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CHEMICAL PATHOLOGY FOR THE BEGINNERS Aamir Ijaz

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CHEMICAL PATHOLOGY FOR THE BEGINNERS

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Dedicated to My Great Teachers

Lt Gen (Prof) Syed Azhar Ahmad, HI(M) (Retd)

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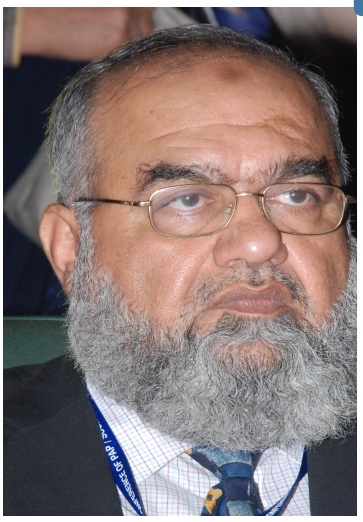
A scientific scholar, great mentor and patronising teacher; he is truly the 'Father of Chemical Pathology' in Pakistan. He was the only candidate who passed FRCPath in the very first examination held in London. "- May Allah grant him long life and prosperity.



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Gen Farooq is the founder President of PSCP. His contributions to Chemical Pathology are countless and it is a unique honour, that all fellows of Chemical Pathology in Pakistan are either his direct students or trainees of his students. Such an example is hard to find in any other specialty.

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He is Past President of PSCP and is affiliated with society since its inception. With his continuing efforts the society progressed to a higher level and he continues his endeavors for the betterment of the specialty to date. He is a role model not only for the students of Chemical Pathology but also for other disciplines. He is supervisor of many FCPS, MPhil and PhD students in Chemical Pathology

The Man Behind……

Professor Asim Mumtaz, President PSCP

Prof Asim Mumtaz conceived the idea of a professional organization for Chemical Pathology in 2004. He worked untiringly to make this a reality by getting it registered in Lahore, a job that could not have been possible without him. It was due to his untiring and continuous support to the society that he was elected as President of the PSCP in 2016. He had a huge challenge of re-registration of the society and restoration of its bank accounts. He got both the tasks accomplished after a lengthy bureaucratic process. Not only this, he also promoted the functional divisions of research, education, publication, IT and international collaboration by developing their terms of references.

PSCP has been an active organization since its inception. Regular scientific conferences and courses are conducted every year. It has a very vibrant website with a lot of educational material available to the students and consultants of Chemical Pathology. Scientific books and PSCP Newsletter *"The Spectrum"* are important publications of PSCP. Recently local chapters of PSCP have also started academic activities at Lahore, Karachi, Rawalpindi, Bahawalpur and Rahim Yar Khan. Dr Nusrat Alavi, Secretary PSCP has played vital role in these activities. Present book and other books planned in near future are all educational projects of PSCP.

Prof Asim worked hard with a vision and mission with commitment and dedication for the upbringing of the specialty of Chemical Pathology. May Allah bless him always (Ameen)!!!

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Preface

This book is a compilation of academic material generated during the PSCP educational activities e.g. Distance Learning Programmes (DLPs), Quick Assessment of Data Interpretation Skills (QADIS) and Zoom Lectures, as well as, teaching material that was prepared for MBBS, BDS, MLT students.

I always wished to compile this material in a book form to make life easy for students carrying large folders of DLP lessons, QADIS and lecture presentations during their trainee period. This is the first step towards this goal as many topics and details have not been included in this book and will be part of the "***Advance Chemical Pathology"***, the next level book that will be available soon, In Shaa Allah. All efforts have been made to include the names of Chemical Pathologists who have contributed, but there may be unsung heroes from whom I really apologize.

The term "*Beginners"* in the title means those students who have just entered in the field of Pathology e.g. IMM (Pathology) of College of Physicians and Surgeons Pakistan (CPSP), MPhil (Part 1), BS MLT and many other courses. Undergraduate Medical / Dental Students and candidates of FCPS Part I examination can get immense benefit from Section I of this book. Section II is purely of laboratory sciences and hence suitable for students dedicated to pathology.

While selecting topics for this book I have kept in mind the curricula of courses for which we have recommended this book (list on page 2). Consultants will also find it a very useful book for a quick reference. Some technical topics and details are missing in this book e.g. Basic Laboratory Instruments and Methods of Analyses. For these topics, please consult ***"Manual of Laboratory Medicine"*** published regularly for the last 20 years by my Alma Mater, Armed Forces Institute of Pathology (AFIP) Rawalpindi. Latest edition of this manual has been published, under the dynamic leadership of ***Maj Gen Prof Muhammad Tahir Khadim, Commandant AFIP Rawalpindi***.

Many junior and middle level consultants in Chemical Pathology from all over the country have carried out extensive reviewing and editing process of this book. Every chapter has been reviewed and edited 2 -3 times before going to press. Their names have been mentioned not only in the list in the initial pages but also in the beginning of each chapter. I am especially indebted to these young fellows for their valuable time and energy. Many senior consultants have contributed in terms of scientific material as well as academic techniques.

Each chapter of this book is an independent learning unit with a uniform organization. Chapter starts with 'One Best Type' MCQs from 10-20 in number with an objective to activate the prior knowledge and provide an opportunity to

self-assess before starting the actual text of the chapter. The text is mostly in bullet form, for easy comprehension and students can retain the salient points like a hand-book. While going through the text the students are expected to find the best answers of the MCQs. Thus providing an opportunity to compare their answers with the best options given at the end of the chapter. A unique column of *'Explanation'* has also been added with the best options. I enjoyed writing these explanations, because similar words and sentences were coming in my mind, which I used to utter during my teaching sessions. I hope students will enjoy these explanations, too. Last part of each chapter contains the summary, which is the gist of the chapter for students to take home. In some chapters a feature *"Animated Chemical Pathology"* has been added to present some concepts in interesting and humorous way. Some of these scripts I wrote for the 'The Spectrum', PSCP Newsletter.

At the end, I wish all the students (direct or indirect) a brilliant success in their academic pursuit.

Acknowledgements

Thanks to Allah Subhan Watalaa for giving me strength and motivation to complete this task. Alhamdolillah

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I am extremely grateful to my younger brother ***Prof Tahir Ijaz,*** Principal Punjab College, Lahore for arranging very professional publishers for this book.

Special thanks to ***Dr. Aysha Habib Khan***, AKU, Karachi, for reviewing and editing the initial pages of this book on my request.

I am deeply indebted to my Residents RMI, ***Drs. Kalsoom, Sidra, Sheharyar and Bilal*** for assisting me in proof reading and indexing.

I want to say thanks to my sons ***Dr. Ahmed, Wajeeh and Maahin*** for encouraging me to write this book, and my sweet daughter-in-law ***Dr. Maria Abassi***, who would always ask me "Baba aap ki book kahan pohnchi…? (Father what is the progress of your book?)"

Last but not the least my better half, my wife, ***Mrs. Zill-e-Huma Ghauri***, whose precious time I stole for this book. She would not mind me working on the laptop during our holidays in Bhorban or in the plane while going to the Holy Places for Umrah.

Aamir Ijaz

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Chapter No 1

Diabetes Mellitus and Hypoglycaemia

Reviewed and Edited by:

Safia Asim, Sumbal Nida, Humaira Ashraf and Usman Ali

MCQs

(Please find key at the end of the chapter)

Definition, Classifications and Diagnosis

1. A 10 years old male has just been diagnosed as a case of Type 1 Diabetes Mellitus (T1DM) with very high plasma glucose. He was found positive for Islet Cell Cytoplasmic Autoantibodies (ICA). One of his siblings was also found positive for ICA.

Based on plasma glucose results, what will be the diagnosis of this particular sibling?

a. A carrier of T1DM if plasma glucose is normal
b. Chemical Diabetes if plasma glucose is normal
c. Potential Diabetes if plasma glucose is normal
d. T1DM even if his plasma glucose is normal
e. T1DM if his plasma glucose is high

2. A 21 years old male has been diagnosed to have Diabetes Mellitus (DM). His father, paternal uncle and grandfather were also diabetic. His BMI is 21 Kg/m^2. He responded very well to sulfonylureas and did not require insulin for control. At the time of diagnosis his important laboratory investigations showed following results:

   - Fasting Plasma Glucose (FPG): 9.2 mmol/L (166 mg/dl)
   - Urine Glucose: +++

   What is the most probable diagnosis in this patient?

   a. Brittle DM
   b. LADA

c. Maturity Onset Diabetes of the Young (MODY)
d. T1DM
e. Type 2 DM (T2DM)

3. An 18 years old obese boy being investigated for DM had FPG 6.7 mmol/L (121 mg/l). He underwent OGTT, which showed 2 hours post glucose load value as 9.2 mmol/L (166 mg/dl).
   What is the most probable diagnosis in this patient?
   a. Impaired Fasting Glucose (IFG)
   b. Impaired Glucose Tolerance (IGT)
   c. Normal Result
   d. T1DM
   e. T2DM

4. FPG of a 48 years old senior male officer during his "*Annual Check-up*" was found to be 7.9 mmol/L (142 mg/dl). He was advised re-testing for DM by Glycosylated Haemoglobin (A1c). The result of A1c was found to be 6.8%. Now he wants your opinion. Keeping in view the latest recommendations by international bodies, which of the following you think is the best option for this patient:
   a. DM is confirmed
   b. Requires repeating of FPG
   c. Requires repeating of A1C
   d. Requires OGTT
   e. Requires urinalysis

5. A 48 years old male was diagnosed to be having IFG about 4 years ago but now his physician has informed him that he has developed T2DM. His BMI is 31 Kg/m^2 and his compliance to physician`s advice about lifestyle has always been poor. Which of the following pathological processes is most important in this patient leading to worsening of his disease:

a. Decline in β-cell function
b. Increased insulin resistance
c. Increased gluconeogenesis
d. Increased Glycogenolysis
e. Increasing body weight

Complications and Monitoring

6. In diabetics, there is an increase in ***Advanced Glycosylation End-products*** production. The damage to which of the following organs /tissues leads to the start of a vicious cycle:
    a. Adipose tissue
    b. Kidney
    c. Liver
    d. Lungs
    e. Muscles

7. A 54 years old male has IGT for the last 10 years. Which of the following is the ***most important complication,*** his physician should keep in mind while monitoring him?
    a. Cardiovascular disease
    b. Gangrene of the foot.
    c. Nephropathy
    d. Neuropathy
    e. Retinopathy

8. A 42 years old male has following features:
    - FPG: 5.9 mmol/L (109 mg/dl)
    - Waist Circumference: 105 cm (42 inches)
    - Triglyceride: 2.32 mmo/L (205 mg/dl)
    - Blood Pressure: 145/95 mmHg
    - HDL-C: 1.2 mmol/L (40 mg/dl)

What is the most ***probable diagnosis***?

a. Hyperlipidaemia
b. IFG
c. Metabolic syndrome
d. Obesity
e. Systolic hypertension

9. As per National Cholesterol Education Program (NCEP) guidelines DM has been assigned which of the following category of coronary artery disease risk:
    a. Major risk factor
    b. Minor risk factor
    c. Non-modifiable risk factor
    d. Not a risk factor
    e. Risk Equivalent

10. A 56 years old male is known patient of T2DM for the last 10 years. He has never been investigated for chronic complications of DM. Which of the following is the most sensitive test for the early detection of diabetic nephropathy?
    a. Estimated GFR
    b. Radioactive renal scan
    c. Urinary Albumin: Creatinine Ratio
    d. Urinary N-GAL estimation
    e. Urine routine examination
11. In which of the following patients, highest levels of Blood Glucose will be found when they have not received any treatment:
    a. Acute destruction of Islet cells
    b. Diabetic ketoacidosis
    c. Diabetic nephropathy
    d. Diabetic Rrtinopathy
    e. Hyperglycaemic hyperosmolar non-ketotic coma

12. A 73 years old male known patient of T2DM and Ischaemic Heart Disease is being nursed in a Coronary Care Unit. Which of the following is the most important complication ***to be avoided in this*** critically ill patient?
    a. Diabetic ketoacidosis
    b. Hypertension
    c. Hypoglycaemia
    d. Muscle paralysis
    e. Sepsis

13. An 8 years old boy is a newly diagnosed patient of T1DM. His parents are from very high socio-economic group. They want to adopt a very efficient and safe method for monitoring of insulin dosage for their child. Which of the following modes of monitoring of DM you will recommend to his parents?
    a. Glucometer using whole blood
    b. Glucometer using plasma
    c. Continuous glucose monitoring
    d. Glycosylated Haemoglobin
    e. Average estimated glucose

Diabetes in Pregnancy

14. A 29 years old female is pregnant for 25 weeks. Her BMI is 22 Kg/m^2. OGTT (75 g glucose) was carried out for screening which showed:
    - Fasting: 6.4 mmol/L
    - 1-hour: 8.5 mmol/L
    - 2-hour: 7.7 mmol/L

    What is the most probable diagnosis?

    a. Gestational Diabetes Mellitus (GDM)
    b. IFG
    c. IGT
    d. MODY

e. Overt Diabetes Mellitus

15. A 33 years old female is pregnant for 15 weeks. Her BMI is 33 Kg/m^2. Her father was also diabetic. Her screening tests showed:
    - FPG: 7.8 mmol/L
    - HbA1c: 7.5 % (59 mmol/mol)

    What is the most probable diagnosis?
    a. GDM
    b. IFG
    c. IGT
    d. MODY
    e. Overt Diabetes Mellitus

16. A 24 years old pregnant patient has been subjected to a test in which three blood samples have been collected at hourly intervals after ingestion of 75 grams oral glucose. This test is part of which of the following diagnostic strategies for DM in pregnancy?
    a. Glucose challenge test
    b. Initial antenatal screening
    c. One step approach
    d. O'Sullivan and Mahan criteria
    e. Two step approach

17. The most important pathogenic mechanism of GDM is:
    a. Increased diabetogenic hormones
    b. Increased maternal weight
    c. Failure of beta cells mass to increase
    d. Insulin resistance
    e. Mal-nutrition

18. The Biochemical pathway leading to macrosomia in GDM mothers includes:

a. Foetal hyperinsulinaemia causing lipogenesis
b. Foetal hypoglycaemia causing adrenal hormone secretion
c. Human placental lactogen causing lipid deposit in foetus
d. Maternal hyperglycaemia causing fluid retention in foetus
e. Maternal hyperinsulinaemia causing lipogenesis in foetus

Hypoglycaemia

19. A 62 years old male who is a known diabetic treated with sulfonylureas complains of attack of sweating, tremors and headache. He could not get his blood test done during this attack but these symptoms were relieved with some sweet thing. As per recent guidelines which of the following is the best strategy for his management:
    a. Documentation of Whipple's triad
    b. Frequent lab measurement of glucose
    c. Serum insulin and C-peptide estimation
    d. Reassurance of patient
    e. Tailoring his treatment to reduce risk
20. A newborn with hepatomegaly has recurrent hypoglycaemic attacks. His biochemical findings are:
    - Fasting Glucose: Low
    - Lactate: Normal
    - Acidosis: +
    - Ketones: Negative
    - ALT Raised
    - Fasting Insulin: Markedly increased

    What is the most probable diagnosis?
    a. Fatty acid oxidation defect
    b. Galactosaemia
    c. Idiopathic neonatal hypoglycaemia
    d. Persistent Hyperinsulinaemic Hypoglycaemia of Infant (PHHI)
    e. Urea cycle defect

Chapter No 1

Diabetes Mellitus and Hypoglycaemia

Definition of Diabetes Mellitus (DM)

- By definition DM is a state of "Chronic Hyperglycaemia"
- Deficiency of insulin is NOT an essential feature of DM.
- In fact, many patients of type 2 DM are *Hyperinsulinaemic.*

Classification of DM

Classification of DM mentioned in American Diabetes Association (ADA) Standards of Medical Care in Diabetes—2017 is reproduced here[1]:

1. Type 1 diabetes mellitus (T1DM): Due to autoimmune beta-cell destruction, usually leading to absolute insulin deficiency.
2. Type 2 diabetes mellitus (T2DM): Due to a progressive loss of beta-cell insulin secretion frequently on the background of insulin resistance.
3. Gestational diabetes mellitus (GDM): Diabetes diagnosed in the second or third trimester of pregnancy that was not clearly overt diabetes prior to gestation.
4. Specific types of diabetes due to other causes, e.g., monogenic diabetes syndromes (such as neonatal diabetes and maturity-onset diabetes of the young [MODY]), diseases of the exocrine pancreas (such as cystic fibrosis), and drug- or chemical-induced diabetes (such as with glucocorticoid use, in the treatment of HIV/AIDS, or after organ transplantation).

New Names

Why the older names "Insulin Dependent DM" and "Non-Insulin Dependent DM" have been replaced by new terms like "T1DM" and "T2DM"?

Answer:

- T1DM and T2DM are etiological classes recommended by WHO in 1999.

- Older terms indicate treatment modalities. A patient of T2DM may become dependent on insulin at later stages.

Another Important Question

- Does a patient of T2DM, when on high dose of insulin, become a patient of T1DM?

Answer:

- No. T1DM and T2DM are two different etiological classes.

Clinical Stages of DM[1]

1. Normoglycaemia
2. Impaired Glucose Regulation
    a. Impaired Fasting Glycaemia (IFG)
    b. Impaired Glucose Tolerance (IGT)
3. Diabetes Mellitus (DM)
    a. DM not requiring insulin
    b. DM requiring insulin for Control
    c. DM requiring insulin for Survival

- Very recently ADA has described clinical stages of T1DM (Table 1.1)[1]

Table 1.1: Clinical Stages of T1DM

(*Adapted from American Diabetes Association Standards of Medical Care in Diabetes 2017)*[1]

	Stage 1	**Stage 2**	**Stage 3**
Stage	• Autoimmunity • Normoglycaemia • Presymptomatic	• Autoimmunity • Dysglycaemia • Presymptomatic	• New-onset hyperglycaemia • Symptomatic
Diagnostic Criteria	• Autoantibodies • No IFG or IGT	• Autoantibodies • IFG • IGT • A1c: 5.7-6.4%	• Clinical Symptoms • DM by standard criteria

T2DM or Pre-Diabetes in Children and Adolescence[2]

- T2DM / Pre-Diabetes is no more a disease of adults
- It is due to increasing obesity and low fibre diet (fast food).
- T1DM usually presents with very severe hyperglycaemia, though may present with prediabetes in some patients (Table 1.1)

Genetics of T2DM

- Family studies have revealed that first degree relatives of individuals with T2DM are about 3 times more likely to develop the disease than individuals without a positive family history of the disease.
- It has also been shown that concordance rates for monozygotic twins, which have ranged from 60-90%, are significantly higher than T1DM (30-40%)
- Type 1 A DM has much greater genetic susceptibility than Type 1 B DM.
- Thus, T2DM has a strong genetic component as compared to T1DM.

Diagnostic Criteria of DM[3-9]

- Fasting plasma glucose (FPG) ≥ 7.0 mmol/l (>126 mg/dl)

  Repeat at interval of at least one week or when stress-free

  OR

- Two-hour plasma glucose ≥ 11.1 mmol/l (200 mg/dl) on OGTT
- The Oral Glucose Tolerance Test (OGTT) should be performed by a glucose load of 75 g dissolved in water
- In a patient with classic symptoms of hyperglycemia, a random plasma glucose ≥ 11.1 mmol/L (>200 mg/dl) *OR*

  Glycosylated Haemoglobin (A1c) ≥ 6.5%

Pre-diabetes:

- Impaired Fasting Glucose (IFG): FPG: 5.6-6.9 mmol/l (100-126 mg/dl)
- Impaired Glucose Tolerance (IGT): 2-h plasma glucose in the OGTT: 7.8-11.0 mmol/l (140 – 200 mg/dl)
- A1C: 5.7-6.4 %

OGTT

Indications

- FPG between 5.6 mmol/L (100 mg/dl) and 7.0 mmol/L (126 mg/dl)
- Patients with family history of diabetes mellitus
- Patients with previous history of large babies

Contraindication

- FPG > 7.0 mmol/L (126 mg/dl)

Interpretation of OGTT

Based on 2 h Post Glucose Load:

- Normal: < 7.8 mmol/L (140 mg/dl)
- IGT: 7.8 – 11.0 mmol/L (140-200 mg/dl)
- DM: > 11.1 mmol/L (200 mg/dl)

A1c as Diagnostic Test of DM[10-12]

- In 2009 ADA recommended A1c to be used as diagnostic test.
- After a lot of resistance, WHO also endorsed this recommendation in 2011 and A1c is now widely used as diagnostic test.
- The biggest advantage is *fasting state is not required.*
- Merely dispensing with this requirement of fasting has led to broadening of screening base and many of those patients can be screened who cannot get their test done in fasting state.
- A1c is standardized and aligned to the DCCT/UKPDS (see below) while measurement of glucose is less well standardized
- Better for overall glycemic exposure and risk for long-term complications
- Substantially less biologic variability
- Substantially less pre-analytic instability
- Relatively unaffected by acute perturbations
- Currently used to guide management and adjust therapy

Estimated Average Glucose (eAG)[13]

- eAG is a new way to talk to patients about diabetes management.

- The measurement of A1c—expressed as a percentage—is usually not very clear to patients who use glucometer or lab values.
- This may make A1c targets difficult for patients to translate into action.
- Doctors can now report A1c results to patients using the same units (mg/dl or mmol/l) that patients see routinely in blood glucose measurements.
- It can be calculated by following formula:

$$eAG\ (mmol/L) = A1c\ (\%) x 1.59 - 2.59$$

Challenges of Using A1c

TWO major challenges of using A1c for diagnosis in our country:

1. High cost: Several hundred per test as compared to PKR 10-20 per test.
2. Non-Standard methods: probably our lab persons are not even aware of the standardization etc.

Limitation of A1c[14, 15]

- A1c values are influenced by red cell survival. Thus, falsely high values can occur in patients with iron, vitamin B_{12}, or folate deficiencies.
- Rapid red cell turnover leads to a greater proportion of younger red cells and falsely low A1C values. Examples include patients with hemolysis or anemia and those treated for iron, vitamin B_{12}, or folate deficiency, and patients treated with erythropoietin.
- Depending upon the methodology, the values may be falsely high in patients with abnormal hemoglobins (such as hemoglobin F [HbF]) or low with hemoglobin S (HbS)

Latent Autoimmune Diabetes in Adults (LADA)[1]

- Rapid development of diabetic Symptoms.
- Positive Anti-GAD (Glutamic Acid Decarboxylase Antibodies) or Islet-cell cytoplasmic antibodies (ICA)
- Does not require insulin at diagnosis but progresses to insulin dependence after several months to years.

- Family history of autoimmune disorders is usually present
- It is T1DM of adults.

Pathogenesis of T2DM (Insulin Resistance or Impaired Insulin Secretion?)

- Most of the available evidence favours the view that the ultimate triggering event is impaired insulin secretion, which when superimposed on a background of insulin resistance, leads to overt T2DM.
- Insulin resistance is neither necessary nor sufficient for the development of T2DM but that defective insulin secretion is the primary defect responsible for unmasking overt diabetes.
- Although most patients with T2DM had some degree of insulin resistance, there are patients with a confirmed diagnosis of diabetes who had no evidence of insulin resistance.
- These individuals remain normoglycemic by compensating for the reduction in insulin sensitivity with increased insulin secretion
- In summary, insulin resistance is an early and characteristic feature of the natural history of T2DM in high-risk populations. Overt diabetes develops only when the beta cells are unable to appropriately augment their secretion of insulin to compensate for the defect in insulin action.
- Obesity is a very important predisposing factor in the pathogenesis of T2DM but studies have shown that many obese patients remain normal or with pre-diabetes (IFG or IGT) throughout their lives and never become overt diabetic.
- Only those obese go into T2DM phase who have in-built insulin secretion defect due to one or more of the genetic and environmental factors.
- Obesity is a contributory factor mainly to insulin resistance.
- Here again the real culprit is impaired insulin secretion[16,17]

An Analogy

- During Oct 2005 earthquake, only Margala Tower, Islamabad, Pakistan, collapsed and many precious lives were lost but other buildings were unaffected or minimally damaged.
- Probably there was a construction defect in Margala Tower that other buildings did not have.
- In construction industry earthquakes cannot be avoided but buildings can be made safer, while in human body building defect (insulin secretion) cannot be avoided but earthquakes (insulin resistance) can be avoided.
- Insulin resistance can be reduced by Total Life Style Change i.e. reducing weight and physical activity.

Complications of DM

Chronic Complications of DM

Macrovascular

- Cardiovascular Disease (CVD)
- Peripheral Artery Disease

- Cerebrovascular disease

Microvascular

- Retinopathy
- Nephropathy
- Neuropathy

Mechanisms of Tissue Damage due to Hyperglycaemia

- Non-enzymatic glycosylation that generates Advanced Glycosylation End-products
- Activation of Protein Kinase C (PKC)
- Acceleration of the aldose reductase pathway.
- Oxidative stress seems to be a theme common to all three pathways

Advanced Glycosylation End-Products (AGEs)

- In chronic hyperglycemia, some of the excess glucose combines with free amino acids on circulating or tissue proteins.
- This non-enzymatic process initially leads to formation of early glycosylation end products in the glomeruli of the kidneys.
- Later AGEs are formed which are irreversible.
- Circulating levels of AGEs are raised, particularly due to renal insufficiency, since they are normally excreted in the urine.
- The net effect is tissue accumulation of AGEs that contributes to the associated renal and microvascular complications.
- AGE receptors called RAGE have also been found in various tissues

Macro-vascular Complications of DM

- IGT serves as a marker for the state of insulin resistance and predicts both large- and small-vessel vascular complications, independent of a patient's progression to diabetes.
- Patients with IGT are at significantly increased risk for macrovacular disease i.e. myocardial infarction and stroke than to microvascular complications (nephropathy and retinopathy)

- Cardiovascular complications associated with T2 DM begin to develop well before T2DM is diagnosed. By that time, macrovascular damage may already be well-advanced.
- IGT is more predictive of cardiovascular morbidity than IFG, probably because it is a better surrogate for the state of insulin resistance.

Diabetes Complications and Control Trial (DCCT)[18]

- A land-mark study carried out on T1DM and published in 1993.
- It involved several thousand patients from several states of US.
- The two chronic complications as the study outcome in this trial were retinopathy and nephropathy
- Investigation: A1c

What conceptual change in the management of T1DM came after DCCT?

Answer: DCCT showed that keeping blood glucose level as close to normal as possible slows the onset of progression of eye, kidney and nerve complication.

Can you name a subsequent study, which was carried out in T2DM with similar results?

Answer: United Kingdom Prospective Diabetes Study (UKPDS). This study was carried out on T2DM patients and results were same as DCCT[19].

Glycaemic Control and Vascular Complications in T2DM

- The importance of tight glycaemic control for protection against microvascular and cardiovascular disease in diabetes was established in the DCCT study for T1DM.
- Although the role of glycemic control on microvascular disease in T2DM was documented in the UKPDS, its role in reducing cardiovascular risk has not been established as clearly for T2DM.

Hyperglycemia and Microvascular Disease[20-23]

- The result of the UKPDS, Kumamoto, ADVANCE, and ACCORD trials showed that intensive therapy improves the outcome of microvascular disease (primarily retinopathy, nephropathy) as DCCT showed in T1DM.
- So, improved glycemic control improves the risk of microvascular complications in patients with T1DM and T2DM

Hyperglycemia and Macrovascular Disease

- To date, no randomized clinical trial has convincingly demonstrated a beneficial effect of intensive therapy on macrovascular outcomes in individuals with long-standing T2DM
- But in ACCORD trial the patients on intensive blood glucose lowering therapy had a higher number of total and cardiovascular deaths (257 versus 203).

Target Glycaemic Levels in Type 2 DM

- Fasting glucose of 3.9 to 7.2 mmol/L (70 to 130 mg/dL)

or

- Postprandial glucose (90 to 120 minutes after a meal): < 10 mmol/L (180 mg/dL).

or

- A1c value of ≤ 7.0% for most patients

Cardiovascular Risk Factors[24]

- Advancing age
- Diabetes and other high blood glucose conditions
- Dyslipidaemia
- Genetic background
- High alcohol consumption
- Hypertension

- Insulin resistance
- Left ventricular hypertrophy
- Male gender
- Menopause
- Obesity
- Sedentary lifestyle
- Smoking

The Risk Equivalence

- People with T2DM have the same risk of heart attack as people without diabetes who have already had a heart attack.
- Women with diabetes are subject to sudden death 300% more often and men with diabetes 50% more often than their counterparts without diabetes of the same age.
- Strokes occur twice as often in people with diabetes and hypertension as in those with hypertension alone.
- A person with diabetes has a two to three-folds greater risk of heart failure compared to a person without diabetes.

Microalbuminuria[25]

- Microalbuminuria is detection of small quantity of albumin in the urine i.e. 30-300 mg/day (and NOT small-size albumin)
- Urinary Albumin: Creatinine ratio is more useful.
- The normal rate of albumin excretion is less than 30 mg/day (20 µg/min).
- Persistent albumin excretion between 30 and 300 mg/day (20 to 200 µg/min) is called microalbuminuria
- In patients with diabetes it may be indicative of early diabetic nephropathy, unless there is some coexistent renal disease.

- Protein excretion above 300 mg/day (200 µg/min) is considered to represent macroalbuminuria (also called overt proteinuria, clinical renal disease, or dipstick positive proteinuria)

Albumin: Creatinine Ratio

- The effect of variations in urine volume on the urine albumin concentration can be avoided by calculation of the urine albumin-to-creatinine ratio in a spot urine specimen.
- Values > 300 mg/g of creatinine (or 34 mg/mmol of creatinine) are indicative of macroalbuminuria.
- To label a particular type, at least two of three specimens should fall within the microalbuminuric or macroalbuminuric range over a three- to six-month period.

Three Common Acute Complications of DM[26]

1. Hypoglycaemia
2. Diabetic Ketoacidosis
3. Hyperosmolar Non-Ketotic Coma

- Please hypothesize two patients (A & B) admitted in an intensive care unit in semi-comatose condition. Capillary blood glucose of both patients is very high.
- Patient A is a known case of T1DM and Patient B is known case of T2DM. Now please answer following questions about these two patients:

Question 1: Name the most probable acute complication of DM in Patients A and Patient B.

Answer:

- Patient A Diabetic Ketoacidosis (DKA)
- Patient B Hyperglycaemic Hyperosmolar Non-Ketotic (HONK)

Question 2: Which of these two patients will be having higher level of Blood Glucose as compared to the other

Answer:

Patient B with HONK (may be around 40 mmol/L as compared to DKA in which it may be around 20 mmol/L)

Question 3:

- Give biochemical reason of higher blood glucose in one patient than the other.

Answer :

- In Patient B, there is relative insulin deficiency that is sufficient to take care of lipid metabolism. So, in this patient glucose goes on increasing leading to hyperosmolality and osmotic diuresis.

Question 4:

- Name one biochemical abnormality that, if not treated, can lead to fatal outcome in each of these patients.

Answer:

- Patient A: Hyperkalaemia
- Patient B: Dehydration

Metabolic Syndrome[27-29]

Three of these *Five* criteria should be fulfilled:

- Waist Circumference: > 87.5 cm (35 inches) in women and > 100 cm (40 inches) in men (abdominal obesity)
- Triglyceride: > 1.70 mmol/L (150 mg/dl) or higher
- Blood Pressure: >130/85 mmHg or higher
- Fasting blood glucose: > 5.6 mmol/L (100 mg/dl) or higher

- HDL-C: < 1.29 mmol/L (50 mg/dl) in women and <1.1 mmol/L (40 mg/dl) for men

Homeostatic Model of Assessment of Insulin Resistance (HOMA-IR)[30-31]

HOMA-IR is a convenient and non-invasive method for the demonstration of insulin resistance. Its formula is:

Fasting Plasma Glucose x Serum Insulin /22.5

Normal Sensitivity = up to 2.24

Glycaemic Control in Critically Ill Patients

NICE-SUGAR Study[32]

- Randomized, prospective un-blinded clinical controlled trial of *6104 patients* in 42 centers.
- The 2 groups:
    - Tight Glucose Control: 4.4-6.1 mmol/L (80-108 mg/dL)
    - Conventional Control Target: 10 mmol/L (180 mg/dL or less).

Results of NICE SUGAR

- 27.5% patients died in the intensive control group and 24.9% in the conventional-control group
- Severe hypoglycaemia (< 2.2mmol/L) was recorded in 6.8% of patients in the intensive control group, vs. 0.5% in the conventional group.
- The recommended target glycaemic range: 8.0-10 mmol/L (144-180 mg/dl

Self-Monitoring of Blood Glucose (SMBG) (Glucometers)[33]

- All patients with DM who use insulin should use SMBG to help maintain safe, target-driven glucose control.
- Patients on oral hypoglycaemic agents should use SMBG during the titration phase of their dose.

- Not required in patients on dietary regimen or anti-diabetic which don`t cause hypoglycaemia

Whole Blood or Plasma in Glucometers

- In the past, glucometers reported whole blood glucose values, which made it difficult to compare with results of plasma samples from a laboratory.
- However, majority of instruments now provide plasma equivalent values rather than whole blood values.
- Thus, results from most available glucometers and clinical laboratories should now be comparable.

Continuous Glucose Monitor (CGM)[34]

- A device that provides “real-time” glucose readings and data about trends in glucose levels
- Reads the glucose levels under the skin every 1-5 minutes (10-15 minutes delay)
- Provides alarms for high and low glucose levels and trend information

Advantages of Continuous Glucose Monitoring

- Prevention of low blood glucose alarms)
- Prevention of high blood glucose (ketones)
- Minimize wide glucose fluctuations
- Behaviour Modification
- Prevention of Complications

Hyperglycaemia in Pregnancy

Diabetes mellitus (DM) has dreadful complications in pregnancy. It can present with more than one ugly face (as per new criteria of ADA-2017)[1, 35-37]:

1. Pre-gestational Diabetes: diabetes in the first trimester should be classified as having preexisting pregestational diabetes (Overt diabetes usually T2DM or, rarely, T1DM).

2. Gestational Diabetes Mellitus: is defined as diabetes that is first diagnosed in the second or third trimester of pregnancy that is not clearly either preexisting T1DM or T2DM

- There is a need to diagnose pre-existing DM or overt DM as early as possible because hyperglycaemia at this stage can lead to foetal anomalies.
- If left undiagnosed and untreated the patient may not only have abnormal foetus but also complications related to increased diabetogenic hormones and increased insulin resistance after 24th weeks.
- These guidelines have been formulated to minimize the risk of hyperglycaemia by advocating early and efficient diagnosis and monitoring[1].

Identification of High Risk Patients

- Ethnicity (e.g. Sub-continent countries)
- Strong family history of diabetes
- Prior history of GDM
- Manifestations of Insulin Resistance e.g. Metabolic Syndrome or PCOS
- Other manifestations of glucose intolerance

Recommended Screening Protocol[39,40]

Screening at first antenatal visit:

- If TWO or more ***risk factors*** are present then screening should be done at first visit (before 24 weeks) by protocols used for non-pregnant adults (see below).
- If overt DM or pregestational is diagnosed at this stage patient should be managed not only for her glycaemic control but also for diabetic complications.

Screening at 24-28 weeks:

- All women who have not been diagnosed as GDM or overt DM earlier should undergo a 75 g OGTT as one step at 24-28 weeks of gestation.

Diagnostic Criteria for Overt DM:

Overt DM is diagnosed at any stage of pregnancy if one of the following is present:

- A1c*: ≥6.5%

- Fasting Plasma Glucose: ≥7.0 mmol/L (126 mg/dl)
- Random blood glucose**: ≥ 11.1 mmol/L (200 mg/dl)
- 2-h plasma glucose (following a 75g oral glucose load): ≥11.1 mmol/L

** Should be carried out only in first trimester by an NGSP certified method.*

*** Should NOT be advised except in situations where suspicion of DM is strong*

Diagnostic Criteria for GDM:

- At 2nd and 3rd trimester GDM is diagnosed if any one of the following is present following a 75g oral glucose load:
- Fasting: Between 5.1-6.9 mmol/L (92 -125 mg/dl)
- 1 hour: >10.0 mmol/L (180 mg/dl)
- 2-hour: Between 8.5 – 11.0 mmol/L (153-199 mg/dl)

Monitoring of Hyperglycaemia in Pregnancy:

Following glycaemic targets are recommended in GDM and pre-existing DM in pregnancy which may be achieved by metformin or insulin

- Pre-prandial ≤ 5.3 mmol/L (95mg/dl)
- 1 hour post meal ≤ 7.8 mmol/L (140mg/dl)
- 2-hour post meal ≤ 6.7 mmol /L (120mg/dl)

Follow-up after pregnancy:

At 6-12 weeks test after delivery, FPG or A1c or 75g OGTT should be done.

Further monitoring depends on the results:

- Normal: Reassess after every 3 years
- Pre-Diabetes (7.8 to 11mmol/L): Reassess every year
- Diabetes (>11.1mmol/L): Reassess quarterly

Hypoglycaemia

Categories: Hypoglycaemia can be broadly divided into two categories:

1. Hypoglycaemia in Adults
    a. Hypoglycaemia in non-diabetics
        i. Ill or medicated individuals
        ii. Seemingly well individuals
    b. Hypoglycaemia in diabetics

2. Hypoglycaemia in neonates

Hypoglycaemia in Non-Diabetics[41]

Whipple's Triad: Helps to establish the existence of a hypoglycaemic disorder. Only those patients in whom Whipple's triad is documented require evaluation and management of hypoglycaemia:

1. Symptoms consistent with hypoglycaemia
2. A low plasma glucose concentration measured with a precise method (not a home glucose monitor) when symptoms are present
3. Relief of those symptoms after the plasma glucose level is raised

Causes of Hypoglycaemia in Adults

A. Ill or medicated individual

- Drugs: Insulin or insulin secretagogue, sulphonylureas, alcohol/ethanol
- Others: Beta blockers, Salicylates, quinine, indomethacin, Lithium, getifloxacin
- Critical illnesses: Hepatic, renal, or cardiac failure, Sepsis (including malaria), predisposing illness, hospitalized patients
- Hormone deficiency: Cortisol, glucagon and epinephrine and pituitary hormones deficiency
- Non-islet cell tumour hypoglycaemia

B. Seemingly well individual

Endogenous hyperinsulinism:

- Insulinoma
- Islet hyperplasia
- Nesidioblastosis
- Post - gastric bypass hypoglycaemia,
- Autoimmune hypoglycaemia
- Severe exercise

Accidental, surreptitious, or malicious hypoglycaemia

Investigations of Endogenous hyperinsulinism:

1. Fasting Hypoglycaemia: Observe during fasting up to 72 h.
2. Post-prandial Hypoglycaemia: Observe for 5 h after mixed meal test

Causes of Hypoglycaemia in Childhood[42]

1. Transient neonatal hypoglycaemia
2. Hyperinsulinaemia
    a. Islet cell hyperplasia
    b. Insulinoma
3. Inherited metabolic disorders including
    a. Glycogen storage diseases
    b. Galactosaemia
    c. Hereditary fructose intolerance
    d. Fatty acid B-oxidation defects
    e. Prematurity
    f. Small-for-dates
    g. Endocrine disorders
    h. Starvation
    i. Drugs
    j. Ketotic hypoglycaemia

Hypoglycaemia in DM[43,44]:

All episodes of an abnormally low plasma glucose concentration (with or without symptoms) that expose the individual to harm (Table 1.2).

Blood glucose (SMBG) level ≤ 3.9 mmol/L (70 mg/dL)

At this level patient should take defensive options including:

- Repeating the measurement
- Avoiding critical tasks such as driving
- Adjusting the subsequent treatment regimen
- Ingesting carbohydrates.

Table 1.2 Clinical Classification of Hypoglycaemia in DM (ADA)

(Adapted from: Glycemic Targets. Diabetes Care 2017)

Level	Glycaemic Criteria	Description
Level 1: Glucose alert value	< 70 mg/dl (<3.9 mmol/L)	Sufficiently low for treatment with fast acting carbohydrates and dose adjustment of glucose-lowering therapy.
Level 2: Clinically significant hypoglycaemia	< 54 mg/dl (<3.0 mmol/L)	Sufficiently low to indicate serious, clinically important hypoglycaemia.
Level 3: Severe hypoglycaemia	No specific threshold	Hypoglycaemia associated with severe cognitive impairment requiring external assistance for recovery

Chapter No 1

Diabetes Mellitus and Hypoglycaemia

MCQs with Key and Explanation

MCQ No	Best Option	Explanation
Definition, Classifications and Diagnosis		
1.	e. Diagnosed as T1DM if his plasma glucose is high	By definition, hyperglycaemia is essential for the diagnosis of T1DM. Presence of antibodies in the sibling of T1DM patient is not sufficient for the diagnosis of DM. Terms like Chemical Diabetes, Potential Diabetes or Carrier of Diabetes are not used. A new staging of T1DM has been introduced in 2017 (Ref No 1), first stage is normoglycaemic and in second stage there may be prediabetes, but please remember we will not call it DM until the plasma glucose increases to the level required for the diagnosis of DM (126 mg/dl). Similar clinical staging was also described by WHO in 1999, with 'Normoglycaemia' labelled as the first stage.
2.	c. MODY	Maturity Onset Diabetes of the Young (MODY) is a group of monogenic diabetes. It has many types. MODY 3 is characterized by Hepatocyte Nuclear Factor-1-alpha — one of several mutations in the HNF1A gene on chromosome 12, non-insulin dependent DM with lowered renal threshold for glucose (Ref No 2)

3.	b. Impaired Glucose Tolerance (IGT)	Patient in the scenario has both IGF and IGT but IGT is a severe form of prediabetes, so IGT is the best option. Please note the age of the patient. At this young age, he may develop T2DM, so T2DM is no more a disease of the mature age, thanks to changing lifestyle i.e. sedentary life and fast food.
4.	a. DM is confirmed	For the diagnosis of DM, FPG and Glycosylated Haemoglobin (A1c) both can be used. But the test must be repeated at least once more before declaring a patient diabetic. Repeating is not necessary for declaring of pre-diabetes or normoglycaemic. It's a common tendency that patients get A1c done if FPG is high, if both are above the diagnostic criteria, then diagnosis is confirmed.
5.	a. Decline in beta cell function	There are two major factors in the causation of T2DM viz Insulin Resistance (IR) and Insulin Secretory Defect (ISD). It has been clearly shown that ISD has the permissive role in onset of DM. IR can expedite this process but cannot cause DM alone without the presence of ISD. Unfortunately ISD is beyond the control of the patient but IR can be overcome by lifestyle modifications. Analogy of Margala Towers collapse during earthquake because it had manufacturing defect, explains this phenomenon.
Complications and Monitoring		
6.	b. Kidney	Glycosylation is the basis of almost all the chronic complications of DM. The reaction is non-

		enzymatic, so goes on uninhibited. Advanced Glycosylation End-products (AGEs) are produced in many organs of the body but in kidney it starts the viscous cycle, as damaged kidney cannot excrete these AGEs and leads to further accumulation.
7.	a. Cardiovascular disease	Pre-diabetes and DM both lead to microvascular and macrovascular complications but pre-diabetes is more strongly associated with macrovascular complications (i.e. cardiovascular disease- CVD) as pre-diabetes is usually a part of the metabolic syndrome. Other components of this syndrome are also atherogenic (e.g. dyslipidaemia, obesity and hypertension) and contribute to causation of CVD.
8.	c. Metabolic Syndrome	Metabolic syndrome is the best option as it encompasses all the other components present in the patient and mentioned as other options (distractors). This is a good example of a One Best MCQs as the distractors are also correct but *not the best* and you have to choose the best.
9.	e. Risk Equivalent	DM is now categorized as 'risk equivalent', means patient is assumed to have CVD even if no such event has occurred. The recommended levels of cholesterol, LDL-Cholesterol and Non-HDL-Cholesterol in a diabetic patient are same as in the patient of CVD for secondary prevention.
10.	**c.** Urinary Albumin: Creatinine Ratio	This test has replaced 'Microalbuminuria' which required 24 hours urine collection. ACR is done on spot urine and is a useful test for the early

	(ACR)	detection of diabetic nephropathy but is an expensive test and is not available in smaller labs.
11.	e. Hyperglycaemic hyperosmolar non-ketotic coma	In diabetic ketoacidosis, lipid metabolism is also effected as insulin is absent in T1DM, while in T2DM, sufficient insulin is available to maintain lipid metabolism, glucose level goes on increasing until it is very high (nearly 40 mmol/L) and patient becomes dehydrated to get rid of such high load of glucose by osmotic diuresis. The cause of mortality is usually dehydration
12.	c. Hypoglycaemia	After the ACCORD study, a lot of emphasis is given to avoid hypoglycaemia in diabetics of advanced age and with co-morbidities especially CVD. In such patient hypoglycaemia can be fatal due to arrhythmias, and is more hazardous as compared to neuroglucopenia occurring in other patients.
13.	c. Continuous glucose monitoring (CGM)	CGM is a modern method of real-time monitoring of blood glucose. The devise is planted in subcutaneous area and releases insulin according to the requirement.
Diabetes in Pregnancy		
14.	a. Gestational diabetes mellitus (GDM)	As per new ADA Position Statement (2017), GDM is defined as diabetes that is first diagnosed in the second or third trimester of pregnancy and that is not clearly either pre-existing T2DM or T1DM (Ref No 1)
15.	e. Overt Diabetes	The category ‘overt diabetes mellitus’ was started

	Mellitus	by ADA in 2013 and later was adopted by WHO, too. In ADA 2017 position statement it is synonymous with pre-gestational diabetes.
16.	c. One step approach	This is one of the two diagnostic protocols of diabetes in pregnancy. Details can be found in the text of the Chapter 1.
17.	c. Failure of beta cell mass to increase	In normal pregnancy, there is a physiological insulin resistance to keep the maternal glucose level at higher levels to cater for the growing foetus. Consequently there is an increase demand of insulin, which is met with by increasing the size of the beta cell mass.
18.	a. Foetal Hyperinsulinaemia causing lipogenesis	Please remember maternal insulin cannot not cross placental barrier. It is the maternal hyperglycaemia that causes release of more foetal insulin. Foetal lipogenesis causes increased foetal weight.
Hypoglycaemia		
19.	e. Tailoring his treatment to reduce the risk.	It is important to note that the hypoglycaemic attack in a diabetic should be dealt differently as compared to the one in non-diabetic. Occurrence of such episodes implies a need to review the treatment and patient education. On the other hand hypoglycaemia in non-diabetic is first confirmed by applying Whipple`s triad
20.	d. Persistent Hyperinsulinaemic Hypoglycaemia of Infant	This is an Endocrine disorder due to excessive secretion of insulin from pancreatic islet cells. The disease can be focal or diffuse.

Summary

Chapter No 1

Diabetes Mellitus and Hypoglycaemia

- Demonstration of persistent or 'Chronic Hyperglycaemia' is essential for the diagnosis of Diabetes Mellitus (DM)
- DM is classified as Type 1 DM (T1DM), Type 2 DM (T2DM), DM due to other causes and Gestational DM.
- Diagnostic criteria of DM include Fasting Plasma Glucose (FPG) DM > 126 mg/dl (7.0 mmol/L) on two occasions or glycosylated haemoglobin (A1c) > 6.5 % on two occasions or random plasma glucose > 200 mg/dl (11.1 mmol/L), in a symptomatic patient.
- Oral Glucose Tolerance Test (OGTT) should NOT be done in patients with FPG>126 mg/dl (7.0 mmol/L). During an OGTT, 2 hours post-glucose (2hPG)> 200 mg/dl (11.1 mmol/L) is diagnostic of DM
- Criteria for prediabetes include Impaired Fasting Glucose (IFG): FPG100-126 mg/dl (5.6 to 7.0 mmol/L), Impaired Glucose Tolerance (IGT): 2hPG between 144 - 200 mg/dl (7.8-11.0 mmol/L). Based on A1c, 5.7-6.5% is pre-diabetes.
- Non-enzymatic glycosylation is the major biochemical pathway leading to microvascular (retinopathy, nephropathy and neuropathy) and macrovascular (cardiovascular disease) complications.
- Diabetic Ketoacidosis (DKA) is more common in T1DM, while Hyperglycaemic hyperosmolar non-ketotic (HONK) is associated T2DM. Hypoglycaemia occurs in both types and is the most common acute complication of DM. Plasma glucose is much higher in HONK as compared to DKA.
- Gestational DM is defined as glucose intolerance of any degree diagnosed or starting during second or third trimester of pregnancy
- Ant attack of hypoglycaemia in a diabetic requires review of the whole management plan of the patient while in a non-diabetic, hypoglycaemia should be established first using Whiple`s triad.

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Chapter No 2

Disorders of Lipid Metabolism

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MCQs

(Please find answers at the end of the chapter)

21. The highest ***cholesterol*** containing lipoprotein is:
    a. Chylomicrons
    b. HDL
    c. IDL
    d. LDL
    e. VLDL

22. Cholesterol Ester Transfer Protein **(CETP)** is related to which of the following metabolic phenomena:
    a. Transfer of Cholesterol ester to HDL
    b. Transfer of Triglyceride to HDL
    c. Transfer of Phospholipid to HDL
    d. Transfer of Apolipoprotein A1 to HDL
    e. Transfer of Apolipoprotein A2 to HDL

23. ***HDL*** is a good cholesterol and has many good functions, which of the following is ***NOT*** a function of HDL:
    a. Inhibition of glycosylation of proteins
    b. Inhibition of inflammatory activities
    c. Inhibition of oxidation of lipoproteins
    d. Maintenance of endothelial function
    e. Removal of cholesterol from macrophages

24. In routine, HDL and LDL are ***estimated*** in our laboratories by:
    a. Measuring apolipoprotein contents
    b. Measuring cholesterol contents
    c. Measuring phospholipids
    d. Lipid electrophoresis
    e. Ultracentrifuge

25. ***Friedewald Formula*** is used for calculation of:
    a. HDL-Cholesterol
    b. LDL-Cholesterol
    c. TAG (triglycerides)
    d. Total Cholesterol
    e. VLDL-Cholesterol

26. Which type of lipoproteins is most ***atehrogenic*** in causing cardiovascular disease?
    a. Chylomicron
    b. Large buoyant LDL-Cholesterol
    c. LDL-Cholesterol
    d. Small dense LDL-Cholesterol
    e. VLDL-Cholesterol

27. A 65 years old male has ***TAG (triglycerides***) level 19 mmol/L. Which of the following is the most immediate concern?
    a. Cholecystitis
    b. Chronic renal failure
    c. Lipemia retinalis
    d. Pancreatitis
    e. Peripheral neuropathy

28. A 12 years old male has following ***lipid profile***. One of his elder brothers died of "heart attack" at young ages.
    - Serum Cholesterol: 15.5 mmol/L (598 mg/dl)
    - Serum Triglycerides: 1.36 mmol/L (120 mg/dl)
    - Serum LDL-C: 13.2 mmol/L (510 mg/dl)
    - Serum HDL-C: 1.3 mmol/L (50 mg/dl)

    Which of the following is the most likely biochemical defect in this patient?
    a. Deficiency of Apolipoprotein C-II
    b. Deficiency of Lipoprotein Lipase
    c. Hyperbetalipoproteinaemia
    d. LDL-receptor defect
    e. Mutant forms of Apolipoprotein E

29. A 39 years old male is known to have ***high cholesterol and triglycerides*** at multiple occasions in the past. His two brothers have also similar pattern of lipid abnormality. His BMI is 28 Kg/m^2. He is not hypertensive. His recent biochemical profile indicates:

- Fasting Plasma Glucose: 4.4 mmol/L
- Cholesterol : 9.4 mmol/L
- Triglycerides : 7.2 mmol/L
- LDL Chol (measured): 4.3 mmol/L
- HDL Chol (measured): 0.95 mmol/L

What is the most probable diagnosis?

a. Familial hypercholesterolaemia (heterozygous variety)
b. Familial hypercholesterolaemia (homozygous variety)
c. Familial combined hyperlipidaemia
d. Familial hypertriglyceridemia
e. Metabolic syndrome

30. A state-of-the-art laboratory has started offering ***non-fasting lipid profile*** to facilitate the patients. Which of the following cholesterol cannot be determined in non-fasting state, without significant error?

a. HDL-Cholesterol (Direct)
b. LDL-Cholesterol (Direct)
c. LDL-Cholesterol (by Friedwald Equation)
d. Non-HDL-Cholesterol
e. Total Cholesterol

Chapter No 2

Disorders of Lipid Metabolism

Lipoproteins[1]: These are spherical microparticles, circulating in the blood (Figure 2.1). These particles are classified according to their density determined by a very advanced technique called 'Ultracentrifuge' (Table 2.1). All types of lipoproteins essentially contain 4 contents i.e. cholesterol, triglycerides, apolipoproteins and phospholipids. Various types of lipoproteins differ, however, in the quantity of these contents. Similarly, these lipoproteins also differ in the types of proteins (Table No 2.1).

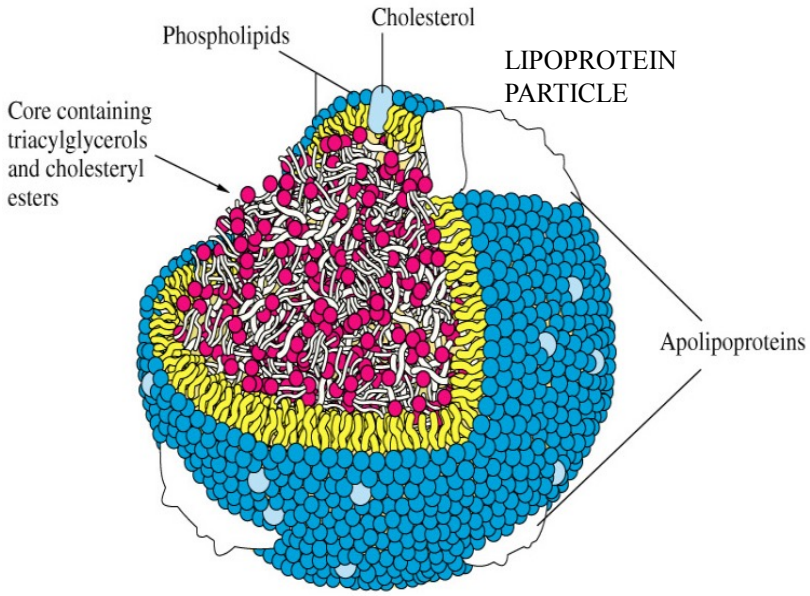

Figure 2.1 Schematic Diagram of a Lipoprotein

(Adapted from: http://www.apsubiology.org/anatomy/2020/2020_Exam_Reviews/Exam_3/CH23_Lipoproteins.htm)

Table 2.1: Major Contents of Various Lipoproteins

Lipoprotein	**Major content**	***Major protein content***
LDL	Cholesterol	Apolipoprotein B100
HDL	Protein	Apolipoprotein A
VLDL, Chylomicron	Triglycerides	Apolipoprotein B-48

HDL (Our Hero)[2-5]

- HDL particles have been studied in vitro, in animal models, and in humans.
- The **classical function of HDL** is to remove cholesterol from macrophages. However, HDL particles inhibit many inflammatory activities, inhibit oxidation of lipoproteins and cell membranes, and help to maintain endothelial function and integrity.
- HDL also exerts anticoagulant effects by inhibiting coagulation proteins and platelet aggregation.
- In addition, HDL plays a role in preserving endothelial function.
- In patients with diabetes, HDL has been found to improve beta cell survival and insulin secretion and to reduce insulin resistance in muscle and adipose tissues.
- HDL particles are formed from lipid-free precursors that continuously acquire lipids from cell membranes with the help of transport proteins.
- Starting with discoidal HDL, cholesterol is esterified in these particles to give rise to small spherical HDL_3 particles.
- Further esterification gives rise to large HDL_2 particles. Cholesterol Esters (CE) are then transferred to VLDL and LDL, which regenerate the lipid-poor Apolipoprotein A1 (ApoA-1) particles that restart the cycle again.
- HDL particle metabolism may seem trivial, but it is vastly more complicated if one looks into other constituents of HDL particles.
- Thousands of different proteins are associated with HDL. In addition, hundreds of different lipids are associated with HDL, and recently even multiple microRNAs were found to be transported in HDL particles (Box 2.1).
- Many of these proteins, lipids, and microRNAs are biologically active and exert the many positive effects of HDL on endothelial cells, beta cells, and smooth muscle cells, and on inhibition of oxidation.

Box 2.1 Heterogeneity of HDL Particle Contents

Proteins or peptides (> 80)	Lipids (100s)	microRNAs
Apolipoproteins: ApoA-I, ApoA-II, ApoA-IV, ApoA-V, ApoC-I, ApoC-II, ApoC-III, ApoC-IV, ApoD, ApoE, ApoF, ApoH, ApoJ, ApoL-I, ApoM, ApoO? **Enzymes:** LCAT, CETP, PLTP, PON1, PON3, GPx, LpPLA2, MPO **Other proteins:** SAA1, SAA2, SAA4, Coe, Hp, Hb, HRP, hPBP, PSP, C3, C4, C4B, lipocalin2?, Oro, α1-AT, α2-HS-GP, α2-AP, PBP, TTR, αFib, A1-PI	**Steroids:** FC, CE, E2, Prg, Pgn, 24OHC, 27OHC **Phospholipids:** PC, SM, PS, PE, PI, CLP, Cer **Etherphospholipids:** Plasmalogen **Glycolipids:** Glc-Cer, Gal-Cer, Lac-Cer, Sulfatid, Gangliosides (GM3), Globosides **Lysophospholipids**: LPC, SPC, LSF, S1P **Vitamins:** α-tocopherol, carotenoids (lutein, canthaxanthin, β-cryptoxanthin), retinoids	miR-33 miR-223 miR-135a* miR-188-5p miR-877 miR-760 miR-375 miR-106a miR-138-1* miR-625a* miR-17

(Adapted from: https://www.medscape.org/noscan/slideshow/760297)

Question: Name THREE adverse effects of Low HDL (other than atherosclerosis)[6]

Answer:

1. Several cancers e.g. breast, lung, colon
2. Bad effects in diabetes mellitus
3. Poor outcome in infections

- Additional epidemiological studies suggest that low HDL-Cholesterol (HDL-C) is not only associated with a risk of coronary heart disease but also of diabetes and several cancers, notably breast cancer, colon cancer, and lung cancer.
- All are inversely associated with HDL-C level and with disease outcomes, whether it is coronary heart disease (CHD) or micro- or macrovascular disease in patients with diabetes.
- This also applies to individuals who have infections; especially in intensive care units, low HDL-C confers an increased risk of a negative outcome.
- In the general population, low HDL-C is associated with being overweight and obese. Physical inactivity and smoking are the main factors linked to low

HDL-C. Conversely, light alcohol consumption is linked to increased HDL-C levels.

- Several drugs modify HDL-C levels. Androgens and anabolic steroids notably lower HDL-C levels, whereas estrogens increase HDL-C levels. Drugs that are used to treat low HDL-C, like fibrins and nicotinic acid, moderately increase HDL-C levels.
- Furthermore, there are several diseases and metabolic disorders e.g. type 2 diabetes, hypertriglyceridemia, and infections that lower HDL cholesterol levels.

Cholesteryl Ester Transfer Protein (CETP)[7-8] (The Traitor)

1. CETP causes transfer of CE from HDL to VLDL
2. Transfer of triglycerides from VLDL to HDL. So CETP which is part of HDL army but carries out an adverse action.

- CETP merges the metabolism of the Apolipoprotein B (ApoB) containing lipoproteins VLDL and LDL, with the metabolism of HDL.
- CETP transfers CE from HDL to VLDL in exchange for triglycerides, which are reciprocally transported to HDL. In an intermediate state, VLDL particles are enriched with CE.
- They are then hydrolysed by hepatic lipase to form small, dense LDL particles, which are believed to be especially atherogenic.
- HDL particles are enriched with triglycerides, but these triglycerides are not stable in HDL because they are quickly hydrolysed by hepatic lipase. As a result, smaller HDL_3 particles are produced and lipid-free ApoA-1 is formed.

CETP in Japanese:

- The importance of CETP has been recognized by an inherited disorder of metabolism, which is not infrequent in Japan.
- There are Japanese patients who carry loss-of-function mutations in both CETP alleles.
- Patients with CETP homozygotes present with very high levels of HDL-C, which exceed 75 mg/dL.
- As a population, the Japanese were considered to be long-lived because it was believed that their CETP deficiency increased longevity and might even contribute to high life expectancy.
- Patients who have one mutated CETP allele – i.e., who are heterozygous -- have moderately elevated HDL-C levels. CHD risk appears to be normal or slightly reduced in carriers with these mutations.

- Early research on CETP has made it a very attractive target for drug therapy. The first CETP inhibitor evaluated in clinical trials was torcetrapib, which nearly doubled HDL-C levels. However, due to the increased incidence of CHD events linked to off-target side effects, clinical development of torcetrapib was discontinued.

HDL and CETP - Animated

HDL and CETP –The Hero and The Traitor: 'H' for hero and 'H' for HDL, our hero has a lot of potentials and plays multiple roles – just like Hercules again with an 'H'. He has a lot of enemies to fight with alone e.g. LDL, VLDL, IDL and Lipoprotein (a). He very successfully fights but unfortunately there is also a traitor in his army and it is Cholesterol Ester Transfer Protein (CETP). When the hero (HDL) is taking cholesterol away from periphery to the liver, the traitor (CETP) smuggles some of its cholesterol to VLDL, which becomes a very dangerous particle i.e. small dense LDL (SDL). In return VLDL gives some cash (triglycerides) to CETP but this money (triglycerides) does not stay long with CETP and is taken up by the liver by the action of hepatic lipase. So, at the end of the day the traitor is left with nothing and vanishes.

Adapted with thanks from "The Spectrum" PSCP Newsletter, 3rd Issue, Dec 2014.

Friedwald Formula[9,10]

- LDL-Cholesterol (mg/dl) = Total Chol – HDL-C – TAG / 5
- LDL-Cholesterol (mmol/L) = Total Chol – HDL-C – TAG/ 2.2

Limitations of Friedwald Equation

- Serum Triglycerides: > 4.5 mmol/L (400 mg/dl)
- Serum Lipoprotein (a): > 40 mg / L
- Increased IDL (broad beta band)
- Lipoprotein lipase deficiency

LDL Patterns[11-15]

Pattern A LDL

- LDL particle with pattern 'A' appearance is the desirable type of LDL (A for acchaaa!!!). Large buoyant fluffy particles
- Not perfectly spherical; they can appear even flat like RBCs.
- Large LDL however fit 'perfectly' into LDL receptors on cholesterol-requiring tissues like our sex organs, adrenal glands, and others

Pattern B LDL

- Pattern 'B' is clearly BAD (B for Bad!!)
- Dense small
- Small dense LDL quickly and rapidly become oxidized LDL which do NOT fit into traditional LDL receptors
- They instead are attracted to many non-LDL receptor surfaces and tissues e.g. endothelium to initiate 'stiffened'/calcified arteries and atherosclerosis

Hypertriglyceridemia and Acute Pancreatitis[16,17]

- Hypertriglyceridemia a common cause of acute pancreatitis. It is associated with:
    - Moderate (Triglycerides > 2.3 to 11.2 mmol/L),
    - Severe hypertriglyceridemia (11.3 to 22.5 mmol/L)
    - Very severe hypertriglyceridemia (>22.5 mmol/L).
- The major cause of pancreatic destruction is release of large amount of free fatty acids (FFA) due to the action of lipase enzyme.
- These FFA have got toxic effects on the pancreatic tissue. In normal circumstances the triglycerides are very low and there is no significant release of FFAs.

Lipoprotein(a)[10]

- It has been found that in some patients of IHD and with normal routine lipid profile, lipoprotein (a) (Lp[a]) is increased
- Lp(a) inhibits fibrinolysis because of its structural homology with plasminogen.
- Plasminogen is an inactive plasma protein that is converted to its active form, plasmin (also called fibrinolysin), which dissolves fibrin
- Lp(a) competes for plasminogen that binds to fibrin and the surface of endothelial cells, inhibiting the breakdown of fibrin. Thus Lp(a) alters fibrinolysis (the breakdown of fibrin) occurring at the cell surface and inhibits plasminogen binding to fibrin. The result is more risk for clot formation

Non-HDL Choelsterol[18-22]

What is Non-HDL Cholesterol (NHDLC)?

- Very simple calculation!!!
- NHDLC = Total Cholesterol – HDL-C
- Does not require triglycerides measurement
- Fasting state is NOT mandatory!!!

NHDLC and Coronary Heart Disease (CHD)

- NHDLC is more strongly related to CHD than is LDL-C
- Focusing only on LDL-C may underestimate coronary risk in an individual
- NHDLC includes all atherogenic lipoprotein particles including LDL-C, Lp(a), IDL-C, and VLDL-C[2]
- VLDL-C: denotes atherogenic remnant lipoproteins

NHDLC is Superior To LDL-C in Predicting CHD Risk

- Non–HDL-C is a more useful predictor of CHD risk than LDL-C because VLDL-C is an independent predictor of CHD risk.
- A strong positive and graded association between non–HDL-C and risk for CHD has been observed.
- Overall, the association with CHD incidence was stronger for non–HDL-C within every level of LDL-C than that for LDL-C within each level of non–HDL-C, regardless of whether triglyceride levels were <200 mg/dL or ≥200 mg/dL.
- NHDLC is a secondary target of therapy when triglycerides > 2.27 mmol/L[1]

A Limitation of NHDLC

- NHDLC is not suitable for pursuing a diagnosis of familial hypercholesterolaemia.
- It is also not suitable for LDL based cascade screening in relatives of an individual with familial hypercholesterolaemia.

Familial hypercholesterolemia (FH)[23,24]

- FH is an autosomal recessive disorder affecting LDL receptors in liver causing uninhibited endogenous synthesis of LDL.
- In homozygous FH, serum cholesterol levels in a young man may be in the range of 1400-1500 mg/dl (Type IIa hyperlipidaemia; Table 2.2)
- In heterozygous variety cholesterol levels may be much less.
- It is an inheritable, autosomal dominant disorder
- Usually due to mutations in LDL receptor gene that result in decreased clearance of LDL particles from plasma
- Other mutations include those in the Apo B and PCSK9 genes

- Clinical manifestations include
  - Severe hypercholesterolemia due to accumulation of plasma LDL
  - May be accompanied by cholesterol deposition in tendons and skin (xanthomas) and in the eyes
  - Evidence of CVD early in life
  - Xanthelasma
  - Corneal arcus (Arcus senilis)
  - Achilles tendon xanthomas
  - Tendon xanthomas
  - Tuberous xanthomas
  - Palmar xanthomas

Table 2.2: Fredrickson Classification[25,26]

Type	Elevated particles	Associated clinical disorders	Cholest	Trig
I	Chylomicrons	Lipoprotein lipase deficiency, apolipoprotein C-II deficiency	Normal	Increased
IIa	LDL	Familial hypercholesterolemia, polygenic hypercholesterolemia, nephrosis, hypothyroidism, familial combined hyperlipidaemia	Increased	Normal
IIb	LDL, VLDL	Familial combined hyperlipidaemia	Increased	Increased
III	IDL	Dysbetalipoproteinemia	Increased	Increased
IV	VLDL	Familial hypertriglyceridemia, familial combined hyperlipidaemia, sporadic hypertriglyceridemia, diabetes	↔↑	↑↑
V	Chylomicrons, VLDL	Diabetes Mellitus	↑	↑↑

Familial Combined Hyperlipidaemia (FCHL)[27,28]

- FCHL is characterized by hypercholesterolemia and/or hypertriglyceridemia in at least two members of the same family with intra-individual and intra-familial variability.
- It's an important predisposing factor for premature CHD
- FCHL is one of the most common genetic hyperlipidemias in the general population
- Characterized by intra-individual or intra-familial variability of lipid phenotype
- Overproduction of hepatically derived apolipoprotein B100 associated with VLDL.
- LDL phenotype B (small, dense LDL particles) make it very strongly atherogenic
- Raised LDL-C levels and triglyceride levels
- Reduced HDL -C
- LDL-C / Apo B ratio < 1.2 (Normal > 1.4)

Metabolism of Lipoproteins in FCHL

- Overproduction of hepatically derived Apo B-100 associated with VLDL.
- LDL phenotype B (small, dense LDL particles) make it very strongly atherogenic
- Raised LDL-C levels and Triglyceride levels
- Reduced HDL -C
- LDL-C / Apo B ratio < 1.2 (Normal > 1.4)

Difference between Metabolic Syndrome (MS) and FCHL

- Apo B is the main differentiating marker, it is high in FCHL, but not in MS.
- LDL-C is usually normal or rather low in MS as compared to FCHL
- The lipid phenotype is more variable in FCHL than in MS (both in individuals and families)
- The inheritance of the disorder is much more evident in FCHL, and life style is much less relevant on FCHL clinical manifestation and prognosis than on MS
- Earlier clinical and laboratory manifestation in FCHL

Secondary Causes of Lipoprotein Abnormalities[2]

Hypercholesterolemia

- Hypothyroidism; obstructive liver disease; nephrotic syndrome; drugs e.g. cyclosporine, thiazides

Hypertriglyceridemia

- Obesity, diabetes, pregnancy, chronic kidney disease, alcohol, stress, sepsis, acute hepatitis, SLE, drugs e.g. estrogen, β-blockers, steroids, acid resins, thiazides

Low HDL

- Type-2 DM, rheumatoid arthritis, malnutrition, obesity, cigarette smoking, beta blockers

Analytical Interference with Ascorbic Acid[9,10]

- In analytical methods with H_2O_2 generation, this problem can arise
- In the step when peroxidase enzyme acts on H_2O_2 to produce coloured compound, ascorbic acid can compete with chromogenic substances
- So, a very high dose of ascorbic acid immediately before sample collection can lead to very low results of certain anlylates.
- Ascorbic acid can also produce 'False Positive' results in urine tests for glucose or occult blood

Lipoprotein Lipase Deficiency[30]

- Type1 hyperlipidaemia (Table 2.1)
- Characterized by very high triglycerides and normal cholesterol
- In a Routine Chemical Pathology laboratory, it can be diagnosed by an overnight tube test i.e. putting the serum sample of the patient at 4^0 C.
- A ring at the top of the tube indicates increased chylomicrons in the sample
- Usually present in children < 1 y of age
- Abdominal pain may be due to pancreatitis
- Hepato-splenomegaly may also be present.

