Barna Vásárhelyi

Lab test for the investigation of major human metabolic disorders

The human body

Contains app. 6 x 10^{14} cells. (100.000-fold higher than the total number of people on Earth).

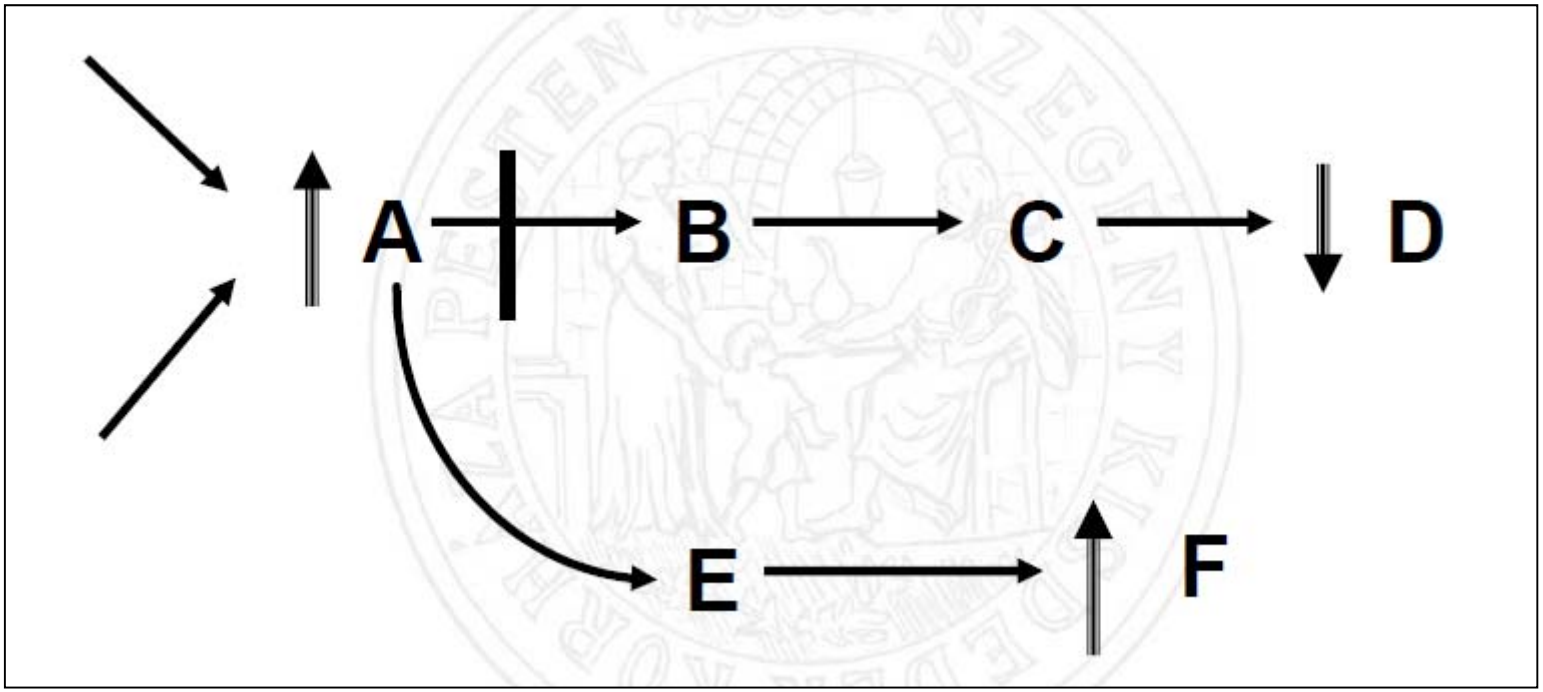
One cell contains 4 x 10^{11} molecules.

The number of possible variations exponentially increase with complexity.

Metabolites in human body

- Several thousands of different structures, classes
- Concentration ranges between mmol and less than pmol
- Distribution varies with tissues
- Blood and urine the most commonly available tissue for analysis – they may indirectly reflect real conditions
- Different analytical approach is required.

Disorder of metabolism: the pathomechanism



Two-sided problem:

(1) metabolites before the block increase (and become toxic)

(2) The level of endproduct decrease

This lecture is about

- Lab aspects of the two most common metabolic disorders:
  - Diabetes
  - Dyslipidemia

- Principles of screening for rare disorders

Disturbed glucose homeostasis

Hypoglycemia (under 2.5 mmol/l)	**Hyperglycemia (above 10 mmol/l)**
Endocrine disorders	Diabetes,
(adrenal insufficiency	Hyperthyreosis,
hypophysis insufficiency),	Overproduction of cortisone / GH / glucagone
Glycogen storage disorders,	
insulinoma,	stress
Alcoholism, liver disorders, cirrhosis	
Severe medical conditions (sepsis, uremia), fasting	
Drug-induced hypoglycemia	
Preanalytical error (use of serum/plasma unseparated from cell compartment).	

Classification of Diabetes Mellitus

1. Type 1 diabetes autoimmune origin; the destruction of beta cells of the pancreas

(6-7%)

2. Type 2 diabetes relative insulin deficiency / insulin resistance (90%

3. Other subgroups (3-4%)

Epidemiology of diabetes (WHO)

1994: 100 millions
2010: 250 millions
2030: 350 millions of diabetics worldwide
(90-95%: Type 2 DM)

