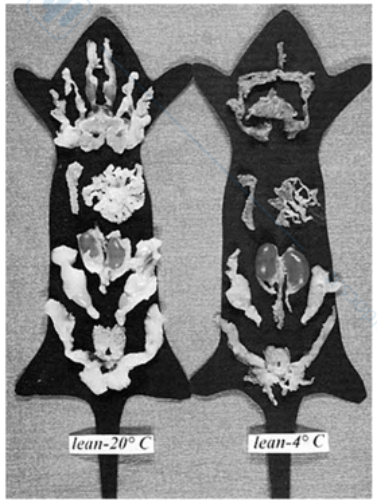
Derivato da Cinti S.
The adipose organ

ADIPOSE ORGAN
in the db/db mouse



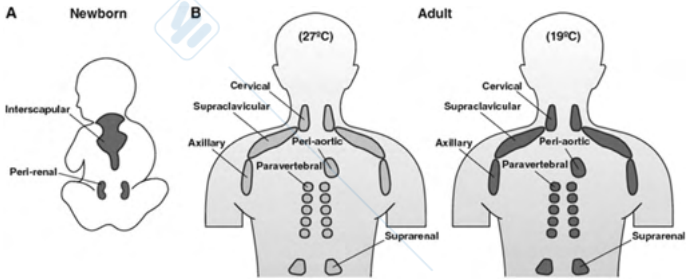
Derivato da Cinti S.
The adipose organ

ADIPOSE ORGAN
in the adult rat housed at different temperature



Derivato da Cinti S.
The adipose organ

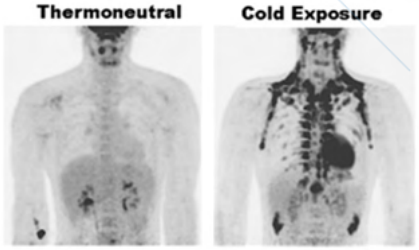
In humans, BAT is mainly found in paracervical, supraclavicular, interscapular, and perirenal areas, in close proximity with blood vessels, so to diffuse heat to the whole body through the bloodstream.



In the past, the presence of BAT was believed to be irrelevant in humans, but several recent researches recognize its presence, and reveal important properties in the cold-induced thermogenesis.

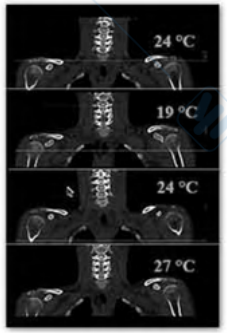
Its main function is to produce heat in response to low temperatures, which is called "cold-induced thermogenesis". In particular, the BAT operates the "non shivering" thermogenesis (NST), which contributes to "shivering" thermogenesis operated by the skeletal muscle, to protect the body from cold stress.

WAT is converted to BAT in response to cold exposure by transdifferentiation



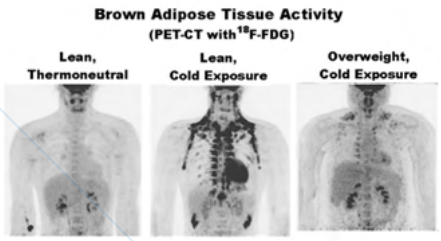
The scientific litterature, still limited, reports that the BAT accounts for the 5% of the basal energy metabolism.
BAT has therefore a role in regulating the body weight.

PET-CT with 18F-FDG studies



Research field trying to generate a drug able to induce wat to bat conversion in order to increase energy waste.

Fluorodeoxyglucose, labelled un radionuclide. Enters the cells by the glucose-receptor, but is not metabolized. BAT tissue is much more metabolically active, thereby internalizing 18FDG



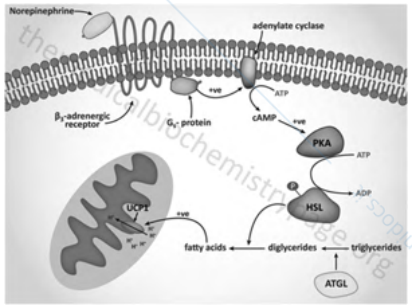
Lean men transforms his WAT in BAT following a cold stimulus

Obese subjects may have an impaired WAT to BAT conversion when exposed to cold.

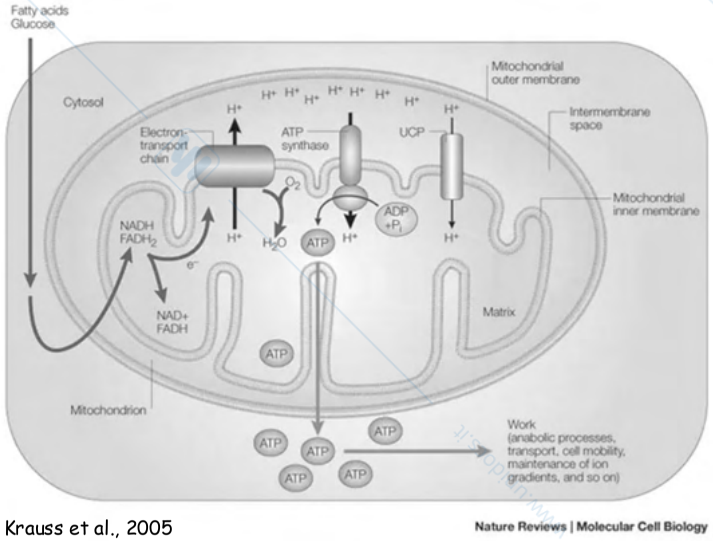
BAT metabolic activity is stimulated by the short-term cold exposure. The non-shivering thermogenesis is based on FATTY ACIDS as substrates.

In the fed state, fatty acids are mainly obtained by LPL action on triglycerides-rich plasma lipoproteins to release free fatty acids, which are then taken up brown adipocytes.

Adipocytes TG stores are another source of fatty acids for NTS, and is obtained by hydrolysis mediated by the hormone sensitive lipase or by adipose triglyceride lipase.



Fatty acids generated by brown adipose cells generally are not secreted; instead, they are used by the cells' mitochondria in order to generate heat (thermogenesis), particularly in hibernating animals and human infants.



Krauss et al., 2005

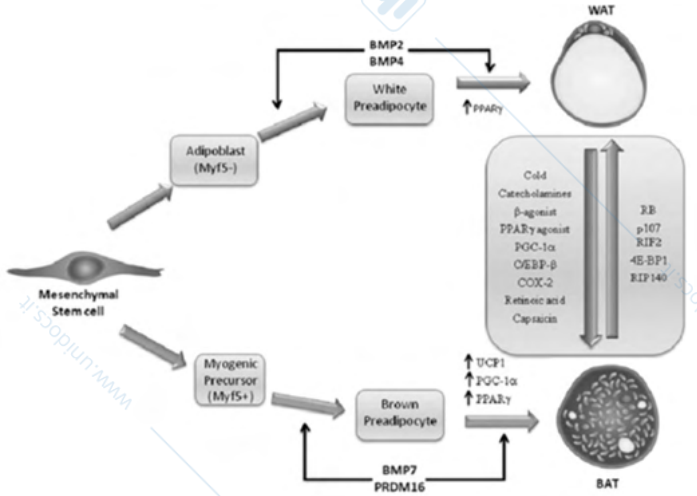
NST mechanism is based on mitochondrial uncoupling, which is the dissociation between the generation of a protone potential across the inner mitochondrial membrane and its use for mitochondria-dependent ATP synthesis.

BAT mitochondria express the Uncoupling Protein 1 (UCP1). This protein has the property of uncoupling the oxidative phosphorylation (the aerobic energy-generation process of cell respiration), by shifting the lipid oxidation process from the generation of ATP (chemical energy) to the generation of heat (thermal energy).

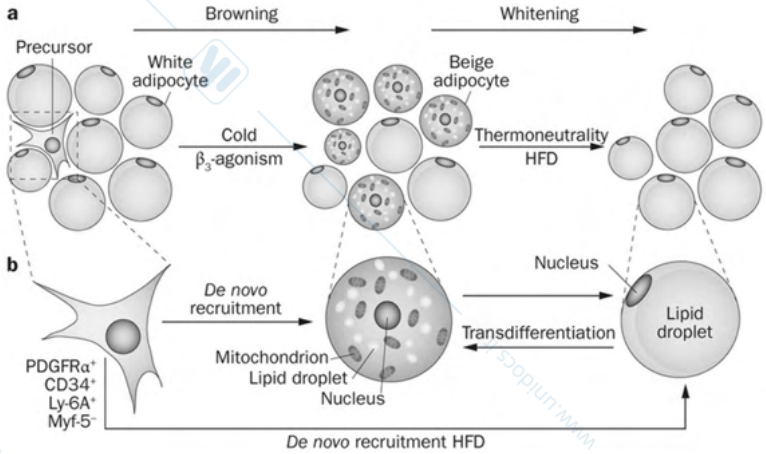
This mechanism is activated in response to cold exposure by noradrenaline β3-receptor signaling, activated through the sympthetic nerves under hypothalamic control. NTS is also supported by the increased release of thyroid hormones T3 and T4. The uncoupling mechanism supporting NST is mainly active, in addition to BAT, in the skeletal muscle and the liver.

Cold sensation is transmitted to BAT primarily via the sympathetic nervous system. Catecholamines activate b3-adrenergic receptors, leading to an increase of cellular cAMP concentrations and activation of protein kinase A (PKA). Upon short-term cold exposure for several hours (acute cold), PKA increases the transcription of UCP-1 and peroxisome proliferator-activated receptor (PPAR)-g co-activator 1a (PGC-1a). PGC-1a, inturn, co-activates PPAR-a, a crucial nuclear receptor orchestrating the transcriptional program for substrate oxidation and thermogenesis in BAT.
A sustained increase in sympathetic tone upon long-term cold exposure for several weeks (cold acclimation) additionally induces the recruitment of new brown adipocytes, leading to tissue hyperplasia and hypertrophy.
Cold-induced adrenergic signaling also induces the hydrolysis of intracellular triacylglycerol (TG) stores (lipolysis) via the activation of the major TG hydrolases adipose triglyceride lipase (ATGL) and hormone-sensitive lipase (HSL). According to the current view, BAT lipolysis is essential for cold-induced NST because BAT-derived fatty acids (FAs) (1) activate UCP-1 and (2) act as primary fuel substrate.

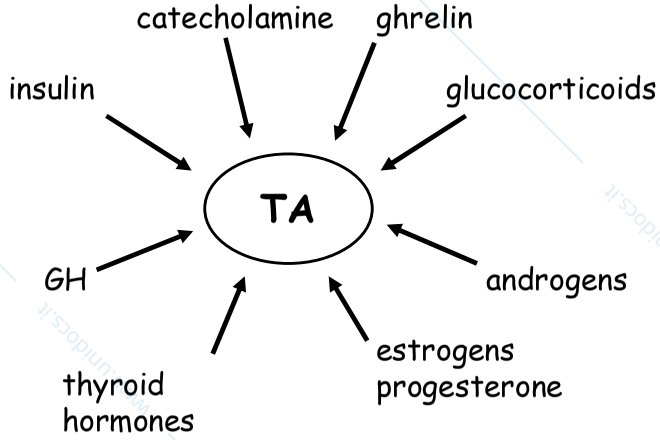
BAT and WAT have DIFFERENT ORIGIN



TRANS-DIFFERENTIATION



ADIPOSE TISSUE PHYSIOLOGY
hormonal regulation

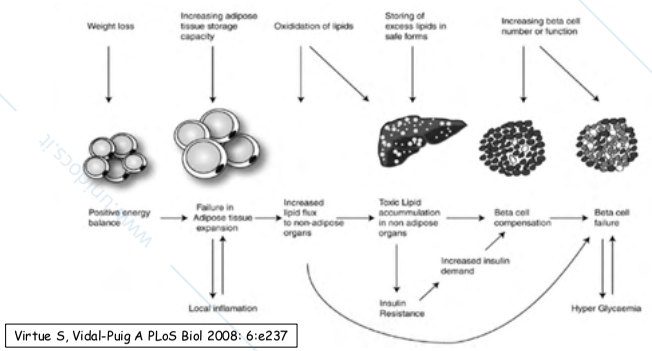


HORMONES FUNCTIONS IN ENERGY METABOLISM

Hormone	Biologic function
Insulin	Adipocyte differentiation + glucose transport + triglycerides synthesis - lipolysis
Catecholamine	+ lipolysis
Glucocorticoids	+ lipogenesis + leptin - catechol-induced lipolysis
Testosterone	+ lipolysis
Estrogens/Progesterone	Contrastanting functions
Thyroid Hormones	+ catechol-induced lipolysis - Oxidative phosphorylation

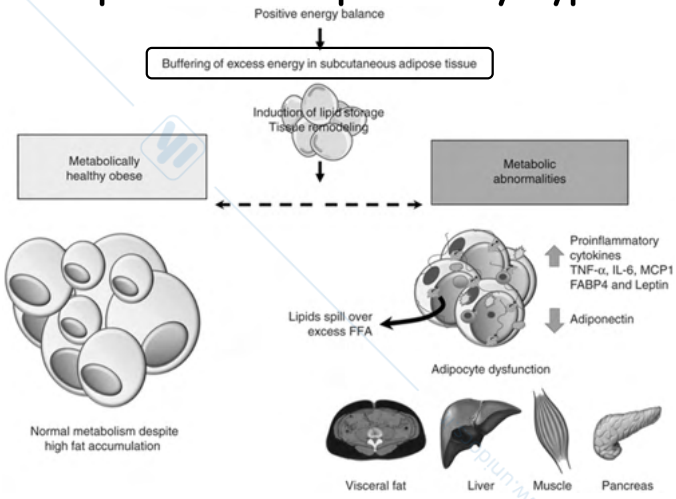
The adipose tissue expandability hypothesis

AT has a limited maximal capacity to increase in mass, which is determined on an individual basis (genes, epigenetic factors). When an individual reaches the limit of AT expansion, then lipids are stored ectopically (liver, muscle) ⇒ insulin resistance



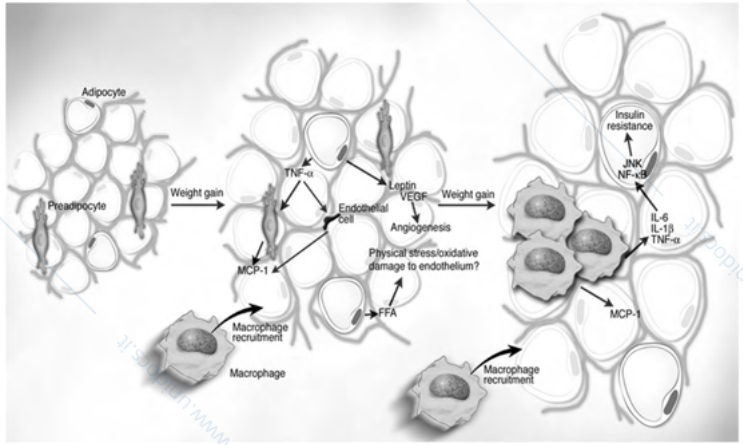
Virtue S, Vidal-Puig A PLoS Biol 2008: 6:e237

The adipose tissue expandability hypothesis



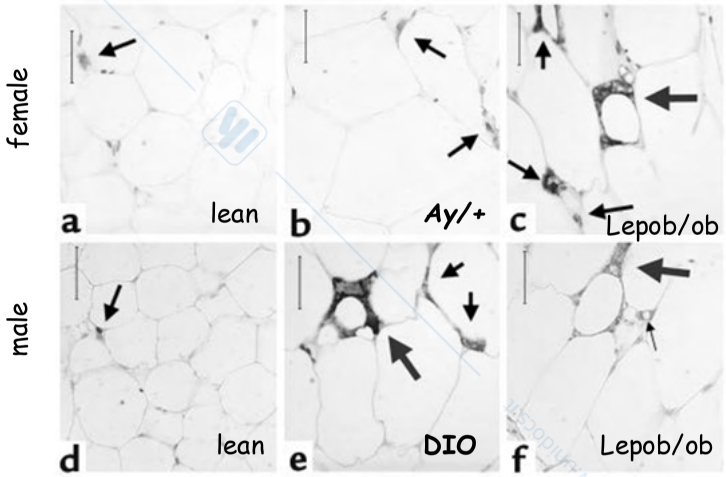
Gaggini M. et al. Hormone Molecular Biology and Clinical Investigation 2017

Dysfunctional Fat: Inflammation and progressive macrophage infiltration



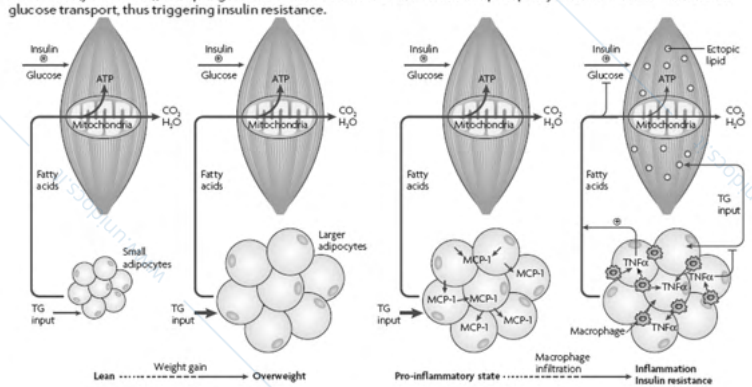
Wellen KE, Hotamisligil GS J Clin Invest 2003; 112:1785-1788

Adipose tissue macrophages (F4/80) in mice with varying degrees of adiposity.

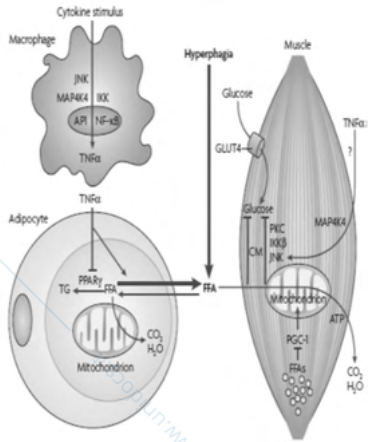


Weisberg SP, et al J Clin Invest. 112: 1796-1808, 2003

Figure 1 | **Chronic inflammation in adipose tissue triggers insulin resistance in skeletal muscle.** **a** | In the lean state, small adipocytes efficiently store fatty acids as triglyceride (TG input, arrow), which can be mobilized and used to generate ATP through the mitochondrial β-oxidation pathway in muscle during periods of caloric need. Insulin-stimulated glucose uptake under these conditions is normal. **b** | Excess caloric intake leads to metabolic overload, increased TG input and adipocyte enlargement. Nonetheless, in non-diabetic overweight individuals, TG storage by adipose cells and β-oxidation in muscle can often be maintained to prevent insulin resistance. **c** | On further overloading with TG, hypertrophy of adipocytes and increased secretion of macrophage chemoattractants occurs, including the secretion of monocyte chemoattractant protein-1 (MCP-1; arrows), which recruits additional macrophages. **d** | Macrophage recruitment in turn results in a pro-inflammatory state in obese adipose tissue. Infiltrating macrophages secrete large amounts of tumour-necrosis factor-α (TNFα), which results in a chronic inflammatory state with impaired TG deposition and increased lipolysis (arrow and plus signal). The excess of circulating TG and free fatty acids results in the accumulation of activated lipids in the muscle (yellow dots), disrupting functions such as mitochondrial oxidative phosphorylation and insulin-stimulated glucose transport, thus triggering insulin resistance.

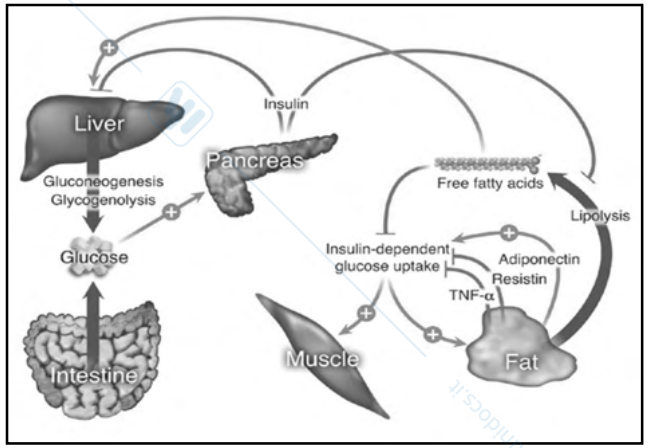


In normal subjects, insulin suppresses the hepatic production of glucose, stimulates glucose uptake of muscle and adipose tissue, and inhibits lipolysis of adipose tissue. With insulin resistance, hepatic glucose production increases, glucose uptake decreases and lipolysis is enhanced.
The increase in FFA due to lipolysis stimulates cellular uptake and lipid oxidation. In the muscle, the increased availability of FFA accelerates the oxidation of fats with the result of a decreased uptake and use of glucose by insulin. In the liver, high FFA levels stimulate gluconeogenesis and increase liver glucose production.



ADIPOCYTES SECRETORY FUNCTIONS

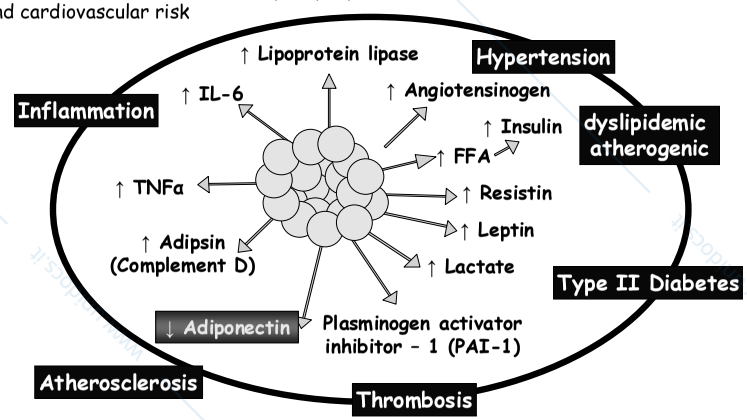
Cross-talk tissue-tissue in glucose and lipids homeostasis



Evans RM, Nature Medicine 2004

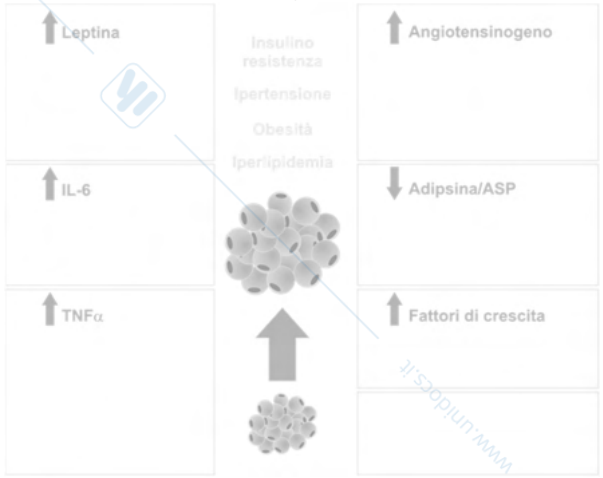
Adverse cardiometabolic effects of products of adipocytes products in excess fat condition

bioactive substances secreted by adipocytes, that modulate insulin resistance and cardiovascular risk

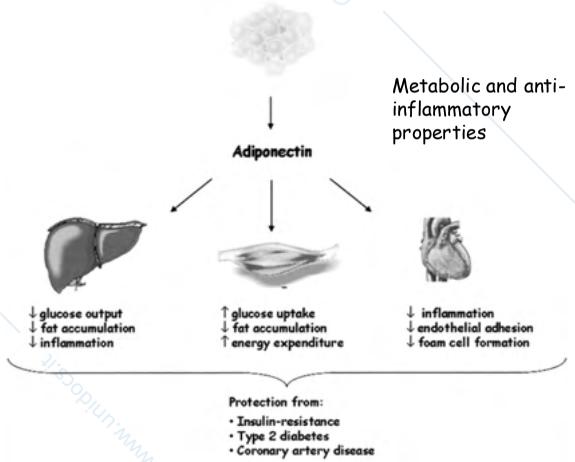


Lyon 2003; Trayhurn et al 2004; Eckel et al 2005

Excess in adipokine productions and consequences



ADIPONECTIN



Menzaghi C et al.Diabetes 2007

ADIPONECTIN
alias GBP-28, apM1, AdipoQ, Acrp30

- Protein hormone MAINLY secreted by the adipose tissue
- Key role in the regulation of glucose and lipid metabolism
- Obese and type II Diabetes patients display low adiponectin levels in the bloodstream
- Low adiponectin levels are associated with low HDL and high triglycerides and small LDL
- High adiponectin levels are associated with weight loss and lower glucose and insulin, eventually leading to a better insulin sensitivity.

ADIPONECTIN

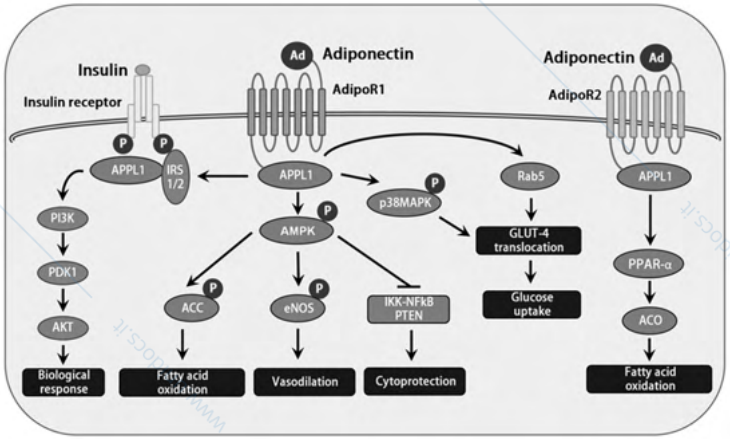
- Adiponectin expression is induced by PPAR-γ
- Adiponectin expression is activated during adipogenesis. At variance, its expression is inhibited in the development of visceral obesity, possibly by TNF-a.
- Adiponectin promotes insulin-sensitivity by increaseing lipid oxidation in tissues, NEFA reduction and triglycerides reduction in the skeletal muscles and liver.

* NEFA= non esterified fatty acids

ADIPONECTIN

- In addition to the insulin sensitivity stimulating functions on liver and muscles, adiponecting shows important anti-inflammatory and athero-protective properties on the vascular tissue.
- In these regards, low adiponectin levels in obesity may not only contribute to insulin resistance and lipid profile derangement, but may also have a significant role in the dysfunction of the vascular endotelium typical of patients affected by the metabolic syndrome.

ADIPONECTIN SENSITIZE INSULIN ACTION IN INSULIN-RESPONSIVE CELLS



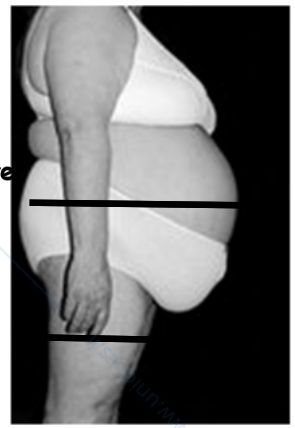
Achari and Jain; Int. J. Mol. Sci. 2017; doi:10.3390/ijms18061321

Obesity and Metabolic Syndrome

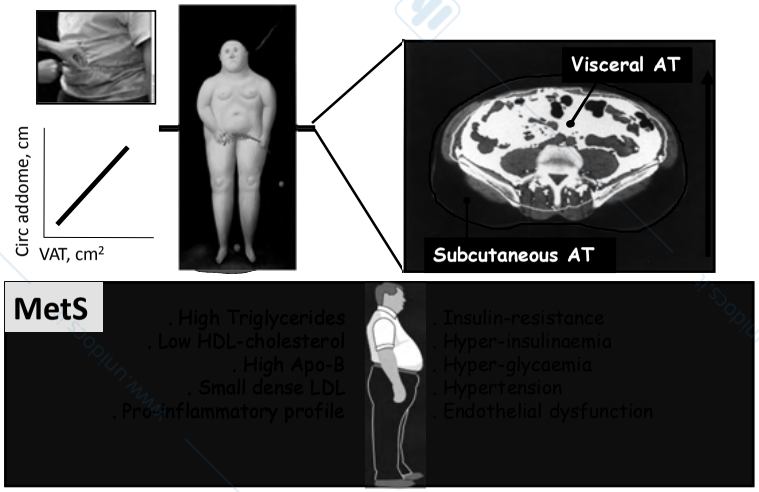
Waist circumference

Punti "chiave"

Waist to Hip ratio



Visceral adipose tissue - intra-abdominal



La suscettibilità genetica condiziona l'espressione della Sindr. Metabolica

ATP III: The Metabolic Syndrome*

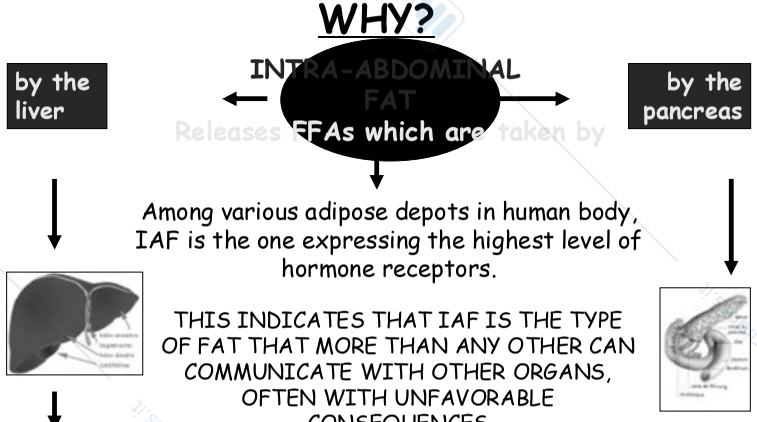
*Diagnosis is established when ≥ 3 of these risk factors are present.

Risk Factor	Defining Level
Abdominal obesity (Waist circumference)	
Men	> 102 cm (> 40 in)
Women	> 88 cm (> 35 in)
TG	≥ 150 mg/dL
HDL-C	
Men	< 40 mg/dL
Women	< 50 mg/dL
Blood pressure	≥ 130/≥ 85 mm Hg
Fasting glucose	≥ 110 (≥ 100)† mg/dL

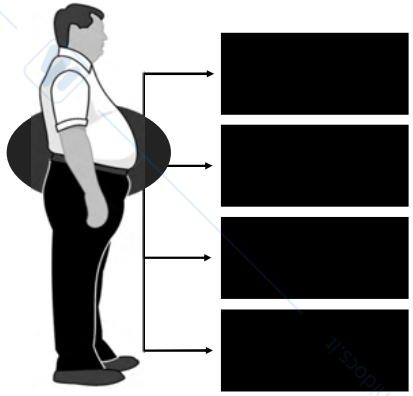
†2003 New ADA IFG criteria

HDL-C, high-density lipoprotein cholesterol.
Expert Panel on Detection, Evaluation, and Treatment of High Blood Cholesterol in Adults. *JAMA*. 2001;285:2486-2497.

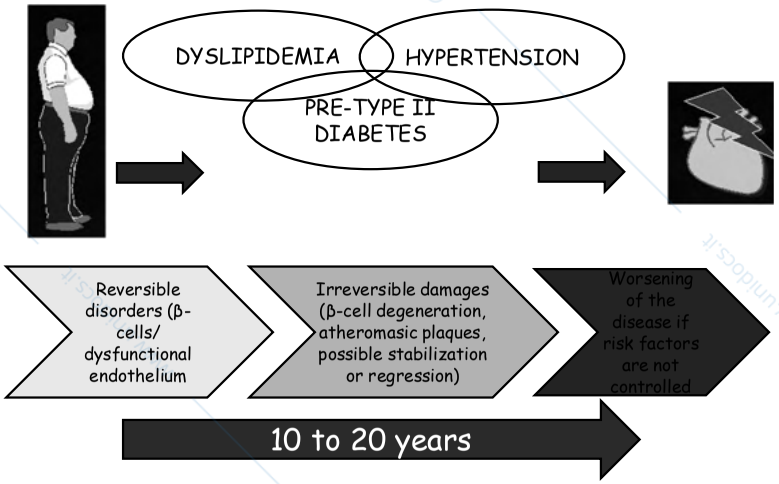
WHY?



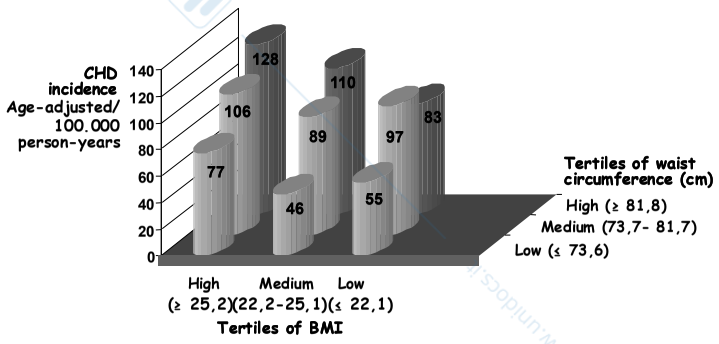
METABOLIC ALTERATIONS ASSOCIATED WITH ABDOMINAL OBESITY



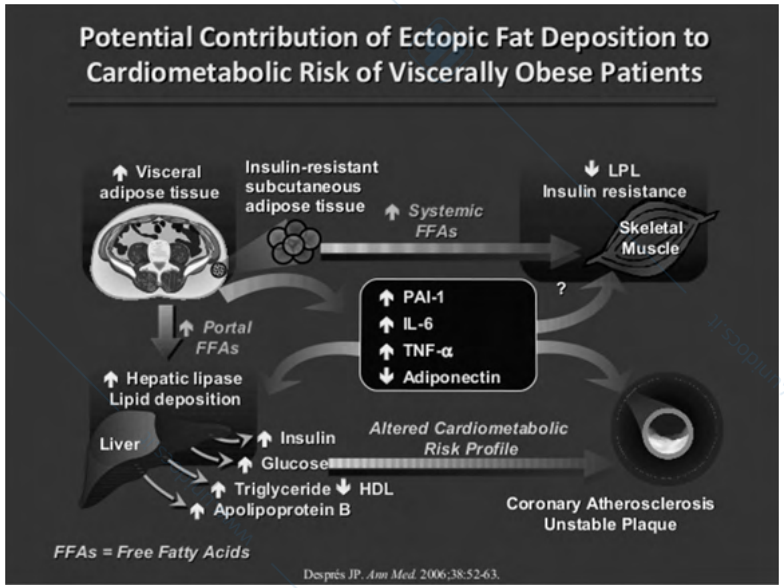
THE PROGRESSION TOWARD CARDIOVASCULAR EVENT

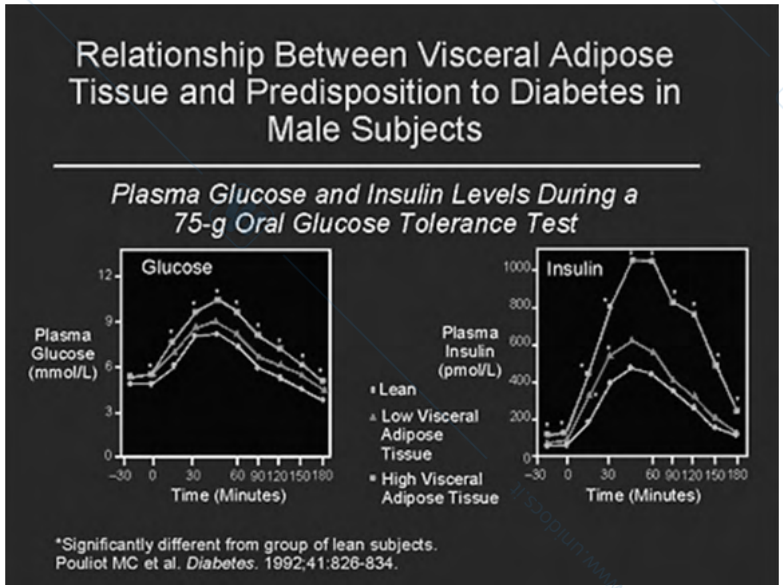


Abdominal obesity increases the risk for coronary hearth disease CHD independently from BMI

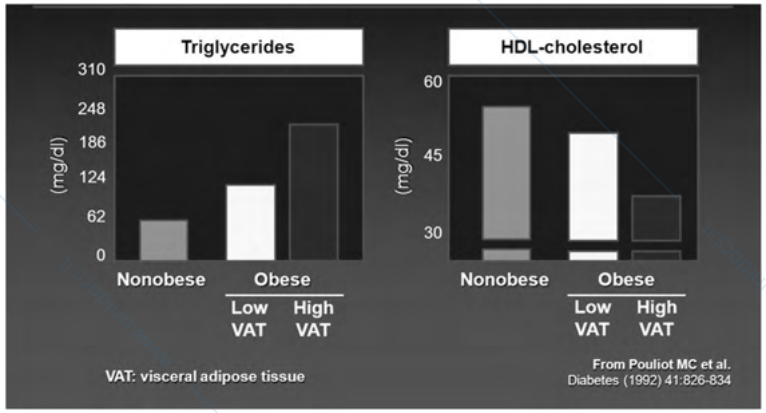


Rexrode KM et al. JAMA 1998; 280: 1843-1848

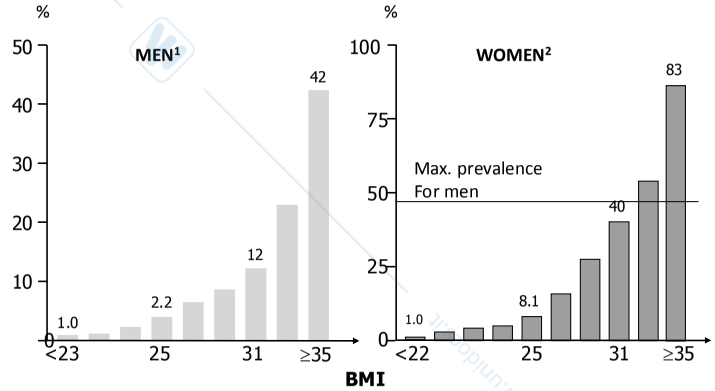




High visceral fat increases cardiovascular risk



Obesity is a major risk factor for developing type II Diabetes



DIABETES MELLITUS

Definition

- Diabetes is a chronic disease featured by hyperglycaemia, a reduction or a total defect in insulin secretion and/or action, and by the increased risk of developing micro- and macro-vascular dysfunction and metabolic, neurologic, bone and more complications.
- The morbidity and overall mortality due to diabetes has increased.

διαβήτης
"water flowing like a siphon"

"Diabetes is a remarkable affliction, not very frequent among men… The course is the common one, namely, the kidneys and the bladder; for the patients never stop making water, but the flow is incessant, as if from the opening of aqueducts.

"Moreover, life is disgusting and painful; thirst, unquenchable; excessive drinking, which, however, is disproportionate to the large quantity of urine, for more urine is passed; and one cannot stop them either from drinking or making water."

https://www.diabetesatlas.org/en/

International Diabetes Federation

https://www.who.int/health-topics/diabetes#tab=tab_1

World Health Organization

Key figures

from the IDF Diabetes Atlas 9th edition

1 in 11 adults (20-79 years) have diabetes (463 million people)

IGT

1 in 13 adults (20-79 years) have impaired glucose tolerance (374 million people)

1 in 2 adults with diabetes are undiagnosed (232 million people)

1 in 5 people with diabetes are above 65 years old (136 million people)

1 in 6 live births (20 million) is affected by hyperglycaemia in pregnancy, 84% of which have gestational diabetes

type 1

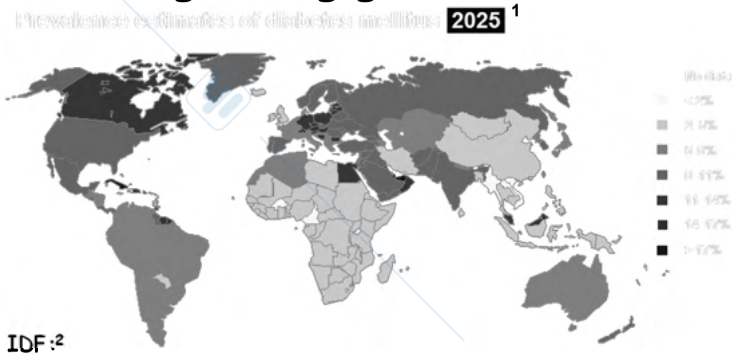
Over 1.1 million children and adolescents below 20 years have type 1 diabetes

10% of global health expenditure is spent on diabetes (USD760 billion)

3 in 4 (79%) of people with diabetes live in low- and middle-income countries

2 in 3 people with diabetes live in urban areas (310.3 million)

Diabetes: the growing global burden

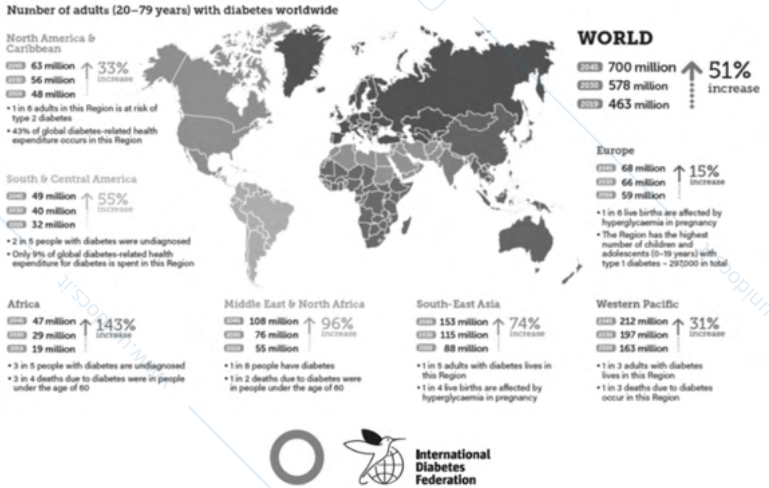


IDF:[2]

- **Diabetes currently affects 246 million people worldwide**
- **It is expected to affect 390 million by 2025**

[1]Adapted from IDF. E-Atlas. Available at: www.eatlas.idf.org (accessed 05.03.07).
[2]Diabetes Atlas, third edition © International Diabetes Federation, 2006.

DIABETES EPIDEMIC



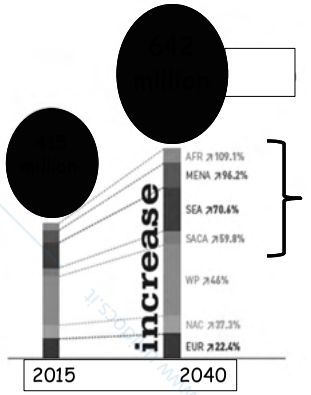
World Health Organization

https://www.who.int/news-room/fact-sheets/detail/diabetes

Key facts

- The number of people with diabetes has risen from 108 million in 1980 to 422 million in 2014.
- The global prevalence of diabetes* among adults over 18 years of age has risen from 4.7% in 1980 to 8.5% in 2014.
- Diabetes prevalence has been rising more rapidly in middle- and low-income countries.
- Diabetes is a major cause of blindness, kidney failure, heart attacks, stroke and lower limb amputation.
- In 2016, an estimated 1.6 million deaths were directly caused by diabetes. Another 2.2 million deaths were attributable to high blood glucose in 2012.
- Almost half of all deaths attributable to high blood glucose occur before the age of 70 years. WHO estimates that diabetes was the seventh leading cause of death in 2016.
- Healthy diet, regular physical activity, maintaining a normal body weight and avoiding tobacco use are ways to prevent or delay the onset of type 2 diabetes.
- Diabetes can be treated and its consequences avoided or delayed with diet, physical activity, medication and regular screening and treatment for complications.

Number of people with diabetes Estimates by IDF regions, 2015–2040

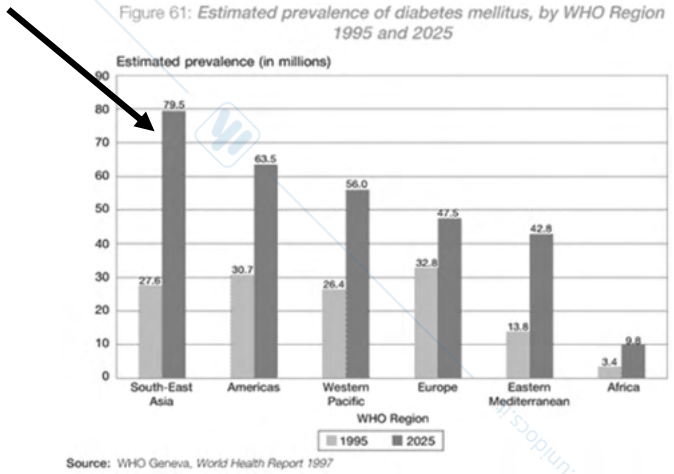


Global increase 55%

The number of people affected by diabetes has increased worldwide, and it has been estimated that it will amount to 55% within 2040.

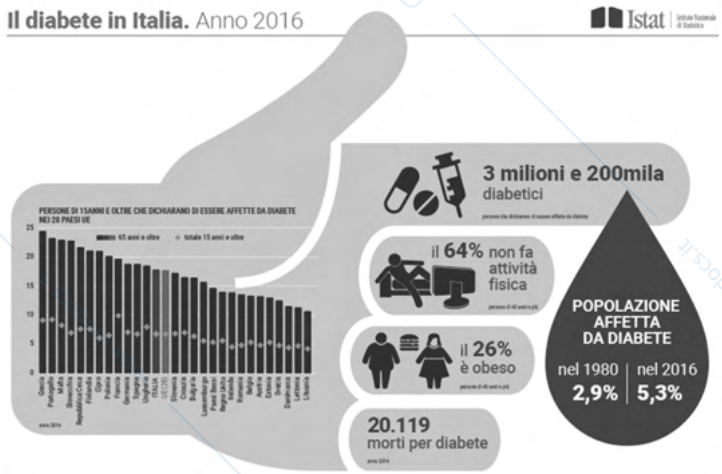
International Diabetes Federation. *IDF Diabetes Atlas, 7th edn.* Brussels, Belgium 2015

THE IMPACT OF ETHNICITY

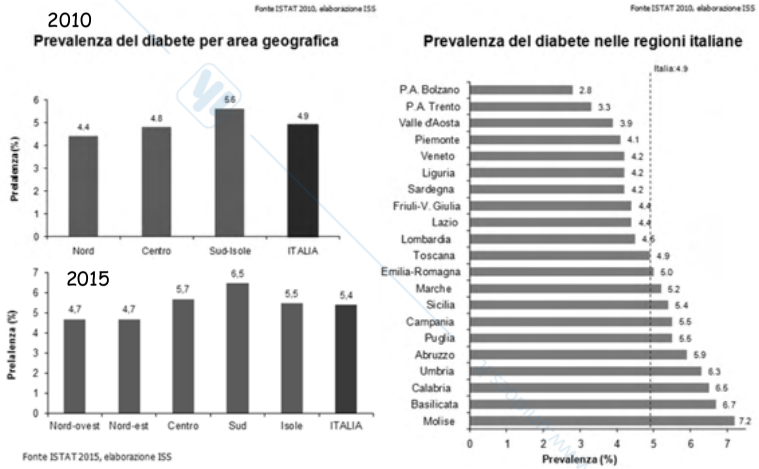


WHO 1997

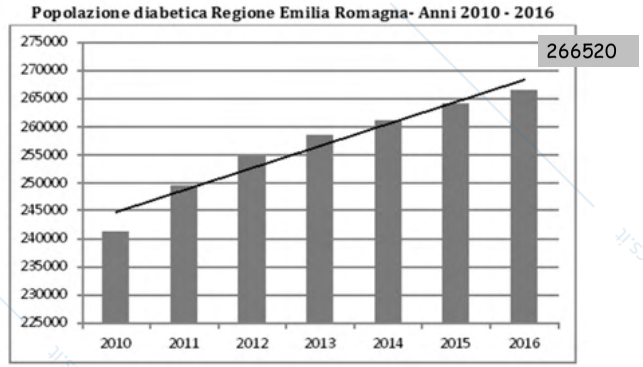
DIABETES IN ITALY



DIABETES IN ITALY



Diabetes in Emilia-Romagna



Anno	2010	2011	2012	2013	2014	2015	2016
Diabetici	241209	249447	254832	258569	261138	263999	266520
% diabetici	6.30	6.50	6.63	6.74	6.81	6.87	6.92

Diabetes Classification

- Type 1: juvenile, autoimmune. Deficient insulin production. Requires daily administration of insulin.
- Type 2: non-insulin-dependent, adult-onset. By far the most diffused world-wide. Associated with obesity and metabolic syndrome.

- Gestational diabetes
- Genetic diabetes
  - Genetic defects of insulin secretion
  - Genetic defects of insulin action
- Diabetes secondary to:
  - Pancreas diseases
  - Endocrinopathies
  - Drugs or chemicals
  - Genetic syndromes
  - Others

Type II diabetes and CVD

➢Type II Diabetes (T2DM) represents the major independent risk factor for the development of cardiovascular diseases (CVD).

➢ Cardiovascular diseases represent the first cause of death in T2DM patients. 65% deaths are caused by stroke or ischemic heart disease.

➢ T2DM increases by 2-4 folds the risk of coronary and cerebrovascular diseases, and of death by cardiovascular disease (Interheart study).

T2DM and CVD look like the two sides of a medal: T2DM has been defined as equivalent to a coronary hearth disease, whereas patients affected by CVD are alse affected by diabetes or pre-diabetes.

CVD generally involves all arterial districts, in particular the coronaric, cerebral and peripheral districts

CVD in diabetes is caused by microangiopathy and by other traditional CVD risk factors

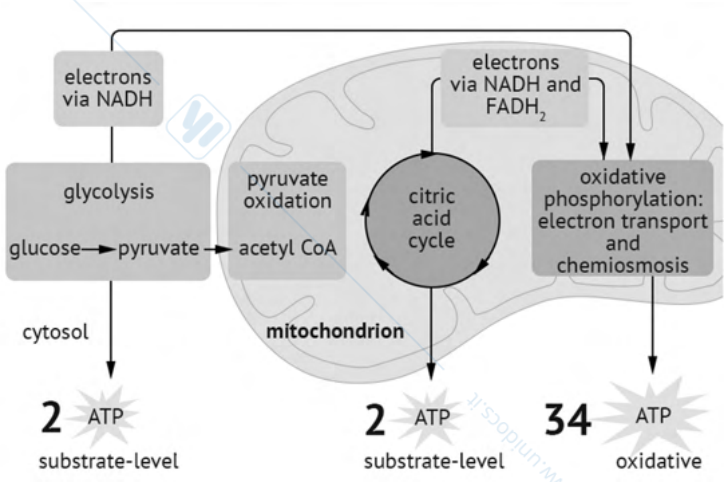
90% of type II Diabetes patients are overweight or obese

66% of type II Diabetes patients have arterial hypertension

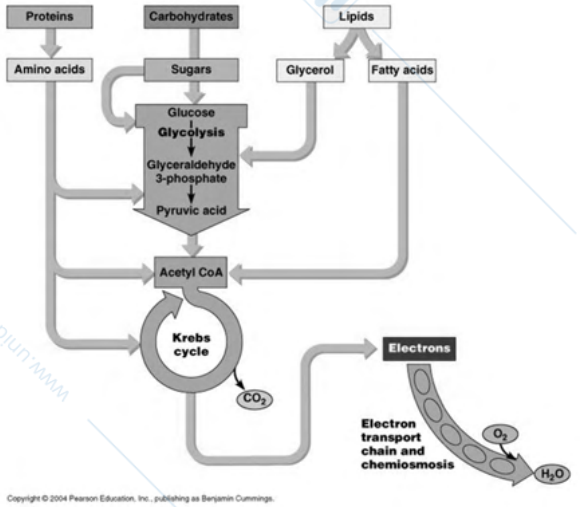
70% of type II Diabetes patients have dyslipidemia

1. http://www.who.int/mediacentre/factsheets/fs317/en/#.
2. http://www.idf.org/diabetesatlas.
3. Nwaneri et al. Br J Diabetes Vasc Dis 2013;13:192-207.

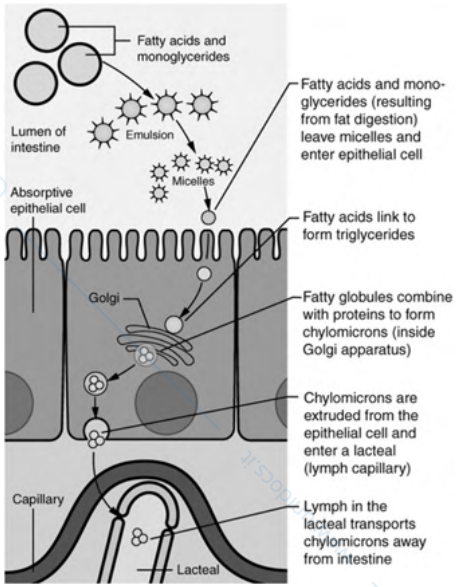
please refresh your knowledge about biochemistry …



please refresh your knowledge about biochemistry …

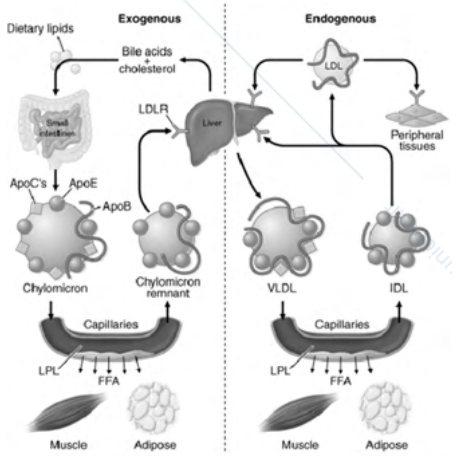


please refresh your knowledge about biochemistry …



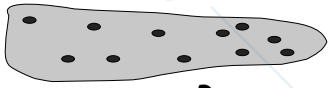
please refresh your knowledge about biochemistry …

Schematic of the exogenous and endogenous lipid metabolism pathways. Cholesterol in the circulation will originate from either the endogenous or exogenous pathway. All cholesterol will be packaged into lipoprotein particles as part of their metabolism pathway and covered with a specific complement of apolipoproteins. As part of the endogenous pathway, the liver is responsible for the packaging of VLDL particles which are hydrolyzed to IDL, returned to the liver so that they may be repackaged as LDL then taken from the circulation by peripheral tissues.

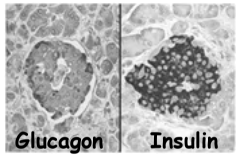


PANCREATIC HORMONES AND GLYCAEMIA

The endocrine pancreas releases 2 hormones involved in glucose metabolism:
-INSULIN: hypoglicaemic effect
-GLUCAGON: hyperglicaemic effect

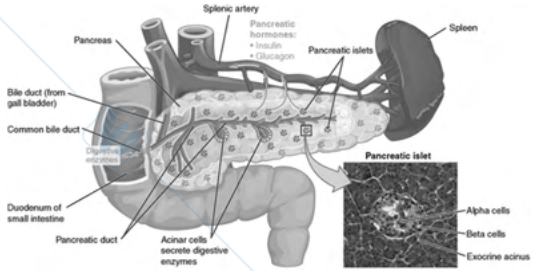


Pancreas



When blood glucose increases, insulin secretion is promoted while glucagone secretion is inhibited.
When blood glocose is low, glucagon is released, while insulin secretion is inhibited.

ENDOCRINE PANCREAS



Endocrine tissue consisting of small isolated formations called Langerhans islets, distributed throughout the gland (1-2 million) but with a higher density in the tail than the body and head.
Endocrine pancreas constitutes about 1-3% of the volume of the whole gland.
Islets are richly vascularized by a network of capillaries, whose endothelium is made up of widely fenestrated cells.
Blood flowing from pancreas passes into the pancreatic veins, then into the hepatic portal circle and finally into the systemic bloodstream.

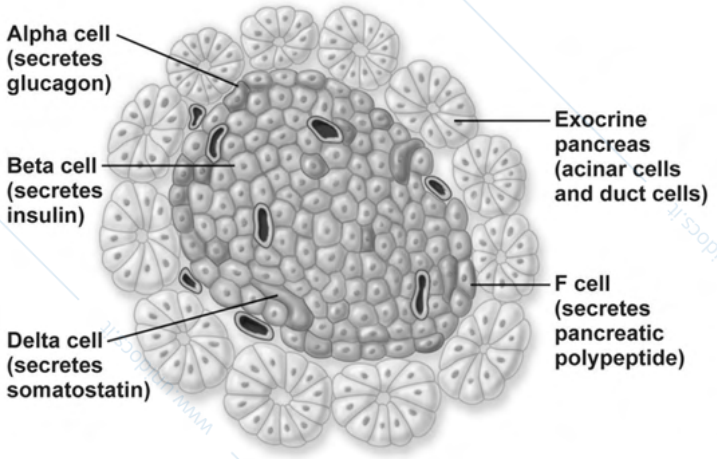
PANCREATIC HORMONES AND GLYCAEMIA

- The release of the two hormones is primarily orchestrated by glucose levels in the bloodstream

HIGH GLUCOSE → Insulin ↓ Glucagon

LOW GLUCOSE → Insulin ↑ Glucagon

LANGERHANS ISLETS



PANCREATIC ENDOCRINE CELLS

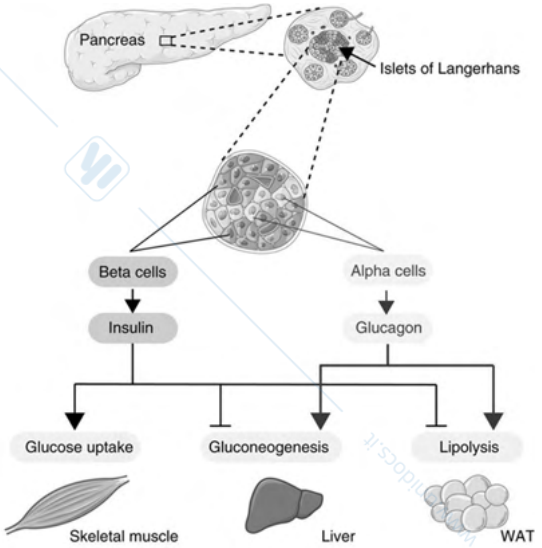
α cells: distributed in the peripheral part, constituting a sort of cortex. 20% of total islet cells. Producing **glucagon**.

β cells: distributed in the central part of the islet. 70 – 75% of total islet cells. Producing **insulin** and **amylin**.

δ cells: distributed in the layer separating internal (β cell) and external (α cells) part of the islet. 3-4% of the total islet cells. Producing **somatostatin**.

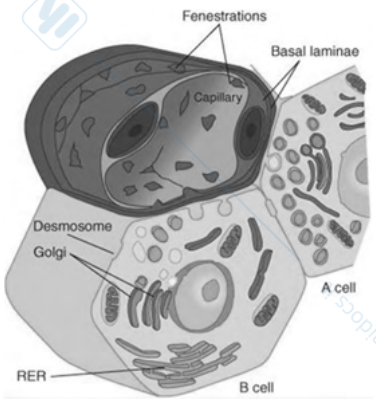
φ cells: distributed in the cortical layer. 1-2% of the total islet cells. Producing the **pancreatic polypeptide (PP)**.

ε cells: producing **ghrelin** (<1%).



α and β cells, showing their relation to a blood vessel.

Insulin from the **β** cell and glucagon from the **α** cell are secreted by exocytosis and cross the basal lamina of the cell and the basal lamina of the capillary before entering the lumen of the fenestrated capillary.



(Junqueira IC, Carneiro J: *Basic Histology: Text and Atlas*,10th ed. McGraw-Hill, 2003.)

Insulin effects

Skeletal muscle
Stimulation of glucose uptake and utilization.

Glycogen synthesis
Aminoacids uptake.

Protein synthesis.

Adipocyte
Stimulation of glucose uptake and utilization.

Stimulation of triglycerides synthesis.

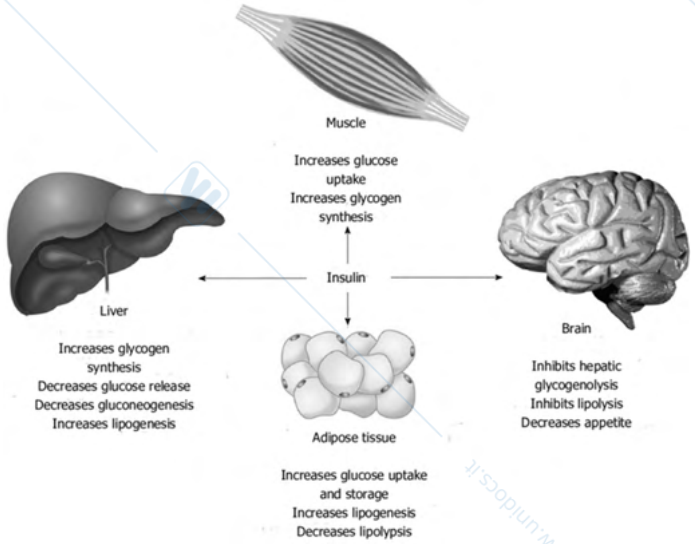
Liver
Stimulation of glucose uptake and utilization.

Glycogen synthesis
Triglyceride synthesis.

Inhibition of ketone bodies synthesis.

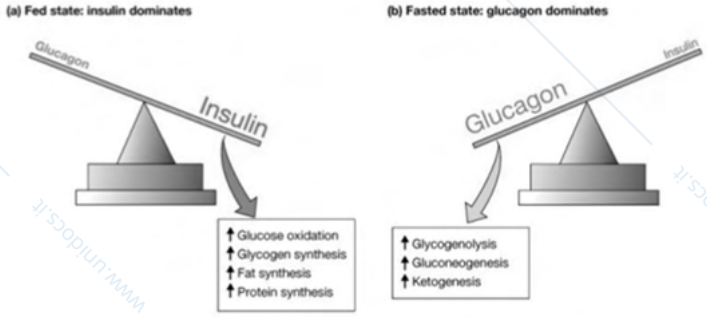
- **Uptake of substrates by cells**
- **Metabolic activation of substrates**
- **Anabolic function**

All processes based on glucose utilization are stimulated

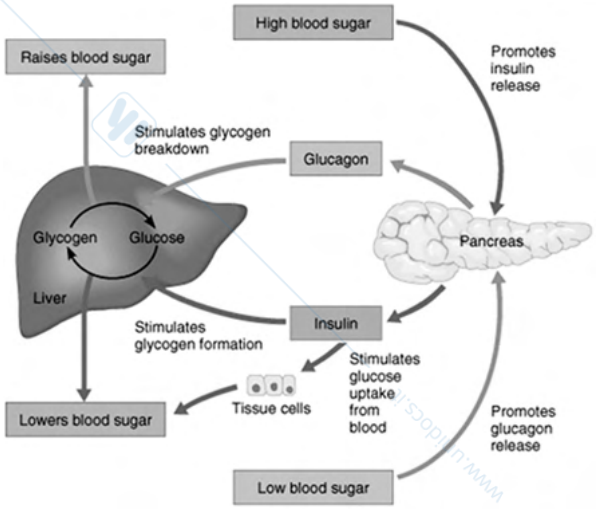


FED STATE
Insulin dominates

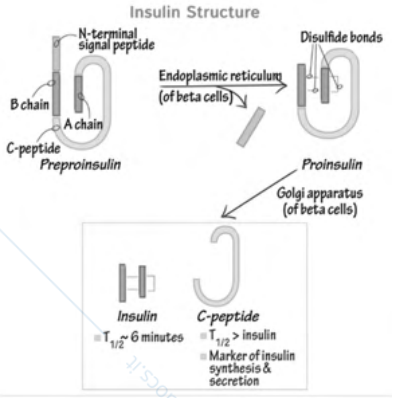
FASTED STATE
Glucagon dominates



GLUCOSE HOMEOSTASIS



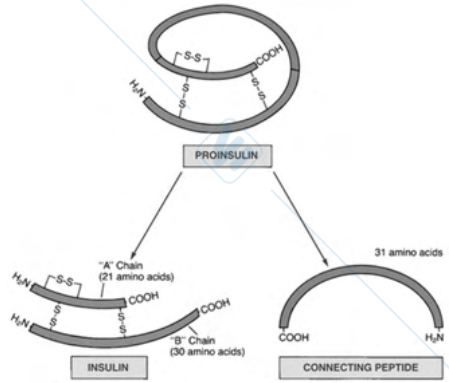
Insulin secretion



β-cells synthesize the preproinsulin which is targeted into the ER and Golgi via a N-term signal sequence, and which is then removed to yield proinsulin. Proinsulin is processed by several proteases in the Golgi apparatus to form 3 separate chains, named B, C and A. Chains B and A are linked through disulfide bonds, and are the components of mature insulin.

Due to portal-hepatic first pass effect, 50% insulin is degraded upon secretion. C peptide is a byproduct used as robust marker of β-cell function.

Insulin secretion

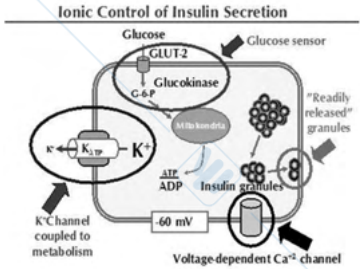


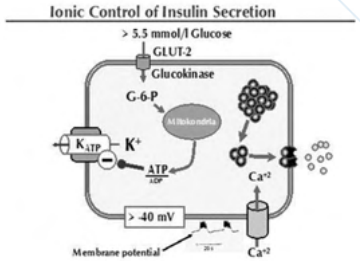
Proinsulin is the immediate precursor of the insulin secreted by our pancreas. Enzymes clip off connecting peptide (C-peptide) to release active insulin, composed of two peptide chains (A and B) connected by two disulfide (S–S) bonds.

Insulin HRT: as C-peptide arises only from endogenous insulin processing, its blood levels indicates that at least some pancreatic insulin is being made.

insulin:
51 aa
6 min half-life

C-peptide:
31 aa, longer half-life
marker of insulin synthesis and secretion





β-cells release insulin following an increase in blood glucose. Soon after the increase in its concentration in the blood (>5.5 mM), glucose is transported in small quantities within these cells. Glucose catabolism within the cell generates ATP, which induces the closure of the ion channels for potassium (ATP dependent). Potassium is a positively charged ion, normally present inside the cell in a concentration of about 150 mM, 20 folds higher than the extracellular matrix. Therefore, when channels are open, potassium flows out according to the chemical gradient. When, as induced by ATP, potassium channels close, the positive charge leakage is stopped, so that the intracellular potential shifts to less negative values, that is, it depolarizes. This depolarization in turn causes the opening of the voltage-dependent calcium ion channels which, by opening, cause the concentration of intracellular calcium to increase. Calcium functions as an intracellular messenger by inducing the fusion of insulin-containing vesicles with the plasma membrane and the consequent outflow of insulin. When the blood sugar decreases, the β-cells return to the resting state.

FACTORS REGULATING INSULIN SECRETION

Blood glucose: when glycaemia is in the normal range (80-90 mg/dL), insulin secretion is low. A raise in blood glucose is followed by a 10 to 30 fold raise in blood insulin, depending on the carbohydrate contents (type and quantity) in the meal.

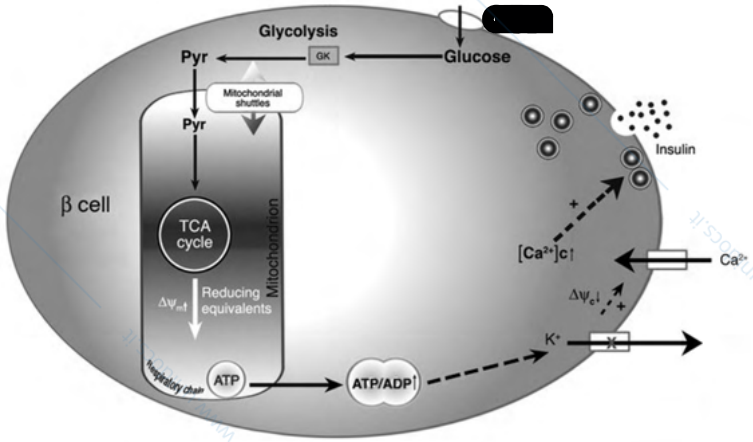
Amino acids, fatty acids, keto-acids: particularly arginin and leucin, are able to stimulate insulin secretion. Protein-rich meal determines the increase in circulating insulin. Same effect by fatty acids and ketone bodies.

Gastro-intestinal hormones (GI): after a meal, GI hormones, particularly GLP-1 and gastrin, signal to pancreatic cells anticipating glucose raise in blood. Others: GIP, secretin, CCK.

Autonomic Nervous System: parasymphatetic neurons projecting at the islets are activated during digestion, inducing acetylcholine-mediated insulin release. Conversely, the activation of symphatetic fibers projecting at the islets and adrenalin release by adrenal medulla during a stress-response, determine the inhibition of insulin release.

Other insular hormones: glucagon enhances the glucose-induced insulin secretion. Somatostatin inhibits insulin secretion.

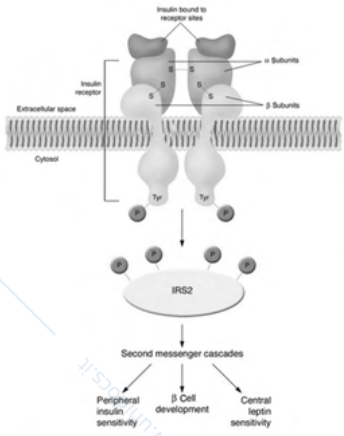
GLUCOSE UPTAKE BY THE B-CELL – INSULIN RELEASE



INSULIN RECEPTOR

Glycoprotein Tyrosine-Kinase composed by 4 subunits:
2 α (insulin-binding) and 2 β (cytosolic catalitic activity)

The autophosphorylation (P) of the IR on tyrosine (Tyr) residues increases the association of various signaling molecules. Insulin receptor directly phosphorylates insulin receptor substrate 2 (IRS2) protein on multiple Tyr residues, which, in turn, initiates a variety of second messenger cascades.
Recent work has demonstrated a critical role for IRS2 in the maintenance of peripheral insulin sensitivity, central leptin sensitivity, and proper β cell development in the islets of Langerhans. Thus, IRS2 plays a central role in preserving insulin action in multiple cell types, while reduction of IRS2 expression and/or function may be a fundamental cause of the development of insulin resistance, obesity, β cell failure, and T2DM.



J Clin Invest DOI: 10.1172/JCI23108

INSULIN EFFECTS ON GLUCOSE METABOLISM

All insulin activities promote the lowering of blood glucose.

In insulin-sensitive cells, in the absence of insulin, glucose cannot enter the cell.

- Insulin promotes glucose utilization:
  - ✓ by increasing glucose uptake by the liver, stimulating glycogenosynthesis and inhibiting glycogenolysis.
  - ✓ by increasing glucose uptake by the muscles and its usage as energy fuel, stimulating glycolysis and Kreb's cycle.
  - ✓ by increasing glucose uptake by the adipose cells promoting glucose storage as fat.
  - ✓ by activating the pentose phosphate pathway, producing NADPH required for fatty acids, nucleotides and aromatic aa synthesis.
  - ✓ Enhances utilization of amino acids

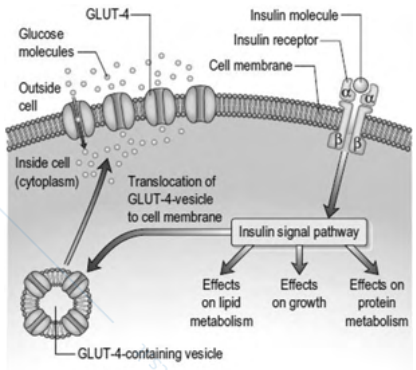
- Insulin inhibits *ex novo* glucose synthesis or gluconeogenesis

INSULIN EFFECT ON GLUCOSE UPTAKE

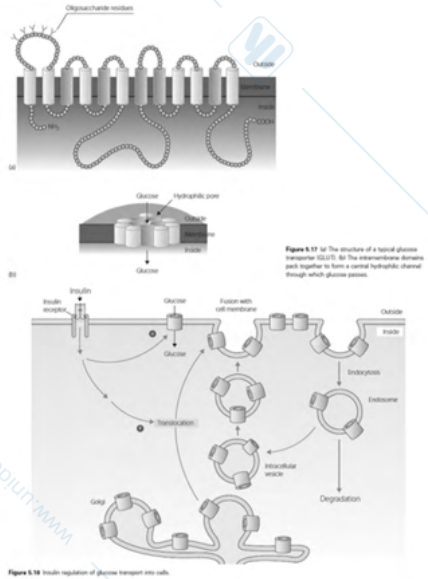
INSULIN-MEDIATED GLUCOSE TRANSPORTER EXPRESSION

Glucose uptake by tissues is mediated by a specific membrane transporter GLUT 4, able to bind glucose in the extracellular space and transport it into the cytosol.

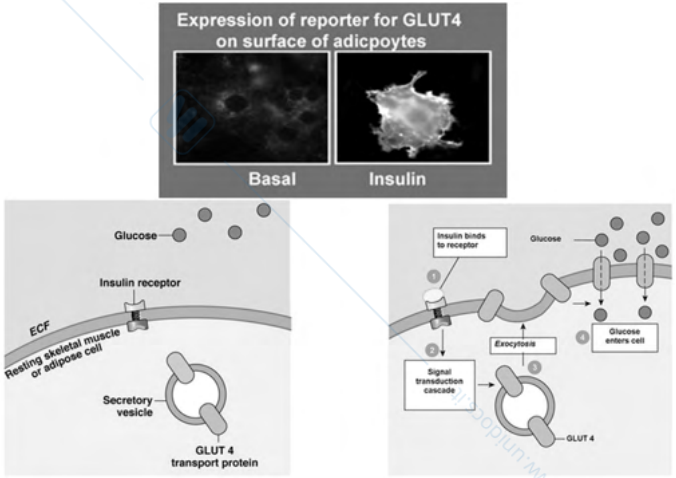
Glut-4 is stored in secretory vescicles, and expressed on the cell surface only after insulin signaling.



In adipocytes from T2DM patients, as a result of impaired insulin sensitvity, reduced glucose uptake is associated with a reduction in number and activity of GLUT-4 transporters.



GLUCOSE UPTAKE BY THE ADIPOCYTE and SKELETAL MUSCLE

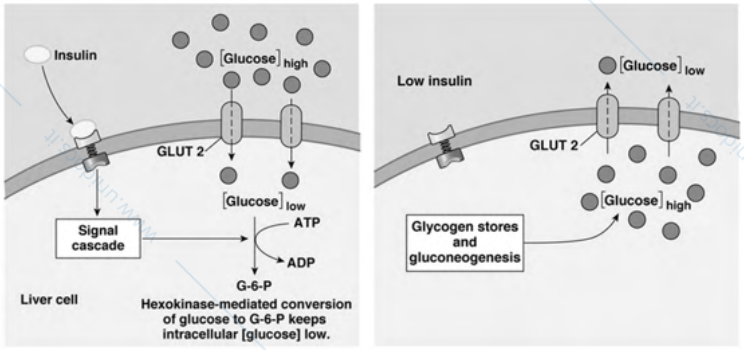


GLUCOSE UPTAKE BY THE HEPATOCYTE

GLUCOSE-MEDIATED GLUCOSE TRANSPORTE

LIVER acts as a glocostat. It senses glucose levels, regulates the availability of glucose and is a major store of glycogen.

GLUT-2 is not insulin-sensitive but it is regulated by glucose level (the same acting on b-cells). GLUT-2 mediate glucose transport in both directions according to its concentration in the inner or outer space.



INSULIN EFFECT ON OTHER METABOLISMS

Insulin has anabolic and anti-catabolic effects

- Insulin promotes lipogenesis at the adipose tissue level by increaseing the uptake of glycerol and fatty acids. It stimulates lipoprotein lipase expression.
- Insulin inhibits lipolysis at the adipose tissue level by inhibiting the hormone-sensitive lipase of the adipocyte, which splits the triglycerides into its constituents
- Insulin inhibits protein catabolism and promote amino acids uptake and protein synthesis.

INSULIN EFFECT overall summary

- Insulin promotes anabolic processes by stimulating glucose storage as glycogen, glycerol and fatty acids storage in triglyceride form,and protein synthesis.
- Insulin stimulates glucose utilization as energy fuel (ATP and NADPH production by stimulating glycolysis, Kreb's cycle and the pentose phosphate pathway); energy is mostly used for anabolic processes stimulated by the hormone.
- Insulin downregulates catabolic processes by inhibiting glycogenolysis, lipolysis and protein degradation.
- Insulin inhibits gluconeogenesis thereby contributing to the glucose-lowering effect, in particual in the post-prandial phase.

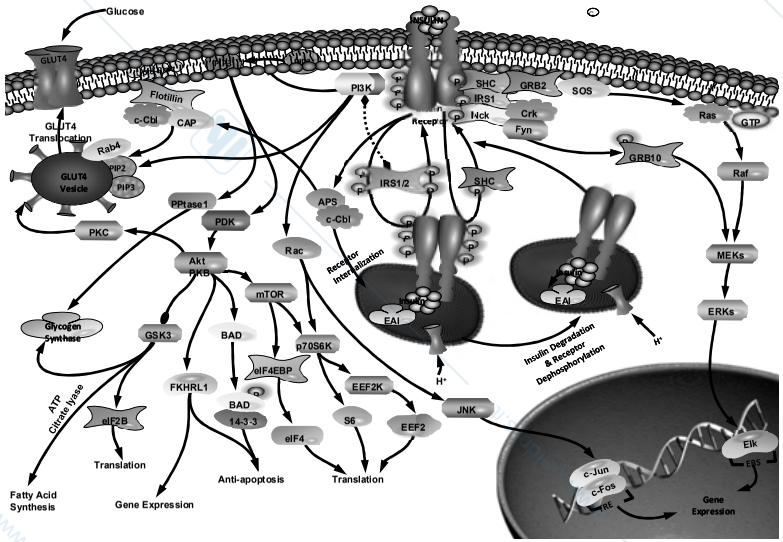
Diabetes Mellitus is commonly considered a disease of sugar metabolism, however, it is much more a multi-metabolism pathology.

A dysfunctional insulin machinery, affecting insulin production / secretion / function, actually affects all metabolic pathways.

DIABETES

Impaired insulin secretion **Insulin resistance**

Insulin Receptor Pathway



Insulin-resistance

Tissues resistance to insulin action may depend on defects at various levels:

- ✓pre-receptor level (autoantibodies)
- ✓receptor level (down-regulation; autoantibodies)
- ✓post-receptor level (multiple and complex, as the insulin down-stream cascade events)

Post-receptor resistance is most often featuring Type II Diabetes and Metabolic Syndrome

REGULATION OF INSULIN SECRETION

- Role of the β-cell
- Role of glucose
- Role of target tissues
- Role of enteropancreatic hormones (glucagon, GLP-1, GIP, etc.)
- Role of counter-insular hormones

β-CELL PHYSIOLOGY OF INSULIN SECRETION

- Insulin secretion is a multistep process requiring the synthesis of a precursors (pre-proinsulin and proinsulin), C-peptide removal and the final conformation of two polypeptides A and B joint by disolfide bridges.
- β-cells are equipped with an efficient glucose transport system (Glut 2) that rapidly equilibrates glucose concententration across the intra- and extra-cellular space.
- The mechanism of insulin secretion depends by the β-cell glucose metabolism, by the availability of Ca^{++} ions, by the permeability of the plasma membrane, followed by mecanisms of extrusion of insulin- and C-peptide-containing granules.

PHYSIOLOGY OF GLUCOSE HOMEOSTASIS

•In normal subjects, blood glucose concentrations are maintained within relatively narrow limits at around 5 mmol/L (90 mg/dL), regardless of the volume and composition of the meal. This is achieved by a balance between glucose release into the circulation from the liver and from intestinal absorption, and glucose uptake by the peripheral tissues such as muscle and adipose tissue.

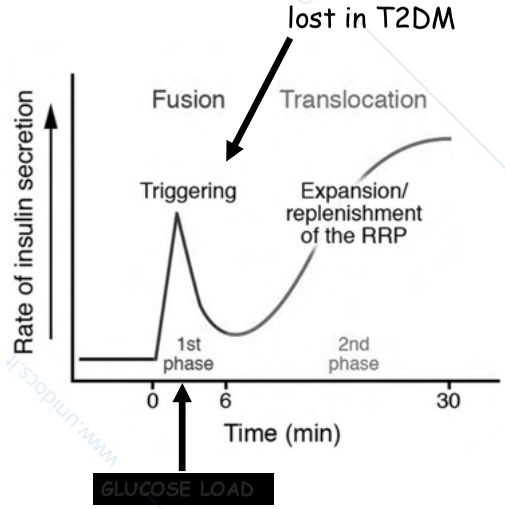
•After a meal, insulin secretory peaks are rapid, proportional in intensity to the glucose load, and transitory, with rapid return to the values of basal secretion.

• Even a small delay in insulin secretion, and the loss of the steep reduction in its levels, is able to cause a substained post-prandial hyper-glycaemia.