Trans Fatty Acids[31]

- Trans fatty acids have been proved to be harmful for lipoproteins .i.e. they increase total cholesterol, LDL-C and triglycerides while decrease HDL-C.
- Polyunsaturated fatty acids and omega 3 (n-3) fatty acids have favourable action on the lipid parameters

Screening Lipid Protocols

Non-fasting lipid profile[32]

- Total Cholesterol
- HDL-C
- LDL-C (Measured)
- Non -HDL-C

Important Note: LDL-C calculated by Friedewald Formula cannot be determined in non-fasting state as chylomicrons are high in this state, while in Friedewald Formula it is assumed that chylomicrons are negligible and VLDL is the only source of triglycerides.

Fasting lipid profile

- If non-fasting lipid profile is abnormal
- Serum sample highly chylous
- Strong family history of CAD

Chapter No 2

Disorders of Lipid Metabolism

MCQs Key with Explanation

MCQ No	Best Option	Explanation
21.	d. LDL	Lipoproteins differ in the quantity of the contents. LDL is high in cholesterol while VLDL and chylomicrons are higher in triglycerides
22.	b. Transfer of Triglyceride to HDL	CETP is one the important proteins of HDL. Unlike HDL, it is an adverse coronary risk factor. The most probable metabolic cause that CETP gives esterified cholesterol to VLDL, which immediately takes a horrible shape of small dense LDL. In turn it takes triglycerides from VLDL.
23.	a. Inhibition of glycosylation of proteins	It is now known that function of HDL is beyond reverse cholesterol transport. It has many other good functions, to say HDL has a pleotropic effect. But in the known literature HDL has not been shown to inhibit glycosylation of proteins. (For multiple functions of HDL please see text).
24.	b. By measuring cholesterol content	In clinical laboratories HDL and LDL are not measured directly, rather the cholesterol contents are measured. In some labs apolipoproteins A and apolipoproteins B are also measured.
25.	b. LDL Cholesterol	Friedewald formula (or equation) basically utilizes the metabolic fact that in VLDL, cholesterol is 20% of the triglycerides. So, if all triglycerides measured in the serum is coming from VLDL (as happens in fasting state), VLDL Cholesterol is calculated. Once we know VLDL Cholesterol, HDL Cholesterol and Total Cholesterol, we can calculate LDL Cholesterol by simple mathematics.
26.	d. Small dense LDL-	Small dense LDL is a sub-type of LDL (L3). It can cross the endothelial lining without LDL-receptor mediation, just

	Cholesterol	because of the small size. Other sub-type 'Large Buoyant LDL' is less dangerous because it requires receptor mediation to cross the endothelial cells and cause atherosclerosis. A person with abundant 'Small dense LDL' is said to have Pattern B (Mnemonic: B for Bad).
27.	d. Pancreatitis	Pancreatitis is second most common complication of hypertriglyceridaemia (after atherosclerosis) but what is the mechanism? Possibly very high triglycerides are digested by pancreatic lipase resulting in release of high quantity of free fatty acid (FFAs). Toxicity of FFAs is well-known in various tissue e.g. liver. In pancreas, the released FFAs start creating havoc right in the place where they were 'born' (Franklin Monsters!!!)
28.	d. LDL Receptor Defect	LDL is produced by the liver and when LDL particles complete the life cycle these particles are taken –up by the liver through the LDL-Receptors on the hepatic surface. A genetic defect in these receptors causes impairment of LDL uptake by liver resulting into very high LDL because of two reasons: 1) LDL particles catabolism is impaired 2) LDL synthesis in the liver, which is normally inhibited by the LDL, goes on uninhibited (Factory goes on producing a product without obtaining the market trend}
29.	c. Familial Combined hyperlipidaemia (FCHL)	In this patient, both cholesterol and triglycerides are increased with a strong family history of hyperlipidaemia in two siblings. These two features indicate presence of FCHL. But a puzzling issue is that according to the definition of FCHL, only cholesterol or triglycerides rise with family history are sufficient for the diagnosis of FCHL. So how to differentiate this condition from 'Familial Hypercholesterolemia' (FH). This can be done only by the presence of xanthomas in FH patient on the face or other parts of the body.
30.	d. LDL-Cholesterol (by Friedwald Equation)	As we saw in MCQ No 25, Friedewald Formula is based on an important assumption that in fasting state, triglycerides come only from VLDL and chylomicrons are negligible. So, if sample is taken in non-fating state, there may be significant contribution from chylomicrons.

Summary

Chapter No 2

Disorders of Lipid Metabolism

- Cholesterol, triglycerides and other lipids circulate in the blood in small spherical particles called lipoproteins, these particles also have proteins in them as carriers
- Lipoproteins are classified based on a separation technique called 'Ultracentrifuge' that is not used in routine clinical laboratories.
- HDL is a 'good cholesterol' because many biochemical facts make it a good cholesterol. HDL has multiple functions because it has dozens of lipids, proteins and microRNAs.
- One of the proteins in HDL called 'Cholesterol Ester Transfer Protein' is an adverse coronary risk factor i.e. it causes atherosclerosis.
- Many abnormalities in lipoprotein metabolism have been found causing various lipid disorders
- Familial hypercholesterolaemia is an autosomal recessive lipid disorders causing ischaemic heart disease in children (homozygous) and adults (heterozygous) because of very high cholesterol level
- Familial combined hyperlipidaemia is another common lipid disorder running in the families. Usually both cholesterol and triglycerides are high in this disorder
- Lipoprotein lipase deficiency causes very high triglycerides because of deficiency of an enzyme involved in the clearance of chylomicrons
- Hypertriglyceridaemia can cause pancreatitis and pancreatitis can cause hypertriglyceridaemia!!
- Among dietary fats, trans fatty acids are most atherogenic, while polyunsaturated fatty acids are good fatty acids.

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Chapter No 3
Plasma Proteins

Reviewed and Edited by:

Ayesha Hafeez, Sobia Irum and Aisha Siddiqi

MCQs

(Please find key at the end of the chapter)

31. Term '***Protein'*** is referred to a specific length of amino acids. Which one of the following residues of amino acids will be a protein?
    a. Decapeptide
    b. Fifty amino acids
    c. One hundred amino acids
    d. Pentapeptide
    e. Tripeptide

32. The ***protein structure*** of haemoglobin is:
    a. Ligandary
    b. Primary
    c. Quaternary
    d. Secondary
    e. Tertiary

33. ***'Alpha Helix'*** and ***'Beta Pleating'*** constitute which of the following protein structural hierarchy?
    a. Ligandary
    b. Primary
    c. Quaternary
    d. Secondary
    e. Tertiary

34. In the process of protein synthesis, 20 amino acids are ***coded*** by:
    a. Sixty-four codons
    b. Sixty-one codons
    c. Sixty-one tRNA species
    d. Three nucleotides in DNA
    e. Two purines and one pyrimidine in mRNA

35. Plasma, proteins are measured by protein mass (g/L) and molar abundance (mmol/L). Which one of the following plasma proteins is ranked at the ***top in molecular abundance?***

a. Albumin
b. Alpha 1 antitrypsin
c. Haptoglobin
d. Immunoglobulin G
e. Transferrin

36. A 62 years old male patient has following ***liver function tests***:
    - Serum bilirubin: 123 µmol/L (<17)
    - Serum ALT: 192 U/L (<42)
    - Serum Albumin: 22 g/L (35-50)
    - Prothrombin Time Failed to clot

    Which of the following amino acids you will like to add in his diet:

a. Alanine
b. Cysteine
c. Cystine
d. Glutamic acid
e. Glutathione

37. Which one of the following is a fast **acute phase reactant?**

a. Alpha 1 antichymotrypsin
b. Alpha 1-acid glycoprotein
c. Alpha 1-protease inhibitor
d. Haptoglobin
e. Procalcitonin

38. A 31 years old female has undergone CSF examination for the exclusion of a neurological disorder lasting for the last few weeks. Her laboratory findings are:

    CSF
    - WBCs: 29 x 10^6/L
    - Lymphocytes: 80%
    - Glucose: 4.1 mmol/L
    - Total Protein: 420 mg/L
    - Lactate: 1.7 mmol/L
    - Serum/CSF Albumin Index: 7
    - IgG: Albumin Ratio: 0.35
    - Oligoclonal band seen in CSF by isoelectric focusing

    Serum
    - Glucose: 5.5 mmol/L
    - No oligoclonal band seen by isoelectric focusing

These findings are most probably suggestive of:

a. Clinically Isolated Syndrome
b. Monoclonal Gammopathy
c. Multiple Sclerosis
d. No Neurological Disease
e. Tuberculous Meningitis

39. A 34 years old woman presents to the Emergency Department with abdominal pain, abdominal distension and dyspnea for 10 days. Clinical Examination of the abdomen shows presence of free-flowing fluid in the peritoneal cavity. In this patient, biochemical analysis reveals:
    - Serum Albumin: 40 g /L
    - Ascitic Fluid Albumin: 10 g/L

    What is most probable cause of the ascites?

    a. Liver Cirrhosis
    b. Pancreatitis
    c. Peritoneal Carcinomatosis
    d. Peritoneal Tuberculosis
    e. Pyogenic Peritonitis

40. A 42 years old male with fever, vomiting, headache, photophobia, altered level of consciousness had following biochemical analysis of CSF:
    - CSF Albumin: 0.90 g/L
    - Serum Albumin: 42 g/L

    Which one of the following is the best matching statement for this patient related to his blood brain barrier?

    a. Intact blood CSF barrier
    b. Moderate impairment of blood CSF barrier
    c. Severe impairment of blood CSF barrier
    d. Slight impairment of blood CSF barrier
    e. Space occupying lesion in the brain

Chapter No 3
Plasma Proteins

Names and Abbreviations of 20 Amino Acids[1]

1. Alanine - Ala - A
2. Arginine - Arg - R
3. Asparagine - Asn - N
4. Aspartic Acid - Asp - D
5. Cysteine - Cys - C
6. Glutamine - Gln - Q
7. Glutamic Acid - Glu - E
8. Glycine - Gly - G
9. Histidine - His - H
10. Isoleucine - Ile - I
11. Leucine - Leu - L
12. Lysine - Lys - K
13. Methionine - Met - M
14. Phenylalanine - Phe - F
15. Proline - Pro - P
16. Serine - Ser - S
17. Threonine - Thr - T
18. Tryptophan - Trp - W
19. Tyrosine - Tyr - Y
20. Valine - Val - V

- **Question:** What is the meaning of term '***Protein'***?
- **Answer:** A chain of 50 Amino Acids[1]

Protein Structure[2]

Four levels of protein structure are distinguished from one another by the degree of complexity in the polypeptide chain. A single protein molecule may contain one or more of the protein structure types (Figure 3.1).

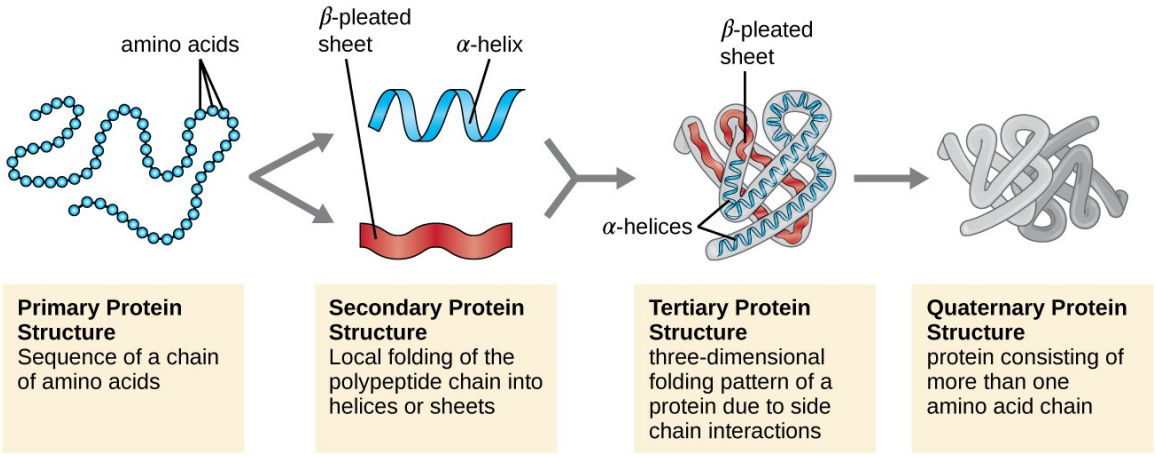

Figure 3.1: Hierarchy of Protein Structure

(Adapted from: https://courses.lumenlearning.com/microbiology/chapter/proteins/)

Primary Structure

- It is the unique order in which amino acids are linked together to form a protein.
- Proteins are constructed from a set of 20 amino acids.
- Carbon (the alpha carbon) is bonded to the four groups i.e.
  - Hydrogen atom (H)
  - Carboxyl group (-COOH)
  - Amino group (-NH2)
  - "Variable" group or "R" group
- All amino acids have alpha carbon bonded to a hydrogen atom, carboxyl group, and amino group.
- The "R" group varies amongst amino acids and determines the differences between these protein monomers.
- The amino acid sequence of a protein is determined by the information found in the cellular genetic code.
- The order of amino acids in a polypeptide chain is unique and specific to a particular protein.
- Alteration of a single amino acid can be a result of gene mutation, which most often leads to a non-functioning protein.

Secondary Structure

- It is the coiling or folding of a polypeptide chain that gives the protein its 3-D shape.
- Two types of secondary structures are observed in proteins (Figure 3.2).
  - Alpha (α) helix structure. This structure resembles a coiled spring and is secured by hydrogen bonding in the polypeptide chain.

- Beta (β) pleated sheet. This structure appears to be folded or pleated and is held together by hydrogen bonding between polypeptide units of the folded chain that lie adjacent to one another.

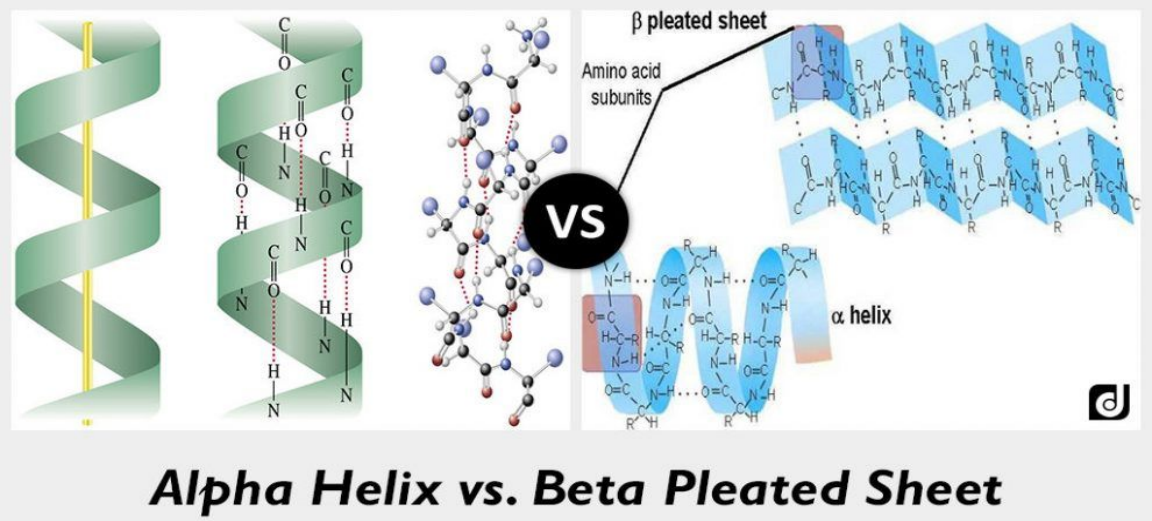

Figure 3.2: Secondary Protein Structure

(Reproduced from: https://www.differencebtw.com/difference-between-alpha-helix-and-beta-pleated-sheet/)

Tertiary Structure

- It refers to the comprehensive 3-D structure of the polypeptide chain of a protein.
- There are several types of bonds and forces that hold a protein in its tertiary structure.
- Hydrophobic interactions greatly contribute to the folding and shaping of a protein.
- The "R" group of the amino acid is either hydrophobic or hydrophilic. The amino acids with hydrophilic "R" groups will seek contact with their aqueous environment, while amino acids with hydrophobic "R" groups will seek to avoid water and position themselves towards the center of the protein.
- Hydrogen bonding in the polypeptide chain and between amino acid "R" groups helps to stabilize protein structure by holding the protein in shape, which is established by the hydrophobic interactions. Due to protein folding, ionic bonding can occur between the positively and negatively charged "R" groups that come in close contact with one another. Folding can also result in covalent bonding between the "R" groups of amino acids.
- This type of bonding form what is called a disulfide bridge. Interactions called van der Waals forces also assist in the stabilization of protein

structure. These interactions pertain to the attractive and repulsive forces that occur between molecules that become polarized.

Quaternary Structure

- It refers to the structure of a protein macromolecule formed by interactions between multiple polypeptide chains.
- Each polypeptide chain is referred to as a subunit. Proteins with quaternary structure may consist of more than one of the same type of protein subunit.
- They may also be composed of different subunits.
- Haemoglobin is an example of a protein with quaternary structure.

Molar Abundance of Proteins:

- Plasma proteins are measured by protein mass (mg/L) and molar abundance (mmol/L).
- Albumin is ranked at the top in molecular abundance*.*

Biosynthesis of Proteins[3-5]:

A brief account of protein biosynthesis is given below and in Chapter 15 of this book.

DNA codes

- For biosynthesis of a protein (chain of upto 20 amino acids) one gene is required
- *"Gene"* is a part of DNA molecule that codes for the synthesis of one specific protein (polypeptide chain) through its sequence of nucleotides.
- Nucleotides of DNA are four i.e. *Thymine, Cytosine, Guanine and Adenine*

Example of DNA codes

T-A-C-A-A-G-C-A-G-T-T-G-G-T-C-G-T-G

Messenger RNA

- Through transcription DNA code is copied into a single stranded '*messenger RNA* (mRNA)'
- mRNA coded is exactly the same as DNA code except for "*Thymine"* which is replaced by "*Uracil"*

Codon

- Codon is part of mRNA, which comprises ***three*** nucleotides

- Each Codon has three positions:
- First position (5\ end)
- Second position (middle)
- Third position (3\ end)

For example, in codon *GAC*, *G* is at 5\ end, *A* at 2nd position while *C* is at 3\ end

- Four nucleotides of mRNA make 64 codons
- Why?

The Genetic Code

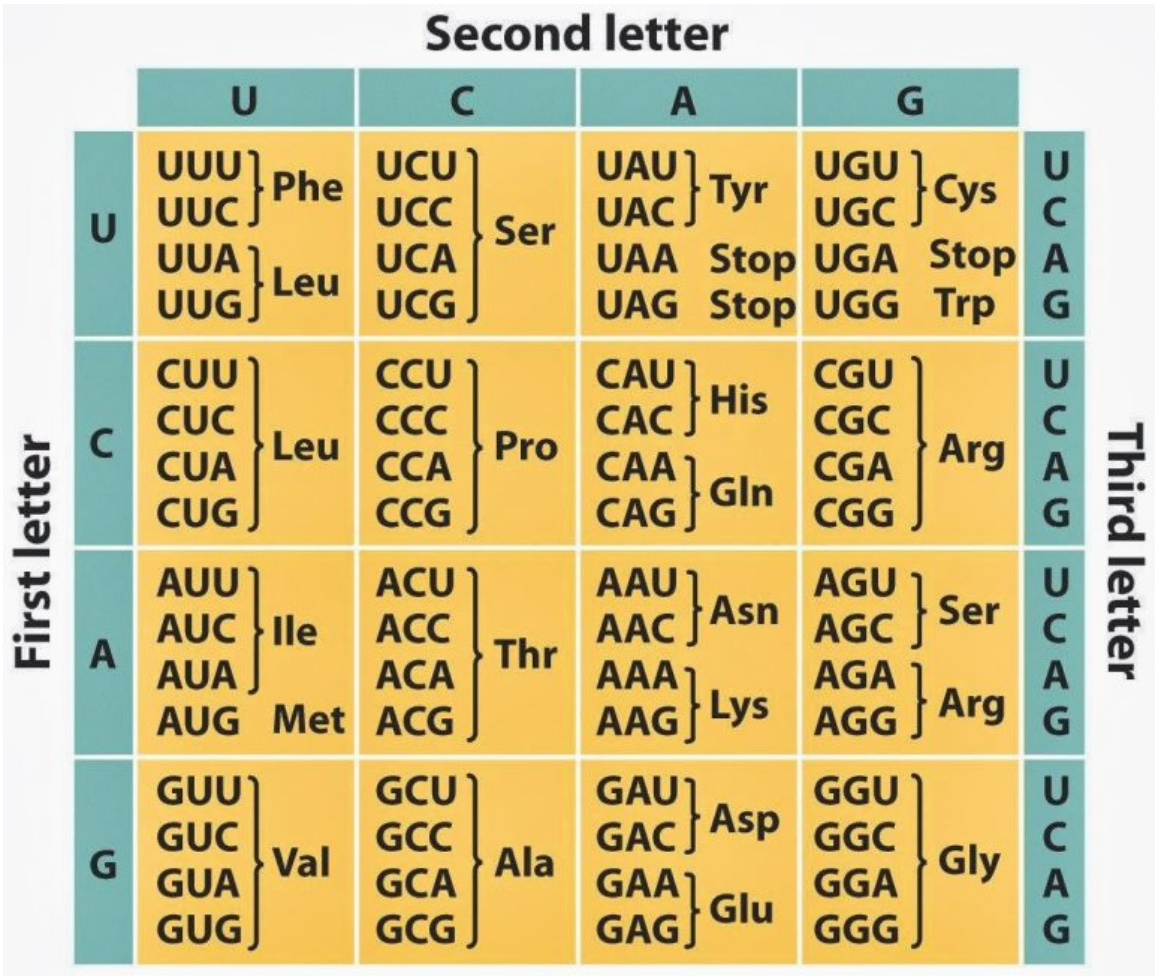

Figure 3.3: the codons as seen in RNA and DNA (U is a T in DNA).

Adapted from: https://sciencetrends.com/codon-chart-table-the-nucleotides-within-dna-and-rna/

64 Codons

- The order of nucleotides in the triplet (i.e. codon) is important e.g.
    - U-U-U codes Phenylalanine
    - U-C-U codes Serine
    - U-A-A codes Tyrosine
    - U-G-G codes Tryptophan
- So, 16 codons will result with *uracil* at First Position.
- Another 16 with *cytosine* at First Position and so on….
- In this way, all four nucleotides will result into 64 codons
- Please note that one amino acid may be coded by one, two, three, four, five or six codons

Why 61 Codons for 20 Amino Acids

- Three codons do not code any amino acids as they are stop codons (Figure 3.3)
- That is why we have 61 codons for 20 amino acids!!!
- *Ribosomal RNA* (rRNA) molecules diffuse from the nucleus to the cytoplasm and into the ribosomes, where it plays an essential role in the protein synthesis
- The amino acids are carried on small RNA molecules called *transfer RNA* (tRNA). Each of these tRNA molecules contains an *anticodon* consisting of 3 unpaired nucleotides
- Every *anticodon* or *base triplet* recognizes its complementary codon in the mRNA. The tRNA deposits its amino acid in the peptide chain. (Translation)

Post-Translational Modifications

- Following ribosomal translation, proteins are modified by addition of carbohydrates, cleavage of bonds within the new protein, shortening or folding. These processes are called *post-translational modification*

Essential Amino Acids[6]

- An essential amino acid is an amino acid that cannot be synthesized by the organism and therefore must be provided in diet.
- Essential amino acids are "essential" not because they are more important, but because the body does not synthesize them.
- If they are not taken through diet, they will not be available for protein synthesis.

Names of Essential Amino Acids

- Phenylalanine
- Valine
- Threonine
- Tryptophan
- Methionine
- Leucine
- Isoleucine
- Lysine
- Histidine

Non-essential Amino Acids

- Alanine
- Arginine*
- Aspartic acid
- Cysteine*
- Glutamic acid
- Glutamine*
- Glycine*
- Proline*
- Serine*
- Tyrosine*
- Asparagine*
- Selenocysteine

*These are conditionally essential amino acids

Conditionally Essential Amino Acids[7]

- The amino acids arginine, cysteine, glycine, glutamine, proline, serine and tyrosine are considered conditionally essential, meaning they are not normally required in the diet, but must be added exogenously in diet of specific populations that do not synthesize them in adequate amounts

Examples:

- Tyrosine: Individuals living with phenylketonuria (PKU) cannot synthesize tyrosine from phenylalanine, so tyrosine becomes essential in the diet of PKU patients.

- Cysteine: L-cysteine is an important protein and a deficiency could cause serious health problems. It helps prevent major organ damage that can be caused by taking acetaminophen.

- In certain dietary supplement form, L-cysteine counteracts with mild pain reliever and its effects on the liver

Alpha-1 Antitrypsin (AAT) Deficiency[8]

- Emphysema of lung is the most common manifestation of AAT deficiency which results due to uninhibited action of elastase in the lung.
- Being member of the Serine Protease Inhibitors (SERPIN) family, it can also present with other manifestations

Acute Phase Reactants (APRs)[9]

Classification of APRs[10]

Positive Acute Phase Reactants

- APRs are defined as proteins which increase by at least 25% during inflammatory state.
- These are synthesized by liver under the influence of cytokines released from inflamed or damaged tissue.

Negative Acute Phase Reactants

- Synthesis of these APR decreases during inflammation (Table 3.1)

Fast Reacting

- Concentration of some proteins rise as early as 4 hours after inflammatory stimulus and attain their maximum levels within 24 to 72 hours and decline very rapidly.
- Examples are Procalcitonin, C reactive protein (CRP), Serum Amyloid A (SAA).

Slow Reacting

- Some proteins begin to increase 24 to 48 hours and reach to their maximum level in about 7 to 10 days and require about two weeks to return to their normal levels.
- Examples are AGP (Alpha 1-acid glycoprotein), PI (Alpha 1-protease inhibitor), ACT (Alpha 1 antichymotrypsin), Hp (Haptoglobin)

Types of APRs according to the involved cytokines

These types are given in Table 3.2

Table 3.1: Positive and Negative APRs

	Positive APRs	Negative APRs
1	Positive APRs are the proteins whose hepatic production is increased to minimize the tissue damage while enhancing the repair process	Negative APRs are those proteins in which synthesis is decreased by liver due to production of many positive APRs
2	These APRs perform various functions like scavenging hemoglobin or other oxidants, activation of complement, destruction or inhibition of microbial growth, protease inhibition, inhibition of inflammatory response etc. •	May serve to divert the protein synthetic machinery of the liver away from the production of relatively non-essential proteins
3	Examples of positive APRs are CRP, haptoglobin, C3, C4, C9, alpha 1 antitrypsin and acid glycoprotein.	Examples are albumin, transthyretin, retinol binding protein and transferrin.

Table 3.2: Types of APRs According to the Involved Cytokines.

Type	Examples	Regulation
Positive APRs Type-I	Serum amyloid A C-reactive protein (CRP) α1-acid glycoprotein Complement component C3	IL-1 type cytokines: IL-1 α and β TNFα and β Potentiated by: IL-6 type cytokines, Glucocorticoids
Type II	Fibrinogen Haptoglobin Hemopexin α1-Antichymotrypsin α1-Antitrypsin A2-Macroglobulin Ceruloplasmin	IL-6 type cytokines: IL-6, IL-11, LIF, OSM, CNTF Potentiated by: Glucocorticoids
Negative APRs	(pre-) albumin Transferrin	IL-1 and IL-6 type cytokines

Multiple Sclerosis (MS)[11]

- A neurological deficit which lasts for < 24 h is called clinically isolated syndrome (CIS) while in MS the deficit(s) may last for > 24 h.
- Most important biochemical finding in MS is demonstration of local synthesis of oligoclonal band (OCB) in CSF.
- Detection of OCB in CSF by Isoelectric Focusing is the gold standard in lab diagnosis of MS.

Serum (to) Ascites Albumin Gradient (SAAG)[12]

How it is calculated?

- SAAG = Serum Albumin – Ascitic Fluid Albumin
- Low and High Grade Ascites are based on SAAG.

SAAG > 11 g/L

- High Grade Ascites

SAAG < 11 g/L

- Low Grade Ascites

Causes of High Grade Ascites

Portal Hypertension leads to fluid accumulation in peritoneum.

- Spontaneous Bacterial Peritonitis
- Liver Cirrhosis
- Alcoholic Hepatitis
- Congestive Heart Failure

Causes of Low Grade Ascites

Protein leaks into peritoneum and fluid follows

- Peritoneal Tuberculosis
- Peritoneal Carcinomatosis
- Pancreatitis
- Nephrotic Syndrome

*S*erum: *CSF Albumin Index*[13]

- This index can be used as a supportive evidence of blood brain barrier status.

How it is calculated?

- CSF Albumin (g/L) / Serum Albumin (g/L) x 1000

Interpretation of Serum: *CSF Albumin Index*

- Index < 9 ----------Intact Blood Brain Barrier
- Index 9 – 14 ---------Slight Impairment
- Index 14 - 30 ---------Moderate Impairment
- Index > 30 ---------Severe Impairment

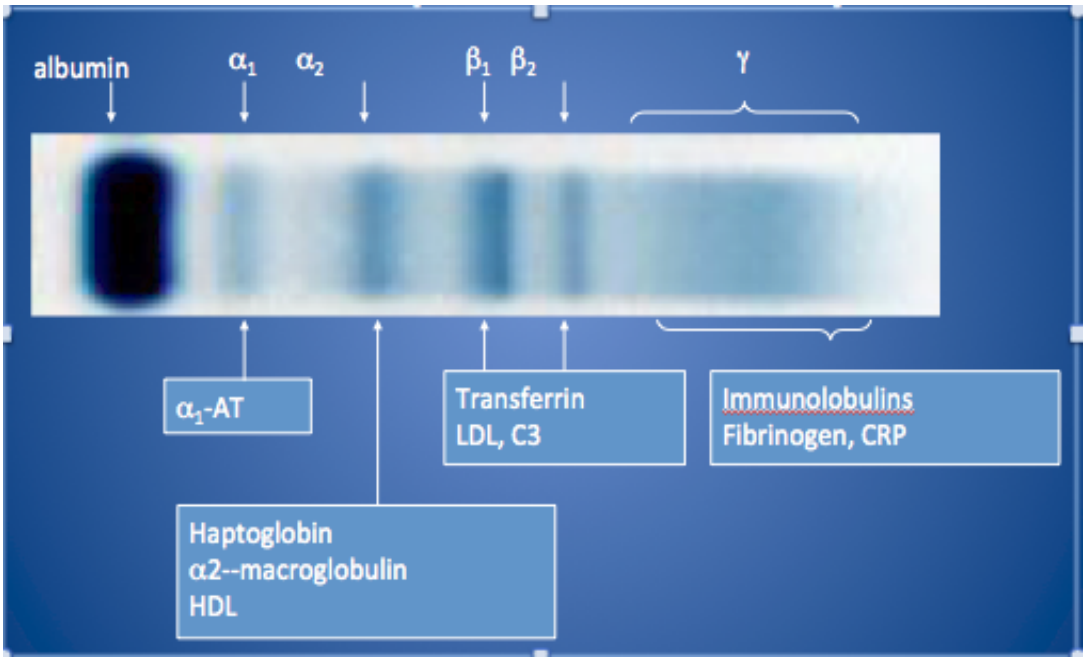

Figure 3.4: Plasma Proteins - Electrophoretic Mobility

(Photograph Courtesy of Dr Ayesha Hafeez)

Chapter No 3
Plasma Proteins

MCQs Key with Explanation

MCQ No	Best Option	Explanation
31.	b. Fifty Amino Acids	Chain of amino acids with shorter length are called monopeptide, dipeptide, decapeptide or polypeptide according to the number of amino acids. Chain of 50 or more amino acids is called protein.
32.	c. Quaternary	Please see details of these structures (Structural Hierarchy of Proteins) in the text.
33.	d. Secondary	Please see details of these structures (Structural Hierarchy of Proteins) in the text.
34.	b. Sixty-One Codons	Four RNA nucleotides i.e. adenine, cytosine, guanine and uracil can make 64 codons comprising three nucleotides e.g. ACG, ACC, ACU etc. Out of these 64 codons 3 are stop codons while 61 codons code 20 amino acids. Another important point to note is that there are more than one codons for each amino acid.
35.	a. Albumin	Concept of relative abundance relates to the size of the protein molecules. Albumin being a small protein has high molecular abundance. In routine clinical practice, however, proteins are measured by their mass i.e. gram per litre (SI Units) or gram per decilitre (traditional units).
36.	b. Cysteine	This is conditionally essential amino acid meaning liver cannot synthesize it if there is liver disease, so it is required in diet in hepatic disease.
37.	e. Pro-calcitonin	Pro-calcitonin is one of the fastest acute phase reactants along with C reactive proteins and

		Serum Amyloid A.
38.	c. Multiple Sclerosis (MS)	In MS, a polypeptide band, called oligo clonal band appears in CSF. Absence of this band in serum is essential for the diagnosis of MS. Isoelectric Focusing is an electrophoretic technique is used for the detection of this band
39.	a. Liver Cirrhosis	Serum Ascitic Fluid Albumin Gradient (SAAG) is calculated by subtracting ascitic fluid albumin from serum albumin. SAAG > 11 g/L (SI Units) or 1.1 g/dl (Traditional Units) is an indicator of transudative fluid that is diagnostic of portal hypertension, which is a complication of liver cirrhosis.
40.	b. Moderate impairment of blood CSF barrier	In this patient, the index is 21.9, indicating a moderate leakage in the blood brain barrier (Please see text)

Summary

Chapter No 3
Plasma Proteins

- Plasma proteins are built from 20 different amino acids, minimum 50 amino acids constitute one protein.
- Proteins have a well-defined hierarchy of structure i.e. primary, secondary, tertiary and quaternary strutures.
- For formation of proteins, DNA code is required, which is in fact a sequence of four types of nucleotides just like the alphabetical passwords generated by computerized systems. The difference is that instead of 26 alphabets, the genetic code is to be created from four nucleotides.
- DNA code then makes messenger RNA (mRNA), which has the same sequence as in the DNA, except that Thymine is replace by Uracil.
- For each amino acid, there are more than one mRNA 'codons'.
- There are 61 codons for 20 amino acids, while three codons are 'Stop Codons'.
- Some amino acids are not synthesized in the body but must be taken in diet. These are called 'essential amino acids', while some amino acids are 'conditionally essential' i.e. these should be taken during certain diseases e.g. tyrosine cannot be synthesized in a congenital condition called phenylketonuria.
- Plasms proteins has multiple functions e.g. they are Acute Phase Reactants (APRs) i.e. the synthesis of these proteins increases whenever there is attack of bacteria etc. from outside. Some APRs are secreted very early e.g. pro-calcitonin and C-Reactive proteins.
- Certain diseases are diagnosed by protein analyses in body fluids e.g. albumin in ascitic fluid or small peptide chains in CSF (for the diagnosis of multiple sclerosis)

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Chapter No 4

Water and Electrolytes Disorders

Reviewed and Edited by:

Majid Latif, Irum Noor and Sibgha Bashir

MCQs

(Please find key at the end of the chapter)

41. A 34 years old male was hospitalized for investigation of fever. He was advised blood counts and biochemical profile. Results of some of his biochemical tests were:
    - Na: 136 mmol/L (132-144)
    - K: 8.7 mmol/L (3.5 - 5.0)
    - Total Calcium: 0.41 mmol/L (2.10-2.55)
    - Urea: 9.2 mmol/L (3.5 – 6.3)

    The patient is not that sick as indicated in the lab results. Which one of the following is the most likely cause of this clinical discrepancy?
    a. EDTA contamination
    b. Haemolysed sample
    c. Prolonged use of tourniquet
    d. Sample collected in lithium heparin tube
    e. Sample taken in plain tube

42. In a clinical audit, it has been found that no case of ***hypokalaemia*** has been reported from your lab while the percentage of patients with hyperkalaemia, and sodium abnormalities is quite reasonable. All your quality checks are within acceptable range. What could be the most likely cause of this anomaly?
    a. A fault in ISE equipment
    b. Delay in separation of serum
    c. Use of expired reagents
    d. Use of liquid heparin causing dilution
    e. Use of collection tubes containing sodium heparin

43. A 48 years old male is a known patient of chronic kidney disease and is on haemodialysis. His serum electrolyte report showed following results:
    - Serum Sodium: 128 mmol/L
    - Serum Potassium: 6.8 mmol/L

    He is prone to which of the following complications?
    a. Cardiac arrhythmias
    b. Cardiac tamponade

c. Myocardial infarction
d. Paralytic ileus
e. Tetany

44. Dr Shakir is carrying out a research project to see the effects of Ramadan Fasting in summer in a rural area of Lower Sindh. His subjects are disease free adults. He has measured several biochemical parameters of his subjects just before iftar. Which one of the following will be the most probable finding?
    a. Decreased ADH
    b. High serum osmolality
    c. High urine osmolality
    d. Low aldosterone
    e. Low haematocrit

45. A 68 years old male is admitted in an Intensive Care Unit with cough and haemoptysis. His chest X-Ray showed an opacity. His investigations revealed:
    - Serum Sodium 112 mmol/L (136-149)
    - Serum Potassium 4.7 mmol/L (3.5-5.0)
    - Imaging studies of the chest: Opacity in the left lung.

    What is the most probable diagnosis?
    a. Excessive water intake
    b. Gastrointestinal loss of sodium
    c. Hypothalamic infarction
    d. Pituitary necrosis
    e. SIADH

46. A 33 years old female is having polyuria (3.5 litres of urine /day). She is not a known patient of diabetes mellitus. She has been referred to you for carrying out 'water deprivation test' to rule out diabetes insipidus.

    Which of the following is the most important parameter to be tested in blood and urine?
    a. Estimation of Aldosterone
    b. Estimation of BNP
    c. Renal function tests
    d. Serum and urine electrolytes
    e. Serum and urine osmolality

47. A 1 year old child has following biochemical profile:
    - Na: 138 mmol/L (132-144)
    - K: 2.6 mmol/L (3.5 – 5.0)
    - Urinary K: Raised

- Urinary Ca: 980 mmol/mol of Creat (565)
- Plasma Aldosterone: 567 ng/dl (5-90)
- Plasma Renin Activity: 15.5 ng/ml/h (0.2-1.6)
- ABGs results indicate: Metabolic Alkalosis

What is the most probable diagnosis in this case?

a. Addison Disease
b. Barter Syndrome
c. Gitelman Syndrome
d. Primary Hyperaldosteronism
e. Renal Tubular Acidosis

48. A 62 years old male has been brought in emergency department with persistent vomiting. Some of his biochemical results are:
    - Na: 128 mmol/L (132-144)
    - K: 2.4 mmol/L (3.5 – 5.0)
    - Chloride: 72 mmol/L (98 – 108)
    - ABGs results indicate: Metabolic Alkalosis

    What is the most probable diagnosis in this case?

    a. Acetazolamide therapy
    b. Alkali administration
    c. Gastric outlet obstruction
    d. Respiratory failure
    e. Uretero-Ileal anastomosis

49. A 2 years old child is admitted in Paediatric Ward with following biochemical profile:
    - pH: 7.31 (7.35 – 7.45)
    - HCO_3: 14 mmol/L (23 – 28)
    - BE: -6.3 <+3- -3
    - PO_2: 108 mmHg (80 – 110)
    - PCO_2: 39 mmHg (35-45)
    - Na: 136 mmol/L (135–150)
    - K: 2.5 mmol/L (3.5 – 5.0)
    - Chloride: 112 mmol/L (98-108)
    - Urea: 7.2 mmol/L (3.3 – 6.6)
    - Anion Gap: 12 mmol/L (7 – 17)
    - Urine K: 9.1 mmol/L
    - Urine K: Creatinine ratio: 0.91 (<1.5)

    What is the most probable diagnosis in this case?

    a. Dehydration
    b. Diarrhoea
    c. Lactic Acidosis
    d. Renal Impairment
    e. Renal Tubular Acidosis

50. A 55 years old male, presented in semi-conscious state. His biochemical profile is as following:

• pH:	7.38		(7.35 – 7.45)
• HCO_3:	24	mmol/L	(23 – 28)
• BE:	2.3		(<+3- -3)
• PO_2:	102	mmHg	(80 – 110)
• PCO_2:	39	mmHg	(35-45)
• Na:	153	mmol/L	(135–150)
• K:	4.5	mmol/L	(3.5 – 5.0)
• Chloride:	118	mmol/L	(98-108)
• Urea:	15.2	mmol/L	(3.3 – 6.6)
• Osmolality:	347	mmol/Kg	(275 – 295)
• Glucose (R):	39	mmol/L	(3.5 – 11.1)

Urine:

- Ketones: Trace

What is the most likely diagnosis?

a. Accidental ingestion of sea water
b. Dehydration due to severe diarrhoea
c. Diabetic ketoacidosis
d. Hyperosmolar Non-Ketotic (HONK) state
e. Primary hyperaldosteronism

Chapter No 4

Water and Electrolytes Disorders

Disorders of Potassium

Causes of Hyperkalaemia[1]

- Chronic kidney disease
- Acute kidney injury
- Potassium sparing diuretics
- Massive blood transfusion
- Transfusion of poorly stored blood
- Mineralocorticoid deficiency
- Increased intake / parenteral
- Diabetic ketoacidosis
- Other metabolic acidosis

Causes of Pseudo-Hyperkalaemia[2,3] (Masking of Hypokalaemia)

- Haemolysis
- Leukaemia / Leukaemoid reaction
- Contamination by K-EDTA salt
- Delay in sample separation

EDTA contamination[4]

Transfer of EDTA crystals by tip of syringe from one sample tube to another

Types of EDTA

- Gross Contamination
- Subtle Contamination

Signs of Gross Contamination

- False hypocalcaemia
- False hyperkalaemia
- Normal renal functions
- No haemolysis (Normal: LD)

Subtle EDTA Contamination

- Very difficult to diagnose in individual cases

- Should be prevented collectively
- Very important in any lab setting

How to prevent EDTA Contamination ?

- Correct filling order
- Estimation of EDTA in each sample
- Single sample tube

Correct Filling Order

- 1st : Blood C/S
- 2nd :Coagulation Profile
- 3rd : Lithium Heparin
- 4th : Plain tube
- 5th: Blood CBC (EDTA tube)
- 6th: Glucose Tube

Water Homeostasis

Question: Which gender has greater body fluids as percentage of the total body mass?

Answer: Males

Action of Anti-Diuretic Hormone (ADH) on Water Homeostasis[5]

A Dialogue:

- A patient has lost large body fluids because of burns.
- His plasma osmolality will tend to?
- Increase
- What reactionary change in ADH will occur?
- Increased ADH secretion!!!
- Leading to?
- Increased water reabsorption in kidney
- ….and what change in Blood Osmolality?
- Decreased
- ……and in blood volume
- Increased
- Change in urine osmolality?
- Increased

Excessive Secretion of ADH[6,7]

- CNS disorders like stroke, infection, trauma, psychosis
- Ectopic production of ADH may be due to small cell carcinoma of the lung
- Less common causes include head neck cancers.

Biochemical Features of SIADH

- Hyponatremia and low plasma osmolality
- Normal potassium
- Normal (or near normal) acid-base status
- Urine osmolality > 100 mOsmol/Kg
- Low serum uric acid

Osmolality of Blood[8]

- Osmolality of blood is controlled by osmo-receptors
- Osmolality of blood is maintained by variations in urinary osmolality

Osmolality Gap

- *Osmolality gap = Measured Osmolality – Calculated Osmolality*
- It is increased in acute alcoholic poisoning

Question:

A 36 years old male was brought to Nishtar Hospital Multan at about 8 a.m. from a nearby village. The relatives narrate that the patient is having diarrhoea and vomiting since 2 am in the morning and that they have given him about 3 bottles of mineral water since then. The patient is now in semi-comatose state. His electrolyte profile carried out on arrival was as following:

Serum Na: 121 mmol/L
Serum K: 3.1 mmol/L

a. What is the most probable diagnosis in this patient?
b. What is the most probable cause of altered consciousness in this patient?

Answer:

a. Patient is suffering from “Water Intoxication’.
b. Mineral water is hypotonic / hypo-osmolar water which has led to dilution of patient‘s ECF compartment. Intravascular hyponatremia causes water to move from vascular compartment into intracellular space to maintain osmotic balance thus resulting in swelling of nerve cells in CNS. The patient may present with CNS symptoms ranging from irritability and mental confusion to severe mental impairment.

Water Intoxication (WI)[9]

- How WI occurs?
- Excessive blood loss replaced with plain water
- What will happen to electrolyte concentration in blood?
- Hyponatraemia
- What will happen to blood osmolality?
- Decreased blood osmolality
- What will be the consequences?
- Water will move from vessels to the interstitial fluid and then into the cells
- What will happen to the cell?
- Cell will swell
- What can be outcomes in severe cases?
- Convulsion, coma and death

Barter and Gitelman Syndromes[10-11]

- Autosomal recessive disorders
- Hyperplasia of the juxtaglomerular apparatus
- Secondary hyperaldosteronism and hyperreninism
- Metabolic alkalosis
- Hypokalaemia
- Increased urinary potassium
- Hypomagnesemia (in some patients)
- Difference between the two disorders are given in Table 4.1.

Table 4.1: Difference Between Barter`s Syndrome Gitelman syndrome

Barter`s Syndrome	Gitelman syndrome
Infant and Young Children	Adults (sometimes late childhood)
Growth and mental retardation	Polyuria and polydipsia alongwith fatigue and weakness
Urinary Calcium Excretion: Increased	Urinary Calcium Excretion: Low or Normal
Mimics Loop Diuretic action	Mimics Thiazide Diuretic action
1 in 1,000,000 (less common)	1 in 40,000 (Much more common)
Impairment in one of the transporters involved in sodium chloride reabsorption in the loop of Henle	Impairment in one of the transporters involved in sodium chloride reabsorption in the distal tubule
Blunted response to a loop diuretic	Blunted response to a thiazide diuretic
Urine concentrating ability is impaired	Urine concentrating ability relatively preserved
Urinary PGE2 excretion increased: so NSAIDS are important part of treatment alongwith K sparing diuretics	Urinary PGE2 excretion appears to be normal: so NSAIDS are of no use and K sparing diuretics are the only treatment

Renin Angiotensin Aldosterone System (RAAS)[12-13]

- A patient has hypotension due to severe diarrhoea (cholera?).
- How will kidney react?
- Increased renin secretion
- What renin will do?
- Convert angiotensinogen to angiotensin I and angiotensin II?
- What will angiotensin II do?
- Angiotensin II will cause increased secretion of aldosterone?
- What aldosterone will do?
- Increased Na reabsorption!!!
- Water will follow Na
- Blood pressure (BP) will become normal

Atrial Natriuretic Peptide (ANP) in Action[14]

- A patient has severe haemorrhage.
- His BP will tend to?
- Decrease
- What reactionary change in ANP will occur?
- Decreased ANP Secretion!!!
- Leading to?
- Decreased water and Na loss in urine
- ….and what change in BP?
- Increased
- ……and in blood volume
- Increased

Chapter No 4

Water and Electrolytes Disorders

MCQs with Key and Explanation

MCQ No	Best Option	Explanation
41.	a. EDTA Contamination	Pseudo-hyperkalaemia is a common condition with many causes. EDTA contamination is characterized by hyperkalaemia and low calcium levels. Haemolysis, another common cause of pseudo-hyperkalaemia, can be ruled out by physical examination of the sample or by carrying out serum LD levels.
42.	b. Delay in separation of serum	Delay in separation of plasma or serum can lead to leakage of potassium from RBCs, as Na-K ATPase pump gets weaker with passage of time and concentration gradient across RBCs membrane cannot be maintained. It is important for the clinical laboratories to collect sample in Lithium Heparin (green top) tube and separate plasma as early as possible for analysis.
43.	a. Cardiac arrhythmias	Hyperkalaemia is a nightmare for the treating physician as it can cause cardiac arrhythmias and instant death of the patient. So, laboratories should be very careful in accurate and precise testing of potassium. Key to good analysis of potassium is proper collection of specimen (as mentioned in MCQ No 42 above).
44.	c. High urine osmolality	Blood osmolality is tightly controlled in a narrow range (280-295 mOsmol/L) by the mechanism involving osmoreceptors, ADH and ADH receptors in the collecting ducts of kidneys. This tight control is carried out at the expense of urine osmolality. So, urine osmolality has wide interval (approximately 50 – 1200 mOsmol/L). In conditions of water deficit (e.g. fasting in summers) water is conserved and urine osmolality is increased i.e. a very concentrated urine is passed.

45.	e. SIADH	Syndrome of Inappropriate Diuretic Hormone (SIADH) is due to excessive secretion of ADH. The condition can co-exist with other diseases e.g. cerebrovascular accident and malignancies. In this patient, the source of ADH is most probably carcinoma lung that is notorious for ectopic ADH secretion as a para-neoplastic syndrome. Excessive ADH leads to retention of water and dilutional hyponatraemia.
46.	e. Serum and urine osmolality	Diabetes insipidus (DI) is disease of ADH, either its deficiency (Central DI) or impairment of its action in collecting ducts of kidneys (Peripheral DI). Polyuria (urine output > 2.5 litres per day) is the most important symptom of DI. In such a patient, diagnosis of DI and differentiation into central DI and peripheral DI is carried out by Water Deprivation Test. Patient is deprived of fluids and food for 8 hours with periodic measurement of urine and serum osmolality (Details can be found in 'Advanced Chemical Pathology' by the same author).
47.	b. Barter Syndrome	Barter syndrome is mainly a childhood disease, affecting sodium chloride transporter of loop of Henle in the kidney. Patient may present with short stature but hypokalaemia and resultant muscle paralysis is the key clinical abnormality. Increase in renin and aldosterone are confirmatory biochemical findings. Stimulus for increased renin and aldosterone is initial hyponatraemia, which in turn brings sodium to normal but at the expense of potassium. So, increased loss of potassium in urine results in hypokalaemia. The adult version of this disease is Gitelman Syndrome (Please see text for differences).
48.	c. Gastric Outlet Obstruction	In vomiting due to gastric outlet obstruction, HCL is lost in greater quantities. So, it is characterized by metabolic alkalosis (due to loss of H^+) and hypochloraemia (due to loss of Cl^-).
49.	b. Diarrhoea	Intestinal fluid is rich in HCO_3^- and K^+. So, loss of these two ions leads to metabolic acidosis and hypokalaemia. In fact, in our set-up diarrhoea is the most common cause of hypokalaemic metabolic acidosis, followed by Renal Tubular Acidosis (RTA). These two conditions can be distinguished by a simple urine test i.e. Potassium Creatinine ratio (K:

		Creat). In diarrhoea K:Creat is < 1.5 while in RTA it is > 1.5.
50.	d. Hyperosmolar Non-ketotic (HONK) state	Very high glucose and blood osmolality is diagnostic of HONK. Please note that osmolality gap is normal as measured osmolality is high as well as calculated osmolality, both sodium and glucose are included in the calculation of osmolality. Sodium is high in this condition due to dehydration.

Summary

Chapter No 4

Water and Electrolytes Disorders

- Serum sodium and serum potassium are the two common electrolytes measured in Clinical Laboratories these days.
- Pseudo-hyperkalaemia should be ruled out by examining the patient and the specimen.
- Increase in serum potassium can be dangerous as it can lead to immediate death due to cardiac arrhythmias
- Osmolality is a very important parameter, its lion share is from sodium (45-50%), so any changes in sodium can cause changes in osmolality.
- Osmolality can be measured directly by an instrument called 'Osmometer' or can be calculated using a formula. The difference between the two is called 'Osmolality Gap'. Widening of osmolality gap is found in 'Alcohol Toxicity' and some other conditions.
- Blood osmolality is maintained in a narrow range while urine osmolality is in wide range.
- Hyponatraemia in a patient with cerebral vascular accident can be due to Syndrome of Inappropriate Anti-Diuretic Hormone (SIADH).
- Barter Syndrome should be suspected if hypokalaemia is found with metabolic alkalosis.
- Hypokalaemia with metabolic acidosis is commonly found in diarrhoea and renal tubular acidosis.
- Metabolic alkalosis and hypochloraemia can be found in upper gastro-intestinal vomiting.

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Chapter No 5
Acid Base Disorders

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MCQs

(Please find key at the end of the chapter)

51. ***Buffer*** is a combination of:
    a. Strong acid and strong base
    b. Strong acid and conjugate base
    c. Weak acid and its conjugate base
    d. Weak acid and strong base
    e. Weak acid and weak base

52. Most abundant ***body buffer*** is:
    a. Ammonium compounds
    b. Bicarbonate buffer system
    c. Haemoglobin
    d. Phosphates
    e. Proteins

53. ***Acidosis*** is defined as:
    a. Decreased HCO_3 in the blood
    b. High blood hydrogen ion concentration
    c. Increased CO_2 in the blood
    d. Low blood pH
    e. Tendency towards decreased pH

54. ***Henderson-Hasselbalch equation*** is most commonly used for the determination of which of the following Arterial Blood Gases (ABG) parameters:
    a. Bicarbonate
    b. H ion concentration
    c. Haematocrit
    d. PCO_2
    e. PO_2

55. ***Anion Gap*** is present in the body due to:
    a. Increased measured anions produced due to disease
    b. Increased measured anions produced during exercise
    c. Increased measured cations normally present in the blood
    d. Unmeasured anions normally present in the blood
    e. Unmeasured cations normally present in the blood

56. Which of the following ***Acid Base Disorder*** is further divided based on Anion Gap:
    a. Double Alkalosis
    b. Metabolic Acidosis
    c. Metabolic Alkalosis
    d. Respiratory Acidosis
    e. Respiratory Alkalosis

57. Which one of the following conditions leads to ***hyperkalaemic Metabolic Acidosis***?
    a. Acetazolamide therapy
    b. Lactic Acidosis
    c. Renal tubular acidosis Type 1
    d. Renal tubular acidosis Type 2
    e. Uretero-illeal anastomosis

58. ***Best site*** for an ABG sample collection is:
    a. Brachial artery
    b. Dorsal pedal artery
    c. External carotid artery
    d. Femoral artery
    e. Radial artery

59. In the ***composite systems*** of analyses ABGs are measured most commonly with which of the following analytes*:*
    a. Cardiac enzymes
    b. Electrolytes
    c. Haemoglobins
    d. Liver function tests
    e. Renal function tests

60. Blood sample for a composite analysis should be collected in which of the following ***containers***:
    a. PET tube rinsed with lithium heparin
    b. PET tube rinsed with sodium heparin
    c. Syringe with lyophilized lithium heparin
    d. Syringe without anticoagulant
    e. Vacutainer with clot activator gel

61. A 32 years old male has Chronic Kidney Disease (CKD) requiring haemodialysis. His ABG values are:
    - pH 7.30 (7.35-7.45)
    - Base Excess -5.0 (<+3- -3)
    - PCO_2 29.2 mmHg (35-45)
    - PO_2 98 mmHg (80-100)
    - Bicarbonate actual: 15.3 mmol/L (22-28)
    - Oxygen saturation: 96.0% (95-98%)

What is your Biochemical Diagnosis?

    a. Metabolic acidosis with partial respiratory compensation
    b. Mixed metabolic alkalosis and respiratory acidosis
    c. Mixed respiratory alkalosis and metabolic acidosis
    d. Normal acid base status
    e. Respiratory acidosis with partial metabolic compensation

62. A 5 months old male baby is failing to thrive. His ABGs are:
    - pH 7.40 (7.35 -7.45)
    - Base Excess -8.4 (<+3- -3)
    - PCO_2 15.0 mmHg (35-45)
    - PO_2 99 mmHg (80-100)
    - Bicarbonate actual: 10.3 mmol/L (22-28)
    - Oxygen saturation: 99.5% (95-98 %)

What is your Biochemical Diagnosis?

    a. Metabolic alkalosis with partial respiratory compensation
    b. Mixed metabolic alkalosis and respiratory acidosis
    c. Mixed respiratory alkalosis and metabolic acidosis
    d. Normal acid base status
    e. Respiratory acidosis with partial metabolic compensation

63. A 50 years old male has gastrointestinal bleeding with nasogastric tube in place. His ABGs are:

• pH	7.52	(7.35-7.45)
• Base Excess	6.9	(<+3- -3)
• PCO_2	45.8 mmHg	(35-45)
• PO_2	52 mmHg	(80-100)
• Bicarbonate actual	32 mmol/L	(22-28)
• Oxygen saturation:	81.2%	(95-98%)

What is your Biochemical Diagnosis?

a. Metabolic alkalosis with partial respiratory compensation
b. Mixed metabolic alkalosis and respiratory acidosis
c. Mixed respiratory alkalosis and metabolic acidosis
d. Normal acid base status
e. Respiratory acidosis with partial metabolic compensation

64. A 22 years old male has multi-organ failure. His ABGs are:

• pH	7.22	(7.35-7.45)
• Base Excess	6.0	(<+3- -3)
• PCO_2	47.2 mmHg	(35-45)
• PO_2	102 mmHg	(80-100)
• Bicarbonate actual	19.8 mmol/L	(22-28)
• Oxygen saturation:	99.2%	(95-98%)

What is your Biochemical Diagnosis?

a. Metabolic acidosis with partial respiratory compensation
b. Mixed metabolic and respiratory acidosis
c. Mixed respiratory alkalosis and metabolic acidosis
d. Normal acid base status
e. Respiratory acidosis with partial metabolic compensation

65. A 4 weeks old female baby had septicaemia. Her ABGs are:

• pH	7.29	(7.35-7.45)
• Base Excess	-22.4	(<+3- -3)
• PCO_2	13.5 mmHg	(35-45)
• PO_2	94.9 mmHg	(80-100)
• Bicarbonate actual	4.3 mmol/L	(22-28)

- Oxygen saturation: 96.4% (95-98%)

What is your Biochemical Diagnosis?

a. Metabolic acidosis with partial respiratory compensation
b. Mixed metabolic alkalosis and respiratory acidosis
c. Mixed respiratory alkalosis and metabolic acidosis
d. Normal acid base status
e. Respiratory acidosis with partial metabolic compensation

66 An 18 years old female is having hysterical breathing. Her ABGs are:

- pH 7.48 (7.35-7.45)
- Base Excess -8.3 (<+3- -3)
- PCO_2 16.5 mmHg (35-45)
- PO_2 97.6 mmHg (80-100)
- Bicarbonate actual 18.1 mmol/L (22-28)
- Oxygen saturation: 98.9% (95-98%)

What is your Biochemical Diagnosis?

a. Metabolic alkalosis with partial respiratory compensation
b. Mixed metabolic alkalosis and respiratory acidosis
c. Mixed respiratory alkalosis and metabolic acidosis
d. Normal acid base status
e. Respiratory alkalosis with partial metabolic compensation

67 A 40 years old male is having vomiting with pneumonia. His ABGs are:

- pH 7.53 (7.35-7.45)
- Base Excess: 5.4 (<+3- -3)
- PCO_2 33.0 mmHg (35-45)
- PO_2 79.4 mmHg (80-100)
- Bicarbonate actual 29.0 mmol/L (22-28)
- Oxygen saturation arterial 95.7 % (95-98%)

What is your Biochemical Diagnosis?

a. Metabolic alkalosis with partial respiratory compensation
b. Mixed alkalosis respiratory and metabolic acidosis
c. Mixed respiratory and metabolic alkalosis
d. Normal acid base status
e. Respiratory alkalosis with partial metabolic compensation

68. A 27 years old male is admitted in HDU for observations. His ABG are:

• pH	7.40	(7.35-7.45)
• Base Excess	0.5	(<+3- -3)
• PCO_2	38.2 mmHg	(35-45)
• PO_2	108 mmHg	(80-100)
• Bicarbonate actual	24.5 mmol/L	(22-28)
• Oxygen saturation	99.1%	(95-98%)

What is your Biochemical Diagnosis?

a. Metabolic acidosis with partial respiratory compensation
b. Metabolic alkalosis with partial respiratory compensation
c. Mixed respiratory acidosis and metabolic alkalosis
d. Normal acid base status
e. Respiratory acidosis with partial metabolic compensation

69. A 78 years old male is admitted with respiratory failure and vomiting. Nasogastric tube is in place. His ABGs are:

• pH	7.40	(7.35-7.45)
• Base Excess	21.8	(<+3- -3)
• PCO_2	80.2 mmHg	(35-45)
• PO_2	80.9 mmHg	(80-100)
• Bicarbonate actual	51.1 mmol/L	(22-28)
• Oxygen saturation arterial	95.1%	(95-98%)

What is your Biochemical Diagnosis?

a. Metabolic alkalosis with partial respiratory compensation
b. Mixed respiratory acidosis and metabolic alkalosis
c. Mixed respiratory alkalosis and metabolic acidosis
d. Normal acid base status
e. Respiratory acidosis with partial metabolic compensation

70. A 59 years old male is in severe respiratory distress. His ABGs are:

• pH	7.26	(7.35-7.45)
• Base Excess	-5.3	(<+3- -3)
• PCO_2	50.7 mmHg	(35-45)
• PO_2	115 mmHg	(80-100)
• Bicarbonate actual	30.1 mmol/L	(22-28)
• Oxygen saturation arterial	99.5%	(95-98%)

What is your Biochemical Diagnosis?

a. Metabolic acidosis with partial respiratory compensation
b. Mixed respiratory alkalosis and metabolic acidosis
c. Mixed respiratory and metabolic acidosis
d. Normal acid base status
e. Respiratory acidosis with partial metabolic compensation

Chapter No 5
Acid Base Disorders

Biochemical Brush-up[1]

Acid: A substance that dissociates in water producing hydrogen ion (H^+).
Base: A substance that can accept H^+.
Buffer: A mixture of weak acid and its conjugate base that attenuates a change in $\{H^+\}$ when a strong acid or base is added to it.
Acidosis: Tendency towards decreased pH i.e. increased {H+}
Alkalosis: Tendency towards increased pH i.e. decreased $\{H^+\}$
Acidaemia: An abnormally decreased pH or raised $\{H^+\}$.
Alkalaemia: An abnormally increased pH or low $\{H^+\}$.
pH: pH is negative log of hydrogen ion concentration. It is actually an indicator of the concentration of hydrogen ions $\{H^+\}$

Henderson–Hasselbalch equation[2]

- $pH = pK + \text{Log } \{HCO_3\} / \{H_2CO_3\}$
  Where pK is the overall equilibrium constant

For bicarbonate system pK is 6.1
or
$\text{Log } \{HCO_3\} / \{H_2CO_3\} = pH - pK$
or 1.3 = 7.40 - 6.1
Anti-log of 1.3 is 20 so:
$\{HCO_3\} / \{H_2CO_3\} = 20 = 7.4\text{-}6.1$

- To keep pH at 7.40 $\{HCO_3\}$ should be 20 times of $\{H_2CO_3\}$.
- An important implication is that H_2CO_3 remains in very low concentration because it constantly dissociates into CO_2 and water So, concentration of H_2CO_3 is directly proportional to Partial Pressure of CO_2 (PCO_2)
- Therefore, we can say: pH = 6.1 + HCO3 / PCO2

Bicarbonate Buffer System[3]

- A mixture of carbonic acid (H_2CO_3) and bicarbonate (HCO_3)
- The most abundant extracellular buffer system.
- Most rapid buffer system but not the most efficient system.

Changes in Bicarbonate Buffer System

a. **Respiratory Component:** PCO_2 is the respiratory component. High blood PCO_2 is respiratory acidosis and decreased PCO_2 is respiratory alkalosis.
b. **Metabolic Component:** Describes HCO_3. High blood HCO_3 is metabolic alkalosis and decreased HCO_3 is metabolic acidosis.

c. **Compensation:** The changes in one component of the buffer system in response to abnormality in the other system to bring the pH normal or near normal.

Bicarbonate (HCO3) in blood Gases[4-6]

a. **Actual HCO_3:** Calculated index (Not measured in any Blood Gas System)
b. **Standard HCO_3:** Calculated assuming PCO_2 is normal (40 mmHg)
c. **Measured HCO_3: (Total CO_2).** Measured in serum by extracting all the CO_2 present in it. Usually part of renal profile along with urea and electrolytes.
d. **Methods of Measurement of Total CO_2**
   i. **Photometric Method**: CO_2 is generated after adding acid and then measured by photometry.
   ii. **Electrochemical Method** using ISE Electrode

Use of Actual HCO3 and Standard HCO3[7]

a. If actual HCO_3 and standard HCO_3 are close to each other, it indicates absence of a respiratory disorder.
b. If actual HCO_3 is *lower* than standard HCO_3, then respiratory alkalosis (compensatory Decrease)
c. If actual HCO_3 is *Higher* than standard HCO_3, then respiratory acidosis (compensatory increase)

Base Excess (BE) [7,8]

- BE can be defined as the concentration of strong acid or base required to return the pH of an in vitro specimen of whole blood to 7.40 while maintaining PCO_2 at 40 mmHg at 37°C by equilibration.
- If plasma pH is >7.40, and PCO_2 is 40 mmHg, BE is the mmol/L of strong acid which brings the pH to 7.40 (while PCO_2 continues to be maintained at 40 mmHg), in such situations BE has a positive sign.
- If pH<7.40, BE is quantified by the strong base required (BE then has a negative sign). A negative BE is sometimes referred to as a “base deficit”.
- The reference values for BE: –3 mmol/L to +3 mmol/L.
- Before applying the BE rules, primary acid-base disturbances should be detected first by inspection of the pH and PCO_2.

Anion Gap[3,9]

a. Anion gap (AG) is a Laboratory Error!!!
b. In routine laboratories fewer anions are measured as compared to cations (HCO_3 and Chloride)
c. So, AG is a measure of anions other than HCO_3 and chloride. Otherwise always: *Cations = Anions*

Major Cations:

- Na = 142 mmol/L
- K = 4.5 mmol/L
- Ca = 2.5 mmol/L
- Mg = 1.0 mmol/L

Total Cations = 150 mmol/L

Major Anions:

- Cl = 100 mmol/L
- HCO_3 = 27 mmol/L
- proteins = 15 mmol/L
- PO_3 = 2 mmol/L
- SO_3 = 1 mmol/L
- Organic Acids = 5 mmol/L

Total Anions = 150 mmol/L

AG =$(Na^+ K^+)$ - $(Cl^- + HCO_3^-)$ or = Na^+ - $(Cl^- + HCO_3^-)$

Range : 7 – 18 mmol/L

AG corrected for low albumin (AG corr)

- Albumin accounts for about 90% of the mean value AG and albumin is reversely correlated with HCO3
- When the level of serum albumin decreases, AG will decrease equally
- AG is concealed by hypoalbuminemia
- HCO_3 will increase accordingly (low Alb alkalosis)
- Albumin: 40 (35-50) g/L×0.25* = 10 (9-13) mmol/L

* Coefficient for converting Alb g/L into Alb mmol/L.

Types of Acid Base Disorders[1, 8, 10]

- Primary acid base disturbance must be determined.
- Primary process is one that if unopposed will shift the pH out of the normal range, and can be either respiratory (PCO_2) or metabolic (HCO_3).
- There can be one, two or less commonly more than two primary processes operating at one time.
- A primary metabolic process should stimulate immediate respiratory compensation (in hours), and a primary respiratory process normally results in metabolic compensation by renal [HCO_3] adjustment (in hours to days).

- Compensation rarely returns the pH to normal except in the case of chronic respiratory alkalosis. With this exception, it can be stated that if only one of either PCO_2 or pH is normal, two primary processes must be operating simultaneously.