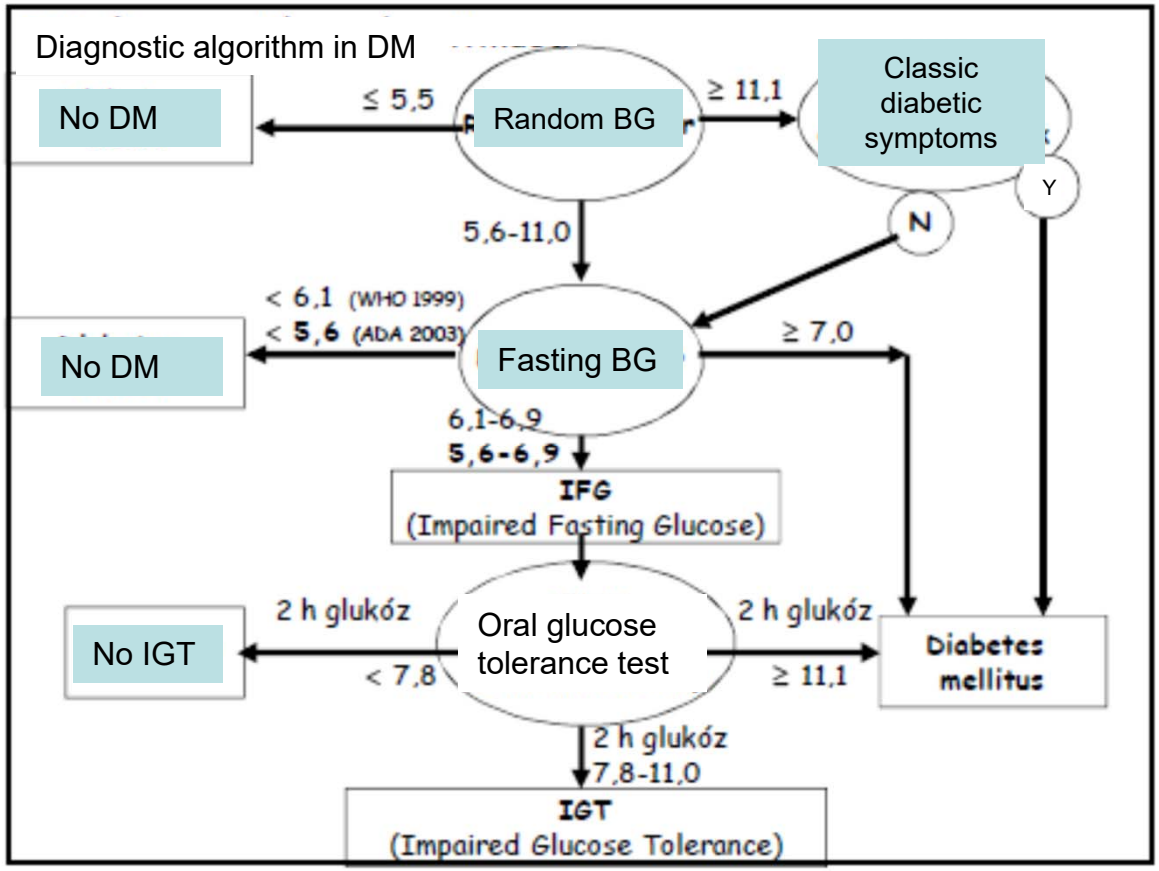
Diabetes is the cause of

50% of end-stage renal disorders
50% of cases with blindness in the elderly
risk factor for stroke (3x), infarction (3-5x)
peripheral artery disease (15-30x)

PROBLEM

- It is characteristic for T2DM that symptoms of disease (thirst, excessive urination, weakness, weight loss etc) occur just in advanced stage if ever
- Hyperglycemia, the leading symptom of diabetes does not cause any complaint

SCREENING IS OF OUTMOST IMPORTANCE



NOTE:
Formula for calculation of mg/dl from mmol/l: **mg/dl = 18 × mmol/l**
Formula for calculation of mmol/l from mg/dl: **mmol/l = mg/dl / 18**

HOW TO PERFORM OGTT

- THE test should be performed in the morning, at fasting state (at least 10 hours after a meal)
- DURING 3 days prior to OGTT the diet should contain at least 150 gram carbohydrate per day.
- PHYSICAL ACTIVITY should be as usual.
- SOME FACTORS (infection, drug, anxiety, stress, smoking) may affect the results
- 75 g (or 1.75 g/kg bw) glucose dissolved in 250 – 300 ml glucose should be ingested during 5 minutes.
- Glucose levels should be measured baseline and in the 120th minute.
- FOR non-cooperating patients: IV glucose 0,5 g/kg bw (up to 35 g), during 3 min; sampling in every 10th min, for 1 hour

GLUCOSE MEASUREMENT

- SAMPLE: recommended: NaF containing tube

WHY?

- FLUORIDE inhibits the enolase enzyme, hence inhibits the consumption of glucose
- In general, glucose levels decrease by 0.5 mmol/l per hour for 3 hours, then stabilise in the presence of fluoride for 3 days.

Analytical approach for glucose level measurement

Glucose-oxidase technique:

Alfa-D-glucose ⟶ beta-D-glucose

beta-D-glucose +H_2O_2 + O_2 —(glucose oxidase)→ géuconate + H_2O_2

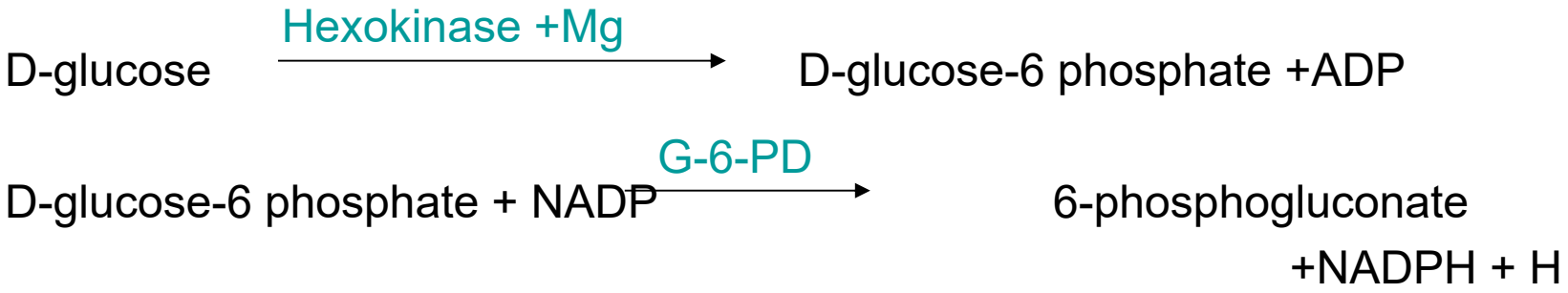
Reduced chromogen (not coloured) +H_2O_2 —(peroxidase)→ Oxidised chromogen (coloured) + $2H_2O$

Warning: cannot be used in reducing environment. Vitamin C decreases by 50% the level.

H_2O_2 can be measured with amperometry; principle for glucometer

Analysis of glucose levels

Hexokinase method (reference method):



Warning: EDTA plasma cannot be used

Important:

- Total blood: Capillary glucose level is lower by 5-10% than that in the venous blood
- Venous plasma glucose: equal to that in capillary whole blood
- Capillary blood: sampling site (finger tip) should be prewarmed before sampling

See FILM 1

- Please, note the low repeatability of POCT blood glucose test results

https://www.youtube.com/watch?v=n7H3KfMNpX4

Urinary glucose levels

- Clinically not sound
- Methods based on the reducing capacity of glucose

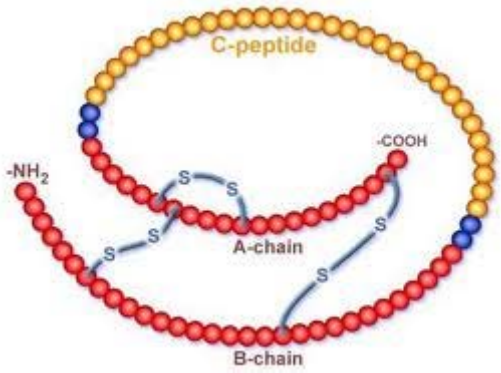
(interferring: uric acid, fructose, lactose, ketone bodies, sulfonamide, cystein, creatinine, salicilate etc.)

- Methods using glucose-oxidase approach

(false positive: hypochloric acid, expired test

false negative: vitamin C, antibiotics, salicylate, ketone bodies)

ADDITIONAL TESTS

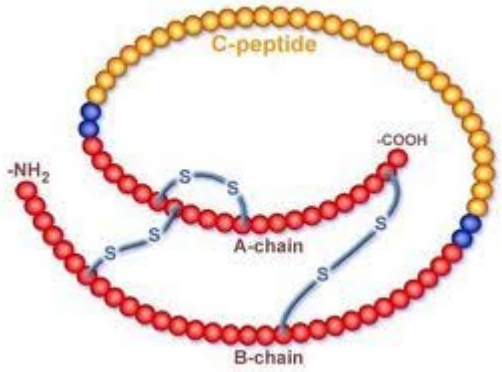


Peptide C

Reference range: 0,3-1,4 nmol/l (0,8-4,2 ng/ml).