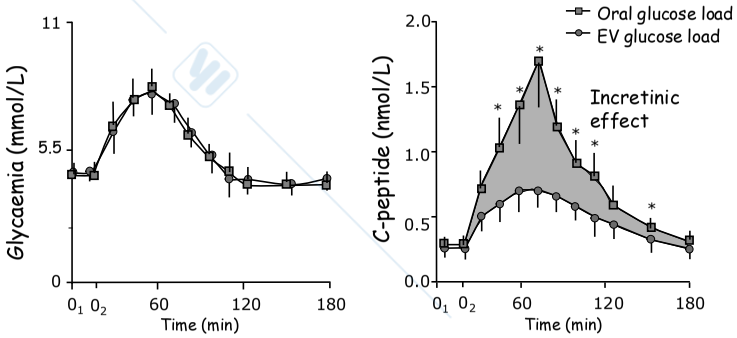
BIPHASIC INSULIN SECRETION

- At normal fasting blood glucose levels of 80-90 mg/dL, insulin secretion is minimal (basal level), in the order of 25 ng/min/kg body weight.
- When the concentration of blood glucose rises sharply to a level 2-3 times higher than normal and remains at these values, insulin secretion increases in two distinct phases:
  - A first raise, up to 10 folds, within 3-5 min from a sharp glucose increase, is obtained by the release of existing insulin stored in granules in β-cells. This peak is short-lasting, and insulin level is halved within 5-10 min.
  - After 15 minutes insulin secretion raise again, reaching a plateau in 2-3 hours, as a consequence of the activation of the enzymatic machinery for insulin synthesis and release from pancreas cells.

BIPHASIC INSULIN SECRETION



INCRETINIC EFFECT: the different response to ORAL vs EV glucose administration



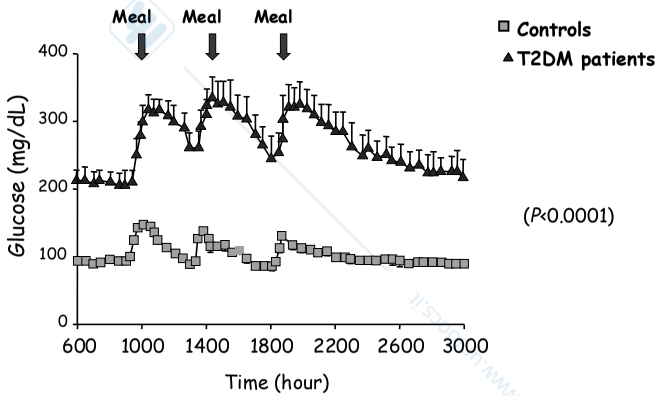
The incretinic effect accounts for the 60% of the total insulin release following a meal

Nauck MA, et al. *J Clin Endocrinol Metab.* 1986;63:492-498

INCRETINS: GLP-1 and GIP

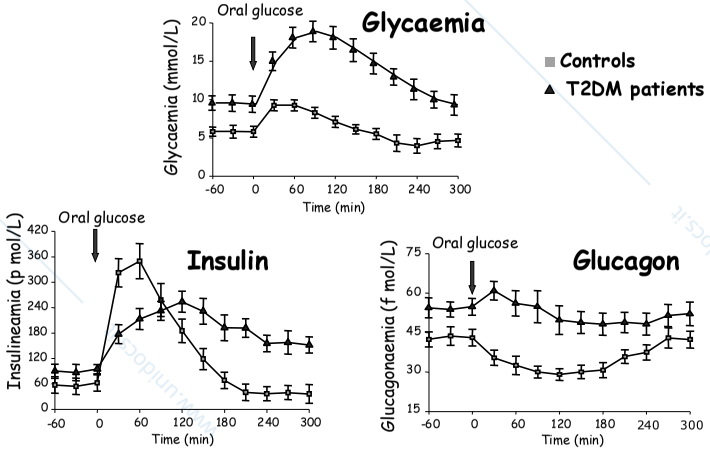
- Major hormones responsible for the incretinic effect are:
  - Glucagon-like peptide 1 (GLP-1)
  - Glucose-dependent insulinotropic polypeptide (GIP)
- GLP-1 is the major determinant of the incretinic effect.
- Incretin hormones :
  - are released by the endocrine cells in the small intestine in response to nutrient intake;
  - increase insulin secretion after a meal, acting on specific receptors at the pancreas level;
  - are rapidly degraded (~2-7 minutes) by the enzyme dipeptidyl peptidase IV (DPP IV)

T2DM patients display higher glucose levels both in fasting and in fed condition



Polonsky KS, et al. *N Engl J Med.* 1988;318:1231-1239.

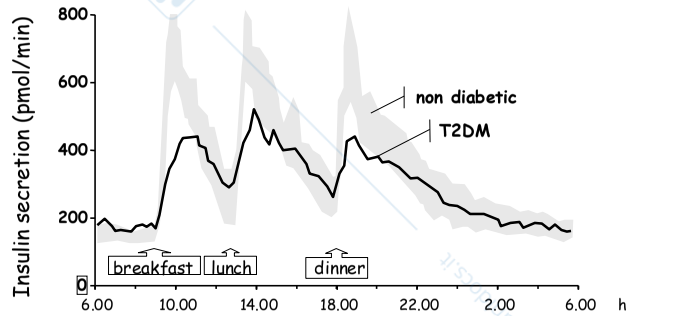
Post-prandial hyper-glycaemia: deficit of insulin secretion and of glucagon suppression



Mitrakou A, et al. *Diabetes.* 1990;39:1381-1390.

Deficit in the early insulin secretion in type II diabetes

The mean 24-hour insulin levels were similar between diabetics and non-diabetic controls. By contrast, the insulin peak following the meal is reduced in diabetic patients.



Polonsky et al. N Engl J Med 1988

Definition

- Diabetes disease is featured by hyperglycaemia, an absolute or relative defect in insulin secretion or action, and by the susceptibility at developing micro- and macro-vascular, metabolic, neurologic, bone sequelae, and others.

Diabetes Mellitus

Hyperglycaemia and related symptoms

Polyuria, polydipsia, dehydration,
weight loss, mental confusion

Acute Complications

- Ketoacidosis
- Hyperosmolar Syndrome
- Hypoglycaemia
- Lactic acidosis

Chronic complications

- Cardiovascular disease
- Nephropathy
- Neuropathy
- Retinopathy

Diabetes Classification

- Type 1
- Type 2
- Gestational diabetes
- Genetic diabetes
  - Genetic defects of insulin secretion
  - Genetic defects of insulin action
- Diabetes secondary to:
  - Pancreas diseases
  - Endocrinopathies
  - Drugs or chemicals
  - Genetic syndromes
  - others

Table 2 – American Diabetes Association diagnostic criteria for diabetes[18]

Test[a]	Threshold	Qualifier
Hemoglobin A_{1c} or	≥ 6.5%	Lab NGSP-certified, standardized DCCT assay
Fasting glucose or	≥ 126 mg/dL (7.0 mmol/L)	No caloric intake for at least 8 hours
2-hour glucose or	≥ 200 mg/dL (11.1 mmol/L)	After 75 g of anhydrous glucose
Random glucose	≥ 200 mg/dL (11.1 mmol/L)	Plus classic hyperglycemia symptoms or crisis

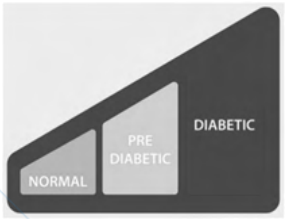
NGSP, National Glycohemoglobin Standardization Program; DCCT, Diabetes Control and Complications Trial.

[a] Results must be confirmed by repeated testing.

PRE-DIABETES

Table-1: Prediabetes - Diagnostic Cut-offs.

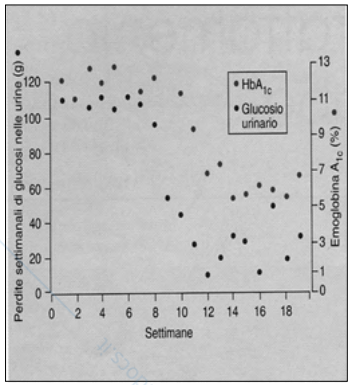
Parameter	Category	ADA Definition	WHO Definition
FPG	Impaired Fasting Glucose (IFG)	100-125 mg/dl	110-125 mg/dl
2hr OGTT	Impaired Glucose Tolerance (IGT)	140-199 mg/dl	140-199 mg/dl
HbA1c	-	5.7-6.4%	-



GLYCATED HEMOGLOBIN A1c

The HbA1c level, expressed as% of total HbA, reflects the average blood glucose concentration in the previous 8 weeks

(i.e. the period corresponding to the half-life of the red blood cells)



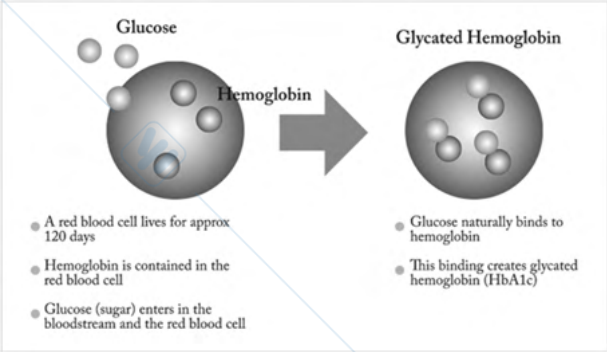
HbA1 is a series of glycated variants resulting from attachment of various carbohydrates to the N-terminal valine of Hb

GHb: glycated hemoglobin

1. HbA1a1: fructose 1,6 diphosphate N terminal valine
2. HbA1a2: glucose 6 phosphate N terminal valine
3. HbA1b: unknown carbohydrate N terminal valine
4. **HbA1c: (60-80%): attachment of glucose to N terminal amino acid valine of the beta chain of hemoglobin**

Total glycated Hb: HbA1c+ sugar Non N terminal sites

GLYCATED HEMOGLOBIN A1c



This test tells us patient average glucose index over a long period of time (2 to 3 months).

This test is not affected by short-term variation like:
1. Food.
2. Exercise.
3. Hypoglycemic agents.
4. Stress.
5. Patient attitude or cooperation.

PITFALLS IN HbA1c DETERMINATION

- Measurement variability (method, laboratory)
- ↓ Erithrocytes turnover (iron, folate, vitamin B12 deficiency)
- ↑ Erithrocytes turnover (hemolysis, iron, folate, vitamin B12 supplementation)
- Hemoglobinopathies (HbF, HbS)

INSULIN MEASUREMENT

DIABETES CARE, VOLUME 33, NUMBER 1, JANUARY 2010
Reviews/Commentaries/ADA Statements
COMMENTARY

Insulin Assay Standardization

Leading to measures of insulin sensitivity and secretion for practical clinical care

MYRLENE A. STATEN, MD[1]
MICHAEL P. STERN, MD[2]
W. GREG MILLER, PHD[3]
MICHAEL W. STEFFES, MD, PHD[4]
SCOTT E. CAMPBELL, PHD[5]
FOR THE INSULIN STANDARDIZATION WORKGROUP

An effort is underway by the American Diabetes Association to standardize insulin assays so that this barrier to advancing research is removed. Once the in-

There are no criteria by which an individual could be classified as being insulin sensitive or resistant or as having mild, moderate, or severe impairment of insulin secretion…

lack of standardized insulin assays

INSULIN MEASUREMENT

…Measurements of insulin sensitivity and secretion are currently done only for research purposes and are only comparable in individual studies. There are no clinical applications for these measures.

…One of the barriers is the lack of standardized insulin assays. Results reported from one study to the next are not comparable, making only qualitative comparisons between studies possible. Larger epidemiology studies have been limited to populations in which the same laboratory was used for all measures of insulin, making it impossible to conduct any quantitative summarization or meta-analysis of the results…

To date, only a few trials have investigated measures of insulin sensitivity and secretion over a period of years in a large number of individuals to be able to determine their predictive power. Even when trials of this type are done, the results are limited to that specific insulin assay and are not transferable to other laboratories or studies due to the lack of standardization of insulin assays.

Type I Diabetes

Type I Diabetes (T1DM)

- Autoimmune disease leading to the distruction of pancreatic islets and to insulin deficiency.
- Usually affects children and adolescents, however, it may also affect adults.
- Prevalence is about 0.4%.
- Though most of the cases do not present a familial hisytory of the disease, 1° degree relatives have increased risk of developing T1DM. Homozygous tweens of T1DM patients have the highest risk.
- The genetic predisposition is determined by the histocompatibility complex HLA class II locus, localized in chromosome 6, in particular HDL-DR3 and HDL-DR4 (most of the cases show heterozygous DR3/DR4 genotypes).
- Environmental factors may increase individual susceptibility (diet, viral infections, others…).

Type I Diabetes

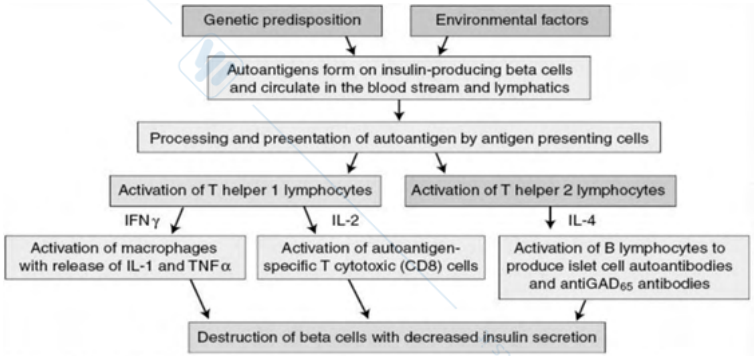


Figure 21-13 Pathophysiology of type 1 diabetes mellitus. GAD_{65}, glutamic acid decarboxylase; *INF*-γ Interferon-gamma; *IL*, interleukin; *TNF*-α, tumor necrosis factor-alpha.

T1DM auto-antigens

- Insulin
- Glutamic acid decarboxylase (GAD 65)
- The pancreatic islet monosialo-ganglioside (GM2-1)
- Sulfatide
- Protein tyrosine phosphatase (IA-2)
- Phogrin (Phosphatase homologue in granules of insulinoma; IA-2β)
- Hsp65
- Peripherin
- Carboxypeptidase H
- Glucose transporter 2

AUTOIMMUNE POLYENDOCRINE SYNDROME TYPE I
Autoantibodies in minor endocrinopathies

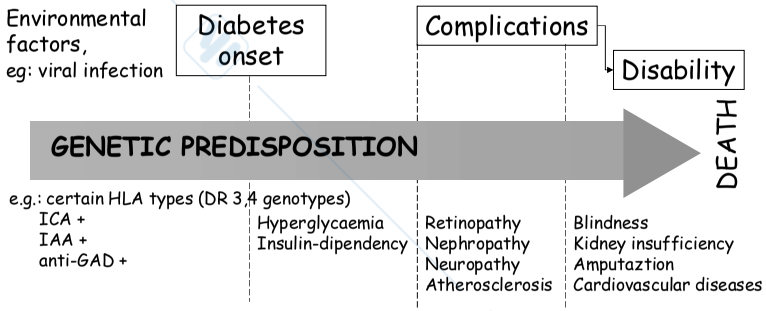
Onset disease	Autoantibodies (AAb)	AAb Presence before disease onset
Hypergonadotropic Hypogonadism	Steroid-producing cells (StCA), 17a-OHAb, P450sccAb	Y
Vitiligo	Complement fixing antibodies to melanocytes; anti-SOX9 and anti SOX10 (transcription factors)	Y unknown
Autoimmune Hepatitis	Anti-microsomes from kidney and liver (LKMA); Anti-P450-IA2, anti-P450-2A6	Y unknown
Celiac disease	Anti-reticulin; Anti-endomysium; Anti-transglutaminase	Y Y Y
Type I DM	Islet Cell Antibody, ICA; Anti-glutamic acid decarboxylase (GAD); Anti-islet tyrosine phosphatase 2 (IA2)	Y Y Y
Thyroid autoimmune diseases	Anti-microsomes thyroid peroxidase (TPO) Anti-thyroglobulin	Y Y
Autoimmune gastritis	Anti gastric parietal cells (PCA)	Y
Pernicious anemia	Anti-PCA + anti-Intrinsic Factor	Y
Malabsorption	Anti-tryptophan hydroxylase Anti-histidine decarboxylase	unknown unknown
Areata alopecia	Anti-thyrosine hydroxylase	unknown

symptom

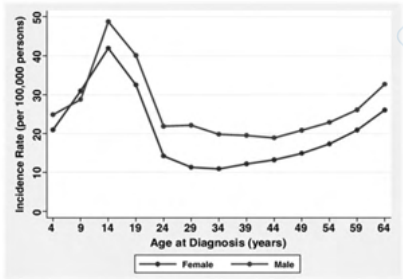
DIFFERENTIAL FEATURES OF TYPE I AND TYPE II DIABETES

	TYPE I	TYPE II
• Insulin level	Absent or low	Normal or high
• Symptoms	Manifest	Usually absent
• Ketosis	Present	Absent
• Body weight	Lean	Obese or overweight
• Age at onset (y)	< 35	> 35
• Chronic complications appearance	Many years after the onset	Most often present at diagnosis
• Prevalence	0.6%	3-7%
• Familiarity	Modest	Strong
• HLA system	Associated with	Not associated with
• Autoimmunity	Present	Absent
• Therapy	Insulin	Diet, glucose-lowering drugs, rarely insulin

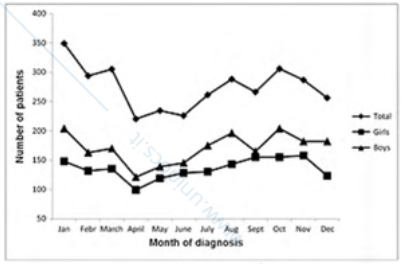
TYPE I DIABETES NATURAL HYSTORY



HLA, Human Leukocyte Antigen; **ICA**, *Islet-cell Cytoplasmic Antibodies;* **IAA**, *Insulin AutoAntibodies;* **anti-GAD** *antibodies (Glutamic Acid Decarboxylase)*



T1DM incidence by age



Frequency of T1DM onset by season/month in children and adolescents

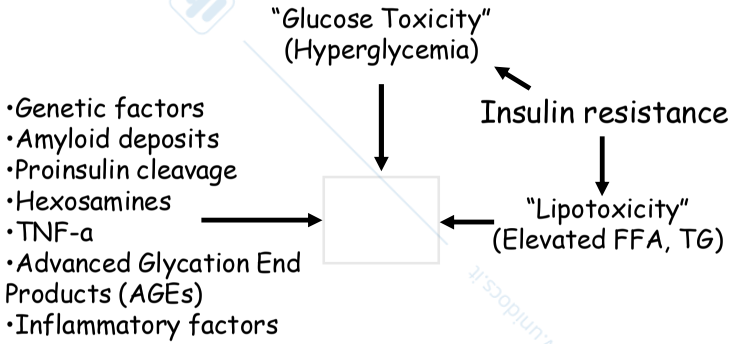
LADA variant of T1DM

Late Onset Autoimmune Diabetes Adult-LADA

Subtype of autoimmune diabetes with slow progression of the insular deficit.

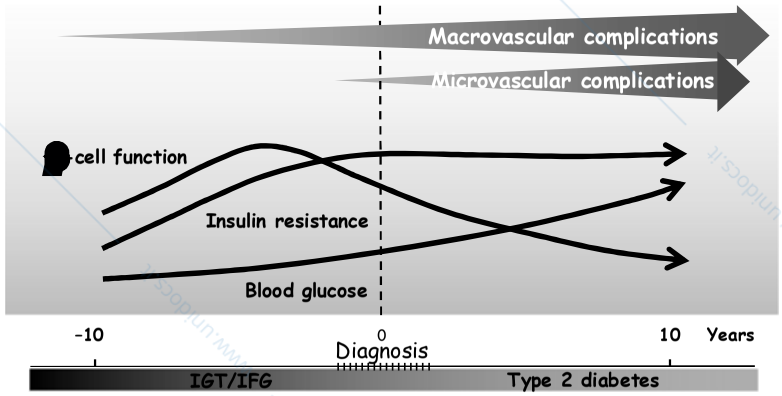
Type II Diabetes

Possible Mechanisms for Decline of β-Cell Function



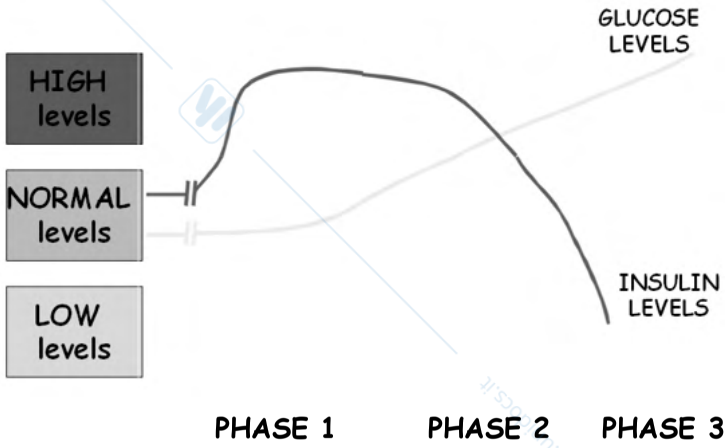
Adapted from Reaven GM. *Physiol Rev* 1995;75:473-486

Insulin resistance and β-cell failure contribute to Type 2 diabetes and risk of complications



DeFronzo RA. *Med Clin North Am* 2004; 88:787-835.

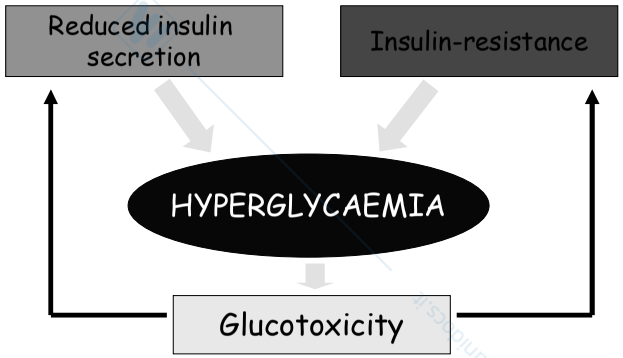
TYPE II DIABETES NATURAL HYSTORY



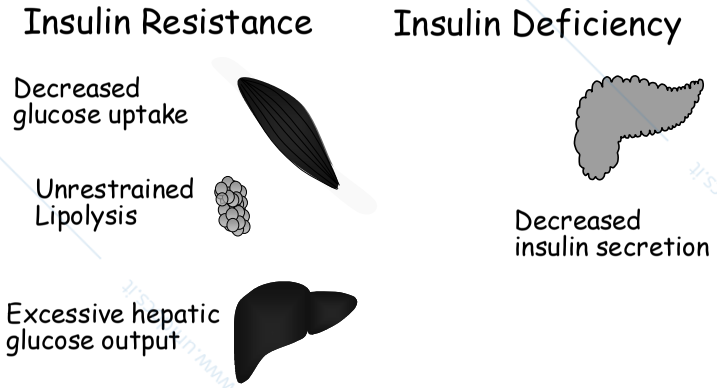
Type II Diabetes: Physiopathology

- Impaired insulin secretion
  - Total or relative insulin deficit
- Impaired insulin action / sensitivity
  - Insulin resistance

PHYSIOPATHOLOGY OF HYPERGLYCAEMIA IN T2DM



Dual Metabolic Abnormalities in Type 2 Diabetes



SUBJECTS AT HIGH RISK OF DEVELOPING TYPE II DIABETES

IFG or IGT or HbA1c at 6 – 6.49%, or previous gestational diabetes,

Age ≥45y, especially with BMI ≥25kg/m^2

Age <45y plus one or more among:

- physical inactivity
- 1° degree familiarity for T2DM
- arterial hypertension
- low HDL-cholesterol (≤35mg/dL) and/or high triglycerides (≥250mg/dL)
- Mothers of newborn baby >4kg
- Low birth weight (<2,5kg)
- PCOS or other insulin-resistance conditions
- Cardiovascular diseases
- High risk ethnicity

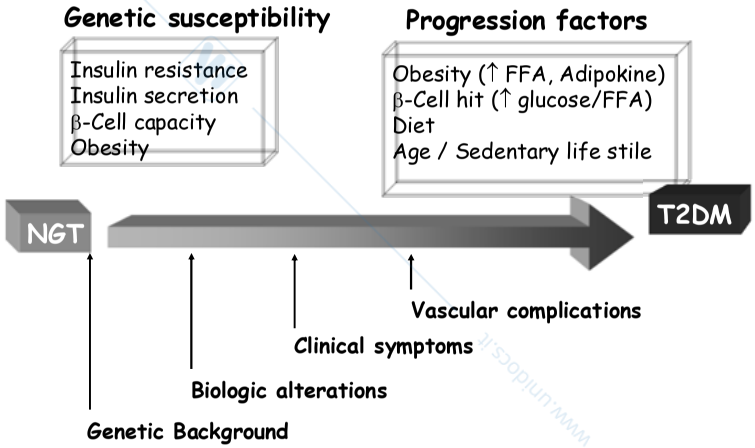
SUBJECTS AT HIGH RISK OF DEVELOPING TYPE II DIABETES

Boys and girls >10y

BMI>85°centile plus two among:

- 1° or 2° degree familiarity for T2DM
- mother had gestational diabetes
- insulin-resistance signs or associated conditions (hypertension, dyslipidemia, acanthosis nigricans, PCOS, low birth weight)
- high risk ethnicity

TYPE II DIABETES NATURAL HISTORY



CLINICAL INDEXES OF INSULIN RESISTANCE

INDEX	FORMULAS	Cut off	REF.
Glu/IRI	Glucose (mg/dl) / insulin (μU/ml)	<6	Caro et al. Jcem 1991
FIRI	Glucose (mmol/L) X insulin (μU/ml) / 25	>2.7	Ducan et al. Lancet 1995
HOMA	Glucose (mmol/L) X insulin (μU/ml) /22.5	>2.7	Mather et al. JCEM 2001
HOMA OGTT ISI COMPLEX	10.000 / (Glu-fast x Ins-fast) X (mean Glu-OGTT x mean Ins-OGTT) Glucose in mg/dl; insulin in μU/ml	<4.7	Matsuda et al. Diabetes Care 1999
QUICKI	1/ (log Insulin (μU/ml) + log glucose) (mg/dl))		Mather et al. JCEM 2001

To change unit from mg/dl to mmol/L, multiply by 0.05551

Standard of Care in Diabetes Mellitus

Diabetes mellitus is a chronic disease requiring continuous treatment combined with patient education and disease awareness, in order to prevent acute complications and to reduce the risk of long-term complications.

The treatment of diabetes is complex and requires, in addition to controlling blood glucose, the achievement of further therapeutic goals.

ADA, Diabetes Care 2006

Metabolic control

- *Definition*
- *How is it measured*
- *What's its meaning*

Complications control

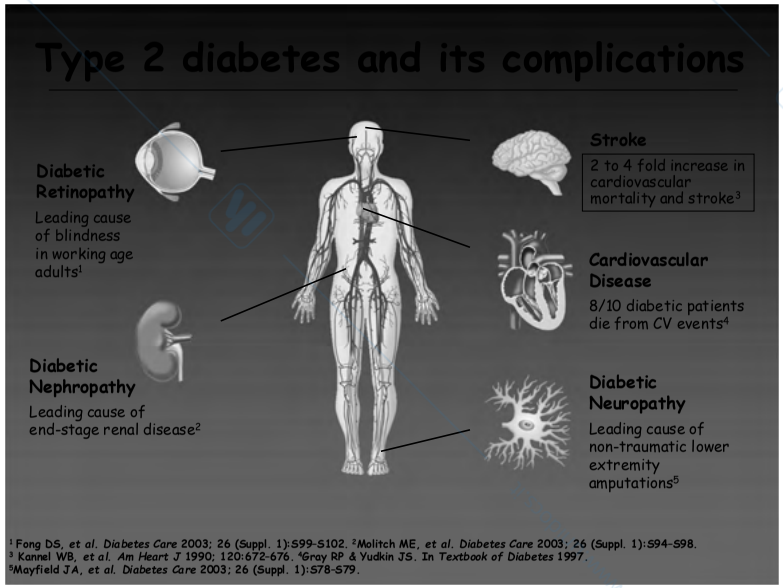
Metabolic control *definition*

Metabolic control is defined as the biochemical condition for which glucose concentrations, in the basal state and in the post-prandial period are normal.

In case of constant hyperglycaemia, metabolic control is defined as inadequate.

Metabolic control *how is it measured*

- Glycaemia at fasting *vs* after a meal
- Glycosuria (24 h)
- Glycated Hemoglobin A1c
- C-peptide (pancreatic reserve)
- Other metabolic parameters (lipids, etc…)



METABOLIC CONTROL OF TYPE II DIATES COMPLICATIONS

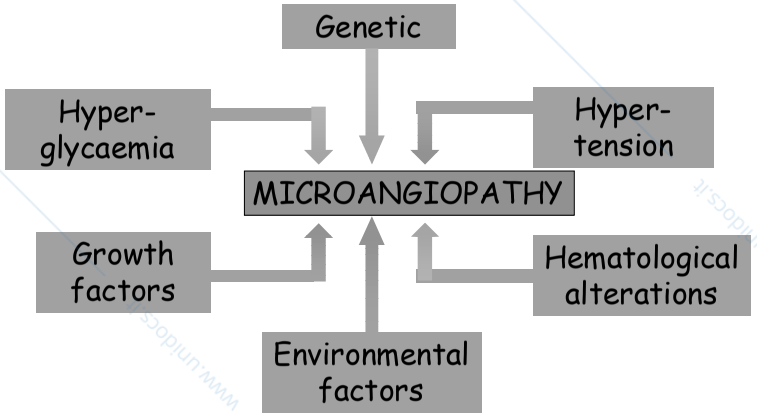
METABOLIC CONTROL	COMPLICATIONS CONTROL
• BODY WEIGHT	• RETINOPATHY
• GLYCAEMIC PROFILE	• NEPHROPATHY
• HbA_{Ic}	• NEUROPATHY
• LIPID PROFILE	• MACROANGIOPATHY
• FIBRINOGEN	• FOOT
• ARTERIAL PRESSURE	• SEXUAL ACTIVITY

COMPLICATIONS OF TYPE II DIABETES

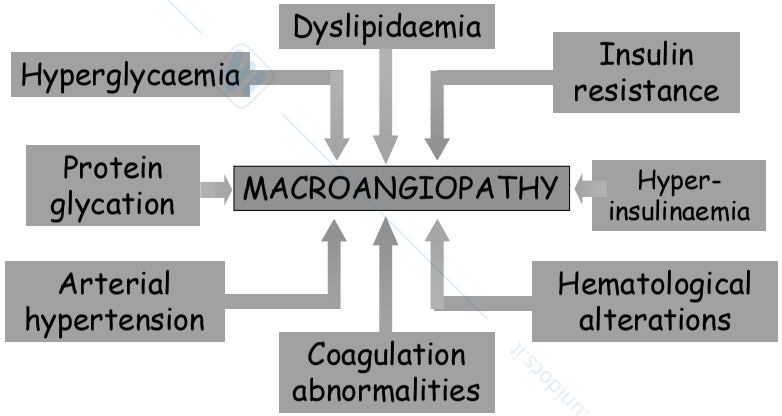
MICROANGIOPATHIC COMPLICATIONS	MACROANGIOPATHIC COMPLICATIONS
• Retinopathy	• Ishemic Cardiopathy
• Neuropathy	• Cerebral Vasculopathy
• Nephropathy	• Peripheral Vasculopathy

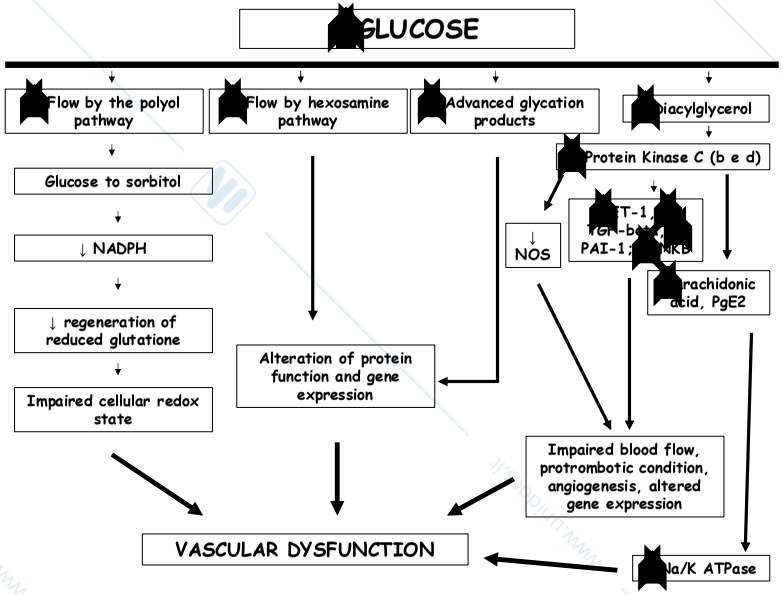
• Sexual dysfunction in men

FACTORS CONTRIBUTING TO THE PATHOGENESIS OF DIABETIC MICROANGIOPATHY

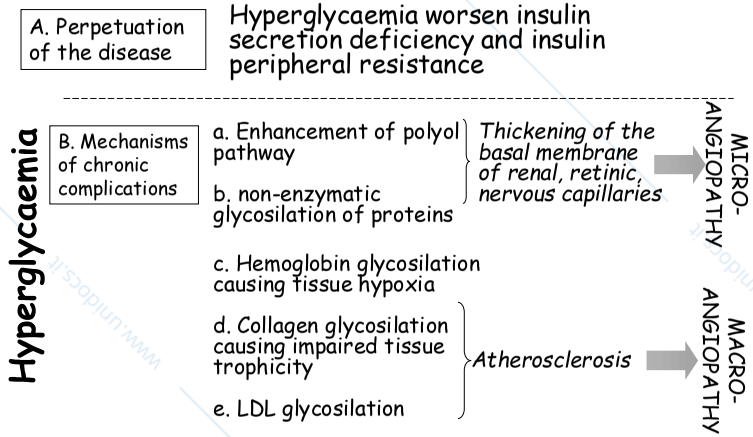


FACTORS CONTRIBUTING TO THE PATHOGENESIS OF DIABETIC MACROANGIOPATHY





GLUCOTOXYCITY ROLE IN T2DM PROGRESSION



POLYOL PATHWAY

The polyols pathway is a particular glycidic metabolism which involves the production of fructose and sorbitol in the cells of the nervous system by the aldose-reductase enzyme; it would be responsible, at least in part, for the onset of diabetic neuropathy.

The polyols pathway is activated in condition of persistent hyperglycaemia, causing the increased glucose levels within the nervous system cells. This surplus can no longer be disposed of via the normal hexokinase route.
The accumulation of sorbitol and fructose produced by the polyol pathway accelerates the metabolism of myoinositol, which is used to transmit nerve impulses along the nervous pathways. A decrease in myoinositol causes the appearance of a diabetic neuropathy.

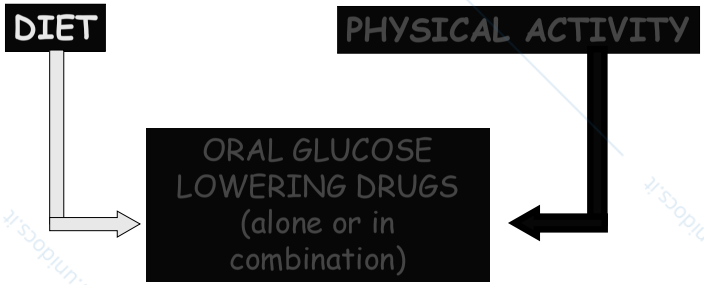
HEXOSAMINE PATHWAY

The hexosamine pathway is responsible for the synthesis of the substrates required for the glycosylation processes. The final product of this metabolic pathway, which uses glucose derivatives as substrates, is UDP N-acetyl glucosamine (UDP-GlcNAc).

This molecule is responsible for molecular mechanism such as the enzymatic post translational modifications of protein, synthesis of glycolipids, protein glycosilation within the Golgi apparatus, and others.

This pathway is tightly associated with glycolysis. Indeed, fructose-6-phosphate, an upstream metabolite of glycolysis, is the first substrate of the hexosamine pathway.

DIABETES THERAPY



Diet

- 40-45 cal/kg/die: lean or young or growing age or physically active subject 30-35 cal/kg/die: middle-age non obese subject
- 25 cal/kg/die: overweight or hospitalized

Calories partitioning through the day

- 2/11 breakfast
- 3/11 lunch
- 3/11 dinner
- 3 snaks of 1/11 each

Calories partitioning among nutrients

- 45-60% sugars (4 cal/g)
    - 80% complex (polysaccarides: cereal or legume starch)
    - 20% simple
- 10-20% proteins (4 cal/g)
    - 50% vegetal source
- <35% fat (9 cal/g)
    - Prefer vegetal sources rich in polyunsaturated fats
    - Saturated fats <7% of total calories

RECOMMENDED DIET IN TYPE II DIABETES

Optimal diet composition for the diabetic patient

Macronutrients	Total recommended amount	Recommended micronutrient quantity	Practical recommendation
Carbohydrates	45-60% tot kcal	Sucrose and other added sugars <10%	Vegetables, legumes, whole cereals, mediterrean diet foods.
Fibres	>40g/die / 20g/1000kcal/die, mostly soluble fibres		5 servings/week of vegetables or fruits or 4 servings/week of legumes.
Proteins	10-20% tot kcal		
Fats	35% tot kcal	Sature <10%, <8% with high LDL; MUFA 10-20%; PUFA 5-10%; Avoid trans fatty acids; Cholesterol <300mg/die, <200 with high blood tot cholesterol.	Among the seasoning fats, prefer vegetable ones (avoid palm and coconut oil).
Salt	<6g/die		Avoid salt intake.

Alcohol (9 cal/g)

- Decrease or avoid: alcohol causes lactic acidosis which can worsen neuropathy, and hypertriglycerides, and hampers glycogen synthesis

Dietary fibers (bran with at least 40% fiber)

- Add 40 g/die: reduces cholesterol, triglycerides, and carbohydrates (mechanism not clear though)

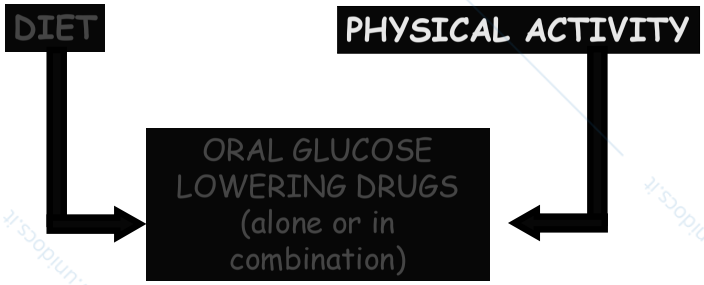
Mineral salts and vitamines

- Based on need

Sweeteners

- Saccharin
- Aspartame (ac. Aspartic acid and phenylalanine)
- Acesulfame K

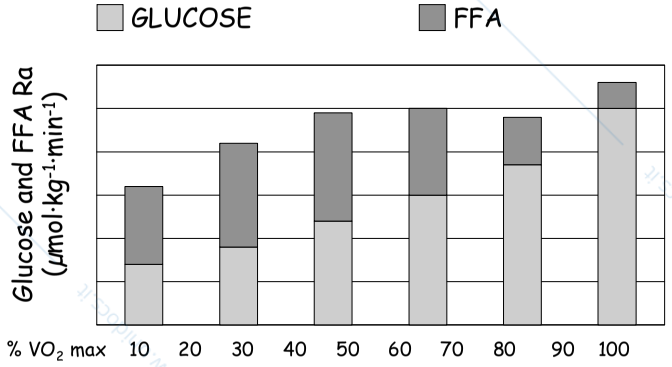
DIABETES THERAPY



RECOMMENDED PHYSICAL ACTIVITY IN TYPE II DIABETES

Physical activity	Body movement generated by skeletal muscle contraction, requiring a higher energy expenditure compared to resting.
Physical training	Programmed, structured and repeated body movement, performed to better or mantain one or more body components in good physical shape.
Aerobic exercise	Rhythmic movement, repeated and continue of large muscle groups for at least 10min each. Walking, slow running, swimming etc…
Resistance training	Activities that use muscle strenght to move a weight or work against a load that opposes resistance

CONTRIBUTION OF GLUCOSE AND FFA IN RELATION TO EXERCISE INTENSITY



Brooks and Trimmer J Appl Physiol 80: 1073, 1996

BENEFITS OF AEROBIC PHYSICAL ACTIVITY

- Body composition improvement
- Insulin sensitivity improvement and prevention of type 2 diabetes mellitus
- Less atherogenic lipid profile (increase in HDL-cholesterol, decrease in small and dense VLDL and LDL)
- Blood pressure reduction
- Reduction of mortality from all causes
- Reduction of coronary mortality
- Likely reduction of brain stroke risk
- Reduction of colon cancer mortality
- Bone density increase and fracture reduction
- Reduction of erectile dysfunction after age 50y
- Improvement of the feeling of physical well-being and quality of life

Physical activity	MET
Light intensity activities	**< 3**
sleeping	0.9
watching television	1.0
writing, desk work, typing	1.8
walking, 1.7 mph (2.7 km/h), level ground, strolling, very slow	2.3
walking, 2.5 mph (4 km/h)	2.9
Moderate intensity activities	**3 to 6**
bicycling, stationary, 50 watts, very light effort	3.0
walking 3.0 mph (4.8 km/h)	3.3
calisthenics, home exercise, light or moderate effort, general	3.5
walking 3.4 mph (5.5 km/h)	3.6
bicycling, <10 mph (16 km/h), leisure, to work or for pleasure	4.0
bicycling, stationary, 100 watts, light effort	5.5
Vigorous intensity activities	**> 6**
jogging, general	7.0
calisthenics (e.g. pushups, situps, pullups,jumping jacks), heavy, vigorous effort	8.0
running jogging, in place	8.0
rope jumping	10.0

INDICATORS of ENERGY EXPENDITURE

- Energy expenditure of various activities (Ainshvorth 1993, Schofield 1990, FAO 2004) are reported as multiples of the MET

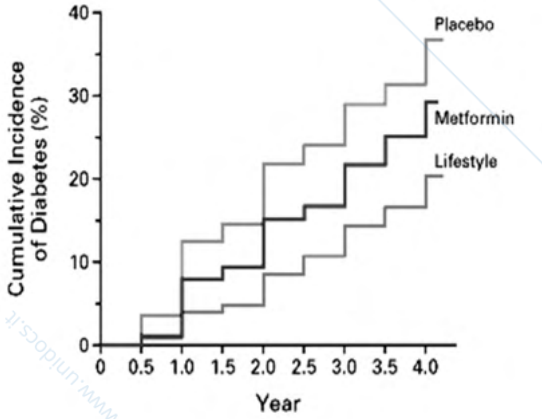
- MET = metabolic equivalent of tasks

  Quantity of energy/time required in resting condition (resting metabolic rate) expressed as oxygen consumption.

  1MET = 3.5mL oxygen/kg*min

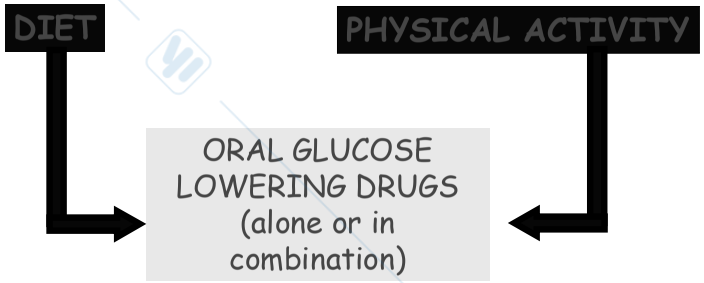
MET is also defined as the ratio of the rate at which a person expends energy, relative to the mass of that person, while performing some specific physical activity compared to a reference, set by convention at 3.5 mL of oxygen per kilogram per minute, which is roughly equivalent to the energy expended when sitting quietly.

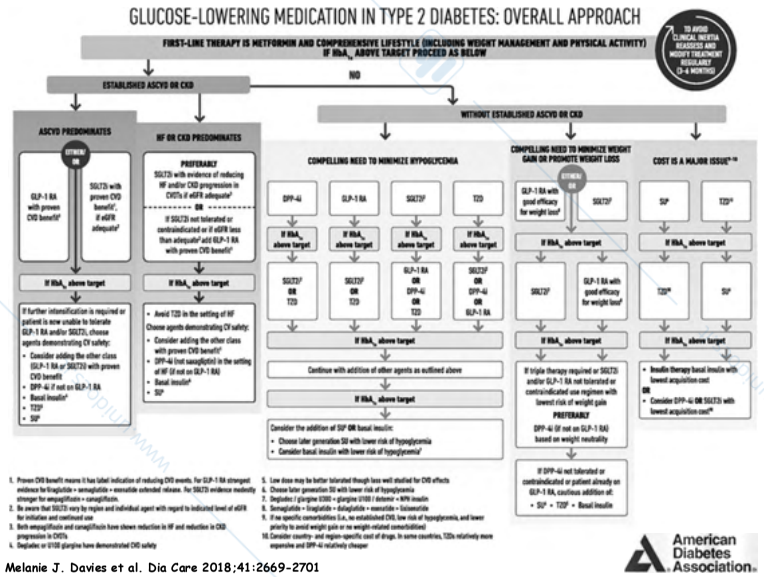
LIFESTYLE INTERVENTION FOR PREVENTING T2DM in at-risk subjects

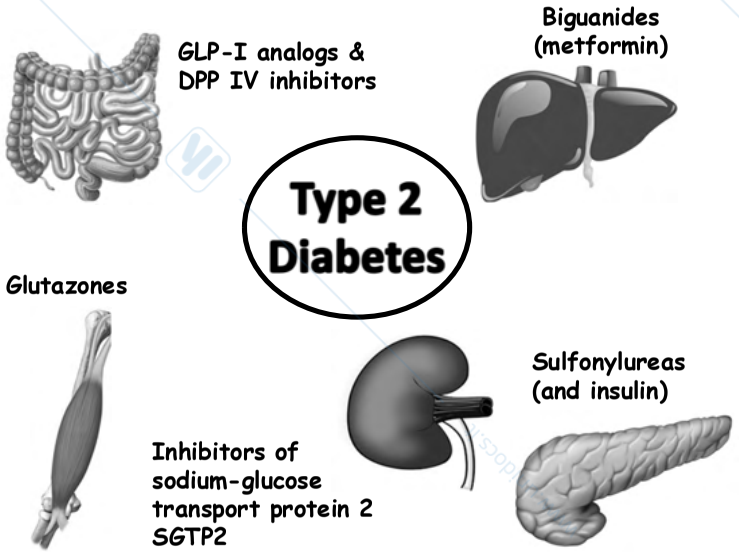


N Engl J Med, Vol. 346, No. 6, February 7, 2002

TYPE II DIABETES THERAPY







Biguanides

INCREASE INSULIN SENSITIVITY
mechanism not fully understood

- Increase glucose uptake at the peripheral level
- Decrease liver gluconeogenesis
- Decrease the intestinal absorption of glucose
- Increase intracellular anaerobic glycolysis and lipolysis in adipose tissues
- Inhibit glucagon

- Indicated in overweight patients
- Only work in diabetic patients
- Do not cause hypoglycaemia
- To be suspended before surgery or investigations with contrast medium (angiography, urography, etc.) which can temporarily reduce kidney function and predispose to lactic acidosis

Biguanides

- Metformin 500 mg: 2-4 tablets/day
- Fenformin (only provided in association with sulfanylureas)

Side effects:

Nausea, asthenia, vomiting, constipation, diarrhea, metallic taste in the mouth, weight loss.

Lactic acidosis can cause death

Contraindications to the use of biguanides:

Liver or kidney failure, tissue hypoxia, alcohol abuse, pancreatitis, after surgery, intensive care, the elderly, pregnancy, history of lactic acidosis, use of iv contrast media

Sulfonylureas (sulfamide derivatives)

Sulfonylureas – 1° generation

- Tolbutamide 0.5 g: 1-4 tablet/day
- Clorpropamide 0.25 g: 1-2 tablet/day

Sulfonylureas – 2° generation

- Glibenclamide 5 mg: 1-2 tablet/day
- Glipizide 5 mg: 1-2 tablet/day
- Glicazide 80 mg: ½ - 1 tablet before meal
- Glimepiride 2 mg: 1 tablet/day

Side effects:

Hypoglycaemic episodes, anorexia, nausea, vomiting, abdominal pain, hyposodyemia, potentiation of insulin action, weight gain, etc.

Drug interference

- Drugs with antagonist effect
  - Thiazide diuretics, corticosteroids, estrogens, phenylhydantoin, etc.
- Drugs with potentiating effect
  - Coumarin, salicylates, phenylbutazone, propanolol, clofibrate, ethanol, etc.

Sulfonylureas (sulfamide derivatives)

- Stimulate the release of insulin by the pancreas
- Decrease glucose release from the liver
- Prolonged treatments increase the number of peripheral insulin receptors

- Indicated in normal weight patients
- Not indicated in pregnancy, breastfeeding, kidney failure, cirrhosis of the liver, allergies, acute conditions of "stress" (infections, acute cardiovascular episodes, surgical interventions)

Inhibitors of intestinal enzymes

Reduce post-prandial glucose level and glycated Hb.
Used alone, or in combination with diet or sulfanylureas.

Acarbose 100 mg tablet

- Oligosaccharide of microbial origin, relents the absorption of sugars by inhibiting intestinal glycosidase enzymes which degrade polysaccharides in the small mucous membrane
- 25-100 mg 10' before meal

Side effects:
flatulence, diarrhea, bloating, reduced Fe absorption
May be used in association with sulfanylureas but not with metformin
Not indicated in pregnancy or enteropathies

Glitazones (or thiazolidinediones)

Pioglitazone and Rosiglitazone

- Glucose and triglycerides lowering.
- Reduce insulin resistance.
- Reduce liver gluconeogenesis and glucose release
- Reduce triglyceride synthesis in liver and free fatty acid release by the adipose tissue.
- Increase glucose uptake and utilization in the skeletal muscle.

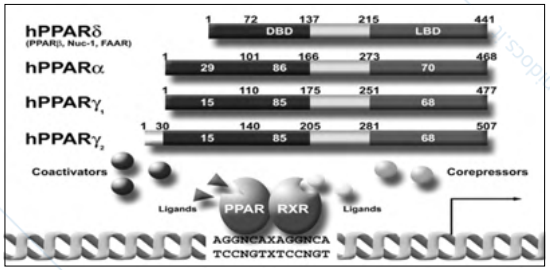
- Do not cause hypoglicaemia events.

- May cause weight gain.
- Heart failure risk

GLITAZONES MECHANISM OF ACTION

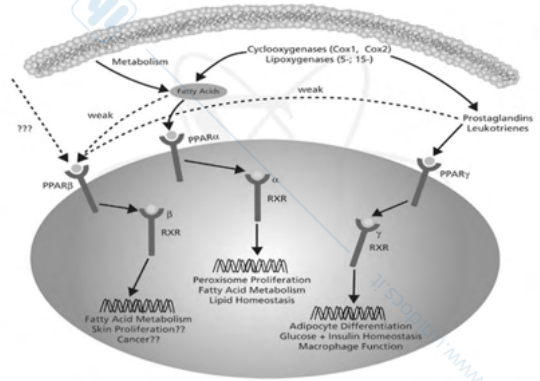
transactivators of the Peroxisome Proliferator-Activated Receptor (PPAR)

- Agonist binding to PPAR induces, in presence of coactivators and corepressors, the formation of an heterodimer between PPAR and Retinoid X Receptor (RXR).
- The PPAR-RXR heterodimer binds specific nucleotidic sequences, called peroxisome proliferator responsive elements (PPRE), usually located in the promoter region of target genes, thereby regulating their expression.



PPAR FUNCTIONS

Stumulates the differentiation of subcutaneous adipocytes, thereby improving insulin sensitivity and inducing weight gain at the same time.



TISSUE DISTRIBUTION OF PPAR and MAJOR BIOLOGIC EFFECTS

	TISSUE EXPRESSION	MAIN BIOLOGIC FUNCTIONS	EXOGENOUS LIGANDS	ENDOGENOUS LIGANDS
PPARα	-LIVER -HEART -KIDNEY -ADRENAL	-SYNTHESIS AND METABOLISM OF TRIGLYCERIDE RICH LIPOPROTEINS -β-OXIDATION - ANTI-INFLAMMATORY	WY-14.643 Clofibrate Nafenopin Bezafibrate	Palmitic Ac, Stearic Ac., Oleico Ac, Linoleic A, Arachidonic Ac., Eicosapentaenoic Ac.
PPARβ/δ	-ALL TISSUES	-ENERGY UTILIZATION - LIPID METABOLISM - ENDOTHELIUM PROCESSES		
PPARγ	ADIPOSE TISSUE (VISCERAL, SUBCUTANEOUS γ2> γ1) -SPLEEN -ADRENAL -COLON -LIVER -SKELETAL MUSCLE -HEART -KIDNEY	-ADIPOCYTES DEVELOPMENT -GLUCOSE HOMEOSTASIS -ANTI-INFLAMMATORY	Indometacin Ibuprofene Piroxicam Trogitazone Pioglitazone Rosiglitazone Ciglitazone	Arachidonic Ac, Eicosapentaenoic Ac, 15d-PGJ2

PPAR: PPRE-REGULATED TARGET GENES

TARGET GENE	FUNCTION
Liver fatty acid binding protein	Intracellular fatty acid binding
Liver-specific type I sugar transporter	Sugar transport
Malic enzyme	Fatty acid synthesis/NADPH production
Medium chain acyl-CoA dehydrogenase	Mitochondrial ß-oxidation
Mitochondrial 3-hydroxy-3-methylglutaryl-CoA synthase	Ketone body synthesis
Phosphoenolpyruvate carboxykinase	Glycerogenesis (adipose tissue)
Scavenger receptor CD36	Uptake of modified LDL in macrophage
Stearoyl-CoA desaturase 1	Desaturation of fatty acyl-CoA
Uncoupling protein 1 (brown adipocyte)	Non-shivering thermogenesis

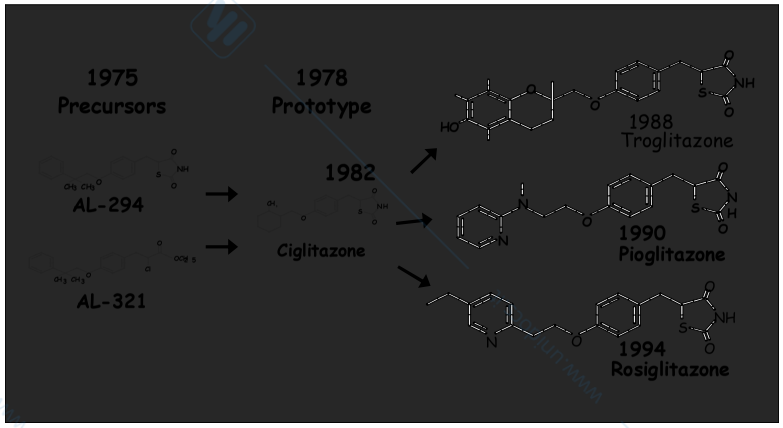
PPAR: PPRE-REGULATED TARGET GENES

TARGET GENE	FUNCTION
Acyl-CoA synthase	Fatty acid activation
Acyl-CoA oxidase	Peroxisomal ß-oxidation
Apolipoprotein A-I	Blood transport of fatty acid
Apolipoprotein A-II	Blood transport of fatty acid
Apolipoprotein C-III	Blood transport of fatty acid
aP2 adipocyte lipid binding protein	Intracellular fatty acid binding
Bifunctional enzyme (enoyl-CoA hydratase/3-Hydroxy-acyl-CoA dehydrogenase)	Peroxisomal ß-oxidation
CPTI carnitine palmitoyl transferase I	Entry of fatty acyl into mitochondria
Cyp4A1/P450 IV family	Microsomal ω -oxidation
Cyp4A6/P450 IV family	Microsomal ω -oxidation
Fatty acid transport protein	Fatty acid transport across cell membrane

THIAZOLIDINEDIONES (TZDs)

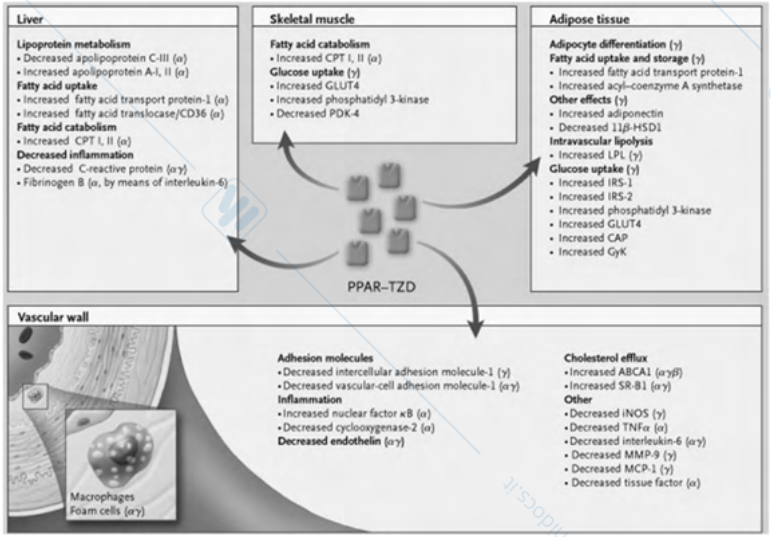
PPAR-γ AGONISTS

Troglitazone, Pioglitazone, Rosiglitazone, Ciglitazone



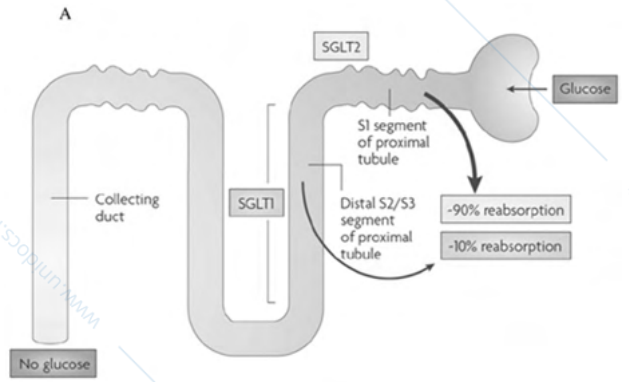
THIAZOLIDINEDIONES (TZDs)

- stimulate adipocytes differentiation
  - reduce FFA
  - inhibit resistin production
  - stimolate adiponectin production (ACRP-30)
  - modulate fat distribution
- improve insulin sensitivity
  - increase glucose transporter expression
- reduce hepatic glucose production
- lower C-reactive protein



H Yki-Jarvinen *NEJM* 351: 1106-1118, 2004

Inhibitors of Sodium-Glucose Cotransporter-2 (SGLT2)

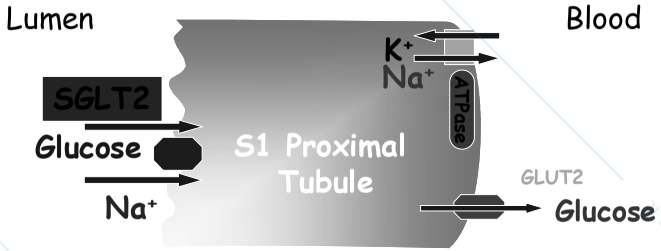


Chao EC and Herny RR. Nat Rev Drug Discov. 2010;9:551-559.

Sodium-Glucose Cotransporters

	SGLT1	SGLT2
Site	Intestine, kidney	Kidney
Sugar specificity	Glucose or galactose	Glucose
Glucose affinity	**High** K_m=0.4 mM	**Low** K_m=2 mM
Glucose transport capacity	Low	High
Role	Dietary absorption of glucose and galactose Renal glucose reabsorption	Renal glucose reabsorption

SGLT2 Mediates Glucose Reabsorption in the Kidney



Major transporter of glucose in the kidney

- Low affinity, high capacity for glucose
- Nearly exclusively expressed in the kidney
- Responsible for ~90% of renal glucose reabsorption in the proximal tubule

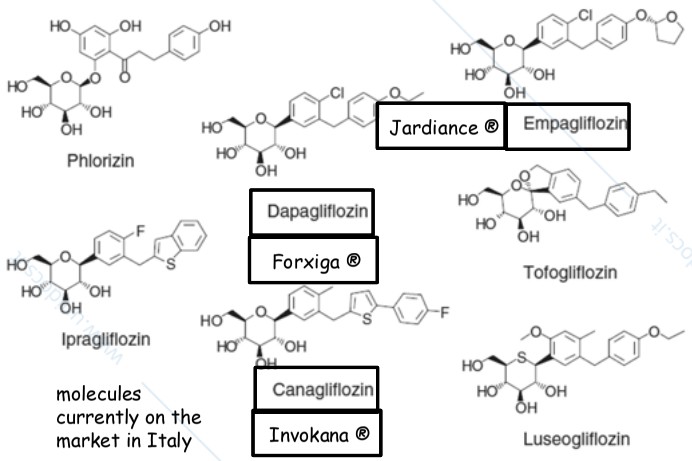
Hediger MA, Rhoads DB. *Physiol. Rev.*

Renal Glucose Reabsorption in Type 2 Diabetes

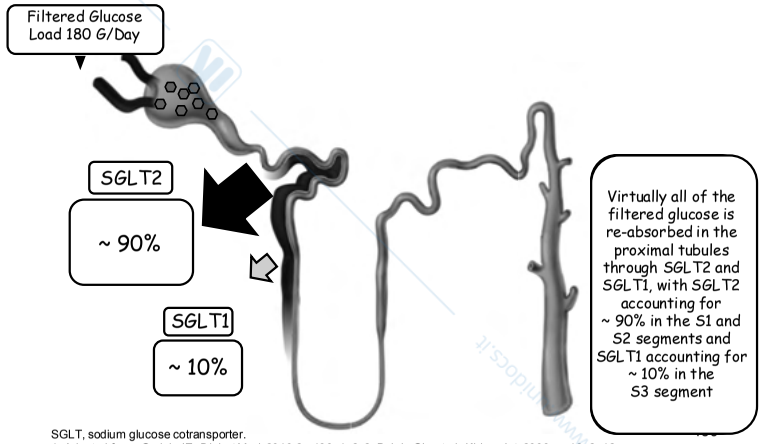
- Sodium-glucose cotransporter 2 (SGLT2) plays a role in renal glucose reabsorption in proximal tubule.
- Renal glucose reabsorption is increased in type 2 diabetes.
- Selective inhibition of SGLT2 increases urinary glucose excretion, reducing blood glucose.

Wright EM, et al. *J Intern Med.* 2007;261:32-43.

SGLT2 INHIBITORS
Glifozin class

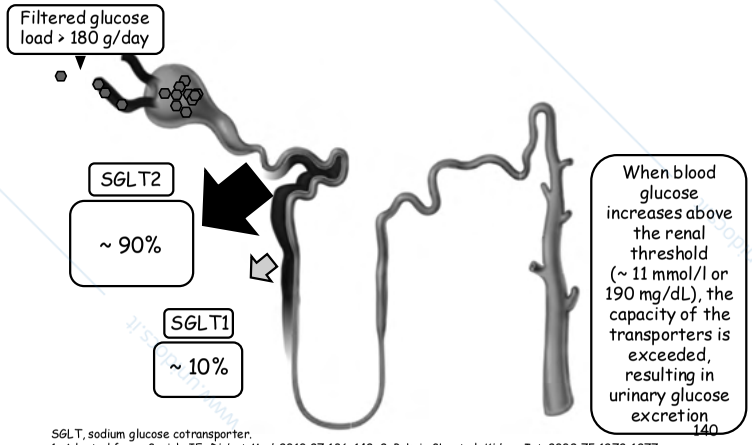


RENAL GLUCOSE RE-ABSORPTION UNDER HEALTHY CONDITIONS



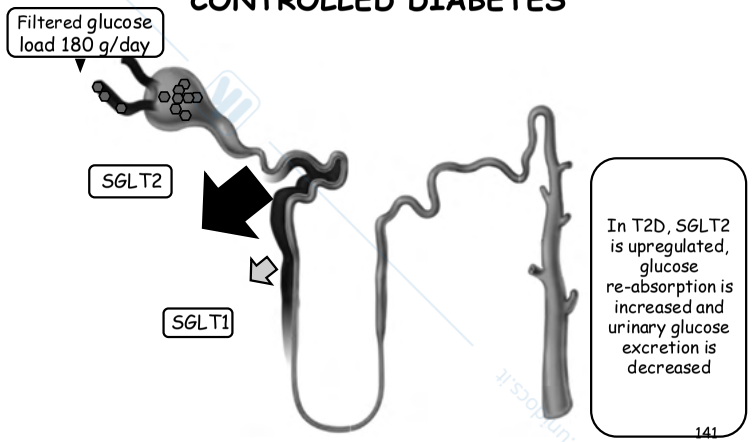
SGLT, sodium glucose cotransporter.
1. Adapted from: Gerich JE. *Diabet Med.* 2010;27:136–142; 2. Bakris GL, et al. *Kidney Int.* 2009;75;1272–1277.

RENAL GLUCOSE RE-ABSORPTION IN PATIENTS WITH DIABETES



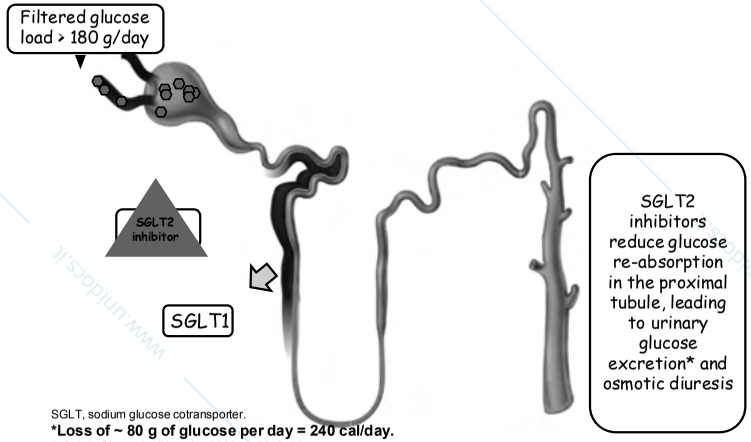
SGLT, sodium glucose cotransporter.
1. Adapted from: Gerich JE. *Diabet Med.* 2010;27:136–142; 2. Bakris GL, et al. *Kidney Int.* 2009;75;1272–1277.

UPREGULATION OF RENAL GLUCOSE RE-ABSORPTION IN PATIENTS WITH POORLY CONTROLLED DIABETES



Gerich JE. *Diabet Med.* 2010;27:136–142.

URINARY GLUCOSE EXCRETION VIA SGLT2 INHIBITION

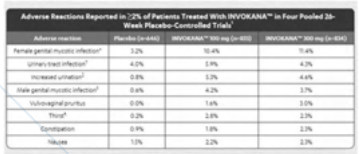


SGLT, sodium glucose cotransporter.
***Loss of ~ 80 g of glucose per day = 240 cal/day.**
1. Bakris GL, et al. *Kidney Int.* 2009;75;1272–1277.

Rationale for SGLT2 Inhibitors

- Inhibit glucose reabsorption in the renal proximal tubule.
- Resultant glucosuria leads to a decline in plasma glucose and reversal of glucotoxicity.
- This therapy is simple and nonspecific.
- Even patients with refractory type 2 diabetes are likely to respond.

SGTL2 inhibitors SIDE EFFECTS



Adverse Reactions Reported in ≥2% of Patients Treated With INVOKANA™ in Four Pooled 26-Week Placebo-Controlled Trials[1]

Adverse reaction	Placebo (n=646)	INVOKANA™ 100 mg (n=651)	INVOKANA™ 300 mg (n=634)
Female genital mycotic infection[2]	3.2%	10.4%	11.4%
Urinary tract infection[3]	4.5%	3.9%	4.3%
Increased urination[4]	0.8%	1.3%	4.6%
Male genital mycotic infection[5]	0.6%	4.2%	3.7%
Vulvovaginal pruritus	0.0%	1.6%	3.0%
Thirst[6]	0.2%	2.0%	2.3%
Constipation	0.9%	1.8%	2.3%
Nausea	1.5%	2.2%	2.3%

Table 1 Adverse Reactions Reported in ≥2% of Patients Treated with JARDIANCE and Greater than Placebo in Pooled Placebo-Controlled Clinical Studies of JARDIANCE Monotherapy or Combination Therapy

	Number (%) of Patients		
	Placebo N=995	JARDIANCE 10 mg N=999	JARDIANCE 25 mg N=977
Urinary tract infection[a]	7.6%	9.3%	7.6%
Female genital mycotic infections[b]	1.5%	5.4%	6.4%
Upper respiratory tract infection	3.8%	3.1%	4.0%
Increased urination[c]	1.0%	3.4%	3.2%
Dyslipidemia	3.4%	3.9%	2.9%
Arthralgia	2.2%	2.4%	2.3%
Male genital mycotic infections[d]	0.4%	3.1%	1.6%
Nausea	1.4%	2.3%	1.1%

[a]Predefined adverse event grouping, including, but not limited to, urinary tract infection, asymptomatic bacteriuria, cystitis

[b]Female genital mycotic infections include the following adverse reactions: vulvovaginal mycotic infection, vaginal infection, vulvitis, vulvovaginal candidiasis, genital infection, genital candidiasis, genital infection fungal, genitourinary tract infection, vulvovaginitis, cervicitis, urogenital infection fungal, vaginitis bacterial. Percentages calculated with the number of female subjects in each group as denominator: placebo (N=481), JARDIANCE 10 mg (N=443), JARDIANCE 25 mg (N=420).

[c]Predefined adverse event grouping, including, but not limited to, polyuria, pollakiuria, and nocturia

[d]Male genital mycotic infections include the following adverse reactions: balanoposthitis, balanitis, genital infections fungal, genitourinary tract infection, balanitis candida, scrotal abscess, penile infection. Percentages calculated with the number of male subjects in each group as denominator: placebo (N=514), JARDIANCE 10 mg (N=556), JARDIANCE 25 mg (N=557).

GLP-1 based drugs

GLP-1 AGONISTS

Administered by sc inj (just like insulin)
once a day (liraglutide, lixisenatide) or
twice a day (exenatide) or
once a week (long-acting exenatide, dulaglutide, semaglutide).

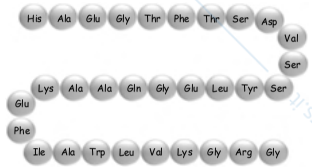
GLIPTINE

Inhibitors of dipeptidyl peptidase IV
preventing GLP-1 degradation.
The progenitor of the class is sitagliptin, approved for the first time by the FDA in 2006.

GLUCAGON-LIKE PEPTIDE-1 (GLP-1)

- GLP-1 is a 31 amino acid peptide
  - Member of incretin family
- Secreted predominantly from L-cells in the gut, but also the brain (nucleus tractus solitarus)

Human endogenous GLP-1

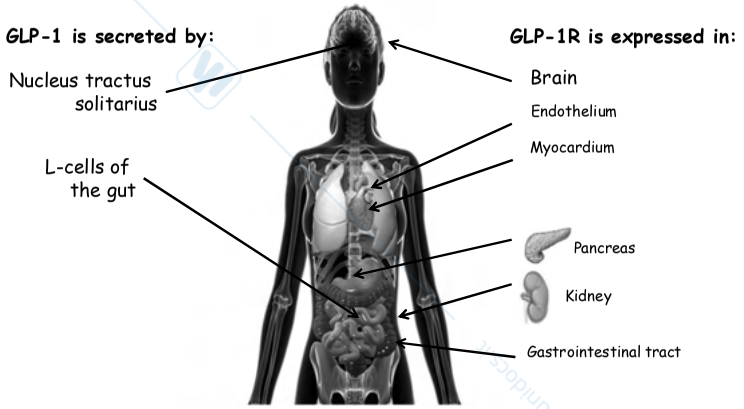


Enzymatic degradation by DPP-4

$t_{\frac{1}{2}}$=1.5–2 min

Merchenthaler *et al. J Comp Neurol* 1999; Baggio & Drucker. *Gastroenterology* 2007; Ducker & Nauck. *Lancet* 2006

GLP-1 SECRETION AND RECEPTOR EXPRESSION

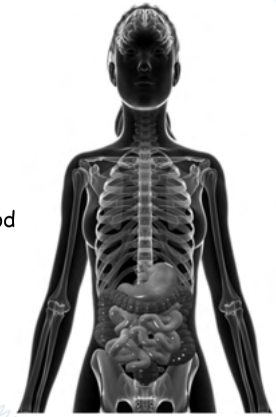


Merchenthaler *et al. J Comp Neurol.* 1999; Baggio & Drucker. *Gastroenterology* 2007; Ban *et al. Circulation* 2008; Vrang *et al. Prog Neurobiol* 2010; Pyke *et al. Endocrinology* 2014.

METABOLIC EFFECTS OF GLP-1

Appetite

- ⇧ Satiety
- ⇧ Fullness
- ⇩ Hunger
- ⇩ Prospective food consumption
- ⇩ Energy intake



Glucose regulation

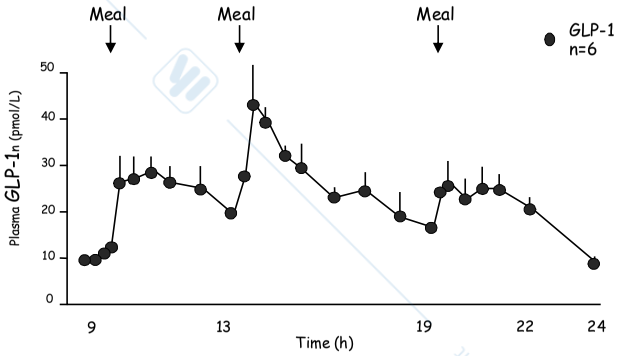
- ⇧ (Glucose-dependent) Insulin secretion
- ⇩ Glucagon secretion

Mild gastric effects

- ⇩ Gastric acid
- ⇩ Gastric emptying

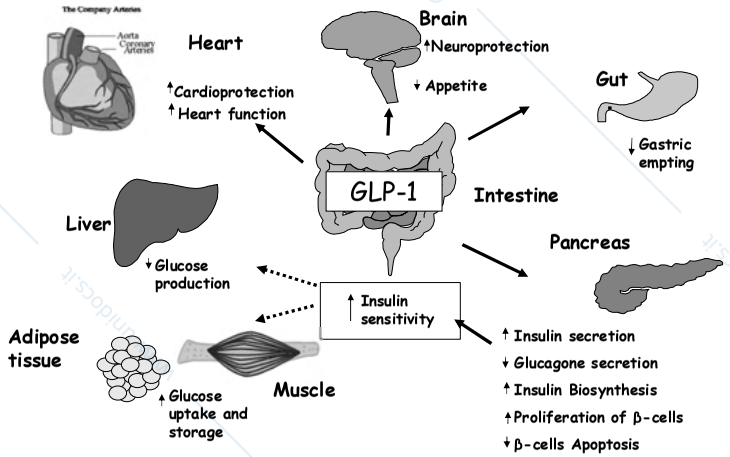
Flint *et al. J Clin Invest* 1998; Nauck *et al. Diabetologia* 1993; O'Halloran *et al. J Endocrinol* 1990; Nauck *et al. Am J Physiol* 1997

GLP-1 IS RELEASED IN RESPONSE TO FOOD INTAKE



Adapted from: Orskov *et al. Scand J Gastroenterol* 1996

GLP-1 effects on peripheral tissues

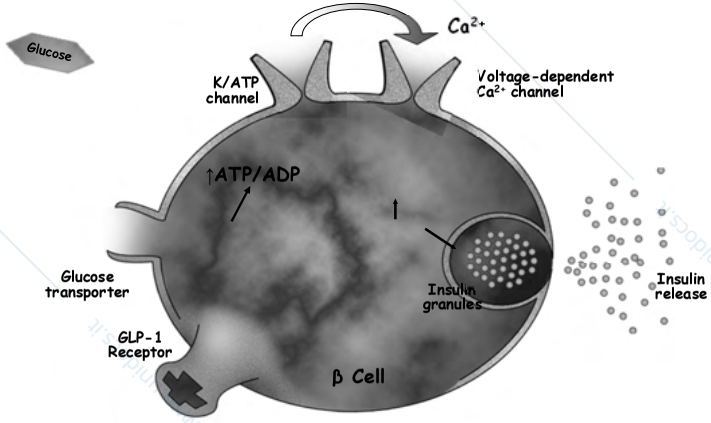


Baggio LL, Drucker DJ. *Gastroenterology*. 2007;132:2131-2157.

Multiple GLP-1 functions

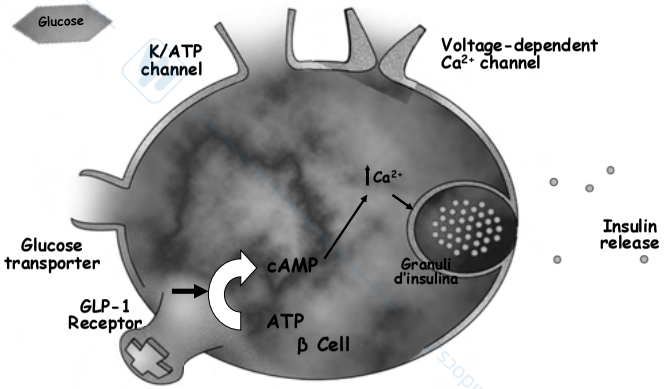
- GLP-1 exhibits multiple functions contributing to reducing hyperglycaemia :
  - Glucose-dependent stimulation of insulin secretion
  - Glucose-dependent suppression of glucagon secretion
  - Slow the gut empting rate
  - Improves β-cell function
  - Increases β-cell mass and the neogenesis of islets (animal models)
- GLP-1 is also associated with the reduced food intake and weight loss

Glucose stimulated insulin secretion



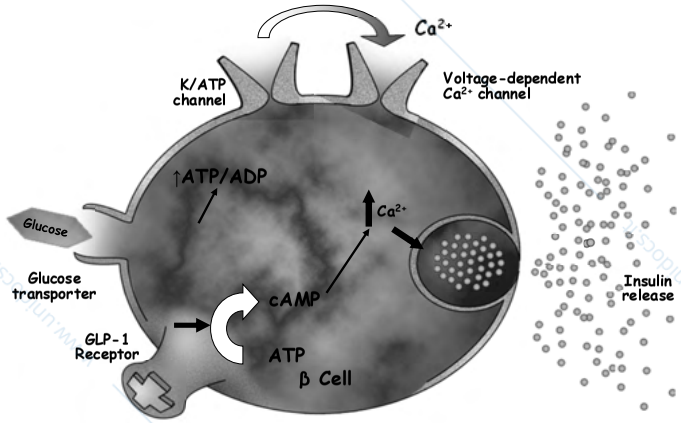
Gromada J., et al. *Pflugers Arch - Eur J Physiol*. 1998;435:583-594.; MacDonald PE, et al. *Diabetes*. 2002;51:S434-S442.

In absence of glucose, the activation of GLP-1 receptor induces the release of only small amounts of insulin



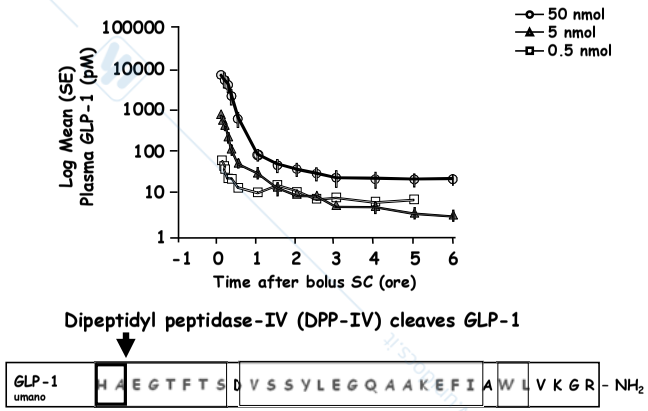
Gromada J, et al. *Pflugers Arch – Eur J Physiol*. 1998;435:583-594; MacDonald PE, et al. *Diabetes*. 2002;51:S434-S442.

The insulinotropic action of GLP-1 is glucose dependent



Gromada J, et al. *Pflugers Arch – Eur J Physiol*. 1998;435:583-594; MacDonald PE, et al. *Diabetes*. 2002;51:S434-S442.

The rapid degradation of GLP-1 by DPP-IV limits the duration of its action



Mean ± SEM;N = 4-7 (rats); $P < .05$.
Adapted from Parkes D, et al. *Drug Dev Res*. 2001;53:260-267.; Eng J, et al. *J Biol Chem*. 1992;267:7402-7405.

GLP-1 THERAPEUTIC POTENTIAL IS LIMITED BY ITS RAPID INACTIVATION

Rapid inactivation (DPP-IV), Short halflife (~1-2 min)

↓

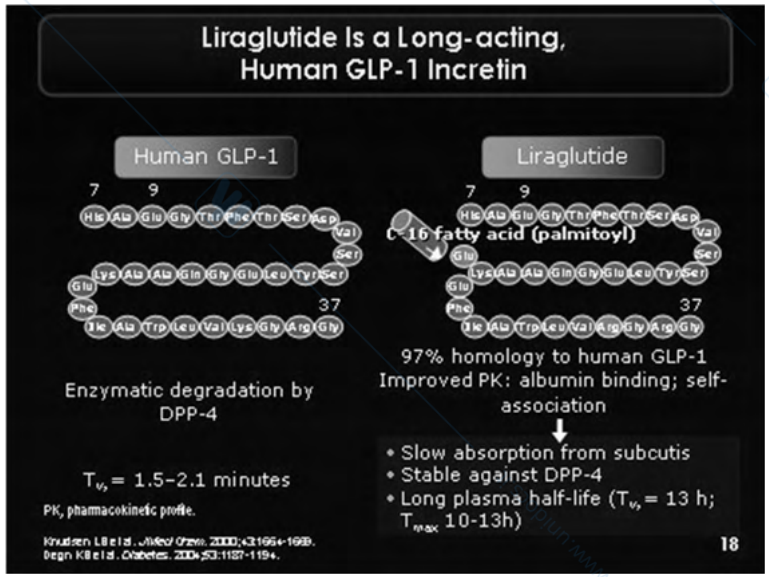
To be effective, GLP-1 should be continuously infused

↓

Drawback for treating a chronic disease such as type 2 diabetes

Drucker DJ, et al. *Diabetes Care*. 2003;26:2929-2940.