Single (Simple) Disorders

Metabolic Acidosis

- Two types of metabolic acidosis
  a. High Anion Gap acidosis
  b. Normal Anion Gap acidosis
- Primary Biochemical Abnormalities:
  a. Low pH
  b. Low HCO_3
- Most important Compensatory Change
  - *Decreased P*CO_2

High AG Metabolic Acidosis

Two distinguishing Biochemical Features?

a. Hyperkalaemia
b. Normal Cl

- In normal situations:
  - Na = HCO_3 + Cl + normal unmeasured anions
  - In High AG Met Acidosis:
  - Na = *Low* HCO3 + Cl + *Increased* unmeasured anions

Three Important Causes

a. Chronic Kidney Disease
b. Lactic Acidosis[11]
c. Diabetic Ketoacidosis

Example (Diabetic ketoacidosis):

- pH: 7.26
- Na 140 mmol/L
- K 6.0 "
- Cl 102 "
- HCO_3 15 "
- Anion Gap: 29 mmol/L (7 – 18)

Normal AG Metabolic Acidosis[12,13]

Two distinguishing Biochemical Features?

a. Hypokalaemia
b. High Cl

- Why Cl is high?
- In normal situations:
- Na = HCO3 + Cl + normal unmeasured anions
- In Normal AG Met Acidosis:
- Na = *Low* HCO3 + *High* Cl + *Normal* unmeasured anions

Three important causes?

a. Diarrhoea
b. Renal Tubular Acidosis
c. Acetazolamide therapy

Example (Severe Diarrhea):

pH: 7.30
Na: 134 mmol/L
K : 2.4 mmol/L
Cl : 118 mmol/L
HCO_3: 12 mmol/L
Anion Gap: 7 mmol/L (7 – 17)

Metabolic Alkalosis

Two Primary Biochemical Abnormalities:

a. High pH
b. High HCO_3

The most important Compensatory Change

- *Increased P*CO_2

Two important causes

a. Gastrointestinal loss of H^+
   i. Vomiting from upper GI
   ii. Gastric drainage
b. Loss in urine of {H+}
   i. Cushing syndrome
   ii. Hyperaldosteronism
   iii. Diuretic therapy
   iv. Ingestion of base

Respiratory Acidosis

Two Primary Biochemical Abnormalities:

a. Low pH
b. High PCO_2

What is the most important Compensatory Change:

High HCO_3

What are five important causes:

a. Respiratory failure
b. CNS lesions
c. Drug over-dosage
d. Bronchial obstruction
e. Inadequate mechanical ventilation

Respiratory Alkalosis

Two Primary Biochemical Abnormalities:

a. High pH
b. Low PCO_2

The most important Compensatory Change

Low HCO_3

One most important Respiratory abnormality leading to Respiratory Alkalosis:

Hyperventilation

Causes of Hyperventilation

a. Drugs e.g. salicylates
b. Anxiety / hysteria
c. Pregnancy
d. Septicaemia
e. Liver failure
f. Pneumonia
g. Asthma
h. CCF
i. Embolism

Mixed Disorders

- Both metabolic and respiratory abnormalities may be present in the same patient.
- Clinically more common than single disorders
- Examples:
  - Renal Failure with pneumonia i.e. metabolic acidosis and respiratory alkalosis.

- Patient with hypoxia with vomiting from upper GI i.e. respiratory acidosis and metabolic alkalosis.

Double Opposing Disorders

a. Normal pH (usually)
b. Grossly abnormal HCO_3 and PCO_2

- Examples:
  - Mixed Respiratory Acidosis and Metabolic Alkalosis
  - Mixed Respiratory Alkalosis and Metabolic Acidosis

Double Disorders

- Metabolic Acidosis + Respiratory Alkalosis
- Metabolic Alkalosis + Respiratory Acidosis
- Metabolic+ Respiratory Alkalosis
- Metabolic+ Respiratory Acidosis

Triple Disorders[9]

- Metabolic Acidosis and Metabolic Alkalosis with
- Respiratory Alkalosis or Respiratory Acidosis

Steps in Diagnosis of a Triple Disorder

a. Diagnose Double Disorder
b. Examine AG
c. Examine $[HCO_3]$

Example 1:

- Double Disorder: Low PCO_2+ High $[HCO_3]$ (i.e. Resp Alk + Met Alk)
- Triple Disorder: Low PCO_2+ High $[HCO_3]$ + AG > 16
  (i.e. Resp Alk + Met Alk+ High AG Met Acidosis)

a. Diagnose Double Disorder
b. Examine AG
c. Examine $[HCO_3]$

Example 2:

- Double Disorder: High PCO_2+ $[HCO_3]$ higher than the upper limit of compensation (i.e. Resp Acidosis + Met Alk)
- Triple Disorder: High PCO_2+ $[HCO_3]$ higher than the upper limit of compensation + AG > 16
  (i.e. Resp Acidosis + Met Alk + High AG Met Acidosis)

Other Situations of Triple Disorders

a. Combined High AG and Normal AG Met Acidosis in a patient with Resp Alkalosis
b. Low Albumin Met Alkalosis with Met Acidosis and Respiratory Alkalosis

Important Fact

Respiratory Acidosis and Respiratory Alkalosis never occur together in the same patient!!!

Delta Ratio

- The delta ratio is used for the determination of a mixed acid base disorder in an elevated anion gap metabolic acidosis

Measured anion gap – Normal anion gap / Normal [HCO_3-] – Measured [HCO_3-]

or

(anion gap – 12) / (24 - [HCO_3-])

Table 5.1: Delta Ratio Assessment Guideline

< 0.4	Hyperchloraemic normal anion gap acidosis
0.4 - 0.8	Renal failure Combined high AG & normal AG acidosis
1 to 2	Uncomplicated high-AG acidosis Lactic acidosis: 1.6 (average value)
>2	A pre-existing elevated HCO3 level due to: A concurrent metabolic alkalosis, or A pre-existing compensated respiratory acidosis

Predicted HCO_3

- Predicted [HCO_3] = 24 - [Anion Gap-12]

If patient's [HCO_3] > Predicted [HCO_3]

Metabolic acidosis + Metabolic Alkalosis

Compensatory response to respiratory acidosis

If patient's [HCO_3] =Predicted [HCO_3]

Simple Metabolic acidosis

If patient's [HCO_3] < Predicted [HCO_3]

Compensatory response to respiratory alkalosis

Blood Gas Analyses

Nevers and Always

(Ten Tips about Blood Gas Analyses)

It is said that *'never' and 'always'* should be avoided in medicine as nothing can be absolute in biology but we dare to use these terms in blood gas analyses (BGA). Here are some 'nevers' and 'always' of Blood Gas Analyses (BGA):

1. ***Never*** transfer blood samples collected for BGA to a sample tube but send the sample to the lab in the syringe used for arterial or venous puncture. Transferring the sample to tubes can mix air gases into the sample[14]
2. ***Always*** transport sample for BGA to the lab within 20 minutes and carry out the test in the lab on top priority[15,16].
3. ***Never*** use sodium heparin for rinsing the syringe used for sample collection and ***always*** use lithium heparin syringes for the collection of blood gas samples. If these syringes are not available then do not report sodium results, as it may be falsely high or falsely normal[14].
4. ***Never*** think that anion gap exists in any physiological or pathological conditions. It is a measure of unmeasured anions[1].
5. ***Never*** use HCO_3 calculated by Henderson-Hasselbalch equation (by a blood gas analyser) for the calculation of anion gap. ***Always*** calculate anion gap with serum or plasma HCO_3 (Total CO_2) measured photometerically or by ISE[2].
6. ***Never*** report PO_2 if blood gas analyses have been carried on a venous sample (which is now one of the samples for BGA)[17].
7. ***Always*** use target values of pH (7.40), PCO_2 (40 mmHg) and HCO_3 (24 mmol/L) while interpreting blood gas reports[18].
8. ***Always*** consider coexisting respiratory acidosis if PCO_2 is > 44 mmHg and coexisting respiratory alkalosis if PCO_2 is < 10 mmHg in a patient with metabolic acidosis (HCO3 < 24 mmol/L)[16,17].
9. ***Never*** think of respiratory acidosis and respiratory alkalosis in the same patient whereas metabolic acidosis and metabolic alkalosis can be present in the same patient (triple disorder)[9]
10. ***Never*** use delta; delta ratio (ΔAG/ΔHCO3) in a patient of respiratory acidosis as it can be > 2 without the presence of a triple disorder[9]

Measured parameters[2]:

a. pH
b. PCO_2
c. PO_2

Calculated parameters:

a. HCO_3 using Henderson Hasselbalch equation
b. Base Excess (BE)

Test Parameters

pH

- Most important investigation
- Ref Values: 7.35 – 7.45
- Some labs also provide direct $\{H^+\}$ in nmol/L.
- Low pH or high $\{H^+\}$ ----Acidaemia
- High pH or low $\{H^+\}$ ----Alkalaemia

Partial Pressure of Carbon Dioxide (PCO_2)

- Ref Values: 4.7 – 6.0 kPa (35 – 45 mmol/L)
- SI unit kPa
- 1 kPa = 7.5 mmHg
- High PCO_2 in respiratory acidosis
- Low PCO_2 in respiratory alkalosis
- In metabolic abnormalities, there is compensatory *change.*

Partial Pressure of Oxygen (PO_2)

- Reference Values: 10.6 – 14.6 kPa (80 – 110 mmHg)
- Abnormal PO_2 may be in:
  - Lung disease
  - Hypoventilation
  - Reduction in the inspired oxygen

Bicarbonate (HCO_3)

a. Reference Values: 23 – 33 mmol/L
b. High bicarbonate in metabolic alkalosis.
c. Low bicarbonate in metabolic acidosis
d. In respiratory abnormalities there is compensatory change.

Samples for Blood Gas Analyses (BGA)*:*

a. **Arterial Whole Blood[14]:**

- Ideal site for arterial puncture is radial artery but blood can be drawn from cubital or femoral arteries.
- Sample can also be drawn from a central arterial catheter. Great care must be exercised when puncturing any artery.
- Only doctors or trained medical students should be allowed to collect arterial samples.

b. **Venous Whole Blood[17]:**

- Any appropriate vein can be punctured for blood gases. Sample can also be drawn from a central venous catheter.

c. **Capillary Whole Blood[17]*:***

- In neonates and children blood sample for BGA can be collected in a heparinized capillary.

Precautions

- *Only **heparinized Syringes** should be used for collection of samples for ABGs and VBGs.* These syringes are prefabricated with Lithium Heparin (Figure 5.1).
- Common syringes rinsed with sodium heparin should NOT be used as the sodium will be added artificially in the sample and electrolyte results will be altered.
- Blood samples collected for BGA should **NEVER** be transferred to any sample tube as it will lead to mixing with the air gases.

Transport of Samples[15,16]:

- After collection of sample for BGA, the filled syringe should reach lab or instrument immediately or within 10 minutes of collection.
- If further delay is expected then the syringe should be transported on ice packs.
- In the lab sample should be immediately sent to the Chemical Pathology Department and performed on top priority.
- *One copy of the print-out of the result should be handed over to the person who brought the sample.*

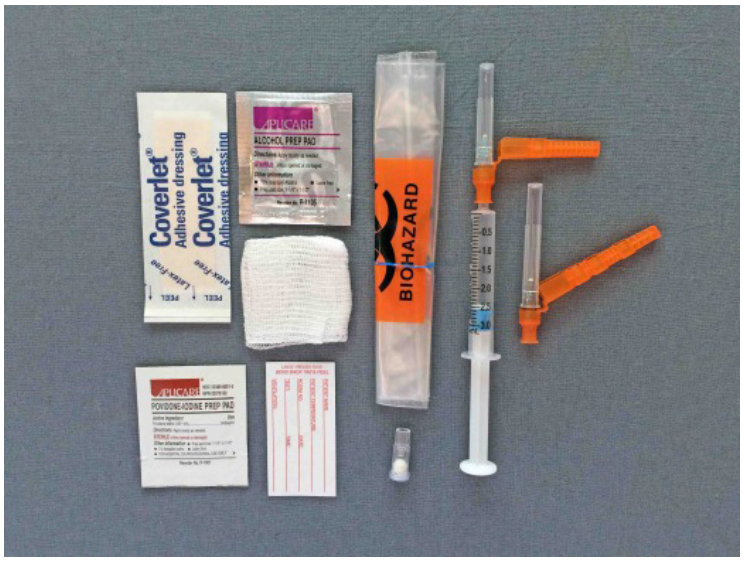

Figure 5.1: Typical ABG Specimen Collection Kit

(Adapted from www.uptodate.com ©2018 UpToDate)

Interpretation of Blood Gases Results

'One Minute Decoder' for Blood Gases Reports

The following decoder has been developed to interpret a blood gases report. It can be used for decoding **Arterial Blood Gases** as well as **Venous Blood Gases** reports.

It answers three basic questions:

Question 1: Acidosis or Alkalosis?

Look at pH.

i. **Low pH**-----Acidosis
ii. **High pH**----Alkalosis
iii. **Normal pH**--- A normal pH does not rule out existence of an acid base disorder (see below)

A. If pH is Abnormal

Question 2: Primary disorder is Metabolic or Respiratory ??

Examine pH and HCO_3 relationship (For single disorders)

a. If pH and HCO_3 change in the same direction primary abnormality is metabolic

Examples:

- *in metabolic acidosis both pH and HCO_3 decrease*
- *in metabolic alkalosis both pH and HCO_3 increase*

b. If pH and HCO3 change in the opposite direction primary abnormality is respiratory

One way to remember this relationship is to use the acronym. **ROME**.

Respiratory
Opposite
Metabolic
Equal

Examples:

- *In respiratory acidosis pH decreases and HCO_3 increases*
- *In respiratory alkalosis pH increases and HCO_3 decreases*

Acid Base Disorders - Animated

pH and HCO_3 Relationship

- pH and HCO_3 are two friends studying in a university.
- Sometimes they are in good terms. They meet daily and go everywhere together. *'M' for 'Meet' and 'M' for 'Metabolic'*
- Other times they are not in good terms and repel each other's presence. If one goes to library, the other goes to cafeteria. *'R' for 'Repel' and 'R' for 'Respiratory'*

Question 3: Single or Double disorder???

This question requires some critical thinking. According to the primary disorder, an appropriate equation is selected (given below). Expected level of CO_2 is calculated for metabolic disorders and appropriate level of HCO_3 is calculated

for respiratory disorders. If acute or chronic nature of the respiratory disorders cannot be ascertained then ***compensatory intervals*** should be used.

B. <u>If pH is Normal</u>

a. All three are normal –Normal Acid Base Status
b. PCO_2 and HCO_3 change grossly in the same direction ----mixed disorder of opposing type e.g. Metabolic acidosis and Respiratory alkalosis
c. Fully compensated Chronic Respiratory Alkalosis if HCO_3 decrease as per equation given below for chronic respiratory alkalosis.

The Equations[8, 19-22]

Following equations can only be applied if Primary Disorder is diagnosed correctly (Please see Step 2 above):

1. **Metabolic Acidosis**

Expected *P*CO_2 (mmHg) = 1.5 x [HCO_3] + 8 (range: +/- 2)

a. Maximal compensation may take 12-24 hours to reach
b. The limit of compensation: ***P*CO_2 = 10 mmHg**

Example: A patient with a metabolic acidosis ([HCO_3] 14 mmol/L) has an actual *P*CO_2 of 30 mmHg. The expected *P*CO_2 is (1.5 x 14 + 8) which is 29 mmHg. This basically matches the actual value of 30 so no evidence of another respiratory acid-base disorder.

2. **Metabolic Alkalosis:**

Expected *P*CO_2 = 0.7 [HCO3] + 20 (range: +/- 5)

In metabolic alkalosis with high [HCO_3] the compensatory response is hypoventilation and an increase in the blood *P*CO_2.

- The response of PCO_2 can be variable due to changes in PO_2
- The limit of compensation: ***PCO2 = 60 mmHg***

Example: *A patient with a metabolic alkalosis ([HCO_3] 30 mmol/L) has an actual P*CO_2 *of 40 mmHg. The expected P*CO_2 *is (0.7 x 30+ 20) is 41 mmHg. This is close to the actual value of 40 mmHg, so compensation is optimal and there is no evidence of another respiratory acid-base disorder.*

3. **Acute Respiratory Acidosis**

Expected [HCO_3] = 24 + {(Actual *P*CO_2 - 40) / 10}

Example: *A patient with an acute respiratory acidosis (PCO_2 60mmHg) has an actual [HCO_3] of 31 mmol/L. The expected [HCO_3] for this acute elevation of PCO_2 is 24 + 2 = 26 mmol/L. The actual measured value is higher than this indicating that a metabolic alkalosis is also present.*

4. **Chronic Respiratory Acidosis**

Expected [HCO_3] = 24 + 4 {(Actual *P*CO2 - 40) / 10}

Example: *A patient with a chronic respiratory acidosis (PCO_2 60 mmHg) has an actual [HCO_3] of 31 mmol/L. The expected [HCO_3] for this chronic elevation of PCO_2 is 24 + 8 = 32 mmol/L. The actual measured value is close to this, so renal compensation is maximal and there is no evidence indicating a second acid-base disorder.*

5. **Acute Respiratory Alkalosis**

Expected [HCO_3] = 24 - 2 {(40 - Actual *P*CO_2) / 10}

Example: *A patient with an acute respiratory alkalosis (PCO_2 15 mmHg) has an actual [HCO_3] of 13 mmol/L. The expected [HCO_3] for this chronic decrease of PCO2 is 24 - 5 = 19 mmol/L. The actual measured value is lower than this indicating that a metabolic acidosis must also be present*

6. **Chronic Respiratory Alkalosis**

Expected [HCO_3] = 24 - 5 {(40 - Actual *P*CO_2) / 10} (range: +/- 2)

Example: *A patient with chronic respiratory alkalosis (PCO_2 15 mmHg) has an actual [HCO_3] of 13 mmol/L. The expected [HCO_3] for this chronic elevation of PCO_2 is 24 - 5 = 12 mmol/L. The actual measured value is close, so there is normal renal compensation and there is no evidence indicating a second acid-base disorder.*

7. **Compensatory Intervals**

In situations when it is not possible to determine the acute or chronic nature of respiratory disorders, Compensatory Intervals (Range) can be used:

How to calculate Compensatory Intervals:

Respiratory Acidosis:

Expected [HCO_3] = 24 + {(Actual *P*CO_2 - 40) / 10} to 24 + 4 {(Actual *P*CO2 - 40) / 10}

Respiratory Alkalosis:

Expected [HCO_3] = 24 - 2 {(40 - Actual *P*CO_2) / 10} to 24 - 5 {(40 - Actual *P*CO_2) / 10}

8. **Anion Gap: (Na + K) – (Cl + HCO_3)** *Ref Values: 7 – 18 mmol/L*
9. **Delta Ratio: (anion gap-12/24-[HCO_3]**: It is used for the diagnosis of three acid base disorders present simultaneously. In triple disorders with respiratory alkalosis Delta Ratio is more than 2.0.
10. **Steps in the diagnosis of Triple Disorder:** Metabolic Acidosis can coexist with Metabolic Alkalosis but Respiratory Acidosis and Respiratory Alkalosis cannot coexist.
    a. Diagnose Respiratory Disorder (e.g. Respiratory Alkalosis)
    b. Examine Anion Gap (if > 18 indicates High Anion Gap Metabolic Acidosis)
    c. Examine HCO3 (if > 20 mmol/l indicates Metabolic Alkalosis)

Table 2: Summary of Acid Base Disorders

Primary Disorder	Defect	Effect on pH	Compensatory Response	Expected Response	Limit of Compensation
Metabolic Acidosis HCO_3^- Decreased	Gain in H^+ or loss of HCO_3^-	Decrease	Respiratory Alkalosis $PaCO_2$ Decreased	ΔPCO_2 (mmHg) = 1.5 x [HCO_3] + 8 (range: +/- 2)	PCO_2 = 10 mmHg
Metabolic Alkalosis HCO_3^- Increased	Gain in HCO_3^- Or loss of H^+	Increase	Respiratory Acidosis $PaCO_2$ Increased	ΔPCO_2 = 0.7 [HCO3] + 20 (range: +/- 5)	*PCO2 = 60 mmHg*
Respiratory Acidosis $PaCO_2$ Increased	CO_2 retention	Decrease	Metabolic Alkalosis HCO_3^- Increased	**Acute:** ΔHCO_3= 24 + {(Actual PCO_2 - 40) / 10} **Chronic:** ΔHCO_3= 24 + 4 {(Actual *P*CO2 - 40) / 10}	**Compensatory Interval** ΔHCO_3 = 24 + {(Actual PCO_2 40) / 10} to 24 + 4 {(Actua *P*CO2 - 40) 10}
Respiratory Alkalosis $PaCO_2$ Decreased	CO_2 Washout	Increase	Metabolic Acidosis HCO_3^- Decreased	**Acute:** ΔHCO_3 = 24 - 2 {(40 - Actual PCO_2) / 10} **Chronic:** ΔHCO_3 = 24 - 5 {(40 - Actual $PCO_{2)}$ / 10} (range: +/- 2)	**Compensatory Interval** ΔHCO_3 = 24 - {(40 - Actual PCO_2) / 10} to 24 - 5 {(40 - Actual $PCO_{2)}$ / 10}

(This table has been designed by Dr Farheen Aslam and Dr Sara Reza)

Chapter No 5

Acid Base Disorders

MCQs Key with Explanation

MCQ No	Best Option	Explanation
51.	c. Weak acid and its conjugate base	Buffer is defined as a mixture of week acid and its conjugate base. Weak acid means acid with minimum dissociation of H^+ e.g. acetic acid, while conjugate base means, the base that is constituent of the acid e.g. HCO_3 is the conjugate base of H_2CO_3.
52.	e. Proteins	Proteins are the most abundant intracellular body buffer. These are charged proteins and not the neutral proteins measured in serum in routine clinical practice. Most abundant extra-cellular buffer is Bicarbonate Buffer System.
53.	e. Tendency towards decreased pH	Acidosis is the tendency towards lowering of pH, alkalosis is the tendency towards increasing pH. Acidaemia and alkalaemia are the terms used for decreased and increased pH in blood, respectively. Please note that acidosis and alkalosis can be without acidaemia and alkalaemia, respectively.
54.	a. Bicarbonate	Henderson-Hasselbalch equation has a great utility in accurately calculating HCO_3 if pH and PCO_2 is known. So, all over the world, HCO_3 is calculated by the microprocessors in the ABGs instruments after measuring pH and PCO_2. HCO_3 is also measured in serum samples by photometric and electrochemical methods along with the electrolytes. It is also called Total CO_2 as CO_2 is released from HCO_3 and then measured.
55.	d. Unmeasured anions normally present in the	Anion gap is a laboratory error!! Body maintains electro-neutrality in all states. In lab, we measure more cations (mainly Na^+) and less anions (Cl^- and

	blood	HCO_3^-). The gap between cations and anions is due to the anions which we do not measure in the lab due to difficult techniques.
56.	b. Metabolic acidosis	The most important significance of anion gap is categorization of metabolic acidosis in high anion gap and normal anion gap metabolic acidosis. The aetiology of these two groups is entirely different and, therefore, the division is clinically very important.
57.	b. Lactic acidosis	As mentioned in MCQ No 56 above, metabolic acidosis can be divided into high anion and normal anion gap metabolic acidosis. Lactic acidosis is an important and common cause of high anion gap metabolic acidosis, also characterized by hyperkalaemia. The other two important causes are end stage renal disease and diabetic ketoacidosis.
58.	e. Radial artery	Advantages of arterial specimen collection from radial artery include easy palpation, exposure and absence of any vein in the vicinity.
59.	b. Electrolytes	Most of the composite systems of blood gas analyses have also facilities for electrolyte estimation. The biggest advantage is simultaneous measurement of ABGs and electrolytes and calculation of anion gap.
60.	c. Syringe with lyophilized lithium heparin	Blood gas specimens must be collected in lithium heparin syringes and should never be transferred to any tubes. Rinsing the plain syringes with sodium heparin is not recommended as it will overestimate sodium estimation.
61.	a. Metabolic acidosis with partial respiratory compensation	*Applying One Minute Decoder will help students interpret the clinical data given in MCQs No 61-70.* Step 1: pH is low –so acidosis Step 2: HCO_3 and pH change in the same direction, so it is metabolic acidosis Step 3: Applying equation for metabolic acidosis, it is

		found that PCO_2 value is within the physiological change. So, it is single disorder i.e. metabolic acidosis with partial respiratory compensation. We use word 'partial' to mark the fact that the compensation is not full (pH is in acidic range).
62.	c. Mixed respiratory alkalosis and metabolic acidosis	When pH is normal, we cannot apply steps as applied in MCQ No 61 above. It is obvious that normal pH is due to two opposing disorders of nearly equal intensity.
63.	a. Metabolic alkalosis with partial respiratory compensation	Step 1: pH is high –so alkalosis Step 2: HCO_3 and pH change in the same direction, so it is metabolic alkalosis Step 3: Applying equation for metabolic alkalosis, it is found that PCO_2 value is within the physiological change. So, it is single disorder i.e. metabolic alkalosis with partial respiratory compensation.
64.	b. Mixed metabolic and respiratory acidosis	Step 1: pH is low –so acidosis Step 2: HCO_3 and pH change in the same direction, so it is metabolic acidosis Step 3: Applying equation for metabolic acidosis, it is found that PCO_2 value is greater than the physiological levels. So, it is a double disorder i.e. metabolic acidosis and respiratory acidosis.
65.	a. Metabolic acidosis with partial respiratory compensation	Step 1: pH is low –so acidosis Step 2: HCO_3 and pH change in the same direction, so it is metabolic acidosis Step 3: Applying equation for metabolic acidosis, it is found that PCO_2 value is within the physiological change. So, it is single disorder i.e. metabolic acidosis with partial respiratory compensation.
66.	e. Respiratory alkalosis with partial metabolic	Step 1: pH is high –so alkalosis Step 2: HCO_3 and pH change in the opposite

	compensation	direction, so it is respiratory alkalosis Step 3: Applying equation for respiratory alkalosis, it is found that HCO_3 value is within the physiological change. So, it is a single disorder i.e. respiratory alkalosis with partial metabolic compensation.
67.	c. Mixed metabolic alkalosis and respiratory alkalosis	Step 1: pH is high–so alkalosis Step 2: HCO_3 and pH change in the same direction, so it is metabolic alkalosis Step 3: Applying equation for metabolic alkalosis, it is found that PCO_2 value is lower than the physiological levels. So, it is a double disorder i.e. metabolic alkalosis and respiratory alkalosis
68.	d. Normal acid base status	All three parameters i.e. pH, PCO_2 and HCO_3 are normal, so it is normal acid base status.
69.	b. Mixed respiratory acidosis and metabolic alkalosis	When pH is normal, we cannot apply steps. It is obvious that normal pH is due to two opposing disorders of nearly equal intensity.
70.	e. Respiratory acidosis with partial metabolic compensation	Step 1: pH is low–so acidosis Step 2: HCO_3 and pH change in the opposite direction, so it is respiratory acidosis Step 3: Applying equation for respiratory acidosis, it is found that HCO_3 value is within the physiological change. So, it is single disorder i.e. respiratory acidosis with partial metabolic compensation.

Summary

Chapter No 5

Acid Base Disorders

- Acidosis and alkalosis are *tendencies* towards decreasing pH and increasing pH, respectively. These two conditions may be present with normal pH.
- Bicarbonate is the most abundant extra-cellular buffer, it is very rapid and represents two different systems but is not very efficient as it rarely brings pH to normal.
- PCO_2 represents carbonic acid (H_2CO_3) and respiratory system. So, increase in PCO_2 means respiratory acidosis
- Bicarbonate (HCO_3) represents base and metabolic (non-respiratory) system, so increase in HCO_3 means metabolic alkalosis
- When an abnormality occurs in one component of the buffer e.g. lowering of HCO_3, the compensatory change in the respiratory system starts immediately i.e. lowering of the PCO_2, and vice versa.
- Acid base disorders can be single, double or triple.
- Single disorders are four i.e. metabolic acidosis, respiratory acidosis, metabolic alkalosis and respiratory alkalosis
- Double disorders can be opposing e.g. metabolic acidosis and respiratory alkalosis, pH may be normal if the intensity of two disorders are similar.
- Double disorders can be one sided e.g. metabolic acidosis and respiratory acidosis can be present in the same patient with very low pH
- Triple disorders can be due to the presence of metabolic acidosis and metabolic alkalosis in the same patient with one respiratory disorder.
- Blood gases can be tested in arterial, venous or capillary specimens.
- Specimen for blood gases should never be transferred into collection tube but should be transported to the lab in the same syringe that was used for collection.
- 'One Minute Decoder' is used for quick interpretation of blood gases results (Please see text for the decoder).

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Chapter No 6
Renal Function Tests

Reviewed and Edited by:

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MCQs

(Please find key at the end of the chapter)

71. A scientist is working on renal function. He wants to determine exact glomerular function rate ***(GFR)*** in humans. Only a substance which is neither absorbed nor secreted after filtration in the renal glomeruli can determine exact GFR. Such a substance is:
    a. Albumin
    b. Creatinine
    c. Inulin
    d. Paramino hippuric acid
    e. Urea

72. ***Renal Clearance*** of a substance is:
    a. 24 hours urinary clearance of that substance
    b. Amount of that substance excreted through kidney in one minute
    c. Concentration of the substance in urine
    d. Ratio of urinary concentration of that substance to urinary creatinine
    e. Volume of plasma cleared of that substance per unit of time

73. The test which is MOST effected by ***extra-renal conditions*** (without renal disease):
    a. Creatinine calculated by Modification of Diet in Renal Disease *(*MDRD*)* formula
    b. Creatinine clearance
    c. Serum creatinine
    d. Serum urea
    e. Urinary creatinine

74. The earliest marker of Acute Kidney Injury ***(AKI)*** is:
    a. Alpha one microglobulin
    b. Neutrophil gelatinase-associated lipocalin
    c. Retinol binding protein
    d. Urinary osmolality gap
    e. Urinary sodium

75. A patient is advised ***Creatinine Clearance***. Which of the following tests you will perform in the lab?
    a. Serum and urine creatinine
    b. Serum creatinine
    c. Serum creatinine and sodium
    d. Serum creatinine and urea
    e. Urine creatinine

76. A 57 years old male has BMI 31 Kg/m2. His serum creatinine remains mildly elevated. Laboratory wants to calculate eGFR. Which ***eGFR equation*** you will prefer to accept in this patient?
    a. CKD-EPI
    b. Cockcroft Gault Equation
    c. Counahan Barratt equation
    d. Modification of Diet in Renal Disease
    e. Schwartz formula

77. A patient with severe ***AKI*** faces an abrupt decline in renal function. According to one of the staging systems which of the following is the first stage of this failure:
    a. ESRD
    b. Failure
    c. Injury
    d. Loss
    e. Risk

78. Latest definition of Chronic Kidney Disease ***(CKD)*** includes presence of kidney damage or decreased kidney function for three or more months. Which of the following is ***NOT*** included in the list of indicators of *Kidney Damage*?
    a. Albuminuria
    b. Decreased GFR
    c. Imaging abnormalities
    d. Kidney transplantation
    e. Urinary sediment abnormalities

79. A 56 years old male has following renal profile:
    - Urea 23.6 mmol/L (142 mg/dl)
    - GFR 52.3 mL/min per 1.73 m^2
    - Albumin Creatinine Ratio: 22.4 mg/mmol of creatinine

Which of the following ***CKD stage*** the patient is most probably suffering from:

a. G1 and A1
b. G2 and A1
c. G3a and A2
d. G3b and A2
e. G4 and A3

80. A 36 years old male has severe diarrhea followed by anuria. His biochemical profile shows:

- pH: 7.32 (7.35-7.45)
- PCO_2: 26.3 mmHg (35 - 45)
- HCO_3: 12.0 mmol/L (22 - 28)
- Serum Sodium 140 mmol/L (136-149)
- Serum Potassium 7.07 mmol/L (3.5-5.0)
- Urea: 24.7 mmol/L (3.3-6.7)

What is the ***most appropriate first-line treatment*** in this patient?

a. Dextrose water with regular insulin
b. Emergency Dialysis
c. IV normal saline
d. Frusemide
e. Ringer`s lactate

Chapter No 6
Renal Function Tests

Glomerular Filtration Rate

Inulin[1]

- Inulin is a substance which is neither absorbed nor secreted and is an exact indicator of GFR
- It is not part of normal human metabolism and has to be injected externally

Renal Clearance of a Substance[1]

- Clearance of substance is defined as volume of plasma cleared of that substance in one minute (or second).
- It is expressed as ml/min or ml/sec

The test which is MOST effected by extra-renal conditions[2]:

- Serum urea can go up to 60 mg/dl (10 mmol/L) due to increased protein diet
- It can go up to 120 mg/dl (20 mmol/L) due to dehydration etc.

Acute Kidney Injury (AKI)[3]

The term acute kidney injury (AKI) is used to represent the full spectrum of renal injury, from mild to severe, with the latter having increased likelihood for unfavourable outcomes e.g. loss of function and *End Stage Renal Disease (ESRD). (AKI Network 2007)*

Definition of AKI

An abrupt (within 48 hours) reduction in kidney function i.e. an absolute increase in serum creatinine of ≥ 26.4 μmol/L (or 0.3 mg/dL); a percentage increase in serum creatinine of 50% or more (1.5-fold from baseline); or a reduction in urine output (documented oliguria of less than 0.5 mL/kg per hour for more than 6 hours) (Table 6.1)

RIFLE Criteria of AKI

Three stages of acute injury :

a. Risk
b. Injury
c. Failure

Two outcome measures:

d. Loss of renal function
e. ESRD

Neutrophil Gelatinase-Associated Lipocalin (NGAL)[4]

- NGAL is a highly predictive biomarker of AKI
- An early marker of AKI

- The sensitivity to predict AKI is 0.815 (95 % confidence interval, 0.732-0.892) like troponin for acute myocardial infarction
- There is evidence for the role of NGAL measurements in a variety of clinical situations leading to AKI e.g. contrast-induced nephropathy, AKI after cardiac surgery or kidney transplantation and AKI in the critical care setting

Table 6.1: RIFLE Criteria of AKI

Stage	GFR** Criteria	Urine Output Criteria	Probability
risk	*Scr increased × 1.5 *or* **GFR decreased >25%	UO‡ < 0.5 mL/kg/h × 6 h	High sensitivity (Risk >Injury >Failure)
Injury	SCr increased × 2 *or* GFR decreased >50%	UO < 0.5 mL/kg/h × 12 h	
Failure	SCr increased × 3 *or* GFR decreased >75% *or* SCr ≥4 mg/dL; acute rise ≥0.5 mg/dL	UO < 0.3 mL/kg/h × 24 h (oliguria) *or* anuria × 12 h	
Loss	Persistent acute renal failure: complete loss of kidney function >4 week		High specificity
ESRD	Complete loss of kidney function >3 months		

**Serum Creatinine; **Glomerular Filtration rate ‡Urine Output*

Chronic Kidney Disease (CKD)[6]

- The term CKD has replaced all the previously used vague terms like chronic renal failure, chronic renal insufficiency, pre-dialysis and pre-end stage renal disease.
- A conceptual change has been implemented in the disease definition. Now no consideration of the cause of the failure in definition.

Definition of CKD[7]

The definition is based on 3 components:

a. An anatomical or structural component (one of the markers of kidney damage, including albuminuria and imaging abnormality)
b. A functional component (based on GFR)
c. A temporal component at least 3 months' duration of structural and/or functional alterations.

Guidelines for CKD[7]

K DOQI : *The National Kidney Foundation Kidney Disease Outcomes Quality Initiative (NKF KDOQI)* ™ provides evidence-based clinical practice guidelines for all stages of chronic kidney disease (CKD) and related complications

KDIGO: *Kidney Disease Improving Global Outcomes*.

These guidelines add albuminuria with GFR for staging of CKD.

Kidney Damage[8-10]

Kidney damage includes pathologic abnormalities in the native or transplanted kidney. Kidney damage is identified in most cases by the presence of one of the following clinical markers:

1. Albuminuria − In clinical practice, albuminuria is the most frequently assessed marker of kidney damage. Albumin-to-creatinine ratio (ACR) in an untimed "spot" urine is the best method to measure it.
2. Urinary sediment abnormalities − Urinary sediment abnormalities such as red or white blood cell casts may indicate the presence of glomerular injury or tubular inflammation.
3. Imaging abnormalities − Kidney damage may be detected by the presence of imaging abnormalities such as polycystic kidneys, hydronephrosis, and small and echogenic kidneys.
4. Pathologic abnormalities − A kidney biopsy may reveal evidence for glomerular, vascular, or tubulointerstitial disease.
5. Kidney transplantation − Patients with a history of kidney transplantation are assumed to have kidney damage whether they have documented abnormalities on kidney biopsy or markers of kidney damage.

Decreased GFR

- GFR is generally considered to be the best index of overall kidney function
- Declining GFR is the hallmark of progressive kidney disease. Measured GFR varies in normal individuals by:
    - Age

- Sex
- Dietary Protein Intake
- Race-Ethnicity

- Threshold defining a decreased GFR is less than 60 mL/min per 1.73 m^2
- Kidney failure is defined as a GFR <15 mL/min per 1.73 m^2 or treatment by dialysis

Staging of CKD

The purpose of CKD staging is to:

- Guide management
- Risk stratification for progression and complications of CKD.
- Select appropriate treatments
- Frequency of monitoring
- Patient education

In patients who are diagnosed with CKD using the criteria described above, staging of the CKD is done according to :

1. Cause of disease
2. Six categories of GFR (G stages)
3. Three categories of albuminuria (A stages)

(Based on KDOQI and KDIGO guidelines)

Cause of Disease

- Diabetes Mellitus
- Drug toxicity
- Auto-immune diseases
- Urinary tract obstruction,
- Kidney transplantation

Staging of CKD[10]

GFR — The GFR (G-stages)

- G1 − GFR >90 mL/min per 1.73 m^2
- G2 − GFR 60 to 89 mL/min per 1.73 m^2
- G3a − GFR 45 to 59 mL/min per 1.73 m^2
- G3b − GFR 30 to 44 mL/min per 1.73 m^2
- G4 − GFR 15 to 29 mL/min per 1.73 m^2
- G5 − GFR <15 mL/min per 1.73 m^2 or treatment by dialysis

Albuminuria — Three albuminuria stages are:

- A1 − ACR < (<3.4 mg/mmol (30 mg/g) (Normal)
- A2 − ACR 3.4 to 34.0 mg/mmol (30 to 299 mg/g) (Microalbuminuria)
- A3 − ACR ≥34.0 mg/mmol (300 mg/g) (Macroalbuminuria)

Sub-acute Kidney Disease[11]

- "Acute kidney diseases and disorders" (AKD) encompasses any decrease in renal function occurring in less than three months.
- Disorders that evolve over more than 48 hours but generally in under three months are informally referred to here as subacute kidney injury.
- AKD includes both AKI and subacute kidney injury, and there is considerable overlap in an acute and subacute presentation.

Markers of GFR[1]

(Dr Salma Ayub has added a significant portion in this section)

- Exogenous
- Endogenous

Criteria for GFR Markers

- Freely filterable at the glomerular barrier
- Not reabsorbed by the tubules
- Not secreted by the tubules
- Present at a stable plasma concentration
- No or minimal extra renal excretion
- Accurate, precise and cost effective assay available

Exogenous Markers
Non-radioactive

- Inulin
- Iohexol
- Iodoacetate
- Diethylenetriaminepentaacetic acid

Radioactive

- Tc-diethylenetriaminepentaacetic acid
- Cr-ethylenediaminetetraacetic acid
- I-hippuran
- I-iodothalamate

Disadvantages

- These methods are labour intensive and not possible routinely

Inulin Clearance[1]

Advantages

- Gold standard
- Metabolically inert sugar
- Neither reabsorbed nor secreted
- Provides good estimation of GFR

Disadvantages

- Time consuming
- Poor specificity of analysis
- Assay not easily available
- Extra-renal clearance =0.83 ml/min/10kg

Measurement of GFR by Urinary Clearance of a Radionuclide[1]

- Renal tubular secretion or reabsorption must not contribute to its excretion
- Plasma protein binding of radionuclide should be negligible
- Complete bladder emptying is essential

Constant Infusion Technique

- Fasting subject is required to drink 500 ml of water 1 hour before the test.
- Takes 200 ml of water every half hour until the end of the study
- Supine position is essential throughout the test
- Intravenous loading dose of marker
- Constant infusion of a given quantity per minute for 3 hour
- Collect a blood sample and timed urine samples

Single Bolus Injection

- Fasting subject to drink 500 ml of water 1 hour before the test
- Supine position is essential throughout the test
- Single marker dose is infused at a constant rate over 5 minutes-peristaltic pump
- Line flushed with saline
- Venous blood samples are collected at intervals 120,180,240 min
- Plasma disappearance curve is measured

Endogenous Markers

- Creatinine
- Urea
- Beta2-Microglobulin
- Retinol-binding protein
- Alpha1-Microglobulin
- Cystatin C
- N-GAL

Advantages

- No injection required
- Only a single blood sample needed
- Simplifies the procedure for the patient, clinician and laboratory
- These proteins are entirely eliminated from the circulation

Disadvantages[12]

- There are many factors, which affect GFR estimation by simply measuring concentration of endogenous filtration markers including generation, renal tubular reabsorption, secretion and extra renal elimination of these markers.
- These factors are collectively known as non-GFR determinants.

Serum Creatinine[1]

- Measuring serum creatinine is a simple test and it is the most commonly used indicator of renal function
- Very accurate and precise cost effective assay easily available
- Lower in women, elderly and persons with malnutrition, muscle paralysis and short stature
- Higher levels are associated with high intake of meat, exercise, corticosteroids and ketoacidosis
- A better estimation of kidney function is given by the creatinine clearance test

Creatinine Clearance[1]

- Test based on the rate of excretion of metabolically produced creatinine
- Amount of creatinine produced by endogenous creatine metabolism is constant (creatine is converted to creatinine).
- It is directly proportional to the body surface area
- Creatinine is freely filtered at the glomerulus
- Not absorbed by the tubules

- Actively secreted by the renal tubules in very small amounts such that creatinine clearance overestimates actual GFR by 10-20% and there is also considerable excretion through gut especially when GFR is decreased
- A 24 hours urine collection is performed but shorter collection periods are acceptable
- A blood sample is drawn during the urine collection period
- Height and weight of the patient is noted

Calculation of Creatinine Clearance

- Creatinine clearance (C_{Cr}) (ml/min) =U_{Cr} x V / P_{Cr}
  (U_{cr}: urine creatinine; V= volume of urine; P_{cr}= plasma creatinine)
- Since the product of urine concentration and urine flow rate yields creatinine's excretion rate, creatinine clearance is also said to be its excretion rate (UCr×V) divided by its plasma concentration:

$$C_{Cr} = \frac{U_{Cr} \times \text{24-hour volume}}{P_{Cr} \times 24 \times 60 mins}$$

- To allow comparison of results between people of different sizes, the C_{Cr} is often corrected for the body surface area and expressed compared to the average sized man as mL/min/1.73 m^2.

Problems Associated with Creatinine Clearance

- Timed 24 hours- urine specimen is burdensome
- Incomplete emptying of bladder
- Failure to collect entire specimen
- Wide inter-and intra-individual variation
- Creatinine secretion increases as GFR decreases
- Excretion through gut
- Interference with Jaffe reaction (used for creatinine estimation in the lab)

Advantage of Creatinine Clearance

- Unlike precise GFR measurements involving constant infusions of inulin, creatinine is already at a steady-state concentration in the blood, so measuring creatinine clearance is much less cumbersome

Creatinine Co-efficient / Completeness of Collection: (C.C)[13]

- It is the ratio of mg of creatinine in urine in 24 hour/ body weight in kg.

$$C.C. = \frac{U_{cr} \text{ in mg/dl}}{100} \times \frac{\text{24 h Volume}}{\text{Body Weight}}$$

Reference Values: Male: 17.6-26.8; Female: 13.4-21.0

- Significance: it depends on muscle mass and remains fairly constant. Since muscle mass is constant in the given individual, the creatinine coefficient serves as a reliable index of the adequacy of a 24- hours urine collection.

Estimated GFR (eGFR) Equations[14-18]

- GFR estimation equations estimate GFR from plasma levels of endogenous filtration markers and demographic and clinical variables which serve as surrogates for non-GFR determinants.
- Numerous formulas have been developed for estimating creatinine clearance from serum creatinine concentration –no need for urine collection

Commonly used Equations

In Adults:

- Cockcroft Gault Equation
- Modification of Diet in Renal Disease (MDRD)
- CKD-EPI

In Children:

Schwartz formula

Counahan-Barratt

Cockcroft Gault Equation

Simplest and most widely used

$$CrCl\ (ml/min) = \frac{(140 - age\ in\ years) \times weight(kg)}{7.2 \times Serum\ creatinine\ (\mu mol/L)}$$

Multiply by 0.85 if female

- Developed in 1973
- Not adjusted for body surface area
- Body weight is used as a marker of muscle mass
- Leads to over estimation of muscle mass and of creatinine clearance in obese
- Calculations are relatively simple and can often be performed without the aid of a calculator

Limitations of Cockcroft Gault

- Muscle diseases
- Malnourished
- Severe renal failure
- Liver disease
- Obese

- Critically ill
- Unstable renal function

Modification of Diet in Renal Disease (MDRD)

- Developed in 1999
- Provides an estimate of GFR that is normalized to a standard Body Surface Area of 1.73m^2
- Since renal size and metabolic rate correlate with body surface area, MDRD is a quite good test

MDRD Equation

- Serum creatinine, age, race, and gender are required
- The original MDRD used six variables with the additional variables being the blood urea nitrogen and albumin levels
- Validated in patients with chronic kidney disease; however, both versions underestimate the GFR in healthy patients with GFRs over 60 mL/min.

MDRD Equation

$$eGFR = 186 \times SerumCr^{-1.154} \times age^{-0.203} \times 1.212 \text{ (if black)} \times 0.742 \text{ (if female)}$$

- Most laboratories in Australia and The United Kingdom now calculate and report the MDRD estimated GFR along with creatinine measurements and this form the basis of Chronic kidney disease staging
- The adoption of the automatic reporting of MDRD-estimated GFR has been widely criticized

Limitations of MDRD

- Accuracy not established in
- Elderly
- Paediatrics
- Hospitalized
- Other ethnic groups besides Caucasians

Comparison of The Two Equations

- Neither is perfect particularly in patients with relatively normal kidney function
- MDRD equation is endorsed by UK Chronic Kidney Disease Guidelines
- MDRD can be calculated without body weight
- Both can only be used if serum creatinine concentration is stable

Schwartz Equation:

- *CrCl (ml/min)= [length (cm) x k] / Creatinine*

  (Patient population: infants over 1 week old through adolescence (18 years old))
  k = 0.45 for infants 1 to 52 weeks old
  k = 0.55 for children 1 to 13 years old
  k = 0.55 for adolescent females 13-18 years old
  k = 0.7 for adolescent males 13-18 years old

- For use in children 1-18 years old.
- The formula was updated in 2009 and is currently considered the best method for estimating GFR in children. It is known as the "Bedside Schwartz" formula.
- It is devised for use with creatinine methods with calibration traceable to Isotope Dilution Mass Spectrometry (IDMS) method. All laboratories should be using creatinine methods calibrated to IDMS traceable.
- Nearly all major global manufacturers in the US and other countries now have calibration traceable to an IDMS reference measurement procedure. Thus, older equations for estimating GFR in children and infants are no longer valid.

Counahan-Barratt:

GFR (ml/min/1.73m2) = (0.43 x length)/ Creatinine

Cystatin C[14, 19-21]

- Cystatin C may offer a more sensitive and specific monitoring of changes in GFR than serum creatinine
- Cysteine protease inhibitor
- More freely filtered than the other proteins-13000 D
- Production rate by all nucleated cells is constant
- Not influenced by muscle mass, diet or sex
- No extra-renal mode of excretion
- More sensitive and specific than creatinine

An index of GFR, especially in patients where serum creatinine may be misleading (e.g. very obese, elderly or malnourished patients); for such patients, use of CKD-EPI (Chronic Kidney Disease Epidemiology Collaboration) cystatin C equation is recommended to estimate GFR.

CKD-EPI Cystatin C Equation

Cystatin C < 0.8 mg/dl

$$eGFR = 133 \times \min(S_{cys}/0.8)^{-0.499} \times 0.996^{Age} \times 0.932 \text{ [if female]}$$

Cystatin C > 0.8 mg/dl

$$eGFR = 133 \times \max(S_{cys}/0.8)^{-1.328} \times 0.996^{Age} \times 0.932 \text{ [if female]}$$

- CKD-EPI creatinine-cystatin C equation was found to be the most accurate GFR estimation equation.

Limitation of Serum Creatinine

- Creatinine also show some false rise due to increased dietary intake of proteins but this false rise is much less than urea.
- Major problem of creatinine is that it depends on the muscle mass of the patient. So, people from Afro-Caribbean origin have normally high serum creatinine (1.70 -186 mg/dl)
- Analytical Interference: Some medicine e.g. cefoxitin and fluectosine interfere in assay especially when the level is just above the upper reference value.
- Endogenous substances like serum bilirubin and acetoacetate also interfere the creatinine assay

Limitations of Serum Cystatin

- False increase due to age, height, weight and male sex
- Diabetes mellitus and hypothyroidism also affect cystatin results.

Other Low Molecular Proteins[1]

- Proteins with molecular weight less than 30kD are cleared by renal filtration
- Freely filtered at glomerulus
- Apart from Cystatin C all other proteins are influenced by non-renal factors

Chapter No 6
Renal Function Tests

MCQs Key with Explanation

MCQ No	Best Option	Explanation
71.	c. Inulin	Inulin is an inert material not produced or metabolized in the body. If injected it is neither reabsorbed nor secreted by the kidney, so accurately measures GFR. Inulin clearance study is not used in routine lab practices, as inulin has to be injected externally, whereas for creatinine clearance nothing is to be given from an external source.
72.	e. Volume of plasma cleared of that substance in one minute	This is the standard definition of renal clearance of a substance. So the unit of clearance is ml/min (or ml/seconds).
73.	d. Serum urea	Serum urea is highly variable marker. As a rule of thumb, it can increase up to 60 mg/dl just because of high protein diet and up to 120 mg/dl because of severe dehydration (without intrinsic renal disease)
74.	b. Neutrophil gelatinase-associated lipocalin (N-GAL)	N-GAL a new marker for early detection of acute kidney injury. It is now available in some labs in Pakistan, too
75.	a. Serum and urine Creatinine	Creatinine clearance is widely advised as measure of GFR. For its estimation, urine and serum creatinine must be measured simultaneously.
76.	a. CKD-EPI	Estimated GFR is calculated after measuring serum creatinine. Many formulas have been reported but CKD-EPI is most suitable for use in patients with mild renal impairment as well as in gross impairment. Other formulae like MDRD are not suitable in mild derangement.
77.	e. Risk	RIFLE criteria are used for the diagnosis of various stages of Acute Kidney Injury (AKI). Risk is the first

		stage, while end stage renal disease is the last stage of AKI.
78.	b. Decreased GFR	Chronic Kidney Disease (CKD) is defined as morphological and functional damage of the kidney in 3 months. Albuminuria, urinary cast, findings on imaging, biopsy findings and history of renal transplant are morphological damages while decreased GFR is an evidence of functional damage.
79.	c. G3a and A2	CKD is now staged according to the values of GFR and quantity of albumin in the urine (Please see details in the text)
80.	a. Dextrose water with regular insulin	Hyperkalaemia is a medical emergency, it can be immediately reverted by giving insulin in dextrose

Summary
Chapter No 6
Renal Function Tests

- Serum urea and creatinine are two most important and commonly used tests for assessment of renal functions. They mainly assess glomerular function.
- Creatinine clearance is a convenient and fairly accurate method for the estimation of Glomerular Function Rate (GFR). There are many other methods available for GFR determination but they are not used in clinical laboratories
- Estimated GFR (eGFR) is a calculated index used to get an idea of GFR. After measurement of serum creatinine of the patient, certain formulae are applied to calculate eGFR.
- Tubular functions of the kidney can be assessed by electrolytes especially by serum and urine sodium
- Two new markers are now available in Pakistan for assessing renal function i.e. N-GAL and cystatin C.
- Acute Kidney Injury (AKI) is new name of acute renal failure. Its definition is based on serum creatinine, urine output, GFR and time (within 48 hour)
- Chronic Kidney Disease (CKD) is the new name of chronic renal failure. Its definition depends on three parameters i.e. chronicity (>3 months), impaired renal function as evident by lower GFR and one of the indicators of renal damage i.e. radiological evidence, albuminuria, history of renal transplant, biopsy findings and abnormal urine sediments.
- CKD is divided into various stages based on two parameters i.e. GFR (G1 to G5) and albuminuria (A1 to A3)

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Chapter No 7
Liver Function Tests

Reviewed and Edited by:

Nusrat Alavi, Uzma Naeem and Asma Hayat

MCQs

(Please find key at the end of the chapter)

81. A 27 years old male complained of generalized symptoms for the last 3 days including malaise and loss of appetite. His Serum ALT was more than 2500 U/L on two occasions but he did not have jaundice.
What is the most probable diagnosis in this patient?
    a. Acute viral hepatitis
    b. Biliary atresia
    c. Non-alcoholic liver disease
    d. Haemochromatosis
    e. Obstructive Liver Disease

82. A 22 years old medical student was upset to find that her sclera sometimes look yellow specially when she is fasting and after taking Panadol for trivial problems. She discussed the problem with her clinical teacher who advised her LFTs. On seeing the report, she assured her that this is due to a benign liver disease which is very common. Her LFTs were:
    - Conjugated Bilirubin: 5 µmol/L (<4)
    - Unconjugated Bilirubin: 30 µmol/L (<14)
    - ALT: 22 U/L (< 42)
    - ALP: 145 U/L (85- 306)

    What is the most probable diagnosis in this patient?
    a. Crigler-Najjar Type I
    b. Crigler-Najjar Type II
    c. Dubin-Johnson syndrome:
    d. Gilbert Syndrome
    e. Rotor Syndrome

83. A 10 days old baby has following LFT
    - Conjugated Bilirubin: 34 µmol/L
    - Unconjugated Bilirubin: 378 µmol/L
    - ALT: 54 U/L (< 42)
    - ALP: 745 U/L (85- 730)
    - Albumin: 49 g /L (35-50)

What is the most probable diagnosis in this baby?

a. Biliary atresia
b. Breast milk jaundice
c. Dubin-Johnson syndrome:
d. Haemolytic Disease of the Newborn
e. Physiological jaundice

84 A 20- hours neonate has following LFTs:

- Conjugated Bilirubin: 3 µmol/L
- Unconjugated Bilirubin: 24 µmol/L
- ALT: 39 U/L (< 42)
- ALP: 670 U/L (85- 730)
- Albumin: 56 g /L (35-50)

What is the most probable diagnosis in this patient?

a. Crigler Najjar Syndrome
b. Gilbert Syndrome
c. Hereditary Spherocytosis
d. Neonatal hepatitis
e. Physiological jaundice

85. A 66 years old male has following LFTs:

- Conjugated Bilirubin: 334 µmol/L (<4)
- Unconjugated Bilirubin: 34 µmol/L (<14)
- ALT: 62 U/L (< 42)
- ALP: 1045 U/L (85- 306)
- Albumin: 49 g /L (35-50)

What is the most probable diagnosis in this patient?

a. Alcoholic liver disease
b. Haemolytic jaundice
c. Hepatitis
d. Obstructive liver disease
e. Wilson's disease

86 A 25 days old male baby presents with jaundice and failure to thrive. Some of his liver tests show:

- Conjugated Bilirubin : 35 µmol/L (<4)
- Unconjugated Bilirubin: 63 µmol/L (<14)
- Gamma-glutamyl transferase: 1334 U/L (<385)

What is the most probable diagnosis in this patient?

a. Biliary atresia
b. Breast milk jaundice
c. Haemolytic disease of the newborn
d. Physiological jaundice
e. Rotor syndrome

87. A new-born developed jaundice at four days of life. His serial bilirubin reports showed following results:

- At 4th day of Life:
  Unconjugated Bilirubin: 72 µmol/L
- At 14th day of Life:
  Unconjugated Bilirubin: 186 µmol/L
- At 10th week of life
  Unconjugated Bilirubin: 10 µmol/L

What is the most probable diagnosis?

a. Biliary atresia
b. Breast milk jaundice
c. Crigler-Najjar Type II
d. Gilbert Syndrome
e. Physiological jaundice

88. A hepatic marker with following characteristics:

- Less sensitive to hepatocellular damage
- Greater liver capacity to remove it
- Divided in two main types by routine lab methods, while four fractions on more sophisticated techniques like HPLC

Which of following hepatic marker we are talking about?

a. Albumin
b. Alpha-fetoprotein
c. Bilirubin
d. Prothrombin
e. Vitamin K

89. Which of the following hepatic enzymes is in ***highest concentration*** in the cytoplasm of the liver cells:

a. Alkaline Phosphatase
b. ALT
c. AST
d. Gamma Glutamyl Transferase
e. Lactate Dehydrogenase

90. Which of the following enzymes is a ***Membrane Bound Enzyme***?

a. 5 prime Nucleotidase
b. Cholinesterase
c. Glutamyl Dehydrogenase
d. Glutathione S-Transferase
e. Isocitrate dehydrogenase

Chapter No 7
Liver Function Tests

Hepatic Transaminases[1]

- ALT is mainly a cytoplasmic enzyme
- AST has two fractions:
  - Cytoplasmic AST
  - Organellar AST
- In acute hepatitis, only cell membrane is damaged while organelles remain intact
- So, in acute hepatitis ALT is markedly increased as compared to AST
- ALT may be the first or only liver marker to rise
- In chronic hepatitis AST is a better marker
- AST lacks specificity
- So, for diagnosis and monitoring of chronic hepatitis (Hep B and C), ALT is used.

Acute Hepatitis[2,3]

- In Pakistan, hepatitis E virus is the most common cause of acute hepatitis in adults, while hepatitis A (HAV) is a common infection among children and accounts for 50-60% of all cases of acute viral hepatitis in children.
- Almost 96% of the population is exposed to HAV by the age of 5 years and 98-100% at adulthood
- Hepatitis B can also present as acute hepatitis in Pakistan.

Neonatal (Physiological) Jaundice[4]

- Full Term infants: jaundice lasts for about 10 days with a rapid rise of serum bilirubin up to 204 µmol/L (12 mg/dL).
- Preterm infants: jaundice lasts for about two weeks, with a rapid rise of serum bilirubin up to 255 µmol/L (15 mg/dL).
- Phase two: bilirubin levels decline to about 34 µmol/L (2 mg/dL) for two weeks, eventually mimicking adult values. Preterm infants - phase two can last more than one month.

Pathological Neonatal Jaundice[5]

Any of the following features characterizes pathological jaundice:

- Clinical jaundice appearing in the first 24 hours
- Jaundice lasting more than 14 days of life.

- Increases in the level of total bilirubin by more than 8.5 µmol/L (0.5 mg/dL) per hour or 85 µmol/L (5 mg/dL) per 24 hours.
- Total bilirubin more than 331 µmol/L (19.5 mg/dL)
- Direct bilirubin more than 34 µmol/L (2.0 mg/dL)

Causes of Pathological Neonatal Unconjugated Hyperbilirubinemia[4]

- **Haemolytic Disease of the New-born**: The most common cause of pathologic indirect hyperbilirubinaemia is increased bilirubin production due to haemolytic disease processes
- **Decreased Clearance**: Congenital condition like Crigler-Najjar syndrome and Gilbert Syndrome
- **Breast Milk Jaundice**: A very common cause
- **Other Causes** e.g. Gestational Diabetes Mellitus

Congenital Causes of Hyperbilirubinaemia

Unconjugated Bilirubinaemia

- Gilbert syndrome:
- Crigler-Najjar

Conjugated Bilirubinaemia

- Dubin-Johnson syndrome:
- Rotor Syndrome

Gilbert's Syndrome[6]

- The most common inherited disorder of bilirubin glucuronidation (9-10% of the adult population)
- Other names are "constitutional hepatic dysfunction" and "familial non-haemolytic jaundice"

Enzyme Defect:

- Bilirubin uridine diphosphate glucuronosyltransferase (UGTs) which is a family of enzymes that mediate glucuronidation of various endogenous and exogenous compounds

- **Genetic Defect:** The mutation is in the promoter region, upstream to exon 1 of the gene encoding bilirubin-UGT[6]

Haemolytic Disease of the New-born (HDN)[4]

- HDN is a condition in which the red cells of the foetus or new-born are destroyed by maternally-derived alloantibodies.
- These antibodies arise in the mother as the direct result of a blood group incompatibility between the mother and foetus e.g. when Rh(D) negative mother carries an Rh(D) positive foetus

Incidence of HDN

- In developed world, incidence has been reduced due to immunization of Anti D.
- In Pakistan, exact incidence is not known. However, in a local study, ABO incompatibility was known to be the major cause of HDN (71%)

Crigler-Najjar Syndrome (CNS)

Type I

- Very rare; only about 100 cases have been reported.
- Autosomal recessive inheritance.
- Severe neonatal jaundice with Bilirubin Induced Neurological Damage (BIND) and death within 24 months
- Unconjugated bilirubin is high at 342-770 µmol/L (20-45 mg/dL) with almost absent conjugated bilirubin
- Phenobarbital has no effect on bilirubin levels.
- Absence of bilirubin glucuronides in bile.

Type II

- More common than Type I CNS but still rare.
- Predominantly autosomal recessive inheritance.
- Unconjugated bilirubin 103-342 µmol/L (6-20 mg/dL).
- Mild persistent jaundice in childhood, without brain damage.
- Phenobarbital reduces bilirubin levels by ~30%.
- Bilirubin glucuronides present in bile.

Conjugated Hyperbilirubinaemia[7]

Defined as:

- Conjugated bilirubin > 17 µmol/L (1 mg/dl) - when total Bilirubin is < 85 µmol/L (5 mg/dl) OR
- Conjugated bilirubin > 20 % - When total Bilirubin is > 85 µmol/L (5 mg/dl)

Biliary Atresia

- A progressive, idiopathic, fibro-obliterative disease of the extra-hepatic biliary tree that presents with biliary obstruction exclusively in the neonatal period
- It is the most common cause of neonatal jaundice for which surgery is indicated and the most common indication for liver transplantation in children.

Biochemical Findings in Biliary Atresia

- Elevations in bilirubin (conjugated bilirubin ≥ 34 μmol/L (2 mg/dL)
- Mild or moderate elevations in ALT or AST
- A disproportionately increased gamma glutamyl transferase

Breast Milk Jaundice (BMJ)

- Defined as the persistence of physiologic jaundice beyond the first week of age.
- Typically starts after the first three to five days of life
- Peaks within two weeks after birth,
- Progressively declines to normal levels over 3 to 12 weeks
- BMJ is a benign condition, needs to be distinguished from breastfeeding failure jaundice that occurs within the first seven days of life, which is caused by decrease intake resulting in weight loss and fluid loss.
- A factor in human milk that promotes an increase in intestinal absorption of bilirubin may be responsible.
- Beta-glucuronidase is a candidate substance as it deconjugates intestinal bilirubin, increasing its ability to be absorbed (i.e. increasing enterohepatic circulation).
- Approximately 20 to 40 per cent of women have high levels of beta-glucuronidase in their breast milk.
- Blocking the deconjugation of bilirubin through beta-glucuronidase inhibition may provide a mechanism to reduce intestinal absorption of bilirubin in breastfed infants

Serum Bilirubin[8,9]

- It important to know that against the common belief serum bilirubin is not a sensitive test for hepatic dysfunction.

- Concentrations of serum bilirubin may be normal despite moderate to severe hepatic parenchymal injury or a partially or transiently obstructed bile duct.
- This lack of sensitivity can be explained in part by the reserve capacity of the human liver to remove bilirubin.
- It has been demonstrated that the normal liver can remove at least twice the normal daily bilirubin load without the development of hyperbilirubinemia.
- The reserve capacity may be even higher based upon the maximal rate of excretion of bilirubin in bile.
- Bilirubin can be tested in two fractions i.e. conjugated and unconjugated by simple lab methods while by HPLC, four fractions of bilirubin are found i.e. alpha, beta, gamma and delta.

Membrane Bound Hepatic Enzymes[10]

- Alkaline Phosphatase (ALP)
- Gamma Glutamyl Transferase (Gamma GT)
- 5-prime Nucleotidase

Increased in Cholestasis

- Increased synthesis due to enzyme induction
- Bound with cell membranes of endothelial cells of bile canaliculi
- Obstruction and stasis cause digestion of fat content of cell membranes.

Isolated Rise of ALT[11]

- Isolated rise of ALT is a very common and often puzzling problem a Chemical Pathologist is to face.
- Suppose you are working as a Consultant Chemical Pathologist in a tertiary care hospital lab. In a weekly meeting your PG Resident has presented following three patients with isolated rise of ALT to seek your opinion (Please note she has collected clinical information about these patients just from the request forms and has not seen these patients):

Patient No 1: A 44 years known patient of *Hepatitis C (Recent PCR negative for both HCV and HBV) and Diabetes Mellitus* has following LFTs:

• Total Bilirubin:	7	µmol/L	(<17)
• ALT:	94	U/L	(< 42)
• ALP:	145	U/L	(85- 307)

- Albumin: 46 g /L (35-50)

Questions

a. What is the most probable diagnosis?
b. What further laboratory tests you will like to perform in this patient

Answers

a. Non-Alcoholic Fatty Liver Disease (NAFLD)
b. Lipid Profile, Glucose (F) and USG

Non-Alcoholic Fatty Liver Disease[12-14]:

- NAFLD is a spectrum of liver abnormalities i.e. non-alcoholic fatty liver (NAFL), non-alcoholic steatohepatitis (NASH), cirrhosis and hepatic carcinoma.
- In NAFL, hepatic steatosis is present without evidence of significant inflammation.
- In NASH, hepatic steatosis is associated with hepatic inflammation that may be histologically indistinguishable from alcoholic steatohepatitis.
- Major risk factors for NAFLD are central obesity, type 2 diabetes mellitus, dyslipidaemia, and metabolic syndrome.
- NAFLD is a very common condition in the Western World, prevalence reported in our country is very high, too.

Patient No 2: A 39 years old male has severe fatigability, impotence, and hepatomegaly. He has following LFTs:

- Total Bilirubin: 14 µmol/L (<17)
- ALT: 294 U/L (< 42)
- ALP: 345 U/L (85- 307)
- Albumin: 36 g /L (35-50)

Questions

a. What is the most probable diagnosis?
b. What further laboratory tests you will like to perform in this patient

Answers

a. Haemochromatosis
b. Ferritin, Transferrin Saturation and HFE gene studies

Haemochromatosis[15]

Haemochromatosis is a hereditary condition of iron overload. Liver damage is due to the deposit of iron. (Please see details in Chapter 9).

Patient No 3: A 58 years old male is a known patient of *Coronary Artery Disease.* He has following LFTs:

• Total Bilirubin:	09	µmol/L	(<17)
• ALT:	87	U/L	(< 42)
• ALP:	233	U/L	(85- 307)
• Albumin:	47	g /L	(35-50)

Questions

a. What is the most probable diagnosis?

b. What further laboratory tests you will like to perform in this patient

Answers

a. Hepatotoxic effect of statins and NAFLD

b. Lipid Profile to titrate the dose, CK to see the myopathic effects and tests for NAFLD (with some interpretation points)

Statin Induced Hepatotoxicity[16]: Many drugs cause isolated rise of ALT. Statins are notorious for the hepatic damage.

LFTs Animated

AST –The Cheater: Heart expelled AST saying *"go away cheater - you are also loyal to the liver"* (AST has been removed from the list of cardiac markers because of its non-specificity). To add to its insult liver also gives preference to ALT on AST because of its 'relation' with heart. So, poor AST is out of heart and liver, on the accusation of cheating. Do we have a lesson here to learn?

Chapter No 7
Liver Function Tests

MCQs Key with Explanation

MCQ No	Best Option	Explanation
81.	a. Acute viral hepatitis	Acute viral hepatitis may present without jaundice and with normal bilirubin but typical symptoms and raised ALT are sufficient for the diagnosis. So, ALT is the most important test not only for the diagnosis of hepatitis but also for monitoring.
82.	d. Gilbert Syndrome	Gilbert syndrome is a very common but benign disorder. There is a defect in bilirubin glucuronidation leading to unconjugated bilirubinaemia.
83.	d. Haemolytic Disease of the new-born (HDN)	HDN is a very common cause of unconjugated hyperbilirubinaemia. It is mostly due to ABO and Rh incompatibility.
84.	e. Physiological jaundice	In new-born, jaundice is normally present as the conjugation of bilirubin has not established, this is called 'physiological jaundice'. Certain features characterise physiological jaundice e.g. unconjugated bilirubinaemia which clears within 10-14 days of life (Please see text for further details)
85.	d. Obstructive liver disease	Obstructive liver disease is characterized by conjugated bilirubinaemia and markedly raised alkaline phosphatase. ALT may also increase to certain extend due to hepatocellular damage because of cholestasis.
86.	a. Biliary atresia	Biliary atresia is a congenital anomaly of biliary tree, resulting in raised conjugated bilirubin and raised gamma glutamyl transferase (Gamma GT).
87.	b. Breast milk jaundice	A benign condition caused by a factor in the breast milk that promotes an increase in intestinal absorption of bilirubin.

88.	c. Bilirubin	Bilirubin should not be relied upon as a diagnostic marker of liver disease. However, it has a greater role in monitoring the liver disease.
89.	b. ALT	Concentration of ALT is high in cytoplasm of the hepatocytes, where one fraction of AST called cytoplasmic AST is high. In acute hepatitis, there is leakage of the membrane of the hepatocytes which releases both the transaminases in the circulation
90.	a. 5 Nucleotidase	Three enzymes are called membrane-bound enzymes i.e. Alkaline Phosphatase, Gamma GT and 5 nucleotidase. These three enzymes are located on the membranes of the cells lining the biliary canaliculi. When there is cholestasis, the contact time of bile with the cell membrane increases that leads to digestion of fat and release of the membrane chunks in the circulation along with these enzymes.