- Pro-insuline molecule is cleaved for insulin and peptide-C before secretion.
- The quantity of secreted peptide C is identical to that of insulin.
- Indicates endogenous insulin production.
- Indication: diagnosis of islet cell tumors, pre-diabetes, hypoglycemia, insulinoma.
- Increased levels: insulinoma, type 2 DM, renal failure
- Decreased levels: type 1 DM

ADDITIONAL TESTS



INSULIN

Reference range: 18-170 pmol/l (2,6-25 mU/l) in fasting state.

- Classification / prediction of DM
- Beta-cell activity assessment
- Increased levels: insulinoma, insulin resistance / early phase of T2DM, polycystic ovary syndrome, exogenous insulin therapy, sulphanylurea therapy
- Decreased levels: T1DM

ADDITIONAL TESTS

HOMA-INDEX CALCULATION (homeostasis model assessment)

Fasting INSULIN * fasting GLUCOSE

Reference range: <4.4

- High values: Indication for insulin resistance

ADDITIONAL TESTS

AUTOANTIBODIES

- **Identification of patients at risk**

T1DM: 75-85%+ (otherwise: 0,5%)

Islet cell autoantibodies: ICA
Glutamate acid decarboxilase autoantibodies: GADA
Tirosin-phosphatase IA-2 autoantibodies: IA-2A
Insulin autoantibodies : IAA

DETECTION: with immunoassay

Genetic testing

HLA-DR3 (30%)

HLA-DR4 (95%) histocompatibility genes
(also present in 40% of non-diabetics)

ADDITIONAL TESTS

OTHER hormones

Glucagone, IGF, epinephrine, GH, thyroxin, somatostatin

DIABETIC COMPLICATIONS

EARLY onset:
Ketoacidosis, lactic acidosis, hyper (hypo) glycemia
– blood glucose tests, blood gases, electrolytes

LABORATORY TESTS ARE ESSENTIALLY NEEDED

LATE onset:

- Microvascular
- Macrovascular
- Retinopathy
- Neuropathy
- Nephropathy --- microalbuminuria (30 – 300 mg/day)

NO test for detection , just for risk assessment
(EXCEPT: nephropathy)

DIABETIC KETOACIDOSIS: KETONE BODIES

Reference range: serum 20-40 µmol/l (0,2-0,4 mg/dl)

β-hxdroxx-butirate, acetic acid, acetone.

Under normal conditions: peripheral tissues consume acetic acetate and beta-hydroxi-butirate. Acetone is produced from acetacetate by decarboxylation.

In fastaing, disturbed carbohydrate or lipid metabolism: 3-5 mmol/l, increased urinary excretion. In practice, semiquantitative assessment is adequate.

Urinary strips: acetic acetate and aceton produce violet complex with nitroprussid-sodium.

HbA1c assessment: indicator of long-term metabolic state

Self-monitoring of blood glucose levels support the appropriate dosing of insulin and the dietary modifications.

To assess the long-term efficacy of therapy HbA1c determination is needed (reflects the blood glucose levels of the last 2-3 months)

HbA1c helps to decide the appropriateness of therapy and to assess patient's compliance with therapy. More the average glucose higher the HbA1c

HbA1c: what is that and how is to be measured

HbA1c: a labile substance is produced as a result of reaction between aldehide group of the glucose and the free amino group of Hb (Schiff base) that is converted irreversibly for stable ketoamine

No enzyme catalises to this process. The process depends solely on glucose levels.

Healthy range: < 6%

Measurement:

1. HPLC
2. Immunoassay (közel 30 féle teszt)

Problem: different reagents, approaches: standardization is required

IFCC standardisation

- **IFCC** (International Federation of Clinical Chemistry and Laboratory Medicine) :

- **Reference measurement approach** with a pure primary calibrator (β-N1-deoxifructosyl-hemoglobin) *Reagent producing companies are obliged to used this primary reference material to standardise their HbA1c measurement techniques.*

Reference laboratories worldwide

Result parameters

- HbA1c should be reported in

  IFCC units (mmol HbA1c /mol HbA_0) using thes units in **NGSP units (%).**

The conversion formula between IFCC and NGSP unit

(NGSP% = [0,0915 * IFCC mmol/mol]+ 2,15)

- After 2011 HbA1c should be reported as IFCC unit in Hungary

Novel reference range and target values

Reference range	
old	**novel**
4.0-6.0%	20-42 mmol/mol
Therapeutic target value	
old	**novel**
7.0%	53 mmol/mol

- DCCT IFCC
  4.0% = 20 mmol/mol
  5.0% = 31 mmol/mol
  6.0% = 42 mmol/mol
  7.0% = 53 mmol/mol
  8.0% = 64 mmol/mol
  9.0% = 75 mmol/mol

- Kilpatrick's Kludge
  Ann Clin Biochem 2009; 46 (1) 84-5

Using HbA1c: the ADAG (A1c derived average glucose) or eAG (estimated average glucose)

- **Current guidelines**:
  Lab should report the estimated average glucose levels derived from HbA1c

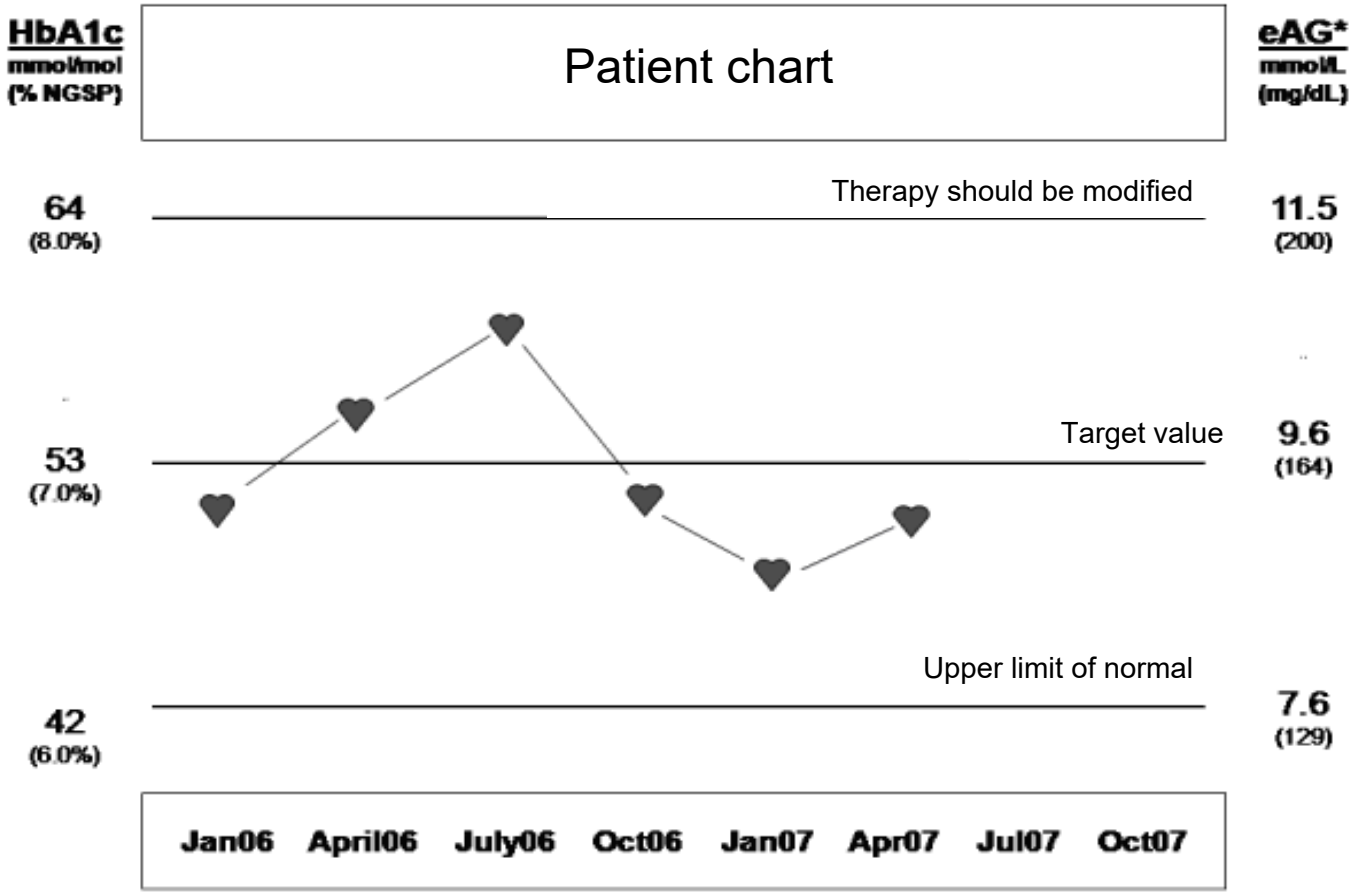
- **In 2008, the following formula was published for the calculation of eAG (estimated average glucose)**