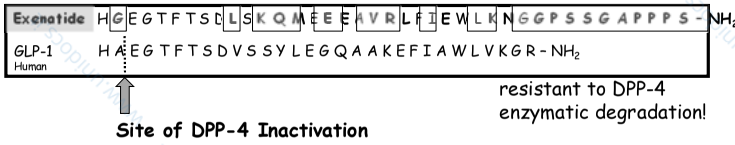
Liraglutide Is a Long-acting, Human GLP-1 Incretin



Development of Exenatide: An Incretin Mimetic

Exenatide (Exendin-4)

- Synthetic version of salivary protein found in the Gila monster
- Approximately 50% identity with human GLP-1
  - Binds to known human GLP-1 receptors on β cells *in vitro*
  - Resistant to DPP-4 inactivation



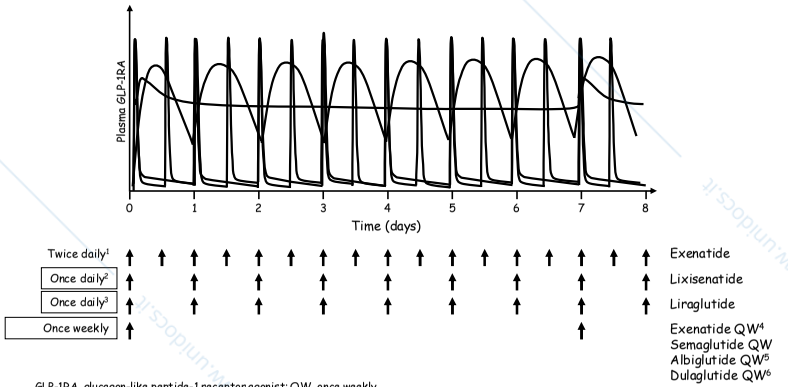
Adapted from Nielsen LL, et al. Regulatory Peptides. 2004;117:77-88. Reprinted from *Regulatory Peptides*, 117, Nielsen LL, et al, Pharmacology of exenatide (synthetic exendin-4): a potential therapeutic for improved glycaemic control of type 2 diabetes, 77-88, 2004, with permission from Elsevier for English use only.

FDA APPROVED GLP-1 BASED THERAPIES for TII-DM

DPP4 inhibitors	GLP-1R agonists
Sitagliptin	Liraglutide
Vildagliptin	Exenatide
Saxagliptin	Lixisenatide
Linagliptin	Albiglutide
Alogliptin	Dulaglutide

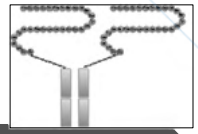
DDP4 inhibitors generally raise concentration of endogenous 7-36a GLP-1 by 2- to 4-fold whilst pharmaceutical circulating levels of GLP-1R agonist can be 10-fold greater than endogenous levels

Different pharmacokinetics of GLP-1RAs at steady state



GLP-1RA, glucagon-like peptide-1 receptor agonist; QW, once weekly
1. Reddy S et al. *AAPS J* 2005;7:M1285; 2. Christensen M et al. *IDrugs* 2009;12:503–513; 3. Elbrønd B et al. *Diabetes Care* 2002;25:1398–1404; 4. Fineman M et al. *Clin Pharmacokinet* 2011;50:65–74; 5. Bush MA et al. *Diabetes Obes Metab* 2009;11:498–505; 6. Kuritzky L et al. *Postgrad Med* 2014;126:60–72

DULAGLUTIDE
Once weekly GLP-1 analogue



AWARD-5: Dulaglutide vs Sitagliptin
~1,100 T2D Patients for 52 weeks

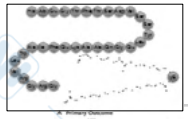
	HbA1c	To Target	BW Loss
Sitaglitpin	-0.39%	33%	-1.53 kg
Dula 0.75 mg	-0.87%	49%	-2.60 kg
Dula 1.5 mg	-1.10%	58	-3.03 kg

AWARD-1: Dulaglutide vs Exenatide BID
~1,000 T2D Patients for 52 weeks

	HbA1c	To Target	BW Loss
Exenatide	-0.80%	38%	-1.07 kg
Dula 0.75 mg	-1.07%	48%	-0.20 kg
Dula 1.5 mg	-1.36%	57%	-1.30 kg

Dulaglutide (Trulicity, Eli Lilly) consists of two fused GLP-1 analogues, with a glycine substitution at position 2, a glutamic acid substitution at position 20 and a glycine at position 30, which protect against DPP-IV degradation. The half-life of dulaglutide is greatly extended by its conjugation to the Fc fragment of human IgG4. This antibody conjugation increases the molecular weight of the molecule well past the renal threshold, providing a half-life of 96 h. In clinical trials, dulaglutide produced a 2.3–3.0 kg body weight loss and a 0.78–1.64%(8.6–18.0mmol/mol) reduction in HbA1c, relative to baseline. Dulaglutide was approved by the US FDA and the EMA in 2014.

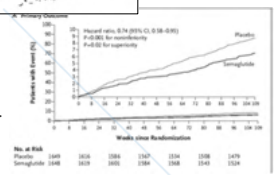
SEMAGLUTIDE
Once weekly GLP-1 analogue
(on going development for oral adm)



stearic-diacid at lysine position

	i.v. administration			sc. administration			
	Cl (L/h/kg)	Vz (L/kg)	$T_{1/2}$ (h)	T_{max} (h)	MRT (h)	F (%)
semaglutide	0.0016	0.1019	46.1	12	63.6	94%
liraglutide	0.0038	0.0674	12.4	7	23.0	66%

Marso SP et al. 2016 NEJM



SUSTAIN 2: Semaglutide vs Sitagliptin
~1,200 T2D Patients for 56 weeks

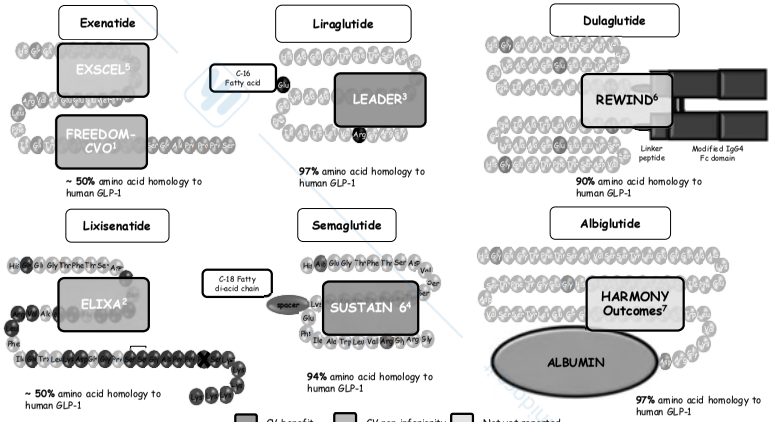
	HbA1c	To Target	BW Loss
Sitaglitpin	-0.5%	36%	-1.9 kg
Sema 0.5 mg	-1.3%	69%	-4.3 kg
Sema 1.0 mg	-1.6%	78%	-6.1 kg

SUSTAIN 3: Semaglutide vs Exenatide LAR
~800 T2D Patients for 56 weeks

	HbA1c	To Target	BW Loss
Exenatide LAR 2.0 mg	-0.9%	40%	-1.8 kg
Sema 1.0 mg	-1.5%	66%	-5.6 kg

Weight loss!

DIFFERENCES IN MOLECULAR STRUCTURE OF GLP-1RAS



CV, cardiovascular; Fc, fragment crystallisable; GLP-1, glucagon-like peptide-1; GLP-1RA, glucagon-like peptide-1 receptor agonist; IgG4, immunoglobulin G4
1. FREEDOM-CVO. Intarcia Therapeutics, Inc May 2016 press release. http://prn.to/15VcaXg; 2. Pfeffer MA et al. *N Engl J Med* 2015;373:2247-2257; 3. Marso SP et al. *N Engl J Med* 2016;375:311-322; 4. Marso SP et al. *N Engl J Med* 2016;375:1834-1844; 5. Mentz RJ et al. *Am Heart J* 2017;187:1-9; 6. Gerstein HC et al. *Diabetes Obes Metab* 2017; 7. HARMONY Outcomes. https://clinicaltrials.gov/show/NCT02465515. All web links: Accessed June 2017

EFFECTS OF NOVEL ANTIDIABETIC DRUGS ON THE CV SYSTEM

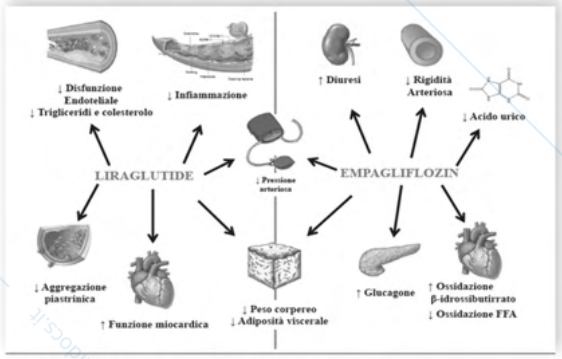
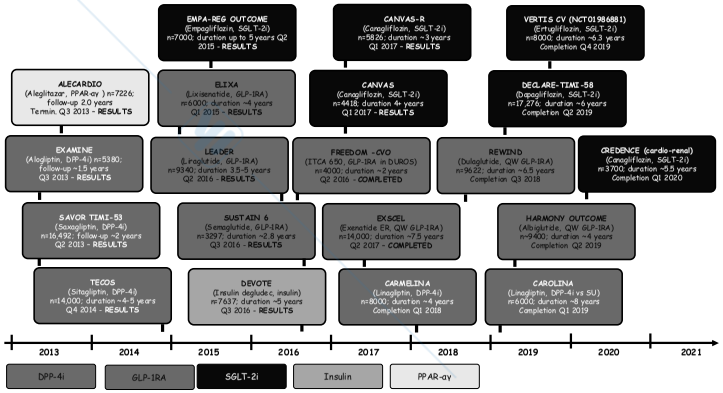


Figura 1. Effetti di liraglutide ed empagliflozin sul sistema cardiovascolare.
FFA, acidi grassi liberi; ICAM-1, molecola di adesione intercellulare-1; VCAM-1, molecola di adesione cellulare vascolare-1.

Fiorentino TV, Sesti G G Ital Cardiol 2016

cardiovascular outcomes trials in T2DM

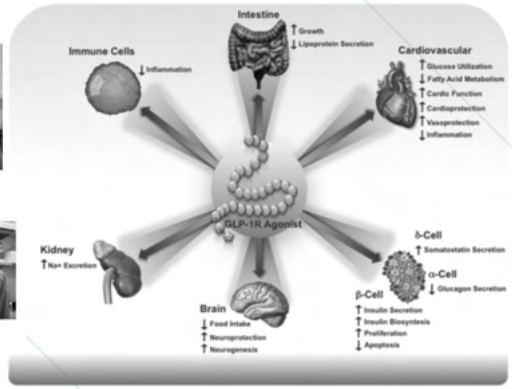


CVOT, cardiovascular outcomes trial; DPP-4i, dipeptidyl peptidase-4 inhibitor; ER, extended release; GLP-1RA, glucagon-like peptide-1 receptor agonist; ITCA 650, continuous subcutaneous delivery of exenatide; PPAR- receptors-αγ, peroxisome proliferator-activated receptors-α and γ; QW, once weekly; SGLT-2i, sodium-glucose cotransporter 2 inhibitor; SU, sulphonylurea; T2DM, type 2 diabetes mellitus
ClinicalTrials.gov. Accessed June 2017

GLP-1 PHYSIOLOGY *vs* GLP-1 PHARMACOLOGY: DIRECT ACTIONS

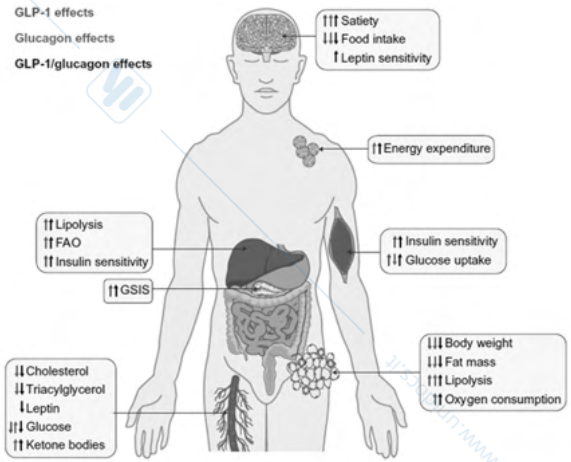
M. H. Tschoep

R. D. DiMarchi



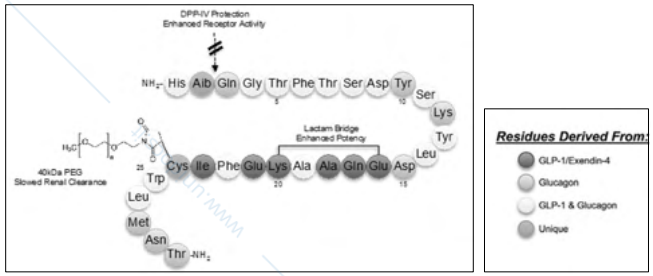
Campbell & Drucker, *Cell Metabolism* 2013

GLP-1 AND GLUCAGON COACTIVATION



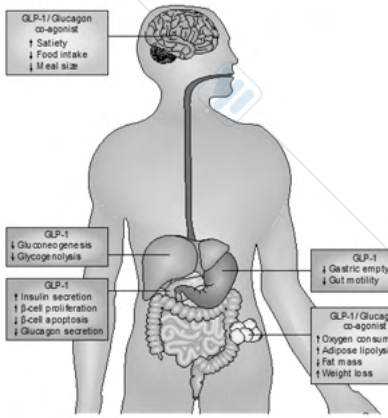
UNIMOLECULAR GLP-1R/GCGR

GLP-1 and glucagon exhibit similar amino acid N-terminal sequences, a domain essential for proper interaction with their receptors.
A variety of dual agonists were built using the glucagon amino acid sequence as a template for the unimolecular GLP-1R/GcgR.
Amino acid substitutions to the glucagon sequence aimed at increasing potency at the GLP-1R and conferring balanced co-agonism at both receptors. Additionally, a 40 kDa polyethylene glycol (PEG) polymer was incorporated at position 24 to extend duration of action and reduce frequency of administration. Amino acid substitution at position 2 eliminated DPP-IV proteolysis



Clinical Prospects of GLP-1/Glucagon Co-Agonism

GLP-1 BASED MONO-AGONISTS AND POLY-AGONISTS IN PRECLINICAL DEVELOPMENT OR USED CLINICALLY FOR OBESITY AND TYPE 2 DIABETES THERAPY



Drug	Company	Target	Phase	Administration
Exenatide (Byetta)	AstraZeneca	GLP-1R	Registered	SC, twice daily
Exenatide (Bydureon)	AstraZeneca	GLP-1R	Registered	SC, weekly
Lixisenatide (Lyxumia)	Sanofi-Aventis	GLP-1R	Registered	SC, daily
Liraglutide (Victoza)	Novo Nordisk	GLP-1R	Registered	SC, daily
Semaglutide	Novo Nordisk	GLP-1R	Phase 3	Oral, daily
Semaglutide	Novo Nordisk	GLP-1R	Phase 3	SC, weekly
Dulaglutide (Trulicity)	Eli Lilly	GLP-1R	Registered	SC, weekly
Albiglutide (Tanzeum)*	GlaxoSmithKline	GLP-1R	Registered	SC, weekly
SAR425899	Sanofi-Aventis	GLP-1R/GcgR	Phase 1	SC, daily
LY2944876/TT-401	Eli Lilly	GLP-1R/GcgR	Phase 2	SC, weekly
HM12525A	Hanmi Pharmaceuticals	GLP-1R/GcgR	Phase 1	SC, weekly
ZP2929	Zealand	GLP-1R/GcgR	Phase 1	SC, daily
MED0382	MedImmune	GLP-1R/GcgR	Phase 1	SC
VPD-107	Spitfire Pharma	GLP-1R/GcgR	Preclinical	SC, weekly
MOD-6031	OPKO Biologics	GLP-1R/GcgR	Phase 1	SC, monthly
Liraglutide + NN9030	Novo Nordisk	GLP-1R + GcgR	Phase 1	SC
Cpd86	Eli Lilly	GLP-1R/GIPR	Preclinical	SC
ZP-DI-70	Zealand	GLP-1R/GIPR	Preclinical	SC, weekly
NN9709/MAR709	Novo Nordisk/Marcadia	GLP-1R/GIPR	Phase 2	SC, daily
MAR423	Novo Nordisk/Marcadia	GLP-1R/GIPR/GcgR	Phase 1	SC, daily

* Also available as Epertinir
GIPR, Glucose-dependent insulinotropic polypeptide receptor; SC, subcutaneous

Sanchez-Garrido MA et al. 2017 Diabetologia

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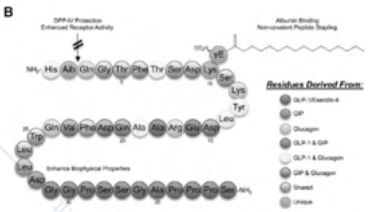
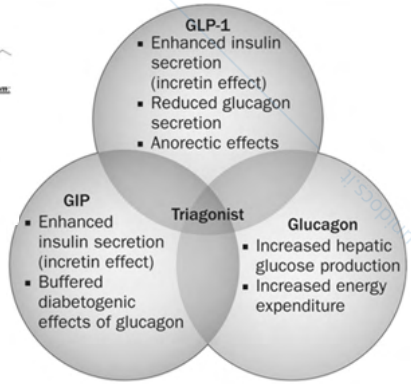
TRIAGONIST ERA

Chimera Glucagon-Glp-1-GIP

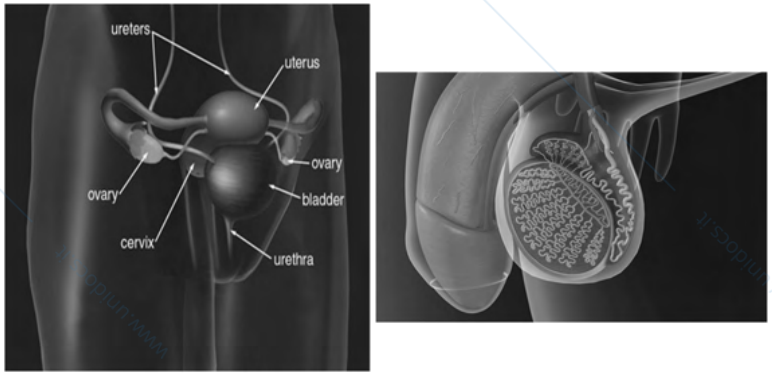


Tschoep M. et al. Cell Metab 2016

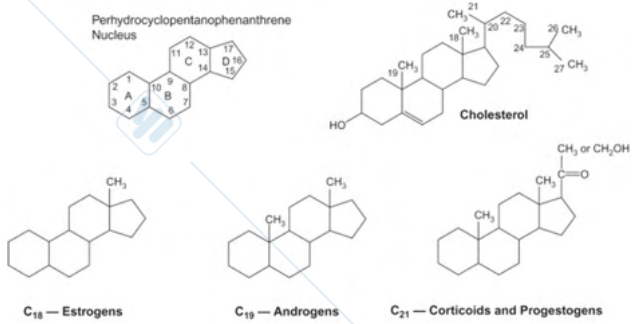


FEMALE HYPERANDROGENISM AND POLYCYSTIC OVARY SYNDROME

HUMAN REPRODUCTIVE SYSTEM



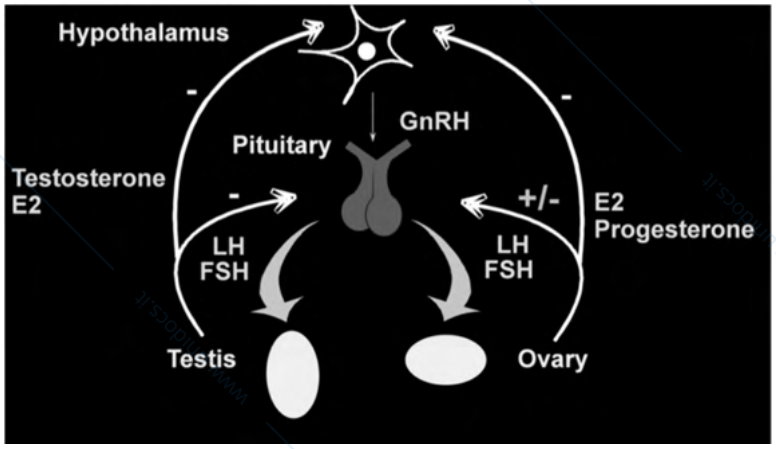
SEX STEROID HORMONES

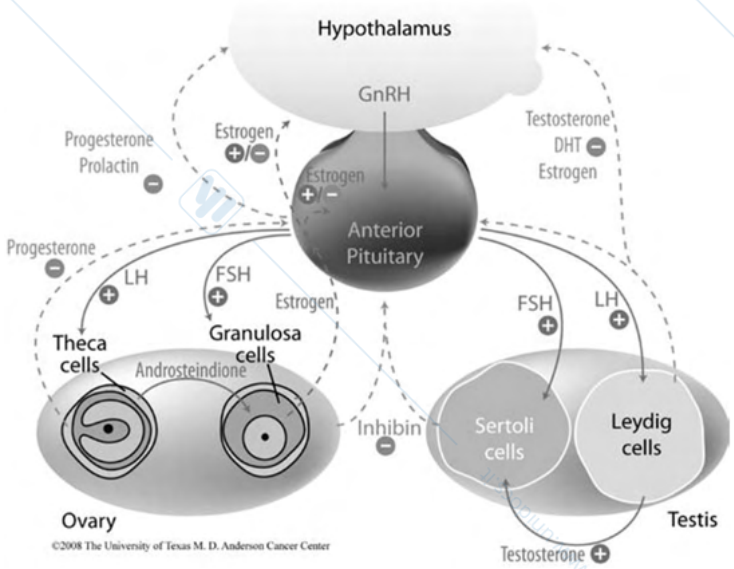


ESTRADIOL TESTOSTERONE PROGESTERONE
ESTRONE DIHYDROTESTOSTERONE

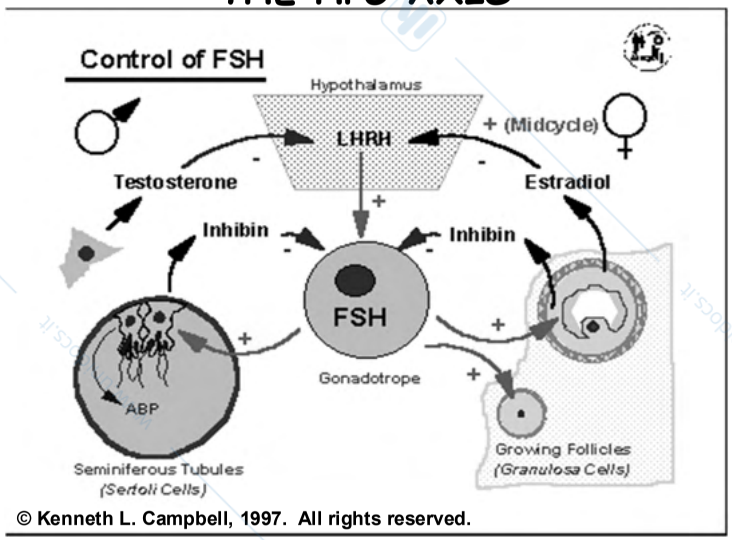
Chemical structures of cholesterol and the three major structural groups of steroid hormones. All of these have the classic steroid nucleus consisting of three six-carbon rings (perhydrophenanthrene) and another five-carbon ring (cyclopentano).

THE HYPOTALAMUS-PITUITARY-GONAD AXIS

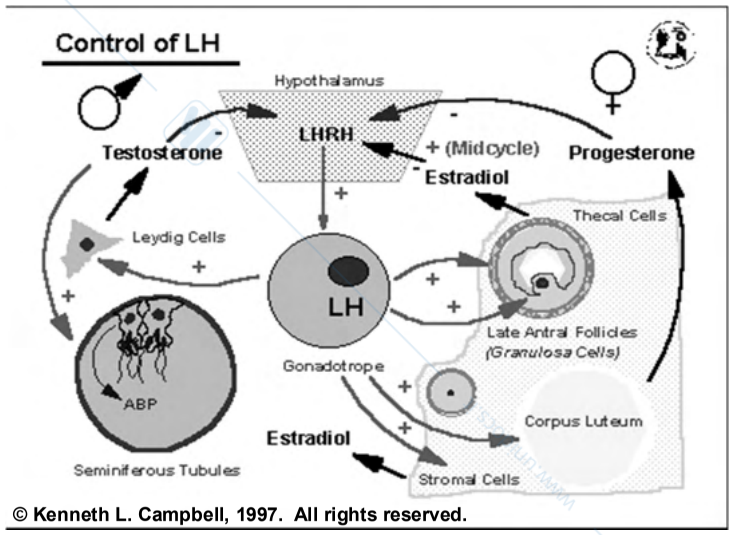




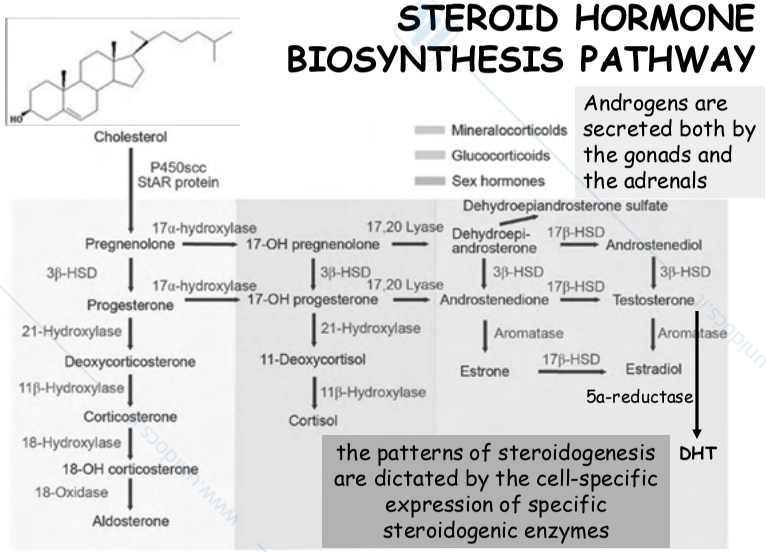
THE HPG AXIS



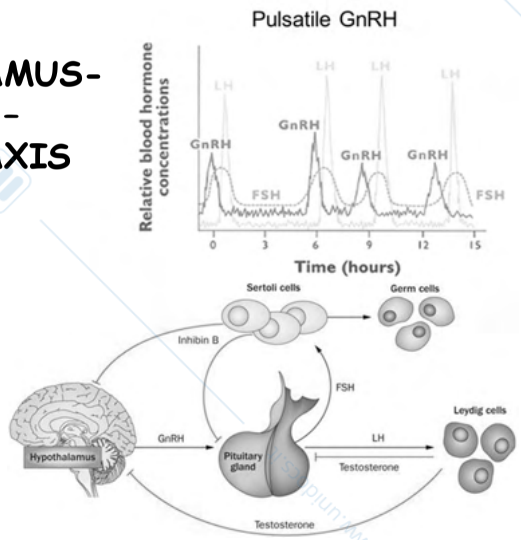
THE HPG AXIS



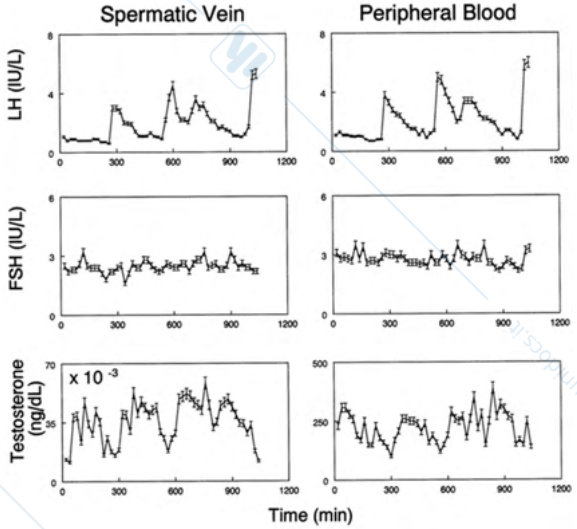
STEROID HORMONE BIOSYNTHESIS PATHWAY



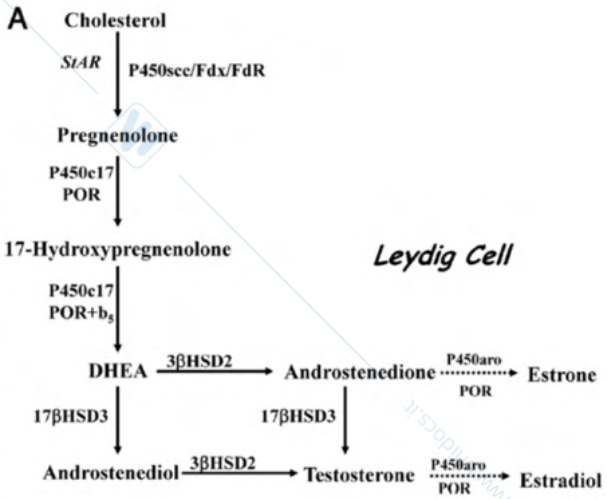
HYPOTHALAMUS-PITUITARY – GONADAL AXIS IN MEN



Illustrative profiles of serum FSH, LH, and testosterone concentrations obtained by sampling blood every 20 min for 19 h from antecubital and left spermatic veins in one young man.



Foresta et al., JCEM 1997

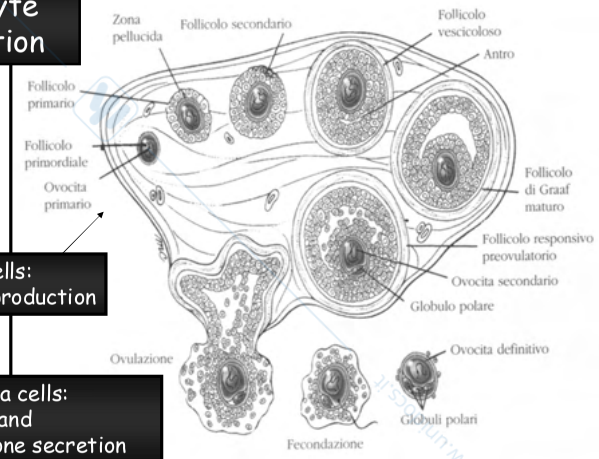


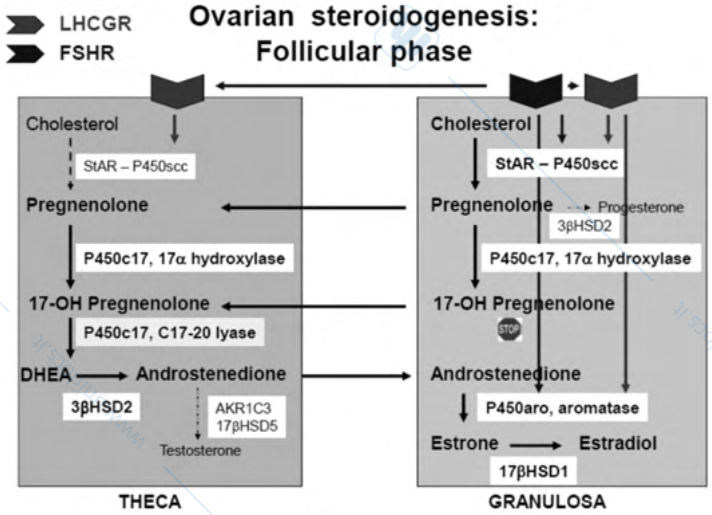
SOURCE OF CIRCULATING SEX STEROIDS IN MEN

	Testicular	Adrenal	Peripheral conversion
Testosterone	95	<1	<5
Dihydrotestosterone	20	<1	80
Estradiol	20	<1	80
Estrone	2	<1	98
DHEA-S	<10	90	,,,

THE FOLLICLE COMPLEX

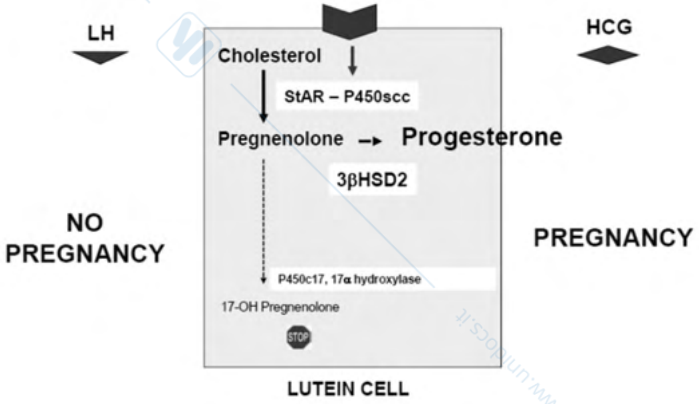
- Ovocyte maturation
- Theca cells: androgen production
- Granulosa cells: estrogens and progesterone secretion





Simoni et al., 2016

Luteal phase: granulosa ⟶ lutein.
Lutein cells are the best known target of hCG



Simoni et al., 2016

SOURCE OF CIRCULATING SEX STEROIDS IN WOMEN IN REPRODUCTIVE AGE

	Ovary	Adrenal	Peripheral conversion
Testosterone	25	25	50 (mainly from DHEA)
Androstenedione	50	50	...
Dihydrotestosterone	...	...	Up to 100 (mainly from T and A4)
Estradiol	predominant	...	Small
Estrone	predominant	...	Small
DHEA-S	2	98	...
DHEA	5	95	,,,

SOURCE OF CIRCULATING SEX STEROIDS IN WOMEN IN MENOPAUSE

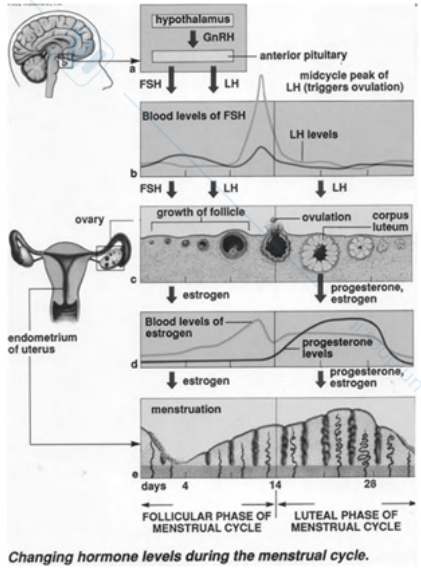
	Ovary	Adrenal	Peripheral conversion
Testosterone	25-35	small	50-75 (mainly from DHEA)
Androstenedione	60	40	...
Dihydrotestosterone	...	...	Up to 100 (mainly from T and A4)
Estradiol	Small/absent		Predominant
Estrone	Small/absent		Predominant
DHEA-S	2	98	...
DHEA	20	50	...

GONADOTROPINS

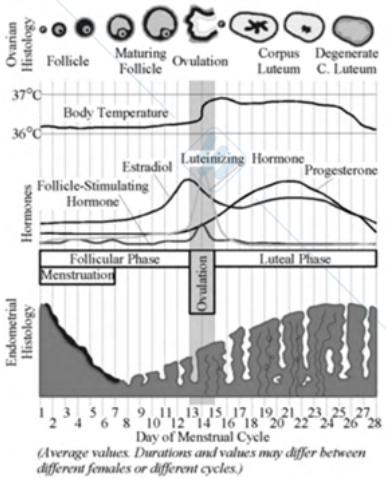
Follicle-stimulating hormone (FSH)

Luteinizing hormone (LH)

orchestrate the MENSTRUAL CYCLE



Changing hormone levels during the menstrual cycle.



(Average values. Durations and values may differ between different females or different cycles.)

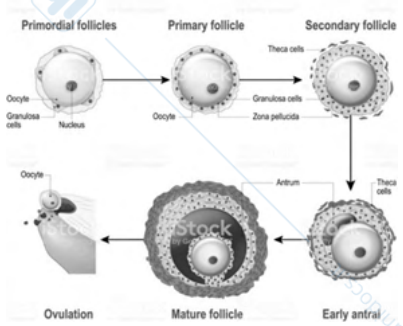
OVARIAN or OVULATORY CYCLE

The cyclical changes of the endometrium are determined by the cyclical changes in the ovarian production of hormones, determined in turn by the cyclical activity of the hypothalamus and the pituitary, starting with puberty.

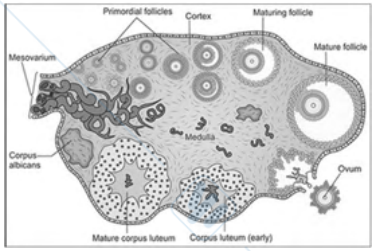
Every month, some of the follicles go through a maturation process as regulated by FSH. Only one follicle will ovulate, while the rest undergo atresia. The maturation of the follicle is completed in 14 days. This phase of the ovarian (and menstrual) cycle is called follicular or even estrogenic phase because FSH stimulates the production of estrogen. Estrogens stimulate the proliferation of the endometrium, so this phase corresponds to the proliferative phase of the uterine cycle.

During the follicular phase the primary follicle undergoes the following modifications:

THE MATURATION OF A FOLLICLE



1) granulosa cells form multiple layers (granular membrane) around the maturing oocyte (secondary follicle); a refractive membrane is formed between the granulosa cells and the oocyte, called the zona pellucida;
2) a liquid (the *liquor folliculi*) is secreted by the follicular cells which determines the formation of the follicular cavity (antrum);
3) the vesicular follicle now consists of a liquid-filled vesicle whose wall (the granular membrane) has a thickening at one pole, called the *cumulus oophorus*, which contains the growing oocyte;
4) the interstitial cells form layers around the granulose membrane, called thecae, which then stratifies in internal and external. The mature follicle is composed by the mature oocyte, antrus and granulosa cells and theca cells. The rupture of the follicle marks the end of the follicular phase. Granulosa and techa cells will transform into the corpus luteum.

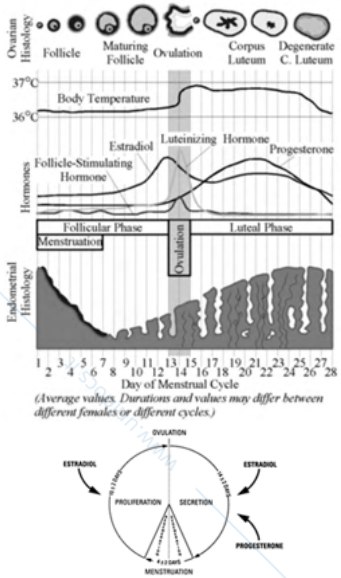


Oocyte rupture defines ovulation, preceded by an increase in pituitary LH (23 hours before ovulation). In menstrual cycles of 28 days, ovulation occurs on the 14th. If the cycle is longer, ovulation occurs later. After ovulation, the secretory phase (also called lutein or progestin phase) of the uterine cycle begins.

LH controls the uterine secretory phase by stimulating and sustaining the development of the corpus luteum. The corpus luteum prepares the uterine mucosa for implantation of the fertilized oocyte by secreting progesterone, which stimulates the secretion of the endometrium glands. When progesterone level in blood reaches a certain concentration, it blocks the production of LH (both directly and by blocking GnRH). Without LH, the corpus luteum begins to degenerate.

If the oocyte is fertilized, the chorial cells produce the human Chorionic Gonadotropin (hCG), which replaces LH in promoting the development of the corpus luteum. The corpus luteum becomes a gravidic corpus luteum, and continues its activity for 4-6 months.

If the no fertilization occurs, in absence of both LH and hCG, the corpus luteum degenerates in 10-12 days. The consequent progesterone reduction causes the degeneration of the endometrium until the menstrual bleeding occurs.



UTERINE CYCLE

Day 1 of the menstrual cycle: bleeding begins.

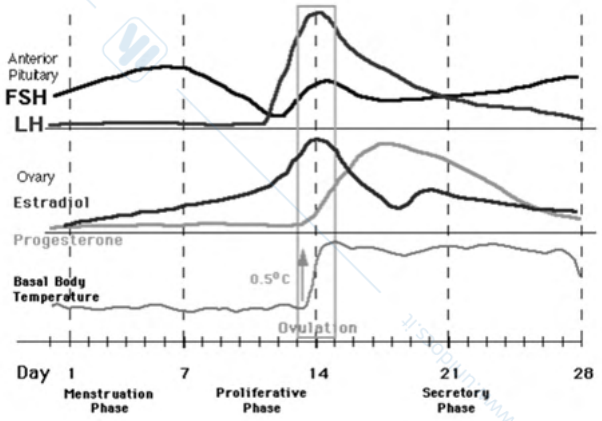
The uterine mucosa called ENDOMETRIUM undergoes 3 main phases:

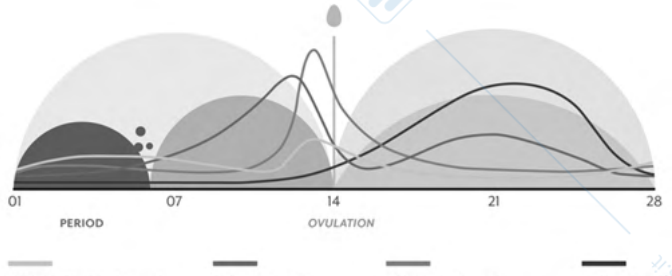
1) Menstrual phase: bleeding, 4-5 days;

2) Proliferative phase: estrogen-stimulated proliferation of endometrium cells and glands, increasing thickness. Corresponds to the estrogenic/follicular phase of the ovary. 10-11 days;

3) Secretive phase: progesterone-stimulated secretive activity of the endometrial glands; 14 days. If no fertilization of the ovocyte occurs, corpus luteum degenerates and stops progesterone production. The reduction of progesterone circulating levels causes endometrium degeneration, followed by its detachment and by a new menstrual bleeding.

Hormonal Control of the Menstrual Cycle





CYCLE	PRE-OVULATION		OVULATION	POST-OVULATION
Ovarian cycle	*FOLLICULAR PHASE*			*LUTEAL PHASE*
Uterine cycle	PERIOD	PROLIFERATIVE		SECRETORY

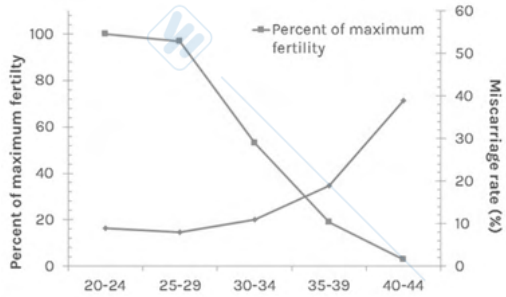
FEMALE INFERTILITY

Italian women have children later than almost all other European women. On average, Italian women have their first child at 30 y, one year later than the European average, and have less children than the other Europeans (1.22 against 1.44).

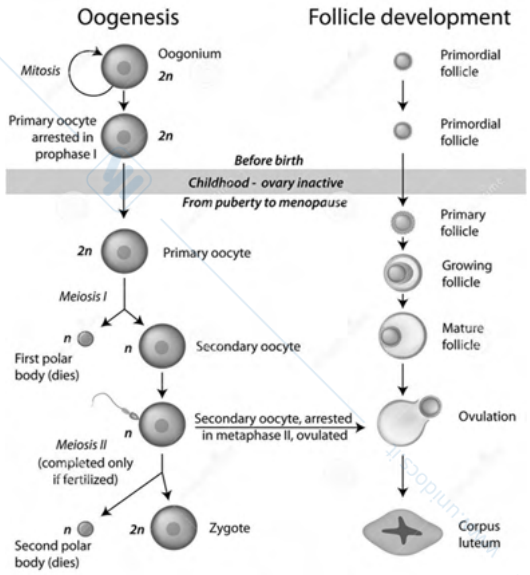
The reasons:
reasonable economic stability,
adequate family organization,
independent choice and no longer a social obligation.

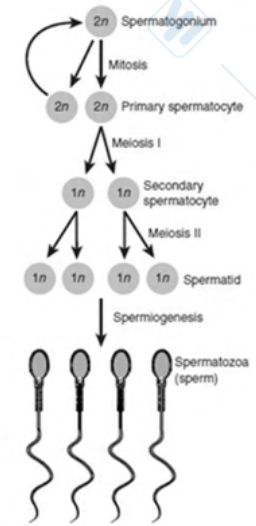
The profound cultural modification of the last fifty years has changed the reasons for filiation, which now is the result of a choice and of individual and couple responsibility as well. This cultural change is allowing women to achieve a more incisive role in the working environment. However, this has a cost in terms of time.
Therefore, unfortunately, when you think you can finally have a child, this is too often too late.

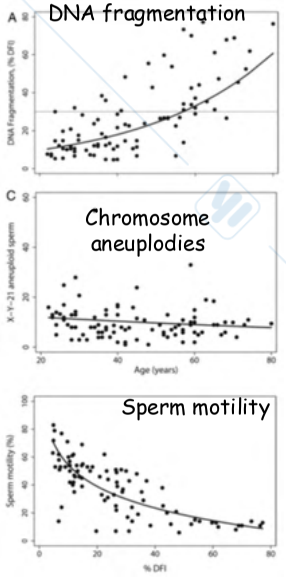
The most fertile period for a woman is between age 20 and 25 yo. Fertility is still good until age 35y, undergoing a remarkable drop at age 35-40y, and becoming very poor over age 40y.



Female gametes age is the same as the woman age: these are all already present in the female fetus, and get into the maturation process anytime during the reproductive age range.
Ageing gametes decrease oocyte quality, thereby increasing the risk of miscarriage, infertility and infertility-related diseases.
Other common diseases that become more frequent with ageing include pelvic inflammatory diseases, tubal pathologies, the development of uterine fibroids, endometriosis.







Ageing men display a worse ejaculate in both qualitative and quantitative terms.

The spermatozoa are less in number, they have poor motility and chromosomal anomalies are more frequent.

An oocyte fertilized by a non-normal spermatozoon may result in a miscarriage or the fetus might be a carrying a genetic diseases.

Aging of the oocytes is a major sterility risk factor. The oocytes of older women display a higher rate of genetic (chromosomal) anomalies and, if fertilized, can generate malformed embryos, often spontaneously aborted.

The incidence of children with Down Syndrome, for example, is:

1 in 2000 in women 20 years old,

1 in 900 in 30 year old women,

1 in 350 in 35 year old women,

1 in 110 in 40 year old women,

1 in 25 in 46 year old women.

Natural selection eliminates most of the embryos with malformations and this explains the high rate of spontaneous abortion in older women:
abortion is 18% for women between age 30 and 39 y,
34% for women around age 40 y,
vs 10% of women under age 30 y.

The factors that reduce fertility are, therefore, both quantitative and qualitative: there are fewer oocytes and are of worse quality.

Over time - especially around 38 years - few oocytes remain in the follicles, which, as seen, in a significant percentage of cases are not able to give rise to a normal embryo. Unfortunately, resorting to medically assisted reproduction in these situations is of little use.

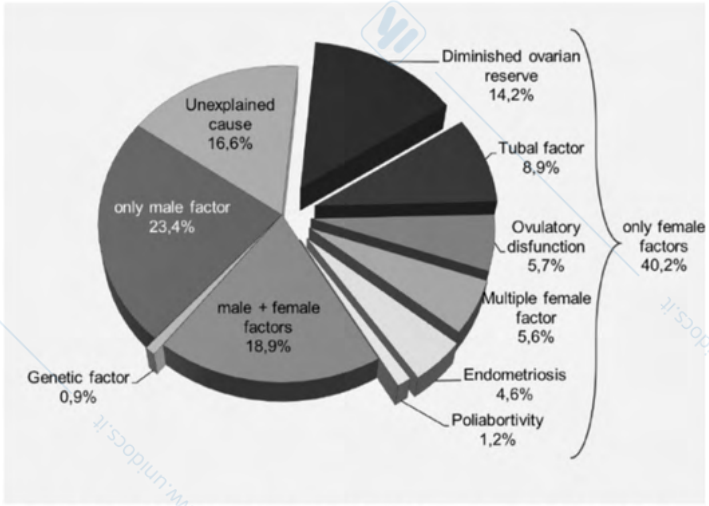


Figure 7. Causes of infertility of couples in ART treatment using fresh cycle, 2017

Studies have shown that even in medically assisted reproduction, the age of the woman greatly influences the chances of success: older women undergoing ovarian stimulation produce fewer oocytes and with chromosomal and/or genetic alterations.

Unfortunately, however, Italian women resort to medically assisted reproduction techniques later and later: data from 2007 show that the average age has even increased over the years, from 35.4 y in 2005 to 36.0 y in 2007.

According to ISS data from 2017, medically assisted reproduction procedures started in women under age 34 y resulted in 26 – 42% pregnancies obtained, while, in women aged ≥43 years or more, 7 – 10% pregnancies were obtained depending on the technique used.

Figure 11 shows pregnancy rate per fresh cycle and cumulative pregnancy rate by women age groups. Overall, embryo and oocyte cryopreservation increased by 57% the chances of achieving a pregnancy per aspiration. It should be emphasized that as we cannot take into account the weight of the "freeze-all" policy in every age group of female patients (data not collected by the Registry), so the pregnancy rates per aspiration for each age groups could be underestimated.

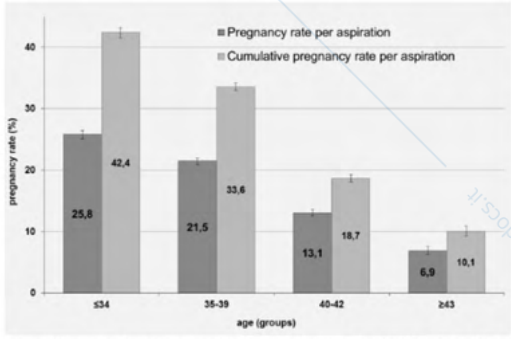


Figure 11. Pregnancy rates per aspiration and cumulative pregnancy rates per aspiration, by female patients age groups, 2017

AVERAGE AGE OF WOMEN RESORTING TO MEDICALLY ASSISTED REPRODUCTION PROTOCOLS

35.3 y in 2005
36.7 y in 2017

Figure 16 shows the distribution of fresh cycles by women age groups, from 2005 to 2017. For women older than 40 the percentage of fresh cycles performed increased from 20,7% in 2005 to 34,3% in 2017, whilst the percentage of fresh cycles performed in women ≤34 years old decreased from 39,3% in 2005 to 27,8% in 2017. Overall, the mean age of women who had fresh cycles increase over time from 35,3 to 36,7 years.

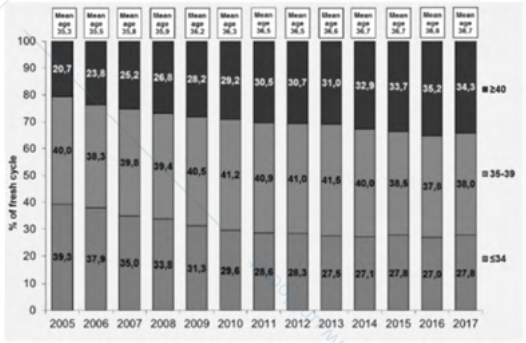


Figure 16. Time-trends of fresh initiated cycles distributions by age classes of female patients, 2005-2017

At variance, ageing impact on uterus function is much less important. Nonetheless, statistical data show a correlation between the age of the uterus and the increase in the percentage of miscarriages, chromosomal abnormalities of embryos, of cases of *placenta previa*, of labor complications, of uterine pathologies such as polyps in the endometrium and uterine fibroids.

An increase in sclerotic lesions in the uterine arteries is also reported, which despite not having a direct impact on fertility, are related to obstetric complications such as placenta abruption, cesarean section delivery, fetus malposition, etc…

FEMALE HYPERANDROGENISM

Causes of hyperandrogenism:
- Polycystic ovary syndrome (PCOS) 70%
- adrenal hyperplasia,
- Cushing's disease,
- certain types of cancers,
- certain medications

PCOS represents 80% of all anovulatory infertility cases

PCOS is the most common endocrine abnormality in women in reproductive-age

HYPERANDROGENISM

BIOCHEMICAL

TESTOSTERONE and other androgens

CLINICAL

HIRSUTISM
Alopecia

LACK OF OVERLAP

Only 50% of hyperandrogenemic women have clinical symptoms

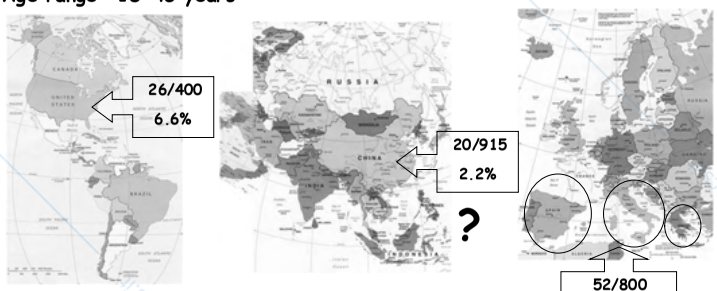
CAUSES OF HYPERANDROGENISM

Adrenal	- Congenital Adrenal Hyperplasia (21-hydroxylase deficit, 11-hydroxylase deficit, 3βHydroxysteroid dehydrogenase deficit) - Cushing's Syndrome - Neoplasms
Ovary	- PolyCystic Ovary Syndrome - Neoplasms - Hyperthecosis
Idiopathic	Idiopathic hirsutism
Genetic	Gonadal dysgenesis
Other	Acromegaly, hypothyroidism, obesity, hyperprolactin, menopause, psychiatric syndromes, drugs

PREVALENCE OF PCOS IN UNSELECTED REPRODUCTIVE-AGED WOMEN

Diagnostic criteria= NIH 1990

Age range= 18-45 years



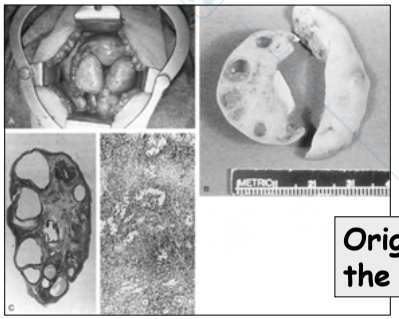
Azziz R et al.
J Clin Endocrinol Metab 2004

Chen X et al.
J Obstet Gynecol 2008

Diamanti-Kandarakis E 1999

PCOS is the most common endocrine abnormality of reproductive-aged women. Up to 10% in some studies.

Irving F. Stein, Sr., and Michael L. Leventhal



Stein IF, Leventhal ML. Amenorrhea associated with bilateral polycystic ovaries. Am J Obstet Gynecol 1935; 29:181.

.

Original descrition of the ovarian morphology

PCOS

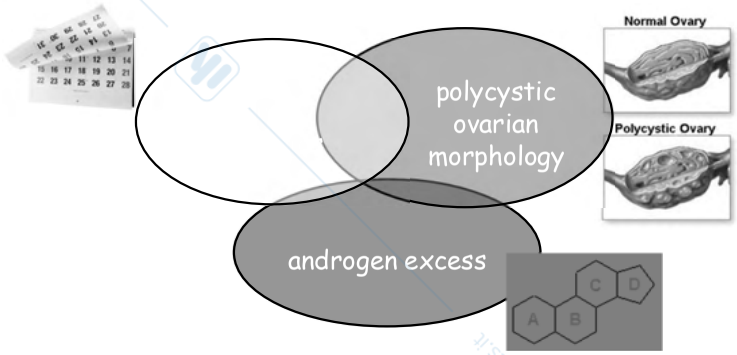
Diagnostic suspicion

Signs of androgen excess (hirsutism, androgenic alopecia)

Menstrual and ovulation disturbances

Unwanted infertility

POLYCYSTIC OVARY SYNDROME



Infertility
Metabolic Syndrome and Obesity
Type II Diabetes

PCOS DIAGNOSIS

Diagnosis of PCOS is set when all other causes of hyperandrogenism are excluded.

PCOS is a syndrome with uncertain etiology.

Diagnosis is based on well defined criteria and paradigms (Consensus statements, guidelines).

Multiple and variegated phenotypes exist which share part of the features.

PCOS: Diagnostic criteria revised

US National Institute of Health (NIH) criteria, 1990	Rotterdam criteria, 2003	Androgen Excess and PCOS Society (AE-PCOS) criteria, 2006
PCOS is diagnosed if both following criteria included*: • Oligo-anovulation • Clinical and/or biochemical signs of hyperandrogenism	PCOS is diagnosed if two out of following three criteria included*: • Oligo-anovulation • Clinical and/or biochemical signs of hyperandrogenism • Presence of polycystic ovaries	PCOS is diagnosed if all of the following criteria included*: • Clinical and/or biochemical signs of hyperandrogenism • Ovarian dysfunction, including oligo-anovulation and/or presence of polycystic ovaries)

All: plus exclusion of other etiologies (CAH, androgen-secreting tumors, Cushing's syndrome, etc.)

PCOS DIAGNOSIS: EXCLUDING OTHER CAUSES OF HYPERANDROGENISM

Cushing's syndrome can be excluded by normal cortisol suppression after an overnight 1-mg DST.

CCAH or NCCAH can be excluded by normal fasting and stimulated (250 mg Synacthen iv) 17-OHP concentrations.

Adrenal/ovarian tumors/hyperthecosis can be suspected by inappropriately (super-) high androgen and imaging techniques.

Severe insulin resistant states have very high insulin fasting/OGTT and often by the phenotype (lipodistrophy, syndromic obesity, etc…).

OVARIAN DYSFUNCTION IN PCOS WOMEN

Irregularities of the menstrual cycle:
- mild-moderate oligomenorrhea (cycle >35 gg)
- severe oligomenorrhea (cycle >60 gg)
- amenorrhea (absence of mestrual bleeding ≥6 months)

Ovulation disorders:
- chronic or cyclic anovulation (> LH)
- polymenorrhea/oligomenorrhea
- early pubarca

In adolescents, irregularities in menstrual cycle and/or ovulation are frequent and often transitory.
In some cases, such alterations express a susceptibility for developing PCOS, especially when associated with excess weight.

Prevalence of menstrual dysfunction in PCOS

TABLE 2
Prevalence of menstrual dysfunction in the polycystic ovary syndrome (PCOS).

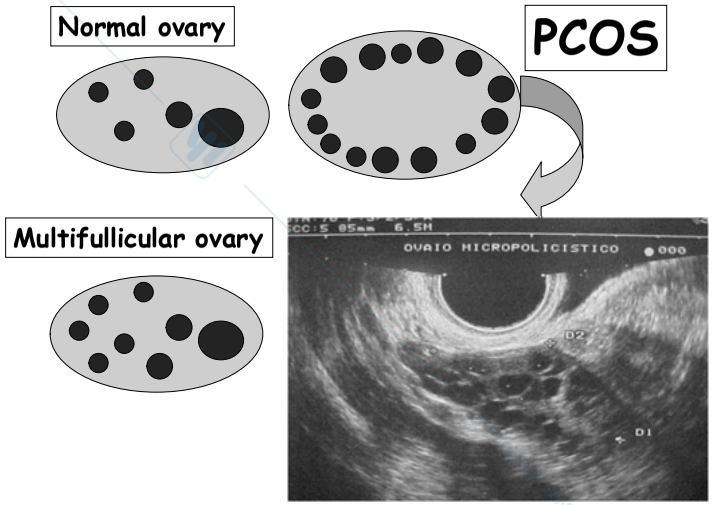
Study	Reference	Total No. PCOS	No. of PCOS patients with oligo-amenorrhea	% of PCOS patients with oligo-amenorrhea	No. of PCOS patients with eumenorrhea	% of PCOS patients with eumenorrhea
Ferriman & Purdie, 1983	83	280	237	84.60%	43	15.40%
Conway et al., 1989	84	556	395	71.00%	139	25.00%
Kiddy et al., 1990	85	263	203	77.20%	60	22.80%
Ardaens et al., 1991	65	144	105	72.90%	39	27.10%
Rajkhowa et al., 1995	86	153	129	84.30%		
Balen et al., 1995	87	1741	1043	59.90%	517	29.70%
Falsetti & Eleftheriou, 1996	88	240	207	86.30%	24	10.00%
Khoury et al., 1996	89	112	112	100.00%	0	0.00%
Talbott et al., 1998	90	244	229	93.90%	15	6.10%
Carmina et al., 1998	91	332	290	87.30%	42	12.70%
Alborzi et al., 2001	92	371	371	100.00%	0	0.00%
Williamson et al., 2001	93	162	144	88.90%		
Haddad et al., 2002	94	146	120	82.20%	26	17.80%
Amer et al., 2002	95	161	149	92.50%	12	7.50%
Glueck et al., 2003	96	138	138	100.00%	0	0.00%
Orio et al., 2003	97	100	100	100.00%	0	0.00%
Chang et al., 2005	98	316	265	83.90%	51	16.10%
Hahn et al., 2005	99	200	200	100.00%	0	0.00%
Carmina et al., 2006	46	685	538	56.60%	147	15.50%
Diamanti-Kandarakis & Danidis, 2007	100	634	545	85.90%	89	14.10%
Total		6978	5520	79.11%[a]	1204	17.25%

[a] Difference in percentage between patients with oligo-amenorrhea and eumenorrhea and anovulation is composed of patients with polymenorrhea or menometrorraghia.

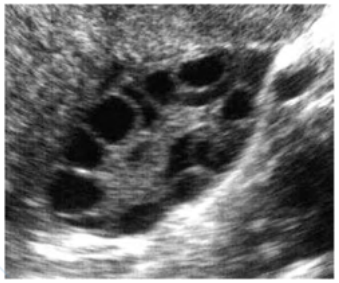
Azziz. AE-PCOS Society report on PCOS phenotype. Fertil Steril 2009.

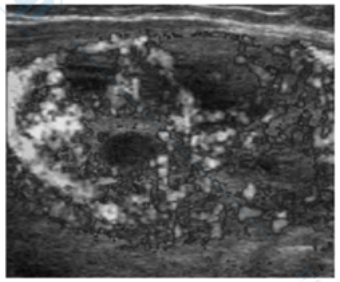
Azziz R et al. Fertility and Sterility, 2009

OVARIAN MORPHOLOGY



The PCO morphology (Rotterdam's)





Criteria providing best compromise in specificity/sensitivity for PCO definition:

(i) presence of ≥12 follicles in each ovary measuring 2–9 mm in diameter, and/or

(ii) increased ovarian volume (>10 ml).

PCO is featured by the increased stromal volume. The measurement of the ovarian volume is a good surrogate for the estimation of stromal volume.

The Rotterdam ESHRE/ASRM-Sponsored PCOS consensus workshop group. Fertil Steril. 2004, Hum Reprod. 2004.

HYPERANDROGENISM

BIOCHEMICAL

TESTOSTERONE and others

CLINICAL

HIRSUTISM
Alopecia

INDISTINGUEDLY used for PCOS DIAGNOSIS
…but…LACK OF OVERLAP
Only 50% of hyperandrogenemic women have clinical symptoms

HYPERANDROGENISM definition

Clinical / subclinical condition characterized by excessive production or action of androgens of adrenal/ovarian origin.

CLINICAL hyperandrogenism

HIRSUTISM

ANDROGENIC ALOPECIA

HIRSUTISM

Hirsutism results by the interaction between androgens availability and activity at the skin level.

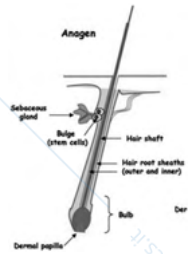
Female condition of androgen-dependent transformation of the vellus hairs into terminal hairs, interesting areas where usually women don't show terminal hairs and corresponding to the distribution pattern of male secondary sexual characters, primarily the face, chest, lower abdomen, inner thighs and back

Terminal hairs differ from vellus hairs for lenght (> 0.5cm), thickness and for pigmentation

HIRSUTISM

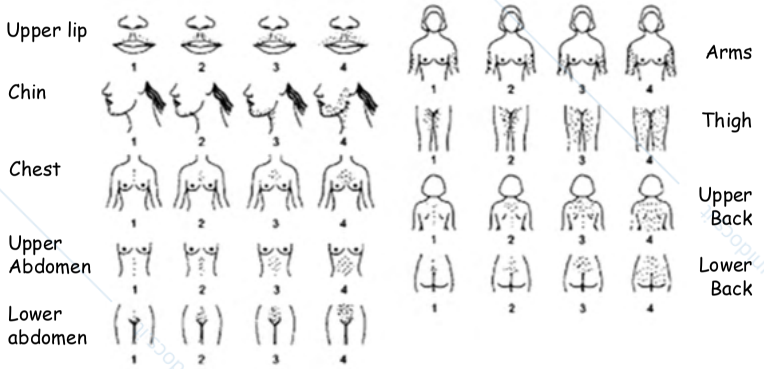
Excessive terminal hair in androgen-dependent areas

Chronic disorder
Affect **Psychological** well being
Impaired **metabolic** profile
Life threatening **tumor**



MULTIFACTORIAL ETIOLOGY
Androgen / proandrogen excess in the bloodstream
Local synthesis at the ***pilosebaceous unit***
Androgen Receptor polymorphism – hyper-responsiveness

Hirsutism quantification: the modified Ferriman Gallwey score

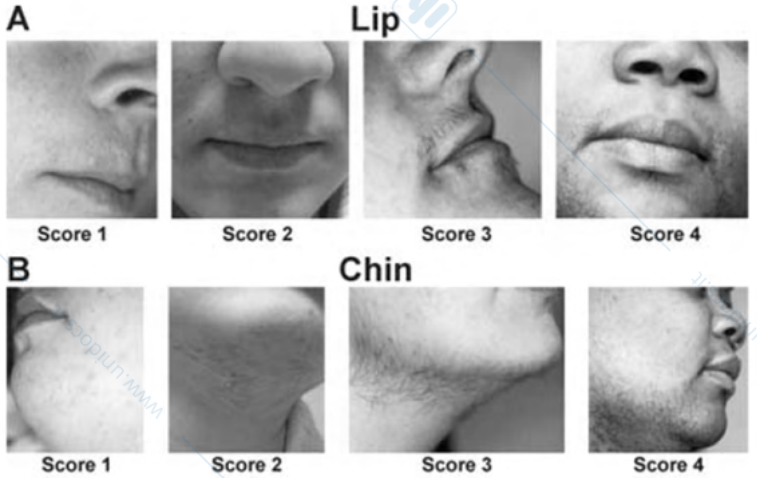


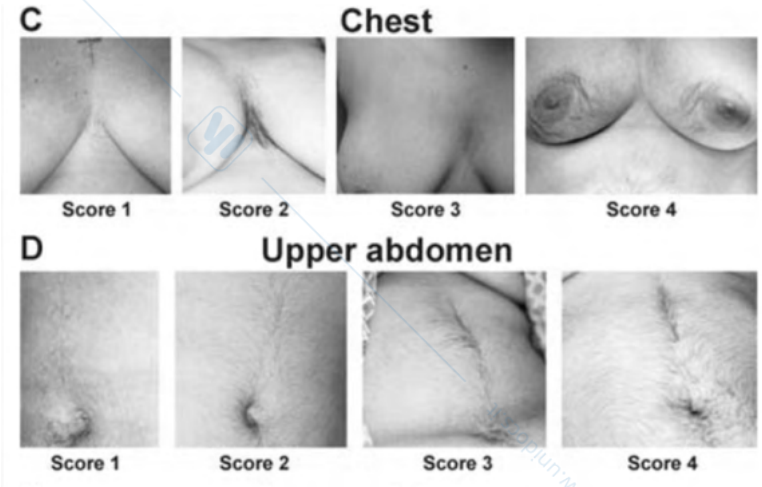
The modified FG score does not take into account the forearm and the leg because these are highly sensitive to very low amounts of androgens even in healthy women, hence being not specific.

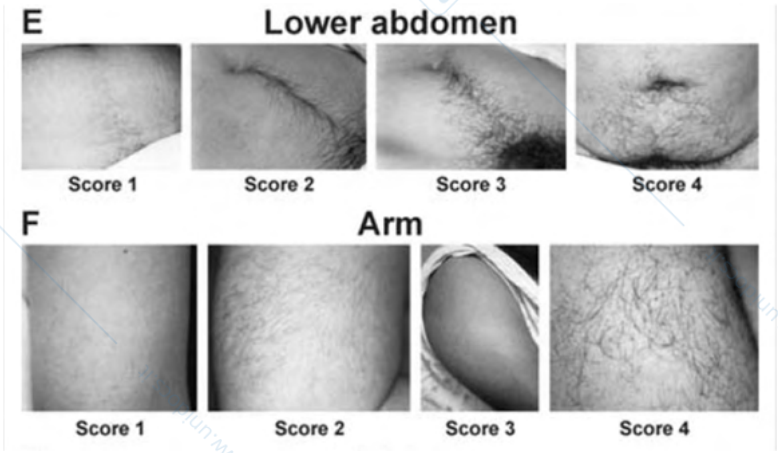
Hirsutism quantification: the modified Ferriman Gallwey score

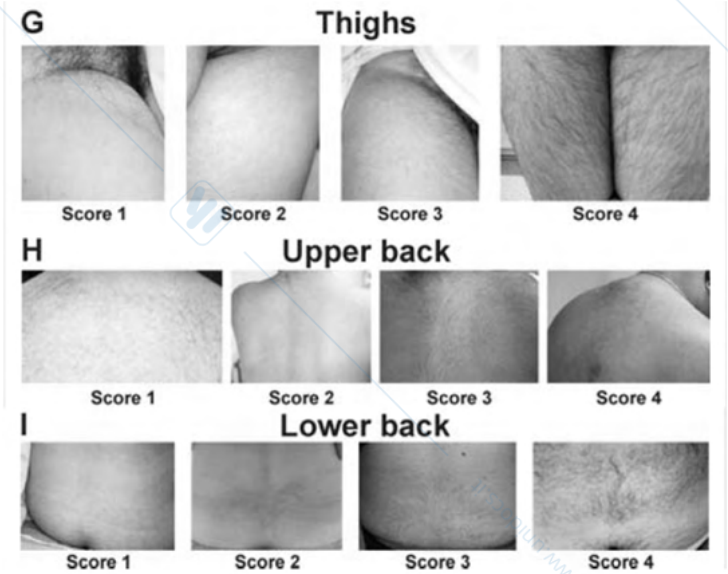
Nine androgen-sensitive areas;
Range mFG score: 0-36;
No hirsutism: <8;
Mild Hirsutism, mFG 8-15;
Moderate Hirsutism, mFG: 16-25;
Severe Hirsutism: >25

Requirements:
No laser depilation or similar in the previous 3 months;
No depilation at all in the previous 4 weeks;
No shaving in the previous 5 days.









mFG score for hirsutism diagnosis: the cut off issue

The cut-off set at 8 was defined by an English study (Hatch et al. 1981) in which only the 4.3% of the female popuation in reproductive age displayed a score above the 8 threshold.

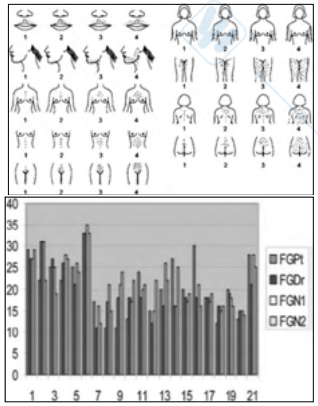
Author, year	Year	Country	Race	Ethnicity	Sample size	Suggested mFG cut-off*
Tellez and Frenkel (1995)	1995	Chile	White	Hispanic	236	≥6
Asuncion et al. (2000)	2000	Spain	White	Mediterranean	154	≥8
Sagsoz et al. (2004)	2004	Turkey	White	Middle Eastern	204	≥9
Cheewadhananaks et al. (2004)	2004	Thailand	Asian	Thai and Chinese	531	≥3
DeUgarte et al. (2006)	2006	USA	White	Caucasian and Hispanic	283	≥8
			Black	African-American	350	≥8
Zhao et al. (2007)	2007	China	Asian	Chinese Han	623	≥2
Api et al. (2009)	2009	Turkey	White	Middle Eastern	121	≥11
Moran et al. (2010)	2010	Mexico	White	Hispanic	150	≥10
Noorbala and Kefaie (2010)	2010	Iran	White	Middle Eastern	900	≥10
Kim et al. (2011)	2011	Korea	Asian	Chinese	1010	≥6
Gambineri (2011, personal communication)	2011	Italy	White	Mediterranean	200	≥9
Escobar-Morreale (2011, personal communication)	2011	Spain	White	Mediterranean	291	≥10

*As defined by the 95th percentile of an unselected population of premenopausal women.

Differences according to ethnicity

mFG score for hirsutism diagnosis: the operator issue

Is the Ferriman and Gallwey score an Objective Method?
Limitations of the VISUAL score system



Wild et al., JCEM 2005, 90:4112-4114

High inter-operator variation.

Inability to detect small variation.

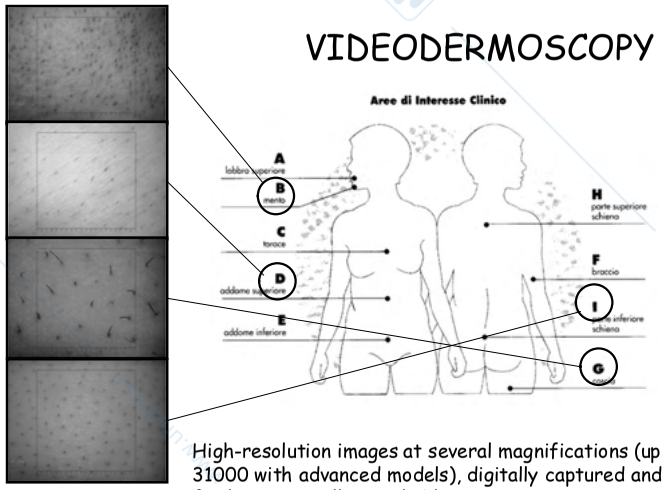
Validated to estimate the total amount of body hair and not the regional distribution.

While the simplicity of this method encourages its use in clinical practice, the visual scoring of hirsutism is not indicated for the assessment of the response to therapy in hirsute women during a randomized control trial.

Comparison of blinded simultaneous FGs estimation by patients (FGPt), physician (FGDr), and research nurses (FGN1 and FGN2). The blinded simultaneously obtained FG scores contrast patient's, physician's, and the two research nurses' scores.

NON INVASIVE OBJECTIVE METHODS

VIDEODERMOSCOPY



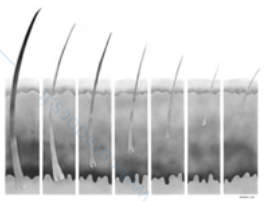
High-resolution images at several magnifications (up to 31000 with advanced models), digitally captured and stored for later use. Allows reliable comparisons over time.

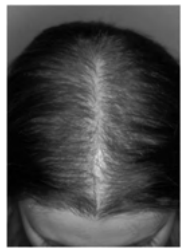
NON INVASIVE OBJECTIVE METHODS
Trichoscan

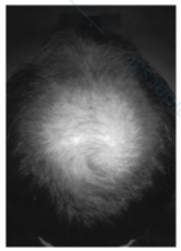
Feature	TrichoScan®
automatic Analysis	yes
analysed area	up to 2 cm²
Hair dye necessary	yes
Hair count	yes
Hair lenght / Hair growth rate	yes
Hair thickness	yes
Terminal hair count	yes
Vellus hair count	yes
Anagen/Telogen ratio	yes
Follicular units	on request
Hide results	yes
Export of results	yes
Artefact correction	yes
Email support	yes
In house training	on request
Camera / Optics	Canon PowerShot with swiss made optics

ALOPECIA DEFINITION

Progressive non-scarring miniaturization
of hair follicles in defined areas of the head,
in males and females genetically predisposed.

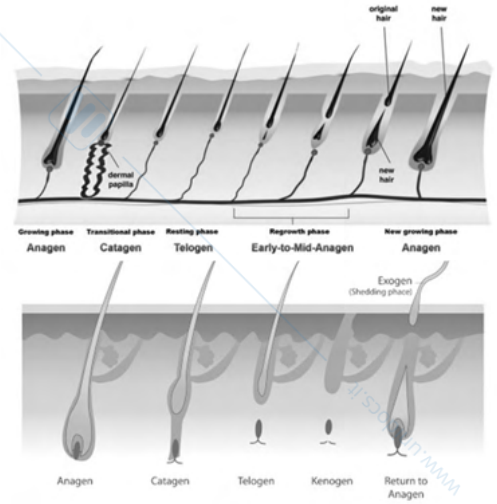






Blume-Peytavi et al. Br J Dermatol 2011, 164: 5-15.