Summary
Chapter No 7
Liver Function Tests

- In most of our medical set ups four tests are grouped together as LFT i.e. Bilirubin, ALT, Alkaline Phosphatase and Albumin
- Serum bilirubin is found in different fractions. Using common laboratory methods, it can be divided into conjugated bilirubin (also called direct bilirubin) and unconjugated bilirubin (also called indirect bilirubin).
- In neonates, certain degree of unconjugated bilirubinaemia is present, called physiological jaundice
- Commonest cause of pathological unconjugated hyperbilirubinaemia is blood group mis-match i.e. Haemolytic Disease of the Newborn (HDN)
- In some neonates, breast milk causes a transient and benign jaundice
- In adults, Gilbert Syndrome is a very common but quite benign cause of unconjugated hyperbilirubinaemia, while a sinister disease called Crigler-Nijjar Syndrome causes severe and morbid unconjugated hyperbilirubinaemia.
- Dubin Jhonson and Rotor Syndromes are congenital causes of conjugated hyperbilirubinaemia
- In neonates, biliary atresia is a congenital cause of conjugated hyperbilirubinaemia requiring surgical intervention
- ALT is the most sensitive and specific marker of hepatocellular damage. It is preferred over AST because of its specificity to the liver (loyalty-see animation)
- Alkaline phosphatase is a marker of cholestasis along with Gamma Glutamyl Transferase (Gamma GT) and 5-prime Nucleotidase.
- In obstructive jaundice, ALT is raised but rise of alkaline phosphatase is much more marked, similarly in hepatitis, alkaline phosphatase is raised but rise of ALT is more marked.

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Chapter No 8
Disorders of Calcium Metabolism

Reviewed and Edited by

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MCQs

(Please find key at the end of the chapter)

91. The most sensitive modality for the diagnosis of calcium abnormalities in the body is:
    a. Calcium in 24 h urine sample
    b. Plasma ionized (free) calcium
    c. Protein-corrected calcium
    d. Serum albumin-corrected calcium
    e. Serum total calcium

92. A 65 years old male has history of smoking for 40 pack years, presented with cough and haemoptysis for last two months. Some of his biochemical tests were as following:

• Serum Calcium:	3.72 mmol/L	(2.15-2.55)
• Serum Phosphorus:	1.34 mmol/L	(0.81-1.45)
• Plasma iPTH:	<1 pg/mL	(10-65)

What is the most probable diagnosis?
    a. Humoral Hypercalcaemia of Malignancy
    b. Hypervitaminosis D
    c. Hypoparathyroidism
    d. Primary Hyperparathyroidism
    e. Secondary Hyperparathyroidism

93. A 41 years old female had four spontaneous fractures (foot, clavicle, upper arm, forearm) four years ago. She also reported poor appetite, nausea and vomiting every day during the last several months.
Her biochemical profile is as following:

• Na:	141 mmol/L	(136-145)
• K:	4.4 mmol/L	(3.5-5.1)
• Urea:	4.3 mmol/L	(2.1-7.1)
• Ca:	3.84 mmol/L	(2.1-2.55)
• P:	0.81 mmol/L	(0.81-1.45)
• Alkaline Phosphatase:	205 U/L	(42-98)

- Plasma iPTH: 336 pg/mL (10-65)

What is the most important diagnosis in this patient?

a. Parathyroid adenoma
b. Parathyroid hyperplasia
c. Primary hyperparathyroidism
d. Secondary hyperparathyroidism
e. Tertiary hyperparathyroidism

94. A 64 years old male is a known patient of CKD. His recent serum biochemical profile is as following:

- Na: 143 mmol/L (136-145)
- K: 4.4 mmol/L (3.5-5.1)
- Urea: 34 mmol/L (2.9- 6.2)
- Ca: 2.34 mmol/L (2.1-2.55)
- P: 2.81mmol/L (0.81-1.45)
- Alk Phosphatase: 205 U/L (56-119)
- Plasma iPTH: 135 pg/mL (10-65)

What is the most probable diagnosis

a. Parathyroid adenoma
b. Parathyroid hyperplasia
c. Primary hyperparathyroidism
d. Secondary hyperparathyroidism
e. Tertiary hyperparathyroidism

95. A 46 years old female complains of backache and generalized weakness. Her biochemical profile was as following:

- Serum Calcium: 1.72 mmol/L (2.10-2.55)
- Serum Urea: 7.5 mmol/L (2.1-6.2)
- Serum Phosphorus: 2.34 mmol/L (0.81-1.45)
- Plasma iPTH: 36 pg/ml (10-65)

The most probable diagnosis is:

a. Congenital hyperphosphaturia
b. Hypoparathyroidism
c. Renal impairment
d. Renal tubular acidosis
e. Vitamin D deficiency

96. An 11 years old boy was being investigated for incidental finding of hypercalcemia. His lab results revealed:

- Serum Calcium: 3.12 mmol/L (2.15 -2.55)
- Serum Phosphate: 1.24 mmol/L (1.29 -2.26)
- Serum Magnesium: 1.1 mmol/L (0.7 – 0.86)
- Plasma iPTH: 55 ng/mL (10-65)
- 25 (OH) Vitamin-D: 64 nmol/L (25 - 162)
- Calcium creatinine clearance ratio: < 0.01

What is the most probable diagnosis in this patient?

a. Familial hypocalciuric hypercalcaemia
b. Primary hyperparathyroidism
c. Pseudohyperparathyroidism
d. Secondary hyperparathyroidism
e. Tertiary hyperparathyroidism

97. A 50 years old male complained of mechanical back and knee pains of a few years duration. On examination, there was mild restriction of the lumbosacral spine movement and patello-femoral crepitus. There was mild bone tenderness too.
His serum biochemical investigations were as following:

- Ca: 2.34 mmol/L (2.1-2.55)
- P: 0.81 mmol/L (0.81-1.45)
- Alkaline Phosphatase: 835 U/L (53-128)
- 25 (OH) D : 35 nmol/L (25 – 162)
- Plasma iPTH : 43 pg/mL (10 – 65

What is the most probable diagnosis in this patient?

a. Familial hypercalciuric hypoclcaemia
b. Hypothyroidism
c. Paget`s Disease of the bone
d. Secondary hyperparathyroidism
e. Vitamin D deficiency

98. A 70 years old female visits a geriatric clinic for routine evaluation. She is rather short and has mildly low IQ. She had lifelong hypocalcaemia and hyperphosphatemia. Recent values are:

- Serum calcium: 5.2 mg/dl (8.4 – 10.2)
- Serum phosphorus: 6.2 mg/dl (2.5 – 4.5)

- Serum Urea: 32 mg/dl (18-38)
- Serum 25-OH D: 30 ng/ml (15 – 60)
- PTH : 130 pg/ml (15 – 65)

What is the most probable diagnosis?

a. Congenital Hyperphosphaturia
b. Familial hypercalciuric hypoclcaemia
c. Pseudo-psuedohypoparathyroidism
d. Psuedohypoparathyroidism
e. Vitamin D deficiency

99. The term *Vitamin D_3* is used for:
    a. 1,25 dihydroxy vitamin D
    b. 25 hydroxy vitamin D
    c. Active form of any vitamin D
    d. Vitamin D of animal origin
    e. Vitamin D of plant origin

100. The metabolite of *Vitamin D most* commonly measured in the lab for the assessment of vitamin D status is:
    a. 1,25 dihydroxy D
    b. 1-alpha hydroxylase
    c. 25 hydroxy Vitamin D
    d. Cholecalciferol
    e. Ergosterol

Chapter No 8
Disorders of Calcium Metabolism

Three Modes of Calcium Estimation in the Clinical Laboratories

1. Total Calcium
2. Ionized (free) Calcium (Ca2+)
3. Albumin Corrected Calcium

- Ionized calcium estimation has been found to be most sensitive as it is not dependent on albumin concentration[1].
- The facility for estimation of ionized Ca is frequently available but if not present then albumin corrected calcium can be used, which is calculated by following formulae[2]:

Corrected" Ca (mmol/L) = Ca measured (mmol/L) + 0.02(40 - albumin (g/L))

or

Corrected" Ca (mg/dl) = Ca measured (mg/dl) + 0.8(4 - albumin (g/dl))

Causes of Hypercalcaemia[2]

Two commonest causes of Hypercalcaemia:

- Malignancy: in hospitalized patients
- Primary hyperparathyroidism: in ambulatory patients

Other Causes of Hypercalcaemia

- Thyrotoxicosis
- Vitamin D intoxication
- Thiazide diuretics
- Sarcoidosis
- Familial hypocalciuric hypercalcaemia
- CKD (Tertiary Hyperparathyroidism)
- Milk alkali syndrome
- Lithium treatment
- Tuberculosis
- Immobilization
- Idiopathic hypercalcaemia of infancy

Hypercalcaemia of Malignancy[3]

Hypercalcaemia of Malignancy is of THREE types:

1. Humoral Hypercalcaemia of Malignancy (HHM): Tumour secretion of parathyroid hormone-related protein (PTHrP)
1. Localized Osteolytic Hypercalcaemia (LOH): Metastases with local release of cytokines.

2. Increased activated Vitamin D. Tumour produces enzyme 1-alpha hydroxylase which in turn increases activated Vit D, though tumour does not produce activated Vit D itself.

Hyperparathyroidism[4-8]

- Primary hyperparathyroidism is due to adenoma or hyperplasia of parathyroid
- Secondary hyperparathyroidism is a *physiological* response of parathyroid gland to hypocalcaemia bringing serum calcium usually to normal levels.
- Tertiary hyperparathyroidism is due to autonomous state of parathyroid gland that develops after long period of secondary hyperparathyroidism
- In primary and tertiary hyperparathyroidism calcium is very high.

Causes of Hypocalcaemia[1]

- Artefactual (blood collected in EDTA tube)
- Associated with low PTH
    - Hypoparathyroidism
    - Hypomagnesaemia
    - Hungry bone syndrome
    - Neonatal hypocalcaemia
- Associated with high PTH
    - Vitamin D deficiency
    - Disorders of vitamin D metabolism (e.g. 1 alpha hydroxylase deficiency)
    - Pseudohypoparathyroidism
    - Acute Pancreatitis
    - Massive Transfusion with Citrated Blood

Hypoparathyroidism[9]

- In hypoparathyroidism, Ca is decreased while P is increased.
- Vitamin D is required for absorption of both calcium and phosphorus, so both decrease in vitamin D deficiency.
- Thyroid surgery
    - It is the most common cause of hypoparathyroidism.
    - During thyroidectomy, parathyroid gland may be removed by planning or accidentally
    - Any surgery in neck area can cause hypoparathyroidism

Disorders of the Calcium Sensing Receptors (CaSR)[10,11]

Gain of Function Mutation:

- It is also called Autosomal Dominant Hypoparathyroidism (ADH)
- This is due to shifting of Ca-PTH curve to the left, so the Ca level goes very low before a PTH response can ensue.

- Following are the biochemical features of ADH:
    - Hypocalcaemia
    - Inappropriately normal PTH
    - High phosphorous

Loss of Function Mutation:

- The inactivating mutations of the *CaSR* make the parathyroid glands less sensitive to calcium. A higher than normal serum calcium concentration is required to reduce PTH release.
- The mutation may occur in heterozygous or homozygous form and the degree of hypercalcemia in these two disorders reflects a gene dose effect.

Familial Hypocalciuric Hypercalcaemia (FHH)

- This is a heterozygous variety with mild hypercalcaemia because of partial loss of function of *CaSR*
- It has following biochemical features:
    - Mild hypercalcaemia
    - Normal PTH
    - High Mg
    - Urine Ca: Creatinine Clearance < 0.01

Neonatal Severe Hyperparathyroidism (NSHPT):

- This is the homozygous variety of loss of function mutation that manifests in neonates with severe hypercalcemia.
- It has following biochemical features:
    - Severe Hypercalcaemia in neonates
    - High PTH
    - High Mg
    - Urine Ca: Creatinine Clearance < 0.01

Paget Disease of the Bone[12]

- One of the most common diseases of the bones
- Prevalence is upto 9% in some European countries
- Usually asymptomatic
- Bone pain and bone overgrowth are common symptoms in aging population
- High Serum Alkaline Phosphatase (ALP) is the hallmark of the disease
- If bone ALP is not available, liver origin can be ruled out by carrying out gamma GT.
- Bone turn-over markers are also elevated

Pseudohypoparathyroidism (PHP)[13]

- Can occur at any age from infancy to senescence

- Biochemical Characteristics
    - Hypocalcaemia
    - Hyperphosphatemia
    - High PTH

Vitamin D

Two types of Vitamin D[14]

- Cholecalciferol (D3) is of animal origin derived from parent molecule, 7-dehydrocholesterol.
- Ergocalciferol (D2) is derived from plant origin precursor ergosterol.

Sources of Vitamin D

- Sunlight is the best source of Vitamin D_3
- 20 minutes daily exposure of 40 per cent of body to the sun equals to 50 glasses of milk!!!

Control of Vitamin D Synthesis[15]

- Hepatic synthesis of 25-hydroxycholecalciferol is only loosely regulated
- In contrast, the activity of 1-alpha-hydroxylase in the kidney is tightly regulated and serves as the major control point in production of the active hormone.
- The major inducer of 1-alpha-hydroxylase is PTH.
- It is also induced by low blood levels of phosphate.

Vitamin D deficiency (VDD)[16,17]

- Vitamin D Deficiency: Serum 25 (OH) D <20 ng/ml
- Vitamin D Insufficiency: Serum 25 (OH) D 21-29 ng/ml
- Desirable level: Serum 25 (OH) D 30-150 ng/ml
- Vitamin D intoxication: Serum 25(OH) D >150 ng/ml
- These values are not universally accepted and some laboratories in Pakistan (e.g. AFIP Rwp) have lowered the values after a PTH-based study[18].
- This pandemic of inadequate vitamin D (VDD, vitamin D insufficiency VDI) has been found in all age groups even in those who are otherwise healthy and are not prone to deficiency.

Vitamin D- A Hormone?[19,20]

- The discovery of vitamin D receptor (VDR) and 25 (OH) D1- alpha hydroxylase have been found in almost all tissue.
- It has led to the identification of role of vitamin D in many organ systems of the body, instead of merely associated with bone disorders.

- Although called a vitamin, in fact it is a hormone, which synthesized in skin or taken in diet and acts on almost every tissue of body. Vitamin D has been postulated to play role in glucose homeostasis e.g. pancreatic beta cell function, insulin sensitivity and up regulation of transcription of insulin receptor gene.

Table 8.1: Summary of Disorders of Calcium Metabolism

Disorders	**Serum Ca**	**Serum P**	**Plasma iPTH**
Primary Hyperparathyroidism	Increased	Decreased	Increased
Secondary Hyperparathyroidism	Decreased to normal	Normal (Increased in CKD)	Increased
Tertiary Hyperparathyroidism	Increased	Decreased (Increased in CKD)	Increased
Vitamin D Deficiency	Decreased	Decreased	Increased
Vitamin D Excess	Increased	Increased	Decreased
Familial Hypocalciuric Hypercalcaemia	Mildly increased Ca Urine calcium to creatinine clearance ratio <0.01	Decreased	Normal
Neonatal Severe Hyperparathyroidism	Marked increased	Decreased	Increased
Hypoparathyroidism	Decreased	Increased	Decreased
Autosomal Dominant Hypoparathyroidism	Decreased	Increased	Inappropriately Normal
Pseudohypoparathyroidism	Decreased	Increased	Increased

Chapter No 8
Disorders of Calcium Metabolism

MCQs Key with Explanations

MCQ No	Best Option	Explanation
91.	b. Plasma Ionized (free) calcium	Ionized calcium estimation has been found to be the best mode of calcium estimation as changes in albumin concentration does not affect this estimation. Secondly, the results are available instantly as the method and equipment is the same used for electrolyte testing. Many studies have shown it to be a sensitive marker for diagnosing calcium disorders.
92.	a. Humoral Hypercalcaemia of Malignancy	Malignancy is the commonest cause of hypercalcaemia in hospitalized patients. Humoral hypercalcaemia of malignancy occurs due to secretion of PTH related peptide (PTH-rp) secreted as a paraneoplastic syndrome in carcinoma of the lungs. It is called 'Humoral' because it is secreted from one site and acts on other sites like a hormone. In another type of hypercalcaemia, malignant tumour erodes the bones locally and causes release of calcium because of bone resorption (e.g. Multiple Myeloma).
93.	c. Primary Hyperparathyroidism	Primary hyperparathyroidism is mostly due to an adenoma of the parathyroid gland. So, hypercalcaemia, low phosphorous with raised PTH is diagnostic of primary hyperparathyroidism.
94.	d. Secondary Hyperparathyroidism	Secondary hyperparathyroidism is NOT a disease of parathyroid. It is in fact a physiological response of parathyroid to hypocalcaemia caused by renal disease or

		vitamin D deficiency. Raised PTH elevates calcium by releasing it from bones. So, in this condition calcium may be normal or just below the lower normal levels. Please note that secondary hypoparathyroidism is not a pituitary disease (unlike secondary hypothyroidism)
95.	b. Hypoparathyroidism	In this patient, PTH is within reference intervals despite hypocalcaemia. This is because of a congenital condition called "Autosomal Dominant Hypocalcaemia". This is due to 'left shifting' of the calcium-PTH homeostasis curve, meaning that PTH starts it response when calcium is much lower. Molecular basis of the disease is gain of function mutation causing defect in Calcium Sensing Receptors (CaSR) in the bones and other tissues. In hypothyroidism due to surgical damage or ablation, PTH is clearly below the reference interval.
96.	a. Familial hypocalciuric Hypercalcaemia	It is a loss of function mutation in CaSR gene (see MCQ No 95, too). Calcium is not excreted normally and accumulates in the body. The diagnostic finding is decreased calcium creatinine clearance ratio in the urine (please note it is not calcium creatinine ratio).
97.	c. Paget`s Disease of the bone	A common disease in Caucasians, Paget Disease of the bones is characterized biochemically by raised alkaline phosphatase.
98.	d. Psuedohypoparathyroidism (PHP)	PHP is due to resistance of PTH in various organs of the body including bones, resulting into low calcium, high phosphorous and appropriately high PTH. In hypoparathyroidism, low calcium is accompanied by low PTH (surgical removal) or normal PTH (autosomal dominant hypoparathyroidism).
99.	d. Vitamin D of animal origin	This MCQ wipes out a very common misconception about Vitamin D_3. It is NOT the active form that is called vitamin D_3 but it is

		Vitamin D of animal origin (cholecalciferol) as opposed to Vitamin D_2 that is of plant origin (ergocalciferol). Vitamin D made in skin by the action of UV light of sun is also cholecalciferol (Vitamin D3).
100.	c. 25 Hydroxy Vitamin D	When some doctor advises 'Vitamin D estimation', labs measure 25 hydroxy vitamin, that is one of the metabolites of vitamin D formed in the liver and acts as vitamin D store. The next metabolite 1,25 dihydroxy vitamin D (calcitriol) is NOT measured in the labs because of its shorter half-life and because of tight control of its synthesis in the kidney (its level tends to be maintained even if there is deficiency in the stores). If you want to check the 'supply and demand' of a food item, you check its stock in the warehouses and not in the market!!

Summary
Chapter No 8
Disorders of Calcium Metabolism

- Ionized calcium is most sensitive modality of calcium estimation.
- Two most common causes of hypercalcaemia are Hypercalcaemia of Malignancy (HOM) and Primary Hyperparathyroidism (PHP)
- HOM can be due to secretion of PTH-related peptide from certain malignancies (Humoral Hypercalcaemia of Malignancy) or erosion of bones by a malignancy such as multiple myeloma (Localized Osteolytic Hypercalcaemia)
- Hyperparathyroidism can be of three types, PHP is due to a parathyroid adenoma in 80% cases.
- Secondary Hyperparathyroidism (SHP) is a physiological response of parathyroid gland to hypocalcaemia most commonly due to renal impairment. SHP is not a pituitary disease as PTH has no control from pituitary.
- Tertiary Hyperparathyroidism is an autonomous response of parathyroid gland to continuous stimuli as a sequel of SHP.
- Most common cause of hypoparathyroidism is removal of parathyroid gland in surgery but it can also be due to an autosomal dominant disorder
- Disorders of Calcium Sensing Receptors (CaSR) are Familial Hypocalciureic Hypercalcaemia (heterozygous variety) and Severe Neonatal Hyperparathyroidism (heterozygous variety). These two disorders are due to loss of function mutation of CaSR gene.
- Psuedohypoparathyroidism (PHP) is due to generalized resistance to PTH.
- Raised serum alkaline phosphatase is hallmark of Paget`s Disease.
- There is worldwide epidemic of Vitamin D deficiency.
- Vitamin D_3 is cholecalciferol of animal origin, while Vitamin D_2 is of plant origin.
- In the laboratories, 25 Hydroxycholecalciferol is measured for the assessment of Vitamin D status.

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Chapter No 9
Disorders of Iron Metabolism

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MCQs

(Please find key at the end of the chapter)

101. A 32 years old female has generalized weakness and early fatigability. Her lab investigations showed normal haemoglobin but low serum ferritin.
What could be the most probable diagnosis?
    a. Anaemia of chronic disorder
    b. Iron refractory iron deficiency
    c. Non-anaemic Iron Deficiency
    d. Sideroblastic anaemia
    e. Thalassemia trait

102. In iron deficiency, serum iron is decreased while Total Iron Binding Capacity (TIBC) is increased. What is the most likely cause of ***increased TIBC*** in iron deficiency?
    a. Decreased pH of blood
    b. Increased absorption of intestinal iron
    c. Increased ferritin causes increased TIBC
    d. Increased synthesis of transferrin
    e. More empty sites on transferrin

103. A 25 years old female has low haemoglobin. Her ferritin was also low. Your physician colleague has called you to ask this question: “What can be the cause of low ferritin other than iron deficiency anaemia in this patient?” Please choose one best answer:
    a. Acute phase reaction
    b. Concomitant B12 deficiency
    c. Congenital disorders of ferroportin
    d. No other cause
    e. Red cell malignancies

104. The major route of ***iron excretion*** from body is:
    a. Bile
    b. Hair
    c. Kidney

d. Shedding of cells
e. Sweat

105. An oral load of 65 mg of *iron* in a healthy volunteer will cause:
    a. Increased ferroportin
    b. Decreased sensing of hepatocytes to transferrin
    c. Decreased signals from other cells to produce hepcidin
    d. Decreased transferrin saturation
    e. Increased hepcidin concentration.

106. A 29 years old female is a known patient of pulmonary tuberculosis. Her haemoglobin is 10.5 g/dl while ferritin is increased. A research group carried out her hepcidin and found it increased. What is the most important ***role of hepcidin*** in this patient?
    a. Causing peripheral destruction of RBCs
    b. Decreasing erythropoiesis
    c. Increasing iron absorption from the intestines
    d. Increasing iron entry into the macrophages
    e. Trapping iron in the macrophages

107. A 55 years old female is a known patient of rheumatoid arthritis and acid peptic disease. She is to undergo endoscopy to evaluate the ulcerating lesion in the stomach. Which of the following laboratory investigations will be most helpful in assessing her ***iron status?***
    a. Serum ferritin
    b. Serum iron
    c. Serum TIBC
    d. Soluble transferrin receptors
    e. Transferrin saturation

108. Which of the following genetic mutations results into **Hemochromatosis with mildest hepcidin deficiency**:
    a. Autosomal recessive Hemojuvelin (HJV)
    b. Autosomal recessive Hepcidin (HAMP)
    c. Autosomal recessive Transferrin Receptor 2 (TfR2)
    d. Bone Morphogenetic Protein pathway (BMP6)
    e. Heterozygous HFE

109. In **Hemochromatosis** which of the following cells are ***iron-overloaded***:
    a. Bone marrow cells

b. Enterocytes
c. Erythrocytes
d. Hepatocytes
e. Macrophages

110. A 49 years old male patient who is a known case of hepatitis C (HCV-RNA PCR Positive) has following biochemical profile:
    - Serum bilirubin: 32 µmol/L (<17)
    - Serum ALT: 267 U/L (<42)
    - Serum Alkaline Phosphatase: 168 U/L (20-178)
    - Serum ferritin: 2698 ng/ml (Iron Overload > 400)

    At this point of time which of the following investigations will be most appropriate ***first line test*** in this patient:

    a. HFE mutation analysis
    b. Repeat HCV RNA
    c. Serum iron
    d. TIBC
    e. Transferrin saturation

Chapter No 9
Disorders of Iron Metabolism

Iron deficiency anemia (IDA) is a common disorder, affecting about 1.2 billion people worldwide[1]. According to WHO global database on anemia 2005, about one quarter of the world's population had iron deficiency anemia, being most prevalent among preschool children and women[2]. The gold standard for determination of depleted iron stores is lack of stainable iron in the bone marrow, however, this is an invasive procedure[3]. In this chapter, we will discuss conditions of 'Iron Deficiency' and 'Iron Overload'. We will evaluate various laboratory tests used for the diagnosis and monitoring of these two conditions.

Tests for Iron Metabolism[5]

- Serum Iron
- Serum TIBC
- Serum Ferritin
- Serum Hepcidin
- Serum Soluble Transferrin Receptors

1. Iron Deficiency

Clinical Features and Haematological Tests:

Iron deficiency is suspected in a patient with typical clinical features of dyspnea, palpitation and easy fatigability. There may be low haemoglobin; MCV, MCH and MCHC, and examination of peripheral blood smear usually allows the clinician to make a presumptive diagnosis of iron deficiency anemia[4].

Changes in Iron Deficiency[5]

- Serum Iron ------ Decreased
- Serum TIBC----- Increased
- Serum Ferritin--- Decreased
- Serum Hepcidin---Decreased
- Serum Soluble Transferrin Receptors---Increased

Serum Iron, TIBC and Transferrin Saturation

- Serum TIBC increases in iron deficiency because synthesis of transferrin is increased[6,7].
- The specificity and sensitivity of serum iron and TIBC are not good. These tests detect IDA when it is already relatively advanced, i.e. when the iron stores of the body are already significantly depleted or even exhausted[8].
- So, if facility of ferritin is available, iron and TIBC should not be carried out.
- The indications of serum iron are situations like iron poisoning.

- There is a misconception that iron and TIBC are not effected by inflammation etc. Iron and TIBC are determined by the same colorimetric kits and are reportedly negative acute phase reactants. Diurnal variation in circulating iron, also decreases its diagnostic value[9].
- Transferrin Saturation (TSat) is calculated by following formula:
    - *TSat = Serum Iron / Serum TIBC x 100*
- Since TSat is calculated from iron and TIBC, which have low specificity and sensitivity, TSat is also not a good test for diagnosis of iron deficiency. Previously it was a recommended test in patients on hemodialysis but now its role has been questioned in these patients, too[10].

Ferritin[11-14]

- A very good test for the diagnosis of iron disorders
- IDA is the ***only cause*** of low ferritin (Specificity: 100%)

What is the major problem with Ferritin?

- Ferritin is an acute phase reactant i.e. it increases in infections and inflammation
- So, in all patients of IDA, it is NOT low (Sensitivity: 70%)

Three Stages of Iron Deficiency[15]

1. Non-Anemic Iron deficiency: It is three times as common as iron deficiency anemia. Haemoglobin is normal but ferritin is low.
2. IDA: Hb drops
3. Severe Iron Deficiency: Hb drops with symptoms

A Dialogue:

- What is the percentage of iron excreted from the human kidney?

  Humans cannot excrete iron from kidney

- Then how human body gets rid of Iron?

  Excreted with cells shed from skin and intestine etc.

- How Iron is regulated in humans?

  Iron is regulated by absorption of iron in the intestines through hepcidin

Hepcidin[16,17]

- Hepcidin was first discovered as a small bactericidal peptide.

- The name 'hepcidin' originates from the organ of synthesis i.e. liver (hep-) and its antimicrobial activity (-cidin)
- The gene encoding hepcidin (HAMP, 19q13) is expressed in the liver, heart, lungs, brain, spinal cord, intestine, stomach, pancreas, adipocytes, skeletal muscles, testis and macrophages

Mechanisms of Hepcidin Action[18]

- It depends on hepcidin interactions with ferroportin.
- Ferroportin is the only iron exporter, which is expressed on the surface of reticulo-endothelial macrophages, hepatocytes, duodenal enterocytes and placenta cells.
- Hepcidin binds to ferroportin and causes its internalization and degradation in endolysosomes, which in turn blocks the iron transport.
- When iron stores are adequate or high, increased hepcidin expression inhibits intestinal iron absorption, release of recycled iron from macrophages and its transport across the placenta.
- On the other hand, when iron stores are low, hepcidin production is suppressed, thus maintaining iron metabolism homeostasis.

Hepcidin Animated

The Iron Master

Hepcidin, holding a stick in his hand, has been assigned the duty of controlling the porters of iron (Ferroportin). On a normal day (with normal iron levels) the iron master is sleeping in his chamber and the porters keep on doing their job of transporting iron from inside the intestinal lumen to the enterocytes (besides other sites). When body has enough iron stock and does not require more iron from intestine, our iron master (hepcidin) gets a telephone call (from liver) to get up and stop his porters (ferroportin). He takes his stick and put all these porters in a cellar (internalization). In people with haemachromatosis, the iron master is off-sick and there is no body to control the porters, with the result that iron is continuously deposited in the body.

Adapted with thanks from "The Spectrum" PSCP Newsletter, 3rd Issue, Dec 2014.

Hepcidin as a Potential Diagnostic Tool

- Greatest practical application of hepcidin assay is the utilization of hepcidin in diagnosis and monitoring of hemochromatosis.
- Distinguishing Anaemia of Chronic Disease (ACD) from IDA, as it is known that hepcidin production is increased induced by inflammation in ACD and reduced in IDA.
- It must be noted that hepcidin performs just like ferritin (ferritin also induced by inflammation and reduced in IDA), so in an inflammatory setting, hepcidin cannot differentiate IDA from ACD

Iron Metabolism in ACD[19]

- Anemia of chronic disease, or anemia of chronic inflammation, is a form of anemia seen in chronic infection, chronic immune activation, and malignancy.
- In case of a threat to the body e.g. bacterial invasion iron is transferred to the ferritin
- All living organisms and cancer cells require iron for survival
- Just enough iron is made available to make red blood cells but no surplus is left to nourish harmful pathogens.
- There is a modest decrease of Hb in ACD (9.0 -10.5 g/dl).
- Should we treat this anaemia with Iron supplement? **NEVER**

Soluble Transferrin Receptor[20,21]

- The soluble transferrin receptor (sTfR) is a truncated form of membrane-associated transferrin receptor.
- sTfR is present on the surface of all iron requiring cells in the body, with highest number on the surface of precursor erythrocytes.
- The circulating sTfR concentration is proportional to cellular expression of the membrane-associated TfR and inversely related to iron status:
    - Elevates in iron deficiency
    - Decreases in response to iron repletion.

Advantage of sTfR over Ferritin

- It is not an acute-phase reactant thus it can identify iron deficiency in hospitalized and chronically ill patient with:
    - Inflammation
    - Infection
    - Chronic disease

- Other conditions in which ferritin concentration does not correlate with iron status, e.g. Cystic fibrosis

- It thus reduces the need for a bone marrow biopsy or trial of iron therapy

Limitation of sTfR

- Cannot differentiate iron deficiency from ineffective erythropoiesis as it is also increased in conditions of increased erythropoiesis e.g. thalassemia, sickle cell anemia and hereditary spherocytosis

Ratio Between Soluble Transferrin Receptor and Log of Ferritin:

- So far, this ratio is claimed to be the best investigation to differentiate IDA from ACD

2. Iron Overload

HFE Hemochromatosis[22,23]

- Occurs predominantly in Caucasians of European descent
- It is classical type of hemochromatosis, previously called hereditary or idiopathic hemochromatosis
- Transmitted as autosomal recessive trait. Caused by common mutations in the HFE gene. The most common deleterious mutation is C282Y.
- HFE protein associates with the transferrin receptor and modulates hepatic expression of hepcidin
- Thus, abnormal HFE protein caused by common HFE mutations can lead to augmentation of iron absorption by the small intestine
- Clinically significant iron overload occurs almost exclusively in adults but is rarely severe before the fourth decade
- The biochemical penetrance of HFE C282Y homozygosity is fairly high
- More than 50% of homozygotes have elevated transferrin saturation values and/or serum ferritin concentrations and about 10% have elevated ALT
- A high proportion of homozygotes diagnosed in families of probands are seriously affected

Table 9.1: Summary of Biochemical and Genetic Features in Various Disorders of Iron Metabolism

Disorder	Transferrin saturation (%)	Serum Ferritin	Serum Hepcidin	Soluble Transferrin Receptor
Iron Deficiency Anemia	Decreased	Decreased	Decreased	Increased
Iron Loading Anemias (Thalassemia, Sidroblastic, Aplastic or Chronic Hemolytic Anemias)	Increased	Increased	Decreased	Increased
Anemia of Chronic Disease	Decreased	Increased	Increased	No change
HFE Hemochromatosis	Increased	Increased	Decreased	Decreased

Chapter No 9
Disorders of Iron Metabolism

MCQs Key with Explanation

MCQ No	Best Option	Explanation
101.	c. Non-Anaemic Iron Deficiency	Patients with low ferritin and normal haemoglobin are far more in number as compared to those with iron deficiency anaemia. Ferritin is a protein responsible for storage of iron but its concentration in blood reflects status of iron in the stores. As iron decreases in the stores synthesis of ferritin decreases because of post-translational change.
102.	d. Increased synthesis of Transferrin	Transferrin is the protein responsible for transport of iron in the circulation. When there is iron deficiency, Total Iron Binding Capacity (TIBC) i.e. total transferrin increases, because of increased synthesis of transferrin in the liver.
103.	d. No other cause	If ferritin is low in a patient, iron deficiency is confirmed!!! So, there is no cause of low ferritin other than iron deficiency (Clinical Specificity: 100%). But in all patient of iron deficiency, ferritin is not low, because ferritin is also an acute phase reactant (Clinical Sensitivity: 70%)
104.	d. Shedding of cells	Body gets rid of iron mainly by shedding of skin and intestinal cells, as all cells of the body contain iron in enzymes and other substances. Please remember iron is not excreted through kidneys. We all know a delicate iron balance is maintained in the body. How does this balance is maintained while cell shedding cannot be controlled? The next MCQ answers this question!
105.	e. Increased hepcidin concentration	Hepcidin controls iron absorption in the intestines, hepatocytes, macrophages and many other tissues according to the iron requirement of the body. In duodenal enterocytes hepcidin inhibits

		ferroportin when the iron stores in the body are adequate (as exemplified in the MCQ).
106.	e. Trapping iron in the macrophages	Hepcidin derives its name from '*hep*atic' and 'bactri*cidin'.* So, in infection, inflammation or malignancy, production of hepcidin is increased but its main function at this juncture is to trap iron in the macrophages and make it unavailable for the bacteria or the cells. Iron is an essential nutrient for any microorganisms or cells. There is some shortage of iron for erythropoiesis, too, resulting in modest anaemia called 'Anaemia of Chronic Disorder'. In other words, *"Hepcidin destroys the enemy by cutting its supply lines but there is some shortage of food for the local population, too, during this war!!"*
107.	d. Soluble transferrin receptors (sTfR)	sTfR is claimed to be a marker for iron deficiency, that is not affected by the acute phase reaction. It is increased in iron deficiency. Biochemically, this is the part of the receptors, which have been shed from the cell surfaces and circulate in the blood.
108.	e. Heterozygous HFE	Many types of haemochromatosis have been described but most common is 'HFE Haemochromatosis'. Previously called 'Hereditary Haemochromatosis', starts in 4^{th} to 5^{th} decade of life and luckily it is the mildest form, too.
109.	d. Hepatocytes	Hepatomegaly and raised transaminases are characteristic features of haemochromatosis. Accumulation of iron in the hepatocytes is the pathological feature that leads to liver disease.
110.	e. Transferrin saturation	Haemochromatosis should be suspected in a patient with markedly raised ferritin, not otherwise explainable because of acute phase reaction. Transferrin saturation (TSat) > 45% is reportedly a good screening test for haemochromatosis. TSat is calculated by *Serum Iron / TIBC x 100*. The confirmation of the disease requires mutation analysis of the HFE gene.

Summary
Chapter No 9
Disorders of Iron Metabolism

- Non-anaemic iron deficiency is three times more common than Iron Deficiency Anaemia (IDA). So, serum ferritin test should precede haemoglobin estimation in clinical practice.
- Iron, TIBC and Transferrin Saturation are not specific and sensitive tests for the diagnosis of iron deficiency anaemia and are now obsolete.
- Serum iron is a negative acute phase reactant, while TIBC and Transferrin Saturation are technically related to serum iron
- Ferritin has 100% specificity for iron deficiency as there is no cause of low ferritin except iron deficiency, but in all states of iron deficiency, ferritin is not low.
- Ferritin is a positive acute phase reactant that means it is high or normal in the presence infection, autoimmunity and malignancy.
- Hepcidin is a major regulator of iron balance in humans, but hepcidin is also a positive acute phase reactant.
- Anaemia of Chronic Disorder (ACD) is a protective phenomenon of body against infections and malignancy, to make iron unavailable for the invading microorganisms or sinister cells. So, iron supplementation can be dangerous in these conditions.
- So far, best investigation to differentiate IDA from ACD is a ratio between Soluble Transferrin Receptor and log of ferritin

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Chapter No 10

Cardiac Biomarkers

Reviewed and Edited by:

S Sabahat Haider, Sibtain Ahmed

MCQs

(Please find key at the end of the chapter)

111. A 44 years old male patient has reported in A&E of hospital with Myocardial Infarction of 1 hour duration. The cardiac marker which will rise first is:
    a. CK-MB
    b. Creatinine Kinase (CK)
    c. Myoglobin
    d. Troponin I
    e. Troponin T

112. A 49 years old male has retrosternal chest pain of about 6 hours duration. His ECG shows changes typical of Myocardial Infarction (MI) i.e. ST elevation and Q waves etc. As per recent guidelines which of the following is the best strategy:
    a. No lab test is required
    b. Rise and fall of troponin should be documented
    c. Serum myoglobin should be done
    d. Three cardiac enzymes i.e. CK, AST and LD should be done
    e. Troponins should be detected by bedside method

113. The best clinical utility of troponins is in:
    a. Non-ST Elevation Myocardial Infarction- Acute Coronary Syndrome (NSTEMI-ACS)
    b. ST Elevation Myocardial Infarction- Acute Coronary Syndrome (STEMI-ACS)
    c. Type 1 MI
    d. Type 2 MI
    e. Unstable Angina

114. A 62 years old male reported in a CCU with chest pain. His troponin I was markedly raised. He underwent primary angioplasty within two hours. Cardiologist has suspected a fresh attack of MI just 12 hour after the procedure.

How troponins can be helpful to diagnose this second attack of MI?

a. Both troponins I and troponin T estimation
b. Cannot use troponins
c. Five times higher cut-off as in fresh first attack
d. Same criteria as in first attack
e. Troponin repeated after every 30 minutes

115. The advantage of performing troponin over CK-MB is:
    a. Also detects left ventricular failure
    b. Cardiac specificity
    c. Cost effectiveness
    d. Earlier rise
    e. User friendly analysis

116. When used for the diagnosis of any form of MI, the most important problem with hs-Troponins is:
    a. Early fall
    b. Higher Limits of Detection (LOD)
    c. Late rise
    d. Poor sensitivity
    e. Poor specificity

117. Troponins levels are interpreted by comparing the patient results with:
    a. 50^{th} percentile (median values) determined in non-diseased population
    b. 99^{th} Percentile values determined in non-diseased population
    c. Medical decision limits given in the guidelines
    d. Qualitative detection of troponin in urine
    e. Reference values determined by mean and 2SD

118. A 55 years old male admitted in CCU for the management of MI. On 3rd day he had a fresh changes in his ECG which forced his treating Cardiologist to think of fresh attack of MI. Which will be the most appropriate test to rule out this second attack?
    a. CK-MB
    b. LD
    c. Myoglobin
    d. NT-Pro BNP
    e. Troponin I

119. A 71 years old male was admitted in CCU with suspected congestive cardiac failure. The most helpful biochemical marker for the diagnosis of heart failure is:
    a. BNP
    b. Plasma aldosterone
    c. Plasma electrolytes
    d. Plasma renin activity
    e. Troponin T

120. Markers of oxidative stress include:
    a. Adiponectin
    b. C–reactive protein
    c. Myeloperoxidase
    d. Troponins
    e. Tumour necrosis factor α

Chapter No 10

Cardiac Biomarkers

Third Universal Definition of Myocardial Infarction (2012)[1]

a. MI has been redefined by consensus of many international organization.
b. A lot of emphasis is given to troponin assay now

New Criteria

Based on ESC/ACC's redefinition of MI *(JACC, 2000)*

- Typical rise and fall of troponin or CKMB with one of the following:
  - Ischemic symptoms
  - Development of Q wave on ECG
  - ST-segment elevation/depression
  - Coronary artery intervention
- Pathologic (morphologic) findings of AMI

Why 'rise and/or fall' of troponins is essential

- To distinguish MI from other cardiac conditions
- In other forms of cardiac disease, the rise in troponins is usually sustained

Types of MI[2]

The Joint ESC/ACCF/AHA/WHF Task Force classified MI into 5 types based on the underlying cause:

- Type 1 (spontaneous MI): Related to atherosclerotic plaque rupture, ulceration, fissuring, erosion, or dissection with intraluminal thrombus in one or more of the coronary arteries,
- Type 2 (MI secondary to an ischemic imbalance): MI consequent to increased oxygen demand or a decreased supply
- Type 3: unexpected cardiac death before blood samples for biomarkers could be drawn or before their appearance in the blood.
- Type 4a: MI related to PCI
- Type 4b: MI related to stent thrombosis
- Type 5: MI related to Coronary Artery Bypass Grafting (CABG)

Acute Coronary Syndrome (ACS)[2,3]

- The term "acute coronary syndrome" (ACS) refers to a spectrum of conditions that occur due to acute myocardial ischemia and/or infarction because of an abrupt reduction in blood flow through the coronary artery circulation.
- There are three types of ACS: ST elevation (formerly Q-wave) MI (STEMI), non-ST elevation (formerly non-Q wave) MI (NSTEMI), and unstable angina (UA).

ST-elevation MI (STEMI):

- The major discriminating feature of STEMI is the presence of symptoms of myocardial ischemia/injury along with persistent ECG ST-segment elevation in addition to the presence of cardiac biomarkers.

Non-ST-elevation MI (NSTEMI):

- NSTEMI is characterized by symptoms of MI but no ST elevation. An elevated troponin is very useful to differentiate NSTEMI from STEMI

Unstable Angina (UA).

- In this condition symptoms of ischemia occurs at rest without elevation in biomarkers with or without ECG changes indicative of ischemia

Box 10.1: Definition of Myocardial Infarction[1]

Definition of myocardial infarction

Criteria for acute myocardial infarction

The term acute myocardial infarction (MI) should be used when there is evidence of myocardial necrosis in a clinical setting consistent with acute myocardial ischaemia. Under these conditions any one of the following criteria meets the diagnosis for MI:

- Detection of a rise and/or fall of cardiac biomarker values [preferably cardiac troponin (cTn)] with at least one value above the 99th percentile upper reference limit (URL) and with at least one of the following:
  - Symptoms of ischaemia.
  - New or presumed new significant ST-segment–T wave (ST–T) changes or new left bundle branch block (LBBB).
  - Development of pathological Q waves in the ECG.
  - Imaging evidence of new loss of viable myocardium or new regional wall motion abnormality.
  - Identification of an intracoronary thrombus by angiography or autopsy.
- Cardiac death with symptoms suggestive of myocardial ischaemia and presumed new ischaemic ECG changes or new LBBB, but death occurred before cardiac biomarkers were obtained, or before cardiac biomarker values would be increased.
- Percutaneous coronary intervention (PCI) related MI is arbitrarily defined by elevation of cTn values (>5 x 99th percentile URL) in patients with normal baseline values (≤99th percentile URL) or a rise of cTn values >20% if the baseline values are elevated and are stable or falling. In addition, either (i) symptoms suggestive of myocardial ischaemia or (ii) new ischaemic ECG changes or (iii) angiographic findings consistent with a procedural complication or (iv) imaging demonstration of new loss of viable myocardium or new regional wall motion abnormality are required.
- Stent thrombosis associated with MI when detected by coronary angiography or autopsy in the setting of myocardial ischaemia and with a rise and/or fall of cardiac biomarker values with at least one value above the 99th percentile URL.
- Coronary artery bypass grafting (CABG) related MI is arbitrarily defined by elevation of cardiac biomarker values (>10 x 99th percentile URL) in patients with normal baseline cTn values (≤99th percentile URL). In addition, either (i) new pathological Q waves or new LBBB, or (ii) angiographic documented new graft or new native coronary artery occlusion, or (iii) imaging evidence of new loss of viable myocardium or new regional wall motion abnormality.

Criteria for prior myocardial infarction

Any one of the following criteria meets the diagnosis for prior MI:

- Pathological Q waves with or without symptoms in the absence of non-ischaemic causes.
- Imaging evidence of a region of loss of viable myocardium that is thinned and fails to contract, in the absence of a non-ischaemic cause.
- Pathological findings of a prior MI.

Adapted from: Third universal definition of myocardial infarction. Eur Heart J 2012; 33: 2551–2567

Ideal Marker to Detect AMI[4]

- High concentration in myocardium
- Absence from non-myocardial tissues
- High sensitivity & specificity in circulation

- Rapid release into blood following myocardial injury
- Remains in blood several days to allow detection
- Blood levels correlate with extent of myocardial injury and prognosis
- Rapid, simple and automated commercial assays available
- Role designed for marker in diagnosis and management based on clinical studies and peer reviewed literature

Troponins[4,5]

- Regulatory proteins in striated muscle
- Responsible for calcium-modulated interaction
- Exist in several isoforms
- Cardiac specific forms immunologically separable
    - Cardiac Troponin T (cTpnT)
    - Cardiac Troponin I (cTpnI)

Use of biomarkers in AMI / ACS

- Diagnosis
- Detecting myocardial damage whether due to MI or other cardiac process
- Risk-stratifying patients
- Commenting on prognosis in:
    - ACS, pre-and post- primary intervention/ reperfusion therapy
    - Congestive cardiac failure
    - Renal Disease

Question

A 50 years old male was admitted in a hospital with retrosternal chest pain of 6 - hours duration. His body temperature, pulse rate, and blood pressure were 38.2 °C, 115 beats/min, and 80/40 mmHg, respectively. Electrocardiography was suggestive of ACS. Investigations showed:

- Cardiac Troponin I: 214 ng/ml (99^{th} percentile 0.1 ng/ml)
- Echocardiography showed a hypokinetic area

a. What advise you will give regarding cTpn I assay?
b. Name a more advanced technical version of cTpn I that can be used in this patient for better clinical results?

Answers

a. Repeat troponin I after 6 hours to document rise and fall.
b. Highly sensitive troponin I (hs cTpn I).

Problem with Cardiac Troponins[6]

- Highly specific for cardiac origin
- Not specific for MI
- Can rise in any cardiac condition

- Can rise in any cardiac intervention e.g. PCI or CABG

What are the Pre-requisite for cTpn assay?[7]

- Should exceed the 99th percentile of cTn concentrations in a disease-free reference population.
- The CV of the cTn assay used should be ≤10% at the 99th percentile concentration.

Troponin Testing as POCT {on a Device)[3]

- POCT Device for qualitative and quantitative determination of Troponin are available
- Should not be used for the diagnosis of MI
- Laboratory-based troponin assay is the preferred testing option when available

High-Sensitivity Cardiac Troponins (HS-cTn)[8]

- Earlier rule-in and rule-out of acute myocardial infarction (AMI)
- Identification of more AMI patients
- Improvement of risk stratification in AMI
- Prediction of long-term prognosis in non-ACS

Highly Sensitive Troponin Assays

- Abbott–Architect Troponin I
- Roche High-Sensitive Troponin T
- Siemens Troponin I (Ultra)
- Backman Coulter Troponin I (3rd generation)

5th Generation hs Troponin Assay?[3]

- The hs-cTnT assay Elecsys® 2010 analyzer (Roche Diagnostics)
- Analytical range from 3 to 10 000 ng/L.
- 99th percentile value 13.5 ng/L
- CV was 9%
- The assay was found specific for cTnT without interferences from human cTnI or cTnC, skeletal muscle TnT, or hemoglobin concentrations up to 1000 mg/L

What is the difference between Troponin I and Troponin T[6]

- Hardly any
- cTrop I is claimed to be better than cTrop T in patients with renal impairment
- cTrop T is a propriety of Roche Diagnostics
- All other manufacturers make cTrop I

Cardiac Biomarkers in Renal Failure[4,5]

- False positives have been reported with use of troponin-T in ESRD patients but not as much with troponin-I

- CK: plasma concentrations are elevated in 30-70% of dialysis patients at baseline, likely secondary to skeletal myopathy, intramuscular injections and reduced clearance
- CK-MB: 30-50% of dialysis patients exhibit an elevation in the MB fraction >5% without evidence of myocardial ischemia
- Therefore, the most specific marker for suspected AMI in ESRD patients is Troponin-I with an appropriate sequential rise

CK-MB[3]

- An isoenzyme of CK.
- A time honoured marker of MI
- In heart, it is the most abundant fraction
- Skeletal muscle injuries also release a large amount of CK-MB. So CK-MB Index can be used for differentiation.

MB Index

- MB Index = (CKMB /total CK) x 100
- Rationale for using MB Index
    - Using CKMB alone (RR < 25 U/L) often yields false positive results
    - Combined use with MB Index helps to rule-out patients with skeletal muscle injury
    - Recommended Cut Off: 6%

Limitation of CKMB

- False positive incidents in perioperative patients without cardiac injury
- False elevations in
    - Skeletal muscle injury
    - Marathon runners
    - Chronic kidney disease
    - Hypothyroidism
- MI detection not timely enough for thrombolytic intervention. MB peaking takes >12h

CK-MB Getting obsolete:

- CK-MB has no additional advantage in any of the clinical situations.
- So, it is now recommended that only troponins should be used as cardiac biomarkers to curtail the cost of the care[3].

Myoglobin[3]

- Earliest marker to rise after MI
- Can reach a peak just after ONE hour
- Highly non-specific. Can rise in any insult to skeletal muscles
- A negative result of myoglobin can be of some use for excluding MI.

- Collect at least 2 samples within 2h for myoglobin determination

New Cardiac Biomarkers

Heart-type Fatty Acid Binding Protein (H-FABP)[9]

- There is a dire need of a marker which can be helpful in diagnosis of MI in the first 2-3 h as troponins typically peak after 10-12 h of the event.
- H-FABP is a small protein which is released in the circulation very early in an event of myocardial ischemia.
- So, H-FABP is being evaluated as early marker of MI but results are variable.

Pregnancy Associated Plasma Protein A (PAPPA)[10]

- PAPPA was first described as a Maternal Serum Marker
- Shown to be implicated in some other conditions like Coronary Artery Disease (CAD) and sepsis etc.
- First time in Pakistan PAPPA was studied as marker of coronary artery Disease at AFIP Rawalpindi[10]

Markers of Heart Failure (HF)

BNP[11]

- The clinical introduction of testing for the natriuretic peptides, including BNP and NT-proBNP, has fueled interest in the determination of biological standards for diagnosis, prognosis determination, and treatment of HF.
- BNP and NT-proBNP have emerged as the workhorse biomarkers to aid in diagnosing HF (Table 10.1)
- Useful in predicting outcomes
- These markers can also help in guiding treatment

Table 10.1 Distinguishing characteristics of BNP and NT-proBNP

Features	BNP	NT-proBNP
Peptide length	77-108	1-76
Molecular weight	3.5 kd	8.5 kd
Hormonally active	Yes	No
Half life	20 minutes	120 minutes
Clearance mechanism	Neutral endopeptidase receptors	Renal clearance

Newer Biomarkers of Heart Failure[11]

Adrenomedullin

- Peptide of 52 amino acids and a component of precursor, proadrenomedullin, which is synthesized and present in the heart, adrenal medulla, lungs and kidneys
- Potent vasodilator, with inotropic and natriuretic properties
- Production is stimulated by both cardiac pressure and volume overload
- Circulating adrenomedullinis elevated in patients with HF and is higher with more severe HF.

ST2

- Member of the interleukin-1 receptor family secreted by cultured monocytes subjected to mechanical strain with stretch
- Increased ST2 seen with severe HF. In patients with STEMI and dyspnea, ST2 levels were strongly predictive of mortality with HF, increased ST2 during a 2-week period was an independent predictor of subsequent death or the need for cardiac transplantation

Chromogranin A

- A polypeptide hormone produced by the myocardium, with potent negative inotropic properties and elevated plasma levels in patients with HF

Galectin-3

- A protein produced by activated macrophages, plasma levels reported to predict adverse outcomes in patients with HF

Osteoprotegerin

- A member of the TNF receptor superfamily implicated in development of LV dysfunction and predicts survival in patients with HF after AMI

Biomarkers of Oxidative Stress in HF

- Oxidized LDL
- Myeloperoxidase
- Urinary biopyrrins

Chapter No 10

Cardiac Biomarkers

MCQs Key with Explanation

MCQ No	Best Option	Explanation
111.	c. Myoglobin	Myoglobin is the most rapid of the cardiac markers in practice today. It rises in first hour and levels off in a few hours. Its biggest disadvantage is non-specificity, as it is not cardiac specific but can be released from skeletal muscles, too.
112.	b. Rise and/or fall of troponin should be documented	As per recent guidelines (Ref no 1) on Acute Coronary Syndrome (ACS), rise and/or fall of troponin is essential for the diagnosis of myocardial infarction (MI), in addition to typical clinical findings, ECG changes or morphological evidence. The purpose of demonstration of rise and /or fall of troponin is to rule out non-MI causes of myocardial injury e.g. myocarditis and myopathy in which there is a sustained rise.
113.	a. NSTEMI-ACS	Non-ST Elevation MI (NSTEMI) is one of the three components of ACS. Other two are ST Elevation MI (STEMI) and Unstable Angina (UA). In STEMI the diagnosis is quite clear on ECG and clinicians use troponin assay for confirmation of diagnosis and monitoring of the disease. Troponin is probably the only strong evidence of MI in NSTEMI and differentiates it from UA. In UA troponin is normal.
114.	c. Five times higher cut-off as in fresh first attack	Troponin levels can rise in Percutaneous Coronary Intervention (PCI), which is any non-surgical procedure carried out to open the stenosed coronary arteries. How to diagnose an attack of MI during or just after the PCI? In

		the guidelines (Ref No 1) it has been shown that if the levels of troponins are 5 times above the cut-off, then fresh attack should be suspected.
115.	b. Cardiac Specificity	Biggest advantage of troponins (troponin I or troponin T) is cardiac specificity. In fact, these troponins are cardiac specific and written as Cardiac Troponin I (cTrop I) or Cardiac Troponin T (cTropT) because these are genetically distinct from the skeletal muscle troponins. A third troponin called trop C is not used because it is same in cardiac and skeletal muscles.
116.	e. Poor Specificity	Connected to the explanation of MCQ 115 (above), troponins are very organ specific but not disease specific. Troponins rise not only in MI but in any cardiac event, even in any cardiac manipulation e.g. PCI (MCQ No 114) or coronary artery bypass surgery. So, troponins are organ specific but not disease specific.
117.	b. 99^{th} Percentile values determined in non-diseased population	In persons without cardiac disease ('healthy individuals') the cardiac troponin levels are almost undetectable, so usual method of determining reference values using mean±SD cannot be used. Instead the highest value found in the non-diseased population is taken as the cut-off. This is the recommended method for determining the cut-off to be used for comparison of patient results.
118.	e. Troponin I	Previously it was thought that CK-MB has an advantage if used for the diagnosis of a second attack in an already diagnosed case of ACS. The logic was that troponin levels remain high for 10-14 days after the first attack. Now it has been shown that troponin levels peak higher on already raised levels and can be conveniently used for the diagnosis of a fresh

		attack of MI. It is now recommended that laboratories should not waste resources on procurement of any cardiac biomarkers other than troponins. So, no need to keep facilities for CK-MB etc. in the lab (Ref No 3)
119.	a. BNP	BNP or B-type natriuretic peptide (not brain natriuretic peptide) is now widely used for the diagnosis of cardiac failure in a patient who presents clinically with dyspnoea. It is particularly helpful in asthmatic patients who may have a mixed picture of bronchial and cardiac asthma.
120.	c. Myeloperoxidase	This is one of the biomarkers of oxidative stress. These markers may be available in future for routine use for the diagnosis and risk assessment of cardiac disease.

Summary
Chapter No 10
Cardiac Biomarkers

- During recent years, cardiac troponins have completely dominated the cardiac biomarkers profile.
- Cardiac Troponin I (cTpn I) and Cardiac Troponin T (cTpn T) are two troponins now commonly used for the diagnosis and monitoring of Acute Coronary Syndrome (ACS)
- cTpn are especially very useful in diagnosing non-ST elevation Myocardial Infarction.
- cTpn are highly cardiac specific but they are not disease specific. Any cardiac condition or cardiac manipulation such as angioplasty can cause rise of these troponins.
- According to the new definition of Myocardial Infarction (MI), demonstration of rise and fall of troponin is mandatory alongwith other clinical, ECG or morphological criteria.
- A laboratory physician must ensure that the assay of troponins is carried out on an FDA-approved immunoassay system employing Highly Sensitive (HS) troponin kits. POCT systems of cTpn should be discouraged.
- For comparison of patients` results, 99^{th} percentile values of cTpn I and cTpn T should be used instead of reference values.
- The Coefficient of Variation (CV) of the troponin assay should be <10%
- BNP or NT Pro-BNP (both tests are the same with different manufacturers), is a marker for the diagnosis of cardiac failure.

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Chapter No 11
Uric Acid Disorders

Reviewed and Edited by:

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MCQs

(Please find key at the end of the chapter)

121. The most important laboratory finding for the diagnosis of ***GOUT*** is:

    a. Hyperuricaemia
    b. Increased WBC count
    c. Monosodium urate crystals in joint fluid
    d. Pyrophosphate crystals in joint fluid
    e. Very high ESR

122. A 47 years old male frequently develops swelling and pain in his ankle and big toe. His biochemical profile shows:

    - Urea: 5.5 (3.3-6.7 mmol/l)
    - Creatinine: 89 (55-100 µmol/l)
    - Uric Acid: 520 (120-420 µmol/l)

All his haematological and imaging investigations are normal.
His physician has informed him that he has got Gout but there is ***no*** '*secondary cause'* of his disease.
What is the most common biochemical mechanism of hyperuricaemia which can lead to these features in this patient?

    a. HGPRT deficiency
    b. Idiopathic overproduction
    c. PRPP synthetase over-activity
    d. Reduced efficiency of urinary urate excretion
    e. Xanthine oxidase deficiency

123. Which one of the following carbohydrates is known to increase uric acid levels?
    a. Fructose
    b. Galactose
    c. Glucose
    d. Lactose
    e. Sucrose

124. Which pathological process increases the ***production of uric acid***?
    a. Acute renal failure
    b. Chronic renal failure
    c. Hodgkin's lymphoma
    d. Hyperparathyroidism
    e. Hypothyroidism

125. A 62 years old male has pain, redness and swelling of his right ankle joint for the last one month. His synovial fluid has shown some crystals. Examination of these crystals under polarized microscopy has shown ***blue interference*** color when oriented parallel to the slow axis of the plate and a ***yellow color*** when perpendicular.

    What is the most probable diagnosis in this patient?

    a. Gout
    b. Osteo- arthritis
    c. Pseudogout
    d. Rheumatoid arthritis
    e. Septic arthritis

126. A 57 years old male, university professor, developed redness and swelling of the right big toe without a recognized antecedent injury. The affected area was intensely painful and tender. Subsequent investigations confirmed the diagnosis of acute primary gout.
    What should be the ***first line of treatment*** in this patient?
    a. Allopurinol
    b. Colchicine
    c. Corticosteroids
    d. Narcotic analgesics
    e. Non-steroidal Anti-inflammatory drugs (NSAIDs)

127. Which of the following conditions is associated with the most pronounced hypouricaemia?
    a. Hereditary xanthinuria
    b. High dietary intake of fructose
    c. Pre-eclampsia
    d. Vitamin C intake
    e. Von Gierke's disease

128. A 12 years old male has uric acid stones. The most important features to be taken care of in this boy is:

a. Dehydration
b. High protein diet
c. Hyperuricaemia
d. Low urinary pH
e. Oxaluria

129. Thiazide causes hyperuricaemia by which of the following mechanisms?
    a. Competes for the same secretory process by which uric acid is secreted into the proximal tubule
    b. Decreases reabsorption of uric acid in the proximal convoluted tubules of kidneys
    c. Increases absorption of uric acid in the intestines
    d. Increases break-down of purines in various body cells
    e. Stimulates xanthine oxidase to increase the production of uric acid

130. All mammals except human beings (and a few others) do ***NOT*** develop gout because of presence of which one of the following enzymes:
    a. Amidophosphoribosyl transferase
    b. Hypoxanthine guanine phosphoribosyl transferase
    c. Phosphoribosyl pyrophosphate synthetase
    d. Uricase
    e. Xanthine oxidase

Chapter No 11
Uric Acid Disorders

Gout

- Gout is defined as a peripheral arthritis resulting from the deposition of *Monosodium urate (MSU) crystals* in one or more joints[1].
- With increase in aging populations globally, the global burden of gout is rising. This evidence makes gout an important disease to be managed in individuals and in the community[2]
- Gout is a heterogeneous disorder that results in the deposition of uric acid salts and crystals in and around joints and soft tissues or crystallization of uric acid in the urinary tract.
  - Uric acid is the normal end-product of the degradation of purine compounds.
  - Major route of disposal is renal excretion
  - Humans lack the enzyme uricase to break down uric acid into more soluble form[3].

American College of Rheumatology preliminary criteria for the classification of the acute arthritis of primary gout[4]

Diagnosis of Gout is Made if:

1. Characteristic monosodium urate crystals in joint fluid, or
2. Characteristic monosodium urate crystals from tophus, or
3. Fulfilment of ≥6 of the following criteria:

    a. More than one attack of acute arthritis
    b. Maximum inflammation developed within 1 day
    c. Monoarthritis attack, redness observed over joints
    d. First metatarsophalangeal joint painful or swollen
    e. Unilateral first metatarsophalangeal joint attack
    f. Unilateral tarsal joint attack
    g. Tophus (confirmed or suspected)
    h. Hyperuricemia
    i. Asymmetric swelling within a joint on x-ray film
    j. Subcortical cyst without erosions on x-ray film
    k. Joint culture negative for organism during attack.

Classification of Gout[5]

- **Primary gout** is caused by inborn defects in purine metabolism or inherited defects of the renal tubular secretion of urate.
- **Secondary gout** is caused by acquired disorders that result in increased turnover of nucleic acids, by defects in renal excretion of uric acid salts, and by the effects of certain drugs

Stages of Classic Gout[5]

The three classic clinical stages in the natural history of gout are:

1. **Asymptomatic hyperuricemia**
- Very common biochemical abnormality
- Defined as 2 SD above mean value
- Majority of people with hyperuricemia never develop symptoms of uric acid excess

2. **Acute Intermittent Gout (Gouty Arthritis)**
- Episodes of acute attacks.
- Symptoms may be confined to a single joint or patient may have systemic symptoms.
- Abrupt onset of severe joint inflammation, often nocturnal, warmth, swelling, erythema, pain andpossibly fever

3. **Inter-critical Gout**

Symptom free interval between attacks. May have hyperuricemia and MSU crystals in synovial fluid

4. **Chronic Tophaceous Gout / Advanced Gout**
- Results from established disease and refers to stage of deposition of urate, inflammatory cells and foreign body giant cells in the tissues. Deposits may be in tendons or ligaments.
- Usually develops after 10 or more years of acute intermittent gout.

Sites of Acute Gouty Arthritis

- 90% of gout patients eventually have podagra i.e. inflammation of the 1st metatarsophalangeal joints.
- Can occur in other joints, bursae and tendons

Laboratory Diagnosis[6,7]

Serum Uric Acid and Gout:

- Measurement of serum uric acid is the most misused test in the diagnosis of gout.

- The presence of hyperuricemia in the absence of symptoms is not diagnostic of gout.
- In addition, as many as 15% of patients with symptoms from gout may have normal serum uric acid levels at the time of their attack.
- Thus, the diagnosis of gout can be missed if the joint is not aspirated. So:
    - All patients with hyperuricaemia may ***not*** have gout
    - All patients with acute gout may ***not*** have hyperuricaemia

Synovial Fluid Examination

- Definitive diagnosis is only possible by aspirating and inspecting synovial fluid or tophaceous material and demonstrating MSU crystals
- Needle shaped crystals of monosodium urate monohydrate that have been engulfed by neutrophils
- In polarized microscopy, the crystals appear as bright birefringent crystals that are yellow when oriented parallel to the slow axis of plate (negatively birefringent)

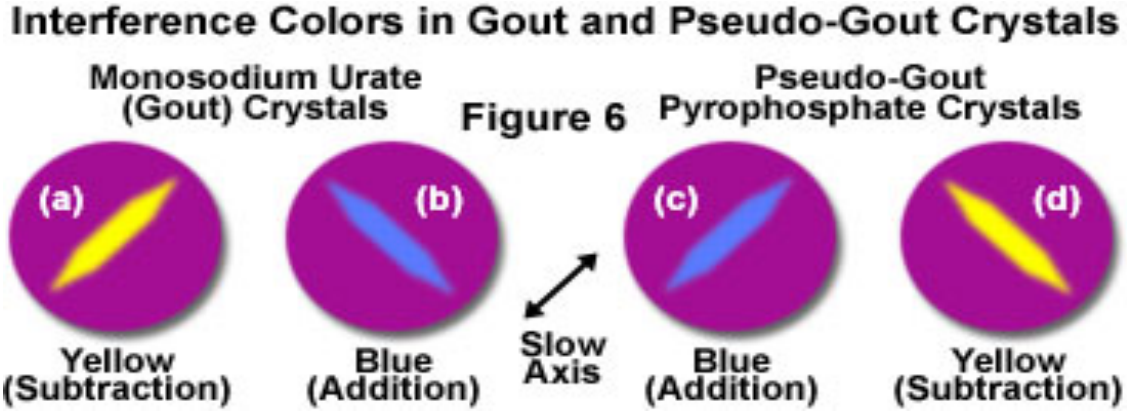

Figure 11.1: Laboratory distinction between Gout and Pseudogout

(Adapted from: http://canacopegdl.com/keyword/pseudogout-crystals.html)

Mnemonic: Double 'L' in yellow and parallel in gout crystals

Differential Diagnosis

- Pseudogout; (Calcium Pyrophosphate Disease)
- Psoriatic Arthritis
- Osteoarthritis
- Rheumatoid arthritis
- Septic arthritis
- Cellulitis

Pseudogout[8]

- Arthritis that is caused by deposits of crystals called *calcium pyrophosphate*
- The crystals that cause pseudogout have a distinct appearance when joint fluid containing them is viewed under a polarized microscope (Figure 11.1).
- Gout and pseudogout can co-exist
- MSU crystals generate a yellow (subtraction) interference colour when oriented parallel to the slow axis of plate (a). Rotating the crystals through 90 degrees changes the interference colour to blue (b).
- In contrast, pseudo-gout pyrophosphate crystals exhibit a blue interference colour (c) when oriented parallel to the slow axis of the plate. A yellow colour (d) when perpendicular.

Primary Gout with No Associated Condition[9]

- Uric acid under secretion (80%–90%)
  - Idiopathic
- Urate over production (10%–20%)
  - Phosphoribosylpyrophosphate amidotransferase (PRPP) over-activity
  - Hypoxanthine Guanine Phosphoribosyltransferase (HGPRT) deficiency
  - Idiopathic

The Most Common Biochemical Mechanism of Hyperuricaemia[10,11]

- Hyperuricemia is due to decreased elimination (under-excretion) (90%). Kidney has increased threshold for uric acid so that excretion is diminished
- Impaired excretion is most often due to abnormalities in the kidney urate transporter called (URAT1) or organic ion transporter (OAT), both of which control the movement of uric acid out of proximal kidney tubules and into urine.
- Idiopathic overproduction cause 10% cases of primary gout.

Secondary Gout[11,12]

With identifiable associated condition

Uric Acid Under-Secretion

- Renal insufficiency
- Polycystic kidney disease
- Lead toxicity
- Drugs
  - Diuretics
  - Salicylates (low dose)
  - Pyrazinamide

- Ethambutol
- Cyclosporine
- Didanosine

Urate Overproduction

- Myeloproliferative disorders
- Lymphoproliferative diseases
- Haemolytic anaemias
- Polycythaemia vera
- Other malignancies
- Psoriasis
- Glycogen storage disease

Fructose and Gout[13]

- Fructose is the only sugar that is associated with gout. In this regard, the most notorious diet is corn syrup containing 45% fructose.
- Very commonly used in Western foods as sweetener, corn syrup has been implicated in causation of gout.

How Fructose is associated with gout?

- Fructose metabolism leads to production of purines and purines produces uric acid and causes gout.
- It has been shown that within minutes after taking high fructose corn syrup-sweetened soda, uric acid levels rise

Urinary Uric Acid[14]

- A 24-hour urinary uric acid evaluation is generally performed if uricosuric therapy is being considered.
- If patients excrete more than 800 mg of uric acid in 24 hours while eating a regular diet, they are over-excreters and thus overproducers of uric acid.
- These patients (approximately 10% of patients with gout) require allopurinol instead of probenecid to reduce uric acid levels.
- Furthermore, patients who excrete more than 1100 mg in 24 hours should undergo close renal function monitoring because of the risk of stones and urate nephropathy.