  eAG mmol/l= 1,59 x HbA1c%-2,59 ; (eAG mg/dl= 28,7 x HbA1c%-46,7)

- **The association is not affected by the age, gender, diabetes, race and smoking**

1. ADAG Study Group Translating the A1c assay into estimated average glucose values Diab Care 2008; 31: 1473-1478
2. David B. Sacks. Translating Hemoglobin A1c into Average Blood Glucose: Implications for Clinical Chemistry Clin Chem 2008; 54:1756-1758.

The information provided by the eAG



IMPORTANT: eAG reflects the average blood glucose level representative for a given period and it is not the same as that measured at home

IMPORTANT

- 1% increase in Hb A1c: 18% and 28% increase in risk of cardiovascular and peripheral artery disease, respectively
- Appropriate blood glucose ontrol is reuired
- Hgb A1c is recommended to be <7%

Problems with HbA1c measurements:

ABNORMALITIES WITH THE QUANTITY / TURNOVER OF HB

- Hemolytic anemia → age of red cells decreases → HbA1c levels may decrease
- Higher age of red cells (iron deficient anemias) → HbA1c levels may increase

ABNORMALITIES WITH THE QUALITY OF HB

- Hemoglobinopathies (Hgb F,Hgb C) → falsely low HbA1c

- Severe renal and liver disorders may interfere

INDICATOR of short-term metabolic status

FRUCTOSE-AMINE

Reference range: 200-285 µmol/l.

Fructose-amine is a protein-ketoamine complex.
It is produced when glucose is attached to albumin.

It indicates the amount of glucose in µmol that binds to albumin in 1 litre of serum.
Half-life is short, it is appropriate to characterize blood glucose levels during the last 2-3 weeks

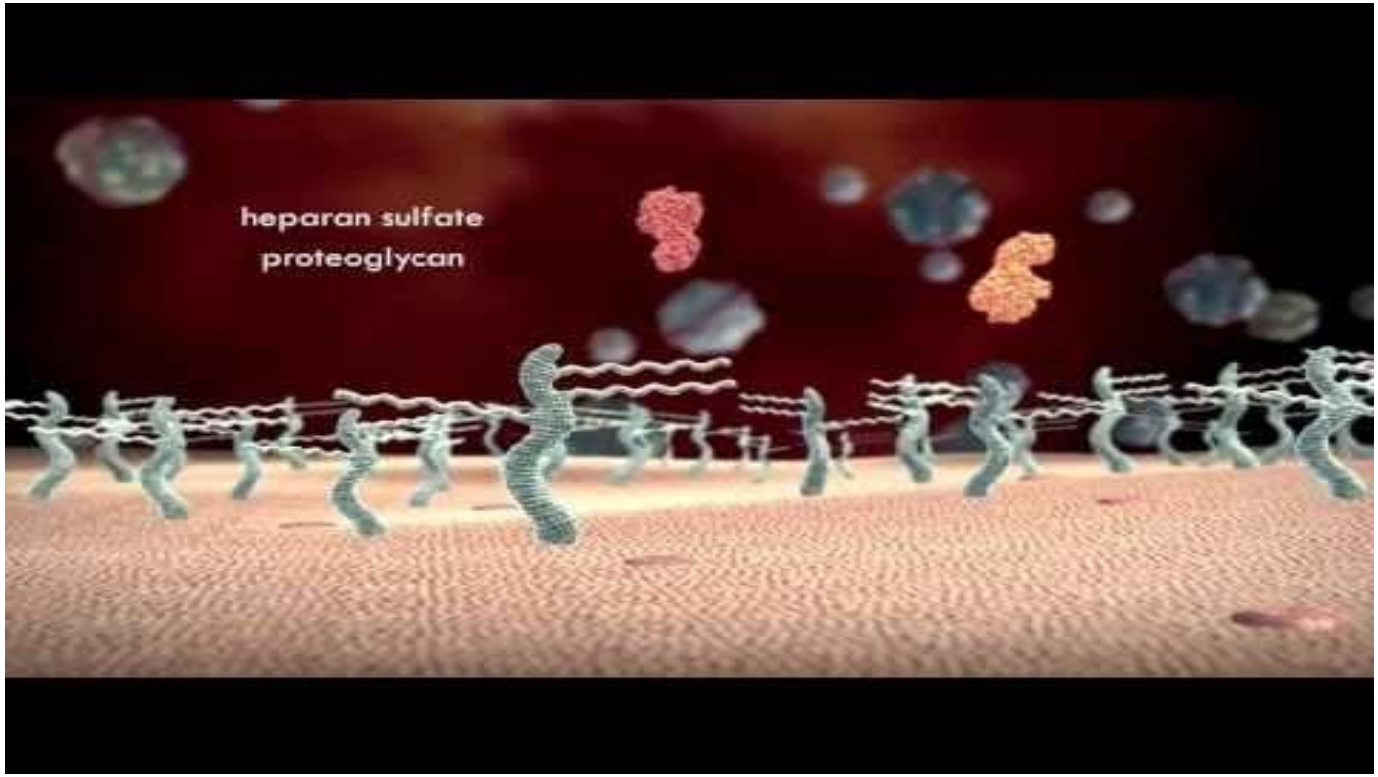
Useful when HbA1c levels cannot be interpreted.