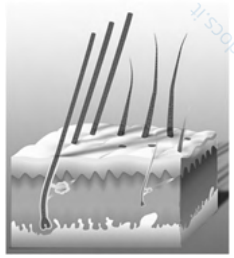
HAIR GROWTH



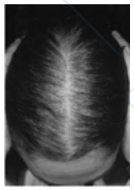
PATHOGENESIS

- Follicular miniaturization
- Extension of the kenogen phase
  (phenomenon of empty follicles)

- Thinner, shorter and lighter hair
- Variabile diameter
- Shorter anagen
- Increased hair loss

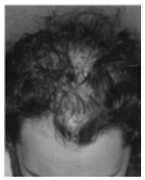


3 CLINICAL PHENOTYPES

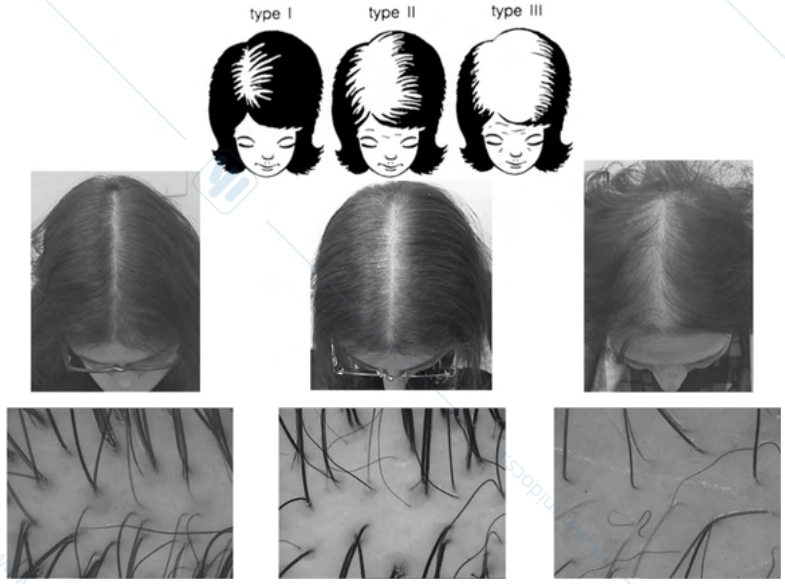


1. Widespread thinning of the "crown" region with frontotemporal hairline maintenance
   (Ludwig and Sinclair scale)

2. Thinning of the central region with front accentuation
   (Olsen scale - Christmas tree pattern)



3. Bitemporal recession
   (Hamilton-Norwood male pattern)



Ludwig scale

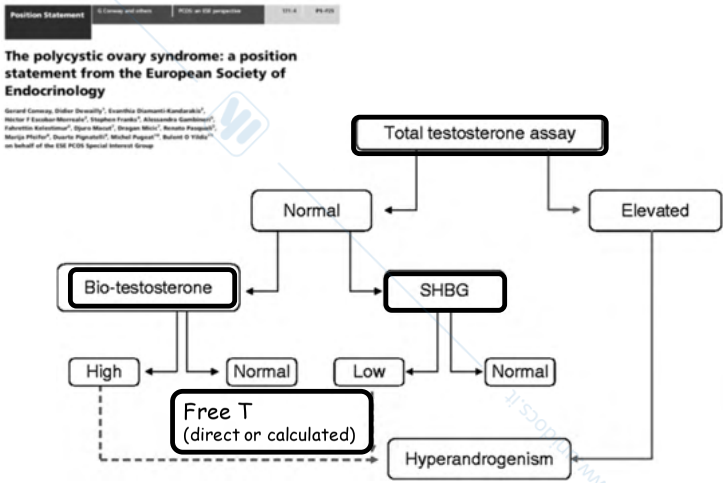
Sinclair scale

BIOCHEMICAL hyperandrogenism

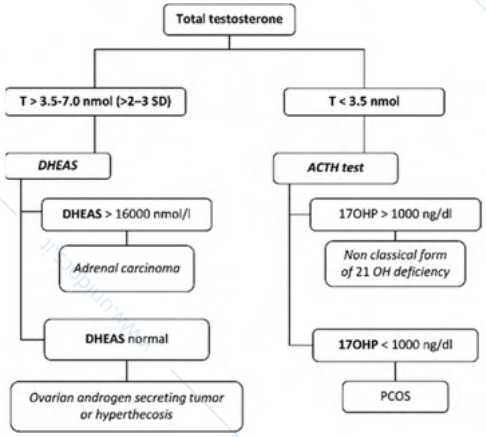
DETECTING ELEVATED ANDROGEN LEVELS

Figure 1

PARADIGM FOR IDENTIFYING HYPERANDROGENEMIA IN PCOS

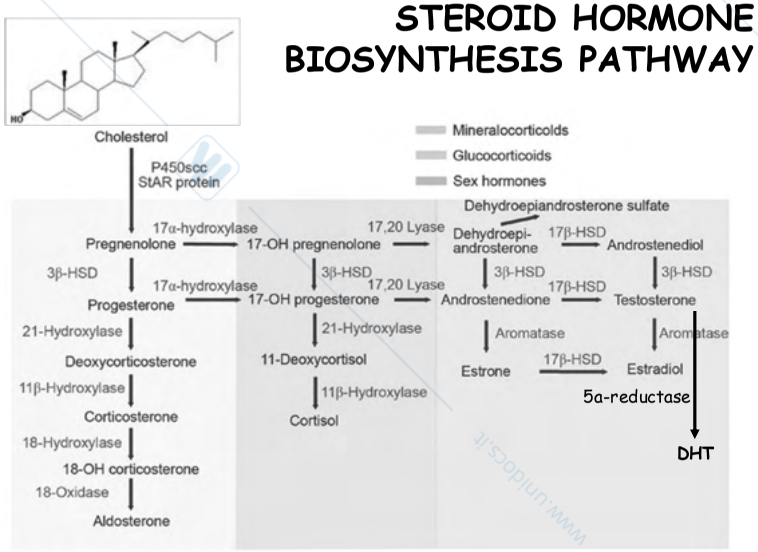


THE ROLE OF THE LABORATORY BEYOND TESTOSTERONE



- ✓ Excluding other causes
- ✓ Source of androgen excess

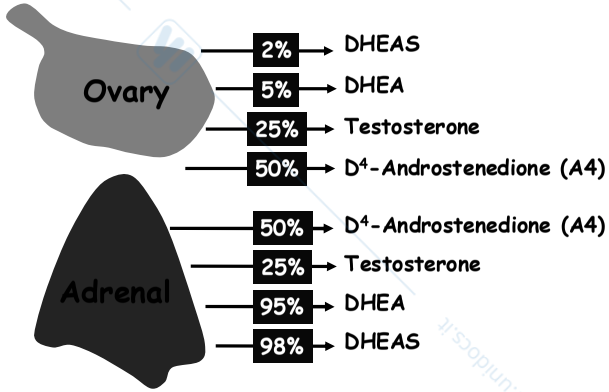
STEROID HORMONE BIOSYNTHESIS PATHWAY



SOURCE OF CIRCULATING SEX STEROIDS IN WOMEN IN REPRODUCTIVE AGE

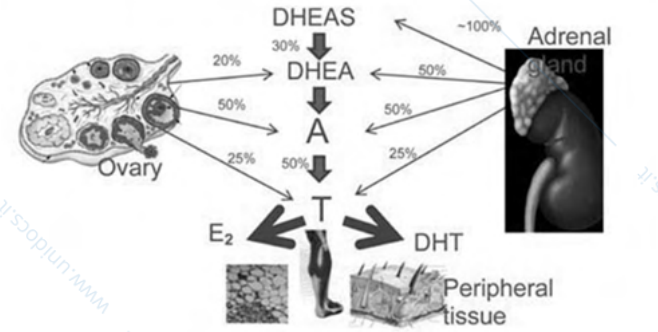
	Ovary	Adrenal	Peripheral conversion
Testosterone	25	25	50 (mainly from DHEA)
Androstenedione	50	50	…
DHT	…	…	Up to 100 (mainly from T and A4)
Estradiol	predominant		Small
Estrone	predominant		Small
DHEA-S	2	98	…
DHEA	5	95	,,,

ORIGIN OF CIRCULATING ANDROGENS IN WOMEN



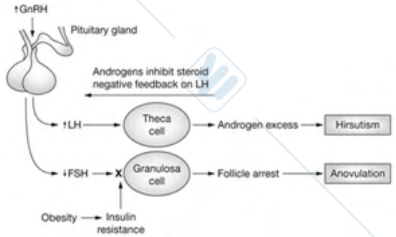
PERIPHERAL ANDROGEN METABOLISM

Figure 1 - steroidogenesis in the ovaries, adrenal glands and peripheral tissues of the principal hormones related to female sexual function



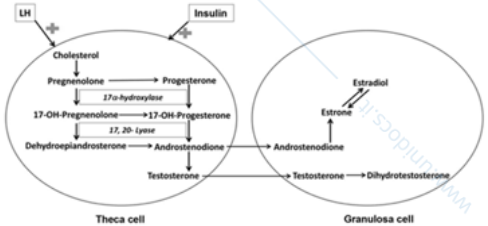
DHT and T, but not androstenedione, DHEA and DHEAS, activate the Androgen Receptor

ORIGIN OF ANDROGEN EXCESS IN WOMEN: DYSFUNCTION OF THE HYPOTHALAMUS – PITUITARY – OVARIAN AXIS IN PCOS

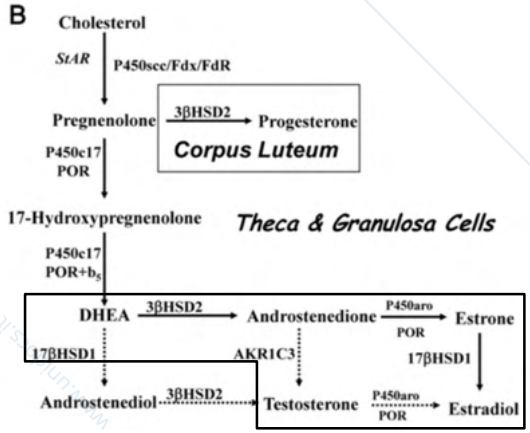


Increased pulsatile GnRH release
Imbalanced LH/FSH ratio

Increased Androgen production
Increased or normal Estrone levels

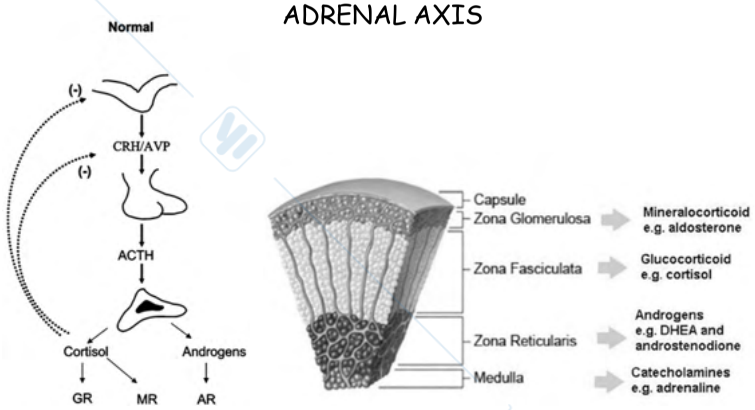


Ovarian sex steroids currently tested in clinical practice:

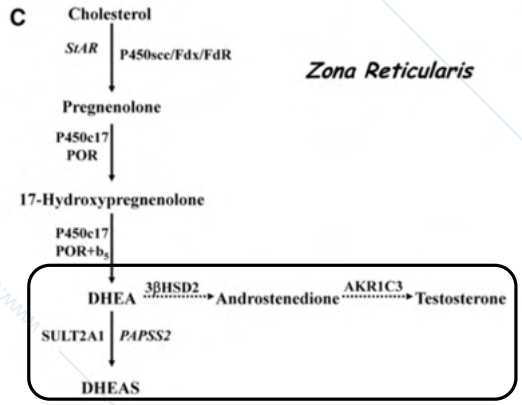


Miller and Auchus, 2011

ORIGIN OF ANDROGEN EXCESS IN WOMEN:
IMBALANCE OF THE HYPOTALAMYS – PITUITARY - ADRENAL AXIS

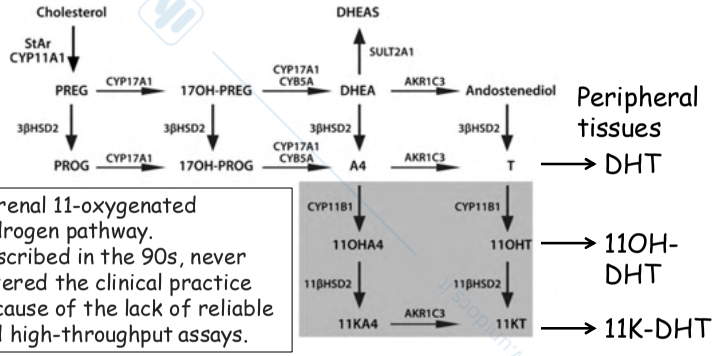


Adrenal androgens and proandrogens currently tested in clinical practice:



Miller and Auchus, 2011

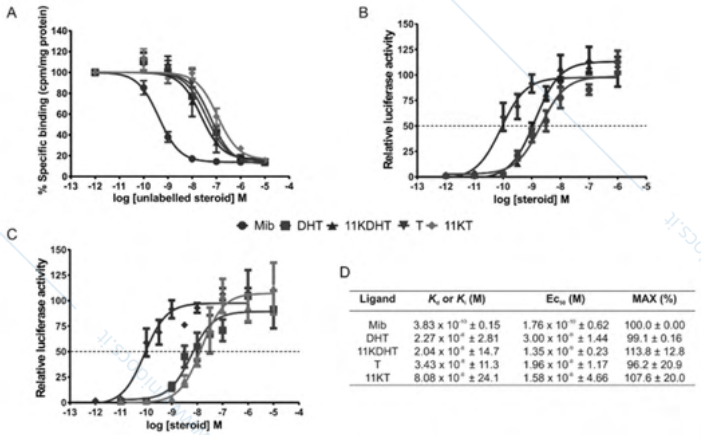
ORIGIN OF ANDROGEN EXCESS IN WOMEN:
IMBALANCE OF THE HYPOTALAMYS – PITUITARY - ADRENAL AXIS
UNCONVENTIONAL ANDROGENS



Adrenal 11-oxygenated androgen pathway. Described in the 90s, never entered the clinical practice because of the lack of reliable and high-throughput assays.

11keto-T and 11keto-DHT have similar activity on the Androgen Receptor

ANROGEN RECEPTOR AFFINITY AND ACTIVATION CAPACITY OF ENDOGENOUS ANDROGENS

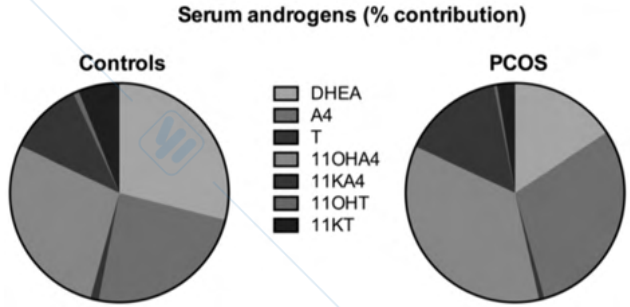


D

Ligand	K_c or K_i (M)	Ec_{50} (M)	MAX (%)
Mib	3.83 x 10^{-10} ± 0.15	1.76 x 10^{-10} ± 0.62	100.0 ± 0.00
DHT	2.27 x 10^{-9} ± 2.81	3.00 x 10^{-9} ± 1.44	99.1 ± 0.16
11KDHT	2.04 x 10^{-9} ± 14.7	1.35 x 10^{-9} ± 0.23	113.8 ± 12.8
T	3.43 x 10^{-9} ± 11.3	1.96 x 10^{-9} ± 1.17	96.2 ± 20.9
11KT	8.08 x 10^{-9} ± 24.1	1.58 x 10^{-8} ± 4.66	107.6 ± 20.0

Pretorius E et al., PLoS One. 2016;11(7):e0159867

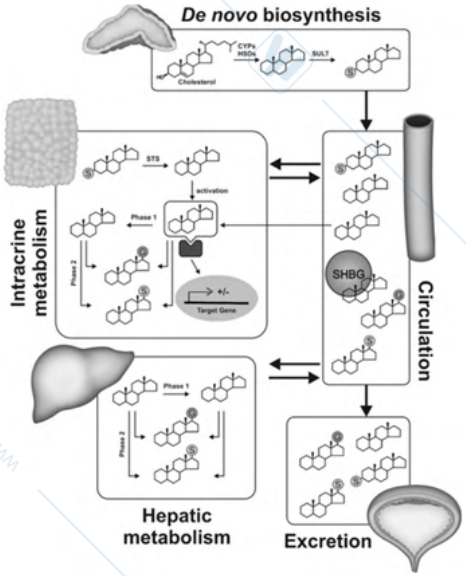
Adrenal 11-oxygenated androgens



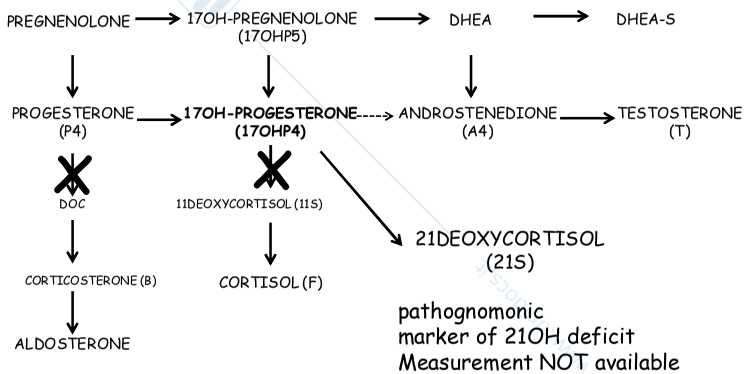
O'Reilly et al., JCEM 2017

Recent studies performed by LC-MS/MS revealed a potential major role for these forgotten adrenal androgens.

AR-inactive precursors 11OH-Androstenedione and 11K-Androstenedione are major contributors to the overall circulating androgen pool, and are increased in PCOS.



ORIGIN OF ANDROGEN EXCESS IN WOMEN:
CLASSICAL / NON CLASSICAL CONGENITAL ADRENAL HYPERPLASIA
(21Hydroxylase genetic defect)



pathognomonic
marker of 21OH deficit
Measurement NOT available

WHAT IS THE MOST SUITABLE ANDROGEN TO BE MEASURED TO ASSESS BIOCHEMICAL HYPERANDROGENISM?

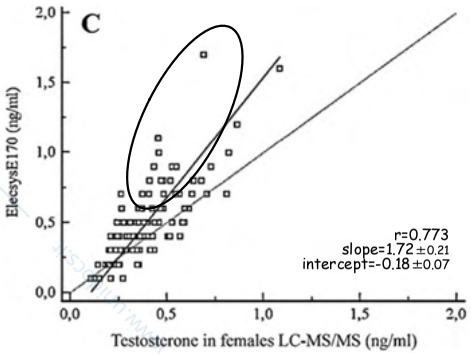
BLOOD TOTAL TESTOSTERONE IS RECOMMENDED AS THE 1° LEVEL TEST

Pros	Cons
• Very limited menstrual fluctuation. • Very high levels put the diagnosis for androgen-secreting tumors (above 1ng/dL)	• Mostly bound to SHBG • Mostly derived by peripheral metabolism of androgen precursors (DHEA, androstenedione) • Do not discriminate the source of androgen production • Do not correlate with symptoms severity (hirsutism) • Requires sensitive and specific assays

What is the most suitable method for measuring total testosterone?

- Radioimmunoassay (RIA) after sample extraction and chromatography
- Electrochemilunesce immunoassays
- Gas chromatography – mass spectrometry (GC-MS)
- Liquid chromatography – tandem mass spectrometry (LC-MS/MS)

COMPARISON OF DIFFERENT TESTOSTERONE ASSAYS



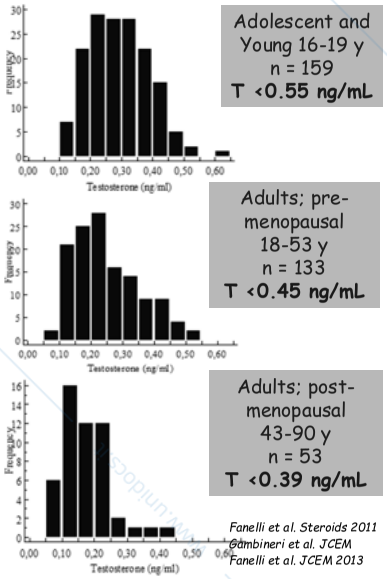
Poor correlation at female range levels

Elecsys T170: direct immunoassay used for routine testosterone measurement in the clinical laboratories

INTERPRETING THE LABORATORY RESULT

DEFINING ASSAY- AND POPULATION- SPECIFIC REFERENCE INTERVALS

TESTOSTERONE distribution in healthy normal weight and eumenorrheic women (by LC-MS/MS)



Adolescent and Young 16-19 y
n = 159
T <0.55 ng/mL

Adults; pre-menopausal
18-53 y
n = 133
T <0.45 ng/mL

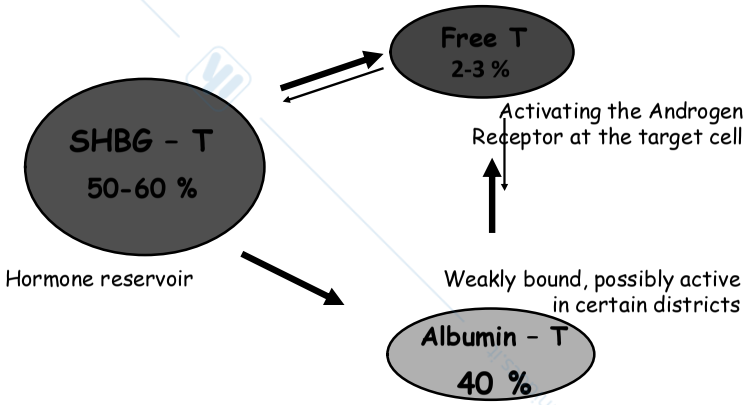
Adults; post-menopausal
43-90 y
n = 53
T <0.39 ng/mL

Fanelli et al. Steroids 2011
Gambineri et al. JCEM
Fanelli et al. JCEM 2013

CIRCULATING FRACTIONS OF STEROID HORMONES IN WOMEN

	Circulating level (nmol/L)	STEROID % DISTRIBUTION in the bloodstream			
		Free	SHBG	CBG	Albumin
Estradiol	0.29	1.8	37.3	0.1	60.8
Estrone	0.23	3.6	16.3	0.1	80.1
Progesterone	0.65	2.4	0.6	17.7	79.3
Testosterone	1.3	1.4	66	2.3	30.4
DHT	0.65	0.5	78.4	0.1	21
Androstenedione	5.4	7.5	6.6	1.4	84.5
Cortisol	400	3.8	0.2	89.7	6.3

PLASMA DISTRIBUTION OF TESTOSTERONE



SHBG FUNCTIONS

- Modulate the free and bioavailable fraction of major sex steroid hormones
- Influence sex steroid clearance
- DHT binds to SHBG with about 5 times the affinity of testosterone and about 20 times the affinity of estradiol
- Testosterone binds SHBG with twice the affinity it has for 17β-estradiol

FREE TESTOSTERONE

Is the most important biomarker of female hyperandrogenism
according to international clinical guidelines.

HOW CAN WE MEASURE FREE TESTOSTERONE LEVELS?

DIRECT MEASUREMENT is possible but not easy!

Gold Standard methods (direct equilibrium dialysis; ultrafiltration) are labour intensive and expensive.

Routine methods (direct RIA) are easy but usually provide unreliable results.

HOW CAN WE MEASURE BIOAVAILABLE TESTOSTERONE LEVELS?

Free T + Albumin-T

Measuring T in SALIVA! non SHBG-bound T fraction can diffuse across capillaries and salivary ducts. Unaffected by saliva flow rates.

High sensitivity assays are required as salivary levels represents 2-3% of circulating levels.

Currently NOT in routine.

CAN WE CALCULATE FREE TESTOSTERONE (cFT) LEVELS?

CALCULATED FREE T is a viable option, but:

various equations are available

the quality of INPUT measurements impact cFT results:

Total Testosterone, SHBG

formula models may deviate at certain T or SHBG values

cFT-Vermeulen: Total-T=[fT]+[alb-T]+[SHBG-T]

is strongly linearly correlated with measured freeT (eqD), independently from serum T, albumin and SHBG concentrations.

FREE ANDROGEN INDEX (FAI): [total testosterone] x 100 / [SHBG]

popular in routine Clinical Biochemistry laboratories because it is easy to calculate. However, the FAI has been shown to overestimate cFT when the SHBG con-centration is low.

The many different versions of formulas and methods for calculating and measuring free T have been shown to cause problems with poor inter-laboratory agreement due to the use of different methods with different reference intervals.

Agreement between cFT or FAI vs measured freeT in women and men, at different SHBG values

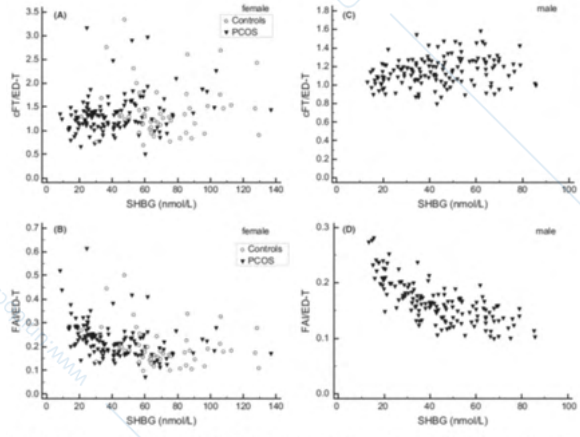


FIGURE 1 (A) Comparison of SHBG against the ratio of cFT/ED-T in women and (B) men (C) comparison of SHBG against the ratio of FAI/ED-T in women (D) and men

Keevil et al., Clinical Endocrinology. 2018;88:706 –710 .

OTHER ANDROGEN BIOMARKERS: ANDROSTENEDIONE

Pros	Cons
– High circulating levels (nM) – Low SHBG affinity – When properly measured, androstenedione is more sensitive than testosterone in detecting biochemical hyperandrogenism – Increased both in ovaric and adrenal hyperandrogenism forms	– Low availability and poor quality of routine immunoassays – Variably increased in androgen-secreting tumors

ANDROSTENEDIONE

O' Reilly et al., JCEM 2014

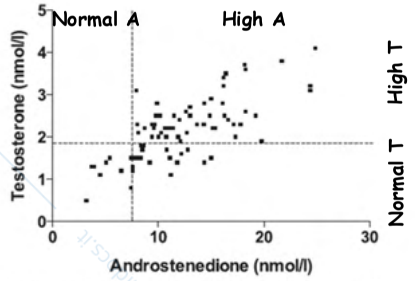


Figure 2. Relationship of serum T and A with stratification of androgenemic subgroups: NA/NT (n = 10); HA/NT (n = 20); and HA/HT (n = 56). No individuals were identified with NA/HT (n = 0).

Good correlation between serum T and A.

High A identifies a relevant number of hyperandrogenic women not detected by T measurement.

ANDROSTENEDIONE

Pasquali et al., JCEM 2016

Table 2. Distribution of Hirsutism, High T, High A, and High FAI, Alone or in Combination, According to the 3 PCOS Phenotypes

Phenotypes	Prevalence		Frequency of Combination of High A and High FAI			
	High A	High FAI	Normal A-Normal FAI	Normal A-High FAI	High A-Normal FAI	High A-High FAI
Overall (n = 156)	87 (56%)	79 (51%)	52 (33%)	17 (11%)	31 (20%)	56 (36%)
HA + OA + PCOm (n = 48)	35 (73%)	31 (65%)	8 (17%)	5 (10%)	9 (19%)	26 (54%)
No hirsutism-high T (n = 14)	13 (93%)	12 (86%)	0	1 (7%)	2 (14%)	11 (79%)
Hirsutism-normal T (n = 29)	17 (59%)	15 (52%)	8 (28%)	4 (14%)	6 (21%)	11 (38%)
Hirsutism-high T (n = 5)	5 (100%)	4 (80%)	0	0	1 (20%)	4 (80%)
HA + OA (n = 65)	38 (58%)	35 (54%)	15 (23%)	12 (18%)	15 (23%)	23 (35%)
No hirsutism-high T (n = 3)	3 (100%)	2 (67%)	0	0	1 (33%)	2 (67%)
Hirsutism-normal T (n = 55)	28 (51%)	30 (54%)	15 (27%)	12 (22%)	10 (18%)	18 (33%)
Hirsutism-high T (n = 7)	7 (100%)	3 (43%)	0	0	4 (57%)	3 (43%)
OA + PCOm (n = 43)	14 (33%)	13 (30%)	16 (37%)	13 (30%)	14 (33%)	0

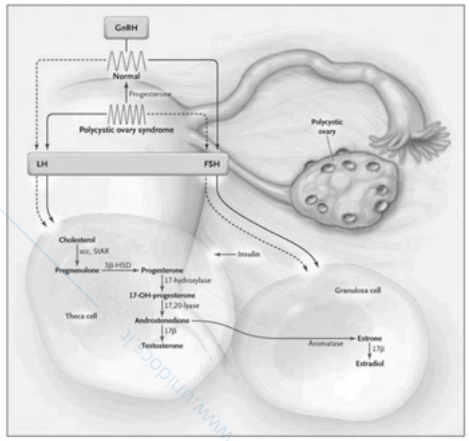
Androstenedione measurement is used in clinical practice since decades, however, its usefulness is now increased thanks to novel accurate and precise techniques (LC-MS/MS)

ROLE OF INSULIN PCOS

OTHER ANDROGEN BIOMARKERS: DEHYDROEPIANDROSTERONE-SULFATE DHEA-S

Pros	Cons
–Very high concentration (uM)	–Variably increased in Cushing and congenital adrenal hyperplasia (non classical 21-hydroxylase deficits)
–Exclusive adrenal origin	–Long half-life
–Diagnostic relevance for adrenal androgen secreting tumors.	–Highly influenced by stress

INSULIN AS A GONADOTROPIC HORMONE



Ehrman DA, NEJM, 2005

Increased ovarian androgen synthesis results from abnormalities at all levels of the HPG axis.

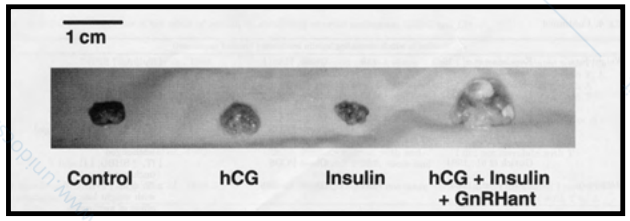
Insulin synergizes with LH to increase androgen production.

Insulin and hyperandrogenism

• **Ovary** - Direct stimulation of androgen secretion (stimulatory effect on the P450c17β enzyme) - Overexpression in LH receptor
• **Pituitary** - Sensitization of LH secreting pituitary cells to GnRH stimulation
• **Proteins** - Decrease of SHBG levels - Decrease of IGF binding proteins
• **Metabolism** - Decrease of androgen clearance - Decrease of the aromatase activity - Increase of the 5α-reductase activity

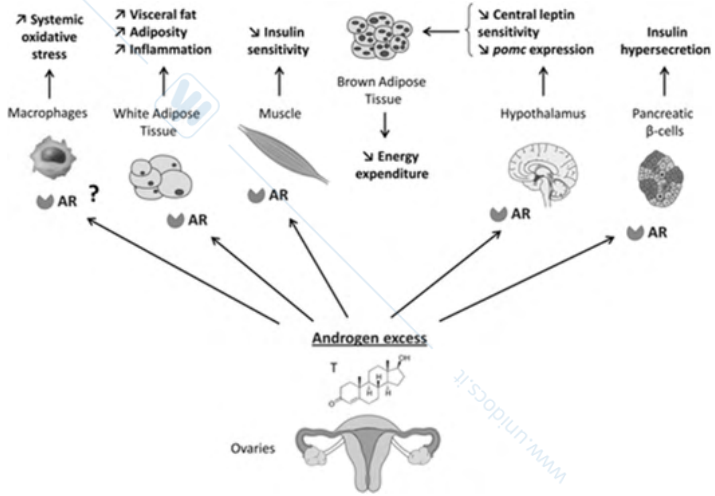
Hyperinsulinemia exerts a central role in the induction of hyperandrogenism in PCOS women

The effects of 23 days injections of normal saline (control), hCG, insulin, or insulin plus hCG and GnRHant on gross ovarian morphology in rats
(female Sprague-Dawley rats)

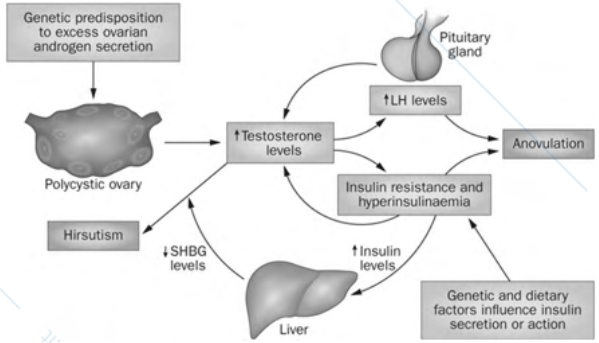


Poretsky L, *Metabolism 1992*

Androgen excess and insulin resistance



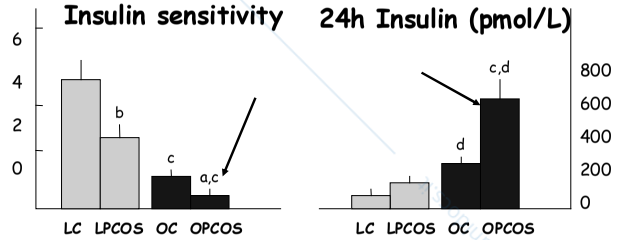
Hyperinsulinemia and hyperandrogenism the vicious cycle:



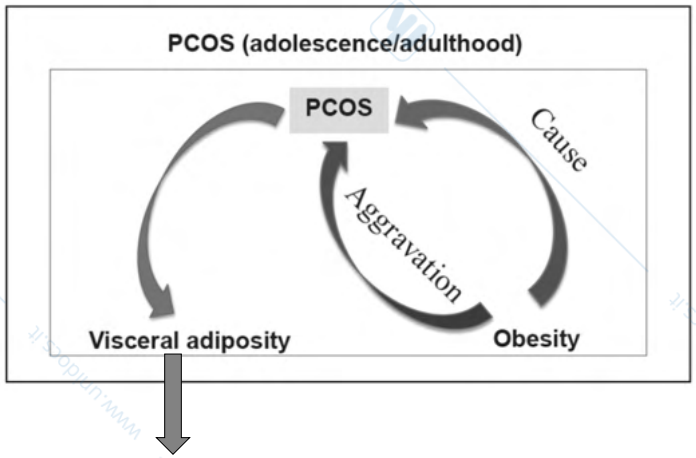
Hyperinsulinemia arises when insulin sensitivity is compromised at the adipose tissue, skeletal muscle and liver.
INSULIN SENSITIVITY IN THE OVARY IS NOT AFFECTED, resulting in further stimulation of androgen synthesis.

Independent effect of obesity on insulin sensitivity in PCOS

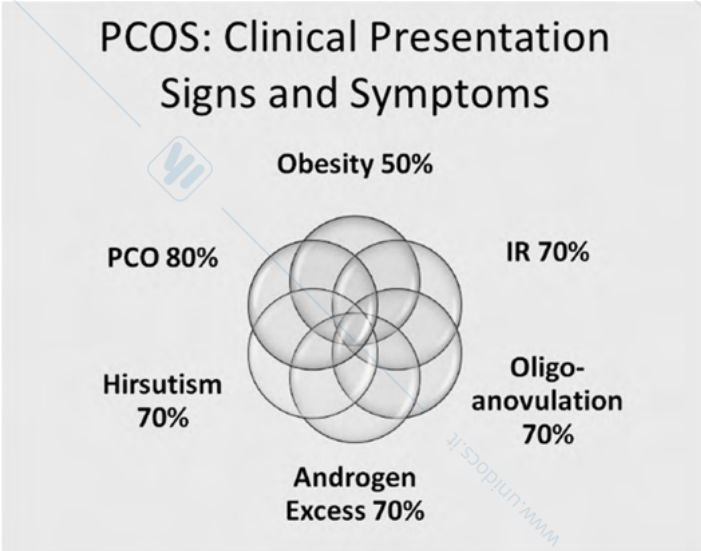
Insulin sensitivity is reduced and insulin levels are increased in PCOS.
Obesity contributes an additional component to insulin resistance in obese PCOS.



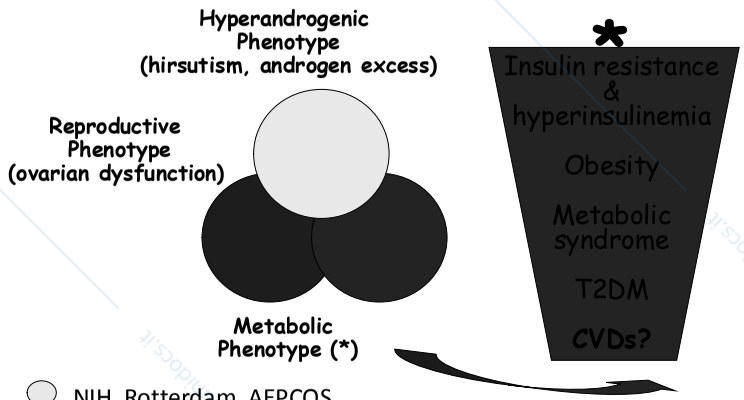
Morales, JCEM, 1996



Pasquali and Oriolo, 2019

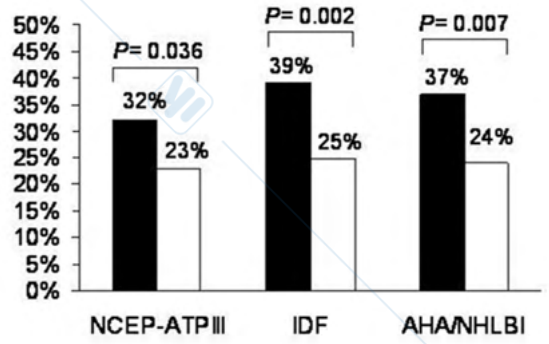


THE CLASSICAL PCOS PHENOTYPE



NIH, Rotterdam, AEPCOS
NIH, Rotterdam, AEPCOS
NIH, Rotterdam, AEPCOS

Prevalence of the metabolic syndrome in PCOS

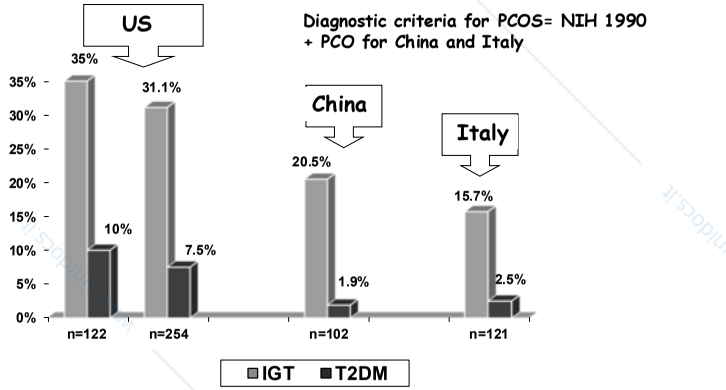


PCOS (n= 200) Controls (n= 200 age- and BMI-matched)

Gambineri et al, NMCD 2008

PREVALENCE OF T2DM IN REPRODUCTIVE-AGE PCOS WOMEN

Ehrmann DA, Diabetes Care, 1999; Legro RS, JCEM, 1999; Chen X, Hum Reprod, 2006; Gambineri A, Diabetes, 2004



Diagnosis of glucose intolerance by WHO criteria 1999

Incidence of T2DM in women with PCOS

Prospective study in 255 women with PCOS, followed for 10-35 yrs (16.9 years). Six women had T2DM at baseline, and another 42 women developed T2DM during the follow-up **(5 times more than the Italian general population).**

Crude incidence rate of T2DM in the cohort, overall and according to BMI class

Variables	n/N (persons-years of follow-up)	Incidence rate (95% CI) x 100 persons-year
Overall sample	42/249 (4.018)	1.05 (0.75-1.41)
BMI class (kg/m^2)		
< 25	3/79 (1.216)	0.25 (0.05-0.72)
25-29.9	8/78 (1.269)	0.63 (0.27-1.24)
≥ 30	31/92 (1.533)	2.02 (1.38-2.86)

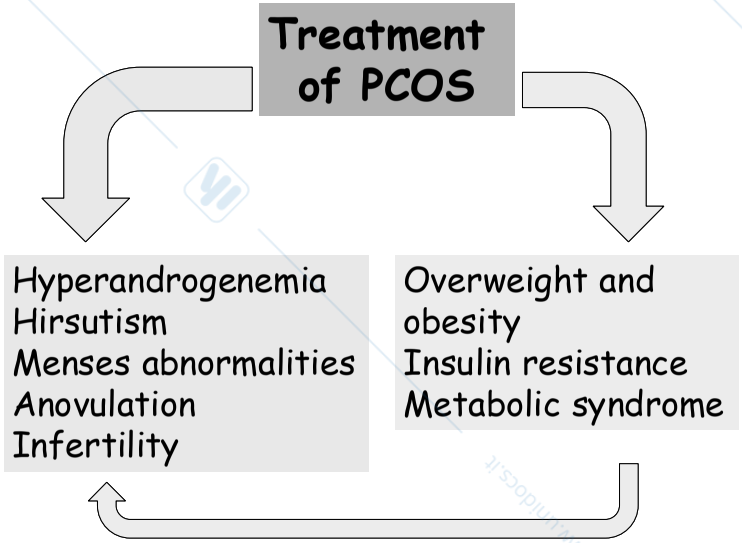
Gambineri A et al, Diabetes 2012; 61: 1-6

The PCOS phenotype is prone to developing Type 2 Diabetes Mellitus - earlier onset and higher incidence -

Mechanisms responsible for the susceptibility to develop T2DM in PCOS women are still unclear.

However, they should derive from the combination of:

- Insulin resistance and secretion
- Obesity (visceral + low grade inflammation)
- Androgen excess
- SHBG (blood levels and genetic)
- Other genetic factors
- Related to the phenotype (classic vs non-classic) ?



CLINICAL PRESENTATION

family doctors, dermatologists, gynecologists…and endocrinologists

Signs/symptoms of androgen excess
- *Hirsutism*
- *Acne*
- *Androgenic Alopecia*
- *Sexual Dysphoria*
- *Habitus androide*

Signs/symptoms of ovarian dysfunction
- *Menstrual disturbs*
- *Anovulation*
- *Infertility*
- *PCOm (ultrasound)*

Other non-specific signs/symptoms
- *Excess weight*
- *Metabolic alterations (blood biochemicals)*
- *Familiarity*
- *Early puberty*
- *Willing to start EP*

TREATMENT OF PCOS

TREATMENT SHOULD BE PLANNED ON THE BASIS OF INDIVIDUAL NEEDS

- **Hyperandrogenism**
  - EPs (antiandrogenic progestins)
  - Antiandrogens
  - Weight loss (in obesity) and /or lifestyle
  - Insulin sensitizers
  - Cosmetic treatment
- **Menses & anovulation**
  - EPs
  - Insulin sensitizers (metformin)
  - Weight loss (in obesity) and/or lifestyle

Treatment of PCOS

Treatment should be planned on the basis of individual needs

- **Infertility**
  - Weight loss (obesity) and/or lifestyle
  - Clomiphene citrate (selective estrogen receptor modulator, SERM)
  - Laparoscopic ovarian surgery
  - Gonadotropins and GnRH analogues
  - ART (IVF, ecc.)
- **Obesity**
  - Weight loss and/or lifestyle; bariatric surgery
- **Insulin resistance and metabolic dysfunction**
  - Weight loss (obesity) and/or lifestyle
  - Insulin sensitizers
  - Antiandrogens

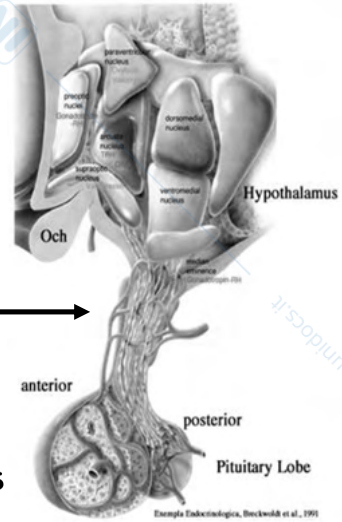
PITUITARY ADENOMAS

Hypothalamus-Pituitary (*hypophysis*) Axis

Hypothalamus
Hormone specific neural nuclei

Connecting stalk: INFUNDIBULUM →

Pituitary LOBES:
- Anterior: ADENOHYPOPHYSIS glandular tissue
- Posterior: NEUROHYPOPHYSIS neural tissue



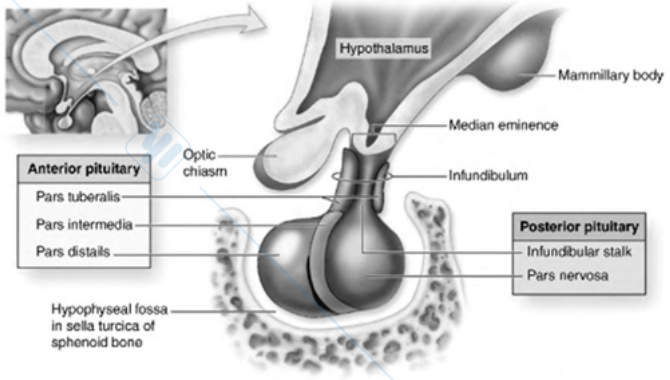


FIGURE 1.5 Diagram of normal pituitary anatomy, including pars distalis and pars tuberalis comprising the adenohypophysis. The infundibular stalk and pars nervosa comprise the neurohypophysis. *Source: Reprinted with permission from Mescher AL. Junqueira's basic histology. text and atlas. 12th ed. New York: McGraw-Hill; 2009, Figure 20.2.*

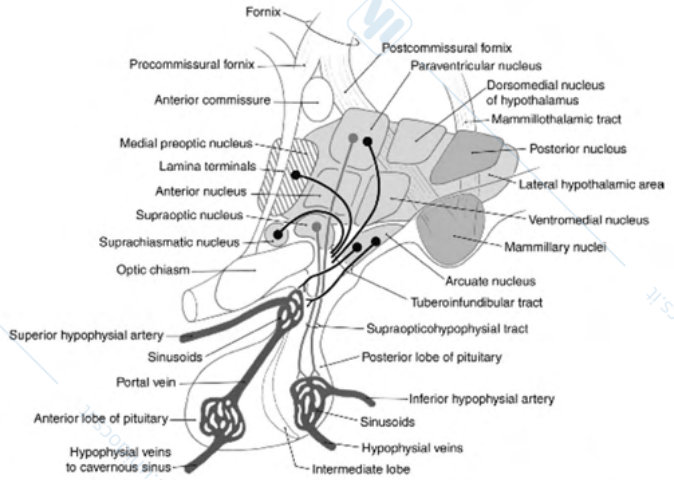


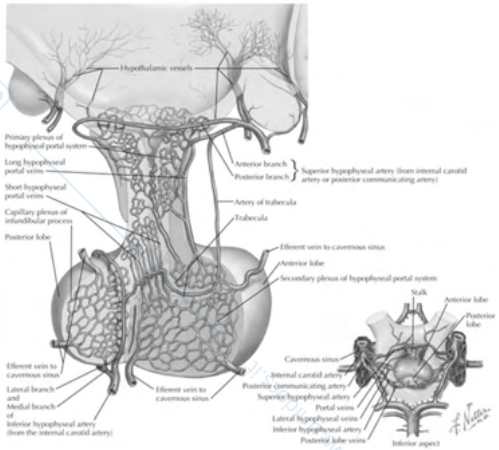
FIGURE 1.8 Sagittal view of the hypothalamus demonstrating the pre-optic, supraoptic, tuberal, and mammillary groups of medial hypothalamic nuclei. *Source: Reprinted with permission from Haines, D. Fundamental neuroscience for basic and clinical applications. San Diego: Elsevier, Chapter 30, Figure 30.5.*

Hypothalamus-Pituitary VASCULATURE

The pituitary gland is supplied by superior and inferior hypophyseal arteries derived by the internal carotid arteries.

Venous drainage is to local dural venous sinuses, mainly to the cavernous sinus, allowing pituitary hormones to diffuse to the whole body and reach target tissues.

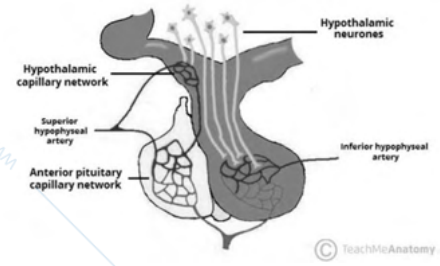
The inferior hypophyseal artery supplies the posterior pituitary lobus.



HYPOTHALAMO-HYPOPHYSEAL PORTAL SYSTEM

The superior hypophyseal arteries arise from the supraclinoid carotid Circle of Willis, and penetrate the median eminence (the lowest part of the hypothalamus) where they break up to form a primary capillary plexus featured by fenestrated endothelium.
At the median eminence level, axons from hypothalamic neurons secrete releasing and inhibitory factors (releasing hormones, RH), that rapidly enter these capillaries through the little windows in the lining endothelia cells.

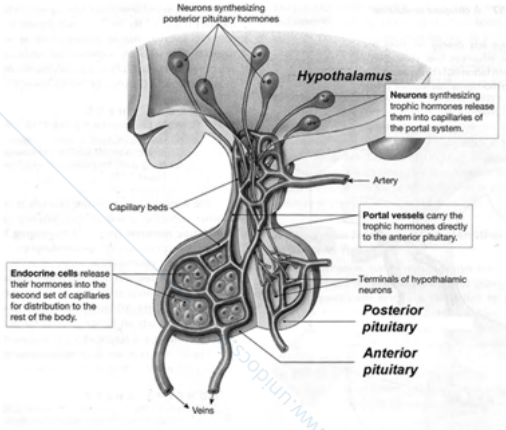
Long secondary capillaries or venules run from the primary plexus down the stalk of the gland and enter the adenohypophysis, where they join the secondary plexus of sinusoidal capillaries around the secretory cells.

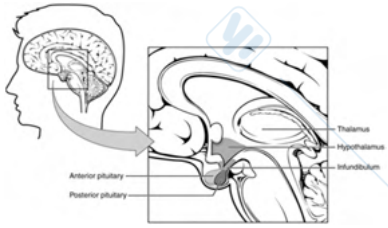


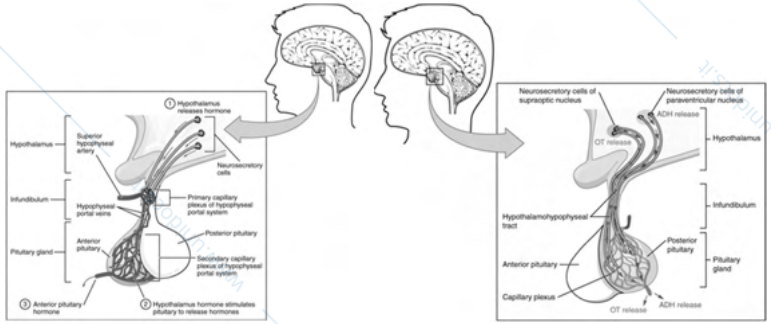
HYPOTHALAMO-HYPOPHYSEAL PORTAL SYSTEM

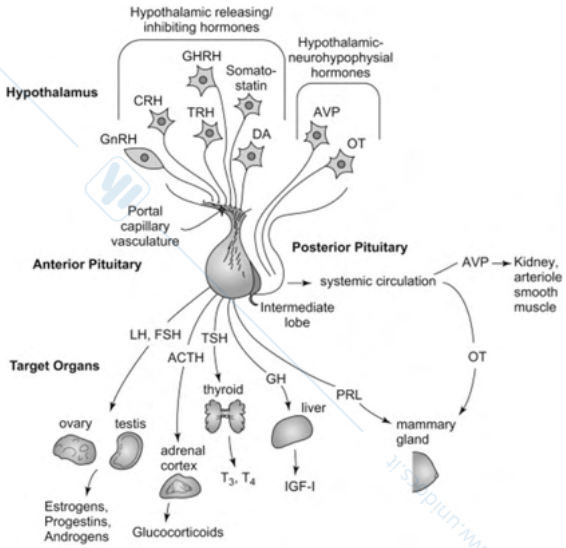
This direct route prevents releasing hormones from being diluted by the entire bloodstream. Consequently, only minimal amounts of active hypothalamic hormones are needed to regulate the cells.

Anterior pituitary cells are bathed in releasing and inhibitory factors in very high concentrations.







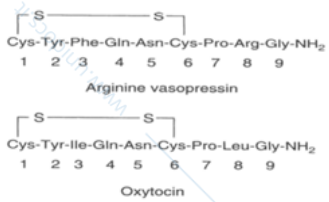


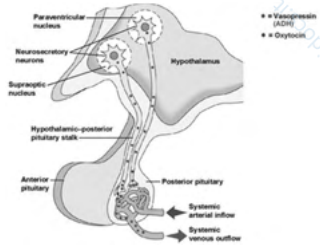
Vasopressin or antidiuretic hormone (ADH)

- Secreted following the activation of osmoreceptors in hypothalamic neurons (high osmolality) and following baroreceptors activation in atrium and large artieries, via vagal afferents (hypovolemia).
- Promotes renal reabsorption of water (antidiuretic effect).
- The lack of ADH or its receptors causes the elimination of large quantities of diluted urine (up to 500-1000 ml/h), causing severe dehydration. This condition is called **diabetes insipidus**, and is featured by polyuria and polydipsia.
- The hypersecretion of ADH (e.g. due to tumors) causes plasma hypo-osmolality (hyponatriemia) which can reach a state of water intoxication after the intake of large quantities of liquids.

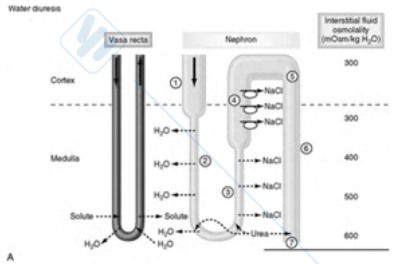
POSTERIOR PITUITARY (neurohypophysis)

- Extension of the hypothalamus.
- Magnocellular neurons having their bodies in the hypothalamic **supraoptic** and **paraventricular** nuclei, project their axons and terminals in the posterior pituitary.
- These neurons synthesize **vasopressin (ADH)** and **oxytocin (OXT)**, respectively. These are stored in vescicles at the terminal level, and are released in the blood when the neurons fire.
- ADH and OXT are cyclic nonapeptides differring for only 2 aa, encoded by adjacent genes on ch. 20.

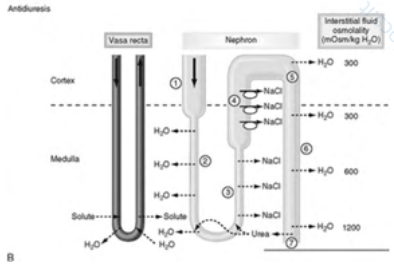




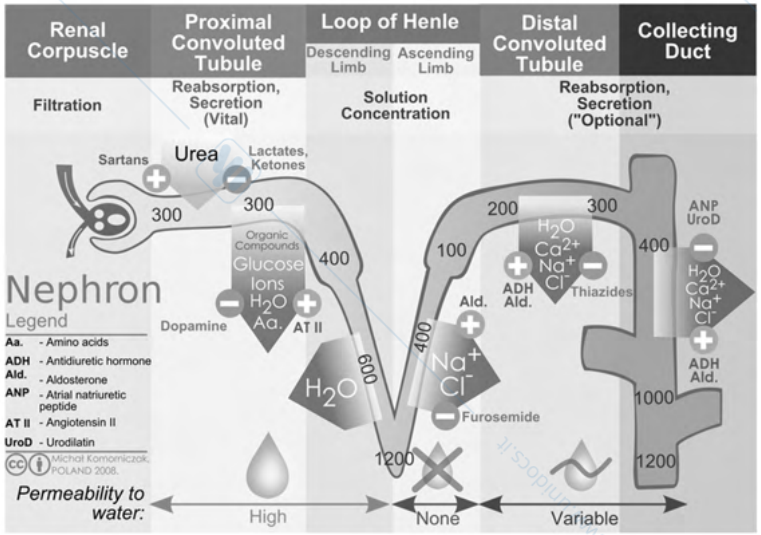
NO ADH
Water diuresis:
The collecting duct is impermeable to water.
Urea moves from interstitium to the duct lumen, reducing osmolality.



ADH
Water diuresis:
The collecting duct is highly permeable to water.
Interstitial osmolality is high.



ADH MECHANISM OF ACTION



REGULATION OF ADH RELEASE

ADH secretion is increased by:

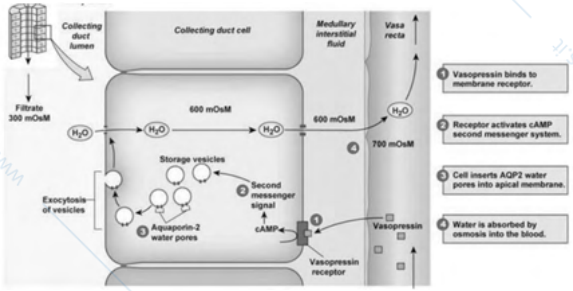
- plasma hyperosmolality (felt by hypothalamic osmoreceptor neurons).
- hypovolemia (felt by aortic and carotid baroreceptors, and by stretching receptors in the left atrium and pulmonary veins).
- Angiotensin II.

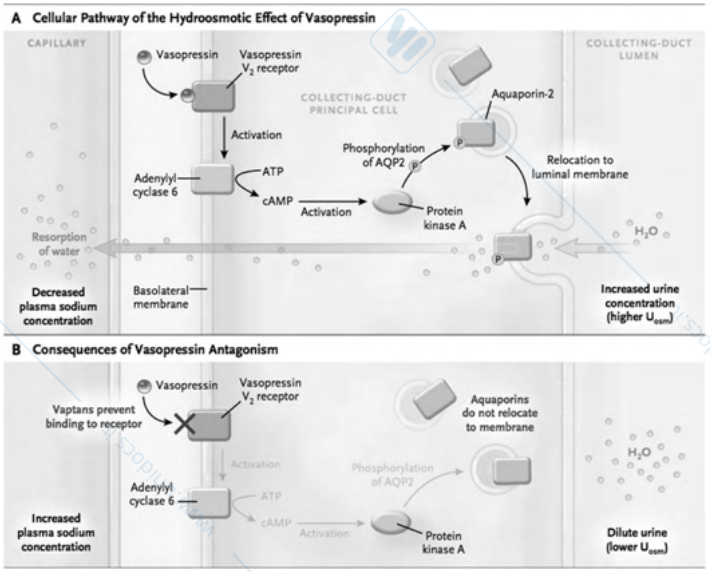
ADH secretion is inhibited by:

- atrial natriuretic factor (secreted in response to blood pressure increases).
- Alcohol.
- cortisol, thyroid hormones.

CELLULAR MECHANISM OF ADH

ADH travels in the blood and binds to V2 receptor on basolateral cell surface.
Vasopressin receptors are G-protein-coupled receptors. This binding stmulates adenylyl cyclase to generate cAMP and activate protein kinases. Activated PKA causes vesicles containing **aquaporin 2 channels to fuse with the apical membrane.**
This increases the permeability of the collecting duct epithelium to water, and water reabsorption increases by osmolarity. As a result, urine becomes more concentrated and water is conserved.



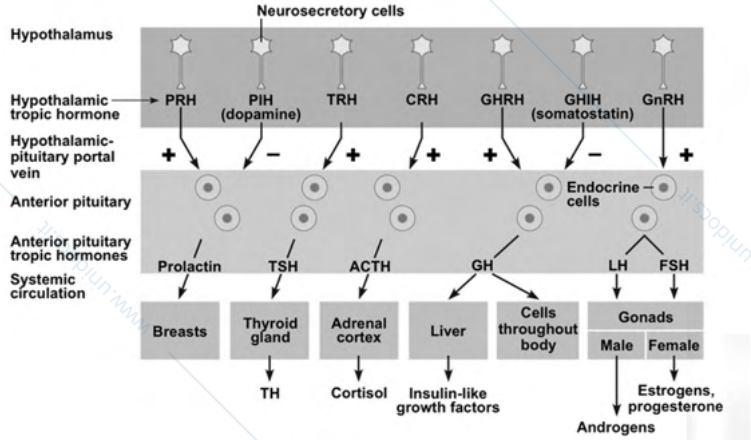


OXYTOCIN

- Induces uterine contractions during the labor.
- Induces milk ejection by contraction of the myoepithelial cells of the mammary gland in lactating breast.
- Is regulated by tactile stimuli (nipple sucking).
- Is regulated by positive feedback.
- It is also present in the male, but its functions are uncertain.
- Recent findings point on the role of oxytocin at the CNS level in mechanisms regulating eating behaviours including anxiety, stress and social interaction.

ANTERIOR PITUITARY (adenopophysis)

HYPOTALAMUS AND PITUITARY TROPIC HORMONES

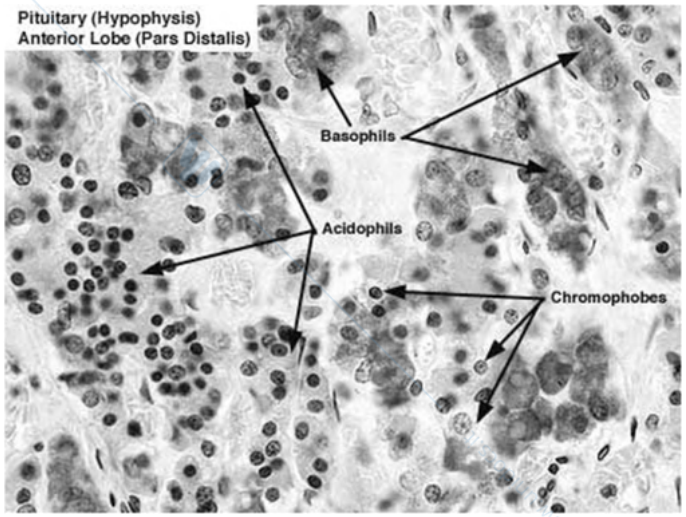


ANTERIOR PITUITARY (adenopophysis)

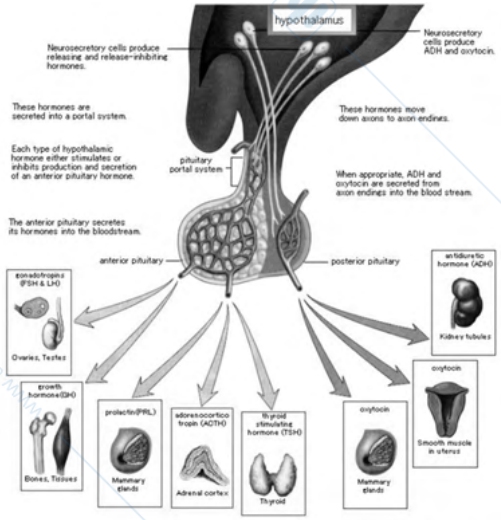
- Epithelial origin, not nervous.
- Composed by three cell types at hem/eox staining:
  acidophils, basophils, chromophobes
- Composed by 5 cell types based on secretory product:
  Lactotropic (PRL), somatotropic (GH), thyreotropic (TSH), gonadotropic (FSH and LH), corticotropic (ACTH)
- All the pituitary hormones are under hypothalamic regulation.
- Three out of six are glycoproteins (TSH, LH, FSH).
- Except for GH, target organs of all other adenohypophisis hormones are other glands.

The differential staining pattern (H&E) reflects the type of hormonal content of the cells

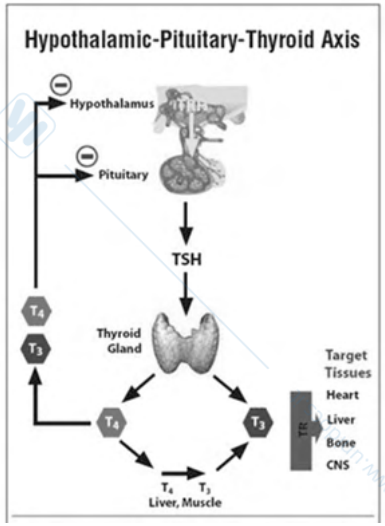
Acidophils	Cells that contain the polypeptide hormones: •Somatotropes which produce growth hormone •Lactotropes which produce prolactin
Basophils	Cells that contain the glycoprotein hormones: •Thyrotropes which produce thyroid stimulating hormone •Gonadotropes which produce luteinizing hormone or follicle-stimulating hormone •Corticotropes which produce adrenocorticotrophic hormone Due the high carbohydrate content, they also stain bright purple with PAS stains.
Chromophobes	These are cells that have minimal or no hormonal content. Many of the chromophobes may be acidophils or basophils that have degranulated and thereby are hormone depleted. Some chromophobes may also represent stem cells that have not yet differentiated into hormone-producing cells.



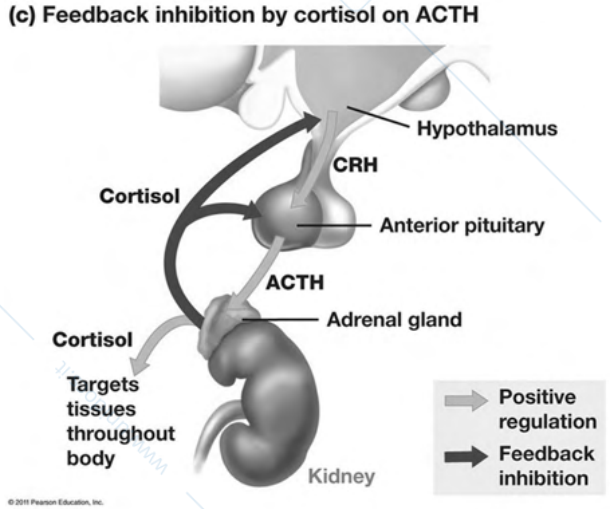
PITUITARY HORMONES



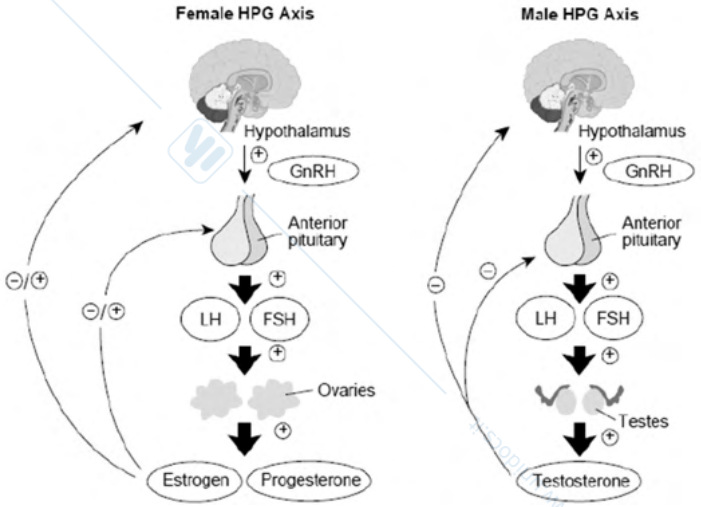
Hypothalamus-Pituitary-Thyroid Axis



Hypothalamus-Pituitary-Adrenal Axis

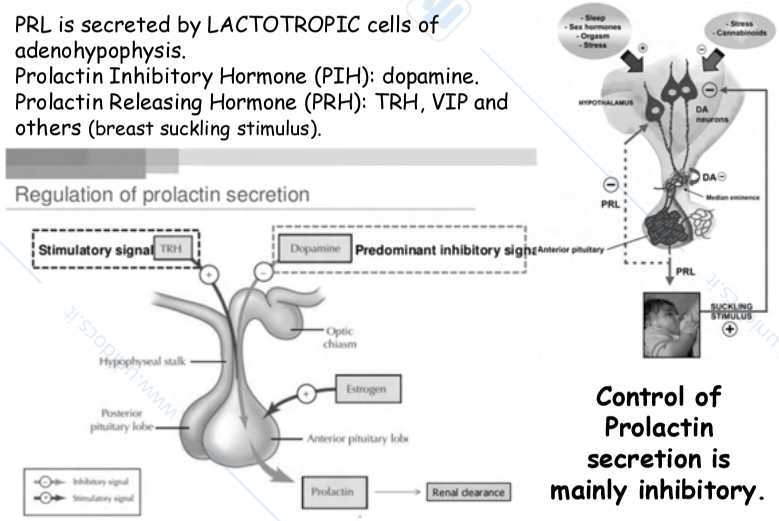


Hypothalamus-Pituitary-Gonadal Axis



Hypothalamus-Pituitary-Prolactin Axis

PRL is secreted by LACTOTROPIC cells of adenohypophysis.
Prolactin Inhibitory Hormone (PIH): dopamine.
Prolactin Releasing Hormone (PRH): TRH, VIP and others (breast suckling stimulus).

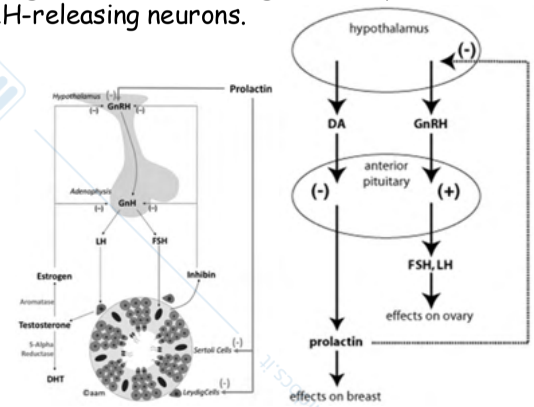


Control of Prolactin secretion is mainly inhibitory.

Hypothalamus-Pituitary-Prolactin Axis

Prolactin plays a negative feedback on gonadotropins axis, by inhibiting GnRH-releasing neurons.

Hyperprolactinemia causes the interruption of the pulsatile secretion of the gonadotropin releasing hormone (GnRH) with inhibition of the gonadal steroids production and can provoke infertility and hypogonadism.

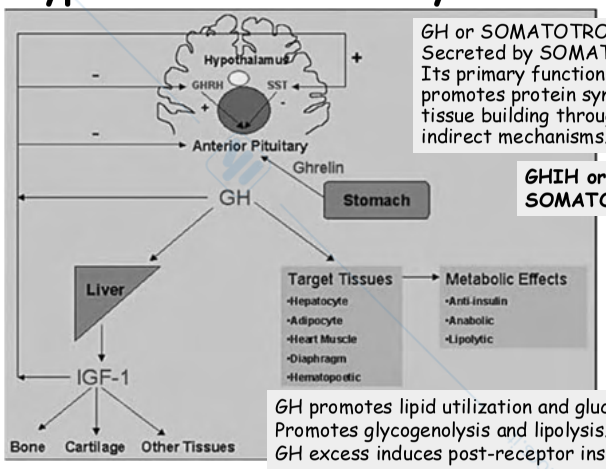


In men, testosterone synthesis is lost, affecting spermatogenesis.
In women, the LH ovulatory peak is lost, resulting in anovulation and amenorrhea.

Regulation of PRL secretion

	STIMULATION	INHIBITION
Physiologic conditions	Pregnancy / neonatal period Brest-feeding/Nipple stimulation Physical activity Hypoglycaemic stress Sleep	Dopamine (constitutive)
Pharmacologic effects	Hormones: TRH, VIP, estrogens Neurotransmitters: oppioids, dopaminergic antagonists, MAO inhibitors Verapamil Liquorice	Neurotransmitters: dopaminergic agonists, GABA
Pathologic conditions	Tumors/lesions of the hypothalamus pituitary unit Lesions of the chest or spinal cord Hypothyroidism Chronic kidney insufficiency Severe hepatopathy	Pituitary destruction or hypophysitis Pseudohypoparathyroidism

Hypothalamus-Pituitary-GH/IGF-1 Axis



GH or SOMATOTROPIN (STH) Secreted by SOMATOTROPIC cells. Its primary function is anabolic: it promotes protein synthesis and tissue building through direct and indirect mechanisms.

GHIH or SOMATOSTATIN (SST)

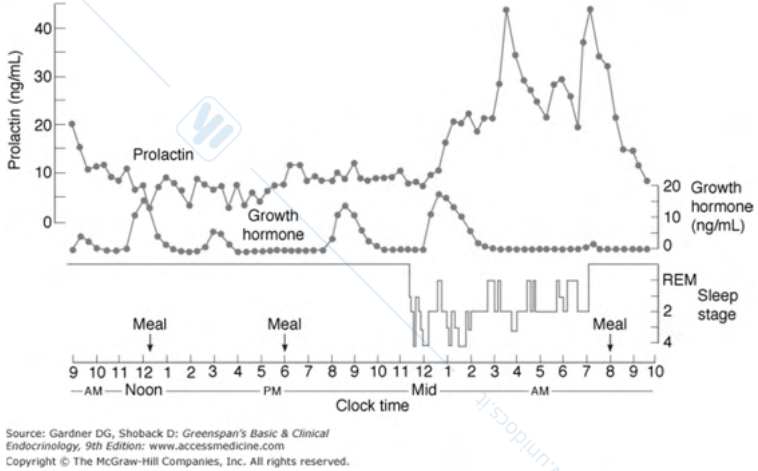
GH promotes lipid utilization and glucose sparing.
Promotes glycogenolysis and lipolysis.
GH excess induces post-receptor insulin-resistance.

ISULIN-LIKE GROWTH FACTOR-1 (IGF-1 or SOMATOMEDIN) promotes cell proliferation and inhibits apoptosis, mainly acting on skeletal muscle and cartilage.

Regulation of GH secretion

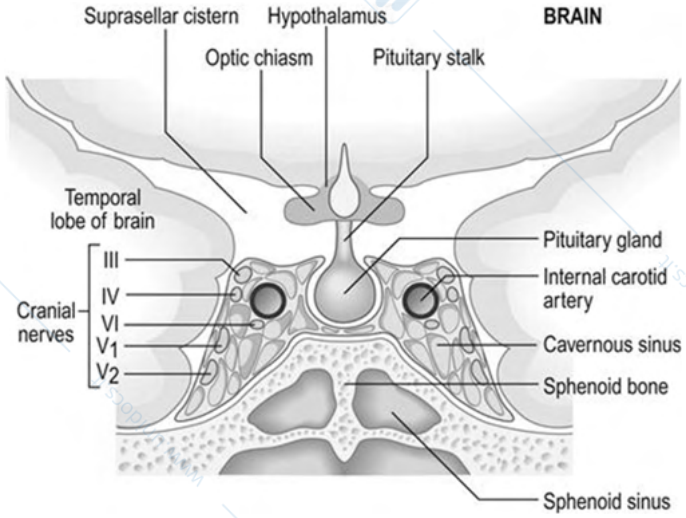
	STIMULATION	INHIBITION
Physiologic conditions	Sleep Physical activity Stress Hyper-aminoacidemia Hypo-glycaemia	Post-prandial hyperglycaemia High circulating free fatty acid
Pharmacologic effects	Hormones: GRH, ACTH, a-MSH, estrogens Neurotransmitters: a-adrenerdic, GABA, dopaminergic agonists B-adrenergic antagonists Serotonin precursors	Hormones: SST, GH, progestrone, glucocorticoids Neurotransmitters: a-adrenergic, serotonin, dopamin antagonist B-adrenergic agonists
Pathologic conditions	Fasting and low protein intake Anorexia nervosa Pyrogens Ectopic GHRH secretion Chronic kidney insufficiency Acromegaly	Obesity Hypo- and hyperthyroidism

24h secretory pattern of PRL and GH



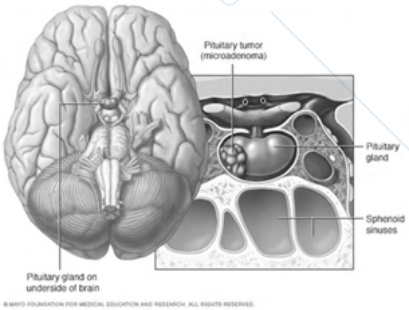
Episodic secretion, mainly during sleep-time

PITUITARY ANATOMY



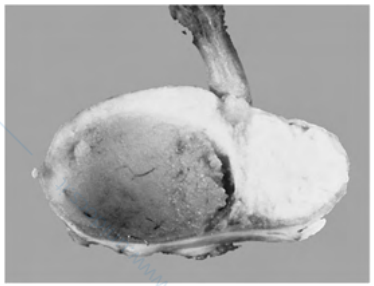
PITUITARY ADENOMAS

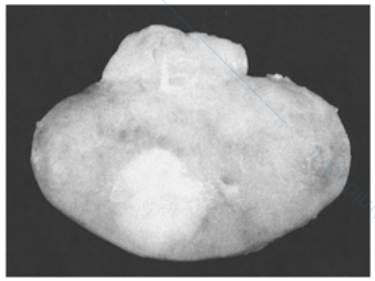
The pituitary gland sits inferior to the hypothalamus. It is surrounded caudally by the sphenoid bone in a basketlike structure called the sella turcica, and superiorly by the optic chiasm. The sella turcica forces an expanding adenoma superiorly, leading to compression of the optic nerve and headaches from mass effect. Additionally, destruction or compression of the pituitary gland may cause complete or partial hypopituitarism.



Pituitary adenomas are benign tumors that arise from one of the five cell types that comprise the anterior pituitary (lactotrophs, gonadotrophs, somatotrophs, corticotrophs, and thyrotrophs). Tumors rarely form from a combination of these cells. Pituitary adenomas are true neoplasms with a monoclonal cell origin. Hypersecretion or diminished inhibition of the hormones of the hypothalamic-pituitary axis can lead to the constellation of endocrine symptoms often seen in patients with pituitary adenomas.

PITUITARY ADENOMAS





PITUITARY ADENOMAS

Pituitary tumors present as:
- incidentalomas following radiographic imaging;
- as a result of mass effect (typically visual impairment or headache);
- signs or symptoms of hypofunction or hyperfunction.

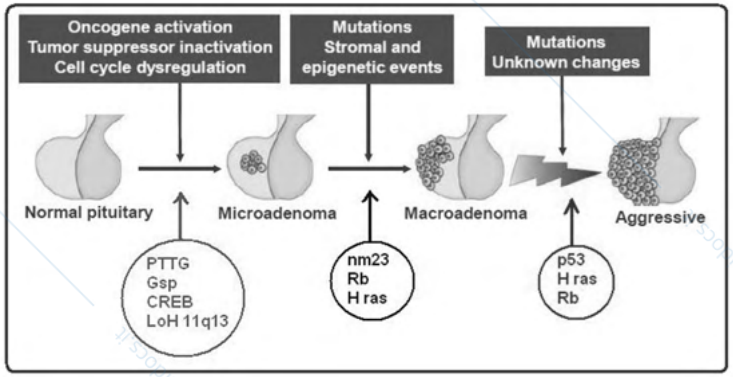
Tumors can be divided according to size into two groups:
- Microadenomas: ≤ 10 mm
- Macroadenoma: > 10 mm

Tumors can be functional or nonfunctional.

Pituitary hyperplasia may occur during puberty and pregnancy, or in response to a chronic lack of feedback, e.g. primary hypothyroidism
Pituitary carcinomas only accounts for 0.1% of all pituitary tumors.

INCIDENTALOMA: incidental finding observed during imaging investigations for unrelated reasons.
Most of the pituitary incidentalomas are non functioning adenomas.

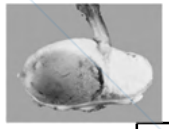
TUMOR PROGRESSION



Melmed S.
Pathogenesis of pituitary tumors
Nature Reviews Endocrinology 2011; 7:257–266 (modificato)

PITUITARY ADENOMAS EPIDEMIOLOGY

- Pituitary tumors represent about 10% of all intracranial tumors.
- MRI studies in unselected populations report microincidentaloma rates of 10–38% and 0.16–0.3% for macroadenomas.
- Autopsy data estimate an average prevalence of 10.7%.
- Prevalence: 80-100/100,000
- Annual incidence: 4 new cases/100,000
- Peak frequency: adult population (fourth-eighth decade)

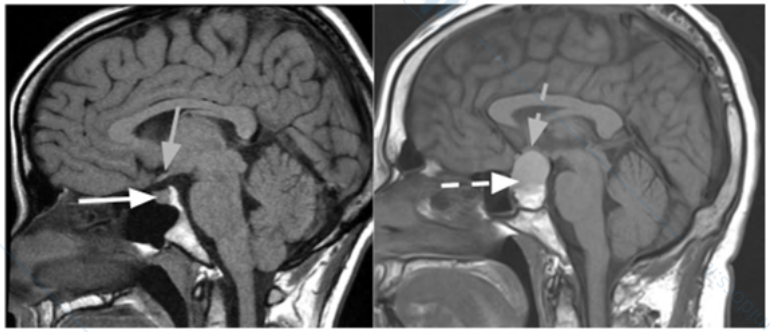


PITUITARY ADENOMAS CLASSIFICATION

Pituitary adenomas are subdivided into six groups based on their immunohistochemical affinities.

- ✓ 40-50% PRL-secreting adenomas
- ✓ 20-25% GH-secreting adenomas
- ✓ 20-25% non-secreting adenomas
- ✓ 8-10% ACTH-secreting adenomas
- ✓ 1-2% gonadotropinomas
- ✓ 1-2 % thyrotropinomas

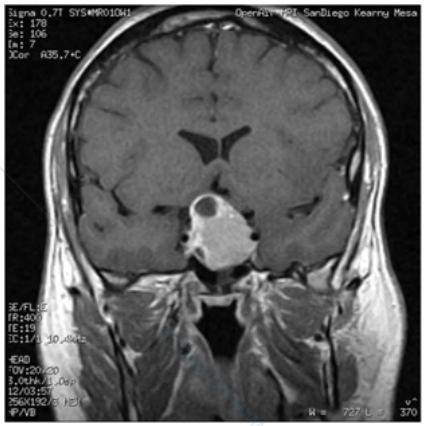
	Microadenomas		Macroadenomas		Overall Incidence of
Pituitary Tumor Type	%	% Invasive	%	% Invasive	Invasion (%)
GH-cell adenoma	14	0	86	50	50
PRL-cell adenoma	33		67		52
Mixed GH/PRL-cell adenoma	26	0	74	31	31
ACTH-cell adenoma (Cushing's disease)	87	8	13	62	IS
ACTH-cell adenoma (Nelson's syndrome)	30	17	70	64	50
Silent ACTH adenoma	0	0	100	82	82
Gonadotrope adenoma	0	0	100	21	21
Thyrotrope adenoma	0	0	l00	75	75
Null cell adenoma	2		98		42
Plurihormonal adenoma	25	31	75	59	52

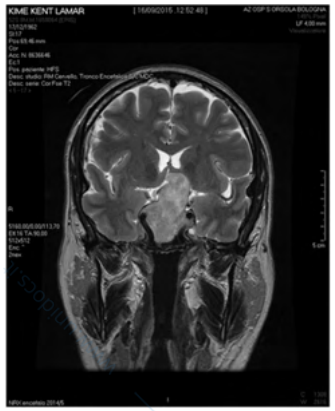


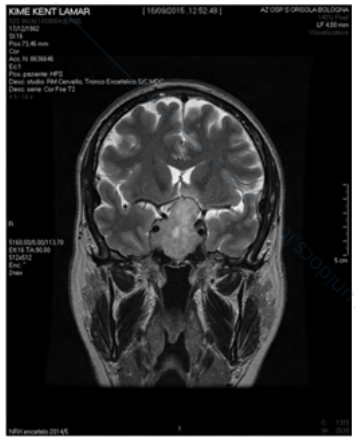
The normal pituitary gland (solid white arrow) and a pituitary tumor (dashed white arrow). The optic chiasm, which is where the optic nerves meet, is shown by the green arrows and is pushed up by the pituitary tumor.

Effects of compression by pituitary adenomas

Magnetic Resounance Imaging (MRI)

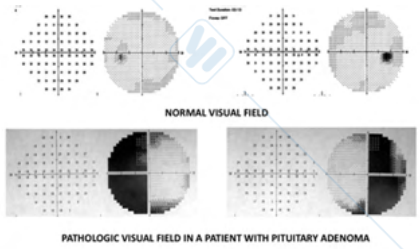




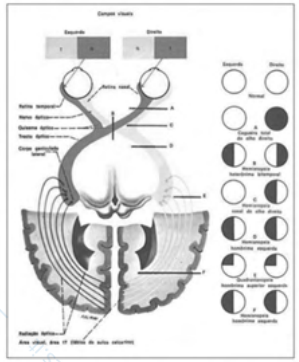


- Headache
- Visus defects
- Rinorrhea
- Cranial nerves paralisis

Visual defect analysis in pituitary adenomas



Estimation of the loss of vision in different spatial areas.