Uric Acid Stones[15]

- Most children with uric acid calculi do not have hyperuricemia.

- Elevated serum uric acid levels are frequently due to dehydration and excessive purine intake.
- Consistently low urine pH is a risk factor for stone formation. As the urine pH level increases above 5.8, uric acid forms the more soluble urate ion.
- Uric acid stones may be faintly visible on radiograph (due to some amount of calcium)

Drugs and Uric Acid Disorders[16]

- Drugs causing increased production of uric acid
  - Xylitol
  - Theophylline
  - Cytotoxic drugs
- Effect of Salicylates on Uric Acid
  - At low doses (≤2.5 g/day) salicylates cause the retention of uric acid by blocking the OAT1/OAT3 tubular uric acid secretors resulting in hyperuricemia.
  - At high doses (>3 g/day) they cause increased urinary excretion of uric acid by competitively inhibiting reabsorption through URAT 1 in the proximal tubule resulting in uricosuria
- Effect of Thiazide Diuretics on Uric Acid
  - Diuretics reduce uric acid excretion by both directly and indirectly decreasing uric acid secretion and increasing uric acid reabsorption
  - Recent studies have shown that thiazides compete for the same secretory process by which uric acid is secreted into the proximal tubule, thus reducing the clearance of uric acid, and raise the levels of uric acid in the blood.
  - Human organic ion transporter 4 (hOAT4), located at the apical membrane of proximal tubule cells, is responsible for facilitation of hydrochlorothiazide-associated hyperuricemia
- Allopurinol Hypersensitivity Syndrome[17]
  - About 2% of patients develop a rash and 0.4%, predominantly those on thiazides, may experience a severe idiosyncratic reaction known as allopurinol hypersensitivity syndrome. This syndrome is characterized by skin reactions, fever, eosinophilia, and multi-organ involvement with a mortality rate of up to 25%.
  - This syndrome can be confused with acalculous cholecystitis, acute viral hepatitis, leptospirosis and ANCA associated vasculitis because of similar presentation.
  - However, acalculous cholecystitis is associated with gall bladder distention on USG,

- Acute viral hepatitis is unlikely to present with renal impairment,
- Leptospirosis is associated with a clear history of exposure to contaminated water
- ANCA associated vasculitis can be ruled out by a normal urine dipstick test

Uric Acid and Metabolic Syndrome[18]

- Uric acid concentrations have also been found to be positively related to the occurrence of metabolic syndrome and its components.
- Moreover, serum uric acid is being presented in different studies as a potential biomarker for patients with cardio-metabolic risk.
- One feature that is common to patients with metabolic syndrome is an elevated uric acid. Although often considered to be secondary to hyperinsulinemia, recent evidence supports a primary role for uric acid in mediating this syndrome.
- Fructose, which can cause metabolic syndrome in rats, also raises uric acid, and lowering uric acid in fructose-fed rats prevents features of the metabolic syndrome.
- Uric acid can also accelerate renal disease in experimental animals and epidemiologically is associated with progressive renal disease in humans.
- It is proposed that fructose-rich and purine-rich foods that have in common the raising of uric acid may have a role in the epidemic of metabolic syndrome and renal disease that is occurring throughout the world

Hereditary Xanthinuria[19]

- Hereditary xanthinuria is caused by a defect of xanthine oxidase and inherited with an autosomal recessive manner.
- Characterized by hypouricaemia and increased levels of xanthine and hypoxanthine in blood and urine.
- May start at any age.
- Approximately 50% of the patients with classical hereditary xanthinuria present with symptoms of urinary tract infection, hematuria, renal colic, acute renal failure, crystalluria or urolithiasis.
- Diagnosis is based on estimation of uric acid in blood and urine
- There is no curative treatment but low purine diet and high fluid intake is recommended.
- Alkalization is of no value as the solubility of xanthine is not affected by urinary pH.

- When calculi are present, a pyelolithotomy might be necessary. The overall prognosis is favorable, even though, in some cases, the disease progresses to end-stage renal insufficiency

Gout is a Disease of Man and a few other Mammals[9]

- Two factors are responsible for high uric acid levels near to its solubility limit (6.8 mg/dl or 408 μmol/l) in man and few other mammals and only they can develop gout.
- Absence of Uricase Enzyme. Majority of mammals and low primates have very low serum urate levels because uric acid is converted by uricase to more soluble and non-toxic allantoin, which is freely eliminated by the kidneys.
- This absence of uricase enzyme in man and few mammals due to modification of uricase enzyme gene to pseudogene status, results in high UA levels.
- Second is renal reabsorption: Another less important factor is that man and some higher primates also have efficient renal mechanism for reabsorbing filtered urate.

Another Question

A 45 years old male is a known patient of Chronic Kidney Disease (CKD). He has following biochemical findings:

- Urea: 27.8 mmol/L (3.3-6.7)
- Creatinine: 874 µmol/L (55-100)
- Uric Acid: 512 µmol/L (120-420)

Why is his uric acid level not very high although kidneys are important source of uric acid excretion?

Answer:

- Failure of the tubular reabsorption of urate in patients with CKD results in clearance of greater proportion of filtered urate.
- Extra-renal degradation of urate by the action of microbial flora of gut increases markedly from 22-25% as in health to as much as 65% in CKD.

Chapter No 11
Uric Acid Disorders
MCQs Key with Explanation

MCQ No	Best Option	Explanation
121.	c. Monosodium Urate (MSU) Crystals in joints fluid	It is important to note that hyperuricemia is neither essential for the diagnosis of gout nor all patients of gout have hyperuricemia. Demonstrating MSU crystals in synovial fluid drained from the gout affected joint is diagnostic of gout. Due to difficulty of joint fluid aspiration and non-availability of polarized microscopy, diagnosis of gout is mostly clinical (Please see text for description of MSU crystals visualization in the lab).
122.	d. Reduced efficiency of urinary urate excretion	Primary gout, meaning gout without any obvious cause, is due to a congenital defect in excretion of urinary urates in nearly 90% patients. This primary gout is due to a mutation in the genes coding uric acid transporters e.g. urate transporter called (URAT1) or organic ion transporter (OAT) (Ref No 11 for details)
123.	a. Fructose	Increased fructose consumption has been associated with gout in Western Society as fructose metabolism produces purines which in turn is converted to uric acid.
124.	c. Hodgkin's lymphoma	In lymphomas, there is secondary hyperuricemia due to increased cell turn-over
125.	c. Pseudogout	A distinct condition with deposition of Calcium Pyrophosphate (CPP) crystals in the big joints is called pseudogout. (For differentiation of CPP and MSU crystals please see the text).

126.	e. Non-steroidal Anti-inflammatory drugs (NSAIDs)	In acute attack of gout anti-inflammatory drugs like NSAIDS should be given. Drugs to lower uric acid levels e.g. zyloric should be avoided.
127.	a. Hereditary xanthinuria	It is an autosomal recessive disease due to a defect of xanthine oxidase enzyme. It is characterized by hypouricaemia and increased xanthine in blood and urine.
128.	d. Low urinary pH	Uric acid becomes soluble urate in higher urine pH. So, low urine pH is a risk factor for forming uric acid stones.
129.	a. Competes for the same secretory process by which uric acid is secreted into the proximal tubule	Thiazide diuretics cause hyperuricemia because of the decreased secretion of the uric acid in the proximal convoluted tubules of the kidneys. Many other drugs also affect uric acid metabolism (please see text for details).
130.	d. Uricase	In other species, uricase is an enzyme that breaks uric acid to 5-hydroxyisourate, allantoin, urea or ammonia and excreted from the body. In humans and some other mammals this mechanism is lacking due to pseudogenization of the uricase gene in the evolution. So, gout is a disease of humans and some other mammals.

Summary
Chapter No 11
Uric Acid Disorders

- Hyperuricaemia is strongly associated with gout but neither hyperuricaemia is necessary for the occurrence of an attack of gout nor an attack of acute gout will always occur in patients with hyperuricaemia.
- Diagnosis of gout can be confirmed only by demonstration of 'monosodium urate' crystals in the synovial fluid aspirated from the affected joints.
- Gout can be of two types; primary and secondary gout.
- Most common cause of primary gout is under-excretion by the kidneys due to a congenital defect in two transporters of uric acid reabsorption URAT1 and organic ion transporter (OAT) required for secretion or uric acid in the renal tubules.
- Secondary gout can be due to impaired excretion as in chronic kidney disease and over production as in malignancies.
- Many drugs cause hyperuricaemia due to decreased excretion; salicylates have unique dose-dependent action, in low dose it causes retention while in high dose it increases uric acid excretion.
- In children, uric acid stones are very common.
- Hypouricaemia can be due to uricosuric drugs or congenital defect of uric acid synthesis e.g. defect in xanthine oxidase enzyme causing 'hereditary xanthinuria.
- Gout occurs only in humans and a few other mammals because of lack of uricase enzyme which converts uric acid to allantoin in other animals.

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Chapter No 12
Thyroid Disorders

Reviewed and Edited by:

M. Shahzad Anwar, Uzma Ansari, Azeema Ahmed and Sadia Bashir

MCQs

(Please find the key at the end of the chapter)

131. A 26 years old Pakistani female has lethargy and bradycardia. Screening of thyroid dysfunction should be carried out by:
    a. T_3 and T_4 estimation only
    b. TSH and free T_4
    c. TSH and T_4
    d. TSH only
    e. TSH, T_3 and T_4 estimation

132. Serum T4 of a patient decreased from 3.0 pg/ml to 1.5 pg/ml. The expected change in TSH is:
    a. Fifty-fold increase in TSH
    b. One-hundred-fold increase in TSH
    c. Ten-fold increase in TSH
    d. Two-fold decrease in TSH
    e. Two-fold increase in TSH

133. A 65 years old female has following thyroid profile:
    - Serum fT3 2.16 pg/ml (1.60-4.20)
    - Serum fT4 1.34 ng/ml (0.70-1.68)
    - Serum TSH 5.62 mIU/L (0.30-4.0)

    The most probable diagnosis in this patient is:

    a. Non-thyroidal Illness syndrome
    b. Normal thyroid profile for the age
    c. Primary hypothyroidism
    d. Secondary hypothyroidism
    e. Sub-clinical hypothyroidism

134. A 68 years old male admitted with *pneumonia and sepsis* has following thyroid profile.
    - Serum fT3 0.16 pg/ml (1.60-4.20)
    - Serum TSH 0.22 mIU/L (0.30-4.0)

    The most probable diagnosis in this patient is:

a. Non-Thyroidal Illness Syndrome (NTIS)
b. Primary hyperthyroidism
c. Secondary hypothyroidism
d. Sub-Clinical hyperthyroidism
e. Thyroid crisis

135. A 45 years old female has vague symptoms. Her serum TSH done with following result:

Serum TSH: 0.22 mIU/L (0.30-4.0)

All the following can be a cause of this low TSH ***EXCEPT:***

a. Choriocarcinoma
b. Incomplete recovery from hyperthyroidism
c. Nonthyroidal illness syndrome
d. Primary hyperthyroidism
e. Resistance to thyroid hormones

136. A 22 years old female had undergone total thyroidectomy after a diagnosis of thyroid carcinoma. She is on thyroid replacement therapy. Which of the following values constitutes an important part of the treatment goals in this patient:

a. Serum free T3 > 3 nmol/L
b. Serum free T4 > 22 pmol/L
c. Serum TSH 2- 3 mIU/L
d. Serum TSH < 0.10 mIU/L
e. Serum thyroglobulin > 40 mg/L

137. A 24 years old female is pregnant for 10 weeks. She has severe nausea, excessive vomiting, electrolyte disturbances, and weight loss of more than 5% of body weight. She has no goitre or exophthalmos. Her thyroid profile shows:

- Serum Free T3 3.26 ng/ml (1.60-4.20)
- Serum T4 1.80 pg/ml (0.70-1.68)
- Serum TSH 0.12 mIU/L (0.30-4.0)

Most probable diagnosis in this patient is:

a. Gestational hyperthyroidism
b. Grave`s disease
c. Overt hyperthyroidism (requiring immediate treatment)
d. Non-thyroidal illness syndrome
e. Sub-clinical Hyperthyroidism

138. In the United States National Health and Nutrition Examination Survey (NHANES III) carried out on 13,344 people without any history of thyroid disease, which finding was most common:
    a. High TSH and low T4
    b. High TSH and normal T4
    c. Low TSH and high T4
    d. Low TSH and normal T4
    e. Raised thyroid peroxidase antibodies

139. During mandatory screening on 4th day of life, a neonate had following TSH result:

TSH: 33.2 mIU/L

What is the most probable diagnosis in this baby?
    a. Congenital hypothyroidism
    b. Mother treated with thyroxin
    c. Normal TSH for the age
    d. Secondary to hypothyroidism in mother
    e. Thyroid agenesis

140. A 34 years old female underwent TRH stimulation test which showed a peak of TSH at 30 minutes which comes to baseline by 60 minutes.
The patient is most probably having:
    a. Hypothalamic hypothyroidism
    b. Normal Axis
    c. Pituitary hypothyroidism
    d. Primary hyperthyroidism
    e. Primary hypothyroidism

Chapter No 12
Thyroid Disorders

TSH Only Strategy for Thyroid Screening[1-3]

- The measurement of TSH in a basal blood sample by a sensitive immunoassay provides the single most sensitive, specific and reliable test of thyroid status in thyroid disorders.
- Thyroid dysfunctional disorders with normal TSH are very rare.
- So, in many countries 'TSH only' strategy is adopted for the diagnosis.

Reflexive Testing for Thyroid Dysfunction[4]

- Serum TSH normal — no further testing performed.
- Serum TSH high — free T4 added to determine the degree of hypothyroidism.
- Serum TSH low — free T4 and free T3 added to determine the degree of hyperthyroidism.

Some exceptions to TSH only Strategy

Measure serum TSH with thyroid hormones:

a. In a young woman with amenorrhea (e.g. Sheehan`s Syndrome).
b. If the patient has convincing symptoms of hyper- or hypothyroidism despite a normal TSH result.
c. In critically ill patients with strong suspicion of a thyroid disorder.
d. Some other rare situations.

TSH – Animated

TSH –The Worried Mother: A worried mother (TSH) brings her two sick children (T3 and T4) to the child specialist. Now whom should the doctor ask to get details? Mother of course, as children are too innocent to tell about their illness. So, if you want to know something about thyroid disease (dysfunction) ask from mother (TSH) and not from the children (T3 and T4). The anxiety of mother cannot be compared with that of children. Do you agree?

Sensitivity of TSH[5]

(Hormone from Mother Gland)

- If T4 halves, TSH increases by 100-fold or even more.
- If T4 doubles, TSH decreases by 100-fold or even less

Subclinical Hypothyroidism (SHO)[6-8]

- Subclinical hypothyroidism is defined biochemically as a normal T4 concentration in the presence of an elevated TSH.
- Clinical symptoms may or may not be present.
- So, it can only be diagnosed based on laboratory test results.
- It is also called 'Mild Hypothyroid Disease'.
- Causes of SHO are the same as of overt hypothyroidism (High TSH, Low T4).
- SHO is far more common than overt disease e.g. among all hypothyroid 70-80% are SHO.

Treatment of SHO

- Patients with TSH > 8-10 mIU/L should be treated.
- Controversy exists over treatment in patients with TSH 4-8 mIU/L (in both children and adults).
- In patients with TSH 4-8 mIU/L treatment should be considered in pregnancy, symptomatic hypothyroidism , high titer of TPO antibody and in patients with hyperlipidaemia and heart disease, etc.

What is Elevated TSH?[2]

- Upper limit of TSH is important in defining SHO.
- Many surveys have recommended upper limit to be 2.5 mIU/L.
- But consensus is on 4.0 to 4.5 mIU/L.
- A higher upper limit is suggested in very advance age (e.g.> 80 y) but without any agreement. So, 4.0 mIU/L is used as upper limit.

Sub-Clinical Hyperthyroidism (SHE)[11]

- Low serum TSH concentrations (<0.5 mIU/mL) but normal free T4 and free T3 concentrations, a constellation of biochemical findings defined as subclinical hyperthyroidism.
- The term overt hyperthyroidism refers to patients with elevated levels of free T4, T3, or both, and a subnormal TSH concentration.
- Differential diagnosis of SHE (suppressed TSH with normal T3 and T4) include nonthyroidal illness, central hypothyroidism and corticosteroid treatment.

Types of SHE[12]

- Exogenous SHE: is the term used to describe hyperthyroidism caused by ingestion of excessive amounts of thyroid hormone.
- Endogenous SHE: Autonomously functioning thyroid adenomas and multi-nodular goiters are the most common causes of endogenous SHE. Nearly 57% patients with multi-nodular goiters have SHE.

Adverse effects in SHE[13]

- Increased Bone Resorption: i.e. Osteoporosis and susceptibility to fractures
- Cardiovascular Effects: e.g.
    - Atrial Fibrillation
    - Coronary Artery Disease
    - Heart Failure etc.
- Poor Quality of Life: e.g. Disturbances in sleep and decreases in some physical functions
- Dementia

Patients of SHE with Higher Risk[13-14]

- Elderly patients >65 years.
- Patients with risk factors for cardiac arrhythmias.
- Postmenopausal women with or at risk for osteoporosis.

Non-Thyroidal Illness Syndrome (Sick Euthyroid Syndrome)[15,16]

Non-Thyroidal Illness Syndrome (NTIS) (sick euthyroid syndrome) is characterized by:

- Low fT_3
- Low fT_4
- High reverse fT_3 (Figure 12.1)
- Serum TSH — TSH results may show some variations:
    - Low but detectable -(greater than 0.05 mIU/L and less than 0.3 mIU/L) will be euthyroid when reassessed after recovery from their illness.
    - Undetectable — In contrast, approximately 75 per cent of patients with undetectable serum TSH concentrations (<0.01 mIU/L) have hyperthyroidism.
    - High — Some hospitalized patients have transient elevations in serum TSH concentrations (up to 20 mIU/L) during recovery from non-thyroidal illness[17,18].
- In NTIS the enzyme 5-deiodinase is blocked which causes conversion of T_4 to T_3.
- This conversion is inhibited in following three conditions:

1) Acute and chronic nutritional problems
2) Poorly controlled diabetes mellitus
3) Drugs such as hydrocortisone and beta blockers

- NTIS is a protective response of body in chronic illness to reduce metabolism
- Thyroid function should not be assessed in seriously ill patients unless there is a strong suspicion of thyroid dysfunction.
- If you suspect thyroid dysfunction in a critically ill patient then TSH assay may be accompanied by fT_4.
- Critically ill patients with low serum T3 and low T4 SHOULD NOT BE TREATED with thyroid hormone.

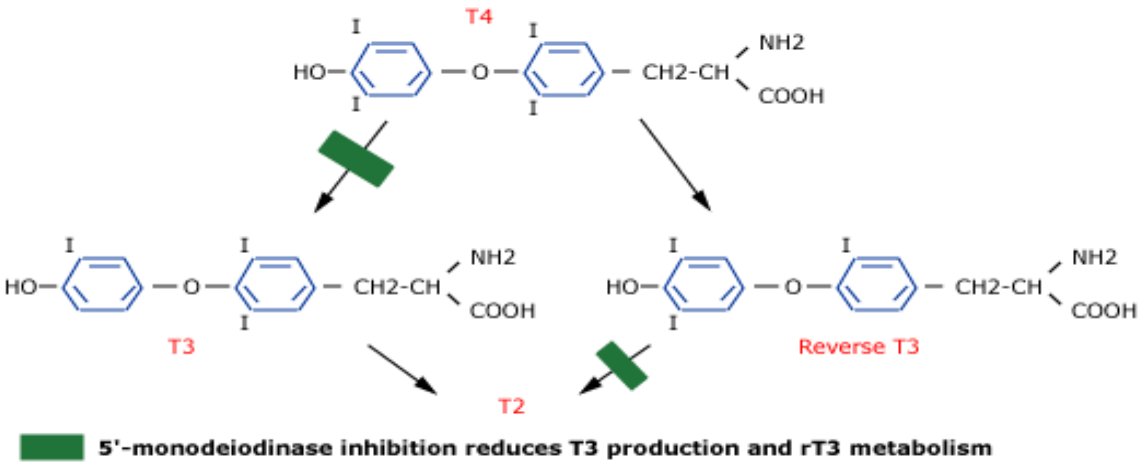


Figure 12.1: Low T3 is common in critical illness[19]

(Adopted from http://www.hormones.gr/723/article/thyroid-function-during-critical-illness%E2%80%A6.html)

Resistance to Thyroid Hormones (RTH)[20]

- Reduced response of peripheral tissues / end organs to thyroid hormones.
- T_3 and T_4 are raised .
- TSH may be raised or normal.
- Differential diagnosis includes TSHoma and familial dysalbuminemic hyperthyroxinemia.

Two types:

- RTH Beta: in which beta receptors are defective.

- RTH Alpha: Much rarer than beta.
- In cardiac tissue, mostly alpha receptors are present, so palpitation may be found in RTH beta.

Thyroid Function Testing in Thyroid Cancer[21]

- TSH should be kept very much suppressed after surgery for thyroid cancer.
- This is done by giving exogenous thyroid hormone.
- British Thyroid Association has recommended "Following initial treatment with total thyroidectomy and radioiodine remnant ablation (RRA), and before evaluation of the patient's response to treatment after 9–12 months, TSH should be suppressed to below 0.10 mIU/l in all patients"
- TSH level suppressed to <0.10 mIU/L.
- The serum fT4 should be elevated .
- So, in these patients TSH and fT4 do not need to be within the 'reference values';
- However, clinical features of over treatment should be noted.
- TSH may provide stimulation of any remnant thyroid secondaries.
- A higher dose of thyroxine is given to the patient to suppress TSH.

Serum Thyroglobulin (Tg) and Thyroglobulin Antibodies (TgAb)

- It is an excellent marker for monitoring treatment of thyroid cancers but only in patients with total thyroidectomy or radio iodine ablation.
- In such patients, detectable serum Tg (>2ug/L) is highly suggestive of residual or recurrent tumour. So, the treatment goal is < 2ug/L (and not the ref values).
- TgAb is recommended to be measured at the same time as Tg to exclude interference of endogenous TgAb in Tg assays
- Tg has no role in diagnosis of thyroid cancer.

Monitoring of Hypoparathyroidism in patients with Thyroid Cancer

- In patients with total thyroidectomy or radio iodine ablation, hypoparathyroidism will be present.
- So, Ca, P and Mg should be monitored and kept within reference values.

Gestational Hyperthyroidism[22]

- This is usually a benign and transient condition due to excessive hCG in first trimester (hyperemesis gravidarum).
- Alpha unit of hCG resembles TSH, so it stimulates thyroid to produce more thyroid hormones
- TSH is suppressed due to negative feedback (Figure 12.2)
- Diagnosis is confirmed in the presence of suppressed TSH and elevated fT4 with no prior history of thyroid disease, no clinical signs of Graves' disease and absent Thyroid Peroxidase (TPO) antibody.

- The condition resolves with the decreased levels of hCG . Anti-thyroid treatment is contraindicated in this condition.

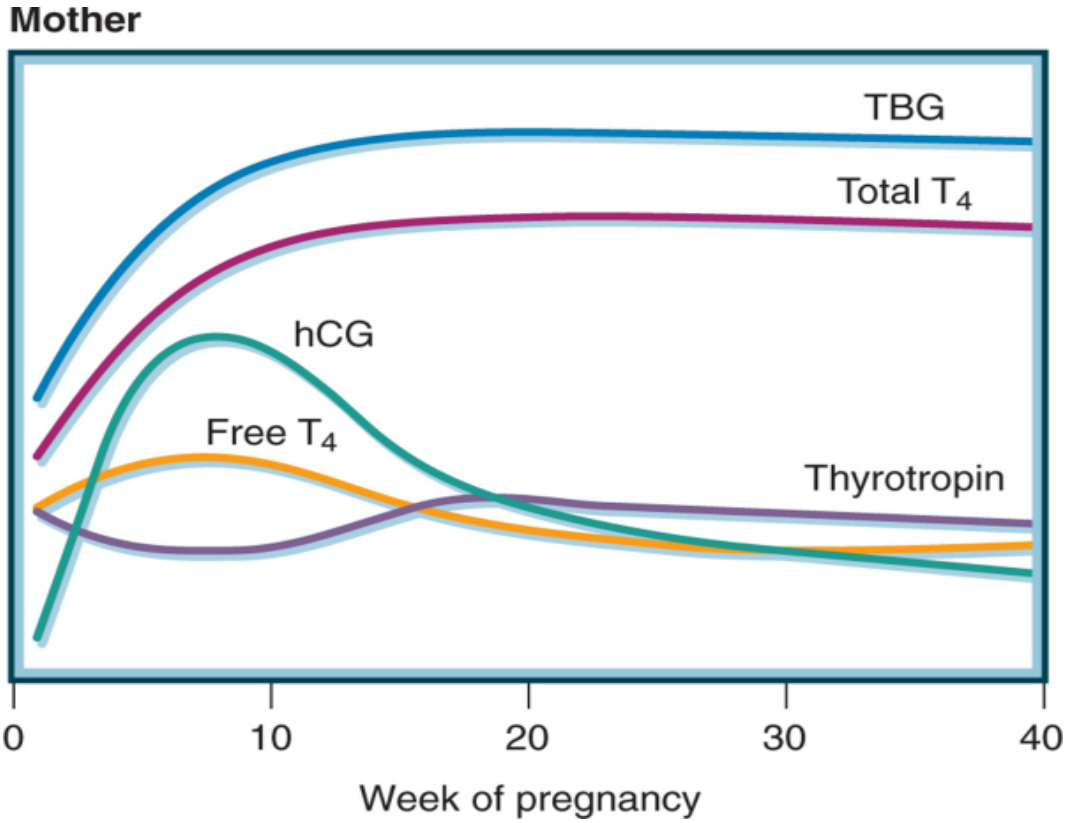


Figure 12.2: Effects of Pregnancy on Thyroid Biochemistry

(Adopted from Thyroid Functions in Pregnancy. https://www.memorangapp.com/flashcards/140014/Week+5,+Block+3/)

Epidemiology of Thyroid Disorders[23]

In the United States National Health and Nutrition Examination Survey (NHANES III), carried out in13,344 people without known thyroid disease or a family history of thyroid disease, the following results were found:

- Sub-clinical Hypothyroidism: 4.3%
- Overt Hypothyroidism: 0.3%
- Sub-clinical Hyperthyroidism: 0.7%
- Overt Hyperthyroidism: 0.5%
- High TPO antibody : 11%

So, it is inferred that TPO antibodies precede biochemical changes and may provide a screening too.

Congenital Hypothyroidism (CH)[24,25]

Clinical Features of CH

The vast majority (more than 95 per cent) of infants with congenital hypothyroidism have few if any clinical manifestations of hypothyroidism at birth. The signs and symptoms may be so subtle that they can be easily missed.

- Constipation
- Lethargy
- Prolonged jaundice
- Hypotonia
- Umbilical hernia
- Large fontanels
- Slow movement
- Hoarse cry
- Feeding problems
- Macroglossia
- Dry skin
- Hypothermia

Cut off limits of TSH for CH

- Normal < 15 IU/L
- Borderline 15 – 30 IU/L
- Hypothyroidism >30 IU/L

Exclusion of Transient CH

- It is important to perform TFT on the mother in cases with abnormal results.
- History of anti-thyroid medication ingestion during the pregnancy should be obtained.
- Exclude the possibility of placental transfer of maternal antibodies that block the action of TSH.

Causes of CH:

- Thyroid Dysgenesis: It is most common cause and includes abnormal thyroid gland development resulting from agenesis, hypoplasia, or ectopy.
- Thyroid hormones synthesis and transport defects
- Thyroid hormone resistance
- Other congenital defects

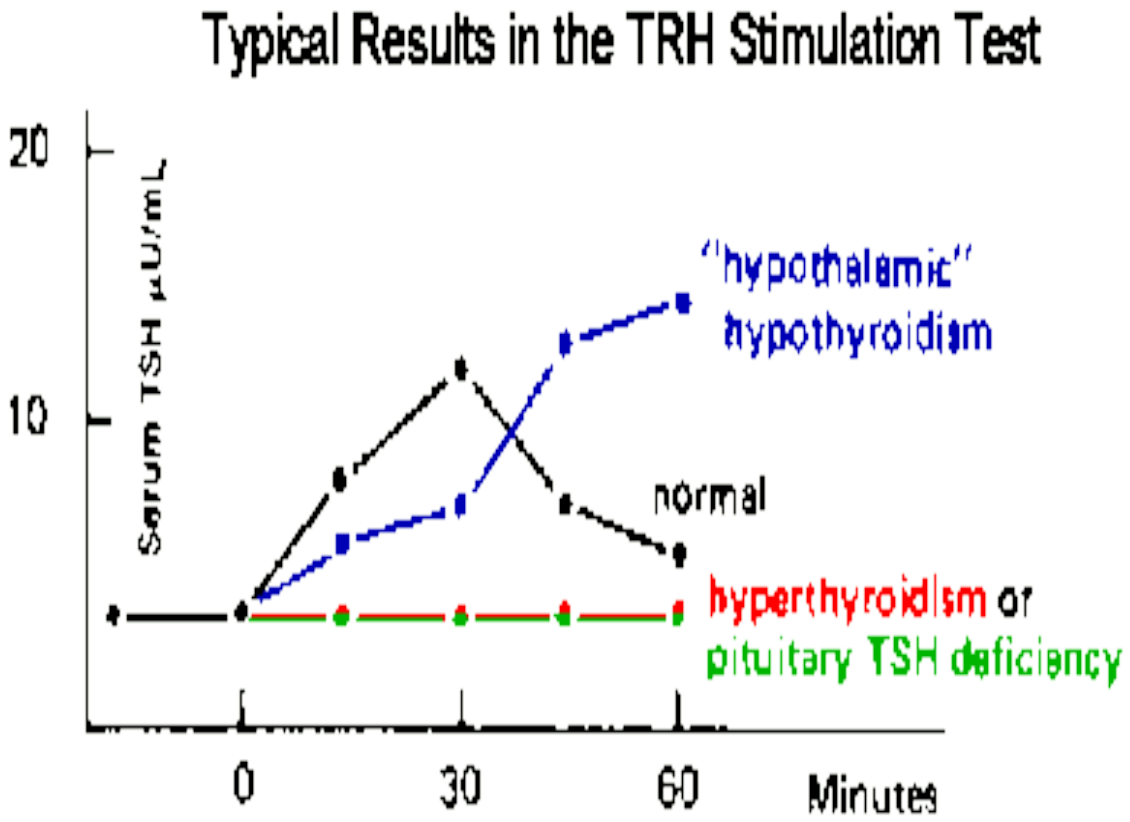
Treatment of CH

- Ideally treatment should be initiated in an infant with a clearly positive screening test as soon as confirmatory blood samples have been drawn, with pending results.
- If screening tests are borderline, a treatment decision can be made after results of the confirmatory tests return.
- Treatment should NEVER be delayed beyond 18 days of life.

- Immediate diagnosis and treatment of congenital hypothyroidism in the neonatal period is critical to normal brain development and physical growth .
- There is an inverse relationship between age at clinical diagnosis and treatment initiation and intelligence quotient (IQ) later in life, so that the longer the condition goes undetected, the lower the IQ
- Treatment for CH is lifelong.

TRH Stimulation Test[5]

- This test is carried out for the diagnosis of a lesion in hypothalamus -pituitary-thyroid axis.
- Thyroid Releasing Hormone (TRH) is injected and samples for TSH estimation are collected at 0, 30 and 60 minutes.
- The pattern of results is given below:



Chapter No 12
Thyroid Disorders

MCQs Key with Explanation

MCQ No	Best Option	Explanation
131.	d. TSH only	With technological improvement in hormone assays, very sensitive TSH assays are now available, carried out on chemiluminescence based auto analysers (Please see chapter 18 for introduction of the technique). So, a normal TSH clearly rules out any thyroid dysfunction. In many countries now, TSH only regime is followed for screening of thyroid functional disorders.
132.	b. One-hundred-fold increase in TSH	TSH is secreted from mother gland i.e. pituitary, that responds vey prominently to changes in the daughter gland (thyroid). It is now established that changes in TSH are more than one hundred folds the changes in thyroid hormones.
133.	e. Sub-Clinical Hypothyroidism	As TSH response to changes in thyroid hormones (T_3 and T_4) is very prominent (MCQ 132), T4 levels may double or half but may remain in the reference values. In this situation TSH may increase or decrease quite prominently. So, increased TSH (Subclinical Hypothyroidism) or decreased TSH (Subclinical Hyperthyroidism) may be seen with normal T4 and T3.
134.	a. Non-thyroidal Illness syndrome	Non-thyroidal illness syndrome, also called sick euthyroid syndrome, is important for interpreting thyroid function tests in a sick patient. First change can be low T3 as body tries to conserve energy by increasing the conversion of T_4 to reverse T_3. Please note that in this MCQ secondary hypothyroidism is

		also a correct answer but not the best one as clinical condition of the patient is more aptly related with option c.
135.	e. Resistance to thyroid hormones (RTH)	RTH is one the more common variety of otherwise rare syndromes of insensitivity to thyroid hormones. The spectrum includes defects of thyroid hormone transport, metabolism and thyroid hormone receptors (RTH). In RTH, T3 and T4 are increased while TSH is normal or increased. The receptor defect is generalized in the peripheral tissues as well as pituitary.
136.	d. Serum TSH < 0.10 mIU/L	In patients of thyroid cancers, when Serum TSH levels are used for monitoring post-surgical thyroid replacement, the levels of TSH are not maintained in reference values but at undetectable values. TSH levels < 0.10 mIU/L is recommendation of British Thyroid Association. The purpose of keeping TSH so low is avoid stimulation of any residual thyroid tissue by TSH and avoid recurrence of tumour.
137.	a. Gestational Hyperthyroidism	In first trimester of pregnancy, hCG level is high. Alpha chain of hCG resembles TSH (and LH and FSH, too). So, high hCG stimulates thyroid to produce more T4 and T3 while Serum TSH level is suppressed as a negative feedback. This condition can get more pronounced in 'Hyperemesis Gravidarum' with higher peaks of hCG. No treatment is required for gestational hyperthyroidism.
138.	e. Raised thyroid peroxidase antibodies	Autoimmune thyroid disease is a very common cause of hypothyroidism. Thyroid Peroxidase (TPO) antibodies are positive in Hashimotos thyroiditis. TPO antibodies are present before biochemical and clinical evidence of hypothyroidism.
139.	a. Congenital Hypothyroidism (CH)	CH is a group of thyroid disorders present at birth due to many of the congenital thyroid defects including thyroid agenesis and metabolic defects of thyroid hormone

		synthesis. CH is an essential part of newborn screening programmes in all the developed countries. Usually TSH is carried out at 2nd to 5th day of life and if raised, immediate treatment with thyroxine should be started to avoid mental retardation.
140.	b. Normal Axis	TRH stimulation test is carried out to diagnose thyroid dysfunctions involving the whole hypothalamus-pituitary- thyroid axis. The test is not commonly used now.

Summary
Chapter No 12
Thyroid Disorders

- Over the last two decades, there is an immense improvement in TSH assay. We can now measure very low levels of TSH with great degree of accuracy and precision. So, diagnosis of hyperthyroidism, in which TSH is very low, is not a problem now.
- TSH should be the first and only test when thyroid dysfunction is to be excluded
- TSH may change 100-folds as a reaction to a two-folds change in thyroid hormones (Please see animated example of mother and child).
- Sub-clinical thyroid disease is the term applied to the situation when TSH is abnormal but T3 and T4 are normal; when TSH is high, it is sub-clinical hypothyroidism and when TSH is low, it is sub-clinical hyperthyroidism. Presence or absence of signs and symptoms is not considered for the diagnosis.
- Non-thyroid illness syndrome is alteration in thyroid hormones or TSH due to a disease other than thyroid gland.
- In pregnancy hyperthyroidism may occur due to high hCG level which mimics TSH.
- While monitoring a patient who has undergone total thyroidectomy due to thyroid malignancy, serum TSH level should not be maintained within reference values but it should be very low.
- Congenital hypothyroidism is a group of disorders that leads to hypothyroidism in newborn, the disorders are usually subtle and have to be screened by testing TSH or T4 after birth.

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Chapter No 13

Other Endocrine Disorders

Reviewed and Edited by:

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Farhan Javed Dar and Shabnum Ramzan

MCQs

(Please find key at the end of the chapter)

Adrenal Disorders

141. A 36 years old female registered nurse is performing night duty for the last 2 years and goes to bed at 0800 h in the morning. She has developed some features suggestive of ***Cushing Syndrome***. At what time of the day (24 h) her sample should be collected:
    a. Afternoon (1500 h)
    b. Early evening (1900 h)
    c. Mid day (1200 h)
    d. Midnight (0000 h)
    e. Morning (0900)

142. Early *diagnosis* of ***adrenal insufficiency*** is of paramount importance to avoid adrenal crisis. Which one of the following investigations should be done at the time of initial presentation:
    a. Estimation of early morning serum cortisol
    b. Estimation of evening serum cortisol
    c. High dose ACTH stimulation test with 250 µg
    d. Low dose ACTH stimulation test with 1µg
    e. Simultaneous estimation of serum cortisol and plasma ACTH

143. A 3 months old baby has presented with ***ambiguous genitalia***. The karyotyping of the baby was found to be 46 XX. Which one of the following is the first most important biochemical investigation in this patient?
    a. Plasma ACTH estimation
    b. Serum 17 hydroxy progesterone estimation
    c. Serum Anti-Mullerian Hormone (AMH) estimation
    d. Serum cortisol estimation
    e. Serum testosterone estimation

Growth Disorders

144. The term ***'Growth Failure'*** is used for which of the following conditions:
    a. Bone age < 20% of chronological age
    b. Height < 2.3rd percentile for the age
    c. Height velocity < 5 cm/y in a 4 years old child
    d. Projected height < 5 cm of mid-parental height
    e. Weight < 3rd percentile for the age

145. On a routine working day 5 children (age 8-12 y) reported in a Growth Clinic. Their preliminary clinical examination and laboratory tests were done for selection of GH testing. Which of the following children is an ideal candidate for GH testing:
    a. Height 4th percentile, Hb: 9.4g/dl; TSH: 2.4 mIU/ml; Anti-gliadin antibodies: negative
    b. Height 5th percentile, Hb: 9.2 g/dl; TSH: 2.4 mIU/ml; Anti-gliadin antibodies: positive
    c. Height 11th percentile, Hb: 13.4g/dl; TSH: 2.4 mIU/ml; Anti-gliadin antibodies: negative
    d. Height 2.5th percentile, Hb: 14.2g/dl; TSH: 2.4 mIU/ml; Anti-gliadin antibodies: negative
    e. Height 7th percentile, Hb: 11.4g/dl; TSH: 10.2 mIU/ml; Anti-gliadin antibodies: negative

146. A 34 years old female suspected of acromegaly has been referred to you to rule out the disease. Which of the following is the single most important first test or screening test to rule out acromegaly in this patient:
    a. CT scan
    b. MRI
    c. Serum GH level basal
    d. Serum Insulin like Growth Factor 1 (IGF-1)
    e. Serum IGF-1-binding protein-3 (IGFBP-3)

Reproductive Endocrinology

147. A 16 years old female presented with very high BMI, abnormal behaviour, low IQ and short stature. On further inquiry, her parents told that she has a voracious appetite. Her puberty is also delayed i.e. no menstrual bleeding, no breast enlargement and absent pubic hair. Her hormonal profile is as following:
    - FSH: 2.0 mIU/mL
    - LH: 1.2 mIU/mL

- Oestradiol: very low
- Progesterone: very low

What is the most probable diagnosis in this patient?

a. Normal profile in non-pregnant
b. Polycystic ovary syndrome
c. Prader Willi Syndrome
d. Primary ovarian insufficiency
e. Turner`s Syndrome

148. A 25 years old female with *Primary Infertility*. Her hormonal profile is as following (LMP not known):
    - FSH: 6 mIU/mL
    - LH: 5 mIU/mL
    - Prolactin: 16 ng/ml (Upper Reference Value: 20 ng/ml)
    - Testosterone: 5.4 nmol/L (0.53 -2.43 nmol/L)

Her Biochemical Profile is indicative of which disorder?

a. Adrenal tumour
b. Cushing syndrome
c. Congenital adrenal hyperplasia
d. Normal Profile in Pregnant
e. Polycystic Ovary Syndrome (PCOS)

149. Clinical situation when result of Beta hCG is ***urgently*** required:

a. Antenatal detection of trisomy 21
b. Detection of ectopic pregnancy
c. Diagnosis of choriocarcinoma
d. Diagnosis of hydatidiform mole
e. Pregnancy test in urine

150. The commonest cause of male infertility is found to be:

a. Anatomical problems
b. Genetic defects
c. Hormonal imbalances
d. Obesity
e. Sperm abnormalities

Chapter No 13
Other Endocrine Disorders

Adrenal Disorders

Serum Cortisol[1]

- Serum cortisol shows excellent diurnal variation-highest in the morning and lowest at mid-night.
- Loss of this diurnal variation is seen in many hypercortisolism states.

Addison Disease (AD)[2]

- Addison disease is chronic hypocortisolism.
- Synthetic ACTH is given to stimulate adrenal gland for the diagnosis of Addison disease.

Timing of Cortisol Sampling for AD[3]

- Morning sample at 0800 hour
- Must be treated as an emergency test

Chronic Adrenal insufficiency

- Inadequate production of glucocorticoids and mineralocorticoids involving destruction of >90% of adrenal cortex
- Secondary adrenal insufficiency: Inadequate secretion of glucocorticoids caused by pituitary insufficiency

Causes of AD[2]

- Idiopathic atrophy (autoimmune adrenalitis)
- Granulomatous disease
  - Tuberculosis (more common in our population)
  - Histoplasmosis
  - Sarcoidosis
- Infectious – viral (AIDS and CMV), fungal
- Neoplastic infiltration
- Haemochromatosis
- Amyloidosis
- Following bilateral adrenalectomy

Lab Investigations

Complete Blood Count:

- Neutropenia
- Relative lymphocytosis

- Eosinophilia
- Mild anaemia

Electrolyte Changes:

- Hyperkalaemia
- Hyponatraemia
- Hypercalcaemia

Other Lab Investigations

- Serum urea
- Serum creatinine
- Plasma glucose
- ABGs

Diagnosis of AD

- Morning serum cortisol < 10 µg/dl
- Plasma ACTH assay > 150 pg/ml
- Dynamic function tests

ACTH stimulation tests[2-4]:

- Short synacthen test
- Long synacthen test

Cushing's Syndrome (CS)[5]

- Iatrogenic is most common cause.
- Other causes are:

ACTH dependent:

- Cushing's disease (70%)
- Ectopic ACTH (15%)

ACTH independent:

- Adrenal hyperplasia (10%)
- Adenoma or carcinoma

Adrenocortical Carcinoma

- Can present as hirsutism
- Characterised by increased metabolites of cortisol and aldosterone
- Increased DHEAS

Timing of Cortisol Sampling For CS

- Late evening sample: To see the loss of diurnal variation i.e. higher cortisol in the evening

- Exact time: One hour after usual sleeping time

Urinary Free Cortisol (UFC)

- 24 h urine sample is required
- Better test in pregnancy and in patients on Oral Contraceptive Pills (OCP) due to increased cortisol binding globulins in these conditions
- Should NOT be done in patients with renal impairment
- In pregnancy dexamethasone test should NOT be done

First line Tests for CS[6]

- Late evening salivary cortisol
- Urinary Free Cortisol (UFC)
- 1 mg Dexamethasone Test (DMT)

Evening Serum cortisol (1 h after sleep)

- Done for confirmation of CS
- Can be done in two ways:
  - Sleeping: Difficult to perform but better method
  - Wakening: by asking the usual time to go to bed

Pseudo-Cushing Syndrome

All the following non-adrenal condition can cause hypercortisolism called Pseudo-Cushing Syndrome:

- Stress
- Obesity
- Depression
- High estrogen state
- Glucocorticoid resistance syndrome
- Drugs (phenytoin, phenobarbital etc.)

Low-dose dexamethasone suppression test[7]

- Oral administration of 0.5 mg dexamethasone every 6 h for 2 days
- Plasma cortisol >5 µg / dl on 3rd day morning ------ hypercortisolism

Determination of the Cause of Syndrome[8]

- Plasma ACTH levels: Plasma ACTH < 20 pg / ml indicates adrenal tumor
- High-dose Dexamethasone suppression test

Overnight 8 mg Test

- Obtain baseline 0800 h plasma cortisol
- Oral administration of 8 mg dexamethasone at 2300 h
- Obtain plasma cortisol on next morning at 0800 h
- Less than 50% of baseline value -------- significant suppression

Two Days Test

- Oral administration of 2 mg dexamethasone every 6 h for 2 days

Congenital Adrenal Hyperplasia (CAH)[9,10]

- Congenital Adrenal Hyperplasia (CAH) is the most important cause of ambiguous genitalia in XX babies
- CAH can be confirmed by 17-hydroxy progesterone (17OHP) estimation.
- In a newborn with ambiguous genitalia and XY karyotyping Androgen Insensitivity Syndrome (AIS) should be suspected.

Growth Disorders

Growth Hormone (GH)[12]

- Most abundant anterior pituitary hormone,
- Produced by the pituitary somatotroph cells, production begins early in foetal life and continues throughout life
- GH secretion is directly controlled by hypothalamic and peripheral factors acting on the somatotrophs.
- Hypothalamic Growth Hormone-Releasing Hormone (GHRH) and somatostatin, stimulate and inhibit GH secretion, respectively
- GH in circulation has half-life of 20 minutes

Factors Causing Increased Release of GH[13]

- GH released in 10-20 pulses throughout the day (basal GH has no value)
- Largest pulse of GH takes place during the beginning stages of sleep
- Exercise
- Fasting (hypoglycemia)
- Protein intake
- Physical and emotional stress
- Hormones like testosterone, estrogens and thyroxine

Insulin-like Growth Factors (IGFs)[12,13]

- There are 7 IGF, IGF-1 to IGF-7
- Most important of IGFs is IGF-1
- The first line test for assessing growth hormone
- Test for screening and treatment monitoring
- Half life is 22 hours
- IGF-1 levels are lower in children, peak at puberty, and gradually decline throughout adulthood.
- IGF-1 reference values must, therefore, be interpreted within the context of the patient`s age.
- Pregnancy and puberty may be associated with abnormally high IGF-1 level.

- Malnutrition, estrogen, hypothyroidism, or liver disease may lower IGF-1 level even in patients with acromegaly.
- Most (approximately 80%) circulating IGF-1 is bound to IGFBP3.
- <1% unbound, remainder bound to other IGF binding proteins.

Insulin-like Growth Factor Binding Proteins (IGFBPs)

- IGFBPs bind circulating IGF with high affinity and specificity
- Functions:
  - Act as carriers of IGF in plasma
  - Prolong the half-life of IGF in circulation
  - Regulate IGF access to receptor in extracellular fluid
- IGFBPs—two main types

1. IGFBP-3

- Most abundant form of IGFBP
- Main carrier of IGF in circulation
- Promotes IGF mediated somatic growth
- High IGFBP-3 associated with growth stimulation

2. IGFBP-1

- Typically, present in small amounts
- High IGFBP-1 associated with growth inhibition

GH Defects

- Overproduction of GH
  - Acromegaly
  - Gigantism
- Underproduction of GH
  - Hypopituitary dwarfism
  - Stress-induced dwarfism
- End-organ resistance

Growth Hormone Deficiency (GHD)[14,15]

- Idiopathic (most common cause in children)
- Damage to the pituitary (most common cause in adults)
  - Radiation
  - Tumor
  - Trauma
  - Ischemic damage
  - Autoimmune inflammation
- Gene mutation (GH, GH receptor, GHBP)
- Congenital malformations

Symptoms of GH deficiency

- Short stature
- Retardation of growth
- Delayed physical maturation
- Delayed bone age

Differential Diagnosis of Short Stature

- Constitutional delay
- Family short stature
- Nutritional
- Malabsorption
- Endocrine
    - Hypothyroidism
    - Hypopituitarism
    - Precocious puberty
- Chromosomal defects e.g. Turner syndrome
- Low birth weight
- Bone developmental disorder
- Metabolic disorder

Growth Failure

- The height-for-age curve has deviated downwards across two major height percentile curves (e.g. from above the 25th percentile to below the 10th percentile).
- Or, if the child is growing slower than the following rates:
    - Age 2-4 years: Height Velocity (HV) less than 5.5 cm/year (<2.2 inches/year)
    - Age 4-6 years: HV less than 5 cm/year (<2 inches/year)
    - Age 6 years to puberty: HV <4 cm/year for boys (<1.6 inches/year) and < 4.5 cm/year for girls (<1.8 inches/year)

Height Velocity (HV)

- The hallmark of pathological short stature is HV
- If HV is normal in a short stature child, it is usually due to a non-pathological cause e.g. Familial Short Stature (FSS) or Constitutional Delay of Growth and Puberty (CDGP)
- If HV is grossly low all pathological causes should be excluded before starting GH investigations.

Diagnosis of GHD

Following are important:

- Detailed history
- Detailed physical examination
- Growth velocity to rule out constitutional delay
- Length / height for age of child
- Weight for age exclude malnutrition / malabsorption
- Growth charts to calculate percentile
- Mid parental height (height of both parents)
- Target height

Laboratory Investigations to Exclude Other Cause of Short Stature

- Blood CP
- Chest X-ray
- Urine R/E
- Stool R/E
- Biochemical profile
- Bone profile
- TSH
- Karyotyping

Delayed or Advanced Bone Age

- Delayed or advanced bone age is defined as 2 SD or more below or above the mean, respectively.
- This is approximately 20 percent below or above the chronological age.
- This translates to a difference between bone age and chronological age of approximately 12 months between 2 and 4 years of chronological age, 18 months between 4 and 12 years, and 24 months after age 12.
- If the bone age is delayed or advanced near or beyond these parameters, then the projected height should be recalculated based on the bone age rather than the chronological age.
- The most important point to differential between FSS and CDGP is bone age. In CDGP bone age is delayed while it is according to chronological age in FSS.

Calculation of Predicted (Target) Height from Mid-Parental Height

- Boys: (father height in cm + mother height in cm + 13 cm)/2
- Girls: (father height in cm + mother height in cm - 13 cm)/2

Growth Hormone Stimulation Tests[15]

- Insulin tolerance test (ITT) “The Gold standard”
- Arginine
- Exercise

- L-DOPA
- Clonidine

GH Insensitivity (GHI)[14]

- Characterized by elevated level of GH, accompanied by decreased level of IGF-1, IGFBP-3 (Figure 13.1)
- Most common form of insensitivity is Laron Syndrome 300 cases have been reported worldwide.

Figure 13.1: Siblings with Growth Hormone Insensitivity Syndrome

(Picture taken and placed after explicit parental consent)

Growth Hormone Excess

Gigantism

- Pituitary gigantism refers to growth hormone (GH) excess that occurs before fusion of the epiphyseal growth plates in a child or adolescent

Acromegaly[16]

- GH excess that begins in adulthood is called acromegaly
- Prolonged exposure to GH excess causes an overgrowth of skeleton and soft tissue
- GH secreting pituitary tumors account most cases of acromegaly

Causes of Acromegaly

- Hypothalamic GH-releasing hormone (GHRH) excess
- Ectopic source of GH or GHRH
- Dysregulation of neuroendocrine control resulting in a diminished inhibitory constraint on GH secretion

Clinical Features

- Acromegaly occurs due to persistently increased GH production, usually because of a pituitary adenoma.

- Excess GH stimulates hepatic secretion of IGF-I, which causes most of the clinical manifestations of acromegaly including:
  - Acral and soft tissue overgrowth
  - Joint pains
  - Diabetes mellitus, hypertension
  - Cardiac failure
  - Respiratory failure

Growth Pattern

- Excessive GH secretion in adults is insidious in nature.
- Dramatic linear growth acceleration in children.
- In other growth parameters, mild to moderate obesity accompanies tall stature
- The abnormal height growth typically precedes or is concurrent with rapid weight gain in children with GH excess.
- Progressive macrocephaly is also seen in children with gigantism
- Large hands and feet, tightening of rings or change of shoes size can occur
- Coarsening of the facial features with frontal bossing and prognathism (projection of the jaw)
- Excessive sweating
- Coexisting endocrinopathies are common in adults

Clinical Presentation

- Soft tissue overgrowth
- Articular overgrowth
- Bone density is increased
- Cardiomyopathy
- Menstrual dysfunction
- Fatigue and weakness
- Sleep apnea
- Neuropathy
- Hypogonadism
- Hyperglycemia
- The clinical diagnosis is often delayed because of the slow progression of disease.
- Biochemically, elevated GH and IGF-1 levels are required for the diagnosis.

The Ideal Screening Test of Acromegaly[17]:

- Unlike growth hormone, serum IGF-I concentrations do not fluctuate but instead reflect integrated GH secretion during the preceding day or longer.
- IGF-1 has much longer half life (18-20 h) than GH (15-20 min)

- Serum IGF-I concentrations are elevated in virtually all patients with acromegaly and provide excellent discrimination from normal individuals
- The most specific dynamic test for establishing the diagnosis of acromegaly is OGTT with 75 g glucose; with GH measurement, up to 120 min.
- GH cannot be suppressed in the presence of liver failure, kidney failure, poorly controlled diabetes, malnutrition, anorexia, pregnancy, oestrogen therapy or in late adolescence.
- Cut off limits for diagnosis are variable depending on type of measurement technique.

Biochemical Findings

- Nearly 30 % of acromegaly will have GH level within reference values
- Nearly 20-50% have elevated IGF-1 level
- IGF-1 best test for screening and treatment monitoring for GH excess
- IGFBP-3 is not a sensitive as IGF-1,
- If IGF-1 and IGFBP-3 are combined, sensitivity is increased

GH Suppression Test (OGTT GH)

- Basal GH
- Then give 75gm oral glucose
- GH after 2 hours
- Paradoxical rise of GH rather than suppression i.e. there is increased level from basal is the diagnostic feature of acromegaly.
- Nearly 20% of the acromegaly will have normal OGTT GH suppression, while their IGF-1 is elevated
- Gold standard for making a definitive diagnosis, should be performed if screening tests (IGF-1) suggests GH excess.
- Measurement of GHRH may also be useful because its elevation is suggestive of ectopic GHRH secretion.

Criteria for Normal Result and Acromegaly (Teitz)[12]

Based on 2 h value after ingestion of 75 g glucose

- Suppression to < 1ng/ml (or 1 µg/L)
- Failure of Suppression i.e. > 2 ng/ml (or 1 µg/L)

Criteria for Normal Result and Acromegaly (UpToDate)[17]

Based on 2 h value after ingestion of 75 g glucose

- Suppression to < 0.3 ng/ml (or 0.3 µg/L)
- Failure of Suppression > 0.3 ng/ml (or 0.3 µg/L)

Figure 13.2: Primordial Dwarfs

(Picture taken and placed after explicit parental consent)

Primordial Dwarfs[18]

- A rare genetic disorder with normal growth hormone secretion and function
- Very small but proportional height
- Typical faces with microcephaly (Figure 13.2)

Reproductive Endocrinology

Hypogonadotropic Hypogonadism[19,20]

- Hypogonadotropic hypogonadism (HH) is characterized by absent or decreased function of the female hypothalamus, pituitary and ovarian hormone.
- Inappropriately low serum concentrations of LH and FSH, which is an effect of GnRH deficiency.
- HH is caused by number of pathological processes, but it can occur as a part of various congenital syndromes.
- HH in which secondary causes had been excluded is called idiopathic HH

Congenital HH

- Idiopathic Hypogonadotropic Hypogonadism (IHH)
  - Kallmann syndrome
  - Adult onset IHH
  - Fertile eunuch syndrome
  - Adrenal Hypoplasia Congenital
- Genetic defects of the gonadotropin subunits
- HH associated with other pituitary hormone deficiencies
- HH associated with obesity
  - Prader-Willi syndrome

- Laurence- Moon-Biedl syndrome

Acquired HH

Structural

- Craniopharyngiomas
- Pituitary adenomas (e.g. prolactinoma, non-functioning tumour)
- Glioma, meningioma
- Infiltrative disorders
  - Sarcoidosis, hemochromatosis, histiocytosis X
  - Head trauma
  - Radiation therapy
  - Pituitary apoplexy

Functional

- Exercise
- Dieting
- Anabolic steroids
- Glucocorticoid therapy
- Narcotics
- Critical illness

Idiopathic HH (IHH)

- IHH also called isolated GnRH deficiency, caused by selective impairments of the hypothalamic-pituitary axis resulting in inappropriately low gonadotropin secretion with otherwise normal pituitary function.
- Characterized by a failure of initiation of puberty due to insufficient gonadotropin release, thus resulting in failure to develop secondary sexual characteristics and a mature reproductive system.
- 30% of IHH cause by genetic defects
- Estimated prevalence of IHH 1 in 4,000 to 1 in 10,000 males
- Females are effected 2-5 times more than males

Kallman Syndrome

- IHH was first reported with anosmia called Kallman syndrome (KS)
- When embryonic migration of GnRH neurons from the nasal placode to their final destination in the hypothalamus is disturbed, resulting phenotype is Kallman syndrome (KS) characterized by HH and anosmia.
- Normosmic IHH (nIHH) has been observed

- Both chromosomal and single gene mutation have been identified in patient with IHH / KS

Diagnosis of IHH / KS

- Presence of both suggestive clinical findings and laboratory findings and absence of secondary causes of HH.
- Detailed history and physical examination with the assessment of secondary sexual characteristics, and family history
- Assessment of olfaction to detect hyposmia, examination of outer ear and hearing to rule out mild CHARGE syndrome
- In nIHH can be revealed by isolated chronic an ovulation, whereas oestradiol secretion is almost normal.
- Ultrasound pelvis for uterus and ovaries
- MRI brain and olfactory bulb
- Renal ultrasound

Laboratory Diagnosis

- Serum oestradiol < 40 pg/ml (110 pmol/L)
- Serum LH <10 IU/L
- Serum FSH <10 IU/L
- GnRH stimulation test

Primary Ovarian Insufficiency (POI)[21,22]

Synonyms

- Premature Menopause
- Premature Ovarian Failure
- Primary Ovarian Failure
- Primary Ovarian Insufficiency

Salient Features

- Affects 1% of women
- Age < 40 years
- May not be permanent
- Spontaneous recurrence of ovarian function and even pregnancy are possible

Diagnosis

- Amenorrhoea
- Climacteric Symptoms
- Raised FSH - Reduced Oestradiol
- Biopsy unhelpful

Causes of Premature Menopause

- Idiopathic
- Chromosomal abnormalities
- Turner's syndrome., X chromosome deletions
- Autoimmune
- Galactosaemia
- Radiotherapy / chemotherapy
- Surgery of the genital system
- Infection - mumps, TB.

The Hormones During Pregnancy[23]

- Oestradiol is increased thousands of folds (in ng/ml rather than in pg/ml in non-pregnancy)
- FSH and LH are markedly reduced
- Placental hormones like hCG, hPL, relaxin, inhibin are increased

Indications of Beta hCG estimation[23,24]

- Detection of pregnancy
- Diagnosis of ectopic pregnancy (as an emergency test)
- Diagnosis and monitoring of abortion (may be an emergency test)
- Tumour marker for hydatidiform mole and choriocarcinoma
- Tumour marker for testicular tumours
- Part of the maternal *triple test.*

Hyperglycosylated hCG (hCG-H)[25]

- An emerging marker related to hCG
- More useful in the diagnosis of pregnancy and Gastric Trophoblastic Disease (GTD).
- Higher percentage of hCG-H indicates more invasive nature of GTD.

Maternal Screening[26]

- Triple Test (B-hCG, a-fetoprotein and estriol) is an example of maternal screening used in developed world.
- Triple test is carried out in maternal serum of all expectants to diagnose foetal abnormalities and placental well-being.

21st Day Serum Progesterone Test[27]

Question: Which is the exact ***day of the menstrual cycle*** when serum progesterone should be tested for assessment of ovulation?

Answer

- Day 21 of the Menstrual Cycle
    - It should really be taken 7 days post ovulation
    - If the cycles are not regular – take sample in the next cycle 7 days before the date of last month bleeding.
    - Excellent replacement of D&C

Question: What ***cut-off value*** of serum progesterone is indicator of ovulatory cycles?

Answer:

- If > 10 ng/ml -ovulatory
- On a medicated cycle >15 ng/ml
- Should be repeated in women > 30 y

Serum Prolactin[28]

Question: Serum Prolactin level in a 24 years female is 245ng/ml (Upper Reference Value: 20 ng/ml). Name ***Physiological*** causes which can cause this much high Prolactin level.

Answer:

- Pregnancy (35 to 600 ng/ml): It is due to very high oestradiol during pregnancy
- First 4-6 weeks of suckling: The level can be as high as in pregnancy. It is again due to high (but dwindling) Oestradiol in post-partum period. With decreasing oestradiol, prolactin decreases and in major part of lactation it remains only mildly increased
- In all Physiological and Associated Pathological causes (e.g. hypothyroidism) there is mild hyperprolactinaemia.

Macroprolactinaemia[29]

- Hyperprolactinaemia without physiological or pathological causes may be because of elevated circulating macroprolactin (isoform/polymeric form of prolactin) have lack of biological activity
- These large molecules are difficult to be excreted and therefore leads to increased but inactive prolactin.
- Precipitation of macroprolactin in the sample with polyethylene glycol (PEG) is the most common laboratory approach to look for presence of macroprolactin.
- Gel filtration chromatography has been used to separate the prolactin isoform but PEG is more practical and efficient.

Hirsutism and Polycystic Ovary Syndrome (PCOS)[30]

- Hirsutism is the commonest clinical presentation of PCOS
- In about 90% cases, the cause of hirsutism is PCOS.
- Menstrual abnormalities and infertility are also common presenting features but there are other causes to be ruled out to reach the diagnosis.

Diagnosis of PCOS[31]

Rotterdam criteria (2003)

Diagnosis of PCOS is made if two out of three criteria are present

1. Chronic oligo-ovulation or anovulation
2. Hyperandrogenism
3. Polycystic ovary morphology

Androgen Excess Society Criteria (2006)

All the following:

1. Hyperandrogenism (clinical or biochemical)
2. Ovarian dysfunction (oligo- or anovulation)
3. Polycystic ovarian morphology
4. Exclusion of other androgen excess or related disorders

Androgen Excess and PCOS Society 2009 requires the simultaneous presence of:

1. Hyperandrogenism (clinical and/or biochemical)
2. Ovarian dysfunction (ovulatory dysfunction)
3. Polycystic ovarian morphology

Oligo- / Anovulation

- Irregular menses, peri-menarchal onset
- < 9 menses / year; cycle length varies > 2d
- Raised prolactin, LH, FSH, and androgens
- Hirsutism, acne, androgenic alopecia
- Gradual onset, stable, chronic

Ovarian morphology

- Helpful for diagnosis if only one criteria present
- Evaluate endometrial thickness
- Ovarian Ultra-Sound Scan (Optional)

PCOS and Metabolic Syndrome[32]

- PCOS is closely associated with metabolic syndrome (43 % in USA, 37.5 % in India and 24.9 % in China)

Male Reproductive Hormones

- FSH-Inhibin axis is a marker of Sertoli cells
- Leydig cell function can be assessed by LH-testosterone axis.

Regulation of Sex Hormone Secretion in Males[27]
Hypothalamus Releases

GnRH or LHRH stimulates pituitary to release LH and FSH.

- In males, LH is also called Interstitial Cell Stimulating Hormone (ICSH) to produce testosterone
- FSH to stimulate sperm cell formation
- Inhibin inhibits FSH secretion from anterior pituitary

Puberty and Testosterone[27]

Puberty

- Age at which individuals become capable of sexual reproduction
- Before puberty small amounts of testosterone inhibit GnRH release
- During puberty testosterone does not completely suppress GnRH release, resulting in increased FSH,LH, and testosterone

Testosterone

- Produced by interstitial cells, adrenal cortex and sustentacular cells
- Causes development of male sex organs in embryo, stimulates descent of testes, causes enlargement of genitals and necessary for sperm cell formation

Three Tiers of Organization

- **Hypothalamus:** GnRH (gonadotropin-releasing hormone)
- **Pituitary gland**: LH (luteinizing hormone), FSH (follicle-stimulating hormone)
- **Testis:**
    - **LH**: stimulates **Testosterone** production by **Leydig** cells in **interstitium**
    - **FSH**: supports **spermatogenesis** by stimulating **Sertoli** cells in the **seminiferous epithelium**
- **Marker of Testicular Function**[33]
    - **Testosterone**
    - **Inhibin B** as marker for impaired Sertoli cell function
    - **Activin:** secreted by Sertoli cells, stimulate transcription of FSH B subunit

Male Reproductive Axis

- **Hypothalamus:**
- **GnRH** - 3 types of rhythmicity
    - Seasonal (in months) – peaks in spring
    - Circadian (in hours) – --highest testosterone levels in AM
    - Pulsatile (in minutes) – peaks occur every 90 - 120 minutes
- Melatonin: modifies seasonal and circadian rhythms from inputs from pineal gland (seasonal) and neural connections (circadian) from suprachiasmatic nucleus (mammalian 24-hour clock)
- Precursors of GnRH neurons migrate to hypothalamus from olfactory placode during development

Development of Male Reproductive Axis

- Sex-Determining Region on Y chromosome (SRY) controls early testis differentiation
- *SRY* gene product, a transcription factor (TF) acts with other TFs (*WT-1, SOX-9, DAX-1*) to initiate male sexual differentiation
- 10% of 46 XX males have no identifiable SRY gene

- Sertoli Cells: secrete anti-mullerian hormone; causes female reproductive structures to regress
- Leydig Cells: secrete testosterone which induces differentiation of the Wolffian duct system (epididymis, vas deferens, sex accessory glands)

Endocrinology of Testis[34]

Leydig cell differentiation

- 1st wave - 7 weeks gestation: stimulated by hCG from placenta; androgens appear in circulation
- 2nd wave - 2-3 months after birth: stimulated by gonadotropin production from neonate's pituitary; briefly elevates Testosterone
- Androgens produced during first 2-6 months of life are thought to hormonally imprint hypothalamus, liver, prostate, phallus and scrotum
- Leydig cells of infants then regress and testes are dormant

Puberty

- Hypothalamus generates pulses of GnRH around 12th year of life
- Onset of GnRH pulses typically occurs at night, due in part to gradual decrease in nocturnal melatonin secretion from pineal gland
- Influenced by nutritional status of body and growth rate
- GH and IGF-1 stimulate reproductive function
- Leptin determines size of fat stores in body

Aging of Hypothalamic / Pituitary Axis

- **Testosterone**: levels decline at > 50 years of age
- **LH**: basal levels increase in older men; LH pulsatility is blunted
- **Leydig cells**: steroidogenic capacity decreases
- **Spermatogenesis**: lower fecundity at > 40 years, 50% lower probability of achieving pregnancy within 1 year compared to men < 25 years of age

Heterosexual Precocious Puberty

- XY child with Congenital Adrenal Hyperplasia presents with precocious puberty but an XX child may also present with precocious puberty of opposite gender called heterosexual precocious puberty. In these children 17-hydroxy progesterone is very high and is the real culprit for the gross androgenic changes[35]

Semen Analyses[36]

WHO Lower Reference Limits
(Human Reproduction Update, Vol.16, No.3 Pp. 231–245, 2010)

5^{th} percentile (lower reference limits and 95% confidence intervals in parentheses), derived from a study of over 1900 men whose partners had a time-to-pregnancy of ≤12 months.

- Volume — 1.5 ml (95% CI 1.4-1.7)
- Sperm concentration — 15 million spermatozoa/mL (95% CI 12-16)
- Total sperm number — 39 million spermatozoa per ejaculate (95% CI 33-46)
- Morphology — 4 percent normal forms (95% CI 3-4), using "strict" Tygerberg method
- Vitality — 58 percent live (95% CI 55-63)
- Motility

Progressive motility — 32 percent (95% CI 31-34)
Total (progressive + non-progressive motility) — 40 percent (95% CI 38-42)

Chapter No 13

Other Endocrine Disorders

MCQs Key with Explanations

MCQ No	Best Option	Explanation
		Adrenal Disorders
141.	e. Morning (0900)	Cortisol has very pronounced diurnal variation i.e. high in the morning and low in the evening. So, random cortisol (without timing) is of no value except in dire emergencies. Estimation of cortisol levels in serum or saliva for the diagnosis of a hypercortisolism state i.e. Cushing Syndrome, should be carried out one hour after start of the sleep. In most patients, it means late evening around mid-night, but in patients who works the whole night and sleeps in the day time, it should be early morning. Please note one hour after the start of sleep can be determined by asking the patient her usual time of sleep.
142.	e. Simultaneous estimation of Serum Cortisol and Plasma ACTH	For hypocortisolism (Addison Disease), cortisol should be tested in the morning (0800 hours) while for Cushing Syndrome it should be done in the late evening. Another important point to keep in mind is that Addison Disease should be diagnosed as early as possible because cortisol and aldosterone are lifesaving hormones and their deficiency may lead to life-threatening hypotension. Simultaneous estimation of cortisol and ACTH will save time for diagnosis of the disease as well as the origin of the disease too (adrenal or pituitary).