Disordered fat metabolism

Friedrichsen-type classification (primary hyperlipidemias)

Phenotype	Chol	Tg	Chy	VLDL	IDL	LDL	Genetic cause
I	+	+++	++			low	LpL deficiency, ApoC-II deficiency
IIa	++	normal		normal		++	familiary hyperchol
IIb	++	++		++	normal or +	++	familiary combined hyperlipemia
III	++	++	+	+	++	low	familiary III típusú hyperlipemia
IV	+	++		++		normal	familiary combined HPL familiary hypertg.
V	+	++	++	++		low	familiary hypertg ApoC-II deficiency

- See FILM 2



https://www.youtube.com/watch?v=of-r3jnzYf0

https://www.youtube.com/watch?v=9dghtf7Z7fw

Secondary hyperlipoproteinaemia:

- diabetes mellitus
- metabolic syndrome
- gout
- obesity
- hypothyreosis
- pregnancy
- estrogen, steroid therapy
- nephrosis syndrome
- alcoholism
- Drug adverse effect
- Obstructive liver disease

4 major parameters:

Parameter	Normal	Intermediate	Pathologic
total cholesterol (mmol/l)	<5,2	5,2-6,2	6,2<
LDL cholesterol (mmol/l)	<3,4	3,4-4,2	4,2<
HDL cholesterol (mmol/l)	>1,6	1 – 1,6	1>
Triglyceride (mmol/l)	<0,9	0,9-1,42	1,42<

Other risk factors should be always considered

Target value depends on risk:

Risk category	**Target**	**LDL-cholesterol**	**total cholesterol**
I.	IHD or equivalent, Risk >20%	2,6	4,0
II.	At least 2 risk factors, risk < 20%	3,4	5,2
III.	0-1 risk factors	4,1	6,5

cholesterol

- 25-40% ‘free’ (unbound)
- 60-75% estherified with fatty acids
- Summa: ‘total cholesterol’
- Circulates exclusively with apolipoproteins

- Sample: serum or plasma (fasting)

Cholesterol: principle of analysis

cholesterol ester + free cholesterol

cholesterol esterase

free cholesterol + free fatty acids

cholesterol oxidase

cholestenone + H2O2

H2O2: colourful complex with phenol and 4-aminoantipyrin

Important

- Strangulation (for more than 3 min) increases cholesterol levels by 10%
- Increase while standing
- 5% of daily fluctuation

Analysis is interferred by:

Hemoglobin, bilirubin (extreme high values), ascorbic acid (extreme high dose)

Triglycerid level analysis

- Free glicerol levels after treatment of the specimen with lipase
- Several techniques

Preanalytical errors:

- Strangulation increases triglyceride levels
- Long-term storage on clotted sample.
- Free glycerol present in the sample.
- Haemolysis; high ascorbate levels: falsely low triglyceride levels.

When should one measure the cholesterol / triglyceride levels?

Guidelines recommend the measurement of lipid panel before 40 years of age as a part of routine lab investigation.

Total cholesterol, triglyceride and HDL-cholesterol levels should be measured in each people with any of the following conditions:

- Known risk factor for CVD (diabetes, hypertension)
- CVD in history
- High prevalence of CVD in young age among relatives)
- the patient or their first level relative have xanthelasma or arcus corneaej
- The serum is lipaemic in fasting state (this time triglyceride should be also measured)
- obese

METABOLIC SYNDROME (if exists at all)

- Hypertension
- Hypertriglyceridemia
- low HDL-cholesterol
- Obesity
- Impaired glucose tolerance
- Microalbuminuria (WHO)

(an entity of lab disturbances)

Conditions frequently associated with metabolic syndrome

- Impaired glucose tolerance
- Atherogen dyslipidemia
- Endothel dysfunction
- Prothrombotic state
- Hemodynamic changes
- Proinflammatory state
- Increased ovarian testosterone production
- Sleeping apnoe

Disorders frequently associated to metabolic syndrome

- diabetes
- hypertension
- polycystic ovary syndrome (PCOS)
- non-alcoholic fatty acid
- sleeping apnoe
- CVD (infarction, PAD, Stroke)
- Cancer (breast, prostata, colorectal, liver)

The diagnosis is largely based on lab tests

Inborn errors of metabolism

- Inherited autosomal recessive
- Young age
- In some case dietary intervention may help to reach symptom free status
- Can be diagnosed from blood (abnormal metabolite levels)
- Screening programs

Neonatal screening for 26 inborn errors of metabolism

***Amino acid metabolism disturbances*:**
Phenylketonuria
Maple syrup disease
Tyrosinemia, type I, II
Argininosuccinate synthase deficiency, ASS)
Argininosuccinate liase deficiency, ASL)
Homocistinuria

Fatty acid oxidation disturbances:
Short-chain acil-CoA dehydrogenase deficiency (SCAD)
Medium-chain acil-CoA dehydrogenase deficiency (MCAD)
Long-chain hydroxi-acil-CoA dehydrogenase deficiency (LCHADa,b)
Very long chain acil-CoA dehydrogenase deficiency (VLCAD)
Multiplex acil-CoA dehydrogenase deficiency (MADD, v. GA II)
carnitin-palmytoil transferase deficiency (CPT-I, CPT-II)
carnitin transport disorders (CT)

Disorders of organic acids:
beta-ketotiolase deficiency
Glutarate acidemia, 1 típus (GA-I)
Isovalerianate acidemia (IVA)
Metilmalonate acidemia (MMA)
Propionate acidemia (PA)
3-Hidroxi-3-metilglutaril-CoA-liase deficiency (HMG)
3-Metilkrotonil CoA carboxilase deficiency (MCC)
Multiplex carboxilase deficiency (MCD)

Endocrine and other metabolic disorders
Hypothyreosis
Galaktosemia
Biotinidase deficiency

Diet / substitution may prevent the complications

Neonatal screening for 26 inborn errors of metabolism

Amino acid metabolism di
Phenylketonuria
Maple syrup disease
Tyrosinemia, type I, II
Argininosuccinate synthase
Argininosuccinate liase defi
Homocistinuria

Fatty acid oxidation distu
Short-chain acil-CoA dehydr
Medium-chain acil-CoA deh
Long-chain hydroxi-acil-CoA del
Very long chain acil-CoA del
(VLCAD)
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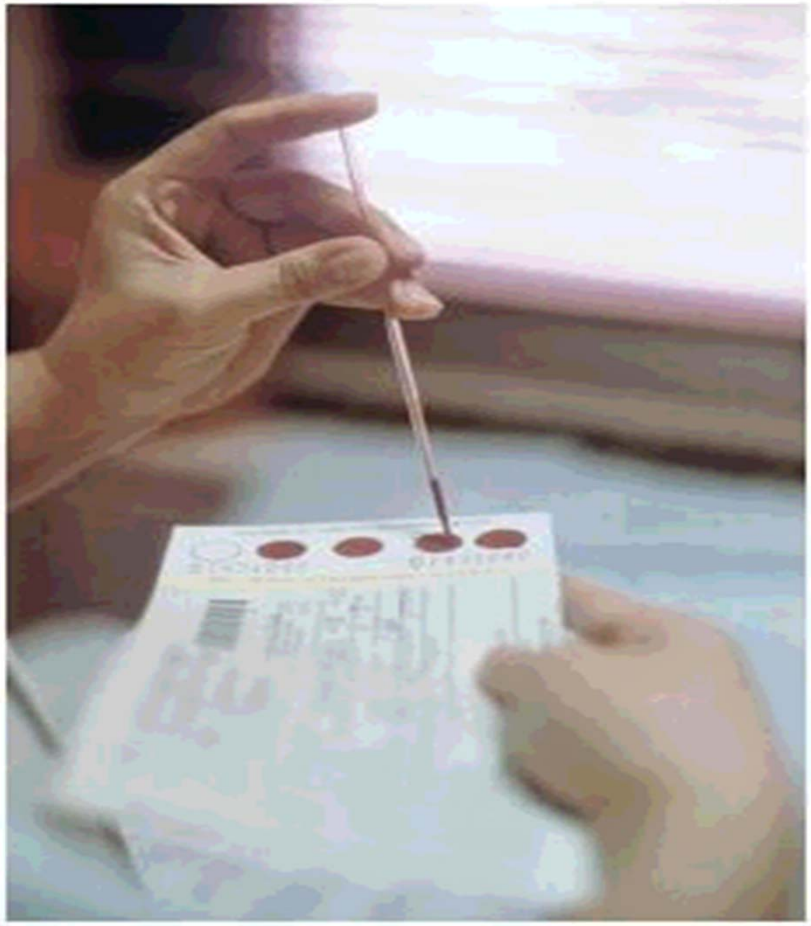
carnitin-palmytoil transferas
carnitin transport disorders (



ganic acids:
deficiency
ia, 1 típus (GA-I)
idemia (IVA)
idemia (MMA)
mia (PA)
glutaril-CoA-liase deficiency (HMG)
A carboxilase deficiency (MCC)
lase deficiency (MCD)