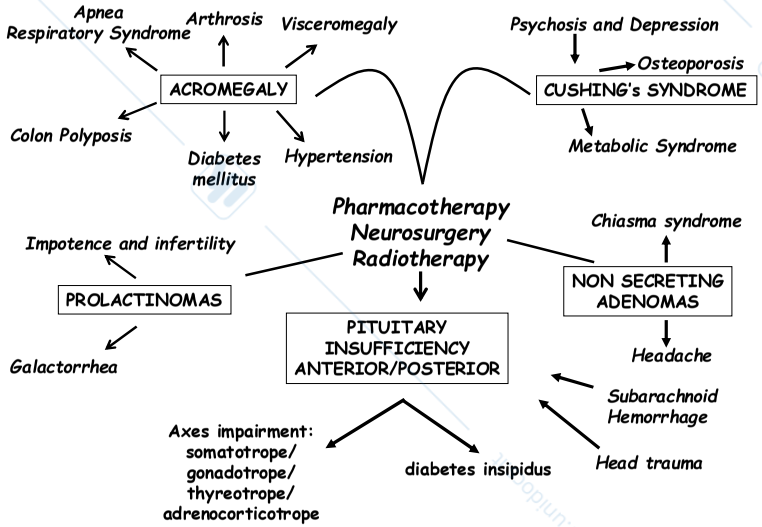
SEX DISTRIBUTION OF THE INCIDENCE OF PITUITARY ADENOMAS

m/f

PRL	NF	ACRO	CUSH	PRL	NF	ACRO	CUSH
1/22	1/6	1/45	3/16	19/84	1	1/96	4/43
Macro-adenomas				*Micro-adenomas*			

Data from a consecutive series of 2137 pituitary tumors

Ambrosi B. et al.: Pituitary adenomas: new trends in basic and clinical research 159, 1991



PROLACTINOMA

- is the most frequent cause of hyperprolactinemia;
- is the most frequent pituitary adenoma (40-55% of all pituitary tumors, 66% in recent reports);
- prevalence is about 100/million (934/million in recent reports) in clinical records;
- prevalence in autoptic series is much higher.

Prolactinomas and Hyperprolactinemia

Micro-prolactinoma (tumor diameter <1 cm) is far more frequent.

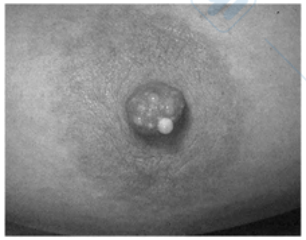
- Women:Men ratio about 20:1.
- Average age at diagnosis is 25-35 years

Macro-prolactinoma

- Frequency is similar in the two sexes.
- Average age at diagnosis is 45-55 years.

Prolactinoma represents the most frequent pituitary tumor even in adolescence.

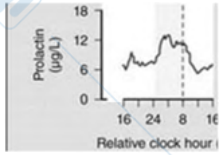
Hyperprolactinemia: clinical signs and symptoms



- Galactorrhea in both sexes (stimulated breast milk discharge)
- Amenorrhea in women
- Hypogonadism in men (low libido, impotence)

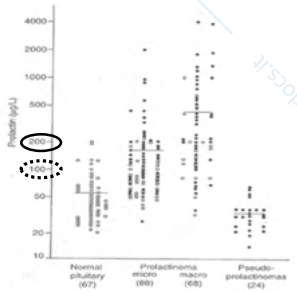
BLOOD PROLACTIN MEASUREMENT

Prolactin levels change throughout the day, being usually highest in early morning. Blood is taken 3-4 hours after waking up → the patient should not fall asleep during withdrawal!



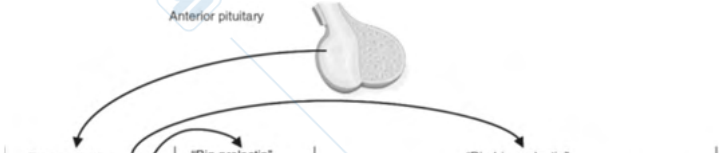
Prolactin levels change with stress, including venipuncture stress → incanulation is required (15min saline infusion)

Some drugs and medications can affect prolactin levels, including: estroprogestins, anti-hypertensive and antipsychotics/ antidepressants/ opiates.



BLOOD PROLACTIN MEASUREMENT

PRL is known to exist in multiple isoforms circulating throughout the body. Current methodologies for measuring the PRL levels typically involve a variety of immunoassays. However, most of these tests are not capable of distinguishing between the different isoforms.

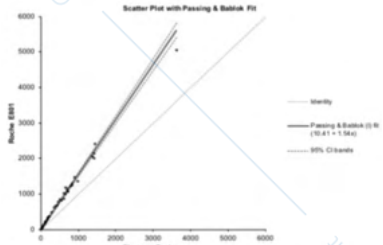


Monomeric	Dimeric	IgG-prolactin	IgA-prolactin	Prolactin aggregates
23 kDa	40–60 kDa	>100–150 kDa		
Active	Inactive	Inactive		
60%–90%	15%–30%	0%–8%	0%–2%	0%–3%

BLOOD PROLACTIN MEASUREMENT

Considerable variability exists among routine prolactin immunoassays as caused by the different reactivity towards monomeric prolactin and macroprolactin.

Macroprolactinaemia is a relatively common cause of interference in the prolactin assay that may lead to incorrect diagnosis and unnecessary investigations. Measurement of prolactin post polyethylene glycol precipitation (PEG), when prolactin levels are above the reference interval, is routinely used to identify macroprolactin.



Saleem M et al. Clin Biochem Rev 39 (1) 2018

Differences in absolute values
plus
falsely normal or elevated values!!

PHARMACOLOGICAL THERAPY

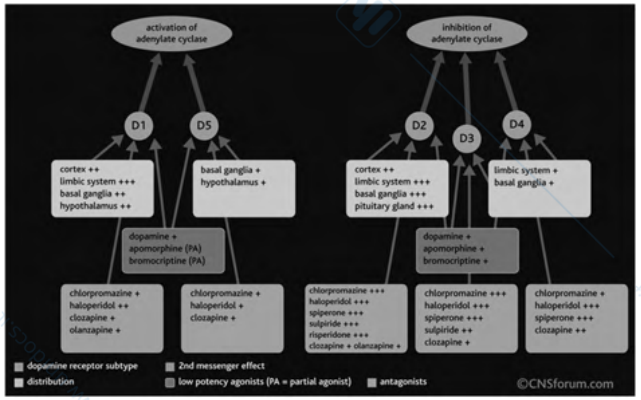
DOPAMINE AGONISTS

FOR pituitary D2 receptor

- **BROMOCRIPTINE**
- **CABERGOLINE**
- **LISURIDE**
- **PERGOLIDE**
- **QUINAGOLIDE (CV 205-502)**

Surgery is usully not necessary for micro- and macro-prolactinomas, as dopamine agonists are usually effective in controlling PRL hypersecretion and in reducing tumor mass.

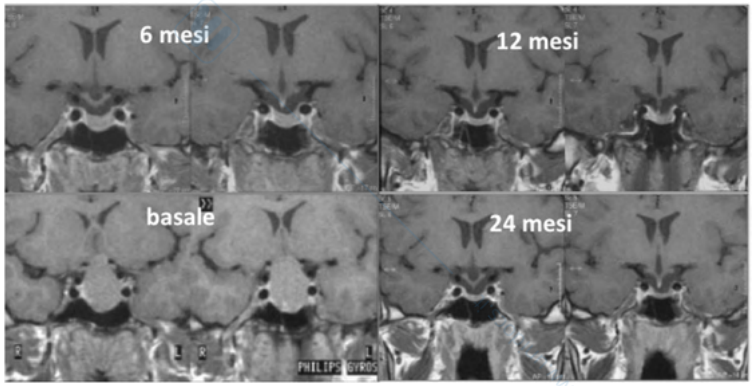
DOPAMINE AGONISTS



Bromocriptine shows side effects due to its action on other dopamine, serotonin, adrenergic receptors.

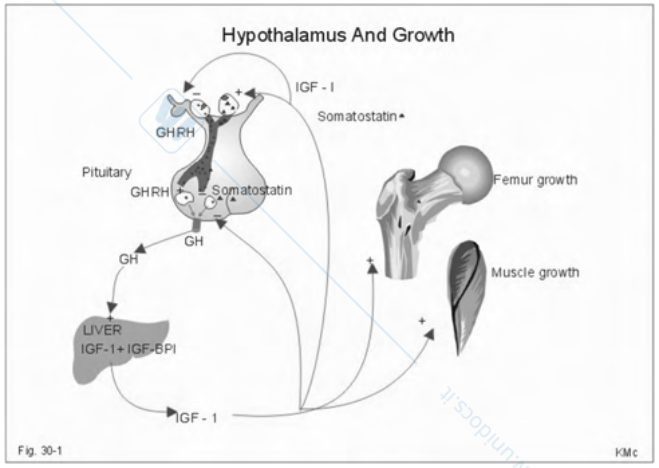
Cabergoline has high affinity for D2 and D1 and serotonine receptors. Low dosage is required, minimizing side effects.

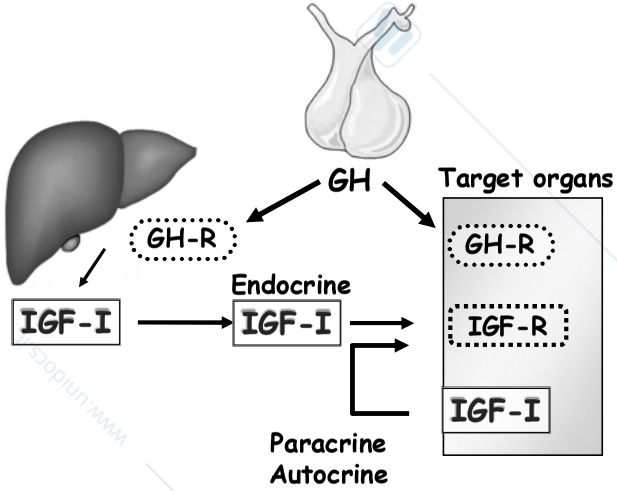
DOPAMINE-AGONIST DRUGS ALSO INDUCE TUMOR SHRINKAGE



GH-omas and ACROMEGALY

Hypothalamus-Pituitary-GH/IGF-1 Axis

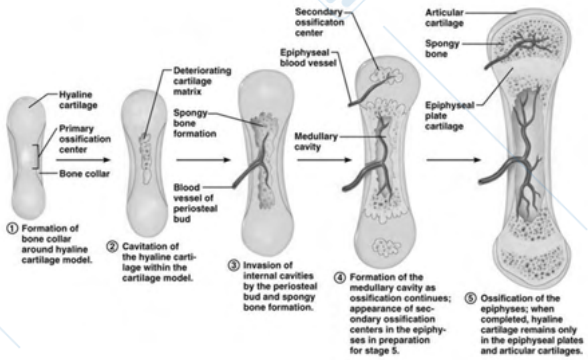




Somatomedins

- Most of GH effects are mediated by the action of somatomedins, also called insulin-like growth factors (IGF).
- Somatomedins (IGF-1 and IGF-2) are mainly produced by the liver and act on tyrosine kinase-coupled receptors.
- Main action is on the regulation of gene transcription.
- GH and IGF induce the production of collagen and chondroitin-sulfate in cartilage chondrocytes (chondrogenesis).

LONGITUDINAL BONE GROWTH



Within the epiphyseal growth plate, chondrocyte proliferation, hypertrophy, and cartilage matrix secretion result in the formation of cartilage that is subsequently invaded by blood vessels and bone cells that remodel the cartilage into bone tissue. A complex network of endocrine signals, including GH, IGF-I thyroid hormones, estrogen, androgen, and vitamin D work seamlessly to regulate longitudinal bone growth.

GH metabolic effect

Actions on protein metabolism:
+ cellular amino acids uptake
+ protein synthesis
- protein catabolism

Actions on lipid metabolism:
+ mobilization from adipose tissue
+ free plasma fatty acids
+ oxidation of fatty acids

Actions on glucose metabolism:
- glycolysis
+ glycogenosynthesis
+ blood sugar

ACROMEGALY

Acromegaly syndrome is determined by the prolonged and chronic exposure of the body to high blood levels of growth hormone (GH) and its peripheral mediator, the somatomedin C or insulin-like growth factor 1 (IGF1).

Excess levels of these molecules arising after the closure of the growth cartilage plates determine the appearance of peculiar somatic stigmata (hands, cranium, jaw) and the typical multisystem involvement, responsible for the higher rate of morbidity and mortality observed in these patients.

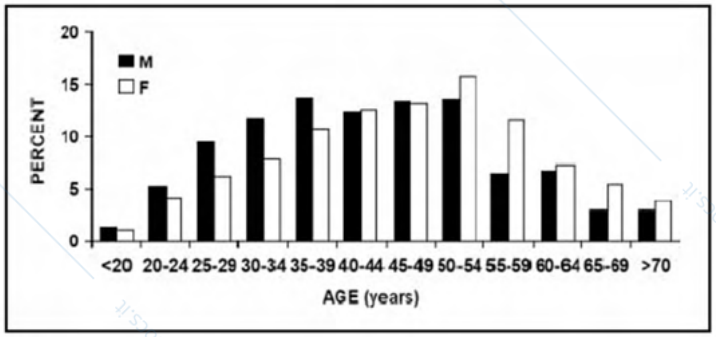
GIGANTISM: excess of GH/IGF1 occurring before or during pubertal development, when cartilage plates are still open, resulting into abnormal longitudinal growth.

INCIDENCE and PREVALENCE

	Cases (n.)	Incidence (cases/million /y)	Prevalence (cases/million)
United Kingdom Alexander et al., 1980	164	2.8	38
Sweden Bengtsson et al., 1988	166	3.3	69
Irland Ritchie et al., 1990	131	4.0	63
Spain Extabe et al., 1993	74	3.1	60
Mean		3.3	58

Adapted from: Epidemiology of Acromegaly, I. M. Holdaway and C. Rajasoorya, Pituitary 1999; 2:29-41

ACROMEGALY PREVALENCE DISTRIBUTION PER AGES



Arosio et al.
Predictors of morbidity and mortalità in acromegaly: an Italian survey
European Journal of Endocrinology (2012) 167 189-198.

ACROMEGALY ETIOLOGY

PITUITARY (98%) (usually >1cm in diameter)

Excess GH secretion: GH-secreting cells adenoma
GH- and PRL-secreting mixed cells adenoma (15% of GH-adenomas)
Multi-hormonal adenoma
GH-secreting pituitary carcinoma (rare)

EXTRA-PITUITARY (<2%)

Excess GH secretion: Ectopic adenoma (sphenoid or parapharyngeal sinus)
Pancreatic islet tumor

Excess GHRH secretion
Central-ectopic (<1%): Hypothalamic tumors (hamartoma, choristoma, ganglioneuroma)

Peripheral-ectopic (1%): Carcinoid, Pancreatic islet tumor, small-cells lung carcinoma

ACROMEGALY SLOW PROGRESSION

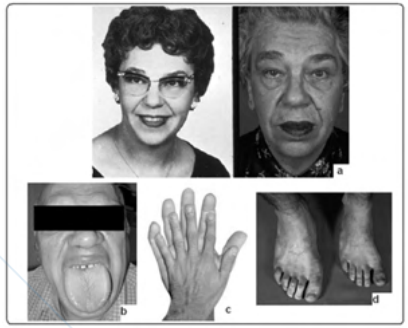
Time lapse between disease onset and clinical diagnosis is about 10 years!

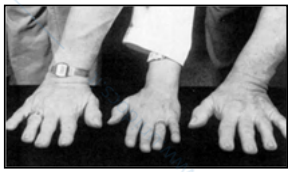
ACROMEGALY SLOW PROGRESSION

ACROMEGALY SLOW PROGRESSION

ACROMEGALY'S CLINICAL SIGNS

- Enlarged hands and feet
- Enlarged tongue
- Coarsened, enlarged facial features
- Coarse, oily, thickened skin
- Excessive sweating and body odor
- Small outgrowths of skin tissue (skin tags)
- Fatigue and muscle weakness
- Deepened, husky voice due to enlarged vocal cords and sinuses

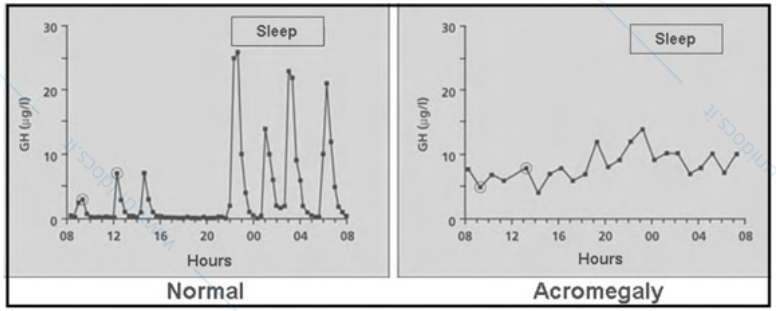




Blood GH level circadian fluctuation

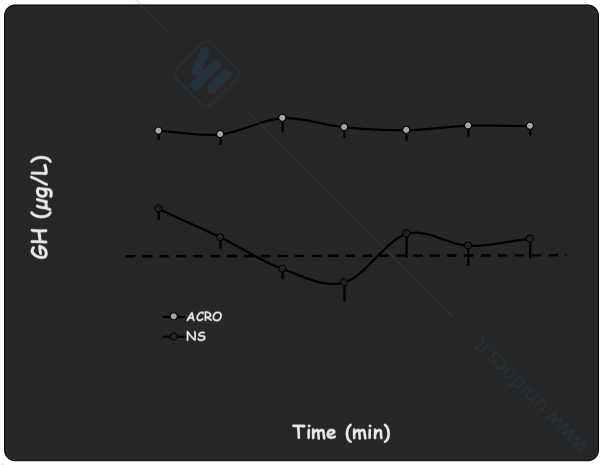
GH pulsatile secretion is normally high in the night-time. In addition, GH is released throughout the day in response to the lack of nutrients (pre-prandial peak).

As GH secretion is episodic, random measurement of blood GH is not useful for either confirming or excluding acromegaly.



Chanson P, Salenave S. *Acromegaly*
Orphanet Journal of rare disease 2008 Jun 25;3:17. doi: 10.1186/1750-1172-3-17

Testing GH levels during an oral glucose load (OGTT; 75 g)



Assay-dependent cut-off

GH vs IGF-1 MEASUREMENT

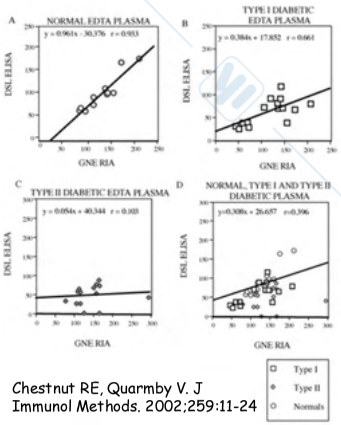
Circulating levels of serum total IGF-I are widely used in the diagnosis of GH disorders, including acromegaly and GH deficiency. Pituitary GH is known to be secreted on a pulsatile manner, in diurnal rhythm, and is subjected to different physiological and environmental stimuli including fasting, exercise and feeding. The short half-life of GH (20-50 minutes) further increases the variability of the circulating level.

IGF-I is synthesized in a more stable manner, does not exhibit diurnal rhythm, has a longer half-time, and therefore is a more reliable biomarker of GH disorders. To highlight the importance of IGF-I in the management of GH disorders, it was recommended by the Endocrine Society that serum level of IGF-I should be used as a first line screening test for acromegaly, followed by GH measurement with an oral glucose loading as a confirmation test.

The management goal of acromegaly was also established biochemically by normalization of serum IGF-I level.

PITFALLS IN IGF-1 MEASUREMENT

"...good agreement when IGF-I levels in samples from normal individuals were measured... agreements were quite poor when IGF-I levels in samples from diabetics were measured."



Chestnut RE, Quarmby V. J Immunol Methods. 2002;259:11-24

Modification of epitope accessibility
IGF-1 circulates bound to IGF-binding proteins (IGFBPs, 6 types). High-affinity IGFBP in the sample can 'cover' epitopes recognized by the antibodies used in an assay.
The amount of each IGFBP varies between samples and can reach extreme values under specific pathophysiological conditions. For example, in individuals with poorly controlled diabetes or in a state of malnutrition, IGFBP-1 and IGFBP-2 levels increase, which might have different effects on measured IGF-I concentrations in different assays, depending on the degree of interference from the IGFBPs.

PITFALLS IN IGF-1 MEASUREMENT

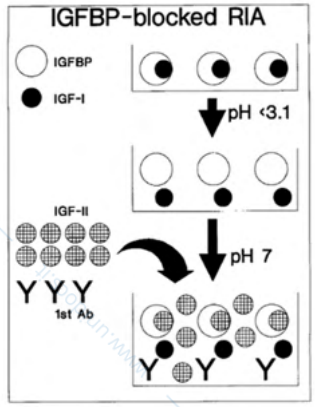
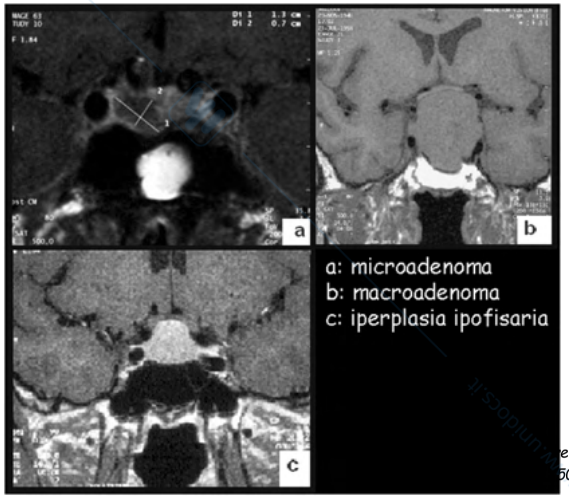


Figure 2.: Principle of the IGFBP-blocked IGF-I RIA

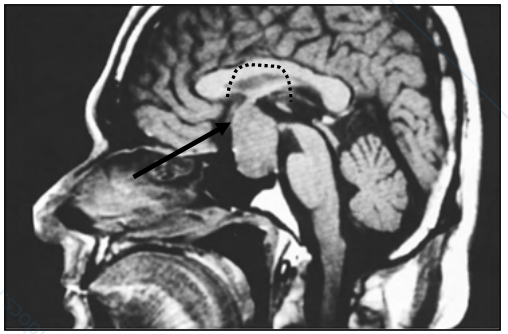
Strategy: Because highly specific antibodies to IGF-I do not cross-react with IGF-II, an excess of IGF-II is added during the acid-ethanol precipitation step and before IGF-I is measured.
The high concentration of IGF-II in this type of assay blocks the IGF-binding sites of the remaining BPs, there-by allowing an unbiased measurement of IGF-I.

PITUITARY MRI



e disease 2008 Jun
50-1172-3-17

GH-secreting macroadenoma: pituitary MRI



THERAPY

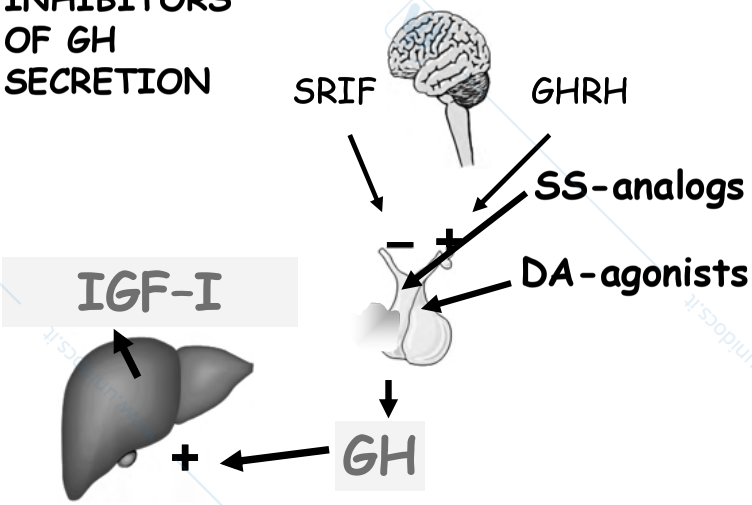
Surgery
- Endonasal Transphenoidal
- Transcranial

Pharmacological
- Somatostatin analogues: Octreotide/Lanreotide
- Dopamine agonists: Cabergoline/Bromocriptine

Novel ligands / novel administration strategies
- GH-receptor antagonists: Pegvisomant

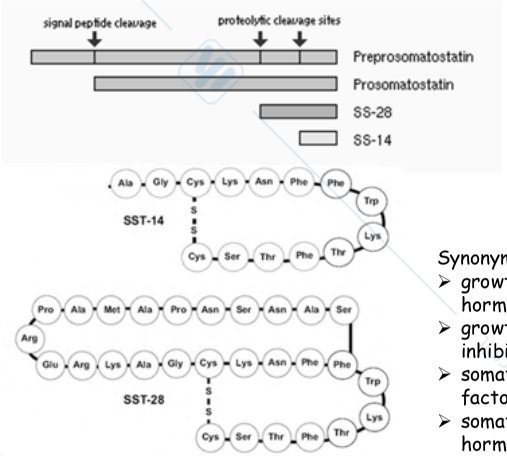
Radiotherapy
- Parallel-opposed fields
- Stereotactic

INHIBITORS OF GH SECRETION



SOMATOSTATIN

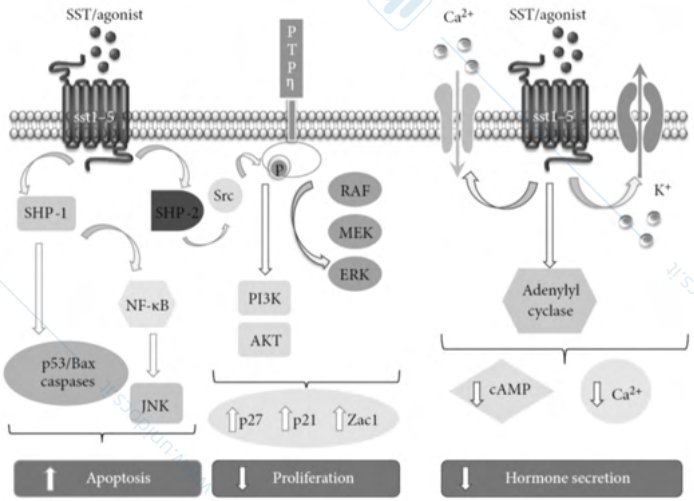
GROWTH HORMONE INHIBITING HORMONE



Two active forms

Somatostatin half-life: 2min!

Synonyms of "somatostatin":
- growth hormone-inhibiting hormone (GHIH)
- growth hormone release-inhibiting hormone (GHRIH)
- somatotropin release-inhibiting factor (SRIF)
- somatotropin release-inhibiting hormone (SRIH)



SOMATOSTATIN ACTIONS

Target	Effect
Hypothalamus Pituitary	↓ basal and stimulated TSH and GH; ↓ PRL in acromegaly; ↓ ACTH in Addison and ACTH secreting adenomas
Gastrointestinal tract	↓ gastric motility; ↓ endocrine cells secretion; ↓ gallbladder motility
Thyroid	↓ FT3 and FT4; ↓ calcitonin
Adrenal gland	↓ Aldosterone stimulated secretion; ↓ Catecholamines
Kidney	↓ Renin
NSC	Neuromodulating actions on cognitive, sensitive, autonomic, locomotor functions
Neoplastic cells	↓ proliferation
Immune cells	↓ proliferation

SOMATOSTATIN ANALOGUES (SSA)

Somatostatin half-life: 2min!

Amino acid substitution makes the molecule resistant to degradation and increases the affinity for the receptor.

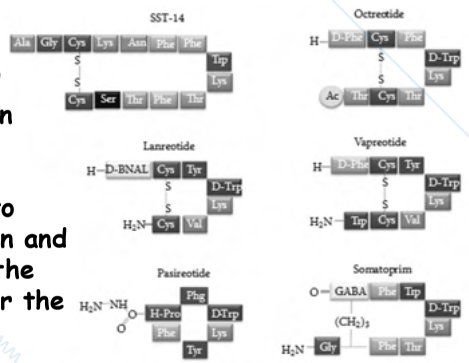
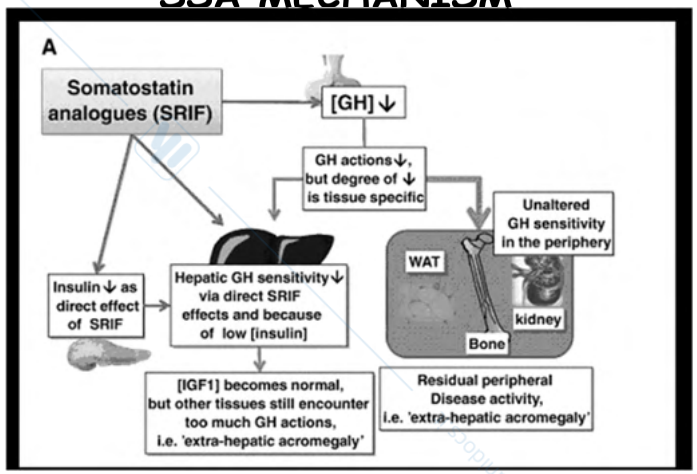


FIGURE 3: Amino acid composition of somatostatin 14 (SST-14) and of the main synthetic analogs (octreotide, lanreotide, vapreotide, pasireotide, and somatoprim).

Pituitary, gastrointestinal and pancreatic tumors (GEP-NETs)

SSA MECHANISM



Neggers, Van Der Lely
Combination treatment with somatostatin analogues and Pegvisomant in acromegaly
Growth Hormone & IGF Research Volume 21, Issue 3, June 2011

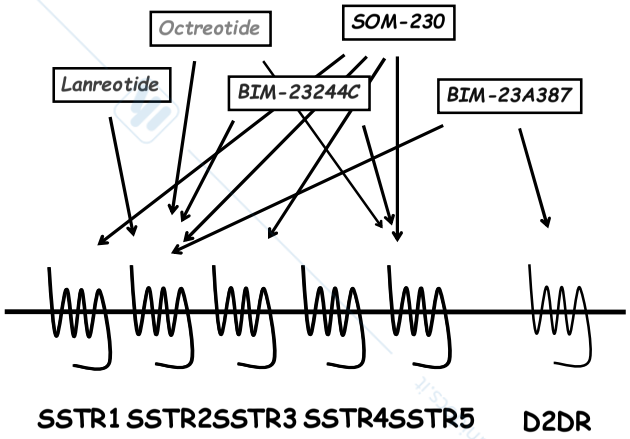
SOMATOSTATIN ANALOGS

Somatostatin agonists receptorial specificity (IC50-nM)

Compound	Receptor subtype					
	SSTR1	SSTR2	SSTR3	SSTR4	SSTR5	DAr2
SST14	1,95	0,25	1,2	1,77	1,41	-
SST28	1,86	0,31	1,3	-	0,4	-
Octreotide	280	0,4	7,1	>1000	6,3	-
Lanreotide	180	0,54	14	230	7	
Pasireotide	9,3	1	1,5	>100	0,16	-
Somatoprim	1000	3	>100	7	6	-
BIM23A760	622	0,03	160	>1000	42	24,5
Vapreotide	-	5.4	31	45	0.7	-

SSTR2 is expressed in GH-secreting cells, specific for acromegaly.
SSTR5 is also expressed in pancreatic islets and mediates the inhibition of insulin secretion.

SOMATOSTATIN RECEPTORS



SSTR subtype espression

- Each of the SSTR subtype is expressed in multiple tissues throughout the body.
- Within a given tissue, multiple, and, indeed quite often all, SSTR subtypes may be expressed.
- The studies of SSTR subtype function have revealed that each of the SSTR subtypes is involved in multiple functions.
- While a particular tissue may express multiple or all SSTR subtypes, it does not necessarily express them all at the same time.
- Expression of specific SSTR subtypes may be dependent on the prevailing environmental conditions or hormonal milieu of the tissue of interest.
- SSTR subtype expression can also change as a result of disease states.

M.D. Culler Digestive and Liver Disease 2004; 36 (Suppl. 1): S17–S25

Targeting multiple receptors

The problem was to produce stable analogues of SST that were effective in suppressing GH secretion without suppressing insulin secretion, one of the other prominent actions of SST.
The realisation that SST acts through multiple receptors suggested the possibility that SST may also achieve functional selectivity by acting through a specific receptor subtype to control a specific action. If true, this suggests that therapeutic specificity could potentially be achieved by producing SST analogues that selectively activate the appropriate receptor subtype.
Different subtypes are responsible for different functions: e.g.
- SSTR 5 directly suppresses insulin secretion from human pancreatic beta cells,
- SSTR 1 is the most efficacious receptor in suppressing out-sprouting of human placental vein explants, a model of angiogenesis.

…multiple SSTR subtypes may interact to regulate a given biological function

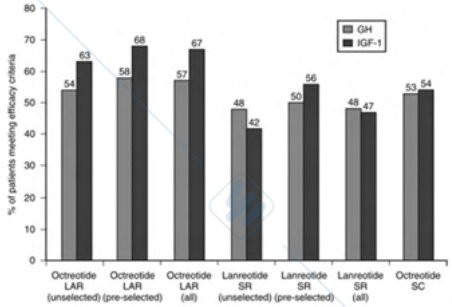
M.D. Culler Evolving concepts in the quest for advanced therapeutic analogues of somatostatin Digestive and Liver Disease 2004; 36 (Suppl. 1): S17–S25

FIRST GENERATION SSA

Table 1: Forms of Somatostatin Analogs, Duration of Action, and Dosage

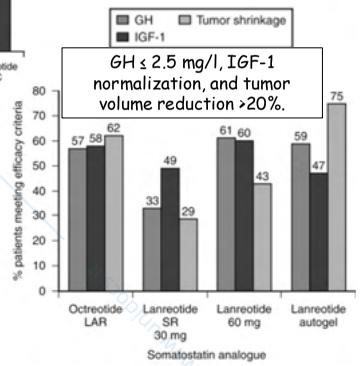
	Mode of Administration	Duration of Action	Dosage Target
Octreotide	Subcutaneous	6–8 hours	150–300 µg/day
Lanreotide SR	Intramuscular	10–14 days	10–20 mg/ 2 weeks
Octreotide LAR	Intramuscular	3–4 weeks	10–30 mg/ 4 weeks
Lanreotide Autogel	Subcutaneous	4–6 weeks	60–120 mg/ 6 weeks

SR: sustained release
LAR: long acting release



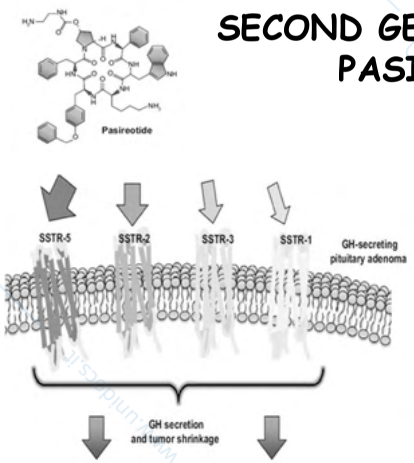
Freda et al., 2005

Efficacy of SST-analogue therapy for acromegaly.



- Biochemical normalization of GH (ogtt) and IGF levels.
- Reduction of tumore volume.

SECOND GENERATION SSA PASIREOTIDE



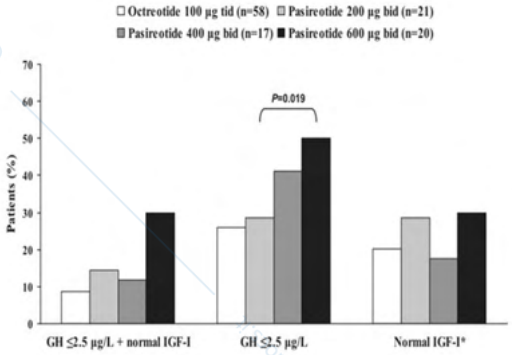
Pasireotide effects	
Effects	**Consequence**
Multireceptor ligand	Higher response rates
Inhibits GH secretion	Disease control
Tumor shrinkage	Surgery resection may be facilitated
Inhibits GLP-1 and insulin secretion	Hyperglycemia and diabetes
Inhibits CYP450	Check for potential drug interactions

Cuevas-Ramos (2016) Drug Design, Development and Therapy

PASIREOTIDE

Efficacy (randomized multicenter phase II trial)

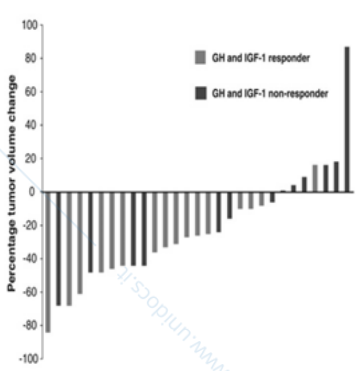
Percentage of patients who achieved a GH, IGF-I, or GH plus IGF-I response after 1 month of treatment with octreotide or pasireotide, by dose.
All patients received octreotide 100 microg tid for 1 month followed by 1 month of pasireotide 200, 400, or 600 microg bid. Results are after 1 month octreotide and 1 month octreotide plus 1 month pasireotide.

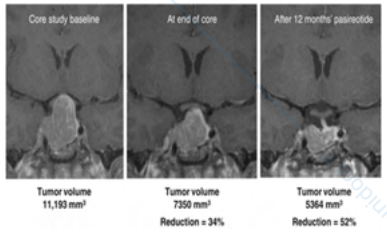


Petersenn et al. J Clin Endocrinol Metab 2010

PASIREOTIDE

Tumor volume change (EXTENSION STUDY 30/60)





Tumor shinkage was obtained in most of the patients though at variable extent. Tumor enlargement was observed in part of the patients.

Petersenn et al. Pituitary 2014

PASIREOTIDE

SAFETY (randomized multicenter phase II trial)

TABLE 4. Adverse events (with a frequency of at least 5%) with a suspected study-drug relationship occurring during 1 month of treatment with octreotide and 3 months treatment with pasireotide

	Octreotide 100 μg sc tid	Pasireotide 200, 400, or 600 μg sc bid
Any suspected drug-related adverse event	30 (50.0)	45 (75.0)
Nausea	9 (15.0)	15 (25.0)
Diarrhea	20 (33.3)	13 (21.7)
Abdominal pain	5 (8.3)	7 (11.7)
Flatulence	5 (8.3)	6 (10.0)
Blood glucose increased	1 (1.7)	4 (6.7)[a]
Dizziness	1 (1.7)	4 (6.7)
Increased HbA_{1c}	0	3 (5.0)[a]
Vertigo	0	5 (5.0)
Diabetes mellitus	0	3 (5.0)[a]

Data are shown as n (%).

[a] These events are reported in different patients.

TABLE 5. Fasting blood glucose levels at baseline and at the end of the study in the 59 patients with post-baseline measurements[a]

Baseline fasting blood glucose level[b]	End of study fasting blood glucose level[c] <5.6 mmol/liter	5.6–7.7 mmol/liter	≥7.7 mmol/liter
<5.6 mmol/liter (n = 38)	28 (73.7)	7 (18.4)	3 (7.9)
5.6–7.7 mmol/liter (n = 17)	2 (11.8)	6 (35.3)	9 (52.9)
≥7.7 mmol/liter (n = 4)	0	0	4 (100)

[a] Data are shown as n (%). Of the 60 patients evaluated for fasting blood glucose levels, one patient did not have a post-baseline measurement and was therefore not included in the analysis.

[b] Based on the American Diabetes Association criteria, 2004.

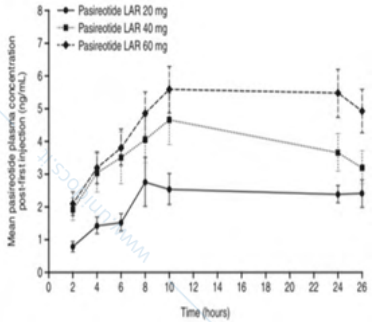
[c] One patient did not have an end-of-study assessment; the last available value was used (visit 7).

Petersenn et al. J Clin Endocrinol Metab 2010

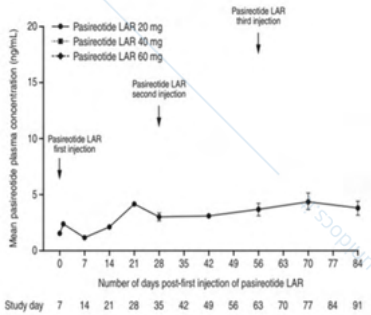
PASIREOTIDE LAR

Bioequivalence

Pasireotide plasma concentration from 0 to 26 hours after the first im injection of pasireotide LAR 20, 40, or 60 mg in patients with acromegaly.



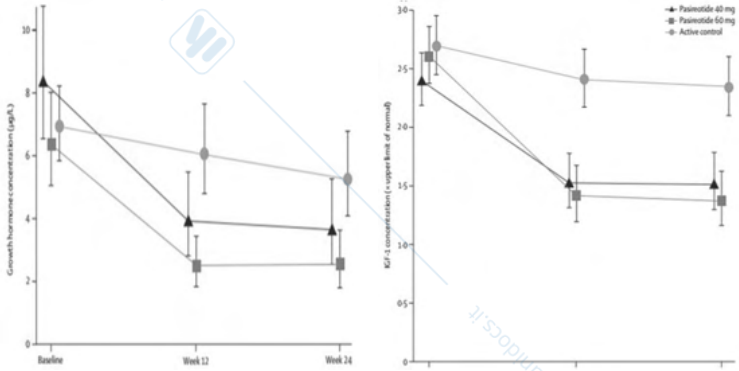
Petersenn et al. J Clin Pharmacol 2014



Pasireotide plasma concentration following monthly im injection with pasireotide LAR 20, 40, or 60 mg in patients with acromegaly /Steady state achieved after the 3rd injection.

PASIREOTIDE LAR

Efficacy



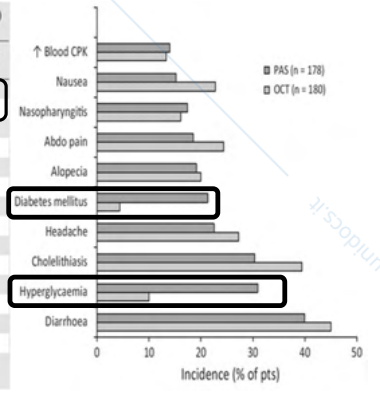
Gadelha et al. (2014) *Pasireotide versus continued treatment with octreotide or lanreotide in patients with inadequately controlled acromegaly (PAOLA): a randomised, phase 3 trial* – Lancet Diabetes Endocrinol

PASIREOTIDE LAR

Side effects

	40 mg pasireotide (n=63)		60 mg pasireotide (n=62)		Active control (n=66)	
	All grades	Grade 3 or 4	All grades	Grade 3 or 4	All grades	Grade 3 or 4
Hyperglycaemia	21 (33%)	7 (11%)	19 (31%)	5 (8%)	9 (14%)	0
Diabetes	13 (21%)	0	16 (26%)	2 (3%)	5 (8%)	0
Diarrhoea	10 (16%)	0	12 (19%)	0	3 (5%)	1 (2%)
Cholelithiasis	6 (10%)	0	8 (13%)	0	9 (14%)	0
Headache	9 (14%)	0	2 (3%)	0	3 (5%)	0
Nasopharyngitis	4 (6%)	0	7 (11%)	0	2 (3%)	0
Abdominal pain	5 (8%)	2 (3%)	5 (8%)	0	2 (3%)	0
Impaired glucose tolerance*	2 (3%)	0	3 (5%)	0	4 (6%)	0
Nausea	2 (3%)	0	4 (6%)	0	2 (3%)	0
Anaemia	4 (6%)	0	2 (3%)	0	2 (3%)	0
Dizziness	5 (8%)	0	1 (2%)	0	2 (3%)	0
Increased blood glucose	3 (5%)	0	4 (6%)	0	0	0
Hypoglycaemia	2 (3%)	0	4 (6%)	1 (2%)	0	0
Alopecia	1 (2%)	0	4 (6%)	0	0	0
First degree atrioventricular block	4 (6%)	0	0	0	0	0

Gadelha et al. (2014) *Pasireotide versus continued treatment with octreotide or lanreotide in patients with inadequately controlled acromegaly (PAOLA): a randomised, phase 3 trial* – Lancet Diabetes Endocrinol –



McKeage et al. (2015) *Pasireotide in acromegaly: a review.* –

PASIREOTIDE LAR vs OCTREOTIDE LAR
Head-to-Head

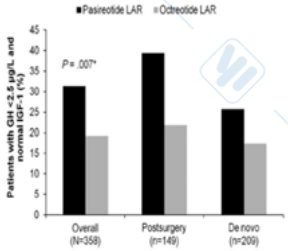


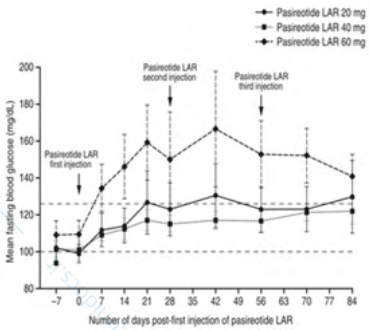
Table 3. Adverse Events Regardless of Study-Drug Relationship Reported in ≥10% of Patients in Either Treatment Group

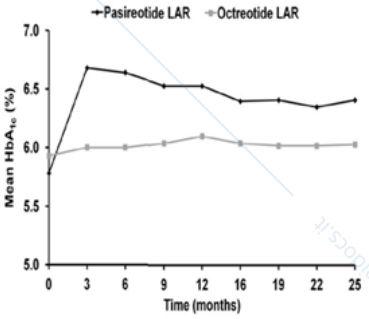
	Pasireotide LAR, n = 178[a]		Octreotide LAR, n = 180[a]	
	All Grades, n (%)	Grade 3/4, n (%)	All Grades, n (%)	Grade 3/4, n (%)
Diarrhea	70 (39.3)	1 (0.6)	81 (45.0)	4 (2.2)
Hyperglycemia	51 (28.7)	6 (3.4)	15 (8.3)	1 (0.6)
Cholelithiasis	46 (25.8)	1 (0.6)	64 (35.6)	2 (1.1)
Diabetes mellitus	34 (19.1)	9 (5.1)	7 (3.9)	0
Headache	33 (18.5)	2 (1.1)	46 (25.6)	5 (2.8)
Abdominal pain	32 (18.0)	1 (0.6)	40 (22.2)	0
Alopecia	32 (18.0)	0	35 (19.4)	0
Nasopharyngitis	28 (15.7)	0	28 (15.6)	0
Nausea	24 (13.5)	1 (0.6)	39 (21.7)	0
Increased blood creatine phosphokinase	23 (12.9)	3 (1.7)	21 (11.7)	4 (2.2)
Abdominal distension	21 (11.8)	1 (0.6)	21 (11.7)	1 (0.6)
Arthralgia	17 (9.6)	1 (0.6)	22 (12.2)	1 (0.6)
Fatigue	17 (9.6)	1 (0.6)	18 (10.0)	0
Dizziness	17 (9.6)	0	19 (10.6)	0
Back pain	14 (7.9)	0	20 (11.1)	2 (1.1)

[a] Two patients randomized to the octreotide LAR treatment arm received pasireotide LAR in error. These 2 patients are included in the pasireotide LAR treatment arm for the purposes of the safety analysis.

Colao AM et al JCEM 2014

PASIREOTIDE LAR
Glucose metabolism





Petersenn et al. (2014) ***Pharmacokinetics, Pharmacodynamics, and Safety of Pasireotide LAR in Patients With Acromegaly: A Randomized, Multicenter, Open-Label, Phase I*** **- The Journal of Clinical Pharmacology - 54 1308–1317**

Sheppard et al. (2015) ***Pasireotide LAR maintains inhibition of GH and IGF-1 in patients with acromegaly for up to 25 months: results from the blinded extension phase of a randomized, double-blind, multicenter, Phase III study*** **– Pituitary – 10,1007/s11102-014-0585-6**

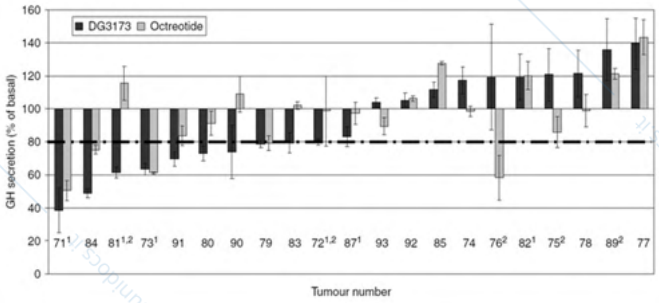
PASIREOTIDE AND T2DM

The short term continuous administration of increasing doses of pasireotide in these subjects increased blood glucose levels associated with decreased insulin secretion, with no significant change in glucagon output.
A significant inhibitory effect was demonstrated on intestinal GLP-1 and GIP secretion as well, whereas hepatic and peripheral insulin sensitivity was unaffected by pasireotide.

Pasireotide has limited applicability in acromegaly patients because it worsen the diabetogenic profile previously caused by the GH-excess.

SOMATOPRIM
(DG 3173 or PTR-3173)

- Affinity for human SSTR2, SSTR4, SSTR5
- Suppress GH secretion in more adenomas than octreotide (10/21 vs 5/21), including 38% (6/16) of octreotide non-responders.

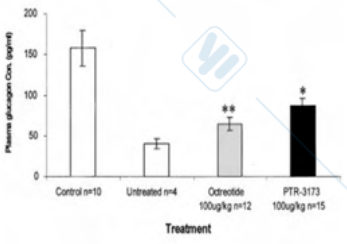


Plockinger et al. (2012) DG3173 (somatoprim), a unique somatostatin receptor subtypes 2-, 4- and 5-selective analogue, effectively reduces GH secretion in human GH-secreting pituitary adenomas even in octreotide non responsive tumors - EJE

SOMATOPRIM

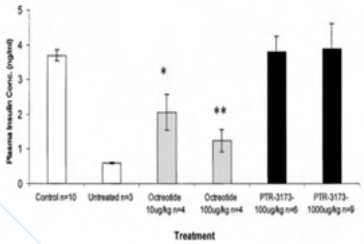
Glucose metabolism

- **No effect on insulin and minimal effect on glucagon secretion**



Octreotide significantly inhibited glucagon release by 70%, whereas PTR-3173 reduced plasma glucagon levels with less potency (55%) than octreotide compared with control values.

Afargan et al. Endocrinology 2001

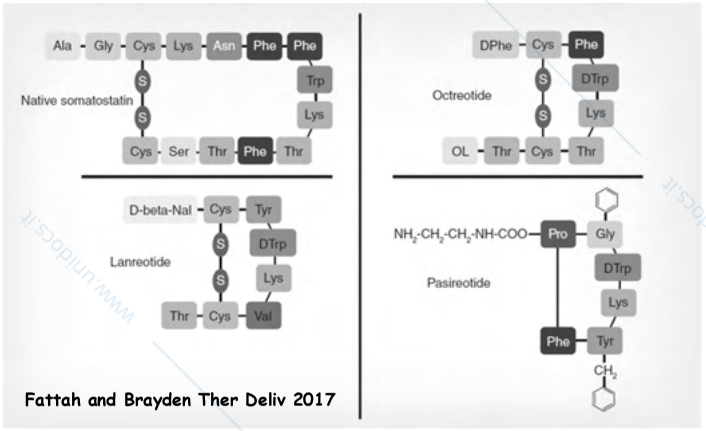


Plasma insulin levels were reduced significantly in the octreotide treated group. PTR-3173, at 100 or 1000 mg/kg, did not affect insulin release.

** P < 0.05; ** P < 0.01; *** P < 0.001 (vs. control)*

NOVEL ADMINISTRATION STRATEGIES

Octreotide is stable against GI enzymes because the amino acid at position 4, the most vulnerable to peptidase, is in the stable D-conformation, which makes it resistant



Fattah and Brayden Ther Deliv 2017

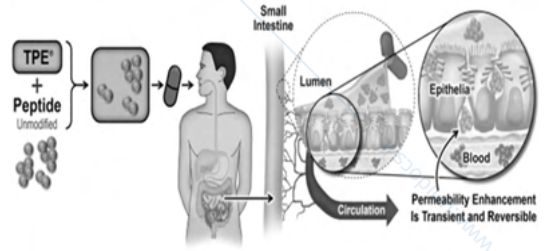
Octreotide capsules

(Octreolin)

Oral ingested octreotide fails to achieve therapeutic drug levels because of low small intestinal epithelial permeability

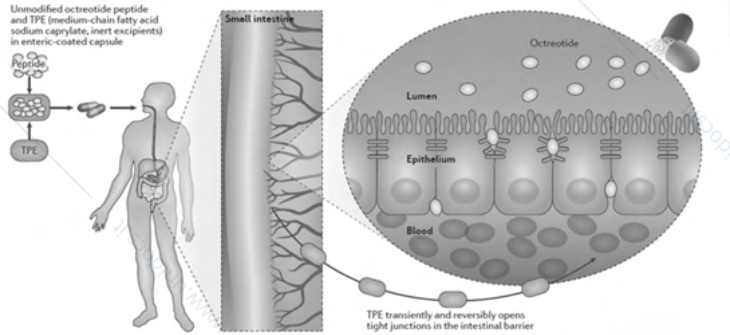
…but….

TPE (transient permeability enhancer) technology



The low bioavailability of octreotide is mostly caused by low small intestinal epithelial permeability (<0.3%)

MYCAPSSA™ or OCTREOLIN™



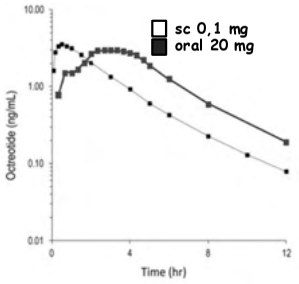
Melmed S Nature Reviews Endocrinol 2016

Octreotide capsules

(Octreolin)

PHASE I study

- **After oral dosing, peak levels were achieved at 2.7 h, whereas the analogous peak after subcutaneous injection was seen at 0.6 h.**
- **The half-life of octreotide was similar regardless of the route of administration.**
- **Therapeutic octreotide levels (≥1 µg/l) were sustained for 6 h with octreotide capsules and for 4 h with injected octreotide.**
- **Subtherapeutic levels (<0.5 µg/l) are reached after 8 h with octreotide capsules and after 6 h with injected octreotide.**



healthy volunteers

Tuvia et al. J Clin Endocrinol Metab 2012

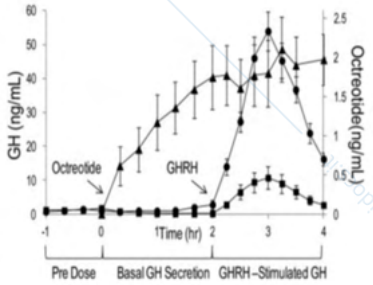
Octreotide capsules

(Octreolin)

PHASE I study

Oral administration of octreotide acetate suppresses the basal GH levels and secretory response to GHRH stimulation.

A single octreotide capsule dose significantly suppressed levels of GH, reducing the average basal GH level by 44% (P <0.05).
After GHRH stimulation, average GH peaks were 80% lower in individuals treated with oral octreotide than in untreated individuals
(12.2 ± 3.6 µg/l versus 56.1 ± 6.0 µg/l; P <0.001).



Plasma GH and octreotide (closed triangles) concentrations (n=16) before and after GHRH/arginine administration, with (closed squares) and without (closed circles) oral octreotide acetate treatment (20 mg).

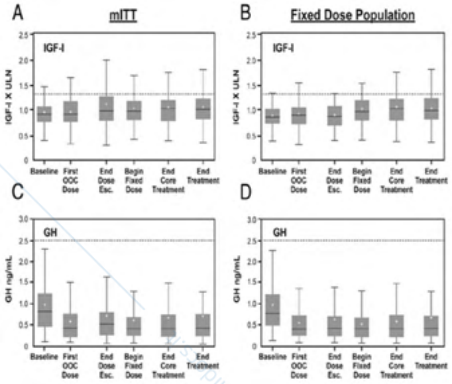
Tuvia et al. J Clin Endocrinol Metab 2012

Octreotide capsules

(Octreolin)

PHASE III study

- Enrolled 155 patients with acromegaly, who had been treated on stable doses of injectable SRL therapy for at least 3 months and then switched to octreotide capsules at least 4 weeks after the last injection.
- The goal of the trial was to determine whether baseline levels of GH and IGF-1 could be maintained with octreotide capsules.
- 65% maintained response and achieved the primary endpoint (IGF-1< 1.3ULN and mean integrated GH < 2.5 ng/mL).
- The effect was durable: 85% of subjects initially controlled on OOCs maintained this response up to 13 months.



Melmed et al. J Clin Endocrinol Metab 2015

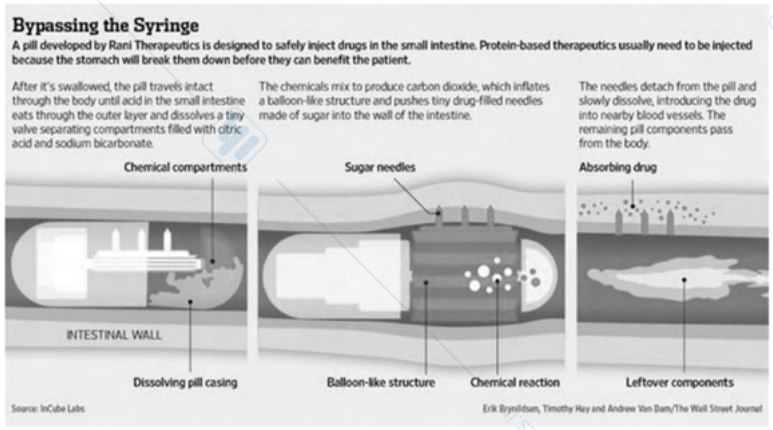
Octreotide capsules

(Octreolin)

…but for FDA and EMA the data are not enough

MPOWERED is ongoing

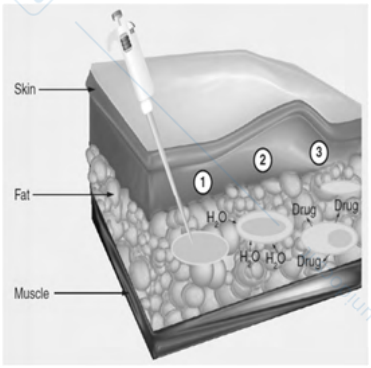
NEXTCOMING FUTURE…… MICRONEEDLE-BASED INTESTINAL DELIVERY SYSTEM



https://www.ranitherapeutics.com/

CAM 2029

- CAM2029 is based on Fluid Crystal R technology, which is administered by the subcutaneous route once a month.
- Octreotide is suspended in a liquid matrix which permits the use of thin 22–27G needles.
- The depot formulation absorbs water from tissue resulting in highly viscous liquid-crystal gel phase from which octreotide diffuses passively at constant rate.



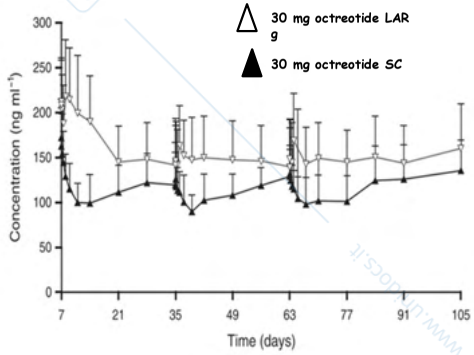
After sc.injection, the formulation absorbs water leading to liquid crystal gel formation (1) followed by biodegradation of depot (2) and release of the active substrate from the matrix (3).

Fattah and Brayden Ther Deliv 2017

CAM 2029

IGF1 reduction

Suppression of mean IGF-1 concentrations was observed during the 24 h post-injection period of single dose sc octreotide, which gradually increased with time until the next injection.
By contrast, octreotide LAR provided a slower and more gradual suppression of IGF-1.



Tiberg F et al. Br. J. Clin. Pharmacol. 2015

CAM 2029

Adverse event, *n* (%)	Oct s.c. depot B 30 mg *n* = 14	Oct LAR 30 mg *n* = 14
Any AE	14 (100.0)	10 (71.4)
Diarrhoea	11 (78.6)	5 (35.7)
Headache	7 (50.0)	6 (42.9)
Abdominal pain	5 (35.7)	2 (14.3)
Injection site pain	3 (21.4)	3 (21.4)
Nasopharyngitis	4 (28.6)	2 (14.3)
Injection site pruritis	1 (7.2)	0 (0.0)
Injection site induration	3 (21.4)	1 (7.1)
Nausea	0 (0.0)	1 (7.1)
Flatulence	2 (14.3)	1 (7.1)
Injection site erythema	2 (14.3)	0 (0.0)
Injection site swelling	3 (21.4)	1 (7.1)
Vomiting	0 (0.0)	0 (0.0)
Injection site haematoma	0 (0.0)	0 (0.0)

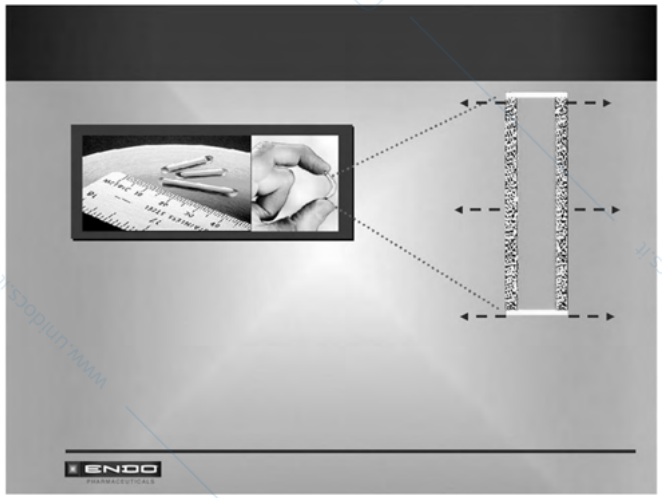
- The results from subsequent Phase II study also showed a well-maintained control of disease symptoms in NET and acromegaly patients.
- Phase III study is planned.

Tiberg F et al. Br. J. Clin. Pharmacol. 2015

INTRANASAL OCTREOTIDE
(DP1038)

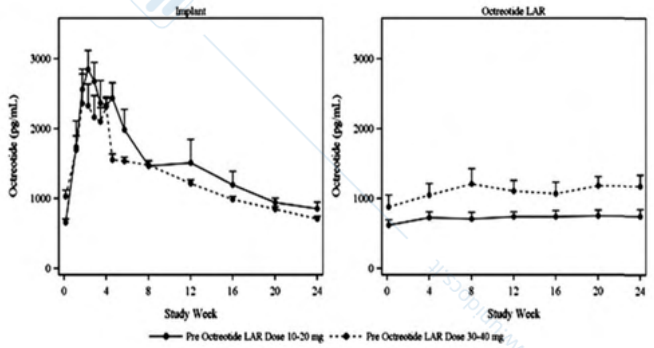
- Intravail R maltoside-based permeation enhancer (PE) technology.
- Breath-actuated metered spray device closes the soft palate when the user blows into the device resulting in substantially increased surface area exposure within the nasal cavity.

OCTREOTIDE IMPLANT



OCTREOTIDE IMPLANT
Bioequivalence

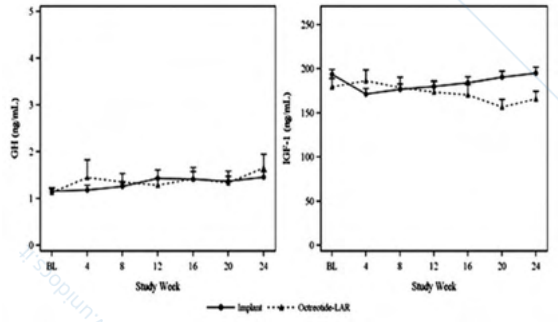
Serum octreotide concentrations after implant insertion increased within 8 days and peaked between days 14 and 28.



Chieffo et al. J Clin Endocrinol Metab 2013

OCTREOTIDE IMPLANT
Efficacy

The success rate of the implant was 86% compared with a rate of 84% for octreotide LAR.



Chieffo et al. J Clin Endocrinol Metab 2013

OCTREOTIDE IMPLANT

Safety

The overall safety of the octreotide implant and octreotide LAR are similar.

- Diarrhea and headache were more frequent with the implant.
- Cholecystitis and hypertension were more frequent with octreotide LAR.

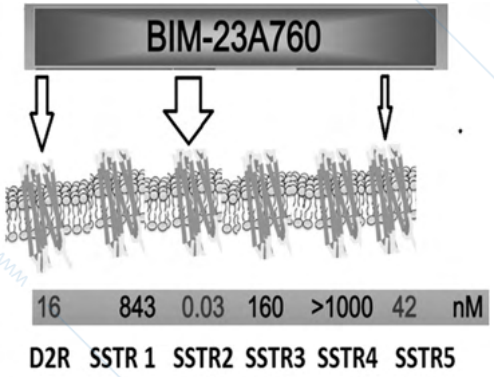
	No. (%) Subjects Reporting Events	
System Organ Class Preferred Term	Implant (n = 122)	Octreotide LAR (n = 41)
Diarrhea	12 (9.8)	3 (7.3)
Headache	12 (9.8)	2 (4.9)
Hypertension	10 (8.2)	6 (14.6)
Cholelithiasis	9 (7.4)	3 (7.3)
Nasopharyngitis	9 (7.4)	1 (2.4)
Arthralgia	6 (4.9)	3 (7.3)
Cholecystitis	1 (0.8)	5 (12.2)

Chieffo et al. J Clin Endocrinol Metab 2013

DOPASTATIN

Bim-23A760

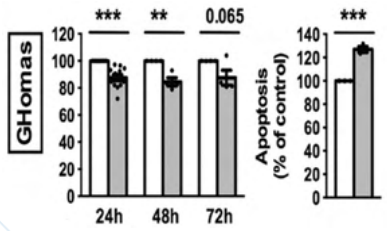
Chimeric somatostatin-dopamine receptors agonist acting on SSTR 2, SSTR 5 and D2



DOPASTATIN

Bim-23A760

Effect of BIM-23A760 on pituitary hormone release and/or expression in primary human pituitary cell cultures from GHomas.



Ibáñez-Costa et al. Scientific Reports 2017

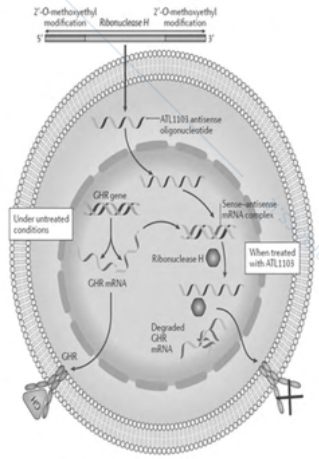
*p < 0.05, **p < 0.01

Results from clinical trials in acromegaly however, provide evidence of relatively weak efficacy of dopastatin injections, so its further development as a therapeutic for management of acromegaly has been halted by Ipsen.

ATL1103

GH receptor antisense oligonucleotide

The modified 20-mer antisense oligonucleotide ATL1103 forms a complex with growth hormone receptor (GHR) mRNA. Cleavage by ribonuclease H breaks apart the hybrid, and GHR mRNA is degraded, which prevents translation of the GHR target protein, thereby abrogating the ability of growth hormone (GH) to signal.

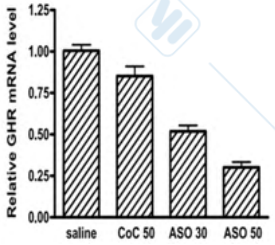


ATL1103

GH receptor antisense oligonucleotide

Preclinical study

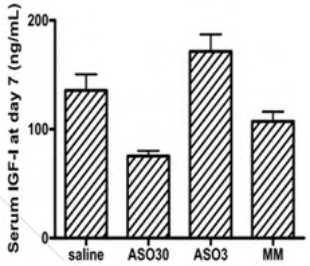
ATL1103 knocked down GH receptor mRNA by 87% in mouse cells *in vitro*, and by 70% in mice *in vivo*.



Relative GHR mRNA levels detected in liver of mice treated with saline, GHR antisense oligonucleotide (ASO) at doses of 30 mg/kg (ASO 30) or 50 mg/kg (ASO 50), or oligonucleotide control (CoC) delivered at 50 mg/kg (CoC50).

Tachas et al. Journal of Endocrinology 2006

Knockdown of GH receptor mRNA in liver tissue resulted into a 59% reduction in serum levels of IGF-1 after 10 weeks.

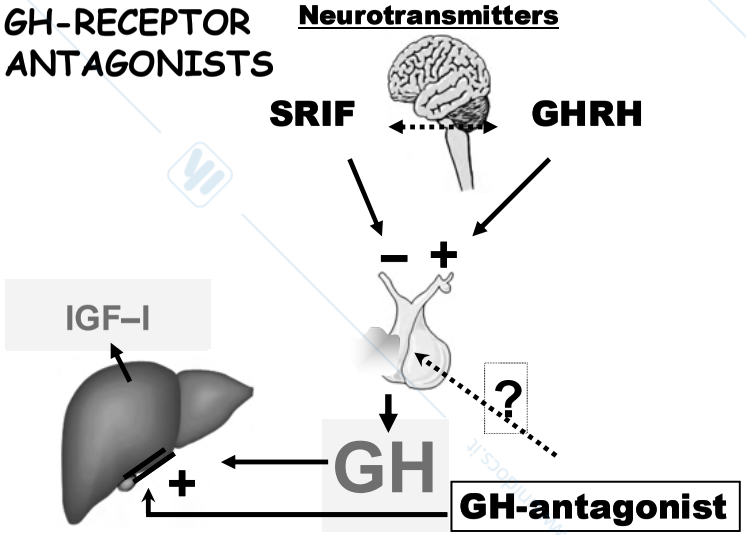


Serum IGF-I levels at day 7, normalised to standard control serum values. Mice were treated s.c. every second day for 7 days with saline, GHR antisense oligonucleotide (ASO) at either 30 mg/kg (ASO30) or 3 mg/kg (ASO3), or oligonucleotide control (CoC) at 30 mg/kg (CoC 30).

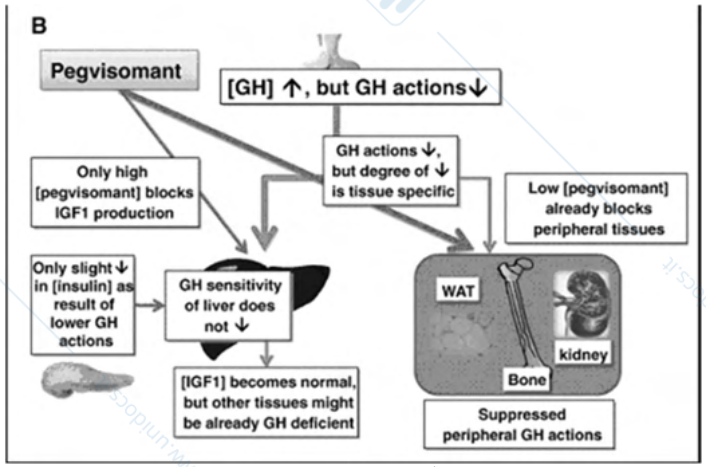
ATL1103

GH receptor antisense oligonucleotide

- Phase I clinical trial: randomized, placebo controlled, double blind study of single ascending doses and multiple doses of subcutaneous ATL1103 (75 mg, 250 mg, 400 mg) in 32 healthy male adults in two stages. In the ascending doses stage, 24 participants were given a placebo or ATL1103 and showed a clear trend for lower mean serum levels of IGF-1 than at baseline by day 14, with a statistically significant effect on day 21.
- Phase II, multicentre study: 26 patients with acromegaly and IGF-1 levels >1.3 × ULN (upper limit of normal) were randomly assigned to receive 200 mg ATL1103 once or twice weekly for 13 weeks, with an additional 4 weeks of follow-up. With twice-weekly dosing, IGF-1 levels were reduced by 26% from baseline by week 14 (577 +/- 198 ng/mL vs 411 +/- 174 ng/mL, p<0,0001), although the normalization had not been reached. Pharmacokinetic modelling indicated that the IGF-1 nadir would be achieved at 21 weeks.
- A trial using a higher dose of ATL1103 than in previous trials (600 mg per week) is planned to determine whether efficacy measures can be improved without affecting the safety profile.
- ...it is as yet unclear whether the drug will yield benefit over the GH antagonist pegvisomant.

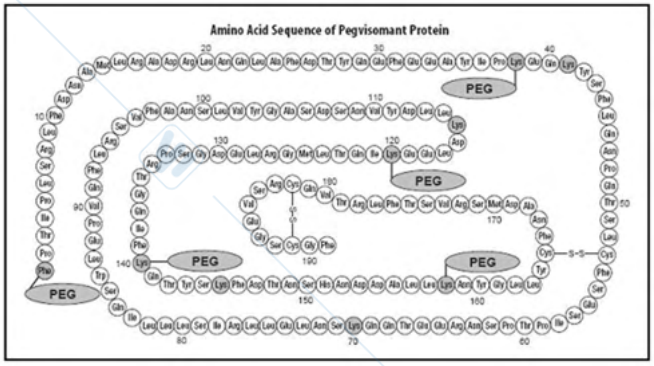


PEGVISOMANT MECHANISM



Neggers, Van Der Lely
Combination treatment with somatostatin analogues and Pegvisomant in acromegaly
Growth Hormone & IGF Research Volume 21, Issue 3, June 2011

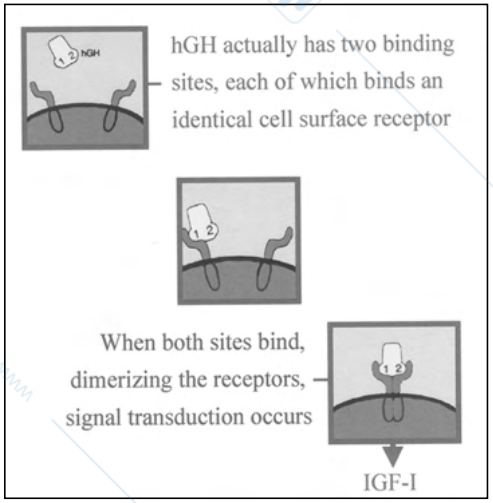
PEGVISOMANT STRUCTURE



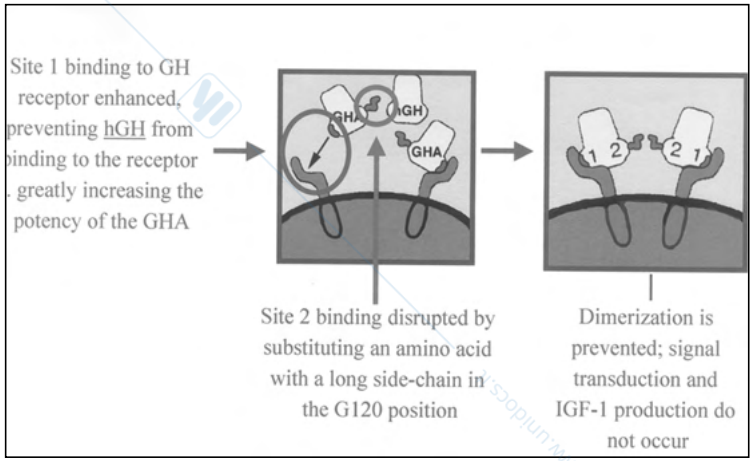
191 amino acid protein covalently conjugated with several polyethylene glycol polymers reducing its clearance.
The protein is a human growth hormone analog designed to bind to and block the growth hormone receptor.

http://images.ddccdn.com/pro/images/f2ea11f2-d3d5-4aec-b46d-9ce13a5d2a64/somavert-01.jpg

GH – GH receptor interaction



GH-Antagonists mechanism



INCIDENTALLY DISCOVERED NON SECRETING ADENOMAS

PITUITARY INCIDENTALOMAS
HOW TO MANAGE IT?

1. DIAGNOSIS
2. EVALUATION OF COMRESSION EFFECT ON SURROUNDING AREAS
3. EVALUATION OF THE PITUITARY HORMONES

Large NFPAs often cause significant hypothalamic/ pituitary dysfunction and visual symptoms.
However, others may be completely asymptomatic, being detected either at autopsy or as incidental findings on head magnetic resonance imaging (MRI) or computed tomography (CT) scans performed for other reasons.
This latter group has been referred to as PITUITARY INCIDENTALOMAS.

A number of other lesions may be found in the sellar area that may mimic a pituitary adenoma, including aneurysms of the internal carotid artery, craniopharyngiomas, Rathke's cleft cysts, meningiomas of the tuberculum sellae, gliomas of the hypothalamus and optic nerves, dysgerminomas, cysts, hamartomas, metastases, sarcoidosis, eosinophilic granulomas, sphenoid sinus mucocoeles and focal areas of infarction. Lymphocytic infiltration of the pituitary can also masquerade as a pituitary adenoma.

CLINICAL-PATHOLOGICAL CLASSIFICATION OF NON FUNCTIONAL PITUITARY ADENOMAS (NFPA)

Table 1
Clinico-pathological classification of NFPA.

Tumor type	Transcription factors	Hormone staining
Gonadotroph adenomas	SF-1, GATA-2, ER	β-FSH, β-LH, α-subunit
Silent adenomas		
Silent somatotroph adenomas	Pit-1	GH
Silent prolactinomas	Pit-1, ER	PRL
Silent thyrotroph adenomas	Pit-1, TEF, GATA-2	β-TSH, α-subunit
Silent corticotroph adenomas	Tpit	ACTH
Null cell adenomas	None	None
Silent subtype 3 adenomas		Multiple

SF-1, steroidogenic factor-1; ER, estrogen receptor; Pit-1, pituitary transcription factor-1; TEF, thyrotropin embryonic factor. Adapted from Asa et al[12].

Greenman and Stern 2009

CLINICAL FEATURES OF NON FUNCTIONAL PITUITARY ADENOMAS

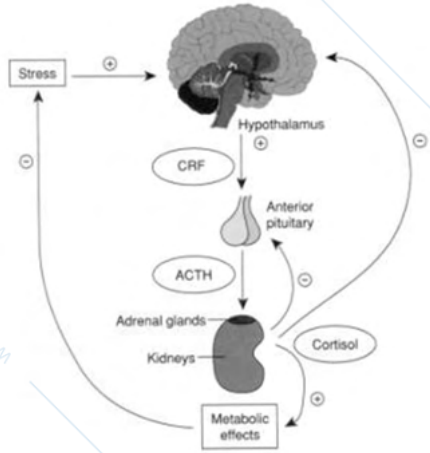
Table 2
Clinical characteristics of NFPA patients.