143.	b. Serum 17 hydroxy progesterone estimation (17 OHP)	Ambiguous genitalia (means anatomic defects in external genitalia) in an XX female is most commonly due to Congenital Adrenal Hyperplasia (CAH) which is a defect of cortisol synthesis and results in accumulation of a metabolite with high androgenic action. This metabolite is 17-OHP. Its level is very high in the serum of these children and is diagnostic of CAH.
	Growth Disorders	
144.	c. Height velocity < 5 cm/y in a 4 years child	Height velocity (HV) is a clinical parameter used to define growth. A child with decreased HV should be considered for Growth Hormone (GH) testing.
145.	d. Height 2.5th percentile, Hb: 14.2g/dl; TSH: 2.4 mIU/ml; Anti-gliadin antibodies: negative	In this MCQ, it is made clear that GH testing should be carried out in short stature children after ruling out all other causes of short stature e.g. anaemia, hypothyroidism, coeliac disease etc. Height percentile greater than 2.5th percentile means child is not truly short.
146.	d. Serum Insulin like Growth Factor 1 (IGF-1)	IGF-1 is used not only for the diagnosis of GH deficiency but also for acromegaly. It will be unequivocally increased in acromegaly.
	Reproductive Endocrinology	
147.	c. Prader Willi Syndrome (PWS)	PWS is an important cause of Hypogonadotropic Hypogonadism (HH). It is due to a defect in 'Genetic Imprinting' in chromosome 15. The disease can occur in both sexes and characterized by pubertal failure, obesity, abnormal behaviour, low IQ and short stature. Early death is also reported due to obesity related issues and endocrine failures. There are many other causes of HH.
148.	e. Polycystic Ovary Syndrome (PCOS)	PCOS is the most common female endocrinal disorder. Its commonest presentation is hirsutism (male like growth of hair on face and other parts). Diagnostic criteria have been suggested by International Organizations

		(Please see text)
149.	b. Detection of ectopic pregnancy	Beta hCG is a very common and important test in any Endocrine Laboratory. Raised beta hCG in a patient with missed periods and severe abdominal pain is diagnostic of ectopic pregnancy and an emergency surgical treatment must be started.
150.	e. Sperm abnormalities	Various abnormalities of sperm count, morphology or motility are the commonest cause of male infertility, demanding it to be first test of infertility in a couple.

Summary
Chapter No 13
Other Endocrine Disorders

Adrenal Disorders

- Cortisol, the most important hormone of adrenal gland, has a very strong diurnal variation
- Cortisol can be tested in blood, saliva or urine.
- For Cushing Syndrome, cortisol should be tested in the late evening, when cortisol is at the lowest level
- For Addison Disease, cortisol should be tested at 8 am in the morning, when cortisol is at the highest level
- Dexamethasone Suppression Test is used for the diagnosis of Cushing Syndrome
- ACTH Stimulation Test is used for the diagnosis of Addison Disease
- Congenital Adrenal Hyperplasia is an important cause of ambiguous genitalia and gastrointestinal symptoms in infants

Growth Disorders

- Short stature can be due to many causes other than Growth Hormone Deficiency (GHD), some of these conditions can be life-threatening, too. So, all these causes should be excluded before testing for GHD.
- Basal level of Growth Hormone (GH) should not be tested for the diagnosis of short stature. GH testing should always be done after some stimulation e.g. exercise or insulin injection.
- Acromegaly is condition of GH excess. It is diagnosed by glucose suppression test.
- Insulin Like Growth Factor (IGF-1) and Insulin Like Growth Factor Binding Protein -3 (IGFBP-3) are now recommended for the diagnosis of GHD and GH excess.

Reproductive Endocrinology

- Hypogonadotropic hypogonadism is a common cause of primary infertility. Low testosterone / oestrogen and LH/FSH are characteristic features of this condition
- Polycystic ovary syndrome is the most common reproductive endocrinological disorder and a common cause of infertility. Hyperandrogenism is the hallmark of this condition
- Primary ovarian insufficiency is diagnosed in a female below the age of 40 years and having menstrual abnormalities with raised FSH.
- Sperm abnormalities is the commonest cause of infertility in a couple.

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Chapter No 14
Miscellaneous Disorders

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MCQs

(Please find key at the end of the chapter)

Pancreatic Functions and GIT

151. A known patient of renal failure presented with severe pain epigastrium pain for the last 48 hours. The most suitable investigation to rule out ***pancreatitis*** in this patient is:

    a. Amylase: creatinine clearance ratio
    b. Plasma ionised calcium
    c. Serum amylase
    d. Serum triglycerides
    e. Urinary amylase

152. Which of the following is a ***direct pancreatic function test***?

    a. $C^{13}O$ Breath test
    b. Cholecystokinin stimulation test
    c. Faecal chymotrypsin and elastase-1
    d. Pancreolauryl test
    e. Serum trypsinogen

153. What is the most common cause of ***pancreatic insufficiency*** in adults?

    a. Bile duct stones
    b. Carcinoma head of pancreas
    c. Carcinoma tail of pancreas
    d. Chronic pancreatitis
    e. Viral infection

154. What is the test for definitive diagnosis of ***celiac disease***

    a. Anti demylinated gliadin antibodies
    b. Endomysial antibodies
    c. Fecal Elastase-1
    d. Jejunal biopsy
    e. TTG Antibodies

Porphyrias

155. In the liver, heme is utilized primarily to produce which of the following compounds:
    a. Catalase
    b. Cytochrome p450
    c. Haemoglobin
    d. Myoglobin
    e. Nitric oxide synthase

156. The ***most common*** human porphyria is:
    a. Acute intermittent porphyria
    b. ALA dehydratase porphyria
    c. Congenital erythropoietic porphyria
    d. Porphyria cutanea tarda
    e. Hereditary corpoporphyria

157. Poisoning of which of the following ***metals*** resembles acute porphyria due to accumulation of ALA?
    a. Arsenic
    b. Copper
    c. Iron
    d. Lead
    e. Zinc

158. The commonest presenting clinical feature of ***Acute Intermittent Porphyria*** is:
    a. Abdominal pain
    b. Dark urine
    c. Muscular weakness
    d. Severe constipation
    e. Tachycardia

Vitamins and Minerals

159. Requirements of vitamins and minerals have been established by various organizations. Which of the following intake is claimed to be sufficient to meet the ***daily nutrient requirements*** of 97 per cent of the individuals of a population?
    a. Adequate Intake
    b. Population reference intake
    c. Recommended dietary allowance

d. The estimated average requirement
e. Upper tolerable level

160. Iodine is an important mineral required for optimum maintenance of thyroid hormones. Which of the following classes of minerals does it belong to?
    a. Macro-minerals
    b. Metallic minerals
    c. Non-essential minerals
    d. Trace-minerals
    e. Ultra-trace minerals

161. A 68 years old male is admitted to a state-of-the-art hospital for investigation of macrocytic anaemia. His vitamin B_{12} and folate results were as following:
    - Vitamin B_{12}: 152 pmol/L (Ref values: 148 - 221)
    - Folate: 8.6 nmol/L (Ref value > 9.1)

    The test most helpful in determining whether B_{12} or folate deficiency (or both) is leading to the macrocytic anaemia in this patient will be:
    a. Bone marrow aspiration biopsy
    b. Mean corpuscular volume
    c. Plasma homocysteine
    d. Schilling test
    e. Serum methylmalonic acid

162. A 35 years old male has developed lower limbs' weakness. Kayser-Fleischer rings were noted in his cornea. Laboratory investigations showed:
    - ALT: 645 U/L (<32)
    - Serum copper: 60 µg/dl (70-140)
    - Serum ceruloplasmin: 238 mg/l (220-400)

    Which of the following tests will be most useful now to confirm the diagnosis in this patient:
    a. Plasma zinc
    b. Serum gamma glutammyl transferase
    c. Serum superoxide dysmutase
    d. Urinary ceruloplasmin
    e. Urinary copper

Inherited Metabolic Disorders

163. A 5 days old male newborn refuses to take feed, has vomiting, and lethargy. On examination he has hepatomegaly, jaundice, and ascites. He has had several episodes of hypoglycaemia on breast feeding. Important biochemical findings are:

- Hypoglycaemia on breast-feeding
- Urine for reducing substance: +++
- Urine by glucose strip: Negative
- Putting the baby on lactose free milk reversed the reducing substance test to negative.

What is the most probable diagnosis?

a. Fatty acid oxidation disorder
b. Galactosaemia
c. Glycogen storage disease type I
d. Maple syrup urine disease
e. Mucopolysaccharidosis

164. An 8 months old male infant presents with progressive neurological impairment with mental retardation (IQ not improving with age). He also has mousy odour, skin pigmentation and abnormal physical growth. His first line biochemical findings did not give any clue towards diagnosis so following screening test was requested:

Plasma amino acids analyses (by HPLC):

- Phenylalanine: 1250 µmol/L (<120)
- Tyrosine: 3 µmol/L (>14)

Which of the following disease you will like to consider for further investigations?

a. Phenylketonuria
b. Pyruvate dehydrogenase complex deficiency
c. Tyrosinaemia type 1
d. Tyrosinaemia type 2
e. Urea cycle defect

165. A 6 months old infant has been brought in a Paediatric Clinic with history of attacks of hypoglycaemic featured by lethargy and vomiting which rapidly progresses to coma within 1–2 h. He also has on and off seizures. His biochemical findings during the attacks are usually like following:

- Hypoglycaemia precipitated by fasting
- Acidosis on ABGs: Positive

- Ketone bodies: Inappropriately Low
- Plasma lactate: Increased
- Fasting insulin: Normal
- Postprandial total acylcarnitine levels: <25-50% of normal

What is the most probable diagnosis?

a. Fatty acid oxidation disorder
b. Galactosaemia
c. Glycogen storage disease type I
d. Mucopolysaccharidosis
e. Pyruvate dehydrogenase complex deficiency

166. All the following disorders are screened in Newborn Screening (NBS) ***EXCEPT***:

a. Biotinidase deficiency
b. Congenital adrenal hyperplasia
c. Gilbert syndrome
d. Medium-chain Acyl-CoA Dehydrogenase (MCAD) Deficiency.
e. Sickle cell disease

Tumour Markers

167. All the following are characteristics of an ideal tumour marker ***EXCEPT***:

a. Can be used for evaluating prognosis
b. Can be used for prediction of therapeutic response
c. Elevated at early stage of the disease
d. High levels at the time of diagnosis
e. Levels decrease without any treatment

168. A 52-year old male who is a known patient of cirrhosis of liver as a sequel of Hepatitis C. His Alpha Fetoprotein (AFP) has been monitored regularly and usually remains below 200 ng/ml. A recent report, however, shows AFP 545 ng/ml and on repeating the test a similar value was obtained. The most probable cause of rise of AFP in this patient is:

a. Carcinoma of colon
b. Carcinoma of the pancreas
c. Extension in cirrhosis
d. Hepatocellular carcinoma
e. Testicular germ cell tumour

169. A 62 years old man has serum Prostate Specific Antigen (PSA) level of 6.9 ng/ml. According to the available evidence, the most promising method of PSA testing to avoid unnecessary prostatic biopsy in this patient is:

a. Free to total PSA percentage
b. PSA assay with age related cut-off values
c. PSA density
d. PSA velocity
e. Serum isoform [-2]proPSA

170. Research in oncogene has led to discovery of certain genes, which can predict certain cancers with reasonable accuracy. A subset of these oncogenes has been found quite commonly in otherwise healthy women raising the moral and ethical issues of prophylactic surgery.

Which of the following oncogenes is a suppressor gene used for inherited prediction of breast cancer in a family with the patients of these cancers:

a. APC mutation
b. BRCA mutation
c. Her-2/neu gene
d. N-ras mutation
e. VHL Mutation

Chapter No 14
Miscellaneous Disorders

Pancreatic Functions and GIT

Acute Pancreatitis (AP)[1]

- Insult of pancreas leading to leakage of pancreatic enzymes into pancreatic and peri-pancreatic tissue resulting into acute inflammatory reaction.

Prevalence of AP

- AP is one of the most prevalent pancreatic/gastrointestinal tract (GIT) diseases, and its prevalence is increasing.

Aetiology of AP[2]

- Two Common Causes

1. *Alcoholism (Ethanol) (30-40%)*
   - Mechanism not fully understood
   - Not all alcoholics get pancreatitis (only about 15%)
   - This suggests a subset of the population predisposed to pancreatitis, with alcohol acting more as a co-precipitant
2. *Gallstones (35%-60%)*
   - Gallstone (GS) pancreatitis risk is highest among patients with small GS < 5mm and with microlithiasis
   - GS pancreatitis risk is also increased in women > 60 years and central obesity

- Other aetiologies of AP
  - Idiopathic
  - Trauma
  - Steroids
  - Mumps (and other viruses: CMV, EBV)
  - Autoimmune (SLE, polyarteritis nodosa)
  - Hypercalcemia and hypertriglyceridemia
  - Scorpion sting
  - ERCP (5-10% of patients undergoing procedure)
  - Drugs (thiazides, sulfonamides, ACE-inhibitors, NSAIDS, azathioprine)

Diagnosis of AP[3]

(American College of Gastroenterology Guideline 2013)

- AP is usually diagnosed by clinical presentation and enzyme levels.

- Pancreatic contrast-enhanced CT and/or MRI should be reserved for patients with an unclear diagnosis or in patients without clinical improvement.
- Two of the following 3 criteria should therefore be present to diagnose AP:

1. Characteristic (severe) abdominal pain
2. Serum amylase and/or lipase levels exceeding 3 times the upper limit of normal
3. Characteristic abdominal imaging findings

Amylase[4]

- Elevates within 5-8 hours of symptoms and can remain elevated for 4-5 days
- High specificity when using serum levels > 3 times normal
- Many false positives (see causes of hyperamylasemia)
- Pancreatic isoamylase (fractionated amylase) is more specific

Causes of hyperamylasemia

Pancreatic Disease

- Pancreatitis due to any cause

Gastrointestinal Diseases

- Biliary tract disease
- Intestinal obstruction
- Gastritis/duodenitis
- Perforated duodenal ulcer
- Appendicitis
- Mesenteric ischemia
- Peritonitis

Genitourinary disease

- Salpingitis
- Ovarian malignancy
- Renal insufficiency

Miscellaneous

- Salivary gland lesions
- Acute alcoholic abuse
- Diabetic keto acidosis
- Macroamylasemia
- Tumours

Urinary Amylase

- Only enzyme excreted through kidneys

- In acute pancreatitis, urinary amylase should also be estimated in addition to serum amylase.
- In renal failure, there may be false increase of amylase in serum
- So, in patients with CKD a correction with GFR is required

Lipase[5]

- The preferred test for diagnosis
- Begins to increase 4-8 h after onset of symptoms and peaks at 24 h
- Remains elevated for several days
- Sensitivity 86-100% and specificity 60-99%
- Three times upper normal level is nearly 100% specific and sensitive.

Other Lab Investigations[4]

- Elevated ALT > 3x normal (in a non-alcoholic) has a positive predictive value of 95% for pancreatitis due to gall stones.
- Other inflammatory markers will be elevated:
  - CRP, IL-6, IL-8. There are studies hoping to use these markers to aid in detecting severity of disease.
- Depending on severity of pancreatitis, one may find:
  - Hypocalcaemia
  - Leukocytosis
  - Deranged renal function tests
  - Decreased haematocrit
  - Hyperglycemia
  - Hypertriglyceridaemia

Chronic Pancreatitis (CP)[5]

- Defined as chronic inflammatory condition that causes irreversible damage to pancreatic structure and function.

Causes of CP

- Alcohol abuse
- Malnutrition
- Hyperparathyroidism
- Ampullary stenosis
- Cystic fibrosis
- Hereditary, trauma and idiopathic

Sequels of CP

- Chronic pancreatitis results in interstitial inflammation, obstruction and dilation leading to parenchymal loss and fibrosis.

- Loss of both exocrine and endocrine pancreatic function.
- Clinically significant malabsorption occurs when 90% of pancreas is lost.

Clinical Presentation of CP

- Mid-epigastric abdominal pain, nausea and vomiting
- Patient appears chronically ill, with signs of pancreatic insufficiency such as weight loss, steatorrhea, clubbing, polyuria
- Differentiating acute vs chronic pancreatitis is difficult because primary distinction is based on disease reversibility

Diagnosis of CP

- Serum amylase and lipase may be normal if pancreas is fibrotic
- CT scan may helpful, if pseudocyst or abscess is present

Pancreatic Function Tests (PFTs)[6]

- Direct PFTs
- Indirect PFTs

Indirect tests measure the consequences of pancreatic insufficiency and are more widely available

Direct Pancreatic Function Tests:

- Direct PFTs are indicated for early diagnosis of Pancreatic Insufficiency (PI) and carried out by stimulation of the pancreas through the administration of a meal or hormonal secretagogues after which duodenal fluid is collected and analyzed to quantify enzymes and bicarbonate.
- *Only a few specialized centers perform these tests:*
  - Secretin stimulation test
  - Cholecystokinin Stimulation (CCK) test
  - Secretin-CCK stimulation test

Indirect Pancreatic Function Tests:

- Indirect tests measure the consequence of exocrine insufficiency (mal-digestion).
- Indirect tests are simpler and easier to perform than direct tests but their main role appears to be in diagnosis of advanced PI since they are much less sensitive than direct tests for diagnosis of earlier stages of CP

Examples:

- Serum trypsinogen
- 72-hour faecal fat excretion
- Faecal chymotrypsin and elastase-1
- Pancreolauryl test

- $C^{13}O$ Breath test

Types of Malabsorption[7]

- Digestive failure
- Structural defects
- Mucosal abnormality
- Infective agents
- Systemic diseases affecting GI tract

Malabsorption due to Digestive Failure

Pancreatic insufficiencies:

- Cystic fibrosis
- Chronic pancreatitis
- Carcinoma of pancreas

Bile salt insufficiency:

- Obstructive jaundice
- Bacterial overgrowth

Malabsorption Due to *Structural Defects*

- Inflammatory bowel diseases e.g. Crohn's Disease.
- Gastrectomy and gastro-jejunostomy.
- Fistulae, diverticulae and strictures.
- Infiltrative conditions such as amyloidosis and lymphoma.
- Eosinophilic gastroenteropathy.
- Radiation enteritis.
- Systemic sclerosis and collagen vascular diseases.
- Short bowel syndrome.

Malabsorption due to Mucosal Abnormality

- Coeliac disease

Malabsorption due to *Enzyme Deficiencies*

- Lactase deficiency inducing lactose intolerance
- Disaccharidase deficiency
- Enteropeptidase deficiency

Malabsorption Due to *Infective Agents*

- Intestinal tuberculosis
- Tropical sprue
- Parasites e.g. *Giardia lamblia.*

Malabsorption due to o*ther Systemic Diseases Affecting GI Tract*

- Hypothyroidism and hyperthyroidism
- Diabetes mellitus
- Hyperparathyroidism and hypoparathyroidism
- Carcinoid syndrome
- Malnutrition.

Clinical Picture

- Diarrhoea, often steatorrhoea is the most common feature. It is due to impaired water, carbohydrate and electrolyte absorption.
- Bloating, flatulence and abdominal discomfort.
- Weight loss
- Growth retardation, failure to thrive, delayed puberty in children
- Swelling or edema from loss of protein
- Anemia, commonly from vitamin B_{12}, folic acid and iron deficiency presenting as fatigue and weakness.
- Muscle cramp from decreased vitamin D, calcium absorption. Also leads to osteomalacia and osteoporosis
- Bleeding tendencies from vitamin K and other coagulation factor deficiency.

Coeliac Disease[8]

- A common cause of malabsorption
- Occurring from the first year of life through the eighth decade.
- Aetiology is not known, but three factors can contribute:

a. Environmental factor:

There is association of the disease with gliadin, a component of gluten that is present in wheat.

b. Immunologic factor:

Serum antibodies are detected such as anti-gliadin.

c. Genetic factor:

-Almost all patients express the HLA-DQ2 allele

Tests for Malabsorption:

- Tests for fat malabsorption
  - 24-hour faecal fat
  - Sudan III stain test
  - Acid steatorrhea test
  - Near infra-red reflectance analysis
- Tests for carbohydrate malabsorption
  - D-Xylose test

- Lactose tolerance test
- H^2 or $^{13}CO_2$ breath test

- Test for protein malabsorption
  - Alpha-1 antitrypsin excretion test

Porphyrias

Haem in the Body[9,10]

- Heme is usually considered significant because of its incorporation into hemoglobin and its role in red blood cells.
- It is also needed for cytochrome P450 function.
- Other uses of haem include its role in:
  - Catalase
  - Myoglobin
  - Nitric oxide synthase

Two Most Important Disorders

- Porphyria Cutanea Tarda (PCT)-------The commonest porphyria
- Acute Intermittent Porphyria (AIP) ---- Most common acute porphyria

What are Porphyrias

- Porphyrias is a set of diseases that result from enzyme deficiencies in the heme synthesis pathway (Figure 14.1)
- Each disease is associated with a deficiency in one of the seven enzymes in the pathway
- δ-Aminolevulinic acid (ALA) is a major metabolite in porphyrins.

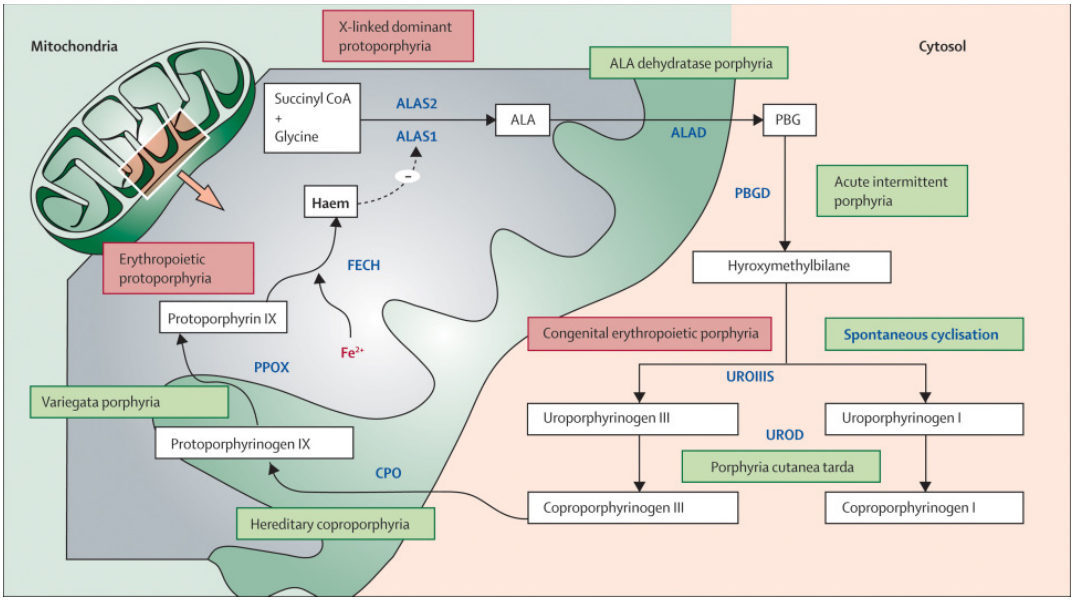

Figure 14.1: Enzyme Defects in Various Types of Prophyrias

(Adopted from: Puy, Hervé et al. Porphyrias. The Lancet , Volume 375 , Issue 9718 , 924 – 937)

Types of Porphyrias[11]

Cutaneous

Bullous lesions

- PCT
- Hepatoerythropoietic Porphyria (HEP)
- Congenital Erythropoietic Porphyria (CEP)

Non-bullous Lesions

- Erythropoietic Protoporphyria (EPP)

Acute Presentation (Hepatic Porphyrias):

- AIP
- ALA Dehyratase Porphyria (ADP)

Mixed Disorders (Acute and Cutaneous manifestations)

- Variegate Porphyria (VP)
- Hereditary Coproporphyria (HCP)

Free Radicals –The Real Culprits

- The respective enzymatic deficiencies lead to the accumulation of porphyrins and porphyrin precursors that ultimately produce free radicals.
- In acute hepatic porphyrias, ALA and porphobilinogen (PBG) accumulate and produce free radicals via autoxidation.
- In erythropoietic porphyrias, uroporphyrins, coproporphyrins and protoporphyrins accumulate and produce free radicals via the absorption of visible light.
- The generated free radicals participate in oxidative stress reactions, such as lipid oxidation and protein crosslinking, that lead to membrane and mitochondrial damage, ultimately promoting cell death.
- Loss of negative feedback of heme leads to further accumulation of porphyrins.

Genetics

Autosomal Dominant

- AIP
- HCP
- VP
- EPP

Autosomal Recessive

- ADP
- CEP

Acquired

- PCT

Environmental Factors

- Due to incomplete penetrance, inheritance of an enzyme deficiency of an autosomal dominant porphyria does not necessarily lead to clinical symptoms.
- Symptoms of acute porphyrias tend to come in the form of "attacks" which may be induced by other genetic factors or environmental factors, such as agents that promote porphyrin and porphyrin precursor synthesis and/or agents that induce cytochrome P450s.

Two Types of ALAS

ALAS-1

- Present in liver and all other tissues for production of haem used for non-haemoglobin substances e.g. cytochrome P450

ALAS-2

- Present in erythrocytes for production of haemoglobin

Lead Toxicity and Porphyrias[12]

- Lead exposure causes increased ALA due to replacement of Zn by Lead in the enzyme ALA Dehydratase.

Clinical Presentation of Acute Forms[13]

- Originate mainly in nervous system
- Symptoms last around 1-2 weeks
- Possible mechanisms include damage by free radicals, direct neurotoxicity of ALA and the deficiency in nervous tissue

Symptoms

- Severe abdominal pain
- Muscle weakness and pain, tingling, or numbness and possibly paralysis
- Pain in arms, legs and back
- Constipation
- Vomiting
- Diarrhea
- Insomnia
- Seizures and confusion
- Anxiety and paranoia
- Fever

Symptoms of Cutaneous Forms

- Occur most commonly with exposure to sunlight
- Predominantly skin symptoms
- Due to excess porphyrins that accumulate in skin surface

Skin Symptoms:
- Fluid filled blisters
- Changes in pigmentation
- Breakdown (necrosis) of the skin when exposed to sunlight
- Overall skin can become scarred, brown, blotchy and fragile
- Occur most commonly with exposure to sunlight
- Due to excess porphyrins that accumulate in surface of skin

Acute Intermittent Porphyria (AIP)[14]
- Second most common form of porphyria
- Caused by deficiency of porphobilinogen deaminase
- Metabolite porphobilinogen accumulates in cytoplasm

Symptoms:
- Localized abdominal pain
- Urinary symptoms
- Peripheral neuropathy
- Raised concentration of urinary porphyrins

Treatment
- Haematin, haeme arginate
- Do not cure but reduce symptoms
- Inhibit ALA synthase which occurs at the beginning of haeme biosynthesis

Treatment for Acute Forms
- Carbohydrate such as glucose helps limit the synthesis of porphyrins
- Phlebotomy reduces excessive iron stores which improves haeme synthesis
- Sedatives to help with anxiety
- Pain medications such as opiates
- Haem arginine inhibits ALA synthase and the accumulation of toxic precursors

Sideroblastic Anaemia[15]
ALA Synthase
- Most important rate limiting enzyme
- Deficiency may cause sideroblastic anaemia
- Bone marrow produces ringed sideroblasts
- X-linked

- Respond to pyridoxine treatment

Congenital Erythropoietic Porphyria (CEP)[16]

- Deficiency of uroporphyrinogen III synthase
- Rare autosomal recessive (1 in 1,000,000)
- Severe photosensitivity

Porphyria Cutanea Tarda (PCT)[17]

- Most common porphyria
- Classified as such when uroporphyrinogen decarboxylase activity <20%
- Hepatic disorder
- May be found in hepatitis C, drugs, alcohol and poisons

Treatment:

- Discourage risk factors and treat symptoms;
- Draw blood to reduce iron in the liver until the serum ferritin reaches normal iron levels.
- Chloroquine or hydroxychloroquine can move excess porphyrins from the liver and promote excretion. Can be used when drawing blood is not recommended.
- Avoid causes of PCT

Hereditary Coproporphyria (HCP)[18]

- Deficiency of coproporphyrinogen III oxidase
- Autosomal dominant
- No cure exists

Variegate Porphyria (VP)[19]

- Deficiency in protoporphyrinogen IX-oxidase
- Autosomal dominant

Erythropoietic Protoporhyria[20]

- Caused by deficiency of ferrochelatase
- Autosomal dominant
- Photosensitivity can be managed by limiting exposure

Vitamins and Minerals

Recommended Dietary Allowance (RDA)[21]

- Requirements of vitamins and minerals have been established by various organizations.
- RDA is claimed to be sufficient to meet the daily nutrient requirements of 97 percent of the individuals of a population:

Categories of Minerals in the Human Body[21](Table 14.1)

- Macro-trace minerals
- Trace-minerals
- Ultra-trace Minerals

Table 14.1: Mineral in Human Body

Macro-, trace-, and ultra-trace minerals in man

Macro-minerals	Trace minerals	Ultra-trace minerals
Sodium	Iron	Arsenic
Potassium	Zinc	Boron
Chloride	Copper	Chromium
Calcium	Manganese	Iodine
Phosphate	Fluoride	Selenium
Magnesium		Silicon
		Nickel
		Vanadium

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Vitamin B_{12} And Folate Deficiency

Physiological Role of Folic Acid and Vitamin B_{12}[22]

- Required for DNA synthesis and red cell maturation
- Two vitamins combine in methionine synthase reaction
- Methyl group is transferred to homocysteine to make methionine
- Decrease in either vitamin leads to increase in homocysteine level
- Premature coronary artery disease and peripheral vascular disease can result.

Deficiency of Folic Acid and Vitamin B_{12}[23]

- Deficiency leads to change in RBC shape
- Megaloblastic anaemia is a subgroup of macrocytic anaemias
- Megaloblastic erythropoiesis when defect in DNA synthesis and the cells are arrested at the G2 phase
- A buildup of cells occurs that do not synthesize DNA, so nucleus develops at a slower rate than the rest of the cell
- Cytoplasm continues to grow due to RNA synthesis
- Cells become larger and megaloblastic

Lab Diagnosis of Vitamin B_{12} Deficiency

- Normal: >300 pg/mL (>221 pmol/L)
- Borderline:200 to 300 pg/mL (148 to 221 pmol/L)
- Vitamin B_{12} Def: <200 pg/mL (<148 pmol/L)

For Borderline cases:

- Homocysteine raised but normal Methylmalonic Acid (MMA) indicate folate deficiency
- Homocysteine and MMA raised: B_{12} Def

Serum or Red Cell Folate[24]

- The serum folate concentration is unequivocally low in patients with folate deficient megaloblastic anaemia but it can be falsely normal in some situations e.g. diet with sufficient folates.
- Red cell folate is free of short term fluctuations and is, therefore, a better indicator of folate status
- Some studies have, however, questioned routine use of red cell folate estimation

Biochemical Evaluation of Vitamin B_{12} and Folic Acid Deficiency[25]

- Serum vitamin B_{12} and folic acid levels
- In hospitalized patients, samples should be obtained immediately before taking any meal and blood transfusion. Even a single meal can normalize the serum folic acid level
- Red cell folate level is more reliable indicator of tissue folate adequacy as it reflects time average value of folic acid availability
- Evaluate for accumulation of specific metabolic intermediates
- Homocysteine levels – increased in both vitamin B_{12} and folic acid deficiency
- MMA increased in vitamin B_{12} deficiency only

Disorders of Copper Metabolism[26]

- Cu is absorbed by the intestinal cells; within the enterocyte an enzyme called ATP7A releases copper into the portal vein which is then transported to the liver.
- In liver lies another enzyme, ATP7B, which has two functions:
    - Incorporates copper into ceruloplasmin and releases it into the bloodstream.
    - Removes excess copper by secreting it into bile.

Wilson Disease (WD)

Wilson disease, also known as hepatolenticular degeneration, is an autosomal recessive disorder of copper metabolism characterized by excessive deposition of copper within the liver, brain, cornea and other tissues

- Caused by mutation to ATP7B gene.

- More than 500 mutations have been described so far which can be detected in 90% of the patients while 10% have no detectable mutation.

Hepatic Changes in WD

- In hepatocytes affected by WD, Cu cannot be transported from the cytosol to the golgi apparatus; so, Cu accumulates in the cytosol.
- Cu deficiency in the golgi apparatus results in reduced secretion of Cu into the blood as well as biliary excretion of Cu is disturbed.
- Accumulated Cu in the hepatocyte is released into the blood as non-ceruloplasmin-bound Cu

Biochemical Tests for WD[27]

- Serum ceruloplasmin---Low (<20 mg/dl in WD)
- Serum free copper—High (>250 µg/L in WD)
- 24 h urinary copper—High (>50 µg/24h)

Penicillamine Challenge Test

- Standardized for children only
- Urinary copper excretion can be increased in a variety of liver diseases, penicillamine challenge has been proposed to increase sensitivity and specificity (only in children).
- Penicillamine greatly increases urinary copper excretion in patients with WD, and to a lesser extent, in patients with other forms of liver disease

Hepatic copper content

- High i.e. >250 µg/g dry weight
- Transaminases—raised due to hepatocyte injury

Menkes Disease (MD)[28]

- Menkes disease, also known as kinky hair disease, is an X-linked neurodegenerative disease of impaired Cu transport.
- Defective ATP7A gene is expressed in almost all cell types except hepatocytes.
- Transport of dietary Cu from intestinal cells is impaired, leading to the low serum Cu levels.
- Abnormal Cu transport in other cells leads to:
    - Paradoxical Cu accumulation in duodenal cells, kidney, pancreas, skeletal muscle, and placenta.
    - Decreased activity of cuproenzymes (any metalloenzyme that contains one or more copper atoms)

Cellular Changes in MD

- In cells affected by MD, copper cannot be transported from the cytosol to the golgi apparatus and thus cannot be excreted from the cells.
- Copper deficiency in the golgi apparatus results in a decrease in the activities of secretory copper containing enzymes

Chromium (Cr)[21,28]

- Trivalent chromium (Cr+3) is an essential trace element
- Enhances the action of insulin on its receptor
- So, its deficiency is reportedly associated to insulin-resistant glucose intolerance and neurological deficits

Proposed mechanism for Activation of Insulin Receptor by Cr

- The inactive form of Insulin Receptor (IR) is converted to active form by binding insulin
- This triggers a movement of Cr (in the form of Cr-transferrin, Cr-Tf) from the blood into insulin-dependent cell
- Cr (Cr^{+3}) binds to apo-chromodulin to form holo-chromodulin
- Finally, the holo-chromodulin binds to IR, further activating the receptor kinase activity

Selenium (Se) and Keshan`s Disease[21]

- It was documented in Keshan and its neighbouring counties in China that a fatal cardiomyopathy is caused by Se deficiency
- Supplementation of diet with Se greatly controls the disease
- A classic example of *"disease-mineral relationship".*

Zinc (Zn)[29,30]

Functions of Zn

- It is second to iron as the most abundant trace element in the body.
- The key roles of Zn are in proteins and nucleic acid synthesis.
- This role explains the failure of growth and impaired wound healing observed in individuals with Zn deficiency

Role of Zn in Hormone Synthesis

Zn has a role in the synthesis and action of many hormones, via zinc transcription factors.

- Testosterone
- Free T4
- IGF-1
- Thymulin, the thymus specific hormone that is involved in T-cell function

Signs and Symptoms of Zn Deficiency

- Depressed growth with stunting
- Increased incidence of infection
- Diarrhoea
- Skin lesions
- Alopecia

Estimation of Zn in Plasma

- Plasma Zn concentrations are most commonly measured by atomic absorption.
- It is essential to consider plasma Zn results along with plasma albumin and plasma CRP or another marker of the acute phase reaction.
- A guidance reference interval is 12 to 18 µmol/L

Inherited Metabolic Disorders

Synonyms[31]

- Inherited Metabolic Diseases (Preferred Term now)
- Inborn Errors of Metabolism
- Inborn Metabolic Diseases

Overview[31]:

- Metabolic disorders result from the absence or abnormality of an enzyme or its cofactor, leading to either accumulation or deficiency of a specific metabolite.
- Optimal outcome for children with inherited metabolic disorders (IMDs) depends upon early recognition of the signs and symptoms, prompt evaluation, and referral to a centre familiar with the evaluation and management of these disorders.
- Delay in diagnosis may result in acute metabolic de-compensation, progressive neurologic injury or death.
- Individual IMD is not very common (1:5,000 to 1:10,000). However, collectively as a group the incidence is around 1:800.
- Management of certain symptoms like hypoglycaemia, hyperammonemia, and seizures must be initiated promptly before exact diagnosis to prevent long-term sequelae

Initial Evaluation:

History*:*

- The history should focus on previous episodes of metabolic decompensation, identification of potential triggering events, and family history of metabolic disease or members with similar presentations.
- Antenatal /birth history should be obtained in all cases and is especially significant for cases like non-ketotic hyperglycinaemia, peroxisomal disorders, some lysosomal storage disorders, and disorders of cholesterol biosynthesis
- Recurrent vomiting and /or diarrhoea
- Intolerance to feeds
- Episodic abdominal pain
- Lethargy
- Failure to thrive/developmental delay
- Fits
- Recurrent hypoglycaemia
- Family history should include history of consanguinity, history of similar complaints in siblings, early neonatal deaths etc.

Age at presentation:

- Diseases like urea cycle disorders, non-ketotic hyperglycinaemia, and branched-chain organic acidaemias often present during the first few hours of life
- During the first or second week, conditions like neonatal hemochromatosis, galactosaemia, tyrosinaemia and maple syrup urine disease are likely to manifest clinically.
- Conditions like alpha-1 antitrypsin deficiency, Niemann-Pick disease, and bile acid synthesis defects usually present after the third week

Triggering events:

- In the evaluation of a child with suspected IMD it is important to ask about the following triggers:
    - Are the symptoms aggravated by ingestion of certain kinds of meals e.g. carbohydrates, protein rich meal, complementary foods (e.g. infant cereals, fruit juice, and pureed fruits, vegetables or meats) ?
    - Are the symptoms related to conditions like fever, infections?
    - Is there any relation of aggravation of symptoms to any anaesthesia, surgery or any specific type of medication?

Physical Examination:

- Examine general body habitus for any dysmorphism. Any coarse facial features, micro –or macrocephaly should alert the physician of any possible IMD.

- Hair and skin should be examined for any signs of alopecia, eczema, hypopigmentation, gingival hyperplasia, xanthomas, oedema, hirsutism and/ or photosensitivity.
- Examine eyes for presence of any cataract, corneal opacities, Kayser-Fleischer ring or lens dislocation.
- Look for any evidence of hepatosplenomegaly
- A complete musculoskeletal and neurological examination should be undertaken to assess for any arthritis, dystonia, myopathy, peripheral neuropathy hearing loss and parathesias.

Laboratory Investigations

- Laboratory investigation in IMD can be routine tests or specialized tests.
- Specialized tests should be carried out in selected patients only (Box 14.1)

Clinical Types of IMDs[32]

Group 1: Disorders That Give Rise to Intoxication:

This group includes IMDs that lead to acute or progressive intoxication from accumulation of toxic compounds proximal to the metabolic block. This group includes

- **Aminoacidopathies** e.g. phenylketonuria (PKU), Maple Syrup Urine Disease (MSUD) and tyrosinaemia,
- **Organic acidaemias** e.g. methylmalonic acidaemias, propionic acidaemias and isovalericacidaemia,
- **Sugar intolerance** e.g. galactosaemia.
- **Urea cycle disorders**

Group 2: Disorders Involving Defects in Energy Metabolism

- Signs and symptoms in these disorders are caused partly by a deficiency in energy production or utilization in the liver, myocardium, skeletal muscle, or brain.
- Signs or symptoms related to accumulation of toxic compounds may be present. This category includes following disorders:
  - Hypoglycaemic disorders
  - Glycogenolysis defects
  - Gluconeogenesis defects
  - Hyperinsulinism
  - Fatty acid oxidation defects
  - Congenital lactic acidaemias
  - Pyruvate carboxylase deficiency
  - Krebs citric cycle defects
  - Mitochondrial respiratory chain defects

Group3: Disorders Involving Complex Molecules

This group includes diseases that involve defects in the synthesis or the catabolism of complex molecules. These include:

Lysosomal Storage Diseases:

- Mucopolysaccharidosis
- Sphingolipidoses
- Glycoproteinosis

Box 14.1: Lab Tests in IMDs[33]

Preliminary Lab investigations	Second line investigations
Arterial Blood Gases (ABGs)	Plasma amino acids by HPLC
Blood glucose	Urine amino acids by HPLC (only for diseases like Fanconi Syndrome etc.)
Electrolytes	CSF amino acids by HPLC (e.g. in NKH)
Plasma ammonia	Organic acids in urine
Plasma lactate	Acylcarnitines in plasma (for fatty acid disorders)
ALT and urea	Orotic acid in urine
Urine reducing substances	Others
Urine ketones	
Complete Blood Count	
Serum insulin if hypoglycaemia is present	

Some Individual Disorders

Galactosaemia[34]

- Galactosaemia can result from deficiencies of three different enzymes, Galactose-1-phosphate uridyl transferase (GALT), Galactokinase or Uridine diphosphate galactose 4-epimerase.
- The most important is classic galactosaemia due to GALT deficiency.

Clinical Features:

- Infants with galactosaemia have normal weight at birth but signs and symptoms appear at initiation of breast milk or cows' milk-based formula feedings and they fail to regain birth weight.
- Symptoms appear in the second half of the first week and include refusal to feed, vomiting, jaundice, lethargy, diarrhoea and hepatomegaly. Symptoms are milder and the course is less precipitous when milk is temporarily withdrawn and replaced by intravenous nutrition.

Diagnosis:

Urine reducing substances detection:

- If symptomatic infant tests positive for reducing substances in urine, it may raise the clinical suspicion enough to trigger further evaluation and empiric treatment.
- A repeat of the test should be negative on switching the baby to soya milk. Due to lack of newborn screening programmes, this test has great utility in our set up, though it has got low specificity and sensitivity.

RBC GALT activity

- The gold standard for diagnosis is the demonstration of nearly complete absence of GALT activity in RBCs. Quantitative assay of RBC GALT activity is necessary to confirm the diagnosis.

Newborn Screening[35]:

- In many countries, newborn with galactosaemia are discovered through mass screening for blood galactose, the transferase enzyme or both.
- This screening is performed using dried blood spots usually collected between the second and seventh days.

Treatment:

- The main goal of long-term treatment of classic galactosaemia is to minimize dietary galactose.
- This must be started immediately after the disorder is suspected clinically or following a positive newborn screening results even before the results of diagnostic tests are available.
- Once solid foods are introduced, ingredients containing lactose and galactose should be minimized.
- When a lactose-free diet is instituted early enough, symptoms disappear promptly, jaundice resolves within days, cataracts may clear, liver and kidney functions return to normal and liver cirrhosis may be prevented.

Phenylketonuria[36]

- Phenylketonuria (PKU), first described by Følling in 1934 as "*Imbecillitas Phenylpyruvicais* is a rare inherited disorder that causes amino acid, phenylalanine (PHE) to build up in body.
- It is due to deficiency of phenylalanine hydroxalase (PAH) enzyme or a co-factor defect (i.e. tetra hydrobiopterin –BH4) which could lead to increase in plasma PHE levels
- It is broadly classified as benign hyperphenylalaninemia and phenylketonuria[31,32].

Clinical features:

- Delayed development
- Behavioural problems
- Dystonia
- Seizures and tremors
- Hypopigmentation
- Blonde hair
- Head size much smaller than normal (microcephaly)
- Poor bone strength
- Musty odour in child's breath, urine or skin.

Causes:

- This heterogeneous group of autosomal recessive disorders is due to deficiency of the enzyme, phenylalanine-4- hydroxylase (PAH) or defects in the metabolism or regeneration of its cofactor tetrahydrobiopterin (BH4).
- Most severe form of the disease is because of PAH deficiency known as classic PKU.

Diagnosis[37]:

Plasma PHE levels:

- Blood PHE is normal at birth in infants with PKU but rises rapidly within the first days of life. Diagnosis is based upon the finding of an elevated plasma concentration of PHE.
- There is variation between different countries and centres in the age at which screening is undertaken (day 1 to day 10), in the methodology used (Guthrie microbiological inhibition test, DELFIA, HPLC, or tandem mass spectrometry).

PHE /Tyrosine ratio:

- The level of blood PHE that is taken as a positive result requiring further investigation (120 to 240 µmol/L) but some laboratories are also using a phenylalanine /tyrosine ratio >3 to be diagnostic.

- The advantage of HPLC is that additional amino acids including tyrosine can be measured simultaneously. A high concentration of PHE together with low to low-normal tyrosine concentration may assist in making the diagnosis of PKU.

Fatty Acid Oxidation defects[38]

- Fatty acid oxidation (FAO) disorders usually present in early infancy as acute life-threatening episodes of hypoketotic, hypoglycaemic coma induced by fasting or febrile illness.
- These include carnitine deficiency, fatty acid transportation defects, and defects of beta-oxidation enzymes.

Classification:

Carnitine cycle defects

- Carnitine transporter Defect
- Carnitine Palmitoyltransferase-1 (CPT-1) deficiency
- Carnitine/Acylcarnitine Translocase (TRANS) deficiency.
- Carnitine Palmitoyltransferase-2 (CPT-2) deficiency

ß-Oxidation Defects

- Very-long-chain Acyl-CoA Dehydrogenase (VLCAD) deficiency
- Medium-chain Acyl-CoA Dehydrogenase (MCAD) deficiency
- Short-chain Acyl-CoA Dehydrogenase (SCAD) deficiency.

Clinical Presentation:

- Clinical presentation of most of the FAO defects is similar.
- The affected children present with signs mainly of hepatic, cardiac, and skeletal muscle involvement.
- Cardiac failure is the major presenting manifestation only in Carnitine Transporter Defects.
- During the first years of life, extended fasting stress may provoke an attack of hypoketotic, hypoglycaemic coma.

Diagnosis:

Hypoglycaemia is precipitated by fasting in FAO defects.

Plasma/Urine acylcarnitine profile:

- The assay of the plasma or urine acylcarnitine profile by triple-Quad (LC-MS/MS) is available.
- In all the other defects, except HMG-CoA synthase deficiency, total carnitine levels are reduced to 25-50% of normal (secondary carnitine deficiency)

Enzyme assays:

Enzyme assays in cultured skin fibroblasts or cultured lymphoblasts are used to measure the in vitro activities of specific steps in the fatty acid oxidation pathway.

Tumour Markers

What are Tumour Marker[39]

Substance produced by tumours found in blood, body fluids, or tissue that may be used to predict tumour's presence, size and response to therapy.

Tumour Suppressor Gene[40]

Gene involved in regulation of cellular growth; loss of tumor suppressor gene has potential to allow autonomous growth

Characteristics of Ideal Tumor Marker:

Tumor markers should be both specific for a given type of cancer and sensitive enough to detect small tumours to allow early diagnosis or use in screening.

Clinical Applications

- Screening for cancer
- Diagnosing cancer
- Evaluating cancer prognosis
- Prediction of therapeutic response
- Tumour staging
- Detecting tumour recurrence or remission
- Localizing tumour or directing radiotherapeutics
- Monitoring effectiveness of cancer therapy

General Categories of Tumour Markers[39,40]

- Enzymes
- Hormones
- Oncofetal antigens
- Carbohydrate markers
- Blood group antigens
- Proteins
- Receptors
- Genetic markers

Alkaline Phosphatase (ALP)

- ALP may arise from liver, bone, placenta or malignant tumours
- Elevated concentrations are seen in primary or secondary liver cancer
- It can be helpful in evaluating metastatic cancer with bone or liver involvement
- Greatest elevations are seen in patients with osteoblastic lesions like in prostatic cancer with bone metastasis
- Malignancies like leukemia, sarcoma and lymphoma complicated with hepatic infiltration may show elevated ALP activities

Lactate Dehydrogenase (LD or LDH)

- An enzyme in glycolytic pathway
- Released because of cellular damage
- Elevations are seen in cancers of liver, non-Hodgkin lymphoma, acute leukemia, nonseminomatous germ cell testicular cancer, neuroblastoma and other cancers like breast, colon, stomach and lung
- Provides prognostic indicator for disease progression
- Elevations of LD_5 isoenzyme is associated with liver metastasis
- LD_5 in spinal fluid is an early indicator of CNS metastasis
- Limited value in monitoring of therapy.

Prostatic Specific Antigen[41]

- It is one of most promising and few organ specific tumour markers
- Prostatic cancer is leading cancer in older men
- Its potentially curable by radical prostatectomy
- Early detection is important for men with life expectancy of at least 10 years
- It is used to screen, stage and monitor treatment and recurrence of prostatic cancer
- It can also be measured in nipple aspirate fluid as a possible tool for breast cancer risk assessment

Improving the Accuracy of PSA

Numerous strategies have been proposed to improve the diagnostic performance of PSA when levels are less than 10.0 ng/ml. These strategies include

- Measuring PSA velocity
- PSA density
- Free PSA
- Complexed PSA
- Using age- and race-specific reference ranges
- Serum isoform [-2]proPSA

Free to total PSA percentage

- The ratio of free-to-total PSA is reduced in men with prostate cancer
- Biopsies should be performed only in men with lower ratios.
- An optimal cutoff selected for biopsy is 25 %
- Men with a normal free-to-total PSA ratio still had an 8% probability of having cancer

PSA density:

- PSA concentration / prostatic volume
- It is determined by trans-rectal ultrasonography
- PSA density measurements better discriminates between cancer and non-cancer groups than PSA levels alone

PSA velocity

- It is the rate of PSA increase as a function of time
- A baseline concentration of PSA in each patient is established, the rate of increase of PSA is then calculated
- Men with a PSA velocity > 0.75 ng/ml/year are at increased risk of being diagnosed with prostate cancer

PSA assay with age related cut-off values

AGE (in years)	CUTOFF
40 to 49	0 to 2.5 ng/ml
50 to 59	0 to 3.5 ng/ml
60 to 69	0 to 4.5ng/ml
70 to 79	0 to 6.5 ng/ml

Serum isoform [-2]proPSA

- Also known as P2PSA
- Is a specific isoform of the PSA proenzyme proPSA
- Increases the detection of prostate cancer for men with PSA values between 2.0 to 10.0 ng/ml
- Reduces the number of unnecessary biopsies by 7.6 % with sensitivity of 95 % for detecting prostate cancer

Genetic Markers[40]

Oncogene

- Oncogenes are derived from proto-oncogenes.
- Activation of proto-oncogenes is found to be associated with cancer.
- These genes code for products that are involved in normal cellular processes, such as growth factor signaling pathways.
- Overexpression of the oncogene will lead to abnormal cell growth, resulting in malignancy
- Most oncogenes code for proteins that function at some stage of activation of cells for proliferation, and their activation leads to cell division.
- Most oncogenes are associated with haematological malignancies, such as leukaemia and, to a lesser extent, solid tumours.
- Of the more than 40 proto-oncogenes recognized, only a few have been shown to be useful tumor markers.

Examples

- *Ras* genes (breast, pancreas, stomach, lung, uterus and colon tumors)
- *C- myc* gene (colon, cervical, gastric and liver tumours)
- *HER- 2 neu (*breast, ovarian and gastrointestinal tumours)
- *Bcl- 2 (*lymphomas, myelomas and chronic leukemia)
- *BCR- ABL* (chronic myeloid leukemia)

- *RET* (papillary thyroid cancer, MEN)

Tumour Suppressor Gene

- Cancerous growth is an inheritable characteristic of cells and is thought to be the outcome of genetic changes.
- Multiple genetic alterations may be necessary for the transformation of a cell from a normal state to a cancerous one and, finally, for metastatic spread.
- Two classes of genes are implicated in the development of cancer**:**
- The suppressor genes, has been isolated from mostly solid tumors.
- The oncogenicity of suppressor genes is derived from the loss of the gene rather than their activation as with oncogenes.
- Deletion or monosomy may lead to the loss of tumor suppressor gene
- The study of suppressor genes may provide a clue as to the development of cancer from normal cell status to benign and cancerous status and to metastasis.

Examples[40]

BRCA 1 and BRCA2

- Two genetic loci have been identified: *BRCA 1* on chromosome 17q and *BRCA 2*, which localizes to *13q12-13*
- A subset of breast cancer patients has been shown to have an inherited predisposition to developing breast and ovarian cancer that is inherited as an autosomal-dominant trait

Retinoblastoma gene (Rb)

- The Rb gene has been localized to chromosome 13q by loss of a chromosomal banding region in peripheral blood lymphocytes of patients with the familial form and by loss of heterozygosity studies in both Retinoblastoma and some osteosarcomas

p53 Protein

- p53 protein may be lost by deletion of the gene or production of a competing mutant protein
- 75 -80% of colon carcinomas show deletion in one p53
- Allelic deletion of p53 occurs only rarely in adenomas (10%), suggesting that p53 inactivation may be a relatively late event in colon carcinogenesis.
- In addition, up to 70% of breast cancers also have deleted p53

Oncofetal Antigens[40]:

- They are proteins produced during fetal life.
- Present in high concentration in sera of fetuses and decrease to low concentrations or disappear after birth.
- Presence of these proteins shows that certain genes are activated because of the malignant transformation of cells.

Alpha-fetoprotein:

- It is glycoprotein.
- Its concentration is less than 10 ug/L in healthy adult.
- It is a marker for hepatocellular and germ cell (non-seminoma) carcinoma.
- Except in the pregnant patient, its concentrations greater than 400-500 ug/L are indicative of hepatocellular carcinoma[41].
- It is useful for determining prognosis and in monitoring of therapy for hepatocellular carcinoma.
- In non-seminoma, along with hCG this marker correlate with tumor volume and prognosis of disease.

Chapter No 14
Miscellaneous Disorders

MCQs Key with Explanation

MCQ No	Best Option	Explanation
	Pancreatic Functions and GIT	
151.	a. Amylase: creatinine clearance ratio	Amylase is the only clinically important enzyme that is excreted through kidney. So, in patients with renal impairment amylase may be falsely high. To overcome this problem, amylase: creatinine clearance ratio has been suggested for use in patients with chronic kidney disease for the interpretation of serum amylase results.
152.	b. Cholecystokinin Stimulation test	These tests are invasive and require parenteral administration of cholecystokinin and endoscopic collection of intestinal fluid. This test and other similar tests provide the direct assessment of pancreatic function
153.	a. Bile duct stones	Pancreatic insufficiency is caused by bile duct stones (38%) and alcoholism (35%). In our country bile duct stones is probably far more common cause.
154.	d. Jejunal biopsy	The diagnosis of coeliac disease can be confirmed only by demonstrating flat mucosa i.e. partial or total atrophy of intestinal villi on jejunal biopsy.
	Porphyrias	
155.	b. Cytochrome p450	Cytochrome P450 (CYP enzymes) are enzymes, which use haem iron to oxidise molecules, often making them more water-soluble for clearance. They are important for clearance of both xenobiotic and endogenous compounds.

156.	d. Porphyria cutanea tarda	It is the most common of all the porphyrias and is the only acquired porphyria.
157.	d. Lead	Lead poisoning is usually a chronic disease due to occupational or environmental lead intake. Lead inhibits δ-Aminolevulinic acid (ALA) dehydratase enzyme resulting in accumulation of ALA.
158.	a. Abdominal pain	Acute intermittent porphyria is most common acute porphyria and abdominal pain is its commonest presentation. It does not present with any skin lesion.
	Vitamins and Minerals	
159.	c. Recommended Dietary Allowance (RDA)	Out of many parameters to measure the requirement of a vitamin in the body, RDA is most commonly used.
160.	e. Ultra-trace minerals	Minerals are divided into macro-trace, trace and ultra-trace according to their concentration in the body
161.	e. Serum Methylmalonic Acid (MMA)	In some patients, serum levels of B_{12} and folic acid are not reliable. Then two metabolites are tested namely homocysteine and MMA. If homocysteine is raised but MMA is normal then its folic acid deficiency. If both homocysteine and MMA are increased then its B_{12} deficiency.
162.	e. Urinary copper	For the diagnosis of Wilson Disease (WD), serum copper and ceruloplasmin are not reliable tests as serum copper depends on its binding globulin while ceruloplasmin is acute phase reactant and can be normal if liver is affected. Urinary copper is free copper and, therefore, gives an important clue in the investigation of WD.
	Inherited Metabolic Disorders (IMD)	
163.	b. Galactosaemia	Galactosaemia is due to deficiency of Galactose-1-phosphate Uridyl Transferase (GALT), enzyme essential for the metabolism of galactose. Typically, hypoglycaemia occurs after feeding. Galactose in high quantities becomes toxic for various organs. Positive

		Benedict`s tests and negative glucose in urine are good screening tests for the diagnosis. Treatment is lactose-free milk as lactose is converted to galactose in the body. With lactose-free milk the urine test should reverse.
164.	a. Phenylketonuria (PKU)	PKU is due to the deficiency of enzyme Phenylalanine Hydroxylase (PAH) required for the conversion of phenylalanine (PHE) (an essential amino acid) to tyrosine. The diagnosis of PKU is confirmed by demonstrating organic acids like phenylalanine acetate, and phenylalanine pyruvate, etc. in the urine. Accumulated PHE becomes toxic for the brain and other tissues. In Classical PKU (PHE > 1200 µmol/L), gross mental retardation is the major clinical feature, while in Benign Hyperphenylalaninaemia, PHE is between 120-600 µmol/L. In fact there is a disease spectrum, milder clinical findings with decreasing levels of PHE. Treatment is dietary restriction of PHE; such diets are now available in Pakistan, too.
165.	a. Fatty acid oxidation (FAO) disorder	Hypoglycaemia with absence of ketone is clinical hallmark of a group of disorders affecting fatty acid metabolism. In other conditions of hypoglycaemia, ketones are available for brain to use for nutrition but in FAO disorders, hypoglycaemia can be quickly fatal. So, one FAO disorder is now part of the newborn screening in Caucasian populations. Treatment is avoidance of fasting periods, longer than 1-2 hours in the affected infants.
166.	c. Gilbert syndrome	Gilbert syndrome, though very common, is a benign disease, therefore, does not qualify for inclusion in the newborn screening programmes.
	Tumour Markers	
167.	e. Levels decrease without any treatment	This MCQ describes ideal tumour marker, which is non-existent at present. Logically a tumour marker should not decrease without

		treatment but should be a good parameter to monitor effectiveness of treatment.
168.	d. Hepatocellular Carcinoma (HCC)	Alpha Fetoprotein (AFP) is tumour marker of HCC, while a lower level is also associated with benign liver disease e.g. cirrhosis, level of AFP >400–500 ng/ml is indicative of HCC. At this level the specificity is nearly 100% but sensitivity is 45%, it means that when the level is >400-500 ng/ml, HCC is almost confirmed but only in 45% cases of HCC, AFP is high.
169.	e. Serum isoform [-2]proPSA	For proper use of Prostate Specific Antigen (PSA) as a tumour marker of prostatic carcinoma, various strategies are used e.g. use of free PSA, PSA velocity and PSA density. But estimation of an isoform of PSA i.e. [-2]proPSA is best known so far. It is not available for clinical use yet.
170.	b. BRCA mutation	Mutations in BRCA1 and BRCA2 have been implicated in the causation of hereditary breast-ovarian cancer syndrome.

Summary
Chapter No 14
Miscellaneous Disorders

Pancreatic Functions and GIT

- Amylase and lipase potentiate each other in terms of specificity and sensitivity. In acute pancreatitis urine amylase is a good test for late presenters.
- Chronic pancreatitis can be diagnosed by direct tests like cholecystokinin test
- Coeliac disease is an autoimmune disorder due to loss of absorptive surfaces of the intestine.

Porphyrias

- Porphyrias is a set of diseases that result from enzyme deficiencies in the haeme synthesis pathway.
- Porphyria cutanea tarda is the most common porphyria and it is the only acquired porphyria, while acute intermittent porphyria is most common acute porphyria, presenting as abdominal pain without skin involvement.

Vitamins and Minerals:

- Blood levels of vitamin B_{12} and folate are diagnostic but some other tests like serum homocysteine and urine methyl malonic acid can also be used for diagnosis
- Wilson disease is due to defective disposal of copper by the liver
- Zinc deficiency can lead to gastrointestinal disease and growth disorders in children

Inherited Metabolic Disorders (IMDs):

- Previously called Inborn Errors of Metabolism, is a group of disorders, which are individually rare but are important as a group.
- IMDs can be divided into intoxication type, defective energy metabolism producing hypoglycaemia and those involving complex molecules.

Tumour Markers

- Biochemical tumour markers are analysed in blood or rarely in urine for the screening, diagnosis and monitoring of the tumours, but unfortunately no single tumour qualifies as ideal tumour marker that could carry out all these functions.
- Prostate specific antigen is a commonly used tumour marker for prostate
- Alpha-fetoprotein is commonly used for hepatocarcinoma
- Some genetic markers are also used as tumour markers e.g. *BRCA or Ras*

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Chapter No 15
Biochemical Genetics

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MCQs

(Please find key at the end of the chapter)

171. Human genome is contained in 23 pairs of chromosomes. How many ***molecules of DNA*** are present in one human cell?
    a. 23 billion
    b. 3.2 billion
    c. Four million
    d. Ninety-two
    e. One billion

172. The famous discovery of ***"The Double Helix"*** by Watson and Crick pertained to which part of the chromatin:
    a. Chromatin loop
    b. DNA string winding the histone octamers only
    c. Leading strand of DNA
    d. Linker DNA
    e. Whole DNA string

173. The basic units of chromatin are nucleosomes, which consist of 140 nucleotide pairs of DNA around a core of histone and linker DNA. ***The linker DNA*** in a chromatin is related to which of the following histones:
    a. H1
    b. H2A
    c. H2B
    d. H3
    e. H4

174. The diversity in cell structures and functions is a consequence of ***gene regulation***. Many processes are involved in regulation of genes. Which of the following processes is post-transcriptional regulation?
    a. Acylation of histones
    b. DNA bending
    c. RNA polymerase II activity
    d. RNA splicing

e. TATA box regulation

175. Somatic cell nuclei and germ cell nuclei differ in their enzyme content. Which of the following enzymes make the ***germ cell "immortal***":
    a. DNA Polymerase
    b. Endonuclease
    c. Ligase
    d. RNA Polymerase
    e. Telomerase

176. The base sequence of codon 57-58 in *cytochrome B5 reductase* gene is 5'CAGCGC3'. What ***messenger RNA (mRNA)*** sequence will be produced by transcription of gene?
    a. CUGCGC
    b. CAGCGC
    c. GCGCTG
    d. GCGCUG
    e. GCCCGA

177. In the process of translation base pairing takes place between complementary codons of mRNA and ***anticodons*** of which of the following RNAs:
    a. Micro RNA (miRNA)
    b. Pre-messenger RNA
    c. Ribosomal RNA (rRNA)
    d. Small Interfering RNA (siRNA)
    e. Transfer RNA (tRNA)

178. Micro RNAs (miRNA) are very small RNAs, which play important role in genetic regulation; miRNAs are transcribed from which of the following regions of DNA?
    a. Enhancers regions
    b. Exons
    c. Inhibitor regions
    d. Introns
    e. Promotor regions

179. A polymorphism is:
    a. Alteration in nucleotide sequence in two alleles
    b. Any change in the DNA sequence.

c. Genetic variations in more than two siblings of a family
d. The most common variation of a gene or marker sequence.
e. Variation of gene present in >1% of the population

180. PCR is a technique that:
a. Allows DNA to coil very tightly in the helical shape
b. Allows purification/isolation of DNA from a sample.
c. Can produce many exact copies of segments of DNA
d. Provides a statistical analysis of the nitrogenous-base pairings
e. Provides information regarding the sequence of nitrogenous bases

Chapter No 15

Biochemical Genetics

Human DNA

DNA Molecules[1]

- One DNA strand is a giant anion (negatively charged molecule). There are two DNA strands in one chromosome which are joined by weak covalent bonds, scientists believe that these are two separate molecules.
- So, in humans 46 chromosomes contain 92 molecules of DNA.
- The human nuclear genome comprises approximately 3.2 billion nucleotides of DNA, the shortest 50 million nucleotides in length and the longest 260 million nucleotides, each enclosed in a different chromosome.
- DNA from a single cell measures approximately 2 meters in length

Genetic Material[2]

- Diploid genome (two sets of chromosomes)
- Packaged in 23 pairs of chromosomes
- 22 homologous pairs (autosomes)
- 2 sex chromosomes (XX or XY)
- 6 billion bases
- Approximately 25,000 genes (1%)
- Out of all the genes, 8% are regulatory sequences for the 1% of DNA that codes for proteins. So, DNA that regulates genes is eight times more abundant (ENCODE project 2012)[3]

Heterochromatin:

- Densely packed region of chromosomes (centromeres), not transcribed

Euchromatin:

- Less densely packed, transcribed regions

Who Discovered DNA Structure?[1]

- James Watson and Francis Crick discovered DNA structure in 1953
- Deoxyribonucleic Acid (DNA) is a double-stranded, helical molecule.

DNA Structure[4]

- Consists of a pair of antiparallel strands (5′ → 3′ and 3′ → 5′)
- Alternating deoxyribose and phosphate groups
- Covalent linkage by phosphodiester bonds
- Strands held together by hydrogen bonds

Components of DNA Structure

- DNA has a double helical structure which is composed of

- Sugar molecule
- Phosphate group
- A base (A,C,G,T)

- DNA always reads from 5’ end to 3’ end for transcription and replication
  - 5' ATTTAGGCC 3'
  - 3' TAAATCCGG 5'

Nitrogenous Bases

- Pyrimidines
  - 6-member ring
  - cytosine, thymine, uracil
- Purines
  - Fused 5- and 6-Member Rings
  - Adenine, Guanine

Structure of RNA

RNA is different from DNA in some aspects given in Table 15.1

Table 15.1: Difference between DNA and RNA

RNA	DNA
RNA nucleotides contain ribose	DNA contains deoxyribose
RNA has the base uracil	DNA has the base thymine
Presence of hydroxyl group at 2' position of ribose sugar.	Lack of hydroxyl group at 2' position of ribose sugar.
RNA is usually single-stranded	DNA is usually double-stranded
RNA moves out to cytoplasm	DNA remains inside the nucleus

Gene Structure[5]

- Most of the genes consist of; short coding sequences or exons, which are interrupted by longer intervening noncoding sequences or introns.
- A few genes in the human genome have no introns (Figure 15.1)

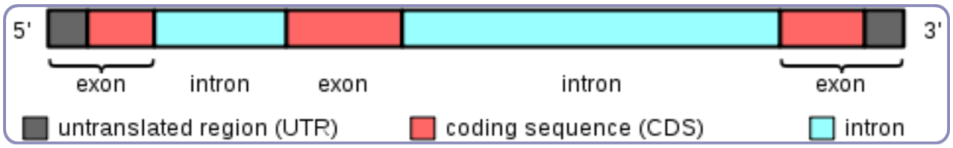

Figure 15.1: Gene Structure

DNA Function

- Carries the blueprint of life for duplication for new cells
- Makes proteins for biological functions (Table 15.2)

Table 15.2: Comparative Genome Sizes of Organisms[4]

Organism	Size (Bp)	Gene Number	Average Gene Density	Chromosome Number
Homo sapiens (human)	3.2 billion	~25,000	1 gene /100,000 bases	46
Mus musculus (mouse)	2.6 billion	~25,000	1 gene /100,000 bases	40
Drosophila melanogaster (fruit fly)	137 million	13,000	1 gene / 9,000 bases	8
Arabidopsis thaliana (plant)	100 million	25,000	1 gene / 4000 bases	10
Saccharomyces cerevisiae (yeast)	12.1 million	6000	1 gene / 2000 bases	32
Escherichia coli (bacteria)	4.6 million	3200	1 gene / 1400 bases	1

Histones[5]

- In humans, DNA molecules are associated with two types of proteins i.e. histone and non-histone proteins.
- The histones play major role in chromosome structure

Classes of Histones

Core Histones

In core histones following families are included

- H2A
- H2B
- H3
- H4
- Two of each of these core histone proteins assemble to form one octameric nucleosome core particle, and **146 base pairs of DNA** wrap around this core particle[6].

Linker histone (H1)[7]

- The linker histone protein H1 **binds the nucleosome at the starting and ending sites of the DNA**, thus locking the DNA into place and help in the formation of higher order structure.
- H1 can stabilize both nucleosome structure and higher-order chromatin architecture.
- In general, H1 molecules consist of a central globular domain with more flexible tail regions at both their N- and C-terminal ends.
- The existence of multiple H1 subtypes and a large variety of posttranslational modifications brings about a considerable degree of complexity

Nucleosome

- Chromatin, under the microscope in its extended form, looks like beads on a string.
- These beads called nucleosomes are the basic unit of chromosome or chromatin fiber.
- Each nucleosome is composed of DNA wrapped around eight histone proteins and linker DNA, functions like a spool and called a histone octamer
- Nucleosomes are repeated every 200 nucleotides.
- Positively charged histones are linked with negatively charged phosphate groups of DNA.
- The histone cores thus act as magnetic forms that promote and guide the coiling of DNA.

Genetic Transcription

- Copying process of one of the two DNA strands into RNA is called transcription.