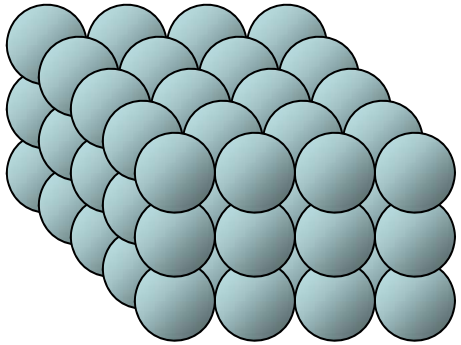
other metabolic disorders

ency

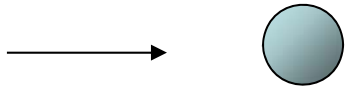
Diet / substitution may prevent the complications

Mass spectrometry for detection

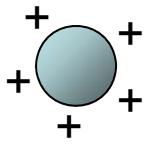
Material



Molecule
•Molecular mass- **m**



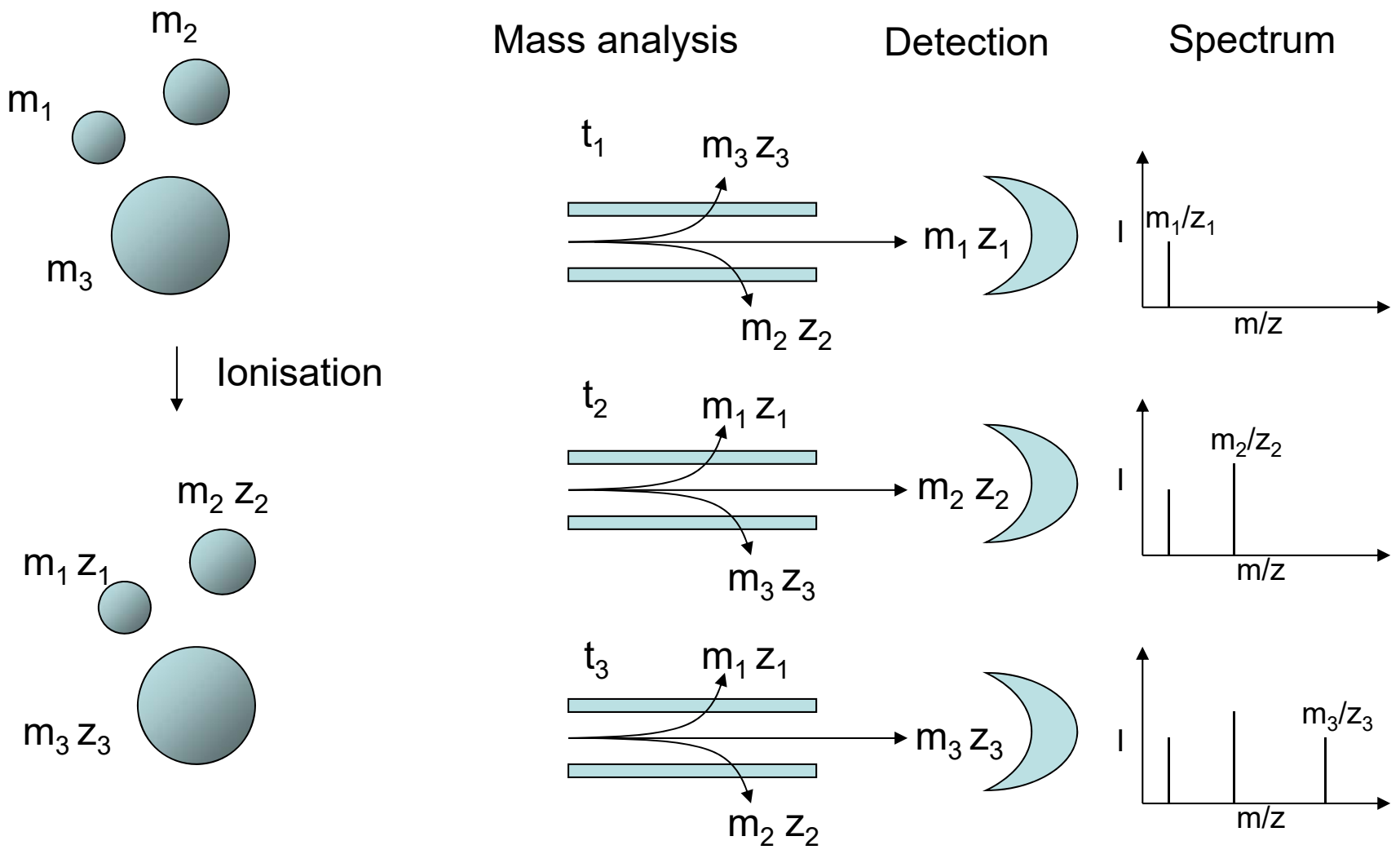
Ionisation



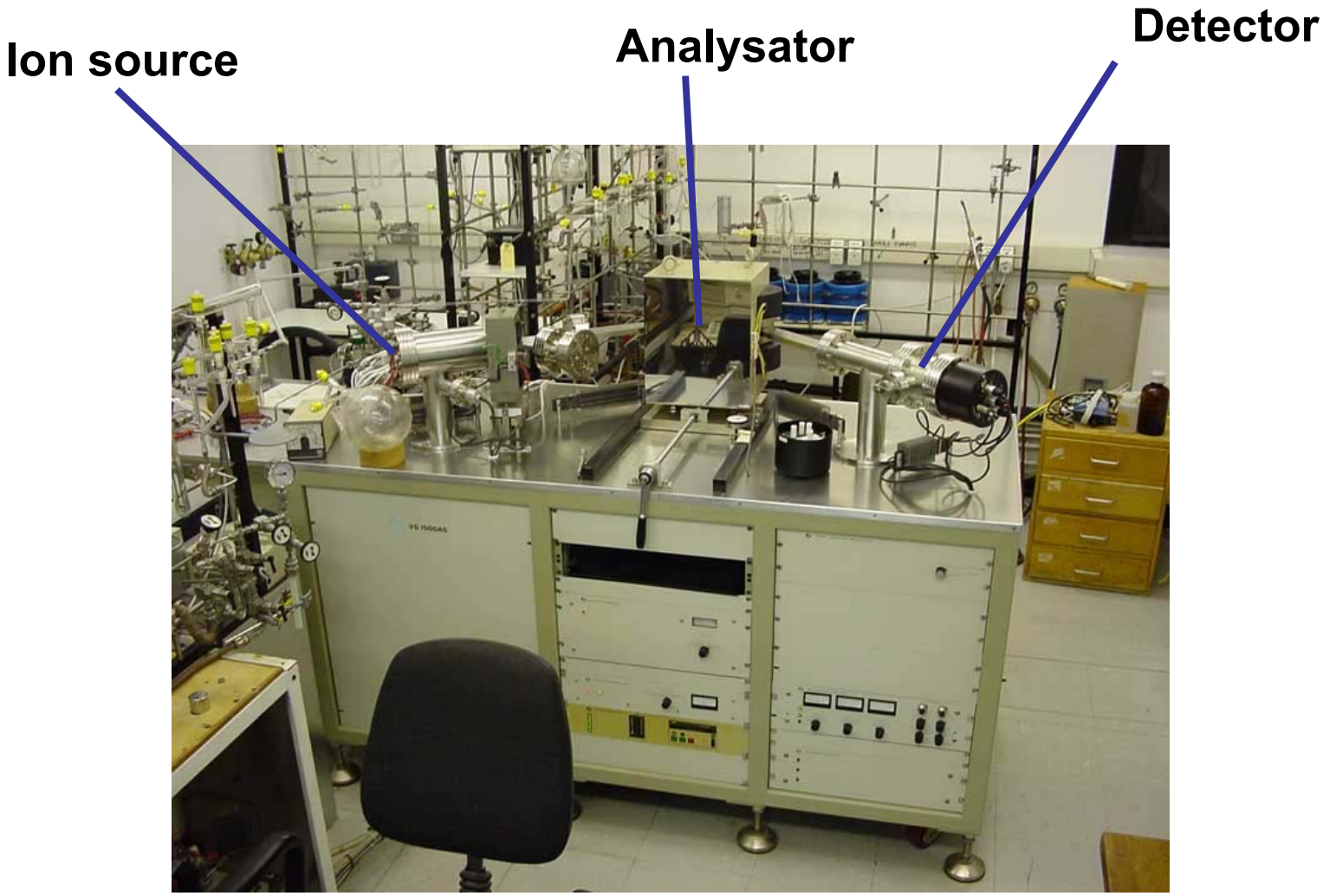
Ion
•Molecular mass– **m**
•Charge – **z**