	Nomikos et al[15]	Losa et al[16]	Chang et al[17]	Ferrante et al[51]	Total
Number of patients	721	491	663	295	2170
Mean age	54.2 ± 19	–	53 (median)	50.4 ± 14.1	
Gender (M/F)	401/320	276/215	394/269	161/134	1232/938 (56.7% M)
Incidental finding	57 (7.9%)	57 (11.6%)	49 (7.4%)	–	163/1875 (8.7%)
Headaches	70 (9.7%)	–	212 (32%)	122 (41.4%)	404/1679 (24%)
Visual deficits	222 (30.8%)	287/486 (59.1%)	327 (49%)	200 (67.8%)	1036/2170 (47.7%)
Pressure on cranial nerves	–	22 (4.5%)	26 (3.9%)	–	48/1154 (4.2%)
Apoplexy	27 (3.7%)	48 (9.8%)	24 (3.6%)	–	99/1875 (5.3%)
Symptoms of Hypopituitarism	345 (47.8%)	–	342 (51.6%)	118 (40%)	805/1679 (48%)
Documented					
Hypopituitarism	614 (85%)		–	183 (62%)	797/1016 (78.4%)
Hypogonadism	512/659 (77.7%)	335/474 (70.7%)		128 (43.3%)	975/1261 (77.3%)
Hypoadrenalism	230 (31.9%)	115/478 (24.1%)		77 (26.2%)	422/1494 (28.2%)
Hypothyroidism	129/658 (19.6%)	116/462 (25.1%)		72 (24.5%)	317/1415 (22.4%)
Hyperprolactinemia	199 (27.6%)	251/462 (54.3%)	–	82 (27.6%)	532/1478 (35.9%)

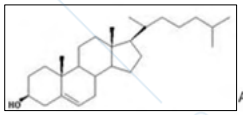
Greenman and Stern 2009

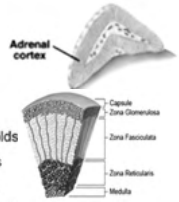
CUSHING'S SYNDROME

HYPOTHALAMUS – PITUITARY – ADRENAL AXIS



STEROID BIOSYNTHESIS

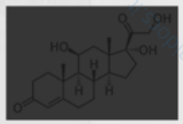




Adrenal steroidogenesis pathway

Mineralocorticoids
Glucocorticoids
Sex hormones

Cholesterol
ACTH
P450scc
StAR protein
Pregnenolone
17α-hydroxylase
17-OH pregnenolone
17,20 Lyase
Dehydroepiandrosterone sulfate
Dehydroepi-androsterone
17β-HSD
Androstenediol
3β-HSD
Progesterone
17-OH progesterone
Androstenedione
Testosterone
21-Hydroxylase
Deoxycorticosterone
11-Deoxycortisol
Aromatase
Estrone
Estradiol
11β-Hydroxylase
Corticosterone
Cortisol
18-Hydroxylase
18-OH corticosterone
18-Oxidase
Aldosterone



CORTISOL EXCESS

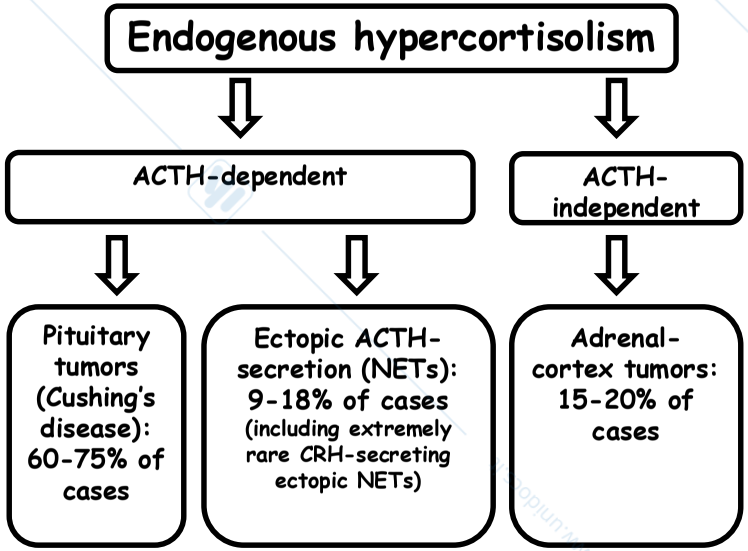
CUSHING's SYNDROME

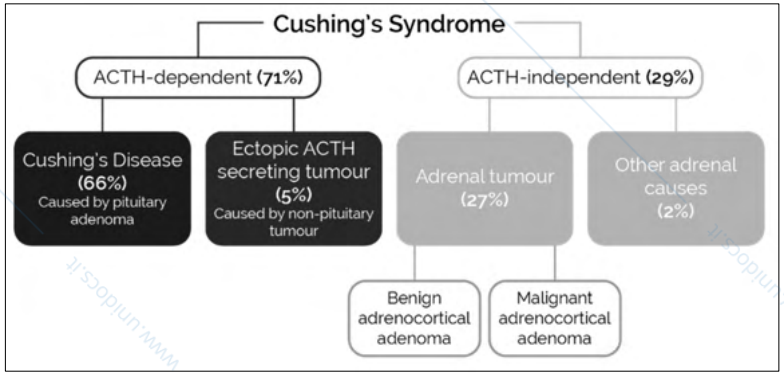
CHRONIC CORTISOL EXCESS - HYPERCORTISOLISM

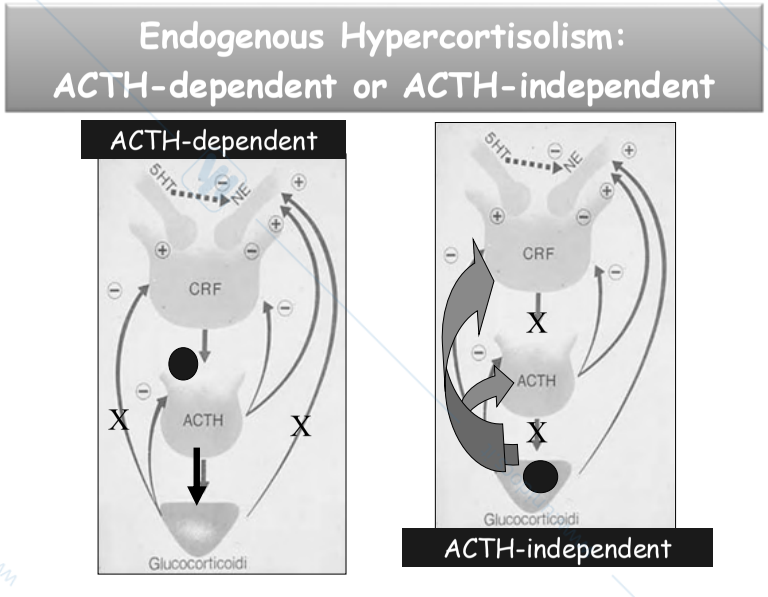
Endogenous
Cortisol overproduction caused by:

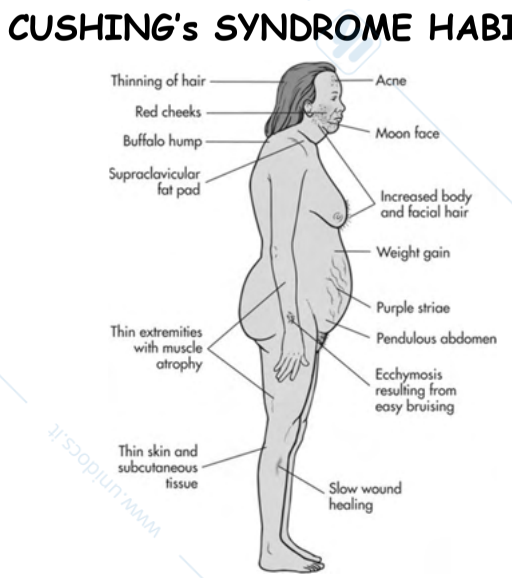
- **Pituitary tumor** ~70% of cases
- **Adrenal-cortex tumor** ~15% of cases
- **Other / unknown** ~15% of cases

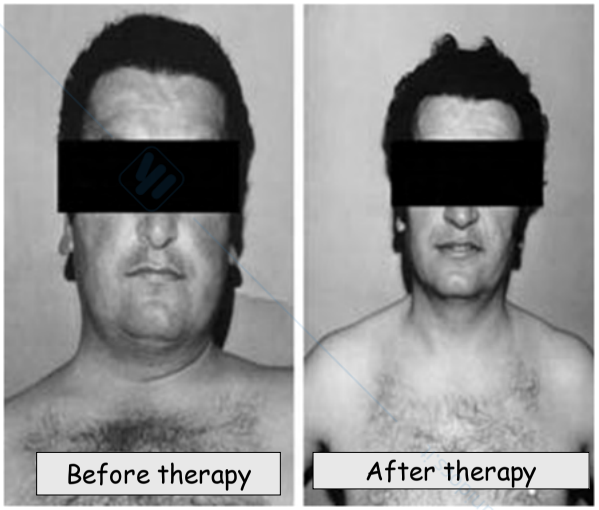
Exogenous / Iatrogenic
Long-term glucocorticoid drugs medications



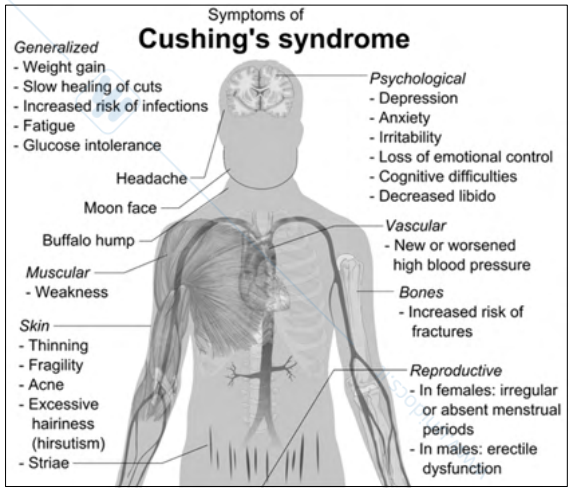




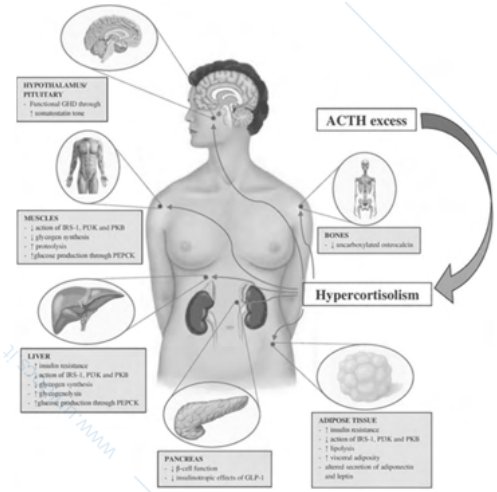


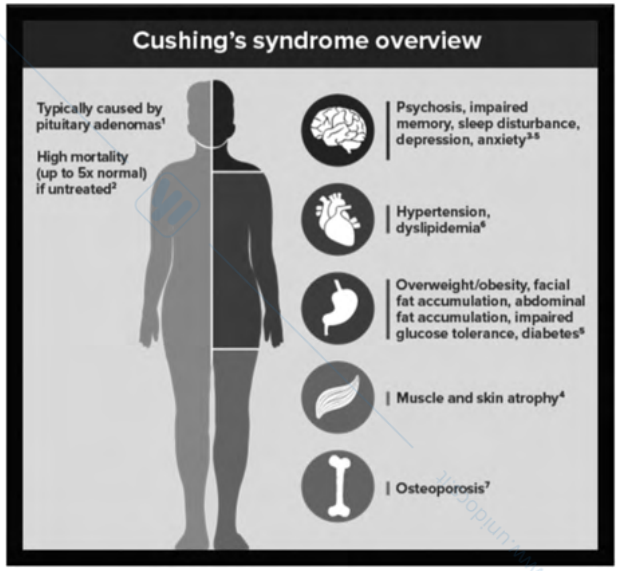


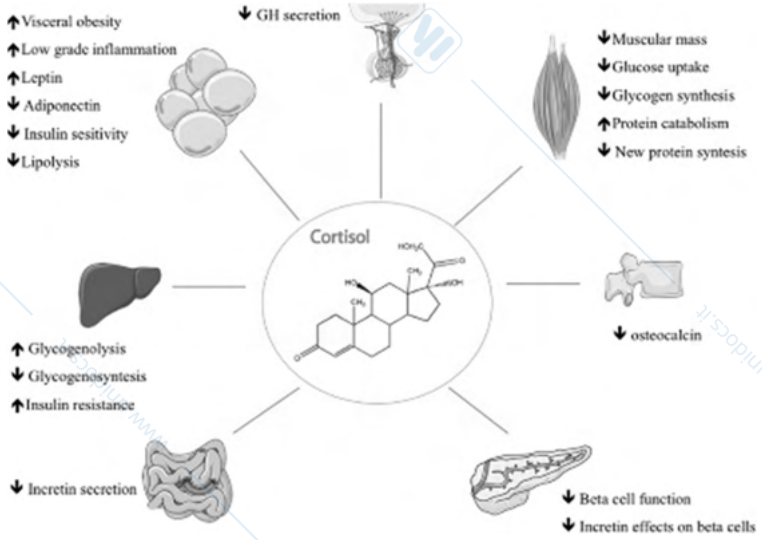
CUSHING's SYNDROME SYMPTOMS



TISSUE-SPECIFIC DERANGEMENTS OF HYPERCORTISOLISM







CORTISOL PHYSIOPATHOLOGY
metabolism

- LIVER stimulating glucose/glycogen turnover;
  promotes glycogen storage
  (+ glycogen-synthase, - glycogen-phosphorylase)
  promotes glycogenolysis
  (promotes adrenaline-stimulated glycogen-phosphorylase action)
  promotes glucose release
  (+ glucose-6-phosphatase e phosphoenolpyruvate carboxykinase)

- SKELETAL MUSCLE inhibits glucose uptake and glycogenolysis;

- ADIPOSE TISSUE stimulates lipolysis and free fatty acid release in the bloodstream;

  stimulates adipocytes differentiation and the increase in visceral fat depots (induces the expression of GR and type I 11ß-HSD).

Visceral obesity
Dyslipidemia
⟷
Insulino-resistance
Diabetes mellitus

CORTISOL PHYSIOPATHOLOGY
blood pressure

- VASCULAR SMOOTH MUSCLE
  increases the sensitivity to vasopressor hormones (catecholamines and angiotensin II)
  reduces NO-mediated vasodilation

- LIVER
  stimulates angiotensinogen synthesis

- KYDNEY
  increases Na retention and K loss

- HEART
  increases the cardiac output

Heart hyperthrophy
⟷
Arterial hypertension

CORTISOL PHYSIOPATHOLOGY
coagulation profile

- BLOOD: increases levels of VIII factor, von Willebrand factor and fibrinogen

→ Thromboembolic events

PATIENTS EXPERIENCE FRACTURES EVEN WITH MINIMAL TRAUMA

Table 3 Fracture energies. Low-energy fractures: fractures occurring after minimal or no trauma; medium-energy fractures: fractures occurring after, for example, a fall at the same level; high-energy fractures: fractures occurring after a fall from one level to another or following, for example, a car accident.

Type	Low-energy fractures	Medium + high energy fractures
Cushing's syndrome	4 (9.5%)	38 (90.5%)
Controls	3 (1.8%)	167 (98.2%)

Fisher's exact test: $P = 0.004$.

Vestergaard P, Eur J Endocrinol. 2002 146:51.

CORTISOL PHYSIOPATHOLOGY
Calcium homeostasis

- BONE
  Stimulates osteoclast activity
  Inhibits osteoblast activity

- BOWEL
  Inhibits calcium absorption

- KIDNEY
  Increases calcium excretion

hypercalciuria ← → osteoporosis

CORTISOL PHYSIOPATHOLOGY
reproduction

- HPG axis: inhibits LH and FSH pulsatility:

Women: amenorrhea,
hypogonadotropic hypogonadism
PCOS-like phenotype

Men: hypogonadotropic hypogonadism

CORTISOL PHYSIOPATHOLOGY
Gynecology / Dermatology

Androgen excess

→ Hirsutism, alopecia acne, ecc.

CORTISOL PHYSIOPATHOLOGY
Central Nervous System

- CNS particularly at the hippocampus, septum and amygdala, centers involved in behaviour, mood, memory and cognitive functions.

⇓ mental concentration
⇓ memory
insomnia

←→

- mood instability
- irritability
- depression

IATROGENIC CUSHING's SYNDROME

Frequent disease caused by the excess intake of corticosteroid medication, often improperly used.

MAJOR SYNTHETIC GLUCOCORTICOIDS

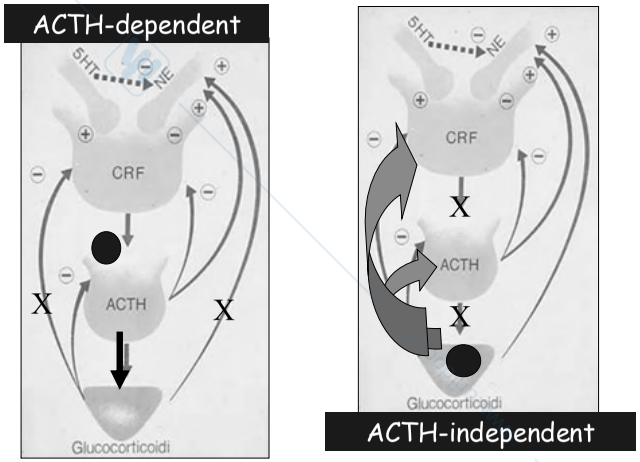
CORTISONE ACETATO	•Cortone
IDROCORTISONE	•Flebocortid, Solucortef
DEXAMETHAZONE	•Decadron
PREDNISONE	•Deltacortene
PREDNISOLONE	•Soludacortin, Metilcortelone
METHYLPREDNISOLONE	•Urbason, Medrol, Solumedrol
BETAMETHASONE	•Bentelan, Celestone
TRIAMCINOLONE	•Ledercort, Kenacort,
DEFLAZACORT	•Ipercortis
BECLOMETHASONE	•Flantadin
BUDOMETHASONE	•Bectide, Clenil, Topster
FLUTICASONE	•Pulmaxan
	•Flixotide, Fluspiral

BIOLOGIC ACTIVITY OF MAJOR GLUCOCORTICOIDS

Steroids	*Anti-inflammatory*	*Na retention*	*Equivalent (mg)*
Short acting			
- Hydrocortisone	**1**	**1**	**20**
- Cortisone	**0,8**	**1**	**25**
Intermediate acting			
- Prednisone	**3,5**	**0,75**	**5**
- Prednisolone	**4**	**0,75**	**5**
- Triamcinolone	**5**	**0**	**4**
Long acting			
- Dexamethasone	**30**	**0**	**0,75**
- Betamethasone	**25**	**0**	**0,60**

Referred to the cortisol activity (100%)

Endogenous Hypercortisolism: ACTH-dependent or ACTH-independent



ENDOGENOUS HYPERCORTISOLISM EPIDEMIOLOGY

Frequency 8-10 cases per million.

Women more affected than men.

This frequency refers to overt cases, but it is current common opinion that hypercortisolism is to be suspect before the characteristic signs and symptoms become evident (SUBCLINICAL HYPERCORTISOLISM).

ENDOGENOUS HYPERCORTISOLISM

ACTH-DEPENDENT CUSHING'S DISEASE

CORTICOTROPINOMA

ACTH-secreting PITUITARY ADENOMA

CORTICOTROPINOMA

Pituitary MRI: "an hypointense formation at the level of the adenohypophysis of the size of 2 mm in all sequences that could be an expression of a pituitary microadenoma ...".

Monoclonal neoplasms that mainly occur sporadically. Cushing's disease is very rarely encountered in genetic familial syndromes.

Oncogenes and tumor suppressor genes commonly associated with other tumor types are only rarely muted in this tumor type.

CORTICOTROPINOMA EPIDEMIOLOGY

INCIDENCE: 1.2 - 2.4 cases per million/year
MAINLY SPORADIC
(familial forms are very rare)

m/f

PRL	NF	ACRO	**CUSH**	PRL	NF	ACRO	**CUSH**
1/22	1/6	1/45	**3/16**	19/84	1	1/96	**4/43**
Macro-adenomas				*Micro-adenomas*			

Data from a consecutive series of 2137 pituitary tumors.

Ambrosi B. et al.: Pituitary adenomas: new trends in basic and clinical research 159, 1991

MOLECULAR BASIS OF CORTICOTROPINOMA

Cushing's disease represents the most frequent form of endogenous hypercortisolism and is caused by ACTH hypersecretion by a pituitary adenoma.

Mutations in the gene encoding the ubiquitin-specific peptidase 8 (USP8) have recently been identified in about 50% of the ACTH-secreting pituitary adenomas and, in particular, in up to 60% of the microadenomas.

These mutations cause the de-ubiquitinization of the Epidermal Growth Factor receptor (EGFR), inducing an excessive synthesis of proopiomelanocortin (POMC) and, consequently, the hypersecretion of ACTH.

MOLECULAR BASIS OF CORTICOTROPINOMA

Glucocorticoid resistance – loss of feedback loop

- Cortisol inactivation: overexpression of 11-β-hydroxysteroid dehydrogenase isoform 2 (11β-HSD2) (cortisol → cortisone).
- Glucocorticoid Receptor (GR): mutations in the GR gene *NR3C1* are extremely rare.
- GR regulation: overexpression of Testicular receptor 4 (repressor of GR activity), contributing to ACTH hypersecretion and tumor growth.
- GR effect: downregulation of Brahma-related gene 1 (Brg1) and histone deacetylase 2 (HDAC2), and overexpression of heat shock protein 90 (HSP90), causing loss of GR inhibition of POMC transcription.

MOLECULAR BASIS OF CORTICOTROPINOMA

Cell cycle dysregulation

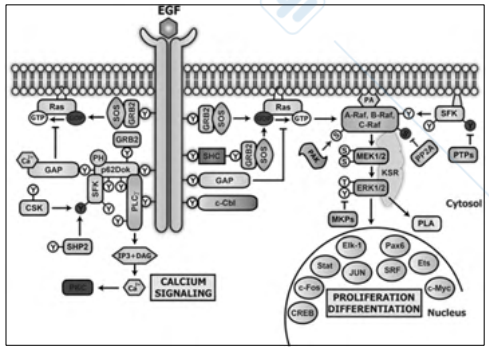
G1/S phase transition

- Cyclin E overexpression → increased E/CDK2 complexes.
- P27/Kip reduction.

Cell proliferation

- EGF-R, FGF-R overexpression.
- Downregulation/loss of tumor suppressors BMP4 and Shh.
- Interleukin 2 and 6 autocrine-paracrine loops.

EPITHELIAL GROWTH FACTOR



EGF is a potent mitogen that enhances DNA and protein synthesis and shifts metabolic activity in epithelial cells, although it does not have cell-transforming capacity *per se*.
EGF-receptor is a tyrosine kinase receptor and is a cellular homologue of the v-erbB oncogene.

EPITHELIAL GROWTH FACTOR

The oncogenic effects of the epidermal growth factor (EGF) have long been established.

EGF-R is overexpressed in many types of tumors and is a target for cancer treatment.

The pituitary gland is a target of EGF action and it is very likely that EGF-R plays a role in pituitary tumor formation and progression.

However, a controversy exists in literature concerning EGF-R expression in the different types of pituitary adenomas.

EPITHELIAL GROWTH FACTOR RECEPTOR

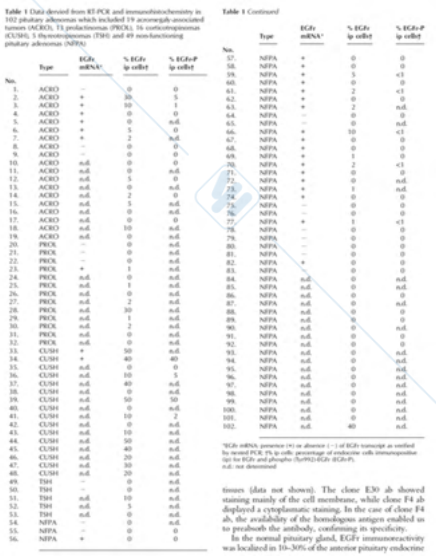
(*…in 2004*)

"AIM: we investigated the expression pattern of the wild type EGFr (EGFrWT) and the constitutively active variant III (EGFrvIII) at the mRNA and protein levels in a large series of pituitary tumors."

Expression of epidermal growth factor receptor in neoplastic pituitary cells: evidence for a role in corticotropinoma cells

M Theodoropoulou, T Arzberger[1], Y Gruebler, M L Jaffrain-Rea[2], J Schlegel[3], L Schaaf, E Petrangeli[2], M Losa[4], G K Stalla and U Pagotto[5]

Journal of Endocrinology (2004) **183**, 385–394



EGF-R in pituitary tumors

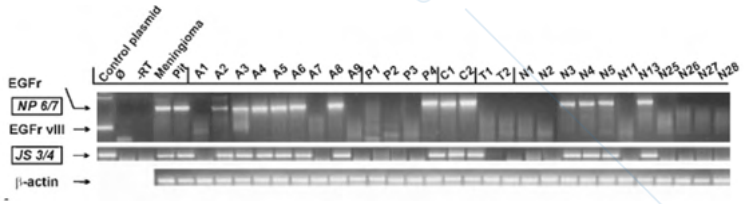
Nested RT-PCR and immunohistochemistry screening of a large series of pituitary tumors including PRL-omas, GH-omas, NFPAs, TSH-omas and ACTH-omas

*EGF-R mRNA: presence (+) or absence (-) of EGF-R transcript;

†% ip cells: percentage of endocrine cells immunopositive (ip) for EGF-R or phospho-(Tyr992)-EGF-R (EGF-R-P).

Theodoropoulou M et al. J Endocrinol 2004

EGF-R in pituitary tumors



Semiquantitative RT-PCR analysis and northern blot showed EGF-R transcript in representatives of all pituitary tumor types, including all the assayed corticotropinomas.

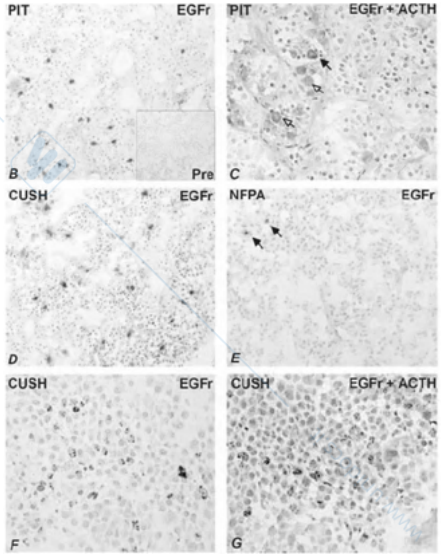
In contrast, all tumors were negative for the constitutively active truncated EGF-R variant III.

Theodoropoulou M et al. J Endocrinol 2004

EGF-R in pituitary tumors

EGF-R: brown
ACTH: red

Cushing: EGF-R is over-expressed



Normal pituitary: EGF-R and ACTH co-localized

Cushing: EGF-R and ACTH over--expressed

Theodoropoulou M et al. J Endocrinol 2004

EGF-R in pituitary tumors

All EGF-R-expressing corticotropinomas showed immunoreactivity for the phosphorylated EGF-R at tyrosine 992, indicative of activated receptor, a phenomenon that tended to be corticotropinoma-specific as it was not observed in the majority of EGF-R-immunopositive acromegalic tumors.

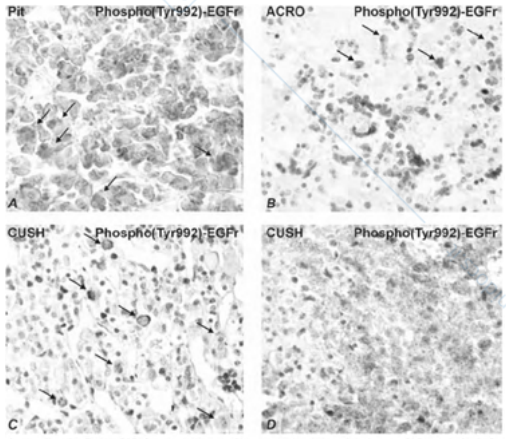


Figure 3 Phospho (Tyr992)-EGFr detection in (A) normal human anterior pituitary (PIt), (B) one ACRO (No. 2) that had 5% immunopositive cells, (C) one CUSH (No. 36) with 5% immunopositive cells and (D) one CUSH (No. 39) with 50% immunopositive cells. Arrows depict the individual immunopositive cells as stained with DAB (brown). All stainings were performed in paraffin-embedded sections. Nuclei are counterstained with Toluidine Blue.

Theodoropoulou M et al.J Endocrinol 2004

EGF-R in pituitary tumors

EGF-R-WT was found in a high percentage of hormone-secreting tumors, but only in a small fraction of non-functioning pituitary adenomas.

No expression of the EGF-RvIII could be detected by nested RT-PCR in any tumor.

Among the hormone-secreting adenomas, the highest incidence of EGF-R expression was found in Cushing's pituitary adenomas.

Furthermore, immunohistochemistry for the phosphorylated EGF-R revealed the presence of activated EGF-R in most Cushing's adenomas, compared with most pituitary adenomas.

EGF-R in pituitary tumors

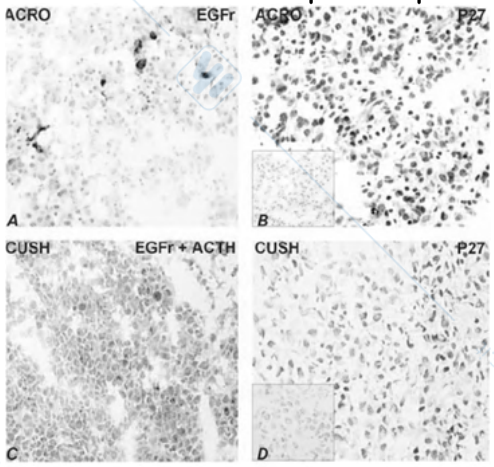
Taking into account that downregulation of p27/Kip1 plays a significant role in corticotrope tumorigenesis and that EGF-R mitogenic signaling results in decreased p27/Kip1, we searched for a correlation between EGF-R expression and p27/Kip1 levels in corticotropinomas.

Low p27/Kip1 immunoreactivity was observed in corticotropinomas expressing EGF-R. On the other hand, somatotropinomas expressing EGF-R had high p27/Kip1 immunoreactivity.

These data suggest a corticotrope-specific phenomenon and indicate that EGF-R may have a role in the unbalanced growth of corticotrope tumoral cells.

EGF-R in pituitary tumors

EGF-R p27/Kip1

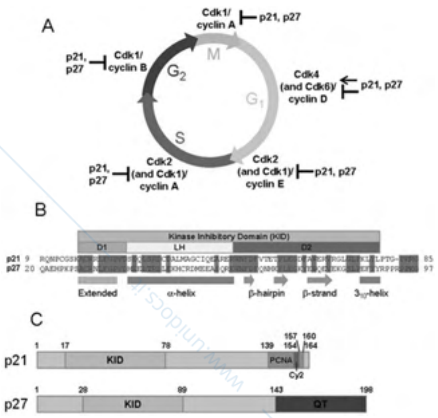


P27 is expressed in GH-omas but not in the ACTH-omas

Theodoropoulou M et al.
J Endocrinol 2004

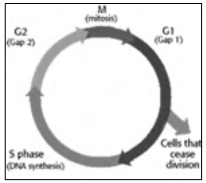
P27/Kip1

CDK-inhibitors family, encoded by the *CDKN1B* gene



p27 protein arrests cell proliferation by regulating the cyclin E threshold level required for the activation of CDK2;

P27 binds both cyclins and CDK preventing the G1→S transition.



MOLECULAR BASIS OF CORTICOTROPINOMA

Somatic mutations (…*since 2015*)

Whole exome sequencing studies revealed that almost half of corticotrope tumors present with somatic mutations in a single mutational hotspot in the *USP8* gene encoding for the ubiquitin-specific peptidase 8.

USP8 mutations were exclusively found in corticotrope tumors, but in no other pituitary tumor subtype, nor in ectopic ACTH-secreting NETs.

USP8 deubiquitinates receptor substrates such as EGF-R and rescues them from proteasomal degradation. The USP8 mutants potentiate EGF-R signaling and its stimulatory action on ACTH synthesis.

UBIQUITIN/EGF-R DYSREGULATION IN CORTICOTROPINOMAS

Ubiquitin is a 76-amino-acid protein highly conserved in eukaryotes.

Ubiquitination regulates the fates or functions of the substrate proteins in multiple ways: it participates in protein degradation in the proteasome, DNA repair, endocytosis and lysosomal trafficking, NFkB signaling.

For plasma membrane proteins, ubiquitination serves as a signal that delivers the protein from the cell surface to lysosomes.

UBIQUITIN-SPECIFIC PEPTIDASE 8

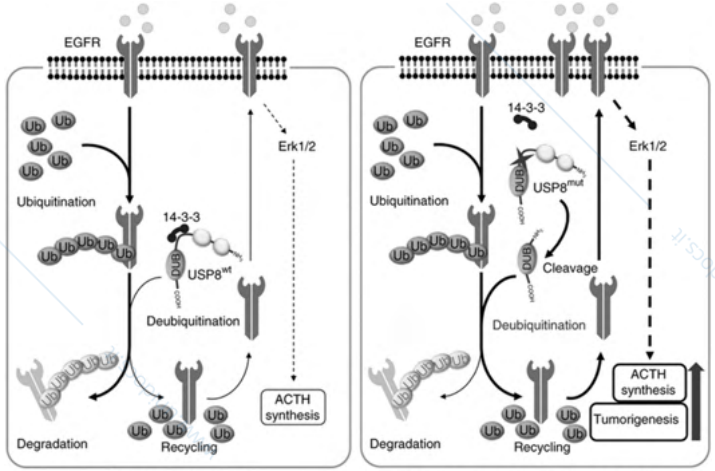
USP8 catalyzes the cleavage of ubiquitin tags (deubiquitination) and is involved in tyrosine kinase receptor trafficking and endosome-lysosome function, leading to receptor recycling to the cell surface.

USP8 is mutated in a single hotspot in 35-62% of sporadic ACTH-omas.

This mutational hotspot is located in exon 14 and ward USP8 off 14-3-3 proteins that prevent the cleavage to a highly active C-terminal fragment.

Reincke M, Nat Genet, 2015
Perez Rivas LG, JCEM, 2015
Huang C, Oncotarget, 2015
Ma ZY, Cell Res, 2015
Hayashi K, EJE, 2016

UBIQUITIN-SPECIFIC PEPTIDASE 8



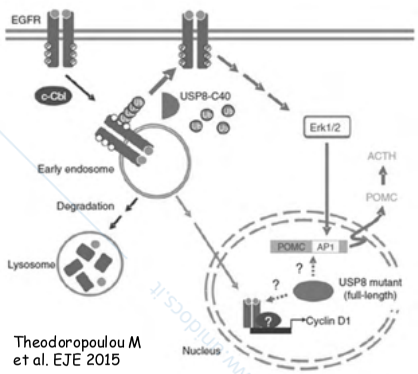
UBIQUITIN/EGF-R DYSREGULATION IN CORTICOTROPINOMAS

Upon EGF binding on the cell surface, activated EGF-R is rapidly internalized and transported, via early and late endosomes, to lysosomes where EGF-R is degraded by proteolytic hydrolases.

This receptor downregulation is a cellular strategy to prevent the cells from responding excessively to EGF due to sustained signals from activated EGF-R.

An E3 enzyme c-Cbl is recruited to ligand-activated EGF-R and ubiquitinates the receptor, adding a tag that targets activated EGF-R to lysosomes for degradation.

USP8 deubiquitinates EGF-R in early endosomes, removing the lysosome-targeting tag and suppressing its downregulation.



Theodoropoulou M et al. EJE 2015

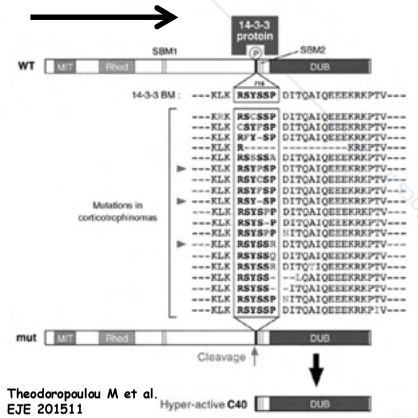
UBIQUITIN/EGF-R DYSREGULATION IN CORTICOTROPINOMAS

The binding of 14-3-3 proteins to the 14-3-3 BM of mouse USP8 suppresses its deubiquitinase activity toward ubiquitinated EGF-R.

USP8 mutations lead to the loss of 14-3-3 protein binding and promote USP8 cleavage immediately N-terminal to the 14-3-3 BM.

The cleaved C-terminal 40-kDa DUB domain (C40) acquires elevated catalytic activity.

USP8 mutation is a gain of function!



Theodoropoulou M et al. EJE 201511

CORTICOTROPINOMA THERAPEUTIC STRATEGIES

SURGERY: Trans-sphenoidal selective adenomectomy results in remission of Cushing's disease in 25–100% of cases.
However, recurrence rates are reported as 22–27% on longterm follow up.

DRUGS: Dopamine receptor D2 and somatostatin receptor (SSTR) 5 have been found in the majority of corticotroph tumors and treatment with their respective ligands decreases ACTH secretion in vitro. Treatment with the SSTR5 ligand pasireotide alone or in combination with the D2 agonist cabergoline was shown to decrease cortisol levels and is approved for the treatment of Cushing's disease.

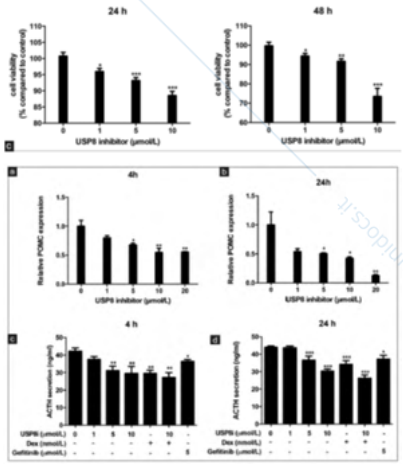
CORTICOTROPINOMA THERAPEUTIC STRATEGIES

NEW AGENTS: USP8 as a novel attractive pharmaceutical target.

Small molecule USP8 inhibitor displayed anti-secretory and anti-proliferation action in immortalized murine corticotrope tumor cells.

9-ehtyloxyimino9H-indeno[1,2-b] pyrazine-2,3-dicarbonitrile,

Jian et al., 2016



ENDOGENOUS HYPERCORISOLISM

ACTH-INDEPENDENT CUSHING'S SYNDROME

ADRENAL TUMORS

INCIDENTALLY DISCOVERED ADRENAL MASS

ADRENAL INCIDENTALOMA

Expansive lesions of various sizes, generally asymptomatic or associated with modest and nonspecific signs / symptoms, found "incidentally" during diagnostic imaging tests such as CT, MRI, ultrasound.

ADRENAL CUSHING EPIDEMIOLOGY

Primary adrenal etiologies account for 15-20% of Cushing cases and are due to unilateral neoplasms in 90-98%.

Of these, adenomas represent 80%, and carcinomas 20%.

The incidence of Cushing's Syndrome secondary to an unilateral adrenal adenoma is 2 cases/million/year.

ADRENAL INCIDENTALOMA EPIDEMIOLOGY

- Prevalence in abdomen CT scans: 3 - 4%.
- Occasional autoptic finding: 2.4% - 10%.
- Marked differences in prevalence according to the selected population (up to 4.4% of cases in cancer patients).
- Higher prevalence in women.
- Incidence increasing with ageing: peak around the 6 -7th decade of life.
- Most frequently affected site: right adrenal gland.

(Mansmann G. et al, Endocr Rev 2004)

ADRENAL INCIDENTALOMA CLINICAL RELEVANCE

EPIDEMIOLOGY LEVEL

~350 new cases/year

EXPECTED AT THE S.ORSOLA POLICLINIC

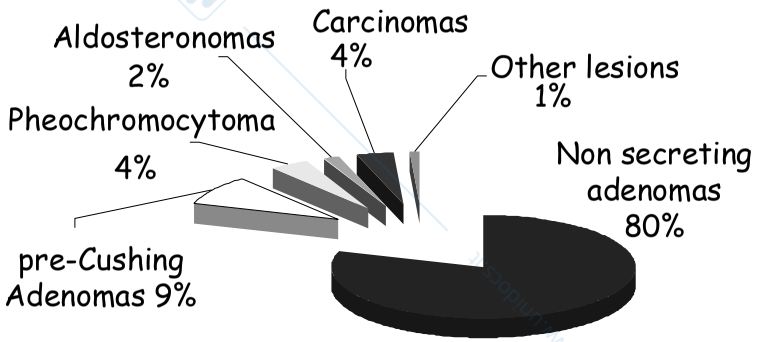
ETIOLOGY LEVEL

POSSIBLE ASSOCIATED FINDINGS:
Subclinical hyperfunction states
Malignant pathologies
Genetic pathologies
Rare pathologies
REDUCTION OF MORBILITY FOR BENIGN PATHOLOGIES

ADRENAL INCIDENTALOMAS

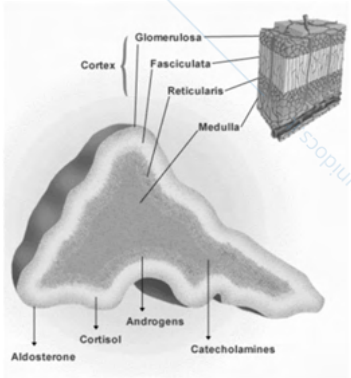
Adrenocortical	Medulla	Others	Metastasis
Adenoma	Pheochromo-cytoma	Myelolipoma	Breast cancer
Nodular Hyperplasia	Ganglioneuroma	Neurofibroma	Lung cancer
Carcinoma	Ganglioneuro-blastoma	Hamartoma	lymphoma
		Teratoma	Leukemia
		Xanthomatosis	Others
		Amyloidosis	
		Cysts	
		Hematoma	
		Granulomatosis	

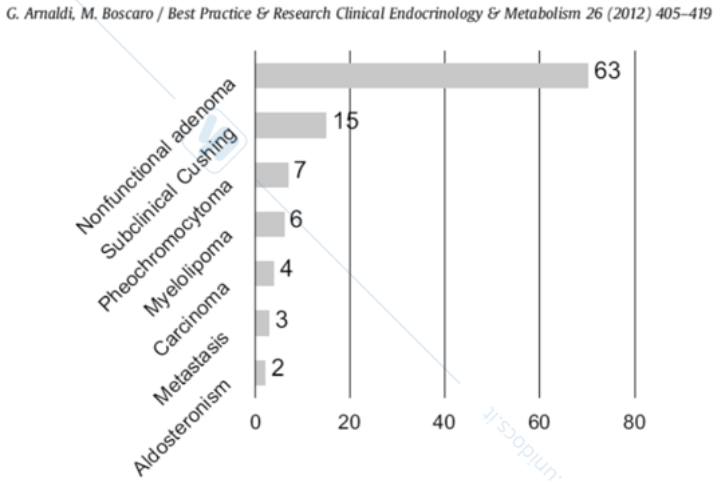
ADRENAL INCIDENTALOMA
Prevalence per subtype



AETIOLOGY

- ✓ Non-functioning adenoma ~80%
- ✓ Functioning adenoma
  - cortisol producing adenoma / (subclinical) Cushings ~5%
  - aldosteronoma / Conn's syndrome ~1%
- ✓ Phaeochromocytoma ~5%
- ✓ Adenocortical carcinoma ~5%
- ✓ Metastatic disease ~2.5%





ADRENAL INCIDENTALOMA MULTIPLE DIAGNOSTIC QUESTIONS

- Differential diagnosis benign vs malignant.
- Differential diagnosis altered vs non altered function.
- To evaluate clinical correlates.
- To provide proper indications for deciding the best therapeutical approach.
- To decide whether performing follow-up and what are its aims.

Morphological diagnostic protocol

- **CT**
- **PET, PET-CT**
- **Scintigraphy**
- **DEXA (dual-energy x-ray absorptiometry or densitometry)**

Genetic diagnostic protocol

- Enzymatic deficits of steroidogenesis
- Sporadic pheochromocitoma
- MEN 2
- Others

IDENTIFYING THE SOURCE OF ENDOGENOUS HYPERCORTISOLISM

At a clinical ground, pituitary, ectopic and adrenal forms of Cushing's syndrome are hardly distinguishable. Localizing the origin of the HPA dysfunction is a hard path…

Up to 7 different biochemical tests
(2 highly invasive)
2 Imaging Approaches

Unstandardized assays
Unstandardized cut-offs
Inter-individual variability

WORKFLOW FOR ENDOGENOUS HYPERCORTISOLISM DIAGNOSIS

1) Diagnosis of endogenous Cushing's Syndrome:

- 24 h Urinary Free **Cortisol** (≥2 tests)
- Serum **Cortisol** Post Overnight 1 mg DST
- Late night salivary **Cortisol** (≥2 tests)

2) Origin of Hypercortisolism: plasma **ACTH**

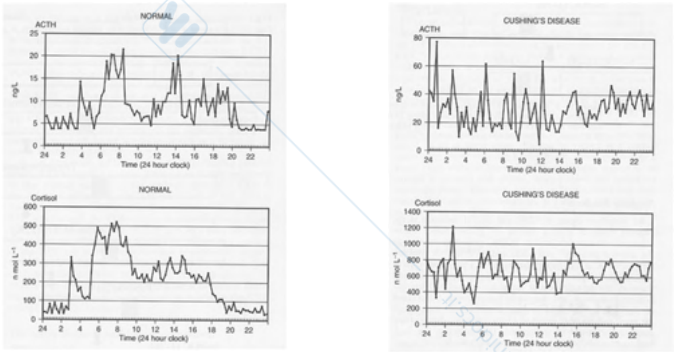
ACTH< 5 pg/mL (>2 tests): ❑ **ACTH- INDEPENDENT CUSHING'S**	5 <ACTH< 15/20 pg/mL: **UNDETERMINED**	ACTH> 15/20 pg/mL: ❑ **ACTH-DEPENDENT CUSHING'S**
⇩ Adrenal Imaging ⇩ ❑ Monolateral, Carcinoma ❑ Monolateral, Adenoma ❑ Hyperplasia: - Adrenal Vein Sampling - NO PERIPHERAL BIOMARKERS	⇘ ⇙	⇩ • 8 mg DST test • CRH test • Desmopressin test • Pituitary MRI • BIPPS ❑ Pituitary ❑ Ectopic

Biochemical assessment of Hypercortisolism

Blood total Cortisol (basal and functional tests)	**24h Urinary Free Cortisol**	**Late Night Salivary Cortisol**
		
Early morning	*24 hour*	*Multiple time points*
✓ Secretion ✓ Complex matrix	✓ Total daily exposure ✓ Steroid interferences	✓ Free – active fraction ✓ Very low levels

Biochemical assessment of Hypercortisolism BASAL BLOOD TESTING

Evaluation of the circadian rhythm of ACTH and cortisol (blood taken at 8 am and 16 am)



Morning cortisol level is not altered in Cushing.
Blood testing in the late afternoon is hard to perform .

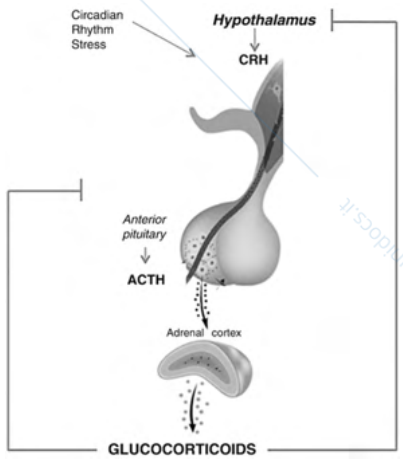
Biochemical assessment of Hypercortisolism DEXAMETHAZONE SUPPRESSION TEST

Exploring the feedback function

Dexamethasone 1mg administration overnight at 11pm

Following morning: blood cortisol measurement

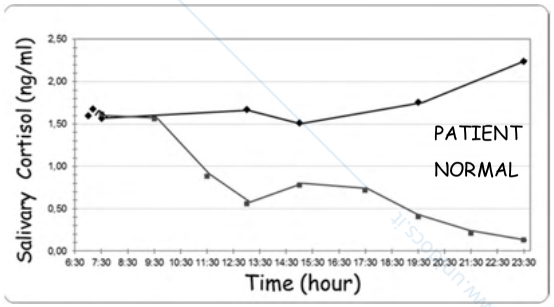
Cut-off: 138 nmol/L



Biochemical assessment of Hypercortisolism
SALIVARY CORTISOL

Exploring the circadian rhythm preservation

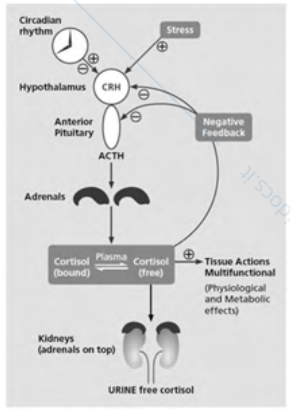
Evaluation of the circadian fluctuation of salivary cortisol throughout the day.
Diagnosis is based on midnight values.



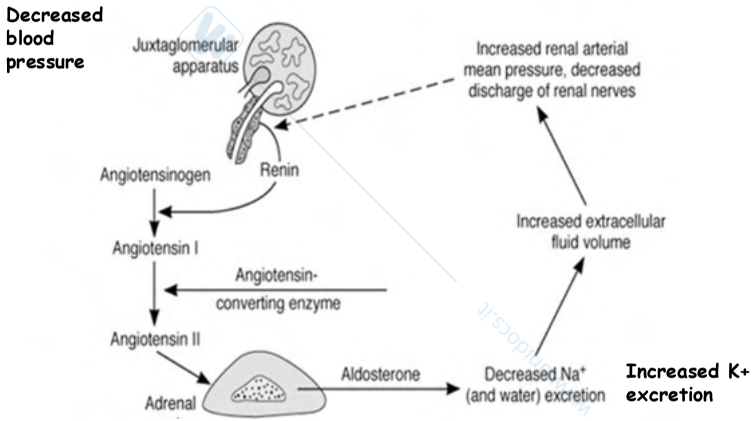
Biochemical assessment of Hypercortisolism
URINARY FREE CORTISOL

Evaluating the total cortisol excretion (and production) over the day.

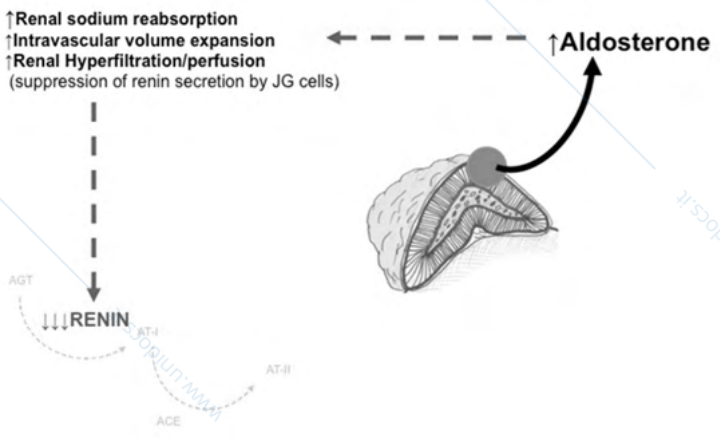
Urine collection throughout the 24h



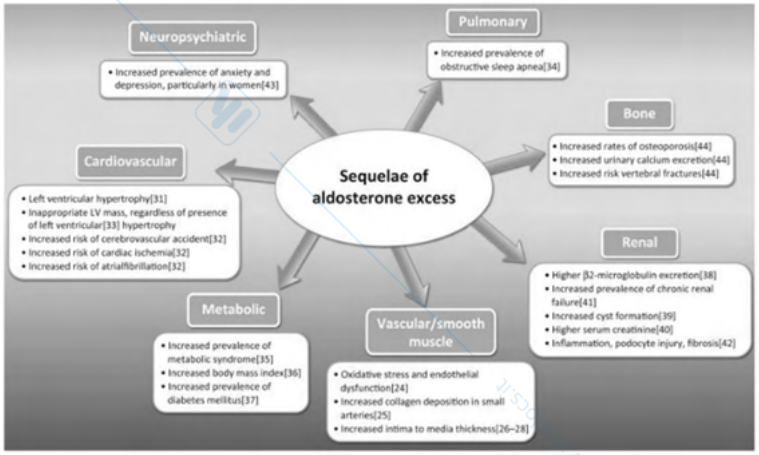
HORMONAL CONTROL OF THE ARTERIAL BLOOD PRESSURE



ALDOSTERONOMA



HYPERALDOSTERONISM

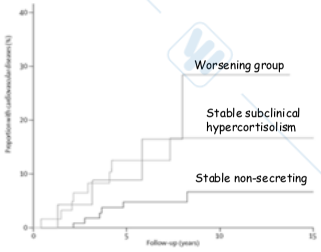


SUBCLINICAL HYPERCORTISOLISM

Functional classification of adrenal mass based on 1mg DST

- Non-secreting: cortisol <50 nmol/L.
- Subclinical hypercortisolism: cortisol >50 nmol/L (<138 nmol/L).

SUBCLINICAL HYPERCORTISOLISM CARDIOVASCULAR RISK



N=198 Mean follow-up 7.5 years (2-15 years)

	Hazard ratio	P value
Age (1 yr increase)	1.01	0.86
Hypertension (yes vs no)	7.36	0.02
Stroke (yes vs no)	10.27	0.01
Myocardial infarction (yes vs no)	29.05	<0.001
Lipid lowering treatment (no vs yes)	0.11	0.02
Delta cortisol baseline-end of follow-up (10 nmol/L increase)	1.13	0.001

Di Dalmazi G et al., Lancet Diabetes Endocrinol, 2014

	SH− Group	SH+ Group	P
n	167	39	
Duration of follow-up, mo	83.2 ± 33.6 (60–186)	79.4 ± 25.2 (60–178)	.826
New CVE	14 (8.4)	8 (20.5)	.040
New CVE in CVE− patients at baseline	11 (6.6)	4 (10.0)	.343
Increased body weight[a]	40 (24.0)	13 (33.3)	.229
Worsened blood pressure control[b]	52 (31.1)	18 (46.2)	.070
Worsened glycemic control[c]	39 (23.4)	12 (30.8)	.334
Worsened LDL[c]	20 (12.0)	7 (17.9)	.303

N=206 - Median follow-up 6 years

	SH -	SH +	P value
Annual rate of cardiovascular events (%)	1.2	3.1	0.004

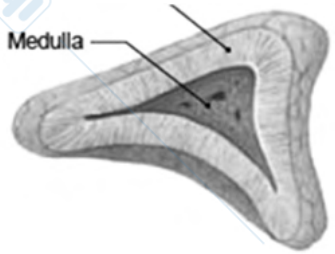
Morelli V eta l., JCE&M, 2014

SUBCLINICAL HYPERCORTISOLISM

Derangement of the overall adrenal steroid secretion.

Excess glucocorticoid is accompanied by increased aldosterone and reduced androgens, overall contributing to the increased cardiovascular risk.

PHEOCHROMOCYTOMA

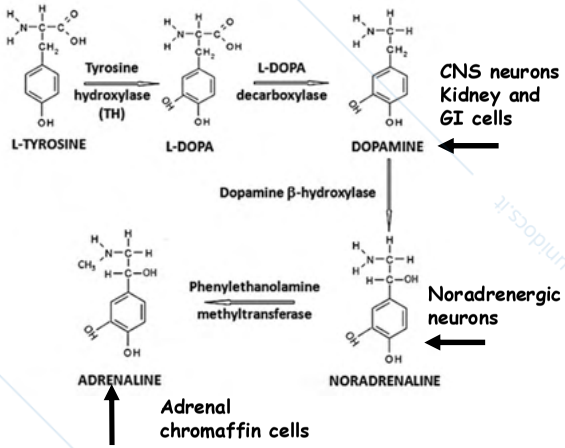


PATHWAY OF CATECHOLAMINE SYNTHESIS

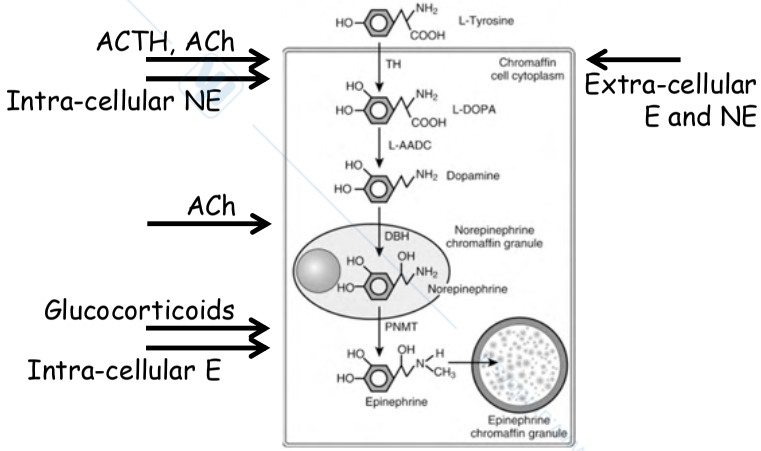
CNS: Dopaminergic and noradrenergic neurons.

Adrenal medulla: sympathetic nerves and chromaffin cells.

Perhipery: paraganglia.



CATECHOLAMINE SYNTHESIS



CATECHOLAMINE FUNCTIONS

Monoamine Type	Site of Monoamine Production	Mode of Transmission	Site of Monoamine Action
Epinephrine	Adrenal medulla (chromaffin cells)	Hormonal	Cardiac muscle, smooth muscle, and widespread effects on cellular metabolism
Norepinephrine	Sympathetic ganglia and varicosities of autonomic nervous system	Neuroeffector cell junction	Effector tissue including cardiac muscle, smooth muscle, vascular endothelium, and exocrine glands
	Adrenal medulla (chromaffin cells)	Hormonal	Primarily cardiac and smooth muscle
	Brain stem (locus coeruleus and reticular formation)	Neuronal synapse	Widespread CNS neuronal connections
Dopamine	Midbrain (substantia nigra)	Neuronal synapse	Neuronal connections in cerebral striatum
	Midbrain (ventral tegmentum)	Neuronal synapse	Cerebral mesolimbic and mesocortical neuronal connections
	Diencephalon (hypothalamus)	Neuronal synapse	Pituitary gland
	Retina	Neuronal synapse	Neuronal connections within the retina
	Olfactory bulb	Neuronal synapse	Neuronal connections within the olfactory bulb
	Gastrointestinal tract	Autocrine/paracrine	Regulation of bicarbonate secretion, gut motility, sodium in exocrine secretions
	Kidney (derived from circulating L-dopa)	Autocrine/paracrine	Regulation of natriuresis

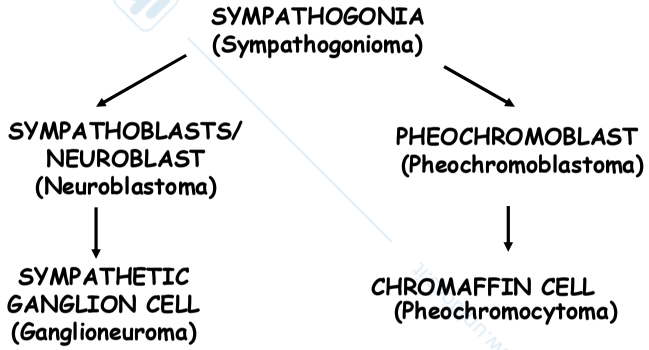
CATECHOLAMINE RECEPTORS

- Alpha-Adrenergic Receptors
  - α_1: vasoconstriction, intestinal relaxation, uterine contraction, pupillary dilation
  - α_2: ↓ presynaptic NE (clonidine), platelet aggregation, vasoconstriction, ↓ insulin secretion
- Beta-Adrenergic Receptors
  - β_1: ↑ HR/contractility, ↑ lipolysis, ↑ renin secretion
  - β_2: vasodilation, bronchodilation, ↑ glycogenolysis
  - β_3: ↑ lipolysis, ↑ brown fat thermogenesis

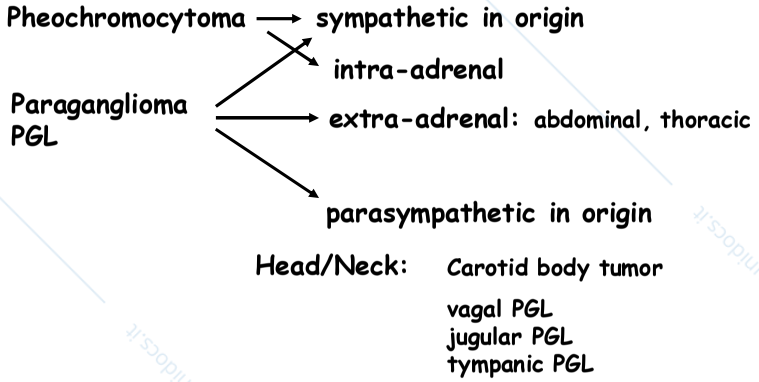
CATECHOLAMINE FUNCTIONS

1. Glucose metabolism: increase glycaemia by promoting gluconeogenesis and glycogenolysis, and by inhibiting glycogen synthesis.
2. Lipid metabolism: promote lypolisis in the adipose cell, the increase in circulating FFAs and their utilization as fuel by heart and muscle cells.
3. Circulatory system: increase cardiac output, blood pressure, oxygen consumption. Smooth muscle relaxation at the bronchi, gastro-intestinal tract and skeletal muscle vessels.

Pheochromocytoma is a rare tumor of the neuroectodermic tissue differentiated toward the chromaffin lineage.

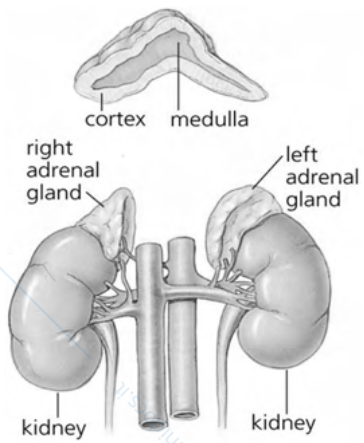


CLASSIFICATION



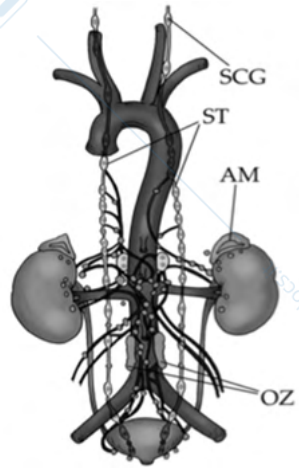
NEURAL CREST-DERIVED TUMORS

Secreting pheochromocytoma (Pheo)



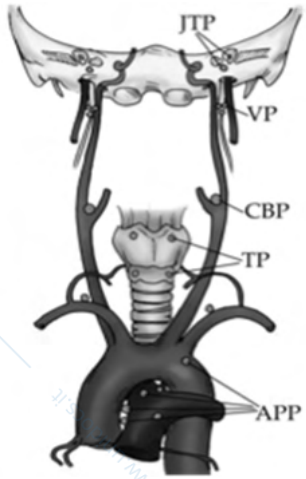
Secreting sympathetic paraganglia (PGL)

✓chest
✓retroperitoneal
✓urinary bladder



Non secreting parasympathetic paraganglia (HNPGL)

✓head
✓neck
✓pulmonary aorta



PHEO/PGL EPIDEMIOLOGY

- Incidence: 2-8 cases / million / year.
- Underlying cause of hypertension in 0.1% of hypertensive patients.
- Estimated prevalence 1:2500 – 1:6500.

- 90% adrenal
- 10% extra-adrenal (PGL)

- 90% benign forms
- 10% malignant forms

- 65% sporadic
- 35% familial

PHEOCHROMOCYTOMA

FAMILIAL FORMS

MEN 2A, 2B
(mutation of *Ret* protoncogene)
Von Hippel Lindau Syndrome
(mutation of *VHL* oncosuppressor)
Von Recklinghausen Syndrome
(mutation of *NF1* gene)
Head and neck Paraganglioma
(mutation of mitochondrial succinate dehydrogenase subunits B, D or C)

Pheochromocytoma is the neoplasm with the greatest inheritance rate

more than 30% hereditary.
more than 10 genes involved
multiple mechanisms, but in connection with each other

the inheritance predictors are:
the presence of a syndrome
age of appearance
bilaterality / multiplicity
malignancy

GENETIC SYNDROMES

Tumor clusters

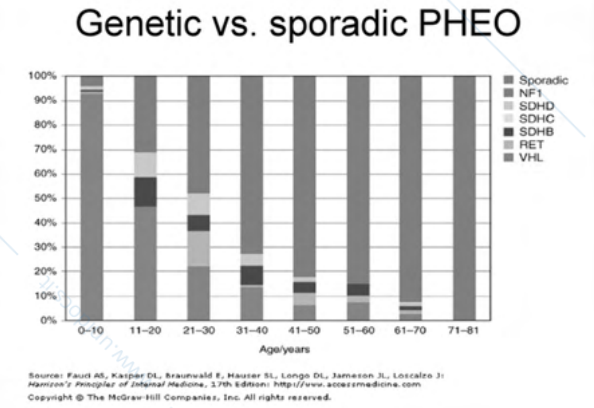
RET: adrenergic phenotype (adrenal), association with: medullary thyroid C, Lychen C, marphanoid habitus, mucosal neuromas

VHL: noradrenergic phenotype, young age association with: hemangioblastomas, renal cell C., pancreatic cysts

SDHB: malignant pheo, association with: thyroid papillary C, renal cell C.

SDHD: multiple head/neck PGL, NCD tumor associated to a head/neck PGL

Age at presentation of Patients with Mutations or Sporadic Disease



Age at presentation of Patients with Mutations or Sporadic Disease

Genetics	0-10	11-20	21-30	31-40	42-50	51-60	61-80	tot
VHL	6	17	2	3	2	0	0	30
RET	0	0	4	4	5	0	0	13
SDHD	1	2	3	3	1	1	0	11
SDHB	0	5	3	2	2	0	0	12
No mutations	3	23	19	32	46	50	32	205
% mutations	70	51	39	27	18	2	0	24.4

A germ-line mutation is present in 24.4% (66/271) of patients affected by an apparently sporadic pheochromocytoma

Neumann et al. Germ-line mutations in nonsyndromic pheochromocytoma. N Engl J Med 2002

PHEO CLINICAL FEATURES

HYPERTENSION is the most frequent symptom: persistent, fluctuating or paroxysmal (ht crysis). Less frequently, orthostatic hypotension.

Crysis may be accompanied by palpitations, sweating, headache, nausea, diarrhea / constipation, dyspnoea, fatigue, fever, dizziness, tremors, abdominal pain, chest pain, anxiety.

Adrenalin hypersecretion can cause metabolic imbalances sych as hyperglycaemia, IGT and DM.

SYMPTOMLESS

Complications of hypertension (hearth, brain, kidney) are the most frequent causes of death.

PHEOCHROMOCYTOMA HARD TO DIAGNOSE

A high percentage of Pheo (30-50%) are not diagnosed in life.

A high number of Pheo are unexpectedly found in autoptic series.

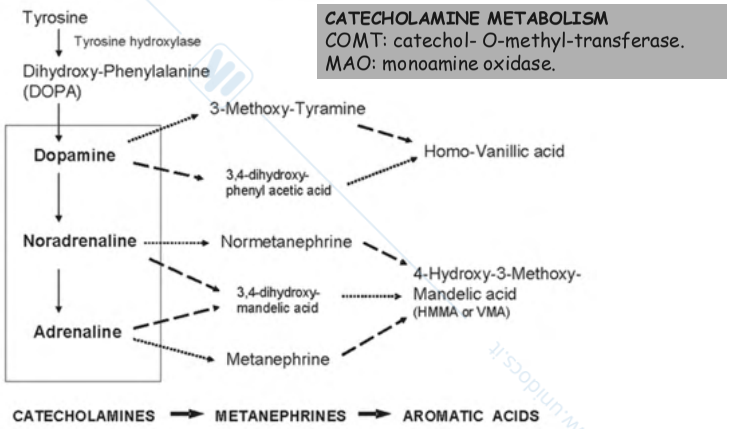
PHEOCHROMOCYTOMA DIAGNOSIS

Should be suspected in presence of:

HYPERTENSION → paroxysmal or not
HYPERTENSION → resistant

ADRENAL MASS → with hypertension
ADRENAL MASS → without hypertension

PHEOCHROMOCYTOMA BIOCHEMICAL DIAGNOSIS



PHEOCHROMOCYTOMA BIOCHEMICAL DIAGNOSIS

DIAGNOSIS OF PHEO REQUIRES BIOCHEMICAL EVIDENCE OF EXCESSIVE CATECHOLAMINE PRODUCTION BY THE TUMOR.

Large heterogeneity exists in catecholamine release in the bloodstream among Pheo tumors.

Short half-life makes it difficult to distinguish pathological overproduction from transient burst of secretion during the stress of blood sampling.

PHEOCHROMOCYTOMA BIOCHEMICAL DIAGNOSIS

Metanephrines levels represent tumor activity independently from catecholamine release.

Urinary excretion or plasma concentrations of metanephrines show strong positive correlation with tumor size and can be useful for judging the extent and progression of disease.

Metanephrine profile can help distinguishing intra- from extra-adrenal location, and can be helpful in distinguishing different genetic etiology.

PHEOCHROMOCYTOMA BIOCHEMICAL DIAGNOSIS

Plasma
- catecholamine (useful during hypertensive crysis);
- metanephrine/normetanephrin (most sensitive markers).

24h Urine collection
- catecholamine;
- metanephrine/normetanephrine (most sensitive markers);
- vanilmandelic acid VMA (not very sensitive, levels might be low if the tumor size is small).

PHEOCHROMOCYTOMA BIOCHEMICAL DIAGNOSIS

SENSITIVITY AND SPECIFICITY OF BIOCHEMICAL TESTS FOR PHEO

SENSITIVITY		SPECIFICITY	
Plasma free metanephrines	99 %	Vanillylmandelic acid	95 %
Urinary fractionated MN	97 %	Urinary total MN	93 %
Urinary Catecholamines	86 %	Plasma free metanephrines	89%
Plasma Catecholamines	84 %	Urinary Catecholamines	88%
Urinary total MN	77 %	Plasma Catecholamines	81 %
Vanillylmandelic acid	64 %	Urinary fractionated MN	69%

Lenders JW, et al JAMA 2002

PHEOCHROMOCYTOMA LOCALIZATION

CT scanning

MRI scanning

I-131-MIBG scintigraphy

PET scan

Venous sampling

PHEOCHROMOCYTOMA THERAPY

SURGICAL EXCISION

First choiche approach, mainly by laparoscopy.

PHARMACOLOCIGAL

- Preparation for surgery.
- Control of hypertensive crysis.
- Intra- or post-surgical complications.
- In inoperable cases.

PHEOCHROMOCYTOMA PHARMACOLOGIC THERAPY

Medical therapy of pheochromocytoma is based on the use of a-antagonists (a-adrenoreceptors blockers), in particular a1-selective, which show lower risk for tachycardia.

These drugs do not interfere with the release of catecholamines, but limit their peripheral effects by blocking alpha vascostrictor receptors. They therefore induce vasodilation and volume re-expansion.

DOXAZOSIN (Cardura®): a1-selective competitive inhibitor.

HYPERPARATHYROIDISM

and

OSTEOPOROSIS

CALCIUM FRACTIONS IN SERUM

Ionized free Ca2+: 50%
Protein bound: 40-50% (90% albumin, globulins)
Complexed: 1% (citrate, phosphate)

Only free ionized Ca2+ is biologically active.

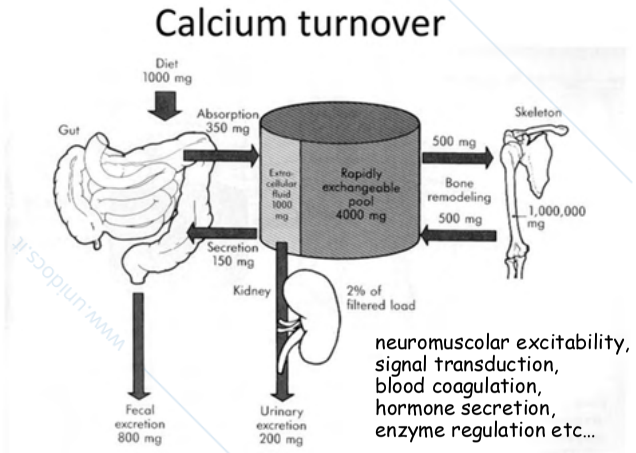
Ca2+ normal range 8.5 – 10 mg/dL in plasma.

99% of Calcium is stored in the bones, mainly as hydroxyapatite crystals.

Thougt it is the major Ca2+ reservoir in the body, very little Ca2+ can be released from the bone.

Because of its greater accessibility, trabecular bone (20% of bone mass) is involved in Calcium turnover more than cortical bone (80% of bone mass).

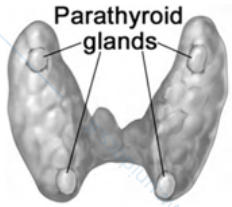
CALCIUM DAILY TURNOVER

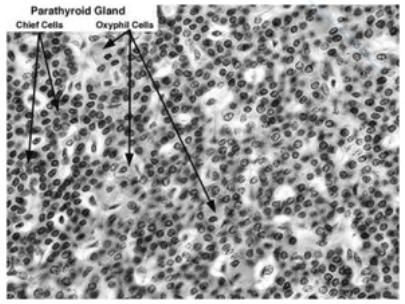


PARATHYROIDS

Parathyroid hormone (PTH) defends the extracellular fluid from hypocalcemia.
PTH is secreted by chief cells.

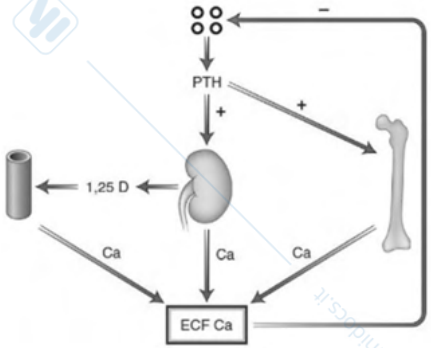
Neuronal, hormonal and local stimulatory control of PTH secretion.





CaSR – PTH – 1,25(OH)2-VitD axis

PTH level is titrated to the plasma Ca^{2+} concentration via a Ca^{2+} sensing mechanism that mediates feedback inhibition of PTH secretion.

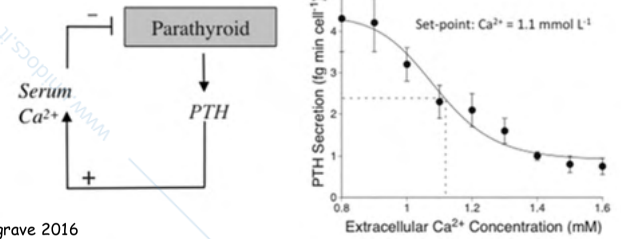


Extracellular "calciostat"

Parathyroid Ca2+-SENSING Receptor

Extracellular "calciostat"

The set-point for this calciostat occurs at a plasma ionized Ca2+ concentration of 1.1–1.2mM, corresponding to plasma total calcium concentrations of around 2.2–2.4 mM, of which approximately half is in an albumin-bound form.
PTH secretion rates rise 2 to 4-fold as Ca2+ drops down to 1.0 mM and are effectively suppressed by >50% as Ca2+ rises toward 1.4 mM.

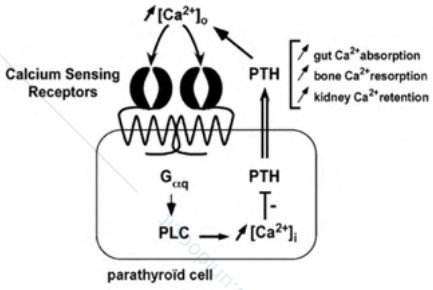


Conigrave 2016

Parathyroid Ca2+-SENSING Receptor

CaSR is a homodimer receptor on parathyroid cell membrane, sensing changes in extracellular [Ca2+] by its external flytrap lobes.
A raise in extracellular [Ca2+] is transduced by the CaSR into intracellular signal cascades leading to a decrease in PTH secretion.

Since PTH acts to increase extracellular [Ca2+], CaSR-mediated inhibition of PTH release prevents hypercalcemia through a negative feedback mechanism.

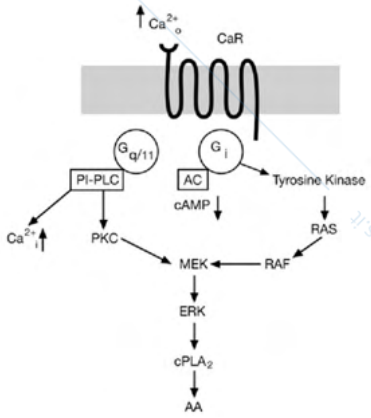


CaSR (3q13.3-21)

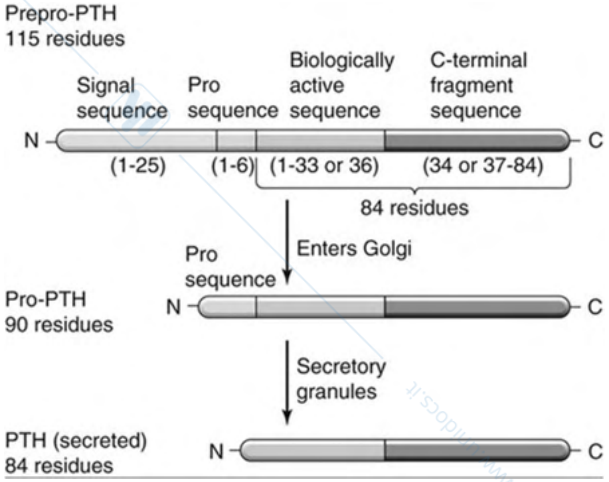
G-protein coupled receptor. Dimerization induced by Ca^{2+} binding activates PI-PLC and inhibits adenylyl-cyclase, leading to downstream events blocking PTH secretion.

Also expressed in oxyphil cells, thyroid C-cells and kidney.

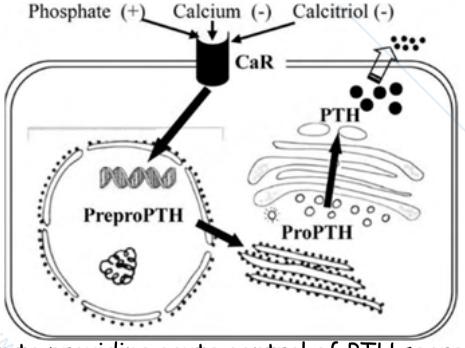
Activating mutations (AD) have been described inducing hypocalcemic hypercalciuria.



PARATHORMONE



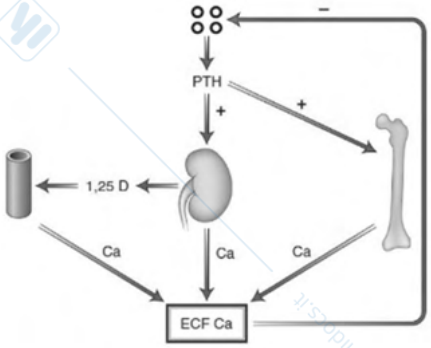
CaSR – PTH – 1,25(OH)2-VitD



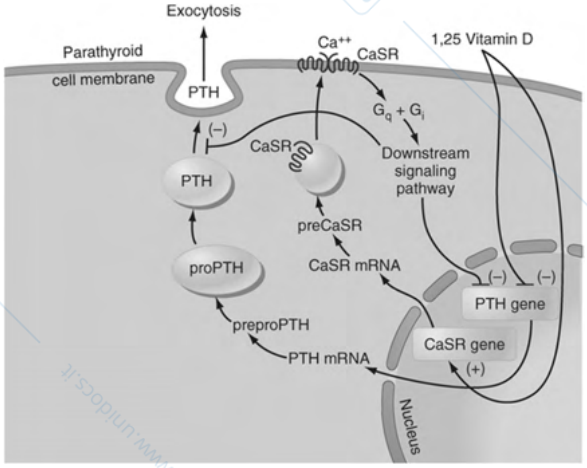
In addition to providing acute control of PTH secretion from both newly-formed secretory vesicles and stored secretory granules, the Ca2+-mediated feedback mechanism also suppresses the transcription of the PreProPTH gene and cell proliferation

CaSR – PTH – 1,25(OH)2-VitD axis

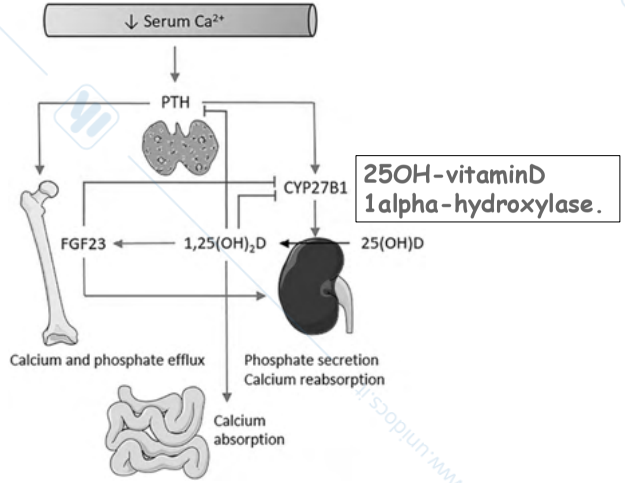
Mutual regulation at the functional and transcriptional level.



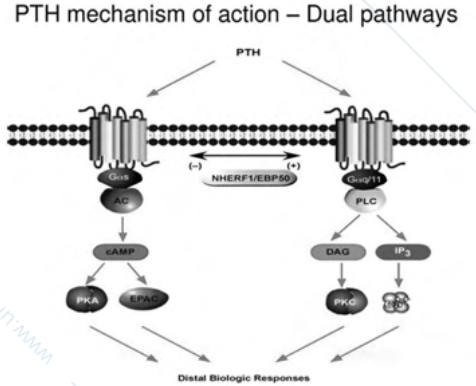
CaSR – PTH – 1,25(OH)2-VitD axis



CaSR – PTH – 1,25(OH)2-VitD axis

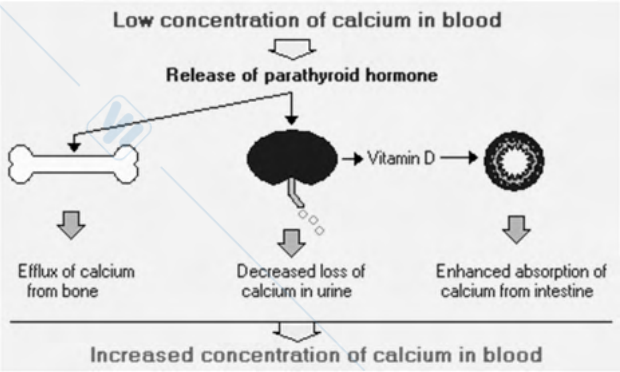


PARATHORMONE RECEPTOR TYPE 1



Type 1 PTH-R expressed in kidney cells, osteblasts, skin, placenta, breast, cartilage, nervous system, smooth muscle.

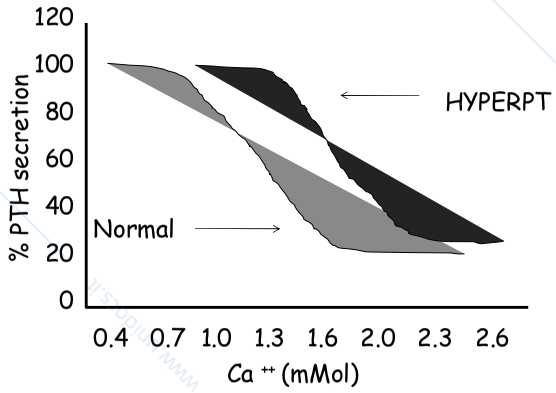
PARATHORMONE ACTIONS



PTH induces Ca mobilization from bones, stimulates phosphates escretion at the kidney, and, via vitamin D activation, Ca absorption at the intestine.

PTH-Ca2+ regulation

The secretion of PTH is inversely proportional to plasma concentration of Ca.
In hyperparathyroidism the curve shifts to the right.



HYPERPARATHYROIDISM CLASSIFICATION

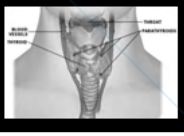
Primary Hyperparathyroidism
- Characterized by normo- or hyper-calcaemia.
- Autonomous secretion of PTH.
- Sporadic forms, familiar forms (FHH, MEN 1 and 2a), iatrogenic (Lithium).

Secondary Hyperparathyroidism
- Appropriate hypersecretion of PTH in response to hypocalcaemia.
- Characterized by normo- or hypo-calcaemia.