- Messenger RNA (mRNA) is formed because of transcription, which is 5% of the RNA in cell
- mRNA encodes message from DNA to ribosomes. Genetic information from nucleus to cytosol
- Rapidly degraded by nucleases

Transcription requirements

- DNA-dependent RNA polymerase
- Promoter sequence
- Ribonucleoside triphosphates i.e. ATP, GTP, CTP and UTP (Adenine, guanine, cytosine and uracil)
- Template DNA strand
- GTF: General Transcription factor (Figure 15.2)

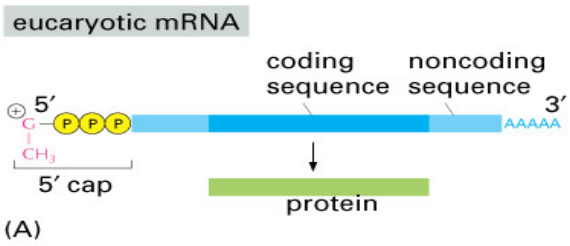

Figure 15.2: Messenger RNA

Genetic Translation

- Genetic translation is the process of making proteins by assembling the amino acids according to the code transcribed from DNA to mRNA.
- From mRNA, a codon of three nucleotides carries the code for each of the 20 amino acids required for protein synthesis (Chapter 3)
- Process of translation involves synthesis of two types of RNAs i.e. Transfer RNA (tRNA), and Ribosomal RNA (rRNA).

Messenger RNA (mRNA)

- mRNA encodes the information for the sequence of amino acids in the protein
- Each mRNA can be reused several times before degradation
- 300-10000 nucleotides
- RNA polymerase II synthesizes mRNA

Transfer RNA (tRNA)

- tRNA are 15% of the RNA in cell
- Carries amino acid to translation machinery
- Stable molecule

- 75-95 nucleotides
- RNA polymerase-III synthesizes tRNA, some ribosomal RNAs and mRNA

Ribosomal RNA (rRNA)

- 80% of the RNA in cell
- 120-4800 nucleotides
- Makes up much of the ribosome
- Very stable
- RNA polymerase I synthesizes majority of rRNA

Gene Regulation[4]

- All cells in an organism have the same genes (Figure 15.3).
- Some genes are turned on while others remain off
- Leads to development of specialized cells and cellular differentiation

Levels of Control of Gene Expression:

1. Chromatin Control

- This is the control at chromosomal level.
- Two processes take place i.e. acylation and methylation according to the requirement of genetic activation and suppression, respectively

Chromatin

- Heterochromatin: Tightly packed
- Euchromatin*:* Less tightly packed

Histone acetylation

- Acetyl groups (-COCH3) attach to lysines in histone tails removing the positive charge on histone proteins
- Breakage of covalent linkage between DNA and histone proteins
- Loosen packing
- DNA is made available for transcription

Histone methylation

- Methyl groups (-CH3) are added
- Tightens packing
- Methylation of histones can either increase or decrease transcription of genes, depending on which amino acids in the histones are methylated

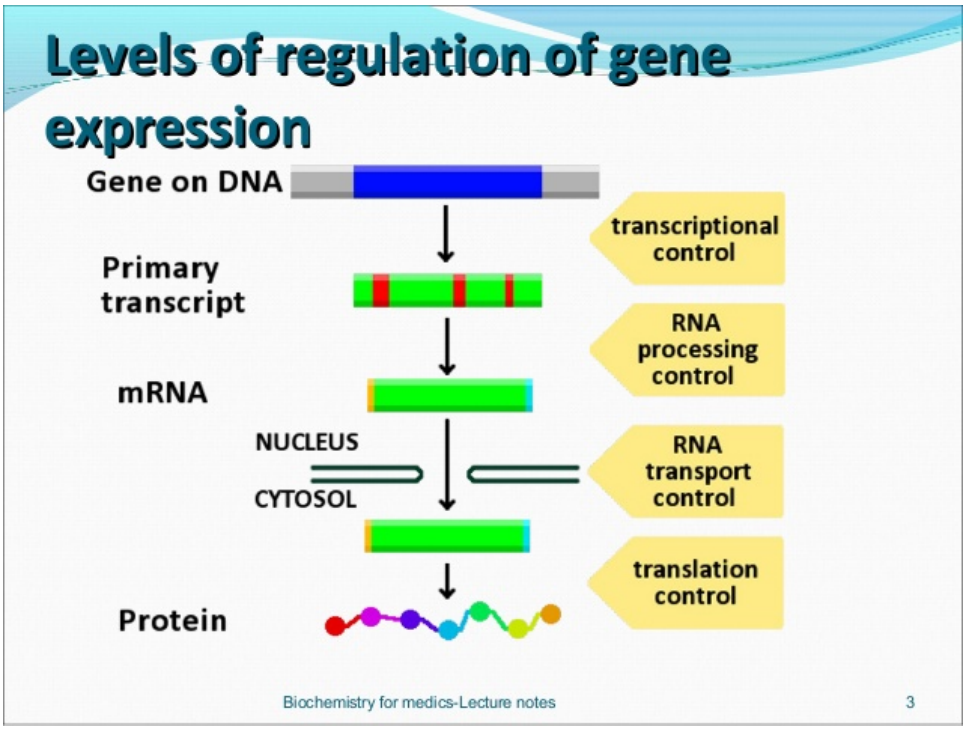

Figure 15.3: Levels of Eukaryotic Gene Regulation

(Adapted from Biochemistry for Medics Available at: https://www.slideshare.net/namarta28/regulation-of-gene-expression-in-eukaryotes-44779699)

2. Transcription Control

- Silencer interferes the binding of transcription factors and blocks the transcription
- RNA polymerase binds to promoter region and transcription begins

Gene expression responds to:

- Environmental conditions
- Type of nutrients
- Amounts of nutrients
- Rapid turnover of proteins

Post-transcriptional control

Three important post-transcriptional controls are:

1. RNA Splicing

- Introns removed from the mRNA
- Splicing plays a role in gene expression
- Exons can be spliced together in different ways.
- Leads to different polypeptides originating from same gene

2. Addition of Nucleotides on 5' and 3' end

- Addition of guanine to 5' end (cap)
- Addition of adenine to 3' end (poly A tail)

3. mRNA degradation by MicroRNA

- Micro RNA regulates complementary mRNA by inducing inactivation and decay of mRNA
- Degradation is done by nucleases or silencing RNA

Telomerase[8]

- Telomerase protects the ends of the DNA strands called 'telomeres'.
- Telomeres are composed of long strings of six nucleotide repeats that cap the ends of chromosomes.
- The repeat sequence is ***GGGTTA***
- With aging, there is attrition of telomeres in somatic tissues, because supply of telomere DNA is exhausted almost in 100 cell divisions. So, mitosis ceases and somatic cell enters a state of 'replicative senescence'
- In germ cells, higher concentrations of telomerase protect these cells from aging, therefore, making them 'immortal'

Mitochondrial DNA[9]

Salient features of mitochondrial DNA are:

- 16,569 base pairs
- 37 genes
- Higher mutation rate
- 128 naturally occurring polymorphisms
- Maternal inheritance

Micro RNA (miRNA)[10,11]

- Very Small RNA (18-25 bp as compared to nearly 1000 bp in messenger RNA) (Figure 15.4)
- They are transcribed from non-coding regions of DNA
- They cause blocking of mRNA or blocking of translation.
- Until now thousands of miRNA have been discovered
- miRNA have been implicated in causation of malignancies and other diseases

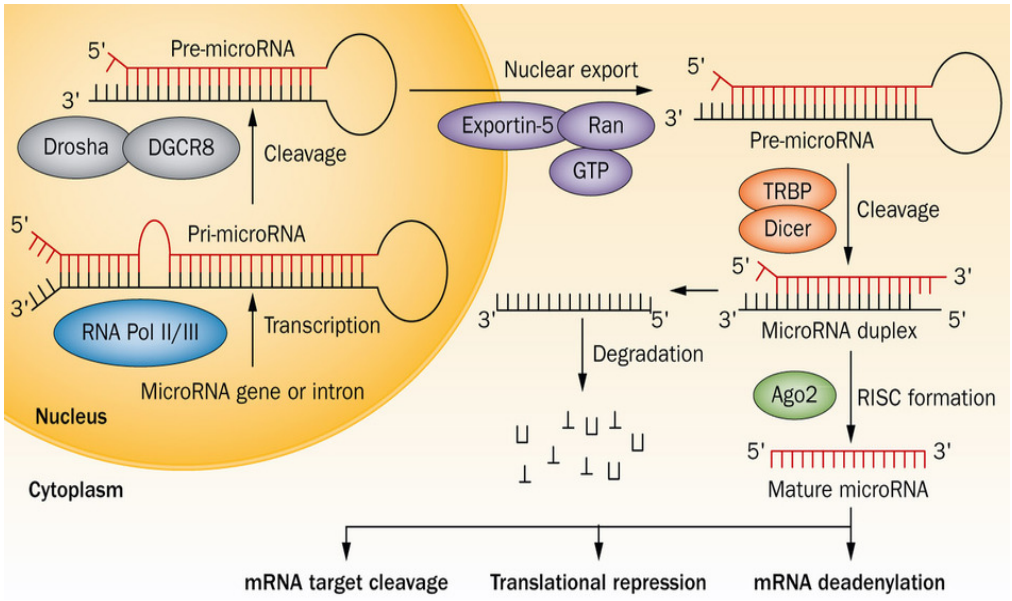

Figure 15.4: Maturation of Micro RNA

Adopted from: https://www.nature.com/articles/ncb0309-228

DNA Polymorphism[12]

- DNA polymorphism is a 'difference' in the nucleotide sequence with a frequency greater than 1% ~~in~~ in a population or species.
- Many types of polymorphism exist e.g. single base pair changes, deletions, insertions, or even changes in the number of copies of a given DNA sequence.
- **SNP:** Most common polymorphism in humans is SNPs (single nucleotide polymorphisms). An example of a SNP would be if a cytosine nucleotide is present at a particular locus in one person's DNA but a guanine nucleotide occurs at the same locus in another person's DNA.
- About 90% of all human genetic variation is due to SNPs. The polymorphism can be:
  - **Transitions:** If both alleles are purines or both alleles are pyrimidines
  - **Transversions:** If one allele is purine and the other is pyrimidine
- Transitions are two times more common as compared to transversions.
- SNP occurs 1 in 1000 nucleotides, meaning there are about 3 million SNP in our genome.

Polymerase Chain Reaction (PCR)[13]

- The polymerase chain reaction (PCR) is the best known and most widely applied of the target amplification methods.
- PCR allows a single DNA sequence to be copied (millions of times).
- Kary Banks Mullis won 1993 Noble Prize in Chemistry for significant improvement in PCR

What is Polymerase

- Polymerase is an enzyme involved in the process of amplification or replication of DNA

Processes of PCR

Three important steps in PCR are:

1. Denaturation: separates parent strands to facilitate new strand synthesis
2. Annealing: "stick" primers to the parent strands to prime DNA synthesis
3. Extension: addition of nucleotides, one at a time, to the growing end of the DNA strand (3' end) using the parent strand as the template

Uses of PCR

Viral Diseases: In our country, most common use of PCR in viral diseases i.e.:

- Hepatitis B
- Hepatitis C
- Human Immunodeficiency Virus (HIV)

Genetic Disorders

- Primers can be created that will only bind and amplify certain sequence of genes or mutations of genes e.g. Cystic Fibrosis, Huntington Disease

Forensic Sciences

- Used for criminal investigations and paternity issues

Phylogenetic analysis

- Study of evolutionary tree of various groups f organisms

Chapter No 15
Biochemical Genetics

MCQs Key with Explanation

MCQ No	Best Option	Explanation
171.	d. Ninety-two	Each DNA strand contains one 1 super long DNA molecule. Humans have 23 sets of chromosomes, 2 chromosomes per set, so 46 chromosomes in each cell and 92 DNA strands or molecules.
172.	e. Whole DNA string	In eukaryotes (including humans) DNA is like a thread wound around the octamer of proteins called histones. The octamer is called nucleosome. Whole of this string is a double helix. (Please see details in the text)
173.	a. H1	There are many types of histones. One of these histones (H1) is not found in the nucleosome but in the linker DNA, that connects the nucleosomes.
174.	d. RNA Splicing	Gene regulation is the key to cell differentiation according to the functions of the tissue where cell is located. This regulation takes place at various levels from chromatin acylation to degradation of messenger RNA by microRNAs (miRNA). RNA splicing is a process, which helps exclude the intronic transcriptions during the formation of messenger RNA (mRNA) from pre-mRNA. So, mRNA consists of only those nucleotides, which are transcribed from exons. Variations in splicing called 'alternate splicing' make variable mRNA according to the need. This is called post-transcription regulation.
175.	e. Telomerase	Telomeres are the DNA sequences located at the end of the chromosomes and telomerase is the enzyme that carries out synthesis of telomeres. In somatic cell this function abandons after 100 cell divisions and cell stops multiplying. In germ cells, due to the abundance of telomerase, the cell division continues the whole life
176.	b. CAGCGC	Transcription process makes mRNA of the same sequence of nucleotides as was present in the original DNA code, except of course, thymine is replaced by uracil in mRNA
177.	e. Transfer RNA (tRNA)	As the mRNA comes out of the nucleus, it carries a codon for one amino acid. Now the tRNA with the specific amino acid attached to it, binds the mRNA according to a nucleotide sequence (anti-codon)

		opposite to mRNA. In this way, amino acid required for the synthesis of protein chain is selected.
178.	d. Introns	Until recently introns were thought as “junk DNA” as they were the ‘non-protein coding’ parts of the DNA (no mRNA is transcribed by them). Discovery of miRNA have changed this concept; miRNAs are transcribed from introns and other non-coding regions of DNA and play a very important role in genetic regulation by controlling the mRNA, either by degradation or blocking.
179.	e. Variation of gene present in >1% of the population	Genetic polymorphism is defined as greater than 1% variations in the genetic material among persons of a population. Any variation lesser than this frequency is called mutation. Single nucleotide polymorphism (SNP- also pronounced as ‘snip’) is the most common and most studied genetic polymorphism.
180.	c. Can produce many exact copies of segments of DNA	Polymerase chain reaction (PCR) reaction is a very commonly used genetic technique, used in almost all the clinical labs for diagnosis and monitoring of the diseases. PCR is basically an amplification technique i.e. it helps in identification of the DNA or RNA by making millions of copies of a particular DNA sequence.

Summary
Chapter No 15
Biochemical Genetics

- Each chromosome in a cell contains two giant DNA molecules (DNA strands) bound with each other by weak covalent bonds.
- DNA is like a thread wrapped around structural proteins called histones with linkers DNA connecting the 8 histones structure called nucleosome.
- DNA thread is in fact two chains of nucleotides wound around each other as double helix
- Nucleotide is composed of a nitrogenous base, sugar and phosphate
- Based on different nitrogenous bases, nucleotides are of four types, thymine, cytosine, adenine and guanine
- Genetic material is coded in a unit of DNA chain called ‘gene’ with protein coding regions (exons) and very large non-coding regions ‘introns’
- From DNA codes, messenger RNA (mRNA) is synthesized containing corresponding code called ‘codon’, except for thymine that is replaced by uracil in RNA (transcription)
- mRNA comes out of nucleus and starts synthesis of amino acid chain with the help of transfer RNA and ribosomal RNA (translation)
- Genetic processes are extensively regulated at various levels of controls
- Micro RNAs (miRNA) are relatively newer discovery, they are formed from ‘non-coding’ regions of the DNA, those were previously called ‘junk DNA’.
- miRNAs and other RNAs are actively involved in genetic regulation
- Mitochondrial DNA is small circular DNA inherited from mothers and involved in genetic coding of a few mitochondrial enzymes.
- Polymerase chain reaction (PCR) is now the most commonly used genetic technique in almost all big and medium-sized laboratories for the amplification of genetic material. It is used for the diagnosis of viral, bacterial and genetic diseases.

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Chapter 16
Laboratory Safety

Reviewed and Edited by:

Aamer Ikram, Erum Khan and Syed Tanveer Abbas Gilani

MCQs

(Please find key at the end of the chapter)

181. Dr Reza has recently returned from USA after obtaining a Fellowship in Clinical Chemistry. He wants to ensure health and safety of all his lab workers. To ensure best professional practice he can follow the guidelines from Occupational Safety and Health Administration (OSHA) in all the following aspects of Lab Safety ***EXCEPT***:
    a. Chemical hygiene plan
    b. Education and motivation of lab workers
    c. Personal protective equipment
    d. Safety inspections
    e. Tuberculosis exposure control

182. For safety of workers against exposure to toxic chemicals, Material Safety Data Sheets (MSDS) must be provided by which of the following stakeholders:
    a. Accreditation bodies
    b. Clinical laboratories
    c. Educational institutes
    d. Employees trade unions
    e. Manufacturers of the hazardous chemicals

183. In the National Fire Protection Association (NFPA) Hazard Rating Diamond, Blue (right small diamond) with 1 written on it, indicates which of the following hazard:
    a. Extremely inflammable
    b. Highly poisonous
    c. Slightly hazardous
    d. Strong shock may detonate it
    e. Water reactive

184. Which of the following fire extinguishers should be used in the event of fire due to flammable metal (e.g. Lithium and Potassium):
    a. CO_2
    b. Dry powder

c. Foam
d. Water
e. Wet chemical

185. A corrosive material spilled onto the hand of a laboratory staff. After diluting the material under running cold water, what should be done next?
a. Consult the MSDS
b. Go to the emergency room
c. Massage lightly with an emollient
d. Rub it with a dry ice pack
e. Wash it with normal saline

186. A new chemiluminescence-based auto-analyser has just been installed in a teaching hospital. As a Consultant Pathologist, you want to create awareness about the potential hazards.

Which of the following is the ***LEAST*** likely hazard to your lab staff while working on this auto-analyser:

a. Aerosols generated from sample probes
b. Effluent contaminated by the pathogens
c. Electric shock
d. Radiation hazard from the reagents.
e. Skin punctures due to high-velocity robotic arms

187. Prof Sikandar is head of the Pathology Department with all modern facilities. Microbiology Section of this department performs diagnostic tests for some important microorganisms. He wants to protect these organisms from ***intentional removal*** from the lab because of a foul play. He develops certain procedures for this purpose. All these procedures come under the category of:
a. Infection control
b. Laboratory biosafety
c. Laboratory biosecurity
d. Risk control
e. Risk mitigation

188. The objective of biosafety programme in a medical lab is:
a. Containment of potentially harmful biological agents
b. Control and monitoring of infectious material generated in the lab
c. Handling of infectious waste
d. Maintain a clean environment
e. Prevention of infections

189. Most important measure for the prevention of Laboratory Associated Infections (LAIs) can be achieved through:
    a. Eye wash
    b. Nasal swabs cultures
    c. Risk assessment
    d. Risk management
    e. Vaccination

190. Let's suppose we symbolize biorisk as a ferocious hungry tiger. The tiger is just a few feet in front of you but closed in a strong steel cage. Which of the following statement best describes this risk:
    a. Consequence: Catastrophic; Likelihood: Rare
    b. Consequence: Insignificant; Likelihood: Rare
    c. Consequence: Minor; Likelihood: Rare
    d. Consequence: Catastrophic; Likelihood: almost certain
    e. Consequence: Catastrophic; Likelihood: likely

Chapter 16
Laboratory Safety

Safety Against Exposure to Toxic Chemicals

Occupational Safety and Health Administration (OSHA) published its Hazard Communication Standard in 1983 to minimize the incidence of chemically related occupational illnesses and injuries in the workplaces. OSHA requires the following[1]:

a. Manufacturers of chemicals should evaluate the hazards of the chemicals they produce and develop hazard communication programs for employees exposed to hazardous chemicals.

b. Clinical laboratories should develop and institute a chemical hygiene plan.

c. Hospitals and laboratories are obliged to maintain an inventory of all hazardous substances used in the workplace.

Laboratory Safety Officer:

Lab leadership (director, in-charge or manager) should appoint a lab safety officer who should endeavour to:

- Create awareness about lab safety among the lab staff
- Provide equipment related to safety e.g. gloves, safety goggles and white coats etc.
- Make a Chemical Hygiene Plan (CHP) with details of hazards of each toxic material used in the lab according to the MSDS (see below) provided by the manufacturers[2,3]

Material Safety Data Sheets (MSDS)[4]

An MSDS is a technical document that provides detailed and comprehensive information on a controlled product related to CHP:

- Health effects of exposure to the product
- Hazard evaluation related to the product's handling, storage or use
- Measure to protect workers at risk of exposure
- Emergency procedures.

OSHA requires that MSDS should be provided for each chemical by manufacturers and suppliers and must be made available on site for laboratory personnel. MSDS will specifically include:

- The chemical identity
- Chemical and common name
- Physical and chemical characteristics
- Signs and symptoms of exposure

- Routes of entry
- Exposure limits
- Carcinogenic potential
- Safe handling procedures
- Spill clean-up procedures
- Emergency first-aid

MSDS contain information on the nature of the chemical, precautions if spilled and disposal recommendations.

The National Fire Protection Association (NFPA)[5]

- NFPA has developed the hazards identification system to provide common, recognizable warning signs for chemical hazards.
- The system consists of four color-coded, diamond-shaped symbols arranged to form a larger diamond shape (Figure 16.1)
  - (top) designates flammability
  - (left) indicates health hazards
  - (right) indicates reactivity-stability
  - (bottom) indicates special consideration

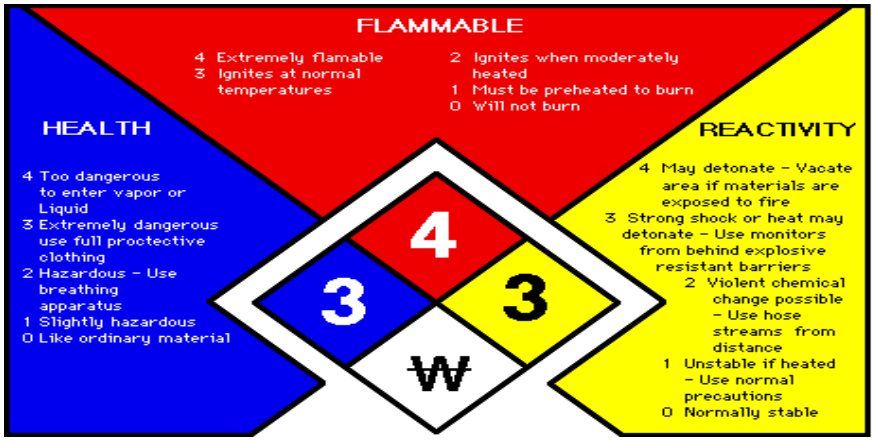

Figure 16.1 The NFPA diamond for Hazard Rating

(Adapted from: https://nationalmarker.com/product/hazardous-materials-classification-sign-spanish-3553/sphmc8p/)

Contained within each color-coded diamond is a number ranging from the respective hazard 0 to 4, indicating the severity of (0 = none and 4 = extreme)

Special Considerations

- Water Reactive
- Oxidizing agent
- Radioactive
- Poison[4]

Hazards in the Clinical Lab[6]

- Biological hazards
- Chemical hazards
- Electrical hazards
- Fire hazards
- Explosives
- Compressed gases
- Inflammable liquids
- Flammable solids
- Oxidizer material
- Toxic materials
- Radioactive materials
- Corrosive materials
- Miscellaneous

Fire Extinguishers[7]

Type (Fire / Extinguisher)	CLASS A Combustible materials (e.g. paper & wood)	CLASS B Flammable liquids (e.g. paint & petrol)	CLASS C Flammable gases (e.g. butane and methane)	CLASS D Flammable metals (e.g. lithium & potassium)	Electrical Electrical equipment (e.g. computers & generators)	CLASS F Deep fat fryers (e.g. chip pans)	Comments
Water	✓	✗	✗	✗	✗	✗	Do not use on liquid or electric fires
Foam	✓	✓	✗	✗	✗	✗	Not suited to domestic use
Dry Powder	✓	✓	✓	✓	✓	✗	Can be used safely up to 1000 volts
CO2	✗	✓	✗	✗	✓	✗	Safe on both high and low voltage
Wet Chemical	✓	✗	✗	✗	✗	✓	Use on extremely high temperatures

Figure 16.2: Classes of Fire Extinguishers in Different Types of Fires

Adopted from: https://surreyfire.co.uk/types-of-fire-extinguisher/

- There are 5 major types of fire extinguisher:
  - Water
  - Foam
  - Dry Powder

- CO_2
- Wet Chemical

- Right type of fire extinguisher should be used to fight the fire started with different types of fuel – these are called 'classes' of fire.
- The fire risk from the different classes of fire in the laboratory premises will determine which fire extinguisher is needed (Figure 16.2).

Laboratory Biorisk Management

Laboratory Biosafety

- A set of preventive measures designed to reduce the risk of ***accidental*** exposure to or release of a biological agent

Laboratory Biosecurity

- A set of preventive measures to reduce the risk of ***intentional*** removal (theft) and misuse of a biological agent– intent to cause harm
  - Lab biosafety + lab biosecurity = laboratory biorisk management
  - Why biosafety practices?

Lab Biosafety is for Protection of:

- Workers
- "Products"
- Co-Workers
- Lab Support Personnel
- Environment

Lab Biosecurity Practice

- Lab biosecurity is protecting the ***organisms*** from misuse
- The biosecurity program should be based on site-specific risk assessment.
- The biosecurity risk assessment can be divided into *five* main steps:

  a. Identify and prioritize the type of microorganism or toxin stored at facility
  b. Identify the type of threat that can be imposed (all possible scenarios)
  c. Analyze the risk associated with specific security scenario
  d. Design and develop overall risk management plan
  e. Regularly evaluate the security risk based on changing laboratory profile (e.g. hiring of new staff, changes in facility design etc.).

Objective of Bio-risk Management

- The objective of biosafety is the ***containment*** of potentially harmful biological agents.
- The purpose of ***containment*** is to reduce/eliminate exposure to lab workers.
- Protection of environment from biohazardous agents[8,9]

Key Elements of Containment[9,10]

- Laboratory practice and technique
- Safety equipment (primary barriers and Personal Protective Equipment)
- Facility design and construction (secondary barriers)
- *Risk Assessment* of the work to be done with a specific agent or under specific circumstances determines the appropriate combination of these elements to employ

Risk Assessment in Diagnostic and Research Laboratory[8-10]

- A risk assessment is a systematic process of evaluating the potential risks that may be involved in a projected activity or undertaking.
- A risk can be based on either a hazard (biosafety) or a threat (biosecurity). A hazard is a source, situation, or act with the potential for harm, while a threat is a person who has intent or ability to cause harm. Collectively, these risks are called biorisks.
- Risk assessments should be fluid, rather than rigid, to allow for evolution with time and changing risks. Ideally, a risk assessment should be performed before any new work is begun. The assessment should be done again when risks change.
- Although there is no singular way to do a risk assessment, there are five basic steps that can be used to perform a risk assessment applied to all laboratories (MMWR, 2012).

Overall Procedure for Risk Assessment[11,12]

- Step 1: Identify hazards
- Step 2: Assess inherent risk (without controls) using consequence and likelihood tables
- Step 3: Determine management controls
- Step 4: Assess residual risk (with controls)
- Step 5: Hierarchy of controls
- Step 6: Monitor control

Step 1: Identify hazards

- Note the potential for infection.
- Ask questions such as what is the most common mode of transmission?

- Is transmission in the laboratory most likely to happen from surfaces, cuts, inhalation, or exposure to mucous membranes? How frequently and in what concentration is the organism isolated?
- If possible, it is important to know the virulence and pathogenicity of the agent when determining the risk, it presents. There are different risk agents in the lab, for example:
    - Microorganism: *Mycobacterium tuberculosis*
    - Chemicals: Formaldehyde, glutaraldehyde
    - Procedure: Frozen section, FNA, trephine
    - Instrument: Chemical analyzer, centrifuge.

Step 2: Determine Inherent Risk

- Determine consequence i.e. what will happen the if exposure occurs (Table 16.1)
- Identify the activities that might cause exposure i.e. the risk that exists without any controls
- Determine likelihood i.e. the chances of exposure (Table 16.2)
- Determine what activities, practices, procedures, and instrumentation will be used to process and test the sample containing the organism
- For example, if centrifugation will be used, the potential for aeroionisation is a risk factor to consider
- Consider the layout of the facility where the specimen will be processed and determine if an open work space is being used or whether separate spaces are required for specific activities. The workflow of the specimen testing from one area of the lab to another should also be considered

Table 16.1 Types of Consequence in Risk Assessment

Level	Descriptor	Consequence – Description
1	Insignificant	No injuries, low financial loss
2	Minor	First aid treatment, on site release immediately contained
3	Moderate	Medical treatment required, on site release contained with outside assistance, high financial loss
4	Major	Extensive injuries, loss of production capability, off-site release with no detrimental effects, major financial loss
5	Catastrophic	Death, toxic release off site with detrimental effect, huge financial loss

Table 16.2 Types of Likelihood in Risk Assessment

Level	Descriptor	Likelihood – Description
1	Rare	May occur only in exceptional circumstances
2	Unlikely	Could occur at some time
3	Possible	Might occur at some time
4	Likely	Will probably occur in most circumstances
5	Almost Certain	Is expected to occur in most circumstances

Example of Risk Agents and Risk Activities[8-13]
Risk Agent (Formaldehyde)

- Formaldehyde (3.7%–4.0%) Surgical Pathology workers are exposed.
- The chemical is volatile and toxic and causes irritation to the eyes, mucous membranes, and skin
- Increased risk for all cancers.
- OSHA exposure limit of 0.75 ppm as an 8-hour time-weighted average
- 2.0 ppm for short-term (15-minute) exposures
- If formaldehyde can be detected by smell, means exposure is occurring at a concentration beyond acceptable limits.

Risk Activity 1 (Automated Analyzers)

- Clinical Chemistry auto-analyzers often have high-velocity robotic arms and samplers that may cause skin punctures and lacerations.
- Sample probes may generate aerosols and droplets.
- Effluents of clinical analyzers are contaminated with pathogens and hazardous chemicals.

Risk Activity 2 (Frozen Sections)

- Performed on fresh tissue and is a high-risk procedure for infectious exposure
- Freezing tissue does not kill organisms
- Use of the cryostat cutting blade creates potentially dangerous aerosols.
- Use of freezing propellant sprays, causes aerosolization of tissues from previously cut specimens that are at the base of the instrument.
- Generate aerosol and droplet contamination, posing an infectious risk to all personnel in the area

Other Risk Activities

- Use of centrifuge
- Use of vortex
- Use of syringes
- Use of pipette
- Risk of breakage (sharp injury)
- Activities generating aerosols and droplets
- Manipulating needles, syringes and sharps
- Sub-culturing positive blood culture bottles, making smears
- Expelling air from tubes or bottles (including Eppendorf pipettes)
- Withdrawing needles from stoppers
- Separating needles from syringes
- Aspirating and transferring body fluids
- Harvesting tissues
- Manipulating inoculation needles, loops, and pipettes
- Flaming loops
- Cooling loops in culture media
- Sub-culturing and streaking culture media
- Expelling last drop from a pipette (including Eppendorff pipettes)
- Manipulating specimens and cultures
- Setting up cultures, inoculating media

Step 3

- Identify control measures
- Determine the residual risk i.e. the risk that remains after implementing measures to reduce it

Step 4

- Determine if existing controls are very good, reasonable or poor
- Management Controls

Step 5

- Act where necessary!
- Use hierarchy of controls when determining any controls that need to be implemented:
- Hierarchy of Controls
    - Elimination
    - Substitution

- Isolation
- Engineering control

- Administrative control
- PPE (Personal Protective Equipment)
- Procedure

Step 6

- Monitor controls

Biosafety Levels (BSL)

- Combination of laboratory practices and procedures, safety equipment (primary barriers) and laboratory facilities (secondary barriers)
- Somewhat related to risk groups
- Focus is on laboratory manipulation of the agent
- Risks groups and biosafety levels are different concepts
- Biosafety levels are also referred to as containment levels

BSL-1

- Dealing with those biological agents which are unlikely to cause disease in healthy humans & are off minimal hazard to the lab
- Laboratory environment and personnel
- Agents not known to cause disease:

  - *Escherichia coli strain K12*
  - *Agrobacterium tumifaciens*
  - *Micrococcus leuteus*
  - Coryneforms
  - *Bacillus subtilis*
  - Brewers yeast

BSL 1 Requirement

- No special air handling requirements.
- Biological safety cabinet not required.
- Separated from public areas.
- Hand washing sinks.
- Eyewash
- Separate hanging areas for outside lab clothes.
- Windows that can be opened protected from fly screens.
- Floors, walls and lab furniture must be washable.

- No eating or drinking in the laboratory
- Decontamination of waste by effective disinfectants

BSL – 2

- Dealing with those pathogens that can cause disease in humans or animals but not a serious hazard.
- Effective treatment is available; limited risk of spread.
- Immunization or antibiotic treatment available
- Agents associated with human disease

Examples:

- *E. coli*
- *Staph aureus*
- *Clostridium perfringens*

BSL2 Requirement

- Proper Signs.
- Doors should be self-closing, access to lab is controlled (only authorized staff has access)
- Coat hooks for lab coats near exit.
- Lab located away from public areas, general areas and patient care areas.
- Floors, walls and furniture must be impervious for disinfection.
- An autoclave must be in or near the lab.
- Inward directional airflow and negative pressure in the lab area is recommended
- Centrifugation procedures must be contained.

Special Procedures

- Decontaminate work surfaces
- Report spills and accidents
- No animals in laboratories

BSL - 3

- Indigenous / exotic agents associated with human disease
- Has potential for aerosol transmission

Examples:

- *Mycobacterium tuberculosis*
- *Influenza virus*

- SARS virus
- *Coxiella burnetii*

BSL - 4

- Dangerous / exotic agents of life threatening nature like:
    - Ebola virus
    - Marburg virus
    - Small Pox virus

Requirements of BSL-3 and BSL-4 are beyond the scope of this book and may be found elsewhere.

Protect Yourself: Universal Precautions[8,9]

Treat all blood and body fluids as potentially infectious.

- Skin protects from pathogens - cuts, dermatitis, chapping, small cracks allow germs to enter the body
- First aid - use gloves, have as little contact as possible with blood or body fluids
- Wash hands with antibacterial soap after contact
- After contact, flush eyes and face with fresh water for several minutes
- Clean-up and safe housekeeping
- After an accident, the entire area must be cleaned with disinfectant
- Cleaning equipment must be disinfected
- Restrict access to the area
- Use disposable towels - dispose of properly
- Other exposure hazards
- Cleaning surfaces contaminated with blood,
- ALWAYS wear gloves and protective apron or clothing
- Be alert for sharp objects, broken glassware, used syringes in trash
- Do not pick up broken glass - use brush or broom & dustpan
- Dispose of glass, sharp objects safely
- Laundry - bloody or contaminated linens or sharp objects

Common Sense Rules

- Wash hands and remove protective clothing before eating, drinking, smoking, handling contact lenses, applying lip balm or cosmetics
- Keep hands away from eyes, nose, mouth while cleaning
- Frequent hand washing is best defense against spreading infection

Chapter 16
Laboratory Safety

MCQs with Key and Explanation

MCQ No	Best Option	Explanation
181.	e. Tuberculosis Exposure Control	Guidelines related to infectious disease are not issued by Occupational Safety and Health Administration (OSHA) but by CDC (Centre for Disease Control and Prevention).
182.	e. Manufacturers of the hazardous chemicals	Material Safety Data Sheet (MSDS) is to be provided by the manufacturer of the toxic chemicals.
183.	c. Slightly Hazardous	National Fire Protection Association (NPFA) (US) provides fire hazards sign for use in any premises.
184.	b. Dry powder	Fire extinguishers must be used according to the nature of the fire.
185.	a. Consult the MSDS	MSDS provides all the data required for nature and potential hazards of a toxic material.
186.	d. Radiation hazard from the reagents.	As compared to radioimmunoassay the reagents used in chemiluminescence do not emit any radioactivity.
187.	c. Laboratory biosecurity	Laboratory biosecurity is relatively newer concept after the incidence of anthrax letters occurred in US. It deals with intentional removal of a hazardous organism from the lab for malicious purposes. In contrast laboratory biosafety deals with the accidental exposure of the harmful organisms.
188.	a. Containment of potentially harmful biological agents	Any laboratory dealing with some potentially harmful biological agents should adopt proper measure to contain this agent.
189.	c. Risk Assessment	Risk assessment is the key to the biosafety. Six steps involved in risk assessment are given in the text of the

		chapter.
190.	a. Consequence: Catastrophic; Likelihood: Rare	By giving the example of tiger, risk assessment is conceptualized. Two main dimensions are *'consequences'*, which means the potential hazard of the organism being dealt in the lab and *'likelihood'* meaning how much the chances are that this organism will harm any lab staff or any person or community after taking appropriate steps.

Summary
Chapter 16
Laboratory Safety

- One of the most important duties of a Pathologist of any discipline is to protect himself / herself, lab staff and community from various hazards associated with laboratory work. All the practices carried out for this protection are grouped together as 'Laboratory Safety'
- Lab safety encompasses various aspects e.g.:
  - Safety from hazardous chemicals, handled in almost all the sections of a lab
  - Fire hazard
  - Electric hazard
  - Lab Bio-risk Management
- Guidelines for the protection from hazardous chemicals have been issued by OSHA (Occupational Safety and Health Administration).
- Every hazardous chemical should be accompanied by a data sheet called Material Safety Data Sheet (MSDS) prepared by the manufacturer.
- Comprehensive SOPs for fire-fighting are necessary including the proper use of fire extinguishers
- Lab Bio-risk management includes two aspects:
  - Biosafety means protection of the lab staff, lab animals, community and the environment from an ***accidental*** exposure of dangerous pathogens dealt in the lab
  - Biosecurity means protection of dangerous pathogens from ***intentional*** removal from the lab for some malicious use.
- Risk assessment is the key to the successful containment of the hazardous material
- Consequences and likelihood are two important concepts in biosafety.

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Chapter 17
Specimen Collection and Pre-Analytical Variables

Reviewed and Edited by:

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MCQs

(Please find key at the end of the chapter)

191. ***Venepuncture*** should NOT be carried out in which of the following patients?
   a. Prone
   b. Sitting for less than 10 minutes
   c. Sitting for more than one hour
   d. Standing
   e. Supine

192. ***Stainless-steel needle*** is required for the collection of which of the following specimens?
   a. Amino acids
   b. Arterial blood gases
   c. Clotting factors
   d. Cortisol
   e. Trace elements

193. A 42 years old female has reported in Laboratory Reception with a request of following tests:
   - Blood CP
   - Blood Culture and Sensitivity
   - Electrolytes
   - Plasma Glucose
   - PT and PTTK

   Which of the following containers the phlebotomist should fill ***first***?
   a. Citrate
   b. Culture bottle
   c. K-EDTA
   d. Lithium heparin
   e. NaF + K-EDTA

194. An acidified 24 h urine sample (pH< 3) is ***most unsuitable*** for which of the following analytes?
   a. Adrenaline
   b. Calcium
   c. Metanephrine
   d. Urate
   e. VMA

195. A researcher of sports medicine is working on the changes in the serum levels of creatine kinase (CK), lactate dehydrogenase (LD), aspartate amino

transferase (AST) and aldolase of healthy volunteers (pre-and post-moderate exercises).
Which category out of following sportspersons, will have greatest increase in these enzymes after the exercise:

a. Most fit marathon runner
b. Oldest female player (31y)
c. Player on his first day of sports
d. Sportsperson of a game of static nature
e. Youngest male player (19 y)

196. As per standard protocol the Chemical Pathology and Endocrine Clinics preferably do not accept patients after 0900 h for Oral Glucose Tolerance Test (OGTT). A clinician request to carry out OGTT in a patient in fasting condition at 1200 hours was not entertained.
The most important physiological reason not to carry out mid-day OGTT is :

a. Basal insulin level is higher
b. Glucose tolerance is higher
c. Insulin response is less effective
d. Insulin response is sharp
e. No Insulin response at all

197. A healthy volunteer has taken a standard 700-kcal meal, which of following serum analytes will be increased to the greatest extent?

a. Albumin
b. Calcium
c. Cholesterol
d. Phosphorous
e. Urea

198. A 47 years old male is smoking for the last two decades. Which of the following parameters will be falsely high?

a. Carboxyhaemoglobin
b. HDL-Cholesterol
c. PCO2
d. PO_2
e. Urea

199. Which of the following hormones will be five times higher in a premature baby (born at 30^{th} weeks of gestation) as compared to a mature baby?

a. Adrenaline
b. Aldosterone
c. Cortisol
d. Insulin
e. Testosterone

200 A group of healthy volunteers have been brought at the height of 5400 meters. Which of the following changes in the lab parameters will take place after 24 hours:

a. Decreased beta 2 globulins
b. Decreased blood haemoglobin
c. Decreased haematocrit
d. Increased erythrocytic 2,3 diphosphoglyecerate
e. Oxygen hemoglobin curve is shifted to the left

Chapter 17
Specimen Collection and Pre-Analytical Variables

Specimen is Appropriate Term[1]

- Avoid using the word "sample"
- Term "sample" is used for the subjects selected out of the population for a research project

Question:

A patient has entered in a laboratory reception for a lab test with a requisition form in hand. Can you name the ***FIRST STEP*** the phlebotomist should take before the specimen collection?

Answer:

1. Confirm at least TWO identifiers e.g., name and age of the patient.
2. Can be done by asking the patient his/her name and age and then tally it with the requisition form[2].

Posture for Specimen Collection[2]

- Sitting for more than 30 minutes is ideal
- In a critically ill patient supine or prone position can also be used
- Specimen should not be collected in standing position
- Arm / limb with intravenous infusion or I/V cannula should be avoided

***TEN* specimens tested in Chemical Pathology Lab for Various Analytes**[2-4]

1. Plasma
2. Serum
3. Urine
4. Semen
5. Feces
6. Saliva
7. Body fluids e.g. CSF, pleural, ascitic and pericardial fluids
8. Amniotic fluid
9. Hair and nails
10. Chorionic Villous Sampling (CVS)

Question

A 47 years old woman has undergone left mastectomy about 5 months ago. Right mastectomy was done one month earlier than left. Which is the ideal site for collection of blood specimen for routine laboratory tests?

Answer

Specimen collection in patients with mastectomy

- Specimen should not be collected from ipsilateral arm
- It is due to impaired lymphatic drainage
- If mastectomy was done > 6 months ago then specimen can be drawn
- If bilateral mastectomy has been done within 6 months then ankle is the best choice[1]

Specimen for Trace Elements

- These include metals like iron, copper, lead, zinc etc.
- Stainless steel is compressed metal and less chances of breakage of particles

EDTA Contamination[5]

Transfer of EDTA crystals by tip of syringe from one specimen tube to another

Types of EDTA contamination

- Gross
- Subtle

Gross EDTA contamination signs:

- False hypocalcaemia
- False hyperkalaemia
- Normal renal functions
- No haemolysis (normal LD enzyme)

Subtle EDTA Contamination

- Can occur as a result of backflow of K^+-EDTA contaminated blood to specimen collection system (e.g. vacutainer) with transfer of minute amounts of K^+-EDTA to subsequently collected samples[6].
- Very difficult to diagnose in individual cases.

How to Avoid EDTA and other Additives Contamination

- Proper order of draw can prevent these types of contaminations (Table 17.1).

Table 17.1: Order of Draw (WHO Recommended)[1-7]

Order of use[a]	Type of tube/usual colour[b]	Additive[c]	Mode of action	Uses
1	Blood culture bottle (yellow black striped tubes)	Broth mixture	Preserves viability of microorganisms	Microbiology – aerobes, anaerobes, fungi
2	No additive tube (Colorless or pale yellow)	None	Blood clots and then serum is separated (by centrifugation)	Blood chemistry, immunology, serology, blood bank
3	Coagulation tube (light blue top)	Sodium citrate	Forms calcium salts to remove calcium	Coagulation tests (PT, PTTK), INR, D dimers. Requires full draw
4	Clot activator (red top)	Clot activator	Blood clots rapidly and then serum is separated (by centrifugation)	Blood chemistry , immunology and serology, blood bank

5	Serum separator tube (red-grey tiger top or golden)	None	Contains a gel at the bottom to separate blood from serum on centrifugation	Blood chemistry , immunology and serology
6	Sodium heparin (dark green top)	Sodium heparin or lithium heparin	Inactivates thrombin and thromboplastin	For lithium level use sodium heparin, for ammonia level use either
7	Plasma separating tube (PST) (light green top)	Lithium heparin anticoagulant and a gel separator	Anticoagulants with lithium, separates plasma with PST gel at bottom of tube	Blood chemistry
8	EDTA (purple top)	EDTA	Forms calcium salts to remove calcium	Haematology, Blood Bank (cross-match) requires full draw
9	Blood tube (pale yellow top)	Acidcitrate dextrose (ACD, ACDA or ACDB)	Complement inactivation	HLA tissue typing, paternity testing, DNA studies
10	Oxalate/fluoride (light grey top)	Sodium fluoride and potassium oxalate	Antiglycolytic agent preserves glucose	Glucose, requires full draw

a "1" indicates draw first, and "10" draw last (if used).
b Verify with local laboratory as colour codes may differ.
c Gently invert tubes with additives to mix thoroughly; erroneous test results may be obtained when the blood is not thoroughly mixed with the additive.

Sodium Fluoride[2]

- Antiglycolytic activity by inhibiting enolase
- Weak anticoagulant
- Higher concentration can cause fluid shifts as ATPase is also inhibited. Inhibits enzymes in analysis of many analytes e.g., Urea

Fluoride- Animated

Fluoride -the RBC Controller: In a sample tube sodium (or potassium) fluoride addresses the RBCs *"Look gentleman this glucose is not for you; so please don`t eat it. Its patient`s glucose and is sacred for us"*. In the tubes without fluoride, there is no body to control RBCs and they party with glucose as the main course!!

Adapted with thanks from "The Spectrum" PSCP Newsletter, 3rd Issue, Dec 2014.

Specimen for Molecular Biology (DNA) Tests[2]

- Heparin interferes polymerase enzymes and should not be used
- ACD (acid citrate dextrose) can be used

Acidified Urine Sample[2]:

Acidified urine (pH< 3) sample is obtained usually in lab by adding N/10 HCl in the empty container that is handed over to the patient for specimen collection. This specimen is required for following tests:

- Adrenaline
- Calcium
- Metanephrine
- VMA
- Most unsuitable for estimation of urinary urates.

CSF collection[2]

During lumbar puncture, physician should collect Cerebro Spinal Fluid (CSF) in three tubes marked as 1, 2 and 3. In Laboratory, CSF specimen in ***Tube No 1*** will be dealt for Glucose estimation, ***Tube No 2*** (CSF) for cell counts and ***Tube No 3*** (CSF) for culture and sensitivity to avoid contamination.

Pre-Analytical Variables
(Controllable factors)

In multiple studies at different time periods: In multiple studies Carraro and Plebani have demonstrated that upto 75% of the laboratory errors are due to pre-analytical factors and pre- and post-analytical steps of the total testing process are more error-prone than the analytical phase[8-10].

Controllable factors, that can influence the Laboratory Tests[11]

1. Posture
2. Prolonged bed rest
3. Exercise
4. Physical Training
5. Circadian variation
6. Fasting and starvation
7. Diet
8. Drugs and substances
9. Smoking
10. Alcohol

Postural Change

- From lying to standing will cause a decrease in blood volume of about 10% and increase in some proteins by about 8-10%.
- This pre-analytical factor may lead to significant variations in certain analytes e.g., proteins like albumin
- Sudden change in posture e.g., from lying to standing is more important
- Lesson for Pathologists / Lab Staff: Patient should be lying/sitting for at least 30 min before specimen collection

Avoid Prolonged Tourniquet Time

- A prolonged tourniquet time may lead to blood pooling at the venepuncture site, a condition called haemoconcentration.
- Ideally, the tourniquet should be in place no longer than one minute to prevent haemoconcentration.
- Application of tourniquet for a few minutes before collection of a blood specimen in an adult result in changes mimicking postural change from lying to standing, as in standing the fluid shifts to the lower parts of the body and haemoconcentration ensues.
- Haemoconcentration can cause falsely elevated results for albumin, glucose, potassium and cholesterol.
- Analytes bound to albumin or other proteins will also be greatly affected by tourniquet application such as thyroxine.

Changes after Exercise

- Exercise leads to changes in many routine analytes and hormones
- Plasma renin: 400% increased
- Catecholamines: double the upper reference value
- Growth hormone and cortisol: markedly raised
- Arteriovenous difference of glucose is increased

- Once again, the bottom line is NOT to take specimen immediately after exercise

A Word of Caution for Pathologists

- Don't` get carried away with patient`s demands
- Your SOPs should be clear and you should be firm on them
- *"Wait for 1 hour after exercise before specimen"* such a notice in the lab will protect you from many potential Clinical Governance Inquiries
- Dynamic function tests after exercise should be followed as per standard guidelines.

Post-Exercise Enzyme Changes:

- Greatest increase in these enzymes after the exercise will occur in the player on his first day of sports.
- More fitness – lesser change!!
- The usual changes in CK, AST and aldolase are less pronounced in fitter athletes
- Basal levels may be higher though

Timing of OGTT

- OGTT should be done only in the morning before 0900 h, preferably after 8 h fast (water is allowed during medical fast)
- At mid-day insulin action becomes more sluggish and less effective if quantitatively unchanged
- Young pathologists should know why our predecessors insisted on OGTT in the morning

Effect of Diet

- Many analytes are affected by diet but phosphorous estimation is affected the most.
- Many parameters are low in vegetarians e.g. cholesterol and LDL-C
- They may have low B_{12} and other vitamins – may be in the deficiency ranges

Effect of Smoking

- Carboxyhaemoglobin is tested for Carbon Monoxide (CO) poisoning
- In smokers it can be high (approximately10%) without CO poisoning.

Effect of Imaging Investigation

- Hypocalcaemia after contrast MRI or CT is due to calcium binding with contrast dye
- Dyes like gadodiamide can chelate these analytes
- Transient hyperprolactinaemia may occur after mammography.

Pre-Analytical Variables

(Non-Controllable factors)

FOUR classes of non-controllable factors affecting laboratory tests[11, 12]

1. Biological factors
   a. Age
   b. Sex

c. Race
2. Environmental factors
   a. Ambient temperature
   b. Seasonal influence
   c. Altitude
2. Cyclical changes
   a. Menstrual cycle
   b. Diurnal / circadian
4. Underlying medical conditions
   a. Obesity
   b. Blindness
   c. Pregnancy
   d. Stress
   e. Fever
   f. Shock
   g. Transfusion

Enzymes in a normal newborn

In a new-born, even in the absence of disease, all the following enzymes are increased:

- Alanine aminotransferase (ALT)
- Aspartate aminotransferase (AST)
- Creatine kinase (CK)
- Lactate dehydrogenase (LD)

Haemoconcentration in newborn

- Haemoconcentration is the most important haemodynamic change during first few days of life
- Main reason is due to fluid shift to extra-vascular space
- Haematocrit and most biochemical parameters are increased
- Please note that higher bilirubin in neonate is because of low quantities of glucuronosyltransferase, a liver enzyme involved in bilirubin metabolism and is not in optimal quantities

Hormones in Premature Neonates

- Many hormones are increased in premature neonates
- Aldosterone tops the list

Changes in Elderly

- Creatinine clearance decreases with passing years, though the creatinine level is reduced in blood due to decreasing muscle mass
- ALP is higher in post-menopausal women as compared to the ‘old boys’
- Thyroxine remains unchanged in an elderly adult

Why Thyroxine is Unchanged?

- In fact, production of thyroxine is also decreased along with testosterone and cortisol
- But thyroxine degradation is also reduced

Gender Difference in Analytes

- Aldosterone, AST, cholesterol and CK are higher in males. Copper is higher in females.
- Haemoglobin, iron, ferritin are lower in females (ferritin 0.33 of males)
- Good cholesterol and apolipoprotein A is higher in females (lucky ones!)

Ethnic Variations

- Afro-Caribbean people have many peculiarities in their blood biochemistry
- Serum creatinine and CK are higher due to high muscle mass
- Serum proteins are higher because of more IgG while albumin is lower.

High Altitude and Blood Analytes

Changes in blood parameters at high altitude have been a topic of active research in our country. These include:

- Increased blood haemoglobin
- Increased haematocrit
- Increased erythrocytic 2,3 diphosphoglyecerate
- Oxygen hemoglobin curve is shifted to the right
- Changes in haemoglobin metabolism is due to hypoxia at high altitude

Seasonal Variations in Analytes

- Some parameters change with seasons e.g. cholesterol but the changes may not be significant
- Calcium level decreases in summer due to increased urinary excretion
- On the other hand, vitamin D levels may increase due to more sun exposure in summer

Obesity Related Changes

- If a patient has increased triglycerides, just try to know his/her BMI
- It may be rewarding, as patient can be advised lifestyle modification instead of resorting to medicines.
- Obesity and hypertriglyceridemia are important components of metabolic syndrome (Chapter 1)

Biochemical Parameters in Pregnancy

- Haemodilution is the keyword to know the biochemical changes
- Most of the parameters are diluted
- Some proteins are increased, however, due to increased production e.g., TBG and CBG etc.
- Glycosylated haemoglobin is also altered in pregnancy due to shortened life span of RBCs.

Biochemical Tests in Critically Ill patients

- If you receive samples from ICU –interpret it carefully
- Many biochemical tests show aberrant results
- Examples are:
    - Non-thyroid illness
    - Stress hyperglycemia
    - Altered lipid parameters (e.g. increased triglycerides and decreased cholesterol)

Chapter 17

Specimen Collection and Pre-Analytical Variables

MCQs with Key and Explanation

MCQ No	Best Option	Explanation
191.	d. Standing	Standing position is most inappropriate for blood specimen collection due to haemodynamic changes e.g. postural hypotension etc.
192.	e. Trace elements	Ordinary iron needle may contaminate the specimen with iron particles which may interfere with the analysis of trace elements
193.	b. Culture bottle	This MCQ is related to 'Order of Draw'. WHO protocol is given in the chapter. Blood culture bottle is filled first to avoid any contamination.
194.	d. Urate	Amorphous urates can be seen as pinkish crystals in acidic pH while in alkaline pH urates get dissolved. SO uric acid or urate will be falsely low in acidified urine. It is opposite in phosphates which are seen as whitish crystals in alkaline pH and get dissolved in acidic pH.
195.	c. Player on his first day of sports	Exercise affects various biochemical parameters e.g. CK, AST and aldolase and many hormones. This effect is more pronounced in the new players. With increased fitness, the effect reduces.
196.	c. Insulin response is less effective	At mid-day insulin effect becomes sluggish, so OGTT should be done in the morning.
197.	d. Phosphorous	False increase in phosphorous levels will occur if blood specimen is drawn immediately after a heavy meal.
198.	a. Carboxyhaemoglobin	If carboxyhaemoglobin is to be estimated e.g. for the diagnosis of carbon monoxide poisoning, history of smoking is very important as this parameter is high in smokers.
199.	b. Aldosterone	Aldosterone is highest in premature neonates as compared to other hormones
200.	d. Increased erythrocytic 2,3 diphosphoglyecerate	High altitude brings changes in many parameters, erythrocytic 2,3 diphosphoglyecerate is one of them, that is increased.

Summary
Chapter 17

Specimen Collection and Pre-Analytical Variables

- Laboratory reception is the place of interphase between the laboratory staff and the patients. Here patient comes to give their specimen for laboratory tests.
- Most common specimen for lab tests is venous blood, so 'phlebotomist' is the term used for the lab staff specialized in specimen collection
- Lab staff including Pathologist should be well-versed with all the aspects of phlebotomy starting from the verification of the patients` identification, then the procedure itself and shifting of the blood specimen to the appropriate collection tubes
- 'Order of Draw' is the most important protocol providing the correct order of filling of the specimen collection tubes. All members of the laboratory team must know this 'order of draw'.
- Many factors effect laboratory tests, a good knowledge of these factors is vital for avoidance of wrong interpretation of laboratory results, which may result into unnecessary panic.
- Some of these pre-analytical factors are controllable e.g. time of the day, fasting state or exercise etc.
- Other factors are uncontrollable e.g. age and gender of the patient, co-morbidities and menstrual changes. Such factors should be kept in mind while interpreting the lab results.

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Chapter 18
Introduction to Analytical Techniques and Methods of Analyses

Reviewed and Edited by:

Sahar Iqbal, Syed Talha Naeem, Shahnaz Noor and Lubna Falak

MCQs

(Please find key at the end of the chapter)

201. ***Absorbance*** of light is:

    a. Difference between incident and transmitted light
    b. Light absorbed in a solution after striking it
    c. Negative log of transmitted and incident light
    d. Molar absorption of light by the solution
    e. Ratio of incident and transmitted light

202. According to ***Beer-Lambert Law*** absorbance of a solution increases with:
    a. Intensity of the light source
    b. Length of the light path
    c. Number of light scattering particles
    d. Percentage transmittance of the light
    e. Reflectance of light

203. An autoanalyser has rejected the blank cuvettes before the start of analysis with the message ***"Cuvette Check Fail" (Dirty Cuvettes)***. Which of the following phenomena is mainly prevented by this function of autoanalyser:
    a. Absorbance of light
    b. Emission of light
    c. Reflectance of light
    d. Scattering of light
    e. Transmission of light

204. ***Immunoassays*** have passed through an evolutionary process of development with decreasing 'Limits of Detection' (Increasing 'Analytical Sensitivity') and ease of automation. Following is a list of labelled immunoassays in a random order. Which of the immunoassay techniques has the highest ***Analytical Sensitivity***?
    a. Chemiluminescence Immunoassay (CMIA)

b. Enzyme Linked ImmunoSorbent Assay (ELISA)
c. Fluorescent Polarized Immunoassay (FPIA)
d. Immunoradiometric Assay (IRMA)
e. Radioimmunoassay RIA)

205. In an Ion-Selective Electrode (ISE) the most important part determining the selectivity of the cell is:
    a. External reference electrode
    b. Inner electrolyte
    c. Internal reference electrode
    d. Membrane
    e. The frit

206. In Clinical Enzymology, ***Michaelis and Menten Equation*** provides the basis of quantitative measurement of enzymes. Which of the following is a basic assumption of this equation?
    a. The effect of product is negligible on the concentration of enzyme-substrate complex.
    b. The enzyme concentration of the enzyme-catalysed reaction is fixed.
    c. The pH is neutral (7.40).
    d. The reaction is first order with respect to substrate
    e. The temperature of the reaction is 37^0C.

207. The discrete auto-analyser are most commonly used these days, which can carry out multiple tests on one specimen or one test on multiple specimens. They are also called:
    a. Batch analyser
    b. Centrifugal analyser
    c. Continuous flow analyser
    d. Discrete analyser
    e. Random access analyser

208. Specimen having analyte of interest is incubated with suitable reagent, reaction takes place and coloured complex thus formed is read for absorbance at specific wavelength. Which type of analytical reaction it is:
    a. Continuous monitored
    b. End-point
    c. End-point kinetic
    d. Two point enzymatic
    e. Two point kinetic

209. Highly purified chemicals that are directly weighed or measured to produce a solution whose concentration is exactly known. Such Reference Material is known as:

a. Certified Reference Material
b. Crystalline Reference Material
c. NIST Traceable Reference Material
d. Secondary Reference Material
e. Standard Reference Material

210. Which of the following is the most accurate method?
a. Definite Method
b. Field Method
c. Gold Standard Method
d. Index Method
e. Reference Method

Chapter 17
Introduction to Analytical Techniques and Methods of Analyses

Basic Definitions[1, 2, 3]

(Authored by Dr. Syed Talha Naeem)

Open System: Analyzer in which the operator can change the parameters related to an analysis and to prepare "in-house" reagents or use reagents from a variety of suppliers.
Closed System: Analyzer in which reagents and calibrators are provided only by the manufacturer and other reagents or methods cannot be used
Throughput: The maximum number of individual specimens or test analyses that can be practically performed per hour by an assay system, with required dwell time considered.
Dwell time: The minimum time required for an instrument to obtain a result, calculated from the initial sampling of the specimen
Test Repertoire: All the different tests that are available on an instrument, including those that can be made available by changing reagents or instrument components
Test Menu: The number of different tests available on an instrument at one time without changes in reagents or components
Carryover: Contamination of a specimen by the previous one
Dead Volume: The volume in the specimen container that must be present for proper specimen aliquot measurement but is not consumed.
Specimen Blank: Specimen plus diluents; used to correct absorbance of a complete reaction mixture for endogenous specimen colour
Reagent Blank: Reaction mixture minus the specimen; used to subtract endogenous reagent colour from the absorbance of the complete reaction (plus specimen)

Introduction to the Chapter

In Chemical Pathology Laboratory, a myriad of analytical techniques and analytical methods are used in different laboratory equipment. This chapter will encompass following aspects:

a. Optical Techniques: Overview of optical techniques and some details of photometry and chemiluminescence
b. Immunoassays: General aspects
c. Electrochemical Techniques: Brief overview
d. Chromatography: Introduction
e. Mass Spectrometry: Introduction
f. Clinical Enzymology: Principles
g. Automation: Evolution of automation in Chemical Pathology
h. Methods of Analyses: General Aspects (Details to be found in "Manual of Laboratory Medicine" Published by AFIP Rawalpindi)

Optical Technique[3,4]

- Optical techniques are a group of analytical techniques based on colours and light. These are the most commonly used techniques in the medical laboratories.

Optical Techniques Used in Chemical Pathology

1. Photometry
2. Reflectance
3. Atomic Absorption
4. Flame Emission
5. Chemiluminescence
6. Fluorescence
7. Light Scattering

Optical Techniques-Animated

The Seven Sisters:

Photometry, fluorescence, light scattering, chemiluminescence, flame emission, reflectance and atomic absorption.

Let's read the story of these seven sisters:

a. *Photometry, fluorescence and light scattering* are never at good terms with each other. They cannot tolerate each other's presence. If one of them is present, the others two can`t work properly (when working on photometry, light scattering and fluorescence are sources of error, for example, particles present on a dirty reaction cuvette can cause light to scatter and error in photometry and natural fluorophores can also cause error. Similarly, absorbance is a source of error in fluorimetry called *inner filter effect*. Each of these techniques works well only if two others are NOT present.
b. *Chemiluminescence* lives in a privileged community. She has financial independence, too (produces her own light and does not require an external source of light). She keeps herself aloof from the politics of other sisters and work in entirely different atmosphere (dedicated instruments and labs).
c. *Flame emission* is in the habit of making a lot of noise (interferences) and gives importance only to the 5% people, which make agitations (i.e. only fewer excited atoms are counted) while *atomic absorption* is a silence-loving girl. She makes minimum noise (interference) and gives importance to people who remain quite (accounts for 95% ground state atoms). This leads to the success of *atomic absorption* and unpopularity of *flame emission.*

Adapted with thanks from "The Spectrum" PSCP Newsletter, 4th Issue, Nov 2015.

Characteristics of Light Used in Optical Techniques

Optical techniques used in Chemical Pathology and other biological sciences use following characteristics of light:

- Emission
- Absorbance
- Scattering
- Transmittance
- Reflectance

Definition of Basic Characteristics of Light

- Emission: The dissipation of energy when electrons return from higher energy state to lower energy state. Some elements possess this property.
- Absorbance: Mathematical expression of absorbed energy (light). It is the capacity of a substance to absorb radiation, expressed as logarithm (log) of the reciprocal of transmittance (T) of the substance.
- Scattering: This is scattering of light in all direction because of collision of light with some particles
- Transmittance: The ratio of the intensity of light entering the specimen to that exiting the specimen at a particular wavelength is defined as the transmittance.
- Reflectance: The ratio of the total amount of light reflected from a surface to the amount of light incident on the surface.

Names of Techniques Based on Characteristic of Light

- Emission: Fluorometry, Chemiluminescence, Flame Emission, Fluorescence Polarization
- Absorbance: Spectrophotometry, Atomic absorption
- Scattering: Nephelometry, Turbidimetry
- Reflectance: POCT testing

Great advantage of Emission (Luminescence) over Absorption

- Luminescence is generally more sensitive than absorption because it is easier to accurately measure a small amount of absolute light than to accurately detect a small decrease in relative energy.
- *Luminescence is like sun*
- *Absorbance is like moon*

Fluorescence and Phosphorescence

- Fluorescence and phosphorescence are two examples of emission (luminescence).
- In these techniques, the emitted light is best detected at a right angle to the incident light.
- Fluorescence and phosphorescence differ in their quantum mechanical mechanism for the dissipation of the stored energy.

- Generally, fluorescence emits UV light and phosphorescence emits visible light.
- However, most importantly, in both cases the emitted light is linearly proportional to the analyte concentration.

Photometry[3-5]

- It is the most commonly used analytical technique used in a Chemical Pathology lab.
- Most of the routine tests, which constitute the bulk of the workload, are carried out by this technique.
- Every Pathologist and Laboratory Technologists must know the basics of this technique.
- The technique is based on the measurement of light emitted from a reaction tube or cuvette.

Difference between Absorption and Absorbance[3]

- Absorption: The phenomena of absorbing light by a solution when an incident light strikes on it.
- Absorbance: It is mathematic expression of absorption. Analogous to pH.
- Absorbance is negative log of ***ratio*** between light transmitted through a material and the light falling upon a material i.e.:

  $A(\lambda) = -\log (I_t / I_0)$

  Where

  A is the absorbance at a certain wavelength of light (λ),

  I_t is the intensity of the light that has passed through the solution (transmitted light),

  I_0 is the intensity of the light before it passes through the solution (incident radiation).

- It must be noted that absorbance is a direct measurement of transmitted light but indirect measurement of absorbed light.

What is the unit of Absorbance? None!! Though some scientists use arbitrary unit known as Absorbance Unit (AU)

What is Transmittance?

- A ***simple ratio*** between the light falling upon a material and the light transmitted through a material i.e.:

  $T(\lambda) = I_t / I_0$

  Where

  T is the transmittance at a certain wavelength of light (λ),

I_t is the intensity of the light that has passed through the solution (transmitted light),

I_0 is the intensity of the light before it passes through the solution (incident radiation).

Absorbance and Transmittance

- Percentage Transmittance (%T): Commonly used in Labs e.g. Estimation of haemoglobin

How to derive:

- Transmittance x 100 = Transmittance and %T
- Absorbance and %T are more commonly used on spectrophotometers
- Absorbance can be derived from %T as following:

A = 100 – %T

A = Log 100 – Log %T

A = 2 - Log%T

- So, if you know %T you can calculate absorbance using this formula

Beer-Lambert Law[4,5]

This law described the basic principle of measurement of concentration of analytes in a solution. It states, ***"Absorption of light is directly proportional to concentration of the absorbing molecule"*** or

A = e**lc**

e is absorptivity (extinction coefficient)

l is the path length

c is the concentration of the absorbing molecule

Complementary Colours[5]

- The concept of complementary colours (Figure 18.1) is fundamental in photometry. It is because every component colour in white light has a complementary colour. So, if a light of complementary colour is thrown on a solution, it will absorb this light to the maximum extent.

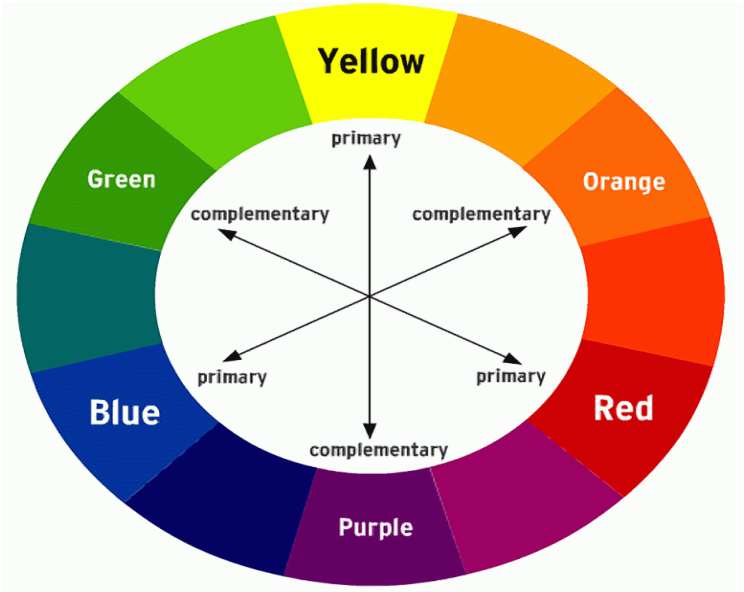

Figure 18.1: The Colour Wheel

An Example

- Red-coloured glucose solution is produced after GOD-PAP reaction.
- Now the complementary colour of red is green. So, if we throw green light on this solution, it will absorb green light to the maximum as green is the complementary colour of red.
- The wavelength of green is 500-520 nm. For glucose by GOD-PAP method, this wavelength is selected for incident light. The light absorbed is directly proportion to concentration of the glucose in the solution as redness of the solution increases with increasing concentration of glucose.
- The amount of green light absorbed will depend on the concentration of glucose present in the solution (Beers-Lambert Law). How to measure the absorbed light?
- It is done indirectly by measuring the ratio of transmitted light and incident light. We take negative log of this ratio called 'absorbance".