Mass spectrometry
Detection of ions according to m/z values

Mass spectrometry



Mass spectrometer



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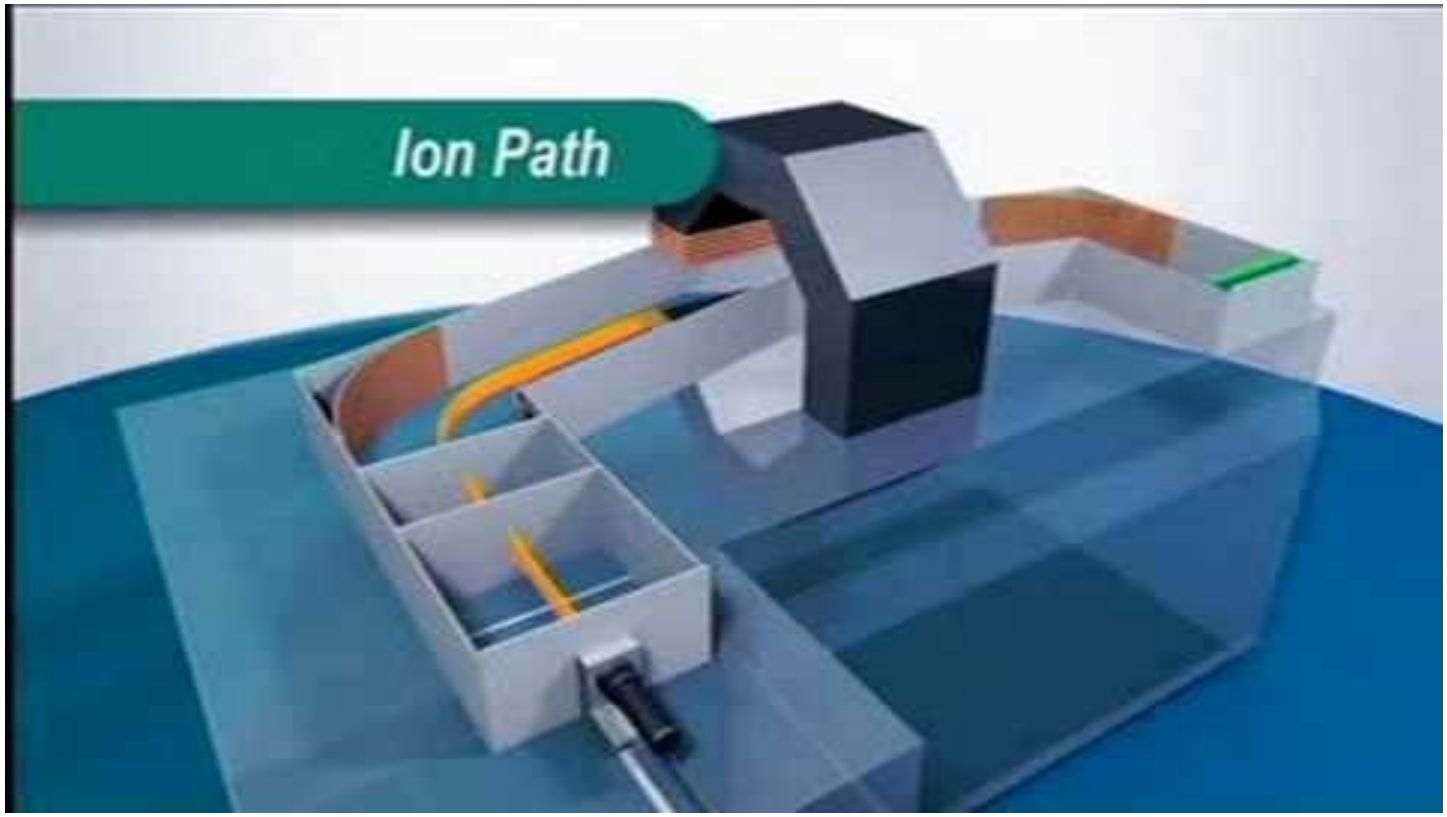
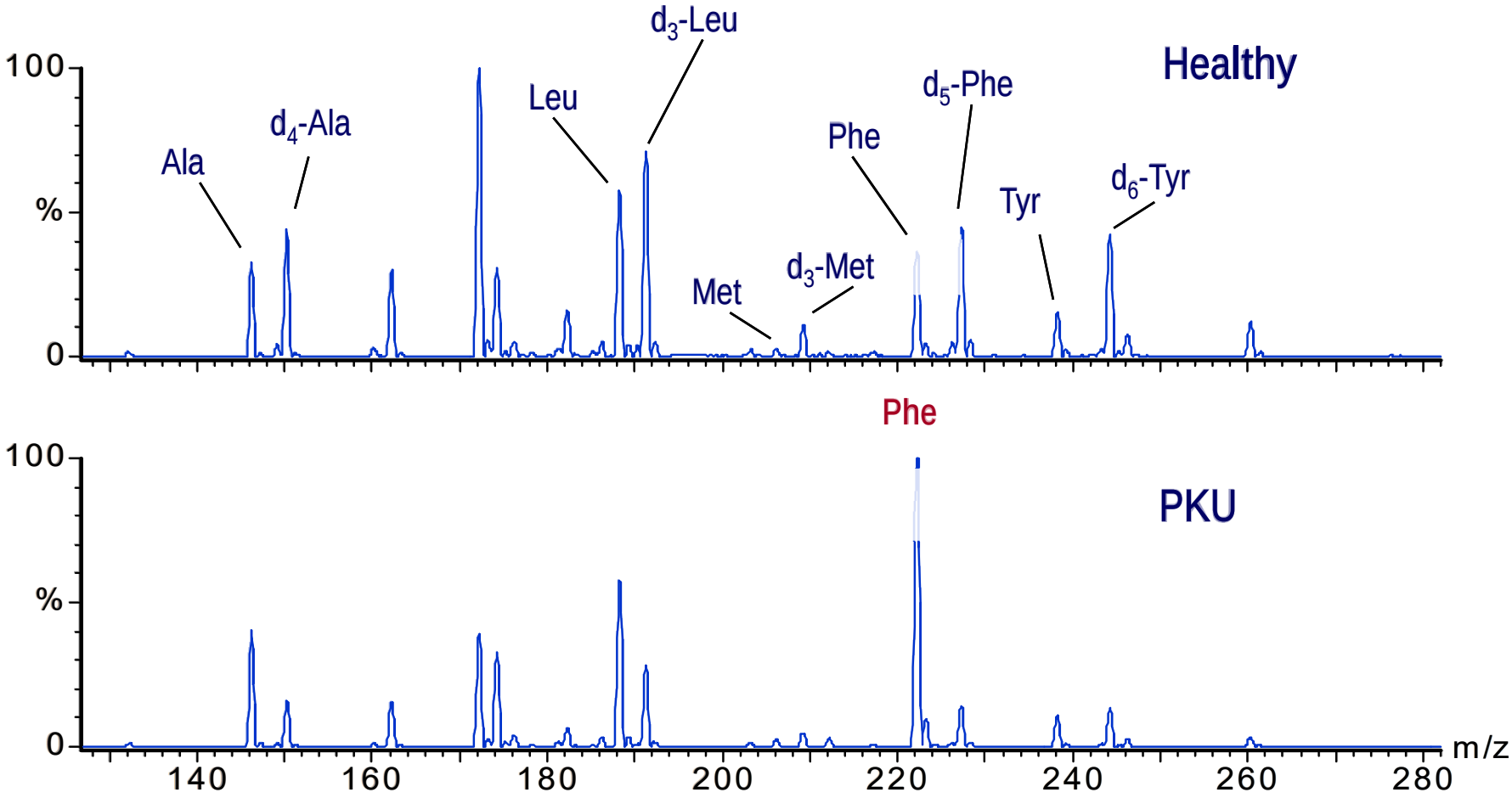
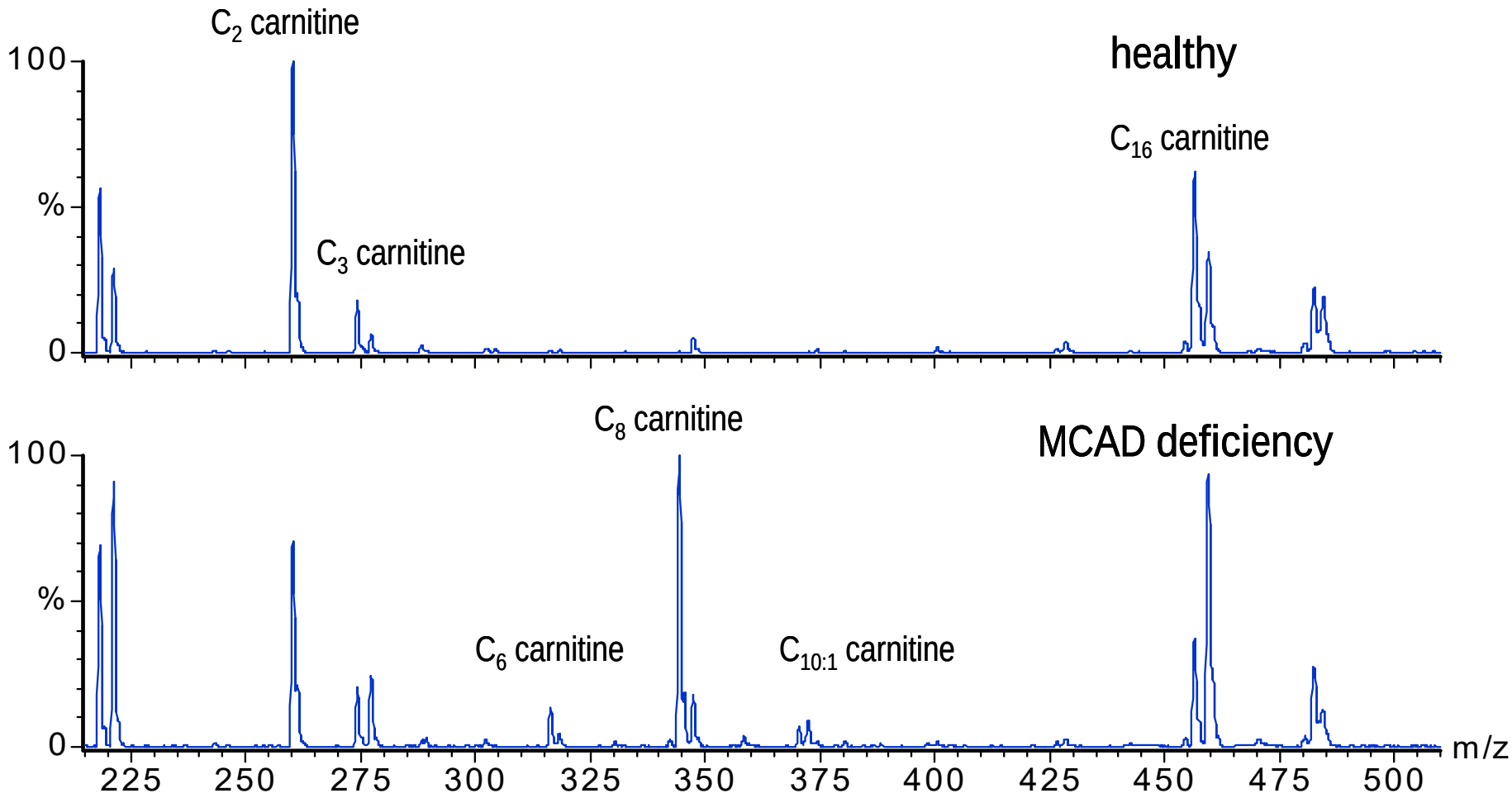


https://www.youtube.com/watch?v=J-wao0O0_qM

Mass spectrum analysis for newborn screening




C2 carnitine
healthy
C16 carnitine
C3 carnitine
100
%
0
C8 carnitine
MCAD deficiency
C6 carnitine
C10:1 carnitine
100
%
0
225
250
275
300
325
350
375
400
425
450
475
500
m/z

Take home messages

1. Lab has a major role in diagnosis and monitoring of metabolic disorders
2. Large variety of techniques
3. The two most commonly ordered groups of tests:
   blood glucose homeostasis
   &
   lipid metabolism
4. Mass spectrometry is used for metabolic screening