Tertiary Hyperparathyroidism
Development of functional autonomy during long lasting secondary hyperparathyroidism.

Primary Hyperparathyroidism (PHPT)

85% PT Adenoma (sporadic and familiar)
5-10% Ectopic neoplasia
15% Hyperplasia (MEN 1 or 2a, FHH)
1% PT Carcinoma



LABORATORY
↑ **PTH**
- Hypercalcemia
- Hypercalciuria (Ca exceeds PTH-induced absorption stimuls in tubules)
- Hypophosphatemia
- Hyperphosphaturia

CLINIC
- SYMPTOMATIC
- SYMPTOMLESS

Primary PHPT, CLINICAL SIGNS

BONE

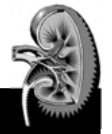
↑ Catabolic processes
↓ Mineralization
↑ Osteoclast activity

Symptomatic forms:
- 90%: Osteopenia/Osteoporosis
  (DEXA for radius, rachis femur);
  Diffused bone pain;
  Local bone pain (disease-related fractures);
- 10% Osteitis fibrosa-cystica, bone deformities.

Symptomless forms:
diffused bone pain and mild osteopenia.

Primary PHPT, CLINICAL SIGNS

KIDNEY



↓ Mild glomerular filtration;
↓ proxymal phosphate reabsorption → hyperphosphaturia;
↑ tubular Ca++ reabsorption with hypercalciuria;
↑ proxymal bicarbonate reabsorption;
↓ sensitivity of distal tubules to ADH and hypercalcemia.

30-40% kidney stones causing kidney colics.
10% Nephrocalcinosis.
Rarely polyuria and polydipsia.

Primary PHPT, CLINICAL SIGNS
GASTRO-INTESTINAL TRACT

- ↑ Gastrine and HCl secretion.
- Calcium salts deposition in pancreatic ducts.

Symptomatic forms:
- dyspepsia up to peptic ulcer
- pancreatitis (acute / chronic)

Symptomless forms:
Diffuse abdominal pain

Primary PHPT, CLINICAL SIGNS
NEUROMUSCOLAR APPARATUS

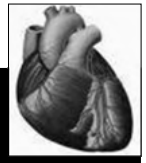
increased neuronal excitability

Symptomatic forms:
- mood swings, depression;
- difficulty in concentrating, reduced memory and weakness;
- With high calcemia: confusion, dullness to lethargy, coma and death.

Symptomless forms: asthenia and headache

Primary PHPT, CLINICAL SIGNS
CARDIOVASCULAR SYSTEM

hypercalcemia-related symptoms



Symptomatic forms:
- high blood pressure (55% of cases);
- left ventricular hypertrophy (82% of cases);
- arrhythmias;
- aortic and mitral valve calcifications (40% of cases);

Symptomless forms:
Penetrance uncertain and still being studied.

Primary PHPT, CLINICAL SIGNS
PARATHYROID CRISIS

Hypercalcemia > 15 mg/dL

PTH 20 folds higher than the normal range

- mental confusion, lethargy, coma;
- severe abdominal pain;
- nausea, vomiting, peptic ulcer, pancreatitis.

DRUG THERAPY IN PRIMARY HPT CALCIMIMETICS

CINACALCET (MIMPARA®)
(2° generation ligand)

- Target = Calcium-sensing receptor on PT cells.
- ↑ CaSR sensitivity to extracellular [Ca2+].

- Normalizes serum Ca in moderate PHPT.
- PTH decreases (though not normalized).

Secondary Hyperparathyroidism

Condition in which a disease outside of the parathyroid glands causes all of the parathyroid glands to become enlarged and hyperactive.

- Chronic renal failure (GFR <40-50 ml/min): reduced phosphate excretion and reduced Vit D activation;
- Osteomalacia (vitamin D deficiency);
- Malabsorption syndromes (celiac disease, inflammatory bowel disease);
- Renal tubular acidosis (< production of vitamin D);
- Pseudohypoparathyroidism (resistance to PTH action).

Tertiary Hyperparathyroidism

Development of autonomous (dysregulated) parathyroid function following a long period of persistent parathyroid stimulation (secondary HPT).

Severe symptomatology justify the surgical option.

HYPERCALCEMIA: OTHER CAUSES

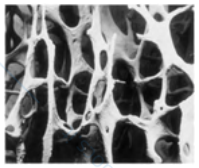
Associated with PTH alteration	Lithium FHH (Familial hypocalciuric hypercalcemia)
Associated neoplasia	Lytic (bone) metastasis in solid tumors (breast, lung, kidney) Hematologic malignancies (mieloma, leukemia, lymphoma) Humoral hypercalcemia of malignancy (lung, kidney)
↑ Vitamin D	vit. D intoxication Granulomatous disease Idiopathic childhood hyperCa
↑ Bone turnover	Hyperthyroidism Immobilization Thiazide vit. A intoxication
Associated with kidney disease	Tertiary Hyper-PTH Milk-alkali syndrome

HYPERPARATHYROIDISM

and

OSTEOPOROSIS

OSTEOPOROSIS: AN EVOLVING PARADIGM



Disease characterized by low bone mass and microarchitectural deterioration of bone tissue, leading to enhanced bone fragility and a consequent increase in fracture risk.

World Health Organization (WHO), 1993

↓

Skeletal disorder characterized by decreased bone strength and increased susceptibility of fractures.
Bone strength reflects both bone density and bone quality.

NIH Consensus Development Panel on Osteoporosis, 2001

OSTEOPOROSIS EPIDEMIOLOGY

WORLDWIDE

Osteoporosis disease has social relevance.

Its incidence increases with age, affecting the majority of the population aged >70 y.

~ 200 million diagnoses /year.

~ 9 million /year osteoporotic fractures.

OSTEOPOROSIS EPIDEMIOLOGY

ITALY

About 3.5 million women and 1 million men are suffering from osteoporosis.

25% of population >65y is affected.

In subjects aged >50y, femoral fractures are >55000/year.

Since in the next 20 years the population >65 years will increase by 25%, we will have to expect a proportional increase in the incidence of osteoporosis.

The lifetime risk of experiencing a typical osteoporotic fracture affecting the distal wrist, vertebral bodies or proximal femur is approximately 15% for each site and 40% for any site.

OSTEOPOROSIS EPIDEMIOLOGY

Vertebral morphological alterations have been found in >20% of subjects aged >65 y of both sexes.

Patients experiencing proxymal femur fracture show a 15-30% mortality rate within one year.

Among the elderly, osteoporotic fractures represent a major causes of mortality, with an incidence comparable to stroke and breast cancer, and 4 times higher than endometrial cancer.

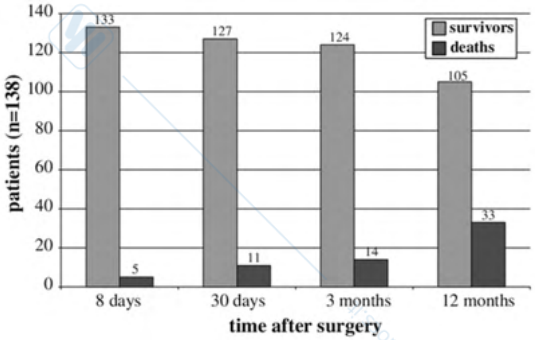
Furthermore, 50% of women with femur fracture have a significant reduction in the level of self-sufficiency and, in about 20% of cases, require long-term full assistance.

MORTALITY by OSTEOPOROSIS

Age (y)	Men	Women
55-64	2	2
65-74	2	13
75-84	9	58
85 e più	7	107
Totale	20	180

ISTAT 1999

Mortality after surgery for proximal femoral fracture



Muhm et al., Eur J Trauma Emerg Surg (2013)

OSTEOPOROSIS BURDEN

In terms of the complexity of treatment and poor prognosis, the annual facility-related hospital cost of osteoporotic fractures is the highest, followed by that of myocardial infarction and stroke.

ITALY

N° of hospitalization for femur fractures: 80.800/year.
Direct costs of hospitalization 394.000.000 €

1 month costs for post-surgical rehab (5% early mortality escluded): 412.000.000 €

Social costs (statal disability insurance for 18.000/year disabled patients) 108.000.000 €

Indirect costs (20% ot total direct costs) 183.000.000 €

Estimated total costs of femoral fractures: 1.097.000.000 €

Adapted from «12ª Commissione permanente del Senato della Repubblica (Igiene e sanità), Sui problemi socio-sanitari connessi alla patologia osteoporotica» 2003

OSTEOPOROSIS EVOLUTION WITH AGEING

OSTEOPOROSIS CLASSIFICATION

Primitive

- Postmenopausal
- Senile
- Juvenile idiopathic

OSTEOPOROSIS CLASSIFICATION
Secondary (1)

➢**Pharmacological causes**
-Corticosteroids
-Alcohol
-Aluminum
-Anticonvulsants, Lithium
-Chemotherapy/immunosuppressive
-Heparin

➢**Kidney disease**
-Hypercalciuria
-Renal osteodystrophy
-Pain reliever nephropathy
-Tubular acidosis
-Idiopathic renal lithiasis

➢**Endocrine diseases**
-Hypogonadism (primitive/secondary)
-Hyperparathyroidism
-Hypothyroidism
-Hypercortisolism

➢**GI diseases**
-Cholestatic diseases
-Malabsorption syndromes (e.g. celiac disease, IBD)
-Parenteral Nutrition
-Subtotal Gastrectomy

OSTEOPOROSIS CLASSIFICATION
Secondary (2)

➢**Hematologic/infectious causes**
- Mastocytosis
- Myeloma, leukemia, lymphoma
- Gaucher's Disease
- AIDS
- Hemochromatosis
- Thalassemia

➢**Connective tissue disorders**
- Ankylosing spondylitis
- Homocystinuria
- Marfan's Syndrome
- Osteogenesis imperfecta
- Rheumatoid arthritis

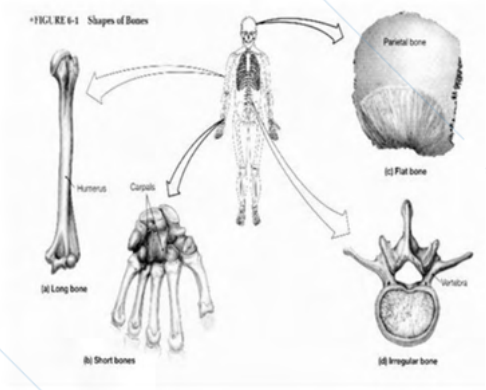
➢**Osteomalacia**
- Vit D deficit/resistance
- Hypophosphatemia

➢**Other**
- Amiloidosis
- Immobilization
- Multiple Sclerosis
- Porphyria
- Low BMI
- Idiopathic scoliosis

PHYSIOPATHOLOGIC MECHANISMS OF OSTEOPOROSIS

BONE TYPES

- Long
- Short
- Flat
- Irregular

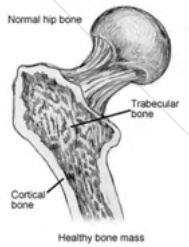


BONE HISTOLOGY

CORTICAL

- 80% of total skeletal mass
- Porosity <15%
- Structural function
  - Protection
  - Locomotion

Consisting of dense and well-organized lamellae has higher strength but a lower capacity to withstand a load that exceeds the elastic deformation range.



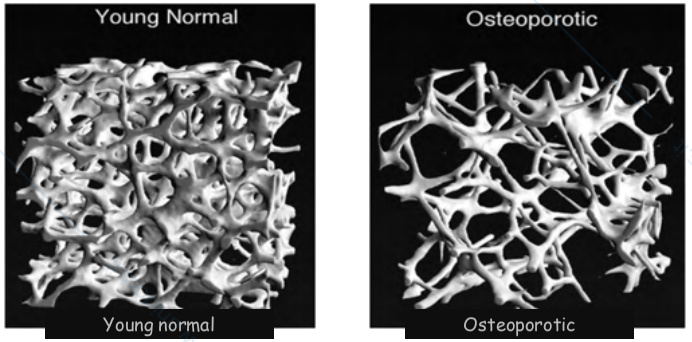
TRABECULAR (or cancellous)

- 20% of total skeletal mass
- Porosity ~30-90%
- Metabolic function
  - High contact surface
  - 6-8 higher metabolic turnover compared to cortical bone

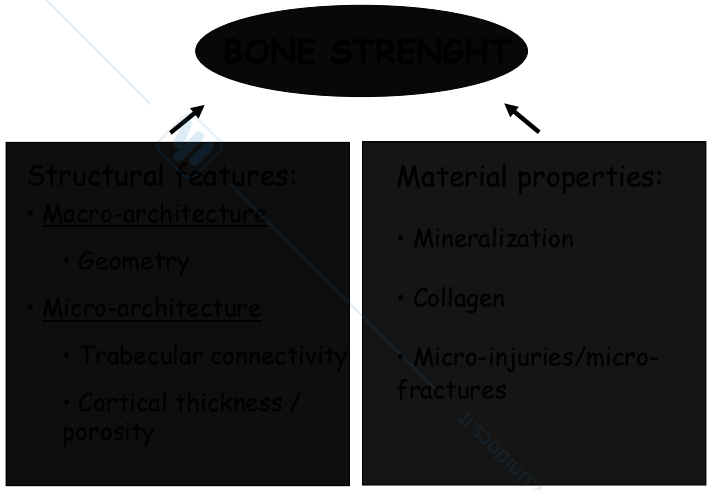
Composed of unparallel lamellar units with variable porosity (50%-90%).
High metabolic rate.

Overview of osteoporosis: pathophysiology and determinants of bone strength

Key pathological feature: Note the greater quantity of normal bone, as well as its greater interconnectivity, compared to osteoporotic bone.



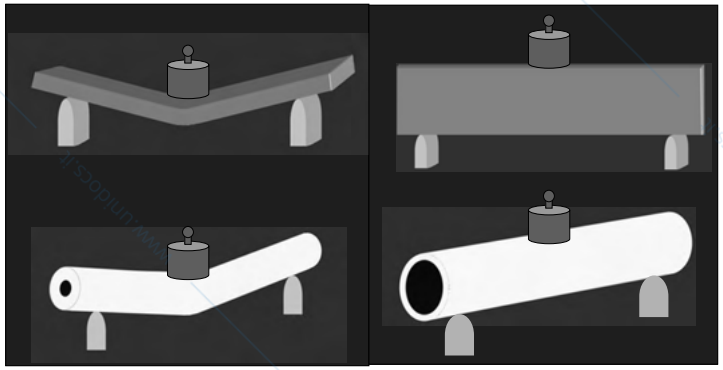
Bono and Einhorn. Eur Spine J. 2003; 12(2): S90 - S96



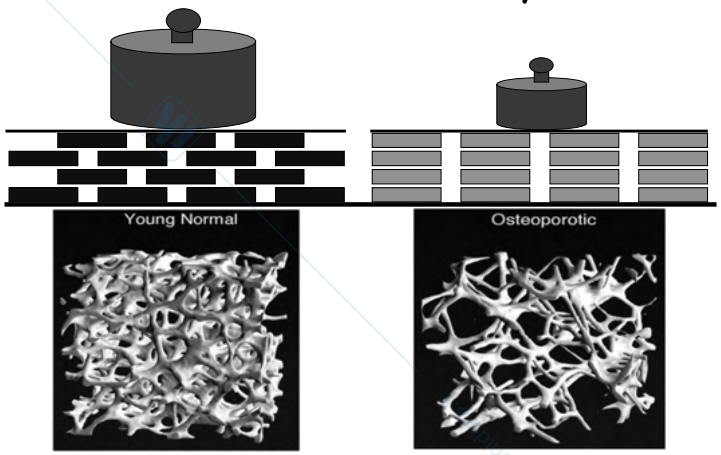
Chesnut et al. JBMR 2001; 16: 2163-72

Same mass, different resistance to a load application

depending on the quality of bone architecture…



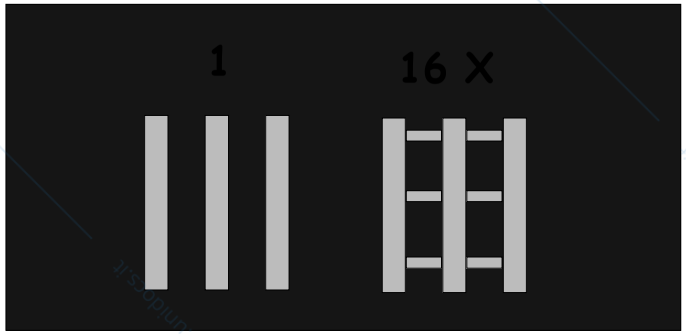
...and connectivity



Bono and Einhorn. Eur Spine J. 2003

TRABECULAE STRUCTURAL ROLE

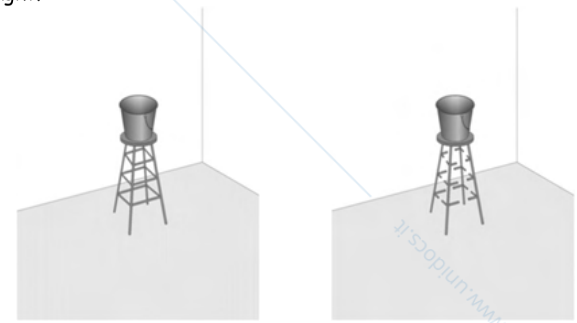
Resistance to compression of interconnected and disconnected trabeculae

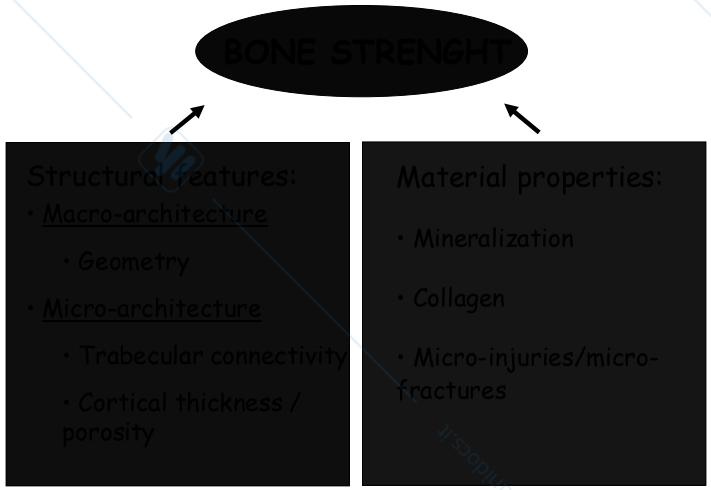


Bell et al. Calcified Tissue Research 1: 75-86, 1967

MICROARCHITECTURE STRUCTURAL ROLE

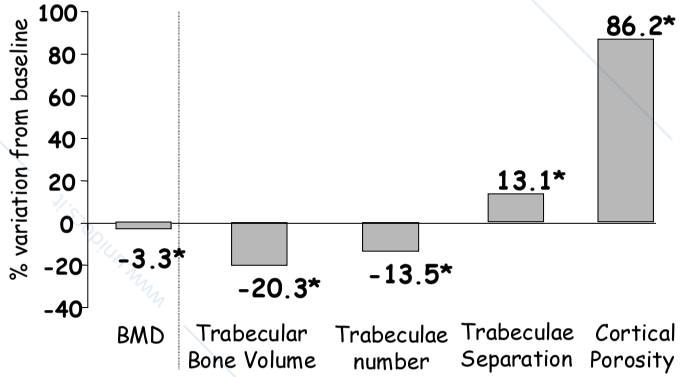
Structural integrity is important for resistance to deformation. This is represented by the network of horizontal pegs, which are at higher risk of losing connectivity.
This picture shows what happens when peg structures disappears. Resistance to deformation of a given bone structure is lowering with the squared lenght of the continuous vertical structure. Therefore, preserving the network is needed for preserving the overall structure strenght.





Chesnut et al. JBMR 2001; 16: 2163-72

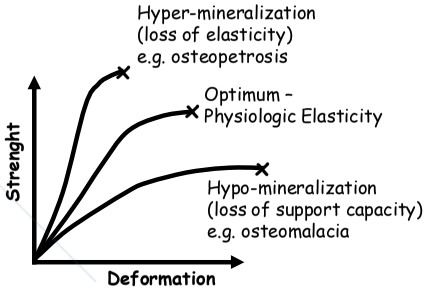
Modifications of micro-architecture one year after menopause



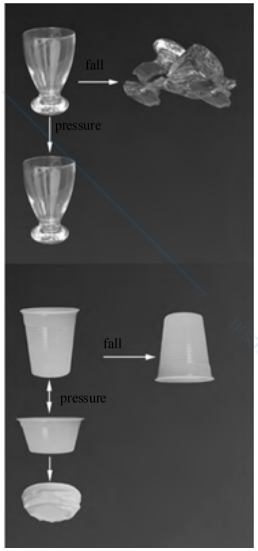
*p<0.05 vs basal

Dufresne TE, et al. Calcif Tis Int. 2003; 73(5):423-32

Bone Mineral Density (BMD)



Turner CH. Osteoporos Int 2002;13:97-104
Seeman E. Osteoporos Int 2003;14 (supp 4) S3



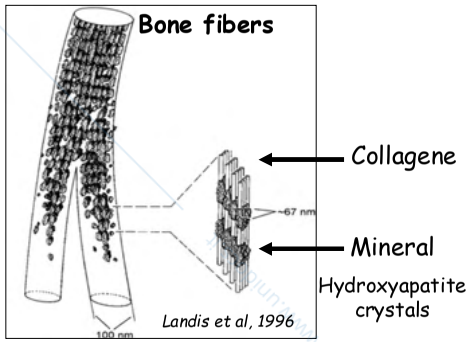
BONE MICROSTRUCTURE:

Thight relation between mineral component and collagen

Bone is a composite structure, consisting of type I collagen and a mineral component, mainly in the form of hydroxyapatite crystals.

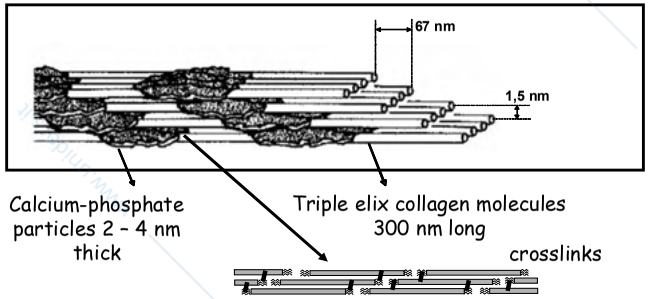
Differences in the amount of collagen or in the type of interconnections, as well as the amount of mineral or the size and shape of the crystals, all contribute to bone strength.

The organic matrix (e.g., collagen and noncollagenous proteins) is thought to control bone ductility and its capacity to withstand an impact without cracking.



MECHANIC PROPERTIES

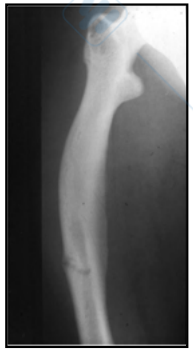
The mineral-collagen layer is the smallest block unit within the bone building structure. It is well conserved among different types of bone tissue and between the different species.



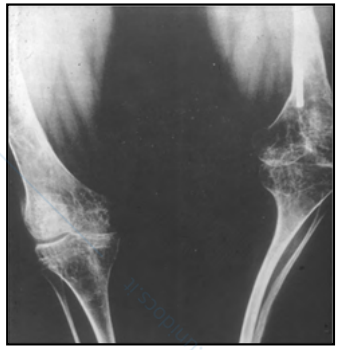
WJ Landis et al., Micr. Res. Tech., 1996

Impairments of mineralization and collagene

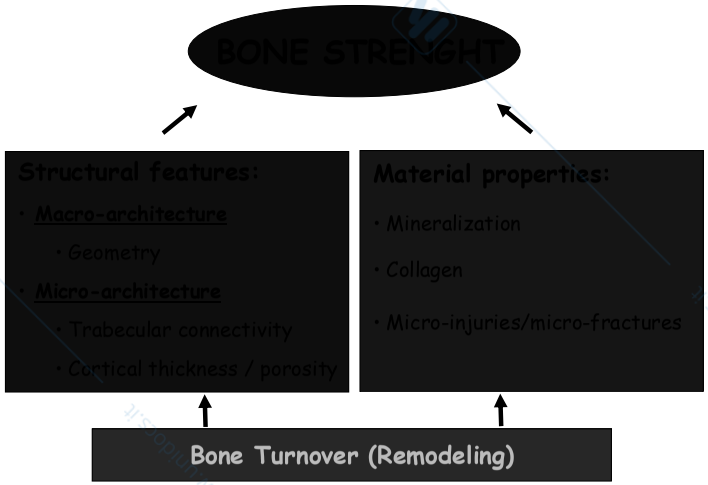
Osteomalacia= deficit in mineralization



Osteogenesis imperfecta = deficit of collagene synthesis



Courtesy of Dr. Papapoulos



Chesnut et al. JBMR 2001; 16: 2163-72

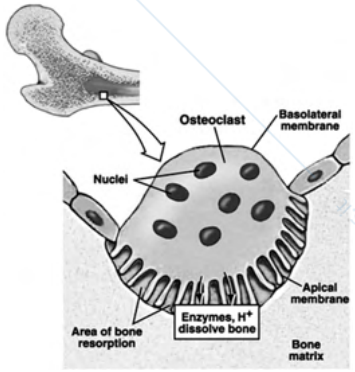
BONE TURNOVER (REMODELING)

About 10% of an adult skeleton is renewed every year due to the continuous succession of 2 opposing, closely related processes.

A complete cycle takes about 100-200 days.
The skeleton is completely renewed every 8-10 years.

BONE DYNAMICS

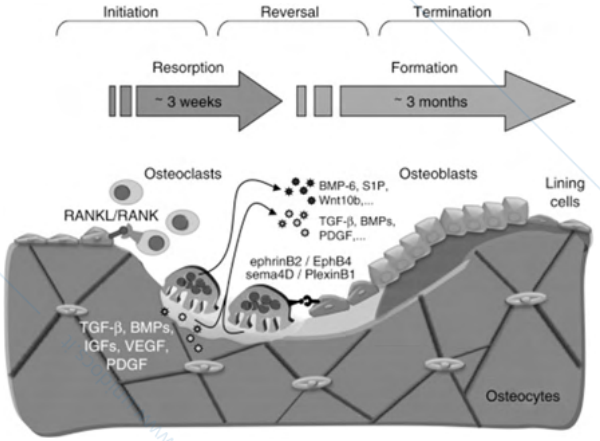
Bone is a complex organ including cells responsible for the continuous remodeling process.
The mineralized bone is reabsorbed by osteoclasts (freeing calcium and phosphate) and is formed by osteoblasts (depositing calcium and phosphate).
Bone remodeling is necessary for Ca++ homeostasis, and is regulated by hormones, growth factors and cytokines.



BONE CELLS

- Osteoclasts resorb bone. Hematopoietic origin. Preostecolasts differentiation is induced by RANKL-RANK binding. Preosteclasts fuse to form multinuclear osteoclasts.
- Osteoblasts synthesize new bone matrix. Mesenchimal origin. Secrete RANKL.
- Lining cells, flatten mature osteoblasts distributed on bone surface.
- Osteocytes are mature osteoblasts trapped in the matrix, equipped wiith dendritic extensions creating a network of communication mediated by gap-junctions (lacuno-canalicular network)

Modeling processes take place in lacunae on the bone surfaces



BONE REMODELING

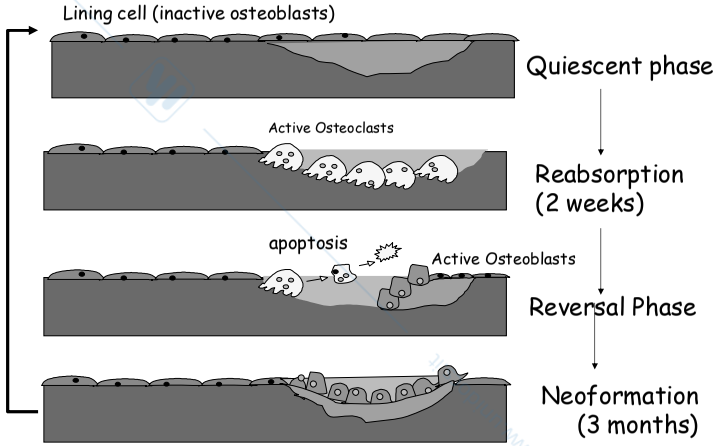
Bone remodeling involves the removal of mineralized bone by osteoclasts, followed by the formation and subsequent mineralization of bone matrix by osteoblasts.

Initiation starts with recruitment of hematopoietic precursors and their differentiation to osteoclasts, induced by osteoblast lineage cells that express osteoclastogenic ligands such as receptor activator of nuclear factor-κB ligand (RANKL). Osteoclasts become multinucleated and resorb bone.

BONE REMODELING

Basic multicellular unit (BMU) or bone remodeling unit (BRU)

In adults 1000000 active BMU at any moment.



BONE REMODELING

Transition is marked by switching from bone resorption to formation via coupling factors, which can stimulate osteoblast recruitment, differentiation, and/or activity. These signals include:

(1) bone resorption–derived signals, most typically comprising growth factors that were embedded in bone matrix and become released upon osteoclastic bone resorption (e.g., TGF-β, BMPs, IGFs, PDGF);

(2) soluble signals either or not osteoclast-derived (e.g., hormones and diffusible growth factors, such as BMP-6, S1P, and Wnt10b);

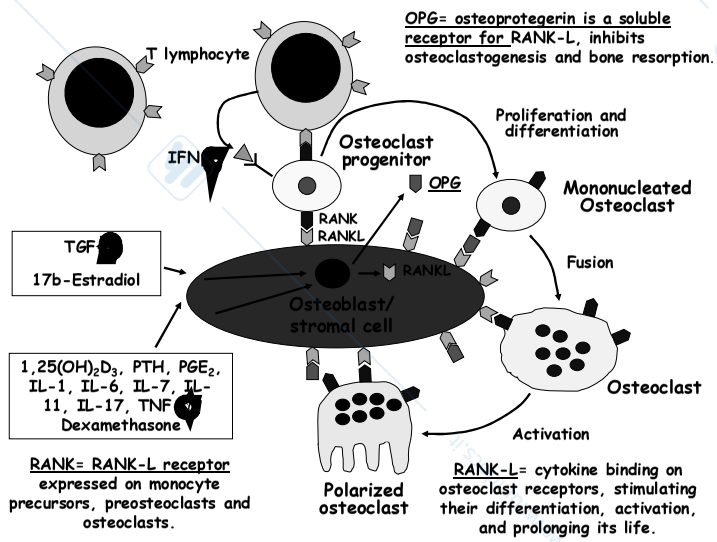
(3) membrane-bound molecules (e.g., ephrins, semaphorins).

During the termination phase, the resorbed lacuna is refilled through bone formation by osteoblasts that later flatten to form a layer of lining cells on the bone surface or become osteocytes connected by canaliculi within the bone.

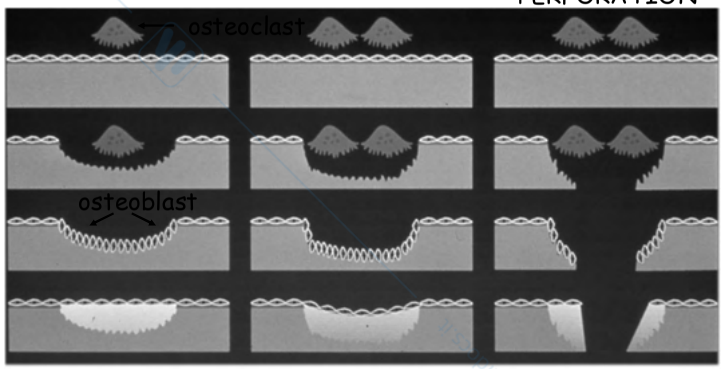
OSTEOBLAST-OSTEOCLAST MUTUAL MODULATION

The osteoblast derives from mesenchymal stem cells. It is responsible for the differentiation of a monocyte precursor to a pre-osteclast.
PTH regulates osteoblast secretion of M-CSF (macrophage-colony stimulating factor) as well as cytokine for which monocytes have a specific receptor, promoting the differentiation of monocyte precursors into osteoclasts.
In particular, RANK-Ligand, a protein expressed on osteoblasts and activated by T lymphocytes, binds to RANK (activating NF-kB receptor), present on osteoclasts and on all cells of the monocytic line leading to the transduction of a direct signal to the NF-kB (nuclear transcription factor regulating the production of many pro-inflammatory cytokines) which, in turn, induces the differentiation, development and activation of osteoclasts. Subsequently, the union of several osteoclasts forms an active multinucleated giant cell, which causes resorption and loss of bone.

To interrupt this circuit it is necessary to block the interaction of the RANKL with the RANK through the Osteoprotegerin (OPG), which is produced by the osteoblasts themselves.
The interaction between RANK-RANKL and OPG is believed to be crucial in regulating bone resorption.



BONE BALANCE



HORMONE REGULATION OF BONE REMODELIN

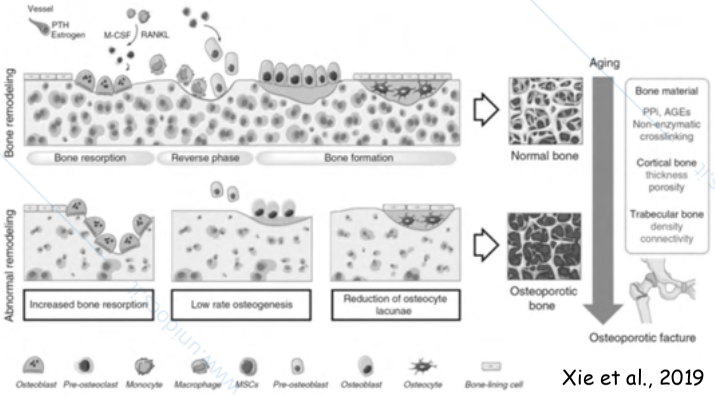
Glucocorticoids increase bone resorption and decrease osteosynthesis.

Thyroid hormones have a catabolic effect, increase collagen degradation and stimulate bone resorption leading to a reduction in total bone mass, as confirmed by the incidence of osteoporosis in hyperthyroid patients.

The **somatotropic hormone (GH)** acts directly on the intestinal absorption of Ca2+ and on the mineralization of the matrix determining an increase in skeletal mass.

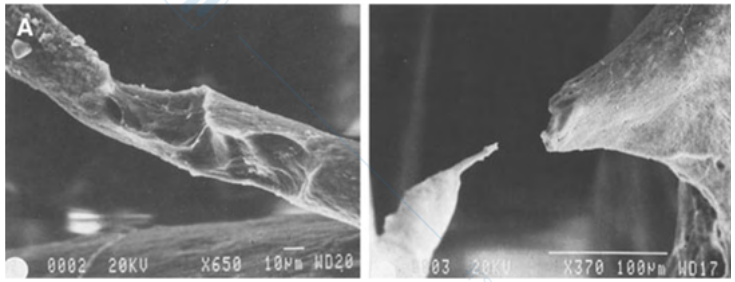
Estrogens and androgens inhibit bone resorption and increase intestinal absorption of Ca2+, the synthesis of vitamin D3 and the secretion of calcitonin.

The aging process in osteoporotic bone would lead to overaccumulation of PPi, AGEs, and nonenzymatic crosslinking of collagen, which disturb the normal organization of bone material. With the increase of bone resorption and low rate osteogenesis, the osteocyte lacunae reduction leads to decreased trabecular thickness and more porous cortical bone.



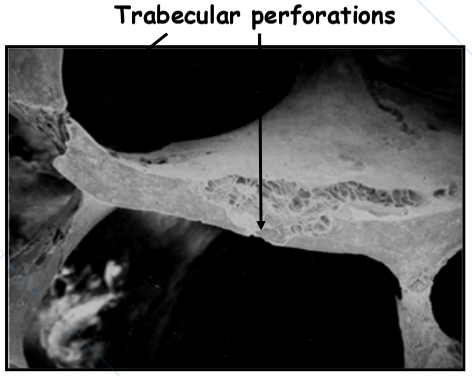
Xie et al., 2019

Imbalance between bone formation and resorption leads to trabecular thinning and eventual loss of connectivity



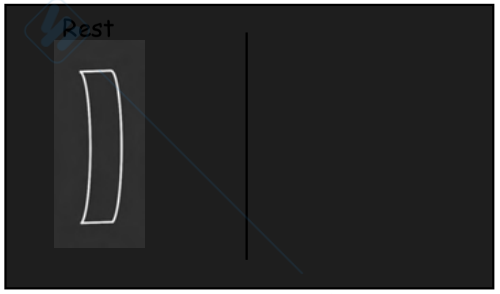
Mosekilde L. Bone Miner 1990

Trabecular perforation could reduce the resistance of the trabecular bone, predisposing to bone fragility.



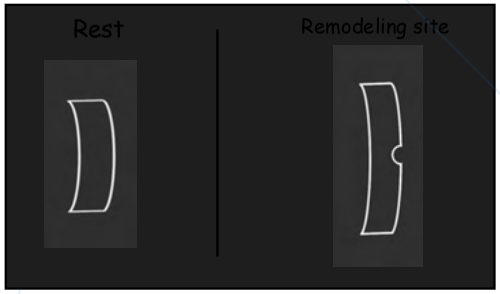
Dempster and Lindsay ,1993

How can remodeling predispose to bone fragility?



Parfitt AM, 1991

How can remodeling predispose to bone fragility?

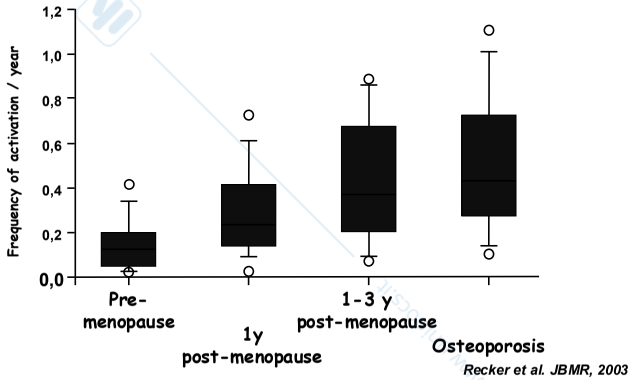


The resorption cavities are weak points on the surface of the trabeculae → increase the proability of breakages

Parfitt AM, 1991

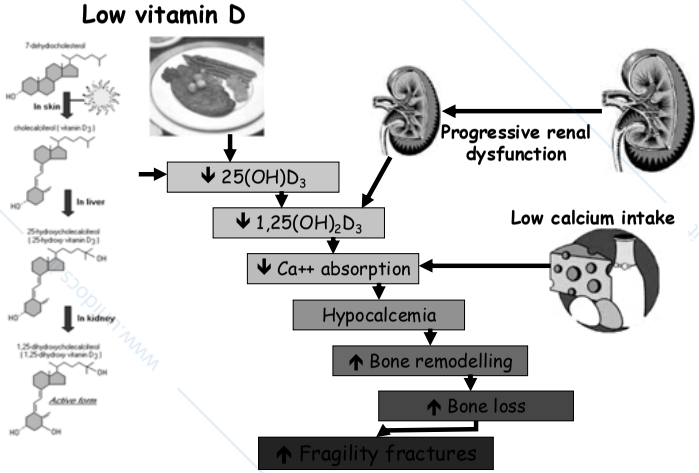
REMODELING INCREASES IN MENOPAUSE AND IN OSTEPOROSIS

Frequency of activation of BRU in human iliac crest biopsies

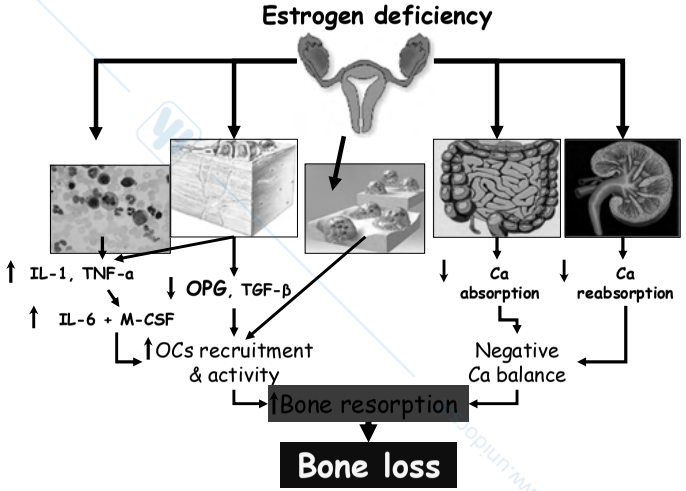


Recker et al. JBMR, 2003

Pathophysiology of senile osteoporosis



Pathophysiology of menopausal osteoporosis



✓ In recent years we begun to understand that osteoporosis is a disease characterized by impaired bone strength, rather than just a reduction in bone mass.

✓ It is clear today that bone quality is involved at least as much as its quantity in determining bone strength.

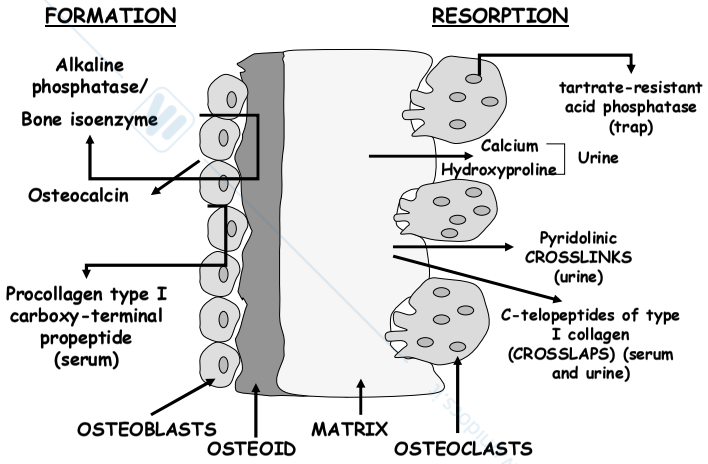
OSTEPOROTIC FRACTURE RISK FACTORS

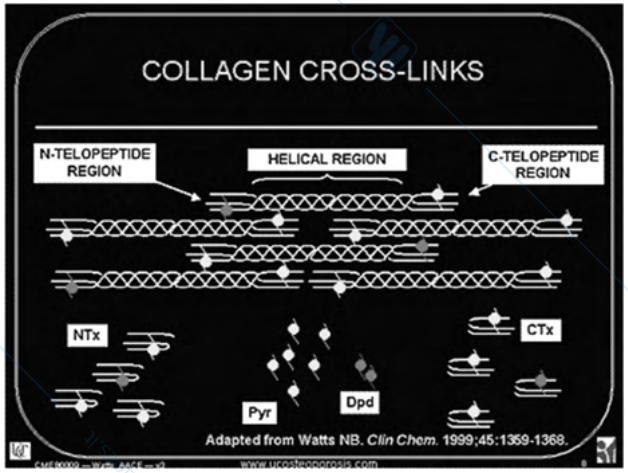
Nonmodifiable	Modifiable
Advanced age	Smoking
Female gender	Inadequate calcium intake
White/Asian race	Inadequate vitamin D
Low peak bone mass	Low body weight (BMI <21 kg/m^2)
Family history of osteoporosis	Estrogen deficiency
Personal history of fracture	Hypogonadism
Low Body Mass Index	Chronic glucocorticoid therapy (see table 3 for other medications)

Clinical management of osteoporosis

- **Femur fracture**
- **Wrist fracture**
- **Vertebral fracture**
  - **Changes in body shape and its consequences:**
    - Kyphosis
    - Height reduction
    - Abdomen protrusion
    - Reduced lung capacity
    - Reflux esophagitis

MARKERS OF BONE TURNOVER



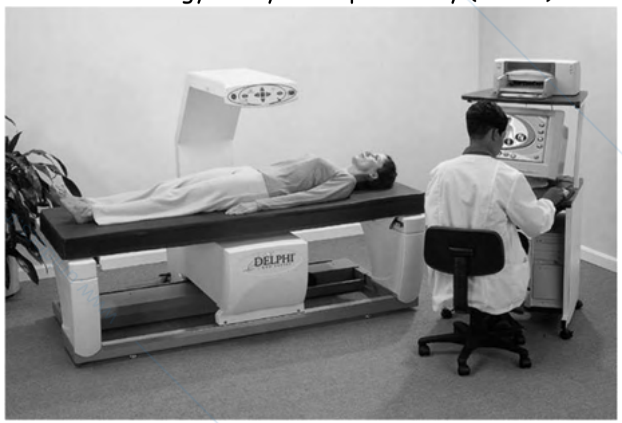


NTx= N-terminal telopeptide ; CTx: C-terminal telopeptide
Pyr= pyridolin; Dpd: desoxypyridolin

Bone turnover markers have an excellent ability both to predict the risk of fx, and to quickly evaluate response and compliance with therapy.

QUANTIFICATION OF BONE MINERAL DENSITY by BONE DENSITOMETRY

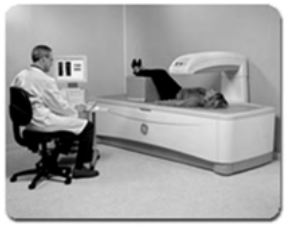
Dual energy X-ray absorptiometry (DEXA)

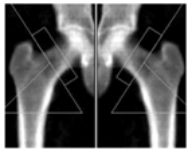


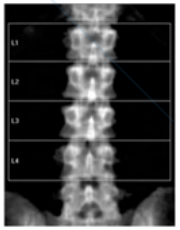
Standard Clinical Assessement of BMD

BMD: bone mineral density
BMC: bone mineral content

$$BMD\ (g/cm^2) = \frac{BMC\ (grams)}{Area\ (cm^2)}$$







T-score - diagnosis

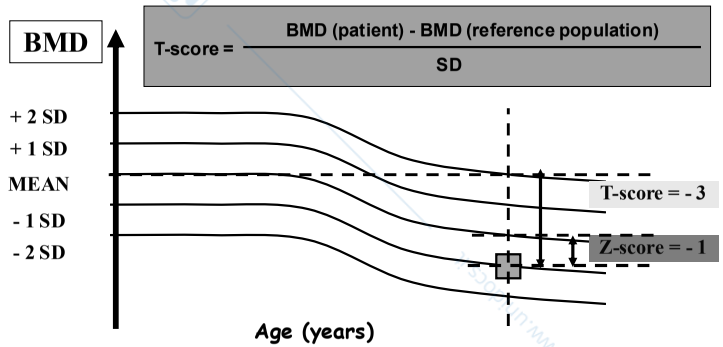
In the adult, the diagnosis is made by the T-score, i.e. by assessing how much the value under examination differs from that of the reference sample (healthy subjects of the same sex and age ranging 25-30 years, i.e. examined in the moment when peak bone mass is reached).

In more precise terms, the T-score is the difference, expressed in number of "standard deviations", between the observed individual value and the average value of the healthy reference population.
T-score values between +1 and -1 indicate normal bone mineralization.

According to the WHO criteria :
- osteopenia T-score < -1,
- osteoporosis T-score < -2.5

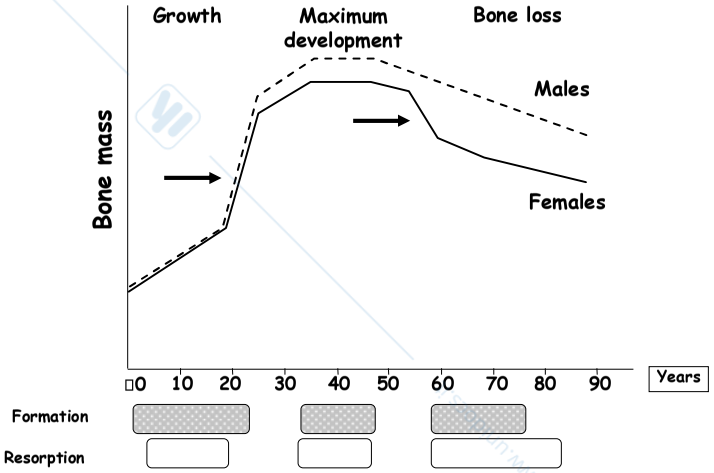
T- and Z-score calculation

T-score: the reference population is represented by healthy subjects aged 30y of the same sex. Expresses the difference between the densitometric value of the patient and the mean theoretical value (peak bone mass).
Z-score: the reference population is represented by normal subjects of the same sex and age. It expresses the difference between the densitometric value measured in the patient and the theoretical mean matched for sex and age.



Suppose we measure the BMD in 50000 women in the given group with the DEXA. We can calculate the average of these measurements and suppose that this value is equal to 300 g / cm3. We can also calculate the standard deviation and assume that this is equal to 40. Statistic tells us that approximately 15.9% of the sample, or 7935 women, will have BMD lower than average - SD, that is 300 - 40 = 260 g / cm3, and that as many of them will have values above the average + SD, 340 g / cm3. Reasoning in the opposite direction we can say that if a woman has a BMD by DEXA equal to 260 g / cm3, then her T-score will be -1. We can also say that a woman having a BMD of 220 g / cm3 will have a T-score of -2, because in this case it is equal to mean – 2xSD.

BONE MASS and AGE



WHO Criteria

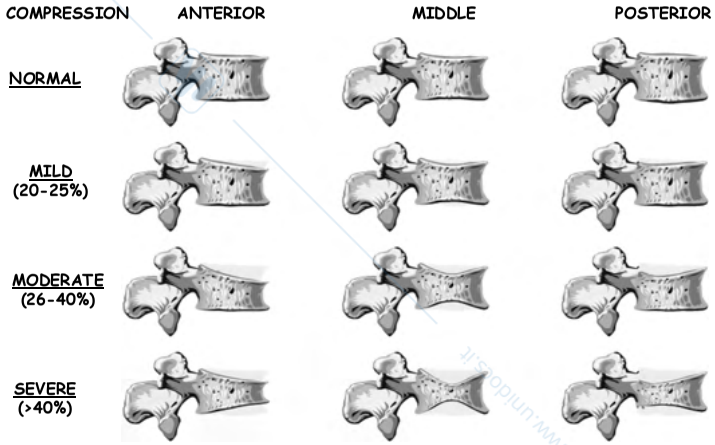
Classification	T score
Normal	-1 or greater
Osteopenia	Between -1 and -2.5
Osteoporosis	- 2.5 or less
Severe Osteoporosis	-2.5 or less and fragility fracture

✓T-score cut-off was set at -2.5 because it defined a % of patients similar to the «life time risk» of fracture of 50yo menopausal women, which is 40%.

✓ These cut-off were only calculated on the female population and on the femur bone.

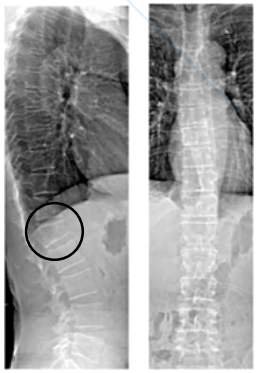
✓ There are no evidences for the applicability of the same cut-off also to the male population.

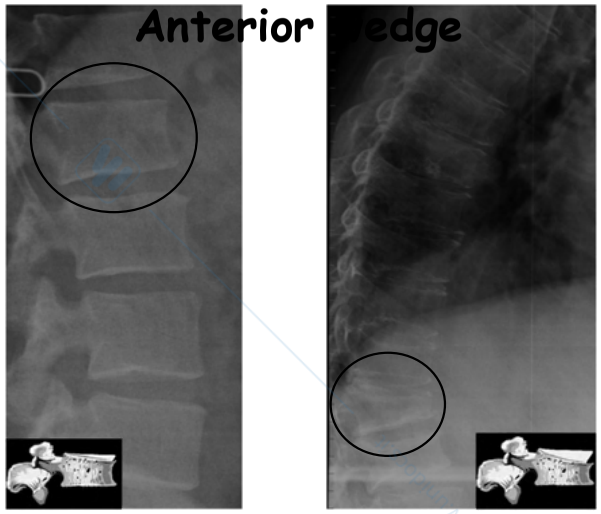
Genant semiquantitative method for diagnosis of osteoporotic vertebral fracture

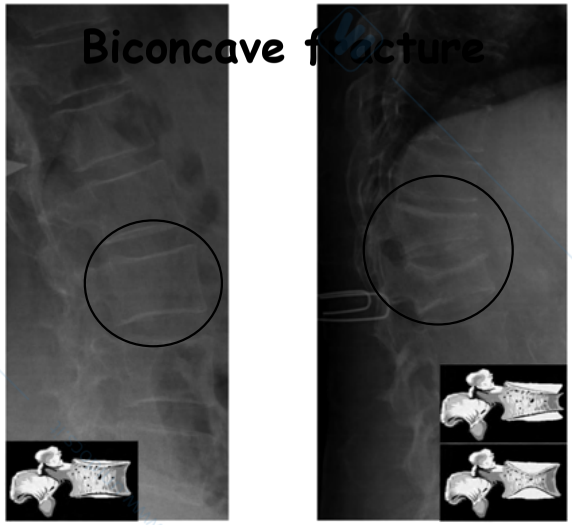


VERTEBRAL MORPHOMETRY
morphometric x-ray radiography

Image is taken on the entire column (T4-L4) in less than 10 seconds and with a dose equivalent to 1% of the dose (<10μ Sv) used in normal radiographic plates.







THERAPY

PILLARS OF BONE HOMEOSTASIS

Alkalis

Calcium

VitD3

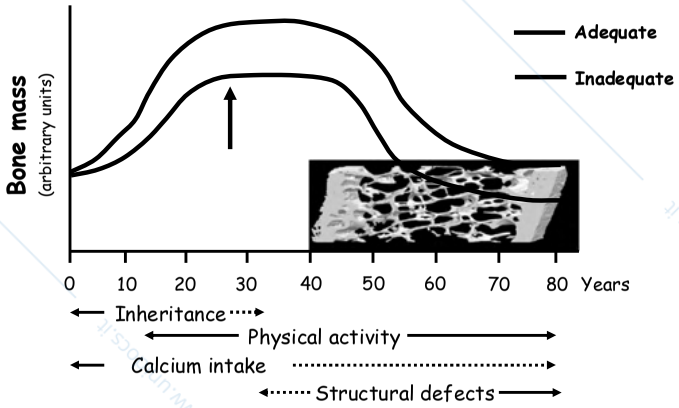
PILLARS OF BONE HOMEOSTASIS

Alkalis

Calcium

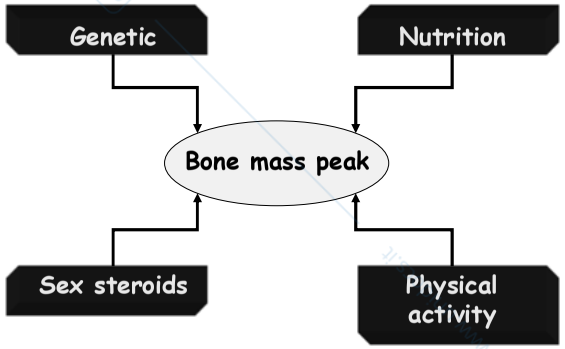
VitD3

DETERMINANTS OF BONE MASS

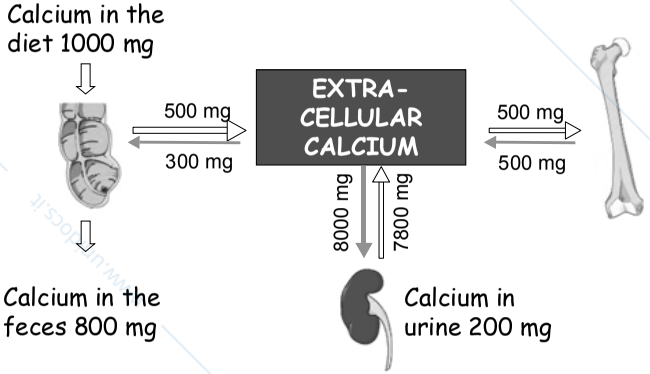


DETERMINANTS OF BONE MASS PEAK

Nutrition is one of the most important modifiable factors in the development and maintenance of bone mass and in the prevention and treatment of osteoporosis.



NORMAL CALCIUM BALANCE



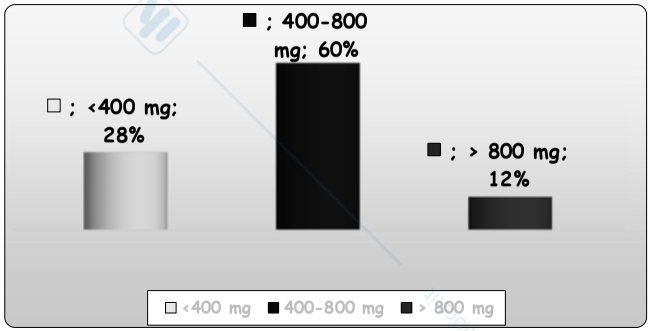
Recommended calcium intake based on different ages and conditions

Age (years)	"RECOMMENDED DIETARY ALLOWANCES" (RDA) *National Research Council* U.S.A. (1989) Calcium (mg/die)	"REFERENCE NUTRIENT INTAKE" (RNI) *Department of Health* UK (1991) Calcium (mg/die)	"ADEQUATE INTAKE" (AI) *Institute of Medicine* U.S.A. (1997) Calcium (mg/die)
0-10	400-800	350-550	210-800
11-24	1200	700-1000	1000-1300
25-50	800	700	1000-1200
> 50	1500	700	1200
Pregnancy	1200	No increment	1000-1300
Breast feeding	1200	+ 550	1000-1300

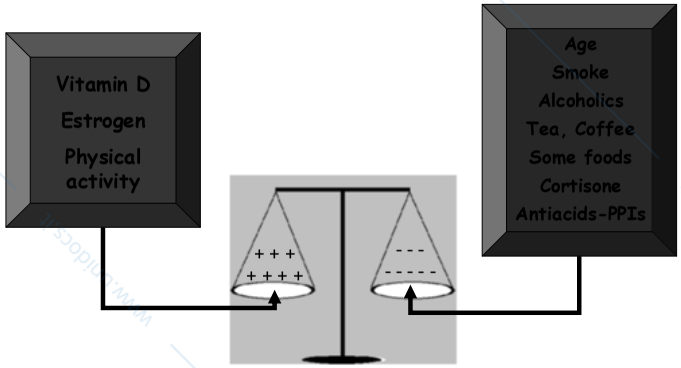
CALCIUM, CHOLESTEROL AND SODIUM AMOUNTS IN MILK AND DERIVED FOODS

100 g di prodotto	CALCIUM (mg)	CALORIES (Kcal)	CHOLESTEROL (mg)	SODIUM (mg)
Latte intero	119	62	14	50
Latte parzialmente scremato	120	50	8	53
Latte scremato	122	37	2	52
Yogurt (latte intero)	121	61	13	46
Mozzarella	484	264	72	350
Fontina	928	362	108	514
Parmigiano	1108	366	62	1498
Provolone	706	330	66	818
Ricotta	194	162	46	80
Pecorino romano	990	362	96	1122
Briè	172	314	66	586
Groviera	946	386	102	314
Fiocchi di latte	73	104	12,8	380

Daily Calcium Intake

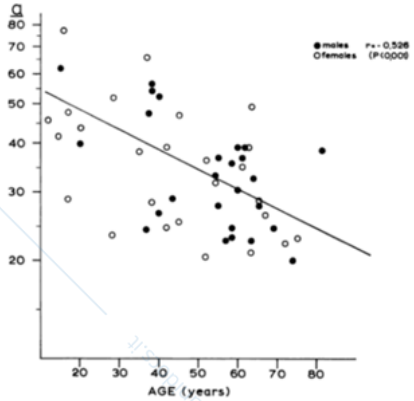


DETERMINANTS OF CALCIUM ABSORPTION



AGE IMPACT on CALCIUM ABSORPTION

Relationship between coefficient of intestinal absorption of calcium and age in normal male and female sublects.



Alevizaki CC. et al. J Nucl Med.

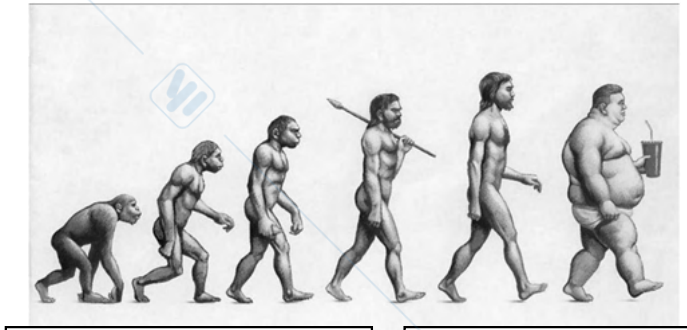
PILLARS OF BONE HOMEOSTASIS

Alkalis

Calcium

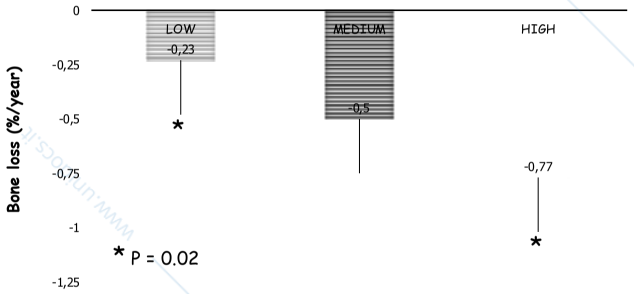
VitD3

Evolution of human diet

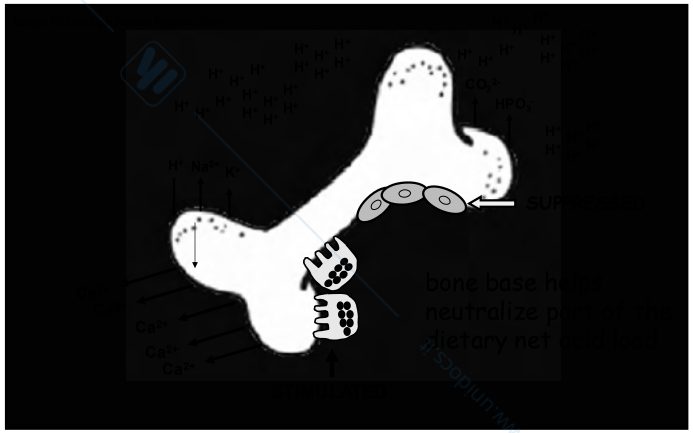


Little meat	A lot of meat
A lot of fruits and vegetables	Little vegetables and fruits
Frequent escapes from predators	Frequent rest in an armchair

Mean rate of femoral neck bone loss by ratio of animal to vegetable protein intake

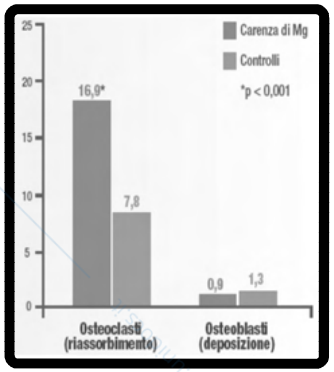


Mechanism by which H+ leads to release of bone calcium and are buffered by the bone mineral during metabolic acidosis



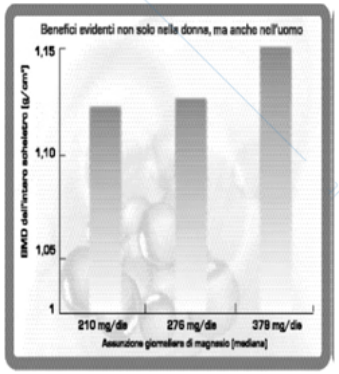
Magnesium role in bone metabolism

RAT



Rude RK. et al. Calcif. Tissue Int. 2003;72: 32-4

HUMAN



Ryder KM. J Am Geriatr Soc. 2005;53:1875-80

PILLARS OF BONE HOMEOSTASIS

Alkalis

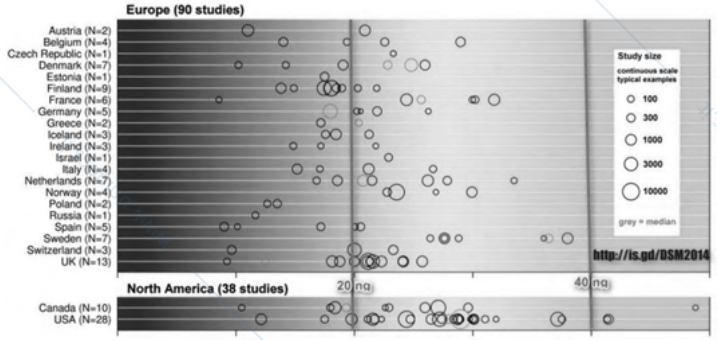
Calcium

VitD3

Vitamin D levels in the world

70% of the Italian population is below the minimum levels of vitamin D in the blood. For hospitalized people the percentage rises up to 100%. Scandinavian citizens, despite not having much sun, have higher levels of vitamin D thanks to the of vitamin D supplementation in popular foods.

Overview of published 25-hydroxyvitamin D mean / median values by countries



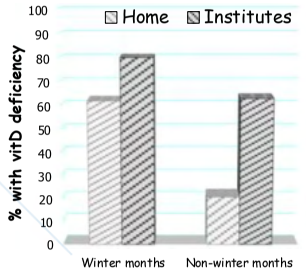
Hilger et al., Br J Nutr (2014)

Prevalence of Hypovitaminosis D in Italian postmenopausal women seasonal factor

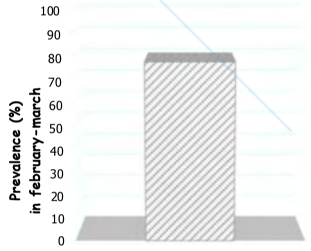
	40-50 years	51-60 years	61-70 years	> 70 years
Dec/May	35.7 %	36.2 %	37.5 %	51.2 %
Jun/Nov	12.0 %	9.6 %	14.9 %	16.7 %

Bettica P et al. Osteoporos Int 1999

Prevalence of Hypovitaminosis D in Italian elderly population



Rossini et al, It J Min Elect Metab, 1990



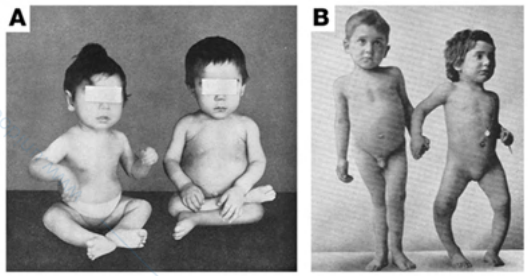
Isaia et al, Osteoporos Int, 2003

Hypovitaminosis D

Vitamin D status	Circulating Concentration 25(OH)D
NORMAL	≥ 75 nmol/L (≥ 30 ng/ml)
SUBOPTIMAL	30-75 nmol/L (12-30 ng/ml)
DEFICIENT	< 30 nmol/L (12 ng/ml)

Early description of rickets

17th century: first descriptions of children suffering from a disease causing growth retardation, widening of the epiphyses of long bones, deformity of the lower limbs, scoliosis, "lumpy" rib cage, weakness and reduction in muscle tone.



Holick, M. F. J. Clin. Invest. 2006;116:2062-2072

Hypovitaminosis D

Increased risk for:

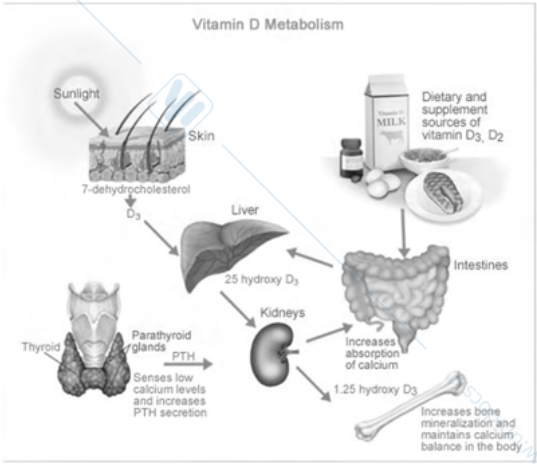
- ✓ Rickets / osteoporosis
- ✓ falls (reduced muscle strength)
- ✓ neoplasms (e.g. colon cancer)
- ✓ type I diabetes
- ✓ cardiovascular disease / hypertension
- ✓ tooth loss
- ✓ autoimmune diseases (systemic lupus erythematosus, multiple sclerosis)

VITAMIN D in FOODS

Aliment	U.I. Vit D/100g
Fresh Salmon	650
Cod liver oil	8500
Eggs	200
Cow milk	0.5-4
Human milk	0.4-9.7
Emmenthal cheese	100
Butter	40

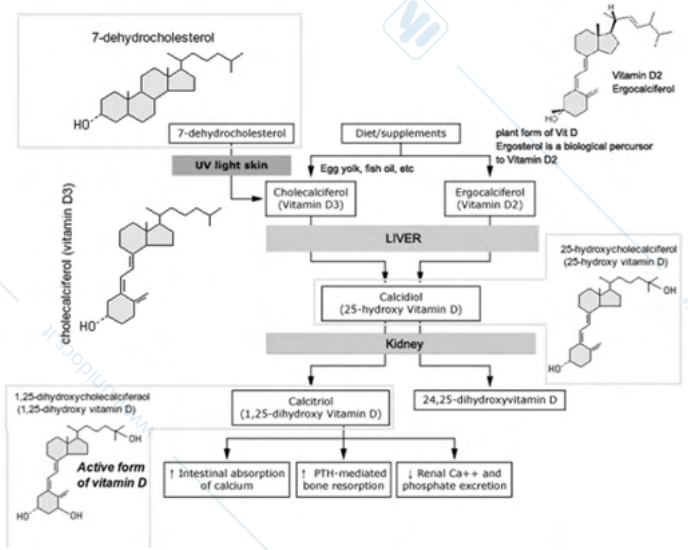
Vitamin D is mostly available in animal fat.

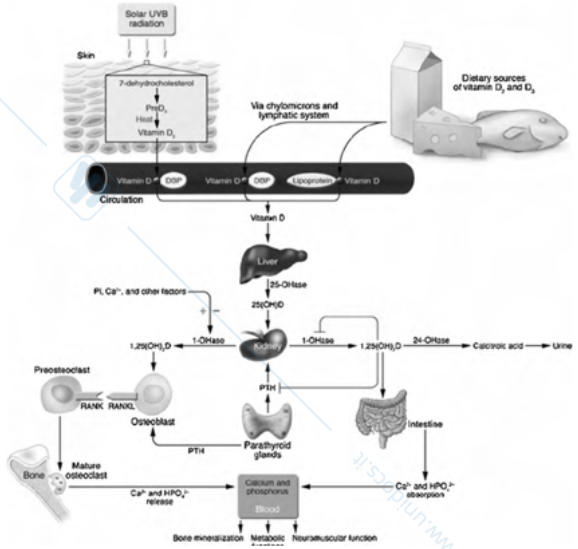
VITAMIN D and SUNLIGHT EXPOSURE

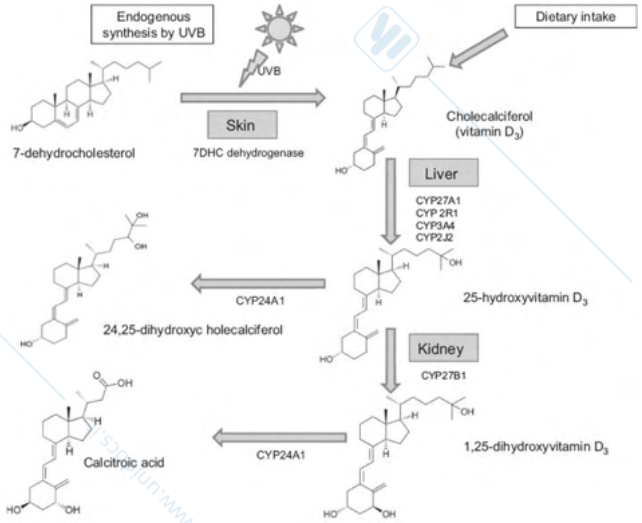


80% of the vitamin D requirement is guaranteed by solar radiation.

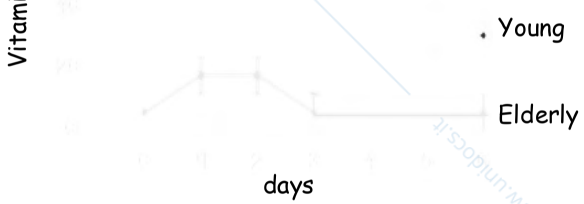
ACTIVE METABOLITE







VITAMIN D level after whole body exposure to UV rays
(minimum dosage causing erythema)

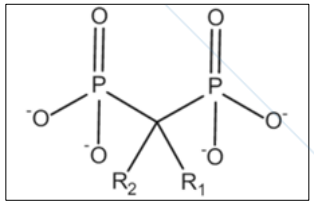


Reduction of the relative risk of fracture in patients treated with cholecalciferol (700-800 IU / day) compared to groups treated with placebo or with Calcium

Fractures	RR vs. placebo or calcium (reduction)	95% CI	NNT
Femur	0.74 (26%)	0.61 – 0.88	45
Non-vertebral	0.77 (23%)	0.68 – 0.87	27

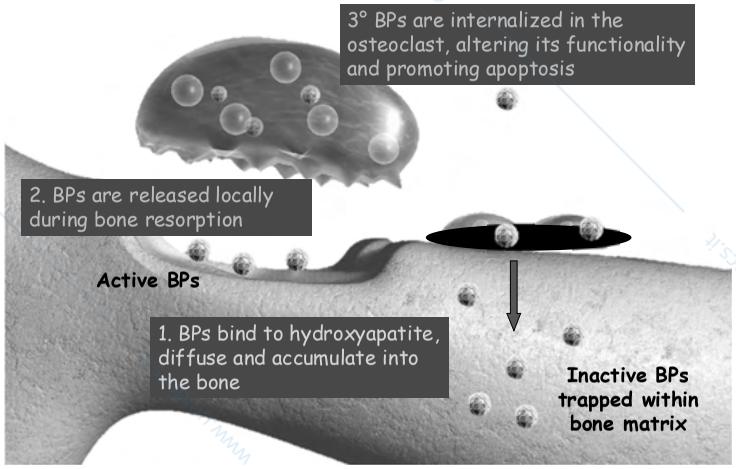
RR = Relativ Risk
95% CI = 95 % Confidence Interval
NNT = Number Needed to Treat (number of patients to be treated to prevent 1 event)

PHARMACOLOGIC THERAPHY
Bisphosphonate

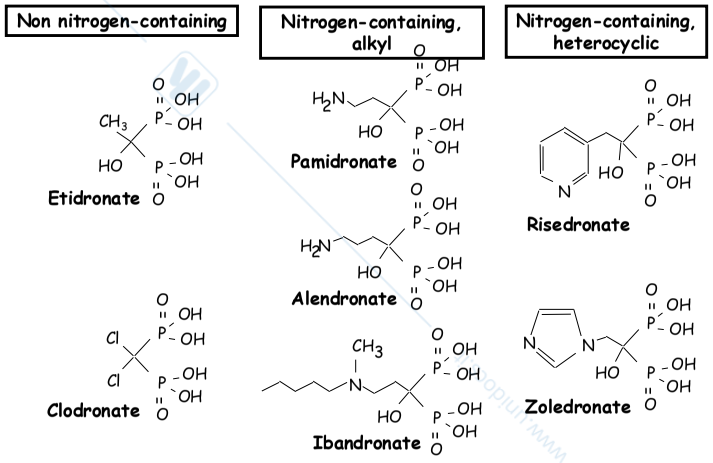


Derivatives of inorganic pyrophosphate (PPi), byproducts of multiple metabolic processes in many tissues, able to bind hydroxyapatite crystals.

Bisphosphonates mechanism of action

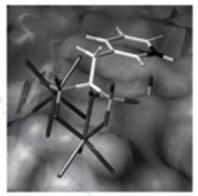


Different structure = different mechanism of action

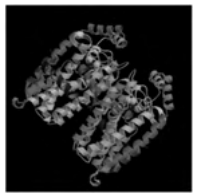


BPs chemical properties determining the anti-resorption activity

Affinity for bone matrix



Intracellular action

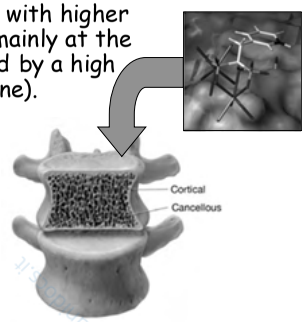


Russel RG. et al. Osteoporosis Int. 2008;19:733–59

High or Low bone affinity
Theoretical clinical implications

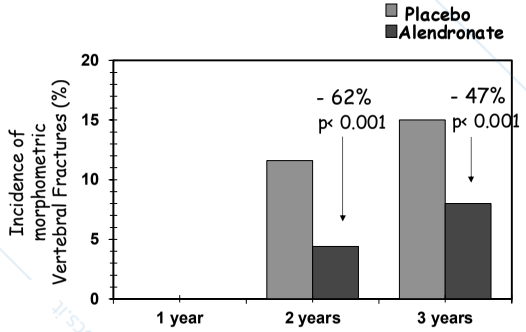
- Since the trabecular bone is characterized by a high turnover (6-8 times higher than the cortical bone), it is conceivable that the BPs with higher affinity for HAP are concentrated mainly at the level of the bone sites characterized by a high content of trabecular bone (e.g.: spine).

The different distribution could influence the effectiveness of BPs in different bone sites



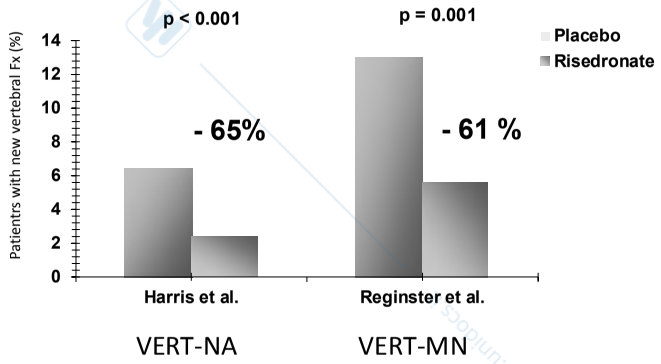
Masarachia et al. Bone 1996; Russel et al. Ann N Y Acad Sci. 2007;1117:209-57

Alendronate: Cumulative New Vertebral Fx Incidence – The FIT Study

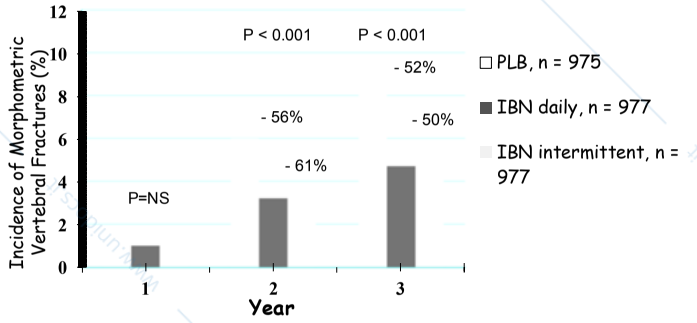


Black DM et al. Lancet. 1996;348:1535-1541

Risedronate: Cumulative 1-Year New Vertebral Fx Incidence – The VERT Studies

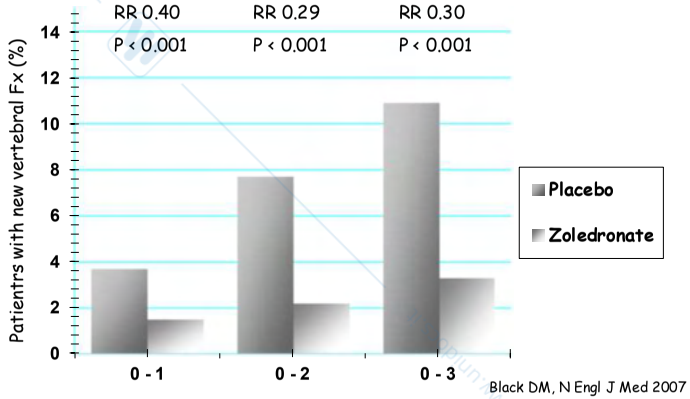


Ibandronate: Cumulative New Vertebral Fx Incidence – The BONE Study



Chesnut III CH et al. J Bone Miner Res 2004

Zoledronate: Cumulative New Vertebral Fx Incidence – The HORIZON Study

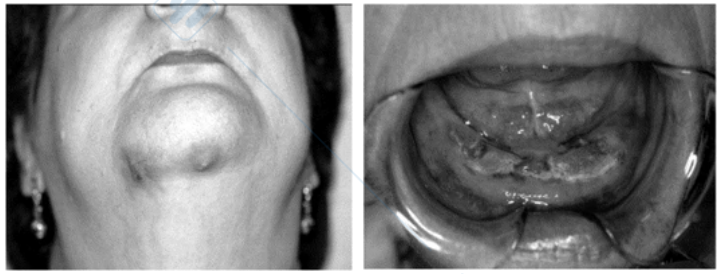


Black DM, N Engl J Med 2007

Bisphosphonate Adverse Events

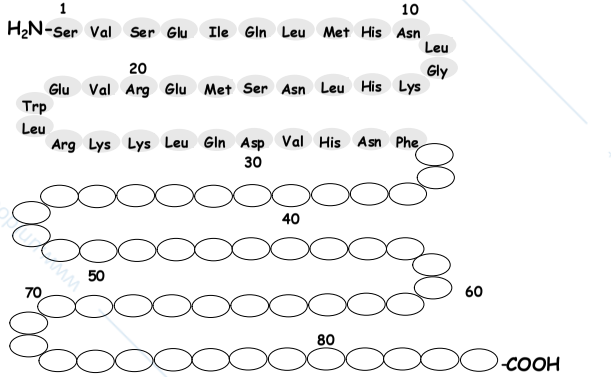
- ✓ Acute phase response
- ✓ Upper GI (gastritis, esophagitis)
- ✓ Rash
- ✓ Iritis
- ✓ Renal impairment
- ✓ Jaw osteonecrosis

BP-associated osteonecrosis of the jaws



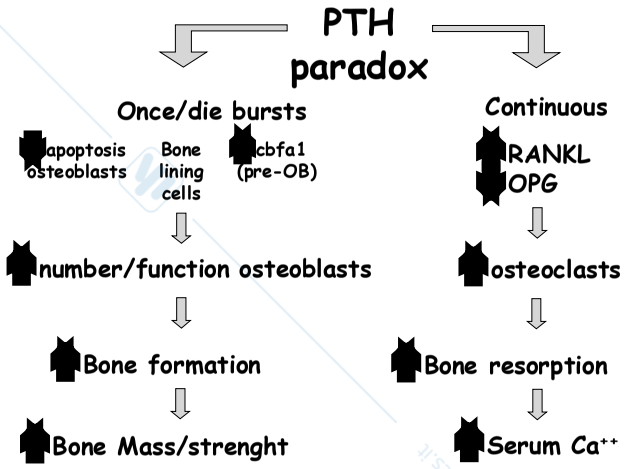
Teriparatide

Teriparatide is a recombinant peptide containing the first 34 amino acids that represent the biologically active sequence of the human parathyroid hormone



Proc Natl Acad Sci USA 1974;71:384-388

PTH paradox



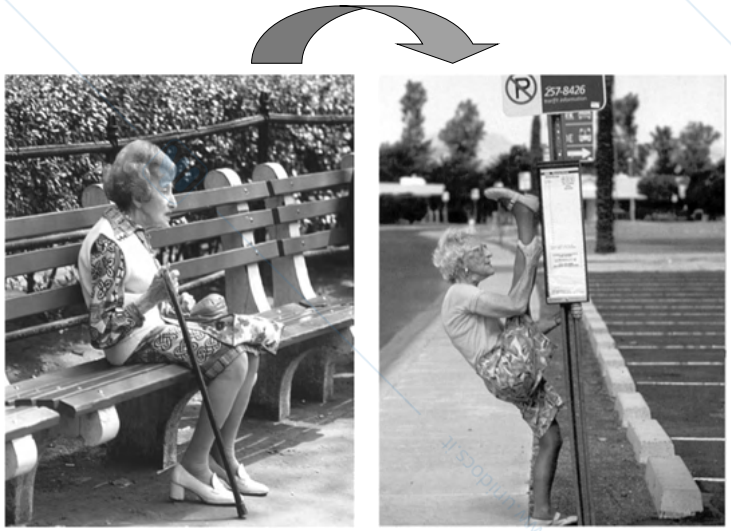
Dobnig and Turner. Endocrinology 1995; Hock. Musculoskel Neuron Interact 2001; Jilka. J Clin Invest 1999; Ma. Endocrinology 2001

Drugs effect on markers of bone turnover

Drug	Time for plateau	Variation
Hormone Replacement Theraphy	3-6 months (resorption)	↓ 50-60%
Alendronate	3 months (resorption)	↓ 70%
Risedronate	3-6 months (resorption)	↓ 60%
Ibandronate	3-6 months (resorption)	↓ 65%
Raloxifene	3-6 months (resorption)	↓ 30-40%
Teriparatide	3 months (resorption) 1 month (formation)	↑ 20% ↑ 55%
Strontium ranelate	3 months (resorption) 3 month (formation)	↓ ~10% ↑ ~ 10%

Adapted from: Briot K. et al. Best Pract Res Clin Rheumatol. 2005

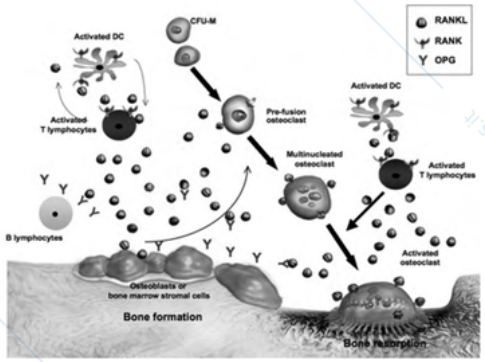
Denosumab



Denosumab

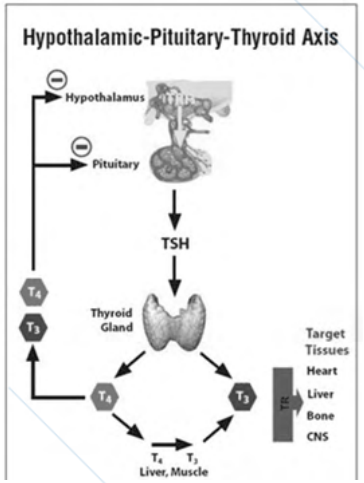
Denosumab acts by forming immune complexes with RANK-L, a protein that acts as a primary signal in promoting bone removal by binding to the RANK receptor.

In a number of conditions featured by bone loss, an imbalance exists between the RANKL (osteoclastic activator) which is increased, and the OPG or Osteoprotegerin (osteoclastic inhibitor).

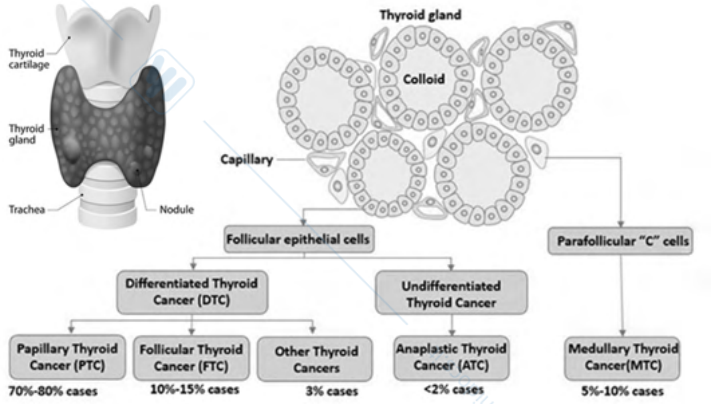


THYROID TUMORS

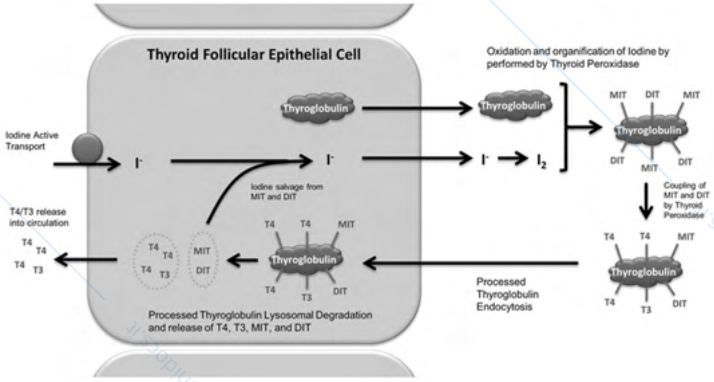
HYPOTHALAMUS-PITUITARY-THYROID AXIS



THYROID TUMORS



SYNTHESIS OF THYROID HORMONES



EPIDEMIOLOGY of THYROID TUMORS

50 – 75 % of the population have a thyroid nodule.

PREVALENCE

World: 2% of all cancers.

USA: 3% of all cancers (630000 in 2013).

INCIDENCE

World: 140.000 new cases in 2002 (74% F, 26% M);
Peaks in Pacific Islands, Australia, Japan, USA
Geographical differences depend on access to screening and possible environmental / ethnic factors.

USA:
- 64.000 new cases in 2016, varying with sex and ethnicity;
overall 8.2 cases / 100.000*year;
max 12.7 in white women, min: 2.4 in black men;
increasing with age: 20.3 in women 45-49y, 11.6 in men 65-69y.

EPIDEMIOLOGY in Italy

	1998-2002	2008-2012
Donne	16,2/100.000	28,2/100.000
Uomini	5,3/100.000	10,1/100.000

Istituto Superiore di Sanità, accessed february 2020

TABELLA 30. Tassi di incidenza (standardizzati sulla popolazione italiana) dei tumori tiroidei

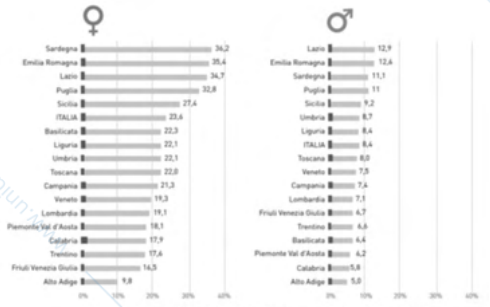


FIGURA 58. Tassi* di incidenza e mortalità per tumori della tiroide in Italia nel periodo 2003-2014 per sesso e Regione. *Standardizzati sulla popolazione Europea 2013

THYROID TUMORS IN ITALY 1998-2012

Incidence per 100000

Table 1
Populations included in the study, observation period, age-standardised incidence rates (ASR)† for incidence of thyroid cancer, Italy, 1998–2012, age 0–84 years.

Region	Cancer Registries (CRs)	Period of registration	Population 2008 (0–84 years) X million	WOMEN ASR 1998–2012	WOMEN ASR 1998–2002	WOMEN ASR 2003–2007	WOMEN ASR 2008–2012	MEN ASR 1998–2012	MEN ASR 1998–2002	MEN ASR 2003–2007	MEN ASR 2008–2012
	All Italian CRs	1998–2012	22.36	22.5	16.2	23.1	28.2	7.7	5.3	7.7	10.1
Piedmont	Torino	1998–2012	0.85	16.4	12.9	17.6	18.8	5.4	3.8	5.6	6.6
Lombardy	Biella	1998–2010	0.18	12.9	10.3	13.8	15.6	5.4	5.2	5.3	5.7
	Brescia	1998–2008	1.08	26.0	24.9	26.0	30.2	9.5	8.1	10.0	11.6
	Mantova	1998–2010	0.39	26.7	21.5	26.9	33.3	9.1	6.8	9.4	11.1
	Milano	1998–2010	1.19	13.6	12.5	13.9	14.5	5.8	5.0	6.5	5.5
	Sondrio	1998–2011	0.18	13.8	13.4	15.1	12.5	5.2	4.4	6.6	4.2
	Varese	1998–2012	0.83	13.9	10.6	13.8	17.4	5.4	4.5	6.0	5.8
Trentino-Alto Adige	Alto Adige/SouthTyrol	1998–2010	0.48	9.5	8.0	10.4	10.5	4.3	4.1	4.2	4.6
	Trento	1998–2010	0.50	15.4	12.4	16.5	18.3	4.9	3.5	5.2	6.2
Veneto	Veneto	1998–2009	2.26	15.5	12.3	16.5	20.7	5.6	4.2	5.8	8.4
Friuli Veneria Giulia	Friuli Venezia Giulia	1998–2010	1.17	15.5	14.1	15.7	17.9	5.2	3.9	5.5	6.6
Ligaria	Genova	1998–2009	0.83	18.1	15.7	19.1	22.3	6.5	4.8	7.1	8.8
Emilia-Romagna	Ferrara	1998–2011	0.34	35.7	28.4	43.1	35.9	12.2	9.7	13.0	14.5
	Modena	1998–2012	0.65	34.2	22.3	35.8	43.8	12.7	8.2	13.6	15.7
	Parma	1998–2012	0.40	36.7	21.7	44.5	43.7	11.5	7.7	13.9	12.8
	Reggio Emilia	1998–2012	0.49	31.2	23.8	28.9	40.0	11.0	7.6	10.8	14.0
	Romagna	1998–2012	1.13	32.6	20.1	33.2	42.3	10.6	6.0	9.0	15.7
Tuscany	Firenze-Prato	1998–2008	1.16	17.0	14.9	16.9	27.5	5.9	5.0	6.6	7.0
Umbria	Umbria	1998–2011	0.84	18.8	14.8	16.6	26.2	6.9	5.2	5.9	9.9
Lazio	Latina	1998–2011	0.51	37.2	25.5	44.2	42.0	11.9	6.5	14.8	14.4
Campania	Napoli	1998–2012	1.15	18.1	12.7	16.6	21.2	7.3	4.6	7.8	8.2
	Salerno	1998–2009	1.07	19.2	17.6	20.5	20.4	5.9	5.0	6.3	7.4
Sicily	Catania-Messina-Enna	2003–2012	1.86	37.3	~	36.2	38.5	12.0	~	9.8	14.0
	Palermo	2003–2012	1.21	23.7	~	23.1	24.4	7.7	~	7.1	8.3
	Ragusa-Caltanissetta	1998–2012	0.56	22.9	16.2	28.8	22.7	6.5	4.6	6.4	7.5
	Siracusa	1998–2012	0.39	22.0	20.0	20.8	24.6	6.2	4.9	6.2	7.2
Sardinia	Nuoro	2003–2012	0.21	44.4	~	35.4	53.1	11.7	~	8.8	14.6
	Sassari	1998–2011	0.46	27.2	18.5	28.5	35.9	8.0	4.7	8.6	11.1

L. Dal Maso et al. / European Journal of Cancer 94 (2018) 1–15

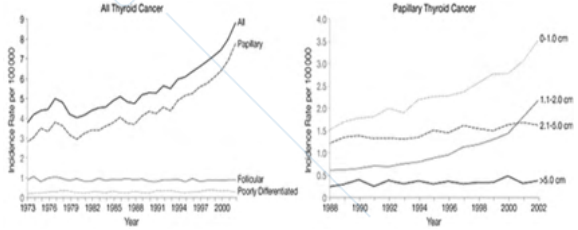
INCIDENCE IS IN INCREASING TREND

- Australia (New S. Wales)	1995	Fahey (Br J Surg
- Sweden and Norway	1997	Galanti (Cancer Causes Control)
- France	2002	Colonna (Eur J Cancer)
- France	2004	Leenhardt (Eur J Endocrinol)
- Scotland	2005	Reynolds (Clin Endocrinol)
- Lithuania	2006	Smailyite (BMC Cancer)
- Australia (Tasmania)	2006	Burgess (Thyroid)
- USA (Los Angeles)	2000	HaselKorn (Cancer Causes Control)
- USA (Florida)	2004	Hodgson (Ann Surg Oncol)
- USA	2004	Jemal (Cancer)
- USA (5 states)	2006	Davies (JAMA)
- Canada	2001	Lin (Br J Cancer)
- Canada (Ontario)	2007	Kent (CMAJ)

INCIDENCE IS IN INCREASING TREND

In USA in 1973 to 2002 the TC incidence grew by 4% per year, from 3.6 to 8.7 cases / 100,000, i.e. 2.4-fold increase in 29 years.
The increase is entirely due to the increase in papillary type, 2.9 times, while the follicular and poorly differentiated are stable.

Papillary tumors increase is almost entirely due to microcarcinomas.



Therefore, the increased incidence of papillary TC reflects, rather than a real increase in the disease, a better diagnostic efficacy for many of the clinically occult cancers whose high prevalence at autopsies is well known but without clinical impact and which could be considered "normal".

INCIDENCE and TREND in HISTOLOGICAL TYPES

Table 2
Number of incident cases and age-standardised incidence rates (ASR)[a] for thyroid cancer by sex, histological type and period. Italy, 1998–2012, age 0–84 years.

Histological type	Women							Men							W/M ratio (1998–2012)
	Period of diagnosis						Variation (%)[b]	Period of diagnosis						Variation (%)[b]	
	1998–2002		2003–2007		2008–2012			1998–2002		2003–2007		2008–2012			
	Cases (%)	ASR	Cases (%)	ASR	Cases (%)	ASR		Cases (%)	ASR	Cases (%)	ASR	Cases %	ASR		
Papillary	5365 (76.6)	**12.37**	10,317 (82.3)	**19.07**	9708 (83.2)	**23.49**	+90%	1438 (66.9)	**3.50**	2988 (75.0)	**5.80**	3033 (76.6)	**7.71**	+120%	**3.2**
Follicular	701 (*10.0*)	**1.63**	959 (*7.5*)	**1.75**	762 (*6.5*)	**1.84**	+13%	265 (*12.3*)	**0.66**	373 (*9.4*)	**0.73**	347 (*8.8*)	**0.88**	+33%	**2.2**
Medullary	227 (3.3)	**0.52**	351 (*2.8*)	**0.64**	301 (*2.6*)	**0.72**	+38%	141 (6.6)	**0.35**	211 (*5.3*)	**0.41**	175 (*4.4*)	**0.44**	+26%	**1.5**
Anaplastic	133 (*1.9*)	**0.32**	194 (*1.5*)	**0.35**	110 (*0.9*)	**0.26**	−19%	86 (*4.0*)	**0.22**	113 (*2.9*)	**0.22**	78 (*2.0*)	**0.20**	−9%	**1.2**
Other	28 (*0.4*)	**0.06**	45 (*0.4*)	**0.08**	38 (*0.3*)	**0.09**	+50%	23 (*1.1*)	**0.06**	30 (*0.8*)	**0.06**	35 (*0.9*)	**0.09**	+50%	**1.3**
NOS	547 (*7.8*)	**1.28**	683 (*5.5*)	**1.26**	749 (*6.4*)	**1.81**	+41%	195 (*9.1*)	**0.48**	271 (*6.8*)	**0.53**	290 (*7.3*)	**0.74**	+54%	**2.4**
Overall	**6981** (*100*)	**16.19**	**12,529** (*100*)	**23.12**	**11,668** (*100*)	**28.22**	**+74%**	**2148** (*100*)	**5.27**	**3986** (*100*)	**7.74**	**3958** (*100*)	**10.05**	**+91%**	**2.9**

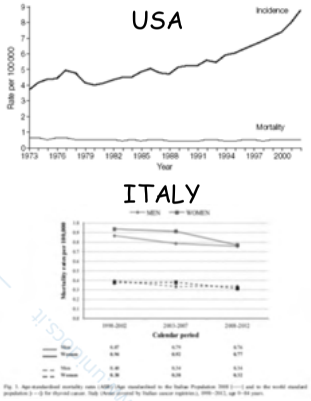
NOS=not otherwise specified.
Bold values signifies the most important information (ASR).
[a] Per 100,000, age-standardised to the Italian population (2008).
[b] ASR in 2008–2012 versus 1998–2002.

Papillary thyroid tumors incidence increased of 90% in women and 120% in men in 1998 – 2012.

MORTALITY by THYROID TUMORS

- WORLD: ~0,5% of all cancer-related deaths;
- Among K with lowest mortality/incidence ratio
- 5 years survival: 98% (similar in M and F)
- Mortality is associated with age:
  - 0.1 % <20 y
  - 3.8 % 20- 44 y
  - 8.8 % 45-54 y
  - 17 % 55-64 y
  - 24% 65-74 y
  - 47% >75 y

Despite increased incidence, mortality is stable.



ANALYTICAL EPIDEMIOLOGY
RISK FACTORS

IONIZING RADIATIONS
the only widely demonstrated risk factor for TC.

EXTERNAL exposure to medical radiation sources in childhood (but not in adults): after a latency period >5 years, the risk increases, peaking at 15 - 19 y, then declines but remains higher than the unexposed population.

INTERNAL exposure to food contaminated with iodine isotopes.

ANALYTICAL EPIDEMIOLOGY
RISK FACTORS

Thyroid is much more exposed (1000 times) to ionizing radiations than extra-thyroid tissues.

This explains why the increase in K post Chernobyl accident (1986) only concerned the thyroid.

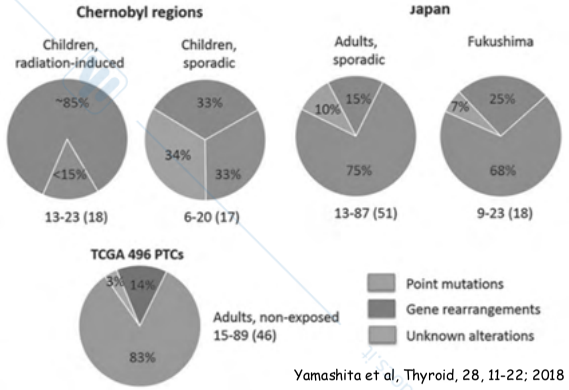
Age at exposure time impacted on the rate of K occurrence:

4.5 times in adults, 12.7 in adolescents, 87.8 times in children, especially <10 years.

Increase started after 4-year latency period.

Histological type: mostly Papillary thyroid cancer.

Distribution of different types of genetic alterations in PTC tissues in Chernobyl areas and Japan, and in the Cancer Genome Atlas project.
Data shown are age range (rounded mean age) of patients. Data are summarized from different publications.



Yamashita et al, Thyroid, 28, 11-22; 2018

Radiation-induced papillary thyroid cancers are most often caused by gene rearrangement.

THYROID CANCER AND CHERNOBYL

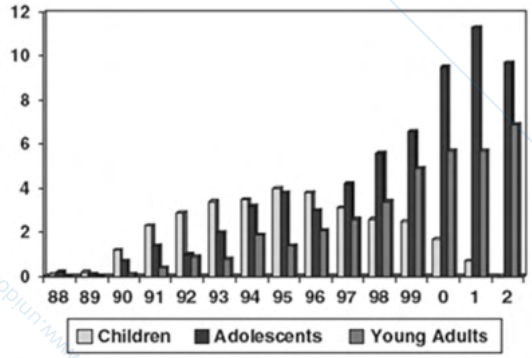


Figure 2 Change in incidence (per 10^5) of thyroid cancer over time after the Chernobyl accident in 1986. The decline in the numbers of children with thyroid cancer is accompanied by a rise in the numbers of adolescents (data from YE Demidchik, in Cardis *et al.*, 2006)

The Japan Times — March 10 2014

Thyroid cancers up in Fukushima

BY MIZUHO AOKI

Screening of Fukushima resident who were 18 or younger at the time of the nuclear disaster (March 11th 2011) had found 26 confirmed and 32 suspected cases of thyroid cancer as of Sept. 30, according to the Fukushima Prefectural Government.

The number of confirmed cases was up by eight from August, while the suspected cases rose by seven, the prefecture-led study found.

About 226,000 people have undergone the screening program since it kicked off in October 2011.

ANALYTICAL EPIDEMIOLOGY
RISK FACTORS

FAMILIARITY

Supposed for some cancers which may present
1) as components of rare hereditary syndromes with recognized genetic defects:
- Familial adenomatous polyposis for papillary TC.
- Carney complex for follicular TC.
- Familial medullary TC / MEN2.

2) as isolated forms: the diagnosis of PTC in a family member involves an increased risk of TC development in sons and siblings, mostly females.
Hypothetical polygenetic predisposition to develop TC in response to (even low) exposure to carcinogens (endogenous or exogenous including radiation) due to defects in the ability to metabolize them and/or to repair DNA damage and/or to control the cell cycle and apoptosis.

ANALYTICAL EPIDEMIOLOGY
RISK FACTORS

SEX

The incidence of thyroid cancers in <u>females</u> prevails males during the <u>reproductive age</u>, but not in the post-menopausal period, therefore hormonal and reproductive female risk factors (including contraceptives) are hypothesized, thought they have not been proven.

ANALYTICAL EPIDEMIOLOGY
RISK FACTORS

IODINE SUPPLY

The occurrence of Follicular and Anaplastic cancer in countries with low iodine supply is higher compared with countries with high supply.

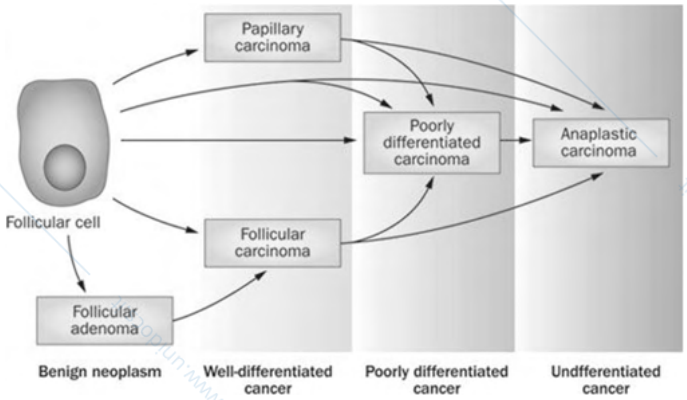
PRE-EXISTING THYREOPATHIES

Goiter and Basedow could be risk factors for Papillary TC, but the prevalence of microcarcinomas (2.2%) in these two diseases is not different from the general population.

OBESITY

Doubles the risk of differentiated TC compared to normal weight.

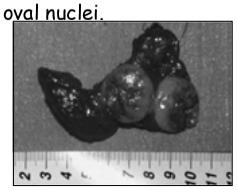
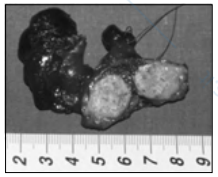
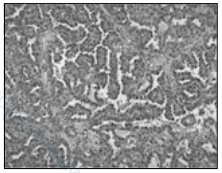
THYROID TUMORS
originating from follicular cells



MALIGNANT THYROID TUMORS
PAPILLARY CARCINOMA

FEATURES:
- typical of young age;
- multifocal and bilateral in 30-40% of cases;
- frequently in occult form (onset with lymph node metastases);
- tendency to metastasize via the lymphatic system.

ANATOMOPATHOLOGY:
Macroscopically it is whitish in color, no capsula is present but fairly clear limits.
Microscopically it presents papillary formations within the follicles, constituted of a central fibrous core surrounded by cells with crowded oval nuclei.

PAPILLARY THYROID CARCINOMA
Variants

- Conventional
- Follicular variant
- Papillary microcarcinoma
- Tall cell
- Oncocytic
- Columnar cell
- Diffuse sclerosing
- Solid
- Clear cell
- Cribriform morular
- Macrofollicular
- PTC with prominent hobnail features
- PTC with fasciitis-like stroma
- Combined papillary and medullary carcinoma
- PTC with dedifferentiation to anaplastic carcinoma

PAPILLARY THYROID CARCINOMA
WORSE PROGNOSIS

- Age >40 y
- Extra-thyroid diffusion
- Dimension
- Multicentric
- Distant metastasis
- Dedifferentiated areas
- Aggressive histologic type

MALIGNANT THYROID TUMORS
FOLLICULAR CARCINOMA

FEATURES:
- Incidence peak 30-40 y
- Metastatic diffusion via vascular system.

ANATOMOPATHOLOGY:
- Encapsulated variant.
- Non-encapsulated variant (frequent infiltration through vascular system).

FOLLICULAR THYROID CARCINOMA

Malignancy Criteria:	Metastasis
Capsula invasion Vessel invasion	1% of cases with minimal capsular invasion 5% of cases with vascular invasion (>4 vessels)

FOLLICULAR THYROID CARCINOMA

Metastasis

- Isolated (single) and never occult.
- Blood diffusion (to lung and bones).
- Bones: often multiple metastasis.
- High affinity for radioiodine.
- Well differentiated.

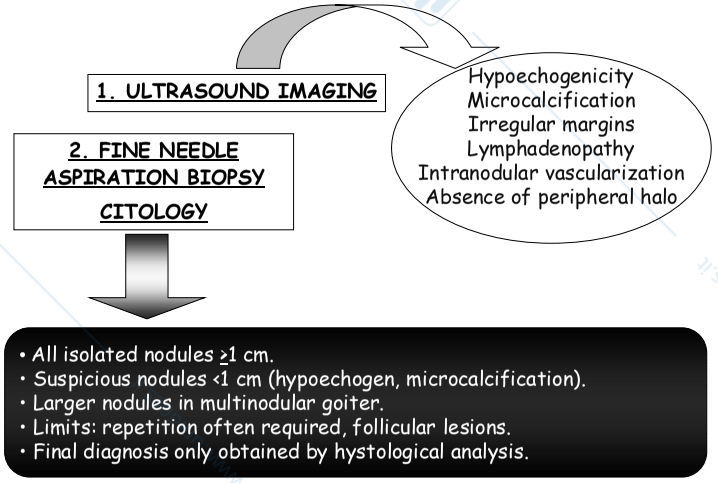
MALIGNANT THYROID TUMORS

ANAPLASTIC CARCINOMA

FEATURES:
- Incidence peak at old age.
- Trend for early metastasis.
- Does not secrete hormones nor thyroglobulin.
- Causes the increase of the serum tissue polypeptide antigen.

ANATOMOPATHOLOGY:
Undifferentiated giant cells carcinoma.

INVESTIGATING THYROID NODULES



Benignity vs Malignancy features of thyroid nodules

Benign	Malignant
• Familiarity of benign goiter. • Diffused multinodular goiter. • Stable dimension. • Benign appearance at FNAB. • Simple cyst at US. • Hyperfunctional nodule. • Volume reduction following levothyroxin (l-T4) therapy.	• Isolated nodule. • Increased consistency, fixed. • Rapidly increasing volume. • Voice alteration, vocal cords paralysis. • Age < 14 or > 65 y. • Suspicious FNAB cytology. • Cyst > 4cm or complex. • Hypothyroidism after ^{131}-iodine treatment. • Hystory of radiation exposure. • Unilateral lymphoadenopathy.

MANAGEMENT OF DIFFERENTIATED THYROID CARCINOMA (DTC)

- PRE-SURGICAL EVALUATION OF NODULES
- SURGICAL TREATMENT
- TNM and PROGNOSTIC CLASSIFICATION
- POST-SURGERY RADIOIODINE ADMINISTRATION
- FOLLOW UP: - ROLE OF TOTAL-BODY SCINTIGRAPHY
  - ROLE Tg and Ab-Tg
  - ROLE OF NECK ULTRASOUND
- SHORT / LONG TERM FOLLOW-UP
- RECURRENCIES THERAPY

SURGICAL TREATMENT DTC

TOTAL THYROIDECTOMY

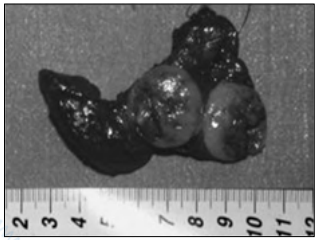
- Surgical complications:

  - Hypothyroidism.

  - Frequent nerve paralysis, uni- or bilateral, permanent (<2%) or temporary (1-6 months, 10%).

  - Hypoparathyroidism (<2% above 3 months).

BIOMECULAR MECHANISMS OF THYROID CARCINOGENESIS



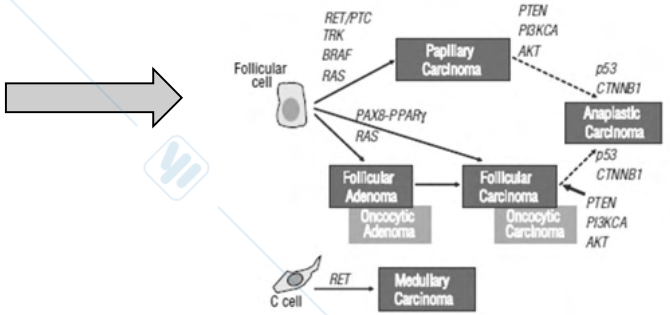


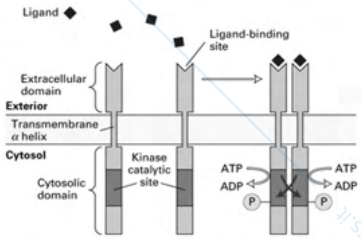
Figure 1. Classification of thyroid carcinoma hystotypes and genetic events involved in their development. RET/PTC and TRK rearrangements and BRAF and RAS activating mutations are involved in initiation and progression of papillary thyroid carcinoma. PAX8-PPARγ rearrangements and RAS activating mutations are involved either in follicular adenoma or follicular carcinoma formation. Loss of differentiation of papillary thyroid carcinomas and follicular thyroid carcinomas involves activation of mediators of the PI3K-AKT pathway. Transition to completely dedifferentiated or anaplastic carcinomas depends on p53 and β-catenin mutation or disregulation. Conversely, activating mutations of the proto-oncogene RET are involved in the formation of all familial medullary thyroid carcinomas and of a part of the sporadic counterpart.

Ret is a tyrosin-kinase receptor

- The *RET* gene encodes a transmembrane tyrosine kinase protein. It has an extracellular ligand-binding domain including a cadherin-like and a cysteine-rich domain, a transmembrane domain and an intracellular tyrosine-kinase domain.
- Chromosomal locus 10q11.2, 21 exons.
- It is expressed in cells derived from the neural crest: in rodents it is present in the embryonic and adult enteric tract, in the sympathetic, sensory neurons and in the excretory system (mesonephric duct during embryogenesis)

Tyrosine Kinase Receptors (TKRs)

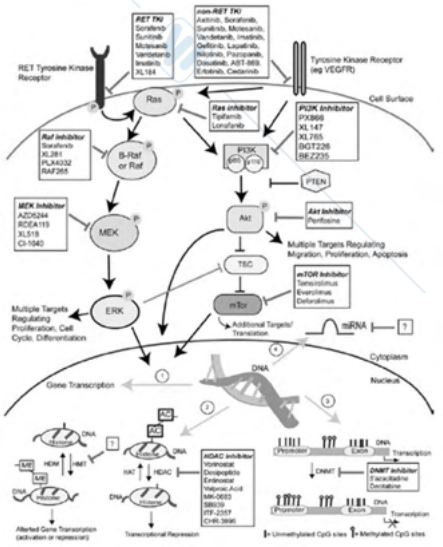
- Tyrosine kinase receptors are involved in cell signaling in processes such as growth, differentiation, survival, and apoptosis.
- In response to binding of extracellular ligands, TKRs generally form **homodimers or heterodimers**. This is usually followed by auto-phosphorylation and signal transduction through the pathway.



RET encodes for a receptor protein which binds GDNF, NTN, PSP and Artemin.

NTRK1 is a TK receptor for the Nerve Growth Factor (NGF) which primarily regulates growth, differentiation and programmed cells death.

RET and TKR transduction pathways



RET and NTRK1 physiology

RET

TK receptor for GDNF (glial-derived neurotropic factor), neurturin, persephin (PSPN), artemin (neuroblastin).

Expressed by the neural crest and by the precursor cells of the urogenital tract.

Key role in kidney morphogenesis, in peripheral NS cell line maturation (enteric, sympathetic, sensory), in spermatogone differentiation.

NTRK1

TK receptor for NGF.

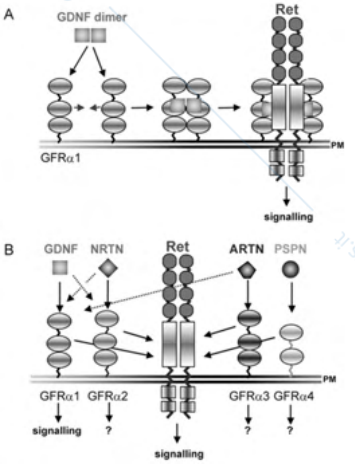
Expressed by sensory ganglia of the neural crest and of the sympathetic peripheral NS, and in some colinergic neurons of the CNS.

Key role for the growth, differentiation and apoptosis of peripheral and central NS cells.

Ret activation and Signaling Pathways

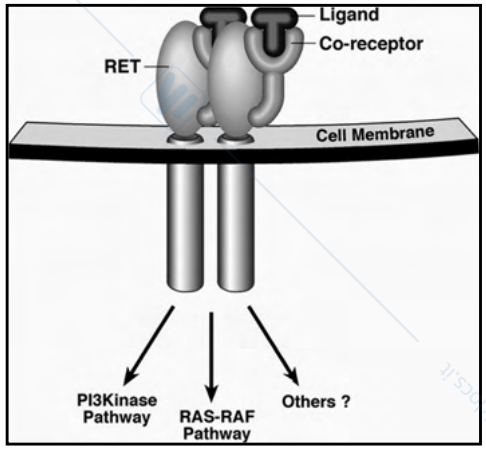
GDNF-family ligand interaction with Ret CO-RECEPTORS.

- Ligands are glial cell line-derived neurotrophic factor (GDNF) family members.
- Ligands bind glycosyl-phosphatidylinositol (GPI)-anchored coreceptors (GFR) 1-4.
- Ret dimerizes as a result of activation by ligand/coreceptor binding, autophosphorylates itself, and activates the phosphorylation cascade.



Hannu Sariola, and Mart Saarma J Cell Sci 2003;116:3855-3862

RET DIMERIZATION ACTIVATES THE PI3 KINASE AND RAS PATHWAYS



Ligand = GDNF

Co-receptor = GFRα-1

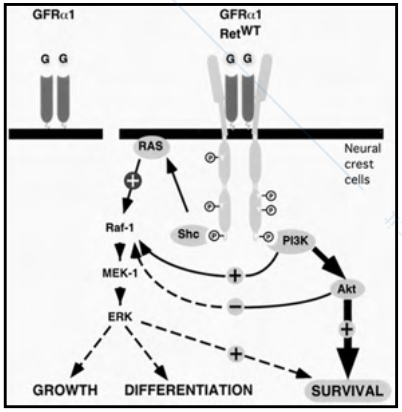
Ret activation and Signaling Pathways

In neural crest cells PI3K and RAS pathways interact to activate cell growth, differentiation and apoptosis.

GDNF/GFR1/RET complex initiates both the RAS and PI3K pathways.

Pathways are activated through Tyr1062, which is a binding site for SHC.

SHC further associates with GRB2/SOS and GAB1/2 complexes in the Ras and PI3K pathways, respectively



Mograbi, B., et.al. *J. Biol. Chem.* **2001**

Wild-type Ret has multiple functions

⌑ Development of the enteric nervous system (ENS) is primarily dependent on GDNF/GFR1/RET

- loss of enteric ganglia if *ret* has a loss-of-function mutation
  Moore, M., et. al. *Nature* **1996**.

- mice carrying *c-ret* homozygous loss-of-function develop an aganglionic phenotype and die because of a lack of ganglia posterior to the stomach.

Taraviras, S, et.al. *Development* **1999**

⌑ Activation of the PI3K pathway by GDNF/GFR1/RET blocks neuroectodermal apoptosis
Mograbi, B., et.al. *J. Biol. Chem.* **2001**

⌑ Renal organogenesis
- GDNF/GFR1/RET null mice show renal agenesis and hypoplastic kidneys due to lack of ureteric bud growth.
Baloh, RH et al.*Curr. Opin. Neurobiol.* **2001**

Papillary thyroid carcinomas are characterized by fusion proteins which are made of a N-terminus part derived from different partners and of a C-terminus of RET receptor carrying the TK domain.

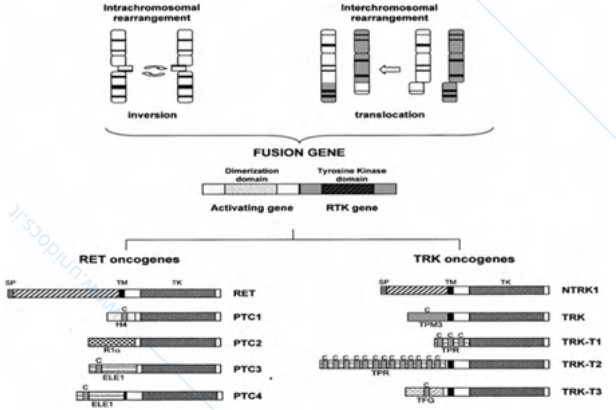
Table 1
RET/PTC rearrangements in papillary thyroid carcinomas.

RET/PTC rearrangement	Fusion gene	Underlying chromosomal rearrangement	References
RET/PTC1	*H4 (Ccdc6, D10S170)*	inv(10)(q11.2;q21)	[37]
RET/PTC2	*Prkar1a*	t(10;17)(q11.2;q23)	[14]
RET/PTC3	*Ncoa4 (Rfg, ele1)*	inv(10)(q11.2;q10)	[78,79]
RET/PTC4	*Ncoa4 (Rfg, ele1)**	inv(10)(q11.2;q10)	[8]
RET/PTC5	*Golga5 (Rfg5)*	t(10;14)(q11.2;q32)	[9]
RET/PTC6	*Htif1 (Trim24)*	t(7;10)(q32~34;q11.2)	[10]
RET/PTC7	*Tif1g (Rfg7, Trim33)*	t(1;10)(p13;q11.2)	[10]
RET/PTC8	*Ktn1*	t(10;14)(q11.2;q22.1)	[13]
RET/PTC9	*Rfg9*	t(10;18)(q11.2;q21~22)	[11]
ELKS-RET	*Elks (Rab6ip2)*	t(10;12)(q11.2;p13.3)	[12]
PCM1-RET	*Pcm1*	t(8;10)(p21~22;q11.2)	[15]
RFP-RET	*Rfp (Trim27)*	t(6;10)(p21;q11.2)	[16]
HOOK3-RET	*Hook3*	t(8;10)(p11.21;q11.2)	[17]

Most frequent

*Breakpoint in *Ret* different from RET/PTC3.

Mechanisms of chromosomal rearrangements generating transforming fusion genes in Papillary Thyroid Carcinoma (chimera oncogenes)



Alberti et al., JOURNAL OF CELLULAR PHYSIOLOGY 195:168–186 (2003)

Mechanisms of chromosomal rearrangements generating transforming fusion genes in Papillary Thyroid Carcinoma (chimera oncogenes)

The specificity of these oncogenic rearrangements as peculiar features for thyrocytes has been related to the higher frequency of proximity RET and H4 loci (and others) in interphase nuclei of human thyroid cells, compared to nuclei of peripheral blood lymphocytes and mammary epithelial cells.

It also appears that thyroid cells are more prone to DNA repair than to favor apoptosis, therefore this increases the probability of rearrangements. This would also explain the high susceptibility of thyroid cells to chromosomal rearrangements following radiation.

THYROID TUMORS AND CHERNOBYL

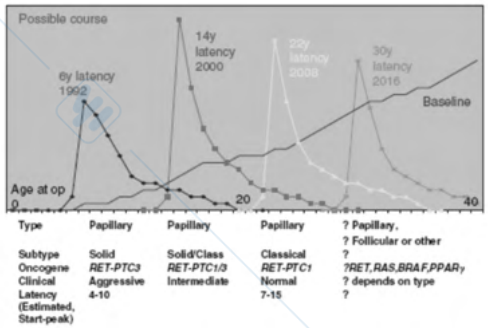


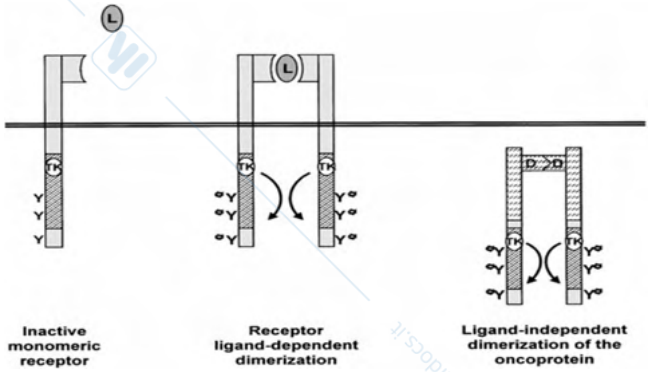
Figure 5 Hypothetical representation of the evolution of the occurrence of thyroid carcinoma in those exposed to fallout after Chernobyl. The baseline incidence is represented from age 0–40 years, and superimposed are the radiation-induced cases for selected years, with correlation with morphology, oncogenes involved and clinical behavior.

Williams D. Oncogene 2009

The rearranged oncoproteins share three major features:

- Capability of self-association mediated by the oligomerization domain.
- Different subcellular localization (cytosolic) with respect to the wild type receptor.
- Ectopic expression of the kinase in the epithelial follicular cells.

Physiological mechanism of activation of the TKR and its oncogenic version



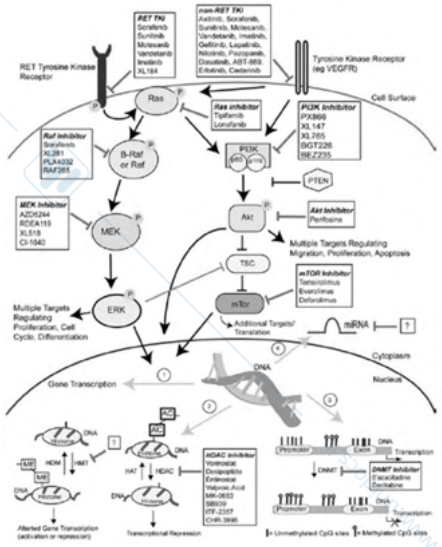
Y=tyrosine residue, L=ligand, D=dimerization domain, TK=tyrosine kinase domain

RET and PTC

RET rearrangements are described in 20-30% of papillary thyroid carcinomas.
RET/PTC1 gene and RET/PTC3 gene are the most frequent rearrangements.

Downstream, Ret activates Ras/ERK and PI3K/AKT pathways.

Moreover RET/PTC1 induces nuclear translocation of beta-catenin in thyreocytes.

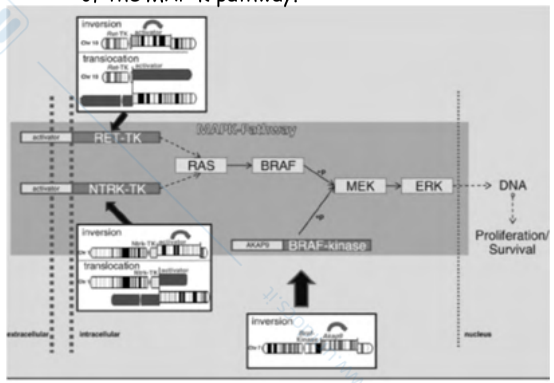


Oncogene mutations in RET / TK pathways

BRAF mutations are the most frequently found in Papillary TC.
Val→Glu substitution in aa 60 (V600E) causes the constitutive activation of BRAF kinase activity, thereby causing a continuous dysregulated posphorilation of downstream effector substrates and to the activation of the MAP-K pathway.

BRAF is one of 3 isoforms of the RAF Ser-Thr Kinase Family.

These proteins are intracellular effectors of the MAP-K cascade. After RAS-BRAF binding, the complex activates MEK, which in turn activates ERK and the MAP-K cascade.



Involvement of Ret mutations in other diseases

Multiple Endocrine Neoplasia (MEN) Type 2
A group of cancer syndromes characterized by medullary thyroid carcinoma.
This condition is the result of a gain-of-function mutation, causing proliferation of thyroid cells.

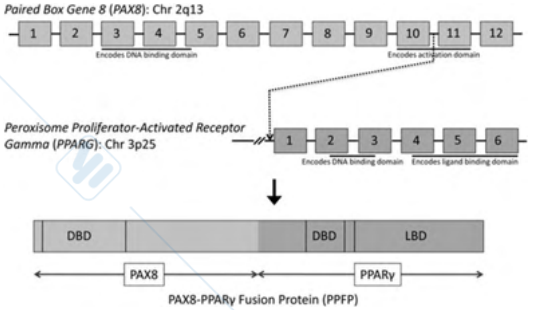
Hirschsprung Disease (HSCR)
A congenital absence of enteric innervation resulting in intestinal obstruction.
The mutations are varied and scattered throughout the Ret coding sequence, which include deletions and a variety of point mutations.
This is the result of a loss-of-function mutation

MUTATIONS DESCRIBED IN FOLLICULAR THYROID CARCINOMAS:

1) Activating point-mutations in RAS (50% of cases)

2) PAX8/ PPARγ REARRANGEMENTS (30-35% of cases) generating a chimera oncogene with

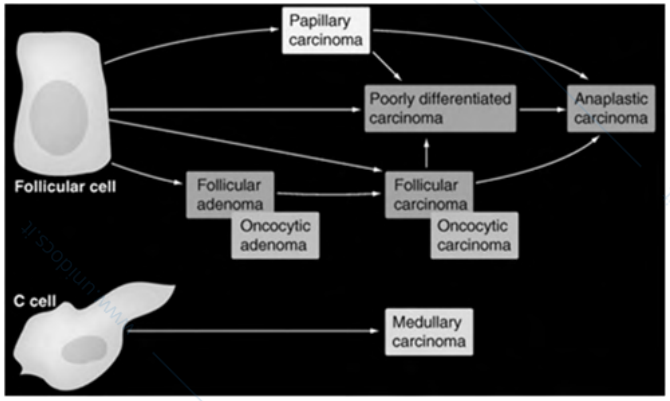
- dominant-negative suppression effect on PPARγ, which has a physiologic anti-neoplastic role (PAX8 thyroid transcription factor);
- transcriptional activating effect on subsets of PPARγ and PAX8 responsive genes.



The PAX8/PPARG rearrangement is created by a translocation between chromosomal regions 2q13 and 3p25. This translocation results in a fusion transcript wherein most of the coding sequence of PAX8 (2q13) is fused in frame with the entire coding exons of PPARγ1 (3p25).
The resulting fusion protein, denoted as PPFP, contains the DNA binding domain and part of the C-terminal activation domain of PAX8, as well as the DNA and ligand binding domains (DBD, LBD) of PPARγ.

Raman and Koenig, Nat Rev Endocrinol. 2014; 10: 616–623

THYROID TUMORS



PARAFOLLICULAR OR C-CELLS

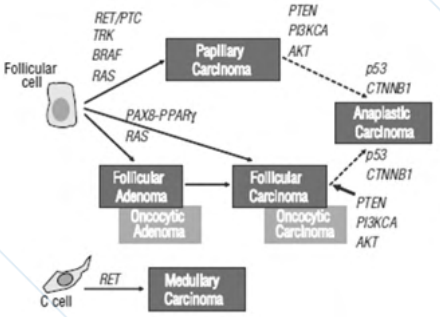
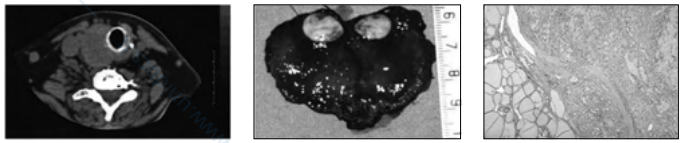


Figure 1. Classification of thyroid carcinoma hystotypes and genetic events involved in their development. RET/PTC and TRK rearrangements and BRAF and RAS activating mutations are involved in initiation and progression of papillary thyroid carcinoma. PAX8-PPARγ rearrangements and RAS activating mutations are involved either in follicular adenoma or follicular carcinoma formation. Loss of differentiation of papillary thyroid carcinomas and follicular thyroid carcinomas involves activation of mediators of the PI3K-AKT pathway. Transition to completely dedifferentiated or anaplastic carcinomas depends on p53 and β-catenin mutation or disregulation. Conversely, activating mutations of the proto-oncogene RET are involved in the formation of all familial medullary thyroid carcinomas and of a part of the sporadic counterpart.

MEDULLARY THYROID CARCINOMA

FEATURES:
- originating from C cells;
- produces thyrocalcitonin and histamine;
- may also produce PG, ACTH, serotonin;
- sporadic form is most frequent in the 5°- 6° decade;
- familiar form is mostly frequent in the 2°- 3° decade;
- featured by increased levels of calcitonin.

- 75% of all MTCs are sporadic; the remaining are hereditary
- present at diagnoses as a mass in the neck or metastatic disease



PARAFOLLICULAR OR C-CELLS

Thyroid neuroendocrine cells in the interstitium, physiologically secreting CALCITONIN and, in low amount, serotonin and somatostatin.

SECRETORY PRODUCTS: calcitonin (CT, 32 aa) and small amounts of precursors (CTpr)

PHYSIOLOGIC REGULATION:

stimulated by ↑ calcemia;

stimulated by Ca oral load preceding ↑ calcemia (gastrin-mediated anticipatory effect)

inhibited by ↓ calcemia

also regulated by other GI peptides, glucocorticoids, GHRH, β agonists

PHYSIOLOGIC FUNCTION:

Overall calcium-lowering effect, achieved by effects on

Bone: osteoclast inhibition

Kidney: inhibition of tubular resorption.

These functions are physiologically poorly determinant, as more potent systems are involved. No ↑CT effect in MTC and no ↓CT following thyroidectomy.

OTHER BIOMARKERS OF MEDULLARY TC

CEA, serotonin, somatostatin, POMC / ACTH, VIP, neurotensin, chromogranin A, neural cell adhesion molecule (NCAM), prostaglandins.

Some of these secreted hormones are responsible for flushing, diarrhea or Cushing Syndrome.

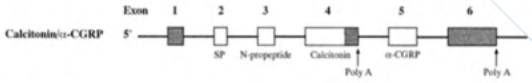
BIOMARKERS OF MEDULLARY TC

The calcitonin/a-CGRP gene is located in chromosome 11p.

6 exones. Coding for at least 2 peptides.

PROCALCITONIN (116 aa) is processed to form:

CT (32 aa) and Calcitonin Gene Related Peptide (CGRP, 37 aa)



CGRP is primarily expressed in CNS and some peripheral neurons.

CT is primarily expressed by C cell, and in low amounts by other neuroendocrine cells (GI, lung, adrenal medulla).

The Medullary TC secretes large amounts of CT, proCT, CGRP and other CTpr, all representing tumor biomarkers.

High CT levels are also found in pregnancy, high physical activity, kidney insufficiency, Hashimoto thyroiditis, follicular lesions (benign, malignant), neuroendocrine tumors (pancreas, lung).

Hereditary Medullary Thyroid Carcinoma

Germline point mutations of RET are responsible for the inheritance of MEN2 cancer syndromes including the three different clinical subtypes:

MEN2A, MEN2B, and FMTC.

- Rare familial cancer syndrome.
- Usually caused by germline mutations.
- Autosomal dominant pattern of inheritance.
- Three types:
  - FMTC (familial medullary thyroid carcinoma),
  - MEN2A,
  - MEN2B

Familiar MTC

- Patient presents with bilateral medullary thyroid carcinoma (MTC).

- Approximately 85% of families with FMTC have an identifiable *RET* mutation.

- Mutations occur at one of the five cysteine residues (codons 609, 611, 618, 620, and 634) with mutations of codons 618, 620 and 634 each accounting for 25 to 35% of all mutations.

- Presents at 20-40 years of age (later compared to MEN2).

- Less aggressive than MEN2A or 2B and prognosis is good.

MULTIPLE ENDOCRINE NEOPLASIA SYNDROMES

MENs

Multiple endocrine neoplasia (MEN)

Occurrence of tumors involving two or more endocrine glands within a single patient. Four major forms of MEN exist, all being autosomal dominant disorders:

MEN type 1 (MEN1), due to menin mutations;

MEN2 (previously MEN2A) due to mutations of a tyrosine kinase receptor encoded by the *rearranged during transfection* (RET) protoncogene;

MEN3 (previously MEN2B) due to RET mutations;

MEN4 due to cyclin-dependent kinase inhibitor (CDNK1B) mutations.

Each MEN type is associated with the occurrence of specific combinations of tumors.

MULTIPLE ENDOCRINE NEOPLASIA

• General definition: tumors in 2 or more endocrine tissues in the same patient.

• Multiple tumor combinations, usually in characteristic patters, caused by a mutation in a defined gene.

• Multiple tumors and early onset compared to isolated forms of the same tumors.

ONCOGENES and ONCOSUPPRESSORS

Oncogenes (e.g. ras, myc, ret) promote the cell proliferation when unproperly activated or overexpressed.

The alteration of a single allele is sufficient to develop a tumor clone.

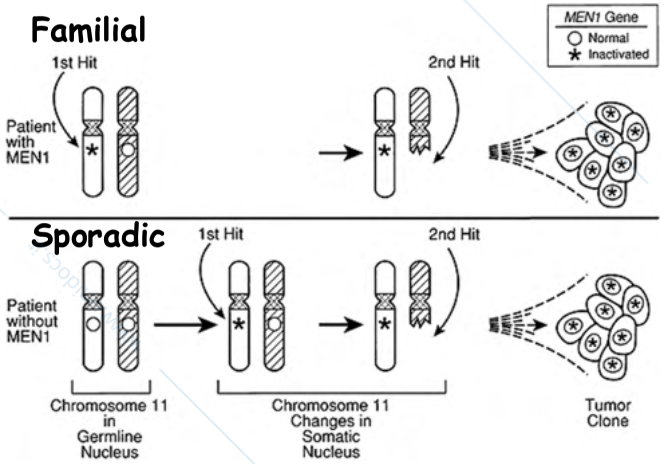
Oncosuppressors (e.g. p53, Rb) arrest cell proliferation and allows cell growth when inactivated. Genes that keep proliferation in check.

Both the alleles have to be inactivated to develop a tumor clone.

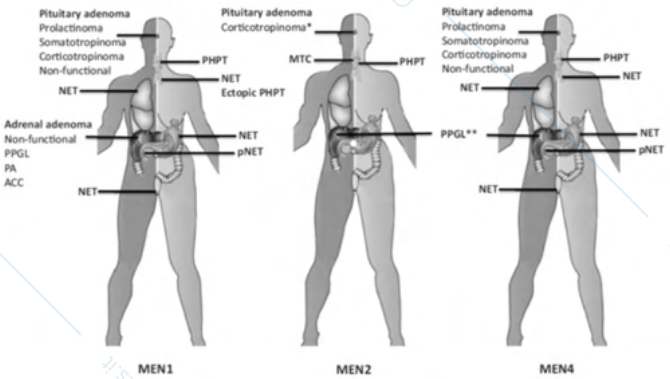
MEN SYDROMES' CANCERS

	MEN1	MEN2A	MEN2B	MEN4
Pituitary adenoma	+			+
Parathyroid adenoma	+	+		+
Medullary thyroid tumors		+	+	
Pheochromocytoma		+	+	
Paragangliomas		+		
Insulinoma	+			
Neuroma			+	

KNUDSON THEORY – TWO HITS HYPOTHESIS



The variable clinical phenotypes of MENs



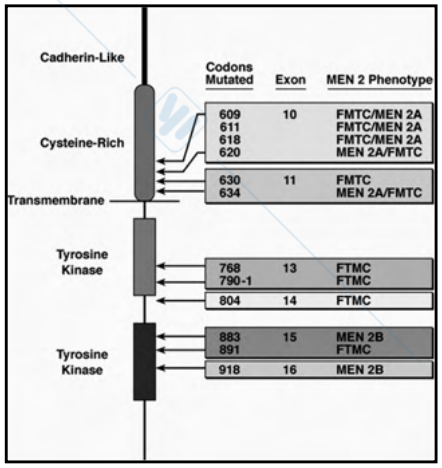
*Recent case report of Cushing disease in MEN2B (Kasturi *et al.* 2017)
**PPGL have been described in mouse models of MEN4

Alrezk et al., 2017

Type (chromosome location)	Tumors (estimated penetrance)	Gene; most frequently mutated codons
MEN1 (11q13)	**Parathyroid adenoma (90%)**	MEN1
	Entero-pancreatic tumor (30–70%)	83/84, 4-bp del (≈4%)
	- Gastrinoma (40%)	119, 3-bp del (≈3%)
	- Insulinoma (10%)	209–211, 4-bp del (≈8%)
	- Non-functioning & PPoma (20–55%)	418, 3-bp del (≈4%)
	- Glucagonoma (<1%)	514–516, del or ins (≈7%)
	- VIPoma (<1%)	Intron 4 ss, (≈10%)
	Pituitary adenoma (30–40%)	
	- Prolactinoma (20%)	
	- Somatotrophinoma (10%)	
	- Corticotrophinoma (<5%)	
	- Non-functioning (<5%)	
	Associated Tumors	
	- Adrenal cortical tumor (40%)	
	- Phaeochromocytoma (<1%)	
	- Brochopulmonary NET (2%)	
	- Thymic NET (2%)	
	- Gastric NET (10%)	
	- Lipomas (30%)	
	- Angiofibromas (85%)	
	- Collagenomas (70%)	
	- Meningiomas (8%)	

Type (chromosome location)	Tumors (estimated penetrance)	Gene; most frequently mutated codons
MEN2 (10 cen-10q11.2)		
MEN2A	Medullary Thyroid Cancer (90%)	RET
	Phaeochromocytoma (50%)	634, missense
	Parathyroid adenoma (20–30%)	e.g. Cys → Arg (~85%)
MTC only	Medullary Thyroid Cancer (100%)	RET
		618, missense (>50%)
MEN2B	Medullary Thyroid Cancer (>90%)	RET
(also known as MEN3)	Phaeochromocytoma (40–50%)	918, Met → Thr (>95%)
	Associated abnormalities (40–50%)	
	Mucosal neuromas	
	Marfanoid habitus	
	Medullated corneal nerve fibers	
	Megacolon	
MEN4 (12p13)	Parathyroid adenoma	CDKN1B
	Pituitary adenoma	No common mutations identified to date
	Reproductive organ tumors (e.g. testicular cancer, neuroendocrine cervical carcinoma)	
	Adrenal + renal tumors	

Ret mutations in MEN2



✧ Codon 634 mutations make the RTK constitutively active.

✧ This is a result of the dimerization of the Ret monomers due to the mutated cysteine. The mutation leaves an unpaired residue, and each mutant Ret monomer forms a disulfide bond with its unpaired counterpart from another mutant Ret.

✧ The same mechanism applies to other mutations in the cysteine-rich region of the protein.

MEN2A

⌑ Most common form of MEN2, accounting for ~90% of all cases.

⌑ Patient presents with medullary TC (up to 90%), pheochromocytomas (~50%) and/or hyperparathyroidism (~15-30%).

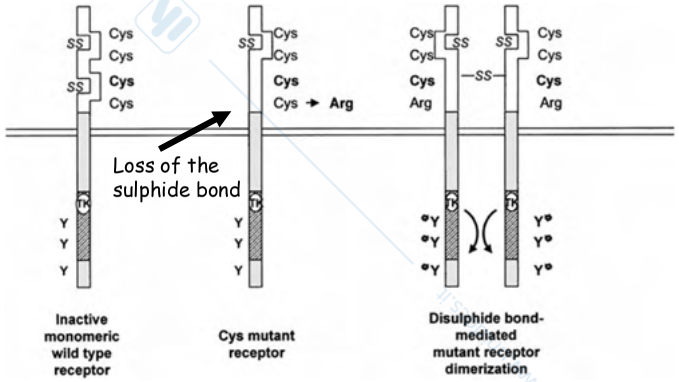
⌑ 50% of individuals with mutations in the Ret gene develop the disease by age 50y, and 50% by age 70y.

⌑ Approximately 95% of families with MEN 2A have a *RET* mutation in exon 10 or 11.

- Mutations of codon 634 Cys occur in about 85% of families; mutations of Cys 609, 611, 618, and 620 overall account for the remaining identified mutations in exons 10 and 11.

Mechanism of disulfide bond-linked RET dimerization in MEN2A

codon 634 cysteine -> arginine C634R



MEN2B

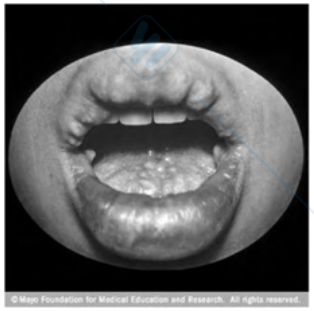
- Patient may present with MTC and bilateral pheochromocytomas, but also with diffuse ganglioneuromas of the intestinal tract, mucosal neuromas (on lips or tongue), and skeletal abnormalities.

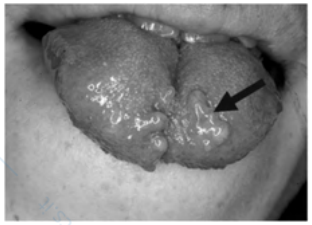
- Approximately 95% of individuals with the MEN 2B phenotype have a single point mutation in the tyrosine kinase domain of the *RET* gene at codon 918 in exon 16, which substitutes a methionine with threonine for M918T, influencing autophosphorilation and interaction with substrates.

- Accounts for ~5% of all MEN2 cases.

- Age of onset is about 10 years earlier than of MEN2A; pheochromocytomas are sometimes detected in childhood.

MOUTH AND TONGUE NEURINOMAS ASSOCIATED WITH MEN 2





MEN2

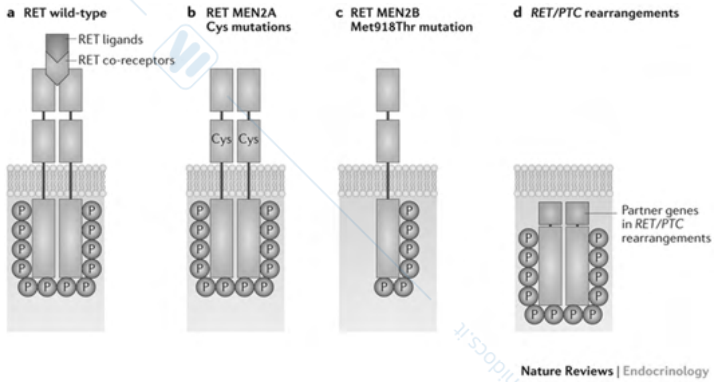
Detection and Treatment Options

- Ret gene testing, elevated calcitonin levels (produced by C cells), elevated blood pressure if pheochromocytoma is present.

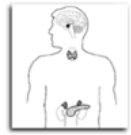
- Prophylactic thyroidectomy by age 6y if mutation is detected (by age of 3 if MEN2B is detected).

- Complete thyroidectomy following the detection of MTC.

RET MUTATIONAL SPECTRUM



MEN-1 DIAGNOSIS



CLINICAL: endocrine disease characterized by tumors in 2 or more endocrine tissues among:

Parathiroids	primary hyperparathiroidism (PHPT)
Pituitary	adenomas (PRL-, GH-, …)
Endocrine Pancreas	gastrinoma, insulinoma, VIPoma, …

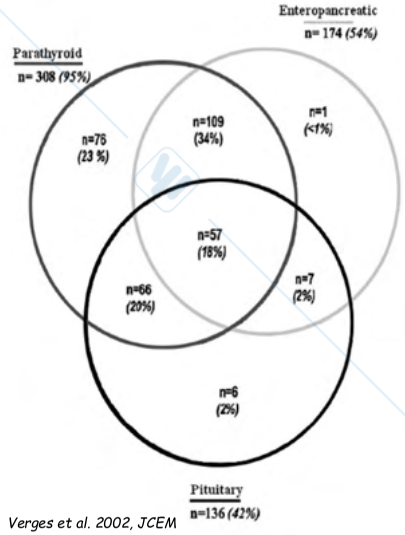
PPP

…plus…Combination of >20 different endocrine and non endocrine tumors

FAMILIAL: a patient with MEN-1 case and one or more 1st degree relatives with tumors in one of the 3 typical organs.

GENETIC: a carrier of *MEN-1* gene mutation not showing clinical or biochemical manifestations.

AUTOSOMAL DOMINANT INHERITANCE pattern



Verges et al. 2002, JCEM

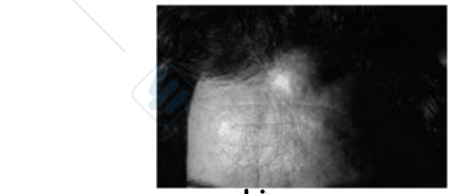
MEN-1

- Penetrance >95% within 5° decade of life.
- Prevalence is 1:30000.
- The FAMILIAL form is the most frequent one.

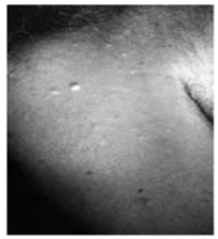
MEN-1 tumor selectivity

Endocrine features	Nonendocrine features
Parathyroid adenoma (90%)	Lipomas (30%)
Entero-pancreatic tumor	Facial angiofibromas (85%)
Gastrinoma (40%)@	Collagenomas (70%)
Insulinoma (10%)	
NF[α] including **pancreatic polypeptide** (20%[b])	Rare, maybe innate, endocrine or nonendocrine features
Other: **glucagonoma**, **VIPoma**, **somatostatinoma**, *etc.* (2%)	
Foregut carcinoid	
Thymic carcinoid NF (2%)	Pheochromocytoma (<1%)
Bronchial carcinoid NF (2%)	Ependymoma (1%)
Gastric enterochromaffin-like tumor NF (10%)	
Anterior pituitary tumor	
Prolactinoma (20%)	
Other: GH + PRL, GH, NF (each 5%)	
ACTH (2%), TSH (rare)	
Adrenal cortex NF (25%)	

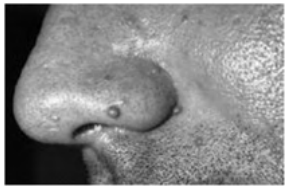
STIGMATA OF MEN-1



Lipomas



Collagenomas



Angiofibromas

Clinical

- The MEN1 syndrome causes tumours in more tissue types than any other syndrome
- Pituitary tumours in MEN1 have the same hormonal profile as sporadic pituitary tumours. All other pituitary tumour syndromes (isolated pituitary tumours, Carney complex, McCune–Albright) favour GH tumours
- Pituitary tumours are larger in MEN1 cases than in sporadic cases
- Germline mutation is the basis for multiplicity of hereditary tumours. Surprisingly, pituitary tumour in MEN1 is rarely multiple
- Most cases of MEN1 have a germline *MEN1* mutation. About 1% have *p27* mutation

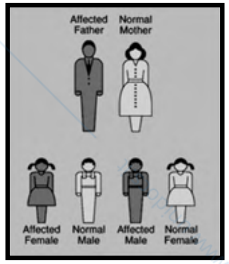
Therapeutic

- The *MEN1* gene contributes by mutation to tumorigenesis in many tissues, mainly endocrine. *MEN1* mutation is less frequent in sporadic tumours of pituitary tissue than of other endocrine tissues. Detecting such a mutation is not important in clinical practice
- More importantly, understanding the molecular pathway of *MEN1* and its encoded menin could lead to new drugs for many endocrine tumours
- Germline *MEN1* mutation testing can provide useful information for patients and caregivers
- For example, early diagnosis of MEN1 cannot guide the prevention of MEN1 cancers due to their critical locations. However, it can lead to the monitoring for emergence of morbid but treatable tumours, such as macro-prolactinoma

MEN-1

Familial: complete autosomal dominant inheritage, high penetrance (100% with ageing), variable expressivity (variable pattern of tumors).

Sporadic: may represent the first case of a familial disease.



Affected parent has 50% chance of passing it on to children, regardless of sex.

Genetic screening at birth. Imaging and hormonal screening starting by adolescence.

MEN-1

- **High inter- ed intra-family clinical variability**
- **No correlation genotype/phenotype**

=

Once the genetic diagnosis has been performed, early monitoring is required, however, it is difficult to predict tumor localization, clinical evolution and prognosis based on the genetic alteration.

MEN-1 GENETICS

MEN-1 causative gene was mapped in chromosome 11q in 1988.

Identification of the MEN1 gene was achieved through positional cloning of chromosome 11q13 in 1997.

Loss of genetic material in the 11q13 region was found in the tumor tissue of affected patients.

According to Knudson's theory, "second hit" mutations usually involve large areas of DNA (e.g. an entire chromosome is sometimes lost).

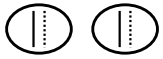
The original 12 mutations discovered consisted of frameshift and nonsense mutations, in-frame deletions, and missense alterations; they were felt to most likely result in loss of function of the protein product which is consistent with a tumor suppressor mechanism.

Pacheco 2016

FAMILIAL MEN-1 TUMORIGENESIS

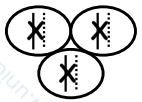
first event

Germ-line mutation inherited from affected parent: abnormal cell function status with high risk of second events (e.g. large DNA losses or chromosomal deletions).

GERM LINE ⇨ 

second event

Somatic mutation: of the allele derived from the unaffected parent

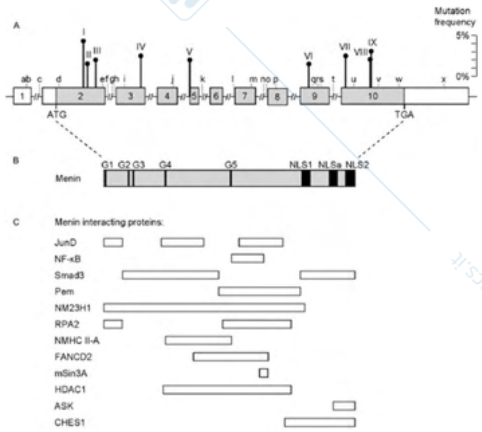
Healthy allele | **Mutated allele** **TUMOR** ⇨ 

MENIN

- Nuclear protein
- Oncosuppressor
- Ubiquitous expression

Role:

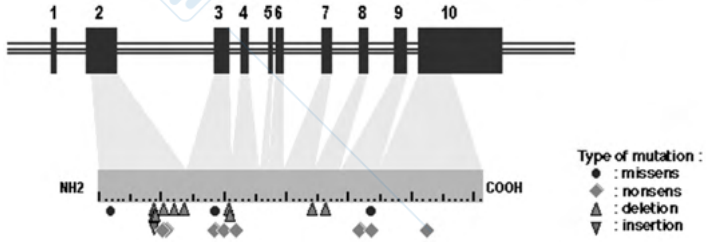
- Control of genetic trascription.
- Control of cell proliferation, differentiation and apoptosis.
- Regulation of genomic stability.



Thakker, 2008 Hum Mutat

MEN-1 GENE

9kb genomic DNA
10 exones, 9 coding for MENIN
2.8kb transcript



MENIN: Protein of 610/615 aa, main nuclear localization

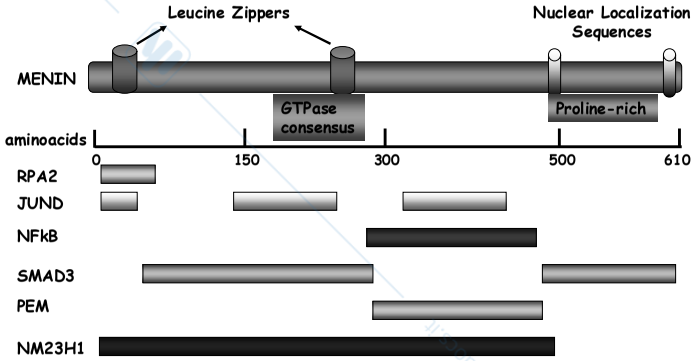
MEN1 GENE MUTATION

Table 1 *MEN1* germline mutations. Data from Concolino *et al.* (2015).

Type of mutation	Percent
Protein truncation	
Nonsense	14.0
Frame-shift	42.0
Splicing	10.5
Large deletion	2.5
Missense	25.5
In-frame deletion/insertion	5.5

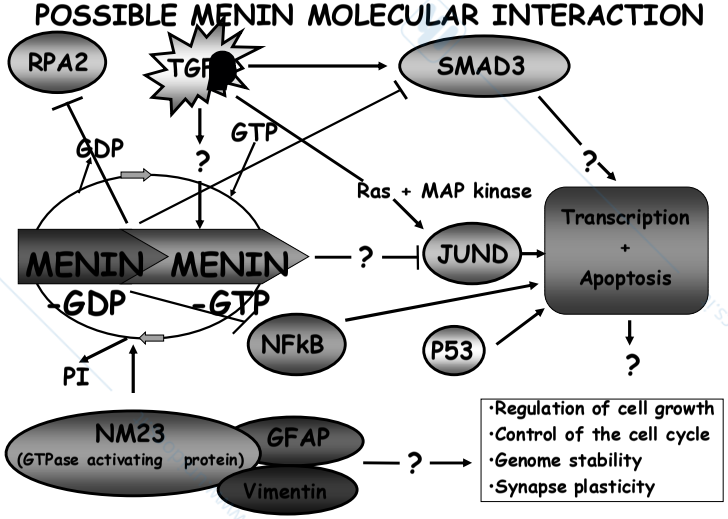
- >550 different mutations of *MEN1* were reported in literature.
- Mutations are scattered over the entire coding region of MEN1 with no hot spots.
- Overall INACTIVATING menin function.

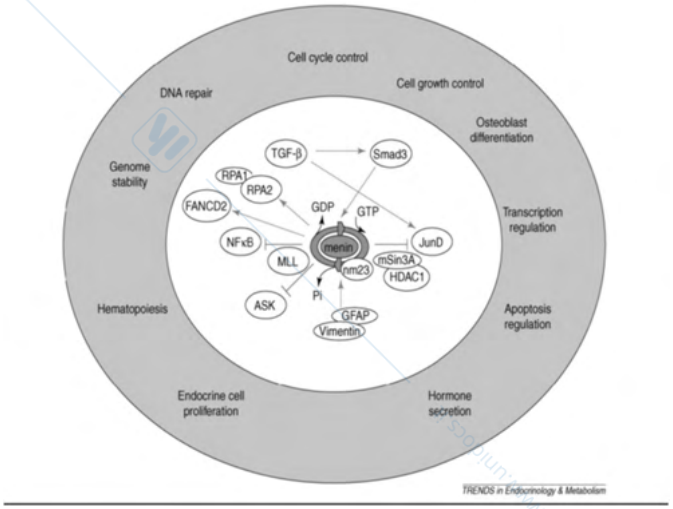
MENIN'S INTERACTION WITH MULTIPLE MOLECULAR PARTNERS



Poisson A et al., *Cancer Letters, 2003*

POSSIBLE MENIN MOLECULAR INTERACTION





UTILITY OF THE MUTATION ASSESSMENT OF *MEN1*

LIMITATIONS

- Does not impact the cancer therapeutic approach nor the life-expectancy of the patient.
- 10-20% of suspected MEN1 mutations have not been identified and are currently not detectable. Actually, these are false negative cases.
- Complex and expensive analysis, targeting a large number of mutations. False negative accounts for 10-20%.
- When no mutation is found, 1° degree relatives of a MEN-1 patient (carrying 50% risk of carrying the mutation) need to be screened by a keen biochemical and imaging evaluation to detect the subtle presence of MEN-1 disease.

UTILITY OF THE MUTATION ASSESSMENT OF *MEN1*

BENEFITS

- No need to repeat the assessment, it is performed only once.
- Mutational test allows the identification of carriers within the family and across generations, and to avoid the clinical screening and monitoring of non carrier relatives.
- Survival benefits based on preemptive management of the potential morbidities.

MEN-4

MEN-X syndrome described in rat strains spontaneously developed a MEN-like phenotype.

A germline mutation in the Cdkn1b gene, encoding the cell cycle inhibitor p27Kip1 was identified as causative for this syndrome.

Mutations in the human homologue CDKN1B were found in some of the MEN1-like cases without mutations in MEN1.

CDKN1B is a novel tumor susceptibility gene for MEN.

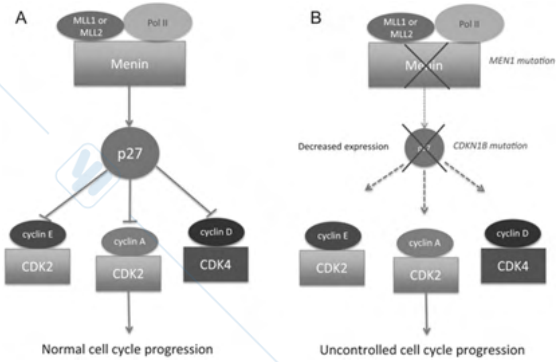
Novel MEN syndrome named MEN4 caused by mutations in p27.

CDKN1B mutations were also identified in sporadic endocrine tumors.

MEN SYDROMES' CANCERS

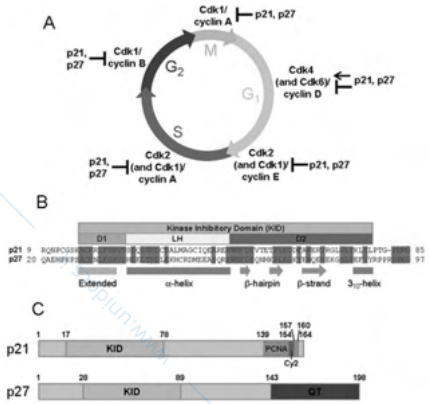
	MEN1	MEN2A	MEN2B	MEN4
Pituitary adenoma	+			+
Parathyroid adenoma	+	+		+
Medullary thyroid tumors		+	+	
Pheochromocytoma		+	+	
Paragangliomas		+		
Insulinoma	+			
Neuroma			+	

Figure 1

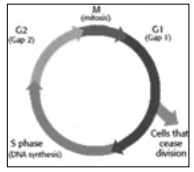


A pathway depicting the alterations in p27 expression in MEN1 and/or MEN4 that lead to tumorigenesis. Menin, encoded by MEN1, regulates the expression of p27 by forming a transcriptional activation complex with methyltransferases (MLL1 or MLL2) and RNA polymerase II (POL II). Menin inactivation (MEN1 mutation) leads to decreased p27 expression. Mutations in CDKN1B, either solely or with MEN1 as a second germline hit, leads to a greater decrease in expression of p27 protein, triggering uncontrolled cell cycle progression.

MEN-4



p27 protein arrests cell proliferation by regulating the cyclin E threshold level required for the activation of cdk2;
P27 binds both cyclins and CDK preventing the transition from G1 to S phase.



P27 (KIP1) belongs to the CDK-inhibitors family, and is encoded by the *CDKN1B* gene

MEN-4

MEN4 is a rare syndrome with clinical features that overlap with the other MENs.

MEN1-negative patients showing a MEN-like phenotype should undergo a careful assessment for possible MEN4. Confirmation of a MEN4 diagnosis should only be made with genetic testing for CDKN1B mutations.

The overall number of MEN4 patients (19 in 2017) is still too small to allow the formulation of guidelines for the clinical diagnosis of the disease or for patient management.

According to recent studies, as compared with MEN-1, MEN-4 showed:

- lower penetrance and disease manifestations later in life with improved life expectancy;
- parathyroid neoplasia in 80% of cases, occurring later in life (~56 vs ~25 years, respectively), with a female predominance;
- pituitary tumors with lower aggressiveness and size;
- possible lower penetrance of pNETs;
- no cases of adrenal tumors.