Light Scattering

- When electromagnetic radiation (light) strikes a particle in solution, some of the light will be absorbed by the particle, some will be transmitted through the solution, and some will be scattered or reflected.
- The amount of light scattered is proportional to the concentration of insoluble particle.
- Many analytical techniques use these phenomena for estimation of analytes e.g. immunoturbidimetry and immunonephelometry (Table 18.1)

Table 18.1: Difference between Turbidimetry and Nephelometry

Turbidimetry	Nephelometry
Measured at 180^0	Measured at 90^0
Suitable for high concentrations	Suitable for low concentrations
Precision and sensitivity much less	High Precision and sensitivity
Mostly used for detection of water impurities In clinical labs used for detection of proteins and haemoglobins	Mostly used for quantification of small protein e.g. IgG etc.
More commonly available with photometric autoanalysers (e.g. Turbidity Inhibition Immunoassay)	A separate dedicated instrument is required (e.g. IMMAGE 800 by Beckman Coulter)

Chemiluminescence[3,4,6]

Some Terminology

- **Luminescence -** general term for the emission of light from a cool object.
- **Incandescence** – Emission of light from a hot piece of metals such as the filament in a light bulb.
- **Phosphorescence**: Absorption and slow re-emission of light. Most commonly observed in minerals.
- **Fluorescence**: Absorption and fast re-emission of light. Seen in deep-sea organisms and some insects and plants.
- **Chemiluminescence**: Emission of light driven by a chemical reaction. In fact, it is the emission of light when an electron returns from an excited or high energy level to a ground or low energy level
- **Electrochemiluminescence:** An electrochemical reaction leading to the emission of light.
- **Bioluminescence** The most common form of natural luminescence. Seen in aquatic organisms, insects and plants.

Question:

Name the most commonly used source of light used in chemiluminescence based auto analysers

Answer: None

What Happens in Chemiluminescence[6]

- The excitation event is caused by a chemical reaction.
- It involves the oxidation of an organic compound such as luminal, isoluminol, acridium esters or luciferin by an oxidant [e.g. hydrogen peroxide, hypochlorite or oxygen].
- The light is emitted from the excited product formed in the oxidation process.
- These reactions occur in the presence of catalysts such as:
  - Enzymes: alkaline phosphatase, horseradish peroxidase and microperoxidase
  - Metal ions: Copper (Cu^{2+}), Iron (Fe^{3+})

- Metal Complexes: Cu $^{2+}$phthalo-cyanidecomplex, Fe $^{3+}$ phthalo-cyanide complex, Hemin etc.

Role of Hydrogen Peroxide(H_2O_2)

- H_2O_2 is produced as a result of antigen-antibody reaction
- Luminol + H_2O_2 → 3--aminophthalate + light

An Example

- A specimen is added to a reaction vessel with goat anti-hTSH-alkaline phosphatase conjugate, buffered protein solution, and paramagnetic particles coated with immobilized mouse monoclonal anti-hTSH antibody.
- The serum hTSH binds to the immobilized monoclonal anti-hTSH on the solid phase while the goat anti-hTSH-alkaline phosphatase conjugate reacts with a different antigenic site on the serum hTSH.
- Separation in a magnetic field and washing removes materials not bound to the solid phase.
- A chemiluminescent substrate, Lumi-Phos 530, is then added to the reaction vessel, and light generated by the reaction is measured with a luminometer.
- The photon production is proportional to the amount of enzyme conjugate bound to the solid support. The amount of analyte in the specimen is determined by means of a stored, multi-point calibration curve.

Quantum Yield and Sensitivity

- Quantum yield is defined as total photons emitted per total reacting moles.
- The quantum yield is approximately 0.1% to 10 % for chemiluminescence and 10% to 30% for bioluminescence.
- Chemiluminescence assays are ultrasensitive and are widely used in automated immunoassays and DNA probe assays.
- Their sensitivity is in the atto to zepto mole range *[10 -^{18} to 10 $^{-21}$ moles].*

Electrochemiluminescence [EC]

- EC differs from chemiluminescence.
- In Electroluminescence, the reactive species that produce the chemiluminescence reaction are electrically generated from stable precursors at the surface of an electrode.
- A commonly used label is Ruthenium [Ru2+]-tris(bipyridyl) chelate and EC is generated at an electrode via a REDOX reaction with tripropylamine.
- This chelate is very stable and relatively small and can be used to label haptens or large molecules [e.g. proteins or oligonucleotides].
- The EC has been used in both immunoassays and nucleic acid assays.
- The advantages of EC include improved reagent stability, simple reagent preparation and enhanced sensitivity.

Instrumentation[3,4,6]

- The instrument that is used to measure chemiluminescence is called as a luminometer.

The basic components of Luminometer are:

- The specimen cell housed in a light tight chamber.

- The injection system to add reagents to the specimen cell.
- The detector (usually a photomultiplier tube) is most commonly used to image chemiluminescence reactions.
- For electrochemiluminescence, the reaction vessel also incorporates an electrode at which the chemiluminescence is generated.
- The other accessory components are digital read out device, a temperature control device, a monochromator [only under special conditions], a microprocessor or computer and a printer.

Applications

4. The chemiluminescence is extremely sensitive technique and is very widely used. In Pakistan, too, most of the middle and large sized labs use instruments based on chemiluminescence. This technique has provided a user-friendly and safer alternate to radioimmunoassays. The only limitation is running cost while the instruments are usually placed free of cost on reagent rental basis.
5. It is used in immunoassays for:
    - Hormones
    - Metabolites
    - Vitamins
    - Biomarkers for cancer and other diseases
    - DNA probe assays for genetic testing and forensic medicine
    - Sensitive drug assays

Limitations

Reagent Purity and the Solvents: The extreme sensitivity of chemiluminescence requires stringent controls on the purity of reagents and the solvents [e.g. water].

Other factors which may degrade analytical performance:

- Light leaks
- Light piping
- High background luminescence from reagents and reaction vessels

Immunoassays[7,8]

- Immunoassays are a large group of analytical techniques base on antigen-antibody reactions.
- These techniques are coupled with many analytical techniques in various sections of the lab e.g. combined with fluorescence (immunofluorescence), chemiluminescence, turbidimetry, photometry.
- One of most powerful of all immunochemical techniques
- Immunoassays are not only used to detect but also quantify analyte of interest
- Analyte being measured may be
    - Naturally present- cortisol, thyroid hormones
    - Not naturally present but disease produces- cancer antigen

- Neither disease produces nor naturally present in body- drugs of abuse

Historical Perspective

- Immunoassays have passed through an evolution leading to the modern most sensitive analytical techniques.
- Immunoassays (In the order of Sensitivity)
  - Radioimmunoassay (RIA)
  - Immunoradiometric Assay (IRMA)
  - Enzyme Linked Immunosorbent Assay (ELISA)
  - Enzyme Multiplied Immunoassay Technique (EMIT)
  - Cloned Enzyme Donor Immunoassay (CEDIA)
  - Florescent Immunoassay (FIA)
  - Florescent Polarized Immunoassay (FPIA)
  - Electrochemiluminescence immunoassay (ECL)
  - Chemiluminescence Immunoassay (CMIA)

Classification of Immunological Techniques[8]

- No definite classification
- Different ways of classification based on different criteria
- Type of analysis- quantitative and qualitative
- Test specimen- antigen or antibody
- Assay system-
  - ✓ Labelled, unlabelled
  - ✓ Competitive, non-competitive
  - ✓ Homogeneous, heterogeneous
  - ✓ Endpoint detection either visibly or by instrumentation
  - ✓ Assay conditions- liquid vs. solid phase
  - ✓ Manual vs automated

Electrochemistry[9,10]

- A significant number of emergency tests in Chemical Pathology are based on the electrical activities of the elements and compounds.
- These techniques are collectively called 'Electrochemistry'.
- It is important that the term electrochemistry, used here, does not include some other techniques, which are based on the movements of ions e.g. electrophoresis and ion exchange chromatography.
- Similarly, electrochemistry deals with only naturally occurring ions, otherwise in some analytical techniques ions are produced by very effective 'ionization' methods and then measured (i.e. mass spectrometry). (see below)

Basic Principles

- Electrochemistry is the group of analytical techniques, which uses the transfer of charge from an electrode to another phase.
- During this process, chemical changes take place at the electrodes and the charge is conducted through the bulk of the specimen phase.
- Both the electrode reactions and/or the charge transport can be modulated chemically and serve as the basis of the sensing process.

Four Major Electrochemical Techniques

a. Potentiometry
b. Amperometry and Voltammetry
c. Coulometry
d. Conductimetry

Potentiometry[9,10]

- In potentiometric sensors, the potential difference between the reference electrode and the indicator electrode is measured without polarizing the electrochemical cell, that is, very small (almost zero) current is allowed.
- The reference electrode is required to provide a constant half-cell potential. The indicator electrode develops a variable potential depending on the activity or concentration of a specific analyte in solution.
- Electric potential is converted into concentration with help of an equation called Nernst Equation
- Membrane junction potential is the most important potential for determination of analyte concentration.

An Analogy: *Potentiometry is like 'basketball throwing'. The ground is reference electrode and the ball gains more power when thrown from a higher building as compared to that thrown from an eye distance[9].*

Four important components of a potentiometric cell.

a. Reference electrode
b. Indicator electrode
c. Salt bridge
d. Ion selective membrane

The most important component of a potentiometric cell

Ion selective membrane

Amperometry

- Amperometry is a method of electrochemical analysis in which the signal of interest is a current that is linearly dependent upon the concentration of the analyte.
- As certain chemical species are oxidized or reduced (redox reactions) at inert metal electrodes, electrons are transferred from the analyte to the working electrode or to the analyte from the electrode.

- The direction of flow of electrons depends upon the properties of the analyte and can be controlled by the electric potential applied to the working electrode.

Amperometry and Voltammetry

- There are many types of amperometries
- Usually term 'amperometry' is used for "constant voltage amperometry"
- Voltammetry is "cyclic amperometry"

Coulometry

- Coulometry is an electrochemical technique, related to amperometry, where the amount of charge (coulombs) passing between two electrodes is measured.
- The amount of charge passing between the electrodes is directly proportional to oxidation or reduction of an electroactive substance at one of the electrodes.

Conductimetry (also called impedimetry)

- It is based on the measurement of electrolyte conductivity, which varies when the cell is exposed to different environments.
- The sensing effect is based on the change of the number of mobile charge carriers in the electrolyte. If the electrodes are prevented from polarizing, the electrolyte shows Ohmic behavior.
- Conductivity measurements are generally performed with AC supply. The conductivity is a linear function of the ion concentration; therefore, it can be used for sensor applications.

Lab Application of Conductimetry

- Conductimetery is used for the measurement of haematocrit, which is part of almost all the ABG analysers
- Blood cells act as electrical insulators because of their lipid membranes, so resistance increases and conductivity decreases as the number of red cells increase. Similar principle is used for counting and differentiating blood cells in common haematology analysers
- The phenomenon is called ***"Coulter Principle"***

Ion Selective Electrode (ISE)

- The ion-selective electrode (ISE) is a commonly used sensor for the measurement of electrolytes (e.g. H^+, Na^+, K^+, Ca^{++} etc.).
- The ISE usually comprises a membrane with a unique composition, either a solid (i.e., glass, inorganic crystal) or a plasticized polymer.
- The composition of ISE is chosen to impart a potential that is primarily associated with the ion of interest via a selective binding process at the membrane-electrolyte interface.

Membranes in ISE

- Membrane is said to be at the 'heart' of ISE.
- Many technical innovations have led to increased selectivity of membranes
- Many types of membranes are in use:
  - Glass e.g.in H electrode
  - PVC
  - Inert metals e.g. platinum
  - Active metals e.g. in Ag/Agcl

Direct Analysis in ABGs

- Base excess and HCO_3 are calculated parameters
- Please note that techniques are available for direct HCO_3 measurement in the form of total CO_2 by ISE but not in routine analysers.
- In the commonly used Severinghaus-style PCO_2 sensor, PCO_2 is NOT measured directly. H^+ are generated as CO_2 reacts with H_2O and these H^+ are then measured.
- pH is negative log of H^+ concentration and again an indirect measurement
- O_2 is measured directly by amperometry

Chromatography[11-13]

(Added by Dr. Syed Talha Naeem)

A physical technique that separates mixture into individual components

Principle:

- Group of separation processes whereby a mixture of solutes, dissolved in a common solvent, are separated from one another by a differential distribution of the solutes between two phases
- *Mobile phase*: ranges from liquids to gases
- *Fixed /Stationary phase*: Ranges from sheets of cellulose paper to capillary glass tubes

Branches of Chromatography

- Planar
  - Paper
  - Thin Layer [TLC]
- Column
  - Gas [GC]
  - Liquid [LC]

Components of Gas Chromatograph:

a. Pressurized carrier gas with ancillary pressure and flow regulators
b. Specimen injection port
c. Column
d. Detector
e. Electrometer and signal recorder

f. Thermostatted compartments encasing the column, detector and injection port

Components of High Performance Liquid Chromatography (HPLC) separation system:

a. Solvent delivery system
b. Specimen introduction system
c. Column
d. Detector

Mass Spectrometry[14]

(Added by Dr. Syed Talha Naeem)

Mass spectrometry uses the creation and analysis of ions to analyze a wide variety of molecules

Principle:

Charged particles moving through a magnetic or an electrical field can be separated from other charged particles according to their mass to charge (m/z) ratios

Mass Spectrometer (Components):

a. System to create and maintain vacuum
b. Device to introduce specimens
c. Ionization source
d. Mass filter or analyzer
e. Ion collection, amplification and detection devices
f. Computer system

Applications:

GC-MS: Drug testing for clinical or forensic purposes, testing for inherited metabolic disorders etc.
LC-MS/MS: Analysis of amino acids, Immunosuppressant drugs, 25(OH) - vitamin D, steroids etc.
MS/MS: Screening and confirmation of genetic disorders and inherited metabolic disorders.

Clinical Enzymology[15,16]

In a routine lab of Chemical Pathology, a large proportion of workload comprises enzyme estimation. Some of these enzymes play vital role in patient care e.g. ALT, alkaline phosphatase, CK, amylase, LD and gamma glutammyl transferase.

Difference in Measurement of Enzymes

- Enzyme measurement is different from other analytes like glucose, bilirubin and cholesterol etc. Some differences are:
- With only a few exceptions, activities of enzymes are measured instead of their concentration.
- Unit of measurement of all the enzymes is same i.e. Unit per litre (U/L) in traditional unit while µKat/L in SI units.
- Enzymes are usually measured by kinetic assays while other analytes (glucose, bilirubin and cholesterol etc.) are measured by end-point methods.
- Kinetic assays of enzymes are quite sensitive to factors like pH and temperature. On the other hand, end-point assays are robust.
- Measurement of enzyme activities are governed by an ingenious biochemical equation called 'Michaelis and Menten Equation' for quantitative measurement of enzymes. (Details of this equation are beyond the scope of this book).
- Two important assumptions of this equation are:
    - The effect of product is negligible on the concentration of Enzyme-Substrate Complex
    - There is no change in factors during the enzyme reaction e.g. pH and temperature

Types of Enzyme-Substrate Reaction

- Zero Order Reaction: When an enzyme activity is to be measured, an excess and fixed quantity of substrate is added.
- First Order Reaction: is the reaction in which quantity of substrate is to be determined. So, the quantity of enzyme is fixed.

Automation[1, 2]

Why Automation

- Increasing workload
- Keep pace with rapidly developing medical field
- Increasing Resources constrains
- Pressing need to maintain high quality
- Dearth of efficient biomedical support

Goals of Lab Automation

- Reduction in costs
- Reduction in turnaround time

- Expansion of lab testing to generate more revenue
- Reduction in lab errors (e.g. *labelling errors*)
- Improvement in lab safety

Important considerations

- Economic and reliable operation
- Reduce human resource cost
- Lab space (*cost of space renovations*)
- Up-gradation/ menu extension
- Service back up
- Availability of on-site biomedical engineer
- Standardization of specimen tubes

Types of Lab Automations

- Subtotal automation (modular integrated automation)
- Total laboratory automation
- Stand-Alone systems

Total Laboratory Automation (TLA)

TLA is the most recent and most exciting development in Chemical Pathology Laboratories.

Parts of TLA

Pre-analytical Automation

- Specimen tubes
- Decapper module
- Centrifuge module
- Specimen transport module
- Resealer module

Analytical Automation

- Input / output module
- Tube storage module

Stand-Alone Systems

Types

Continuous Flow

- Tubing flow of reagents and patient's specimens.
- Chemical reaction is created, and then chromogen solution is pumped into a flow-through cuvette for spectrophotometric analysis.
- There are significant carry-over problems and wasteful use of continuously flowing reagents, which lead to the demise of these analyzers.

Centrifugal Analyzers

- Discrete aliquots of specimens and reagents are pipetted into discrete chambers in a rotor.
- Centrifugal force is used to mix specimen and reagents. The rotary motion is then used to move the cuvettes through the optical path of an optical system

Discrete Analyzers

- Separate testing cuvettes for each test and specimen.
- They have the capability of running multiple tests on one specimen at a time or multiple specimens testing at one time.
- Most popular analyzers and have almost completely replaced continuous-flow and centrifugal analyzers.
- Separate reaction cuvettes, cells, slides, or wells that are disposed of following chemical analysis, are used. This keeps specimen and reaction carryover to a minimum but increases the cost per test due to disposable products.
- These analysers are also called '*random access' analysers*

Four Q Model of Instrument Qualification

The auto analyzers are prepared for "4Q" model according to following guidelines

Design Qualification: documentation of required specifications of design and its function. It also documents qualification of vender or supplier.

Installation Qualification: documented verification that system is installed according to written and preapproved specifications

Operational Qualification: includes verification that system is operating in accordance with preapproved and written specifications under normal and stressed conditions.

Performance Qualification: it includes ongoing monitoring of performance, testing for specified application and periodic updates about the analyzer.

Specimen-to-Specimen Carry Over

- Specimen-to-specimen carry over is an important cause of random error that cannot be detected by any quality control system. It's a nightmare for the lab staff.
- Specimen carry over is determined by analysing 4 identical high level specimens followed by 4 identical low level specimens.
- Beta HCG can be used because it has wide range of concentration ranging from 1 to 10^6 mIU/mL

Situation with Greater Risk of Specimen Carry Over

- The risk of carryover altering patient's results is greater if a primary specimen collection tube is initially processed on the *chemistry analyser and then transferred to an immunoassay analyser* for additional testing.

- Specimen probe washing on a clinical chemistry instrument is sufficient to prevent carryover for chemical analyte, but may not be adequate for immunoassay analyte.

Methods of Analyses (General Aspects)[17-19]

In this section, we will discuss types of methods used in Chemical Pathology and some other Pathology Laboratories. Details of methods of various analytes e.g. Glucose, Bilirubin or ALT etc. can be obtained from other sources e.g. AFIP Manual of Laboratory Medicine or EVOLVE (https://coursewareobjects.elsevier.com/objects/elr/Kaplan/chemistry5e/methodofanalysis)/

There are two basic methods for the estimation of analytes:

1. Endpoint Colorimetric Method
2. Kinetic Method
    a. Endpoint Kinetic
    b. Fixed Time/Two Point Kinetic
    c. Continuous Monitoring[19]

Endpoint Method

- Specimen having analyte of interest is incubated with suitable reagent
- Reaction takes place and analyte would be consumed
- Coloured/non-coloured complex thus formed is read for absorbance at specific wavelength
- This absorbance increases against reagent absorbance, over time
- This increase in absorbance continues till it reaches a stable value, i.e. endpoint of reaction iss reached
- Concentration of analyte of interest can be determined if concentration and absorbance of respective standard are known
- Formula used is;

Concentration of Test = Absorbance of test / Absorbance of standard x Concentration of standard

Examples of Endpoint methods

- Glucose
- Urea
- Creatinine (Jaffe`s End-point)
- Cholesterol
- Bilirubin
- Calcium

Endpoint Kinetic

- Specimen is incubated with buffer substrate for a fixed period
- At the end of reaction, it is stopped
- Concentration of product formed is determined by measuring its absorption spectrophotometrically
- **Example:** Kinetic Jaffe Method for Creatinine

Fixed Time/Two point Kinetic

- Amount of change produced by the enzyme is measured after the reaction is stopped at the end of a fixed time interval
- Reactants are combined. Reaction proceeds for a designated time
- Measurement of amount of reaction that has occurred is made-by measuring difference of absorbance between two points. Rate of reaction is change in absorbance per unit time
- Reaction is assumed to be linear over the reaction time.

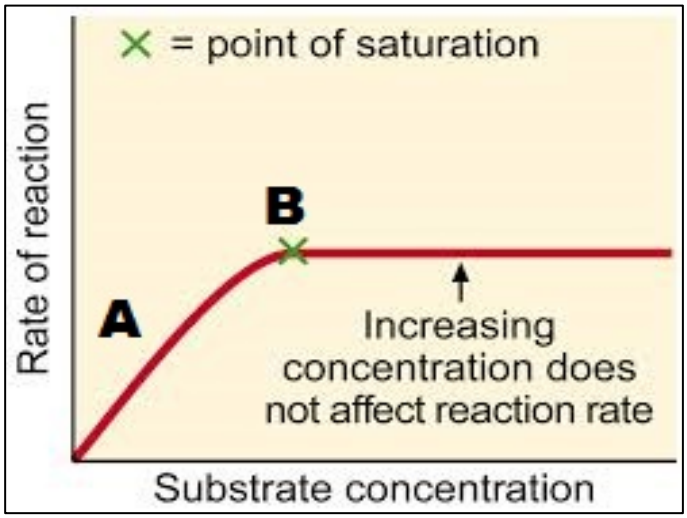

Figure 18.2: Curve showing Rate of Enzyme Reaction

Continuous Monitoring Kinetic Assay

- The progress of reaction is monitored continuously
- A lag phase is observed to let the rate of reaction reach at its maximum (Figure 18.2)
- Multiple measurements, usually of absorbance change are made during reaction, either:
  - At specific time intervals (every 30-60 seconds) or
  - Continuously by continuous recording spectrophotometer
- We measure change of absorbance (delta absorbance) per unit time
- The progress of reaction is monitored continuously

Advantage:

- Linearity of reaction may be more adequately verified.

- If absorbance is measured at intervals, several data points are necessary to increase the accuracy of linearity assessment.
- So, continuous measurements are preferred because any deviation from linearity is readily observable

Examples: Serum ALT, AST, CK

Types of Methods Divided According to Accuracy

- Definitive Methods
- Reference Method
- Field Method

Definitive Method

- An analytical method that:
  - has been subjected to thorough investigation and
  - has been found to have no source of inaccuracy and ambiguity
- Value of an analyte obtained by a definitive method is taken as true value
- Capable of providing highest accuracy among all methods for determining that analyte

Requirement of Development and Performance

- Sophisticated instrumentation
- Superior and well-defined reagents and
- Highly skilled personnel
- Expensive and technically demanding
- Depends in part on the availability of pure reference materials. (e.g. those supplied by the National Bureau of Standards and called SRM's.)
- Uses:
  - Used in the verification of accuracy of field methods
  - Used to validate reference methods and primary reference materials

Reference Method

- Analytical method with thoroughly documented accuracy, precision, and low susceptibility to interfering substances, sufficient to achieve the stated end purpose
- Should show appropriate linearity, sensitivity and specificity
- Accuracy and precision must have been demonstrated by direct comparison with a definitive method

Uses

- To evaluate secondary reference materials and to assign values to control material
- To compare the quality of routine or proposed methods

- To evaluate the bias and interferences in field method, and as an index for the acceptability of field methods that are under development

Field Method

- Any routinely used analytical method that has been shown to have adequate precision, accuracy and specificity for its intended use
- Such method should also have an appropriate analytical range and should be practical and economical
- Field methods that have been endorsed by a qualified body, such ISO are termed as "Recommended," "Standard," or "Selected Methods"
- Field methods are predominantly used in routine service laboratories for measuring analytes in biological fluids

Reference Materials

As defined by the International Standards Organization (ISO) to be "a material or substance, one or more physical or chemical properties of which are sufficiently well established, to be used for:

- Calibrating instruments
- Validating methods
- Assigning values to materials
- Evaluating the comparability of results

Various International organizations have defined different categories of reference materialss:

Primary Reference Materials (PRM)[18-19]

- PRM trace back to *International Bureau of Weights and Measurements –France (BIPM)*
- Well-characterized, stable, homogeneous and highly purified chemicals that are directly weighed or measured to produce a solution whose concentration is exactly known.
- Purity of 99.98% as proposed by International Union of Physical and Applied Chemistry (IUPAC)
- These highly purified chemicals may be weighed out directly for preparation of solutions of selected concentration or for calibration of solutions of unknown strength
- Supplied with certificate of analysis for each lot
- Values assigned by analysis by definitive method

Secondary Reference Materials

- Concentration of Secondary Reference Materials is determined by
    - Analysis of an aliquot of solution by reference method,
    - Using a primary reference material to calibrate the method
- Concentration cannot be prepared by weighing the solute and dissolving a known amount into a volume of solution like primary reference material

Standard Reference Materials (SRM) (NIST)

- *National Institute of Standards and Techniques (NIST) provided 1300* SRMs for clinical and molecular laboratories.
- SRMs are chemical compounds of certified purity, characterized sufficiently well to ensure that if properly used to standardize an analytical method or instrument, no errors in results can be attributed to the calibration of system
- Purity of SRMs is in effect such that none of the residual impurities are present in sufficient quantity to affect materially the results obtained with a system
- NIST provides certificate of analysis and MSDS (see Chapter 16) with SRMs.
- Values assigned by analysis by definitive method
- Each has been well characterized for certain chemical and physical properties, and is issued with a certificate that gives results of the characterization
- May be used to characterize other materials
- Examples of SRMs for use in clinical and molecular diagnostic labs include:
    - Pure crystalline standards
    - Human-based standards
    - Animal based standards
    - Standards containing drugs of abuse in urine and human hair
    - For DNA profiling/crime scene investigations
- NIST also provides some NIST traceable reference material which have well-defined traceability to SRMs.

Certified Reference Materials

- Certified Reference Materials (CRMs) are ‘controls’ or standards used to
    - Check the quality and meteorological traceability of products
    - Validate analytical measurement methods
    - Calibrate instruments
- A certified reference material is a particular form of measurement standard
- CRMs for clinical and molecular laboratories are available from *Institute for Reference Materials and Measurements (IRMM), Belgium*

Chapter 18
Introduction to Analytical Techniques and Methods of Analyses

MCQs with Key and Explanation

MCQ No	Best Option	Explanation
201.	c. Negative log of transmitted and incident light	In this MCQ, absorbance is defined. Please note that absorbance is a mathematical expression as opposed to absorption, which is a physical phenomenon. Absorbance is more closely related to transmittance, which is a simple ratio while absorbance is its negative log (negative log is used because the value is less than 1.0).
202.	b. Length of the light path	Beer-Lambert law states that absorbance of a solution is directly proportional to the concentration of the solution and length of light path. It is fundamental to all photometric analyses carried out in a Chemical Pathology laboratory.
203.	d. Scattering of Light	When the light beam strikes a solution, many optical phenomena take place e.g. absorption, transmission, scattering, fluorescence and reflectance. Since in photometry, we are interested in absorbance (and transmittance) only, we want minimum of other phenomena, as all these phenomena will waste the beam of light and cause error in result. Light scattering can be avoided by removal of any particles in the solution or cuvette. So, cuvettes used in photometry are made of special glass and should be as clean as possible i.e. free of particles.
204.	a. Chemiluminescence	Chemiluminescence is now the most commonly used optical technique for hormone and tumour marker testing in clinical laboratories. Its analytical sensitivity is very high and can detect quantities in zaptomoles (10^{-21}). Its reagents are non-hazardous and can be fully automated. Its disadvantage is very high running cost as compared to traditional radioimmunoassay.
205.	d. Membrane	Ion Selective Electrode (ISE) is a very commonly used electrochemical technique for electrolytes and blood gas analyses. In ISE membrane plays the most important role of selection of the ion from the patient

		specimen (or calibrator or quality control) flowing in the tube. The membranes are designed as such that only a specific ion can pass through. For each analyte, a specific membrane is used e.g. glass membrane for hydrogen ions.
206.	a. The effect of product is negligible on the concentration of Enzyme-Substrate Complex.	Actually, it is a limitation of Michaelis and Menten Equation meaning it does not consider the effect of product on the enzyme-substrate complex. This is the reason that enzyme reactions used for the estimation of enzyme activities are coupled with one or two more enzyme reaction, so that the product is removed immediately without effecting the main enzyme reaction.
207.	e. Random access analyser	Almost all the autoanalysers used in Chemical Pathology laboratories, now-a-days, are random access i.e. they can be accessed right in the middle of the operation. Previous autoanalysers could not be accessed in this manner. A new specimen or reagent could only be placed when the analyser was in idle mode. So, this is a great advancement in lab automation, especially for analysing stat (urgent) specimen.
208.	b. End point test	Generally, two types of photometric methods are used in Chemical Pathology; end-point and kinetic. End-point methods are quite robust as the absorbance reading can be taken after stopping the reaction; on the other hand, in kinetic methods the absorbance readings are taken during the reaction and delta absorbance is then calculated
209.	e. Standard Reference Material	Different hierarchies are followed for various standard materials. NIST (USA) provides Standard Reference Material (SRM) and NIST Traceable Reference Material. While Primary Reference Material (PRM), and Secondary Reference Material are prepared and certified by *International Bureau of Weights and Measurements (France).*
210.	a. Definite method	Hierarchy is also used for methods of analyses. The most superior method of analysis of a particular analyte is called 'Definite Method' e.g. 'Isotope Dilution Mass Spectrometry' is definite method for calcium, next in the hierarchy is 'Reference Method'. In case of calcium it is 'Atomic Absorption Spectrometry'.

Summary
Chapter 18
Introduction to Analytical Techniques
and Methods of Analyses

- Optical techniques are commonly used in various sections of Pathology Laboratories.
- Almost all the tests carried out in a routine Chemical Pathology analysers are based on photometry. Another optical technique used on the same lab autoanalysers is immunoturbidimetry for analytes like glycosylated haemoglobin.
- Chemiluminescence is now a predominant technique in immunoassay systems used for hormones, tumour markers and vitamins etc. It is the most sensitive analytical technique and can detect very low quantities.
- Electrochemical techniques e.g. ion selective electrode is used for electrolytes, blood gases and some other analytes.
- Water monitoring systems and haematology analysers are based on conductimetry, another electrochemical technique
- Chromatography is a physical technique; consists of stationary and mobile phases and separates a mixture into individual components.
- Mass spectrometry uses the creation and analysis of ions to analyze a wide variety of molecules.
- Enzymatic assays are also photometric assays with a bit more complicated methodologies based on Michaelis and Menten Equation.
- Laboratory automation means minimizing human involvement to make the process error free. Pre-analytical processes have also been automated; such an automation is called 'Total Lab Automation or TLA'.
- 'Analytical Methods' are the actual processes required to carry out estimation of an analyte. In routine Chemical Pathology, photometric tests are either end-point or kinetic depending on the time of noting the absorbance values.
- Reference materials and reference methods are used to generate field standards for use in the clinical labs

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Chapter 19
Quality Management

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MCQs

(Please find key at the end of the chapter)

211. A clinical laboratory manager is working on the measures like reducing turnaround time, improvement for specimen and patient identification, and test utility. Which of the following term best describes the ***processes***:
    a. Quality Assurance
    b. Quality Control
    c. Quality Improvement
    d. Quality Laboratory Processes
    e. Quality Planning

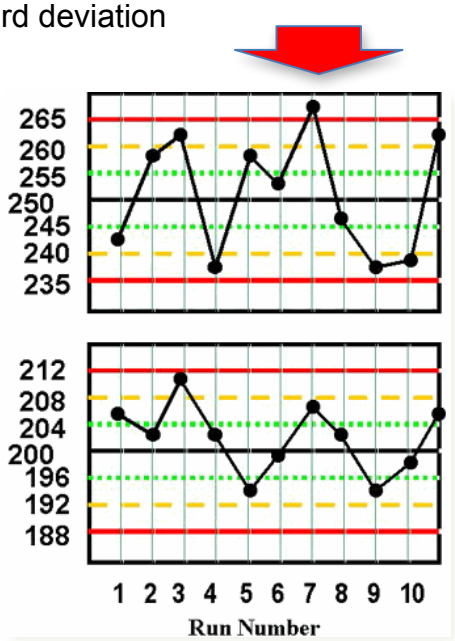
212. Which of the following is most common cause of a ***random error***?
    a. Change in reagent or calibrator lot numbers
    b. Deterioration of reagents or calibrators
    c. Fluctuation in power supply
    d. Improperly prepared reagents
    e. Wrong calibrator values

213. You are working in a state of the art hospital laboratory in Middle East. There is no issue of funds and proper storage of reagents. Which of the following control material you will select for use in your laboratory for ***most precise test***?
    a. Liquid, bovine and assayed controls
    b. Liquid, human, and assayed controls
    c. Lyophilized, bovine, assayed controls
    d. Lyophilized, bovine, un-assayed controls
    e. Lyophilized, human and assayed controls

214. Basic statistics is essential for running a quality control programme in a laboratory. In statistical terms, ***normal data*** means:
    a. Labs own generated values
    b. Result of disease free subjects
    c. Result of healthy individuals
    d. Symmetrical distribution

e. Values within reference values

215. Which of the following is NOT a measure of central tendency?
    a. Average
    b. Mean
    c. Median
    d. Mode
    e. Standard deviation



216. Westgard rules are applied on Levey Jennings plots for the assessment of quality control. Which of the following Westgard rules is violated in Run Number 7 at higher control level (upper chart)?
    a. Four consecutive values have crossed 1SD across the materials
    b. One value has crossed 2 SD on the plus side of the mean
    c. One value has crossed 3 SD on the plus side of the mean
    d. Ten consecutive values have crossed the mean across the materials
    e. Two consecutive values have crossed 2 SD across the materials

217. Which one of the following aspects best describes an External Quality Assessment Scheme (EQAS)?
    a. Detection of imprecision
    b. Detection of random error
    c. Inter-laboratory comparison
    d. Prevention of post-analytical errors
    e. Prevention of pre-analytical errors

218. Result of glucose level of 23.7 mmol/L was forwarded to a Chemical Pathology Resident. Although satisfied with internal quality control and EQAS results, she was hesitant to authorize it. She searched the Lab Information System to find any previous results of the same patient but failed to find any. Then she called the Medical Ward where the patient was admitted. The Resident Medicine told her that the patient is a known diabetic and is admitted with a gangrene right foot.
Which of the following procedures she has carried out to rule out any ***pre-analytical errors***?
    a. Auto-Verification
    b. Delta Check
    c. Lean Management
    d. Proficiency Testing
    e. Quality assurance

219. In a tertiary care hospital, a Consultant Chemical Pathologist has joined the department after getting training from Japan. He launches a new programme to reduce the lab errors and sets a target of < 3.4 errors per million.
Which of the following QM procedure he is trying to introduce in his lab:
    a. Delta Check
    b. External Quality Assurance
    c. Internal Quality Control
    d. Quality Planning
    e. Six Sigma

220. Mr Bilal is an efficient Lab Manager. He has made a Standard Operating Procedure (SOP) indicating the places of storage of the patients` samples in the specified areas of the lab freezers according to the nature of the test e.g. samples of CA-125 will be kept in the Tube Rack No 21 in the compartment No 3 of the freezer No 2. Due to this SOP the time of duty technologist is not wasted in searching of samples for analysis
Which of the following lab management principles is being followed by the manager:
    a. Delta Check
    b. Lean Management
    c. Manpower Management
    d. Quality Improvement
    e. Quality Lab Procedure

Chapter 19
Quality Management

Quality Assurance/Assessment (QA)[1]

- An all-inclusive / comprehensive system monitoring the accuracy of test results where all steps before, during and after the testing processes are considered.
- It includes pre-analytic, analytic and post analytic factors
- Provides a structure for achieving lab and hospital quality goals

Quality Control (QC)

- QC systems monitor the analytical process; detect and minimize errors during the analysis and prevent reporting of erroneous test results.
- It is like *'product testing'* in an industry
- It uses statistical analysis of test system data
- Requires application of Westgard Rules on Levey Jennings Plots for proper interpretation

Difference Between QA and QC:

- The aim of quality control is simply to ensure that the results generated by the test are correct.
- While quality assurance is concerned with much more; that is 'the right test is carried out on the right specimen, and that the right result and right interpretation is delivered to the right person at the right time at right cost.'

Types of QC

1. Internal QC (IQC) (Introduction):

- A **control** is a material which has a known value (predetermined or determined by the users) and which is used for the assessment of precision and accuracy of the laboratory results. It contains the **same constituents as those being analyzed in the patient sample (same matrix).**
- IQC should be run according to regulatory requirements and manufacturers recommendations for a specific analyte.
- The minimum requirement set by Clinical Laboratory Improvements Amendments (CLIA) states that **at least two levels controls should be run in a day**.
- A lab should determine frequency of controls run based on its volume and performance; if performance is good then frequency can be decreased and if poor performance then frequency can be increased to detect errors earlier.
- Apart from daily recommendation IQC should be performed after
  - Calibration/recalibration
  - Change in reagent lot and/or shipment
  - Following instrument repair or change in system

2. External QC (EQC) or Proficiency Testing (PT) (Introduction)

- Determination of laboratory testing performance by means of inter-laboratory comparisons
- There are multiple bodies which provide PT samples e.g. NEQAPP (National External Quality Assurance Program of Pakistan), CAP (College of American Pathologists), CDC (Centre of Disease Control), ERNDIM etc.
- It is an integral requirement of regulatory bodies like CAP, CLIA, The Joint Commission.
- Must be integrated within routine workload and analyzed by personnel who are running the tests.
- Ongoing evaluation of results to correct for unacceptable results
- Can also be utilized to access employee competency
- Please see details in a subsequent section of the chapter

Choice of IQC Materials[2]

The following considerations should be made when procuring QC materials:

- Human or Animal: The control matrix should be selected based on the expected patient population. For human laboratories, human QC materials are preferred as they allow for target values to be set closer to human medical decision limits; while, bovine, ovine or caprine may be optimal for use in veterinary laboratories.
- Liquid or Lyophilized: Liquid is better as there can be error in reconstitution of lyophilized material. However, the advantage of lyophilized is that it usually has more basic storage requirements (room temperature and longer shelf life).
- Assayed or Un-assayed: Assayed are better as the target values are already set while for un-assayed the laboratory should set target values as a first step.
- The best material is human, liquid and assayed but can be expensive. If there are severe financial constraints then one should go for bovine, lyophilized and un-assayed.

Some Basic Statistics[3]

These statistical tools are useful for continuous data generated in Chemical Pathology and some other laboratories. In discrete and categorical data these tools are not used.

Gaussian/Normal Distribution

- All values are symmetrically distributed around the mean(average)
- Characteristic “bell-shaped” curve (Gaussian curve) (Figure 19.1)
- Assumed for all quality control statistics

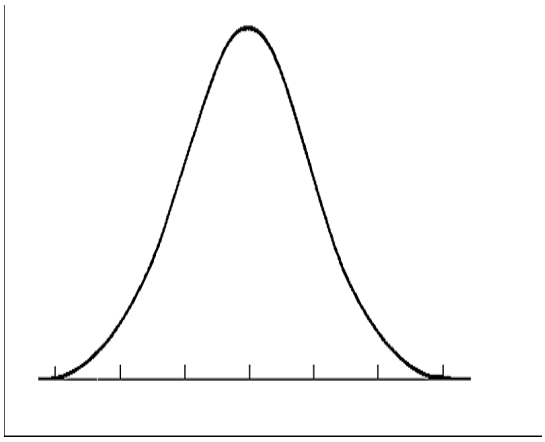


Figure No 19.1: A typical Gaussian Curve

Measures of Central Tendency

- **Mean:** the mathematical average of a group of numbers, determined by adding a group of numbers (events) and dividing the result by the number of events
- **Median:** determined as the 'middle' of a group of values that have been arranged in sequential order. There are an equal number of values on either side of the 'middle' number. In an odd # of observations, it is the middle observation. In an even # of observations, average the two middle values.
- **Mode:** the number that appears most frequently in a group of numbers. There can be more than one mode, or none at all.

Measures of Deviation/Variance:

Standard Deviation (SD):

- SD is a mathematical expression of the dispersion of a group of data around a mean.

Standard Deviation and Probability

The chances of a value to fall within the standard deviation is as under:

- 1 SD: 68.2 %
- 2 SD: 95.5%
- 3 SD: 99.7%

Question: Which is the most precise assay in the following table?

Test	Analyte: FSH Concentration	SD
a.	1	0.09
b.	5	0.25

c.	10	0.40
d.	25	1.20
e.	100	3.80

Answer: e

Explanation: At the face of it Test 'e' has the biggest SD and seems most imprecise but if you calculate the percentage of its SD i.e. CV (see below), it is the most precise test.

Standard Deviation Index (SDI)

1. It is the ratio of difference in means of laboratory and peer group to standard deviation of peer group
2. SDI = 0 Perfect comparison with peer group
3. SDI = 1 -1.49 marginal performance
4. SDI = 1.5 -1.99 marginal performance with trouble shooting required
5. SDI ≥2 unacceptable performance and remedial action required

Coefficient of Variation (CV)

1. Indicates what percentage of the mean is represented by the standard deviation
2. Reliable method for comparing the precision or SD at different units or concentration levels
3. Expressed as a percentage

CV% = Standard deviation / mean X 100

Test	Analyte: FSH Concentration	SD	CV
a.	1	0.09	9.0
b.	5	0.25	5.0
c.	10	0.40	4.0
d.	25	1.20	4.8
e.	100	3.80	3.8

- The smaller the CV, the more reproducible the results: more values are closer to the mean.
- Useful in comparing precision (not accuracy) of 2 or more analytical methods. Ideally should be less than 5 %[7]

Establishment of an IQC System[4]

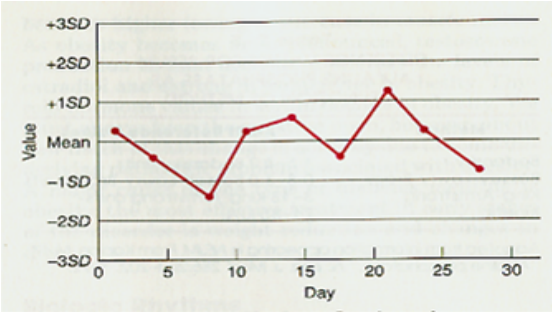
- Two or three levels of control material are usually used e.g. Level 1 (low/abnormal), Level 2 (medium/normal) and Level 3 (high/abnormal). These levels are selected keeping in view the therapeutic decision limits in mind.
- Measure of precision and reproducibility
- For qualitative or tittered assays one positive, one negative and one control with concentration around the cutoff for positive.

Collecting Data

- Run assay on control sample and enter control results on chart
- One chart for each analyte but may be for more than one level of control

Charting Techniques

Levey Jennings chart is a graph that plots IQC values in terms of how many standard deviations each value is away from the mean



Use SD in IQC

- After determination of SD, apply the information to construct a graph.
- Standard deviation is a very useful tool to assess the performance of the control (and therefore, the lab) in terms of precision.
- CV is in fact a better way of expressing SD, as it is a percentage of mean. (CV=SD/mean x100)
- Most labs make use of ± 2 SD or 95% confidence limit. To put this into a workable form, the values of ± 2 SDs should be calculated
- SD is mostly used as a dynamic parameter i.e. mean and SD are calculated at the end of previous month and used for plotting of control data in the current month.

Determining the Acceptability of Results

Scenario

a. Mean of group of control values = 104 mg/dL
b. Standard Deviation = ± 5 mg/dL
c. Determine the values of ± 2SD (which will allow us to evaluate acceptability of performance of the control on subsequent days.)

Is a control value of 100 mg/dL acceptable?
Answer: Yes, because it is within the ± 2SD

Laboratory Errors

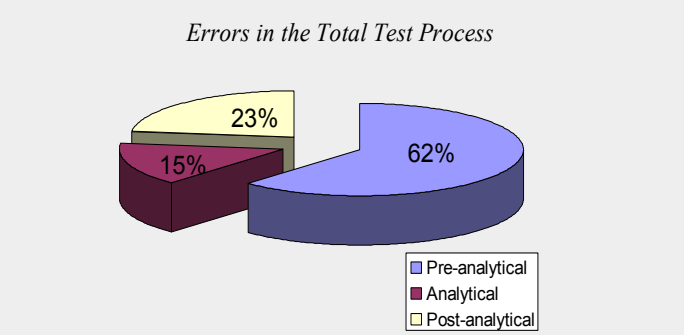


Figure No 19.2: Frequency of Errors in a Stat Laboratory in 10 Years[6]

Adapted from: Plebani M, Carraro P. Mistakes in a stat laboratory: types and frequency. ClinChem 1997;43:1348 –51.

Pre-analytical Errors: Errors in the first phase of laboratory process (pre-analytical or before the analysis) are much more common than analytical errors (Figure 19.2). The importance of pre-analytical and post-analytical errors for quality improvement in the medical laboratories is emphasized in many studies[5-7] Some of the causes of pre-analytical errors are given below (please see Chapter 17 of this book for more details):

a. Specimen collection, handling and processing
b. Physiological variables such as the effect of lifestyle, age, gender, pregnancy and menstruation
c. Endogenous variables such as drugs etc.
d. Patient identification
e. Phlebotomy technique
f. Test collection procedures e.g. applying tourniquet for calcium specimen.
g. Specimen transport
h. Specimen processing
i. Specimen storage before analysis

- Pre-analytical errors cannot be detected by routine IQC or EQA procedures.
- One way of detecting these errors is Delta Check, this will be discussed as a separate topic in this chapter.
- Prevention of pre-analytical errors has also become an important part of the 'Patient Safety' programmes and quality assurance[9]

Types of Analytical Errors[1,2]

Random Error: Imprecision of the test system causing a scatter or spread of control values around the mean.

Causes of Random Error:

- Air bubbles in reagents
- Improperly mixed reagents
- Air bubbles in reagent lines, sampling or reagent syringes
- Improperly fitting pipette tips
- Clogged or imprecise pipette
- Fluctuations in power supply
- Change in Environmental conditions e.g. room temperature
- Pipetting error

Systematic error:

Systematic change in the test system may result in a displacement of the mean from the original value. Systematic error of an analytic system is predictable and causes shifts or trends on control charts that are consistently low or high.

Causes of Systematic Error:

- Change in reagent or calibrator lot numbers
- Wrong calibrator values
- Improperly prepared reagents
- Deterioration of reagents or calibrators
- Inappropriate storage of reagents or calibrators
- Variation in sample or reagent volumes due to pipette misalignments
- Variation in temperature of reaction chambers
- Deterioration of photometric light source
- Variation in procedure between technologists

Difference Between Precision and Accuracy

Precision: refers to the ability to get the same (but not necessarily 'true') result time after time. The degree of fluctuation in the measurements is indicative of the im**precision** of the assay.

Accuracy: The closeness of measurements to the true value is indicative of the accuracy of the assay. An accurate result is one that is the 'true' result[7] (Figure 19.3)

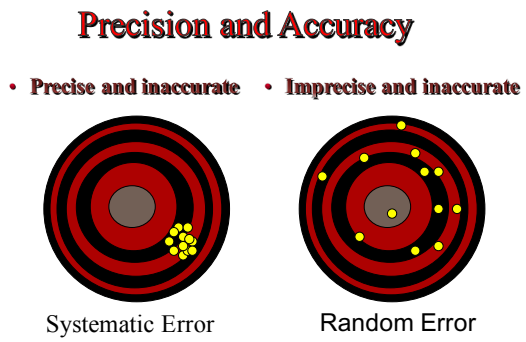


Figure 19.3: Concept of Errors with reference to Precision and Accuracy

Adapted from: http://www.statisticshowto.com/accuracy-and-precision/

Shift and Trend

Shift:

QC data results are distributed on one side of the mean for 6-7 consecutive measurement points. (Figure 19.4). Causes of shift include:

- Inaccurate calibration/recalibration
- Sudden failure or change in the light source
- Change in reagent formulation
- Change of reagent lot number
- Sudden change in incubation temperature
- Failure in the sampling system
- Failure in reagent dispense system

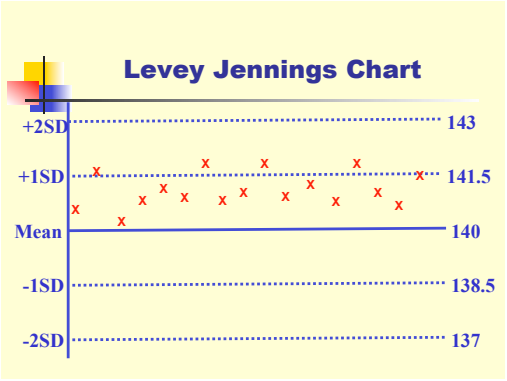


Figure No 19.4: An example of Shift

Trend:

Increase or decrease of QC data points for 6-7 consecutive measurement points (Figure 5). The main causes of trending are as follow:

- Gradual deterioration of control materials
- Deterioration of the instrument light source
- Gradual accumulation of debris in sample/reagent tubing

- Aging of reagents
- Gradual deterioration of incubation chamber
- Gradual deterioration of light filter integrity

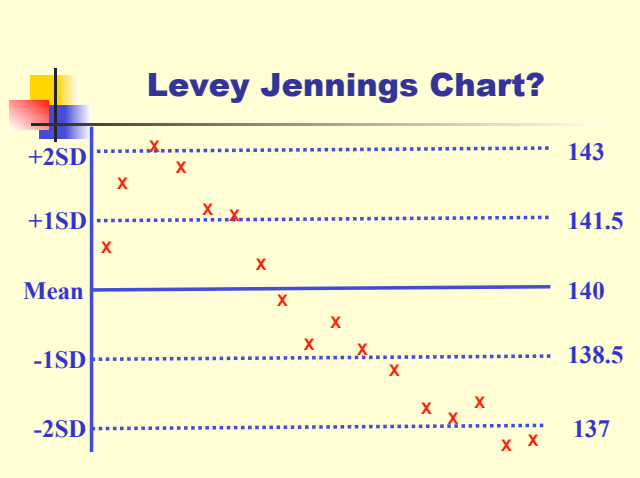
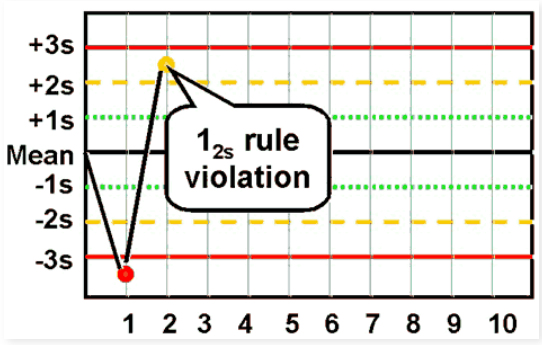


Figure No 19.5: An example of Trend

Westgard Multi-rule System[10,11]

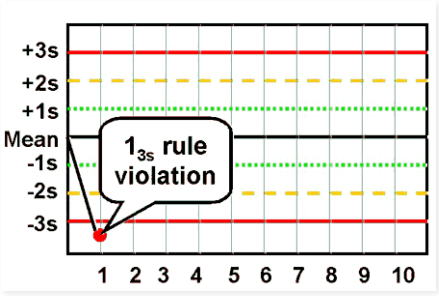
Mr Westgard has described certain rules to check the acceptability of the control results. These rules are briefly mentioned below *(Images adapted from https://www.westgard.com/westgard-rules.htm).*

1_{2s} Rule



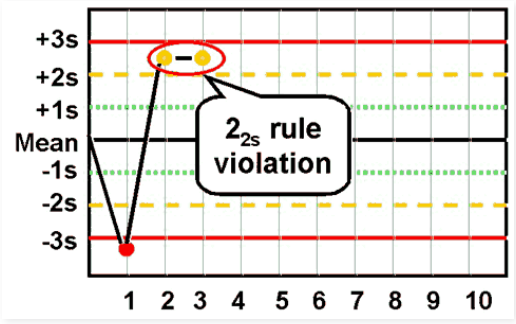
1_{2s} - refers to the situation when value of the one run is above 2 SD but less than 3 SD. It is a rule of caution/warning.

1_{3s} Rule



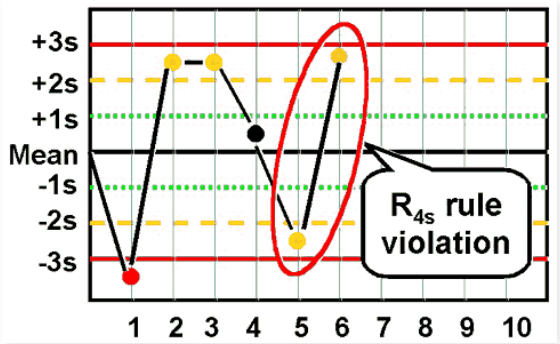
1_{3s} - a rejection rule that is the run has to be rejected if the value of any material (normal or high) exceeds 3 SD on either side of the mean.

2_{2s} Rule



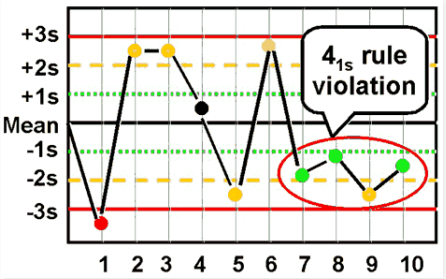
2_{2s} – A rejection rule when values of two consecutive runs cross 2 SD values one side of the mean (plus or minus)

R_{4s} Rule



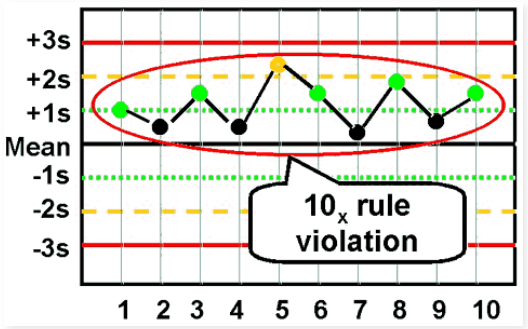
R_{4s} – The run is rejected if the previous runs have exceeded 2SD on plus side of mean and the present exceeds minus side of the mean (or vice versa).

41s Rule



4_{1s} – If ***four*** consecutive values exceed one side of 1 SD (minus or plus), the last (4^{th} run is rejected.

10_X Rule



10_x - If ***ten*** consecutive values exceed one side of mean (minus or plus), the last run is rejected[8].

Principles of Trouble Shooting[12]

Root Cause Analysis[3]

a. One at a Time Principle: When an error is detected in a QC System, it is important to follow the principle of "One at a Time".
b. If all the steps given below are changed together, the root cause of error will not be determined.
   (1) Repeat assays on control specimens using fresh aliquots of QC pool
   (2) Repeat assay on control specimen using newly constituted set of controls
   (3) Look for problems like clots, reagent levels, mechanical fault
   (4) Recalibrate the instrument for out of control analyte and re-assay all controls
   (5) Install new bottle or new lot for one or all reagents, recalibrate and re-assay all controls
   (6) Perform periodic maintenance, recalibrate and re-assay all controls
c. If any of these responses result in acceptable QC data only then patients result can be released
d. Technicians should notify supervisors of QC failure immediately, and a corrective plan determined.
e. Assay different controls of similar known concentration to determine if original control material is at fault
f. Call manufacturer to determine the cause, follow manufacturer's instruction and then re-assay all controls
g. After servicing of instrument by manufacturer recalibrate and reassay all controls

h. Use accuracy based materials to evaluate quality specification of analytical system by checking i.e.
    (1) Linearity
    (2) Accuracy
    (3) Bias
    (4) Precision
    (5) Analytical sensitivity
    (6) Minimal detectable change
i. Determine whether testing system has changed by reassessing reference interval.
j. Consult director or technical supervisor to declare method out of control if above steps fail
k. Final action involves replacement of method or instrument with one that allows laboratory to meet its medical or proficiency goals

Documentation: Quality Control records should include maintenance of Log Book for every analyte and for every instrument. All the information regarding quality control should be recorded as:

a. Date
b. Name of analyte
c. Complete testing system including
    i. Source of reagent
    ii. Instruments
    iii. Calibrators
    iv. Controls
d. Description of problem
e. Problem resolution
f. Name of staff
g. Final corrective and preventive actions

Procedure to Follow During Testing System Failure:

- Out of control conditions can constitute a laboratory emergency
- Laboratory director must be informed
- Use suitable back up method
- Sending test to reference laboratory
- Temporarily discontinuing the test
- Laboratory policy should define how much time the technologist can spend to resolve a trouble shooting before using alternate system

Proficiency Testing[13]

Synonyms: External Quality Control; External Quality Assessment Scheme (EQAS)

Definition

- "A system of objectively checking laboratory results by means of an external agency including comparison of a laboratory's result at intervals with those of other laboratories".
- The main objective is establishment of trueness or accuracy

Objectives of External Quality Control Programme:

Laboratory Oriented Objectives:

- Identifying possible deficiencies in laboratory practice, and guiding participants in any corrective actions to be taken for improvement
- Identifying the reliability characteristics of methods, materials and equipment and personnel under routine conditions and suggest corrective actions as appropriate
- Assessing and monitoring the impact of training; help for the preparation of future trainings (Figure 19.6)

Public Health Oriented Objectives:

- Providing the basis for the comparability of results during epidemiological surveillance and disease control
- Collecting information on laboratory measurements (intra- and inter-laboratory) to alert professionals and/or government bodies about problems related to traceability and harmonization of results, and establish limits of acceptability of results as appropriate for a given purpose
- Collecting information for licensing or accreditation of laboratories.

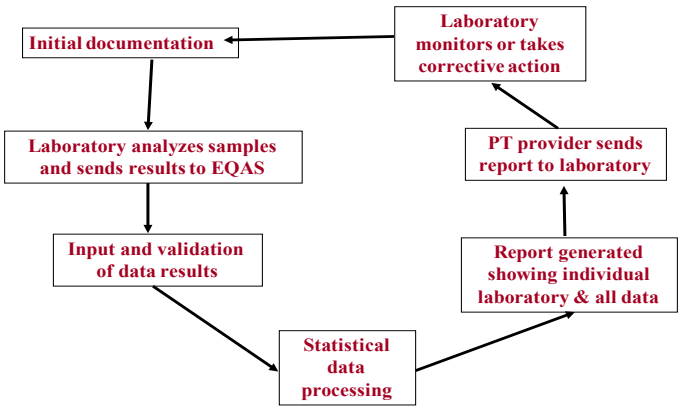


Figure 19.6: Components of a Typical EQAS Cycle

International External Quality Assurance Scheme Available in Pakistan

a. Randox RIQAS (UK)
b. Biorad EQAS (US)
c. CAP (US)

d. NEQAS (UK)
e. CDC provide surveys only for dried blood spot analytes
f. ERNDIM provide surveys for biochemical genetics laboratories

National External Quality Assurance Programme in Pakistan (NEQAPP)[14]

- Designed specifically for the country's needs
- Economical
- Useful in establishing national quality goals
- Fulfilling the requirement of accreditation by Pakistan National Accreditation Council
- Started in 1996, now nearly 200 Laboratories in Pakistan are participating
- Programs are available for Chemical Pathology, Microbiology, Hematology and Histopathology

Delta Check[15]

Introduction:

- Delta check was first described in 1974. It compares the current test result with a previous result from the same test obtained over a short period of time for the same patient.
- Lab can define its own period. For example, at AKU laboratory they take 1 month as the Delta Time.
- Pre-analytical errors that are not detectable with QC can be identified. It is more useful for stable analytes with less biological variation.

Delta Check Alert:

- A "delta check" failure or alert occurs if there is a discrepancy in the patient results.
- When the difference between a patient's present lab result and their previous result exceeds a predefined limit within a predefined length of time, delta alert is shown.

Why Using Delta Checks?

- Delta checks are useful quality improvement measures that can help the lab to identify possible patient-specific errors
- Early error identification has considerable implications for patient care and safety. Incorrect drug dosing, anticoagulation therapy, cardiac intervention, blood transfusion, etc. from erroneous lab results can lead to life-threatening events.
- Predictive value for detecting true specimen errors is between 0.4 and 6%
- Studies have found that most delta check failures (>75%) can be attributed to true changes in the patient's medical condition
- Clinicians need to be alerted to large biological variation in their patients, may indicate need for intervention

Main Goals:

- Delta checks are useful quality improvement measures that can help the lab identify possible patient-specific errors. There are two main goals:
  - Detection of real changes in patient condition or disease state
  - Identification of test quality issues or patient identification problem

Causes of Delta Check Alert (Discrepant Results):

- Causes of the discrepant results giving rise to delta check failure are patient specific and can be grouped into pre-analytical, analytical and post analytical
- Majority of our investigative power lies in detecting the analytical variation (QC, imprecision, bias).
- The analytical variation can be instrument specific or method specific., improper mixing, pH, temperature, reagent and lot changes
- Main goal of the human body is homeostasis. The body attempts to keep essential analytes from fluctuating daily. There are rhythmic changes.
- Then we have changes over the life span and delta check limits may change with patient age. Life style changes cause variation as well like changes in the nutritional status and changes in the activity level.
- Then treatments and medical intervention may cause large fluctuations in overall patient biology, affecting a variety of test results, like IV fluids infusion, Total Parenteral Nutrition, chemotherapy, dialysis, organ transplantation and other medications.

Auto Verification Systems[16]:

- The process of Delta Check has been automated considering increasing workload of laboratories. It is called Auto-Verification (AV) System.
- Similarly, laboratories are expected to increase service quality, simplify processes and decrease the report release or Turn Around time (TAT).
- So, with an AV system, at least 80% of the test reports can be autoverified without the need for manual intervention.
- Test reports are autoverified by medical technologist against report check rules already set on the LIS or middleware[5]
- Instrument manufacturers also offer this middleware AV System (e.g. Roche and Abbott)

These verifications include

a. In an AV system, the verification rules and the criteria of the test results are built into the middleware, so instead of the results requiring a manual check, they are autoverified by computer
b. These verifications include
   (1) Limit check rules
   (2) Critical values
   (3) Comparison with former results (delta check)

(4) Consistency of related results (consistence check)

Six Sigma[17]

Introduction:

- Sigma is the Greek letter representing the standard deviation of a population of data. It is a measure of *variation* (the data spread).
- The concept was initiated in Japan's automobiles industry. Later, it was borrowed by medical field and then to the clinical laboratories.
- Six Sigma is taken as a statistical measure of quality
- It is based on rigorous process based performance for continuous improvement in any business
- A way of thinking that creates a special infrastructure of people within the organization who are trained in executing this program (designated as colours of belts.
- In actual practice errors or defects are counted per million of events
- Processes that operate with "six sigma quality" over the short term are assumed to produce long-term defect levels below 3.4 defects per million opportunities (Figure 19.7)

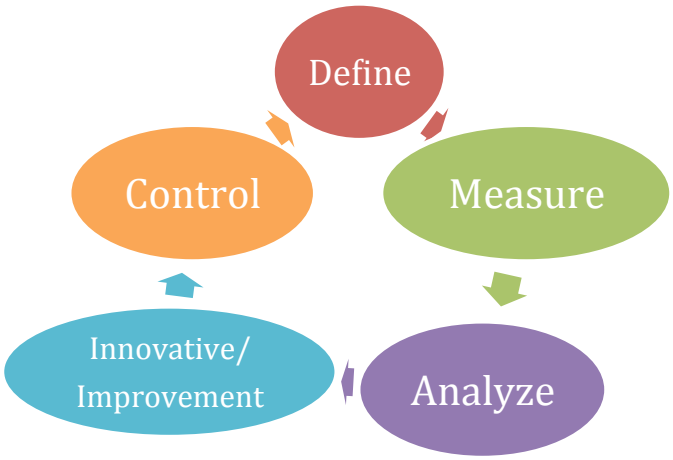


Figure No 19.7: Six Sigma Process

Methods of Six sigma

- Six Sigma projects follow two methodologies inspired by Deming's Plan-Do-Check-Act Cycle
- Composed of 5 phases each
- Bear the acronyms DMAIC and DMADV

Six Sigma and Clinical Laboratories[18]

- Six Sigma is used in the laboratory to monitor and reduce errors in laboratory work. Moreover, laboratory performance can be appraised with the application of six sigma in laboratory functions

- It is the quality performance goal that requires 6 sigma/6 SDs of process variation to fit within the tolerance limits for the process.
- Any laboratory process can be evaluated in terms of a sigma metrics
- Higher sigma values in laboratory indicate better performance
- Lower values indicate a greater number of defects per unit
- Plotted graphically as "**OPSpecs" chart**. This chart incorporates measures like total allowable error, systematic error, imprecision.
- Quality is assessed on the σ scale with a criterion of 3 σ as the minimum allowable sigma for routine performance and 6 σ being the goal for world-class quality
- When the method sigma is ≥6, stringent internal QC rules need not be adopted
- In such cases, false rejections can be minimized by relaxing control limits up to 3s
- A sigma below 3 calls for the adoption of a newer and better method as quality of the test cannot be assured even after repeated QC runs
- For laboratory measurements, the sigma performance of a method can be formulated

Sigma = Total Allowable Error – Bias / CV

How to go about it?

- Launch a six sigma programme
- Multidisciplinary team is formed- include all stakeholders
- Mentored by a six sigma black belt holder well trained person
- Use DMAIC methodology for an existing process and DMADV for a new process

Rules of thumb

- 3 Sigma-considered minimum for any industrial process
- 4 Sigma -typical performance of industry processes
- The first goal of a Six Sigma project in business and industry is to improve from 4 to 5 Sigma
- Some processes never reach 6-Sigma but reaching 5-Sigma may be good enough
- In some cases, the process can be reengineered to achieve 6-Sigma performance

Example of calculations

- Total 500 STAT tests in 1 year
- 116 delayed
- Total defects = 116
- 116/500 (defects/opportunities) =0.232

- Convert to Defects Per Million (DPM) by moving decimal to 6 places to the right (232000 DPM opportunities)
- 2 to 3 sigma

Lean Management

Introduction:

- Quality process focused on creating more value by eliminating activities that are considered waste.
- Increases efficiency; in contrast to Six Sigma which focuses on quality improvement (Table 19.1)

Principles of Lean Management:

a. Any non-value-added activity is waste
b. Eliminating waste is the key goal of lean management

Criteria of Determining Whether an Activity Adds Value or Not:

a. Value adding activities in a lab are:
   (1) Centrifuging the sample
   (2) Testing
   (3) Reporting the results

   Non-value adding activities are:
   (1) Looking for tools
   (2) Waiting to fill a sample rack for a batch
   (3) Suppliers do not add value
b. The activity must change form or function of the raw material or product
   (1) Separating the blood cells from the plasma
   (2) Analyzing
   (3) Arrangement of the specimen in an order.
c. The activity must be correctly done at the first time. Repeated runs do not add value

Lean defines 8 different types of waste[9]

a. Overproduction: Doing more than you need to do.
b. Inspection: Checking and re-checking
c. Motion: Waste of "looking for things". Time spent searching for a part or tool
d. Transportation: Moving samples and supplies.
e. Rework/ Repair: Doing things that could have been done correctly the first time
f. Un-necessary Inventory: Un-necessary equipment or reagents
g. Waiting: Specimen placed around waiting for the next step
h. Non-utilized talent: Underutilization of the talented workers is an important source of waste.

5S and visual management[10]

1. Sort: Eliminate unnecessary materials

2. Store: Stock materials in an organized and clearly labeled manner (example in MCQ No 220)
3. Shine: Clean visually identifiable items and their proper locations within the area
4. Standardize: Set a standard – often a photo of a correctly organized area
5. Sustain: Develop and maintain the discipline needed to keep the area clean and organized - inspect frequently

Table 19.1 Differences Between Six Sigma and Lean Management.

Six sigma	**Lean management**
Aim is to reduce variations	Aim is to eliminate non-valued added steps
Reduces errors	Reduces cycle time.
Improves quality	Increases efficiency

Chapter 19
Quality Management

MCQs with Key and Explanation

MCQ No	Best Option	Explanation
211.	a. Quality Assurance	Total Quality Management (TQM) Framework is a concept, borrowed from industry, describing various aspect of quality management. Quality Control (QC) is one of its components, which corresponds to the 'product checking' of an industry while the processes involved in quality assurance are to minimize the failure of QC and therefore to reduce the manufacturing cost. When we apply TQM in clinical labs we want to assure that all the processes in the lab should be made error free e.g. reagent storage, calibration of instruments and training of the staff etc., the result will be minimum rejection of final results due to QC failure.
212.	c. Fluctuation in power supply	Random errors in any laboratory can be described as an error that cannot be predicted in terms of its direction or in terms of its magnitude. It's a 'mutinous' error. On the other hand, systemic error is usually preceded by a similar error with some variation in the magnitude but in the same direction. Random errors and systemic errors are well illustrated in the text.
213.	b. Liquid, human, and assayed controls	QC material is commercially available in vials, usually 10 vials in a packet for use with almost all the photometric tests and immunoassays (hormones and tumour markers etc.). QC material is in different types with different prices. The most preferred (and costly) is in the option b. Liquid QC material has the advantage that there is no error of reconstitution as in lyophilized material. Using QC material of human origin gives the advantage that values are closer to patients` values as the matrix is same; which is

		not the case, if QC material of bovine origin is used. Lastly assayed QC material means that the target values and ranges of all the analytes are given in accompanied literature. Un-assayed QC material is of lower costs but the lab has to determine the values before use.
214.	d. Symmetrical distribution	This is the type of data, called symmetrical, normal or Gaussian, is characterised by equal distribution of data on both sides of mean. The other type is non-symmetrical or skewed data. In QC, the data is almost always symmetrical.
215.	e. Standard deviation (SD)	SD a statistical parameter used to indicate degree of dispersion of data, which means how much values in a data set differ from one another (or from the mean of this particular data set). Please note that SD can be used only when the data is symmetrical.
216.	c. One value has crossed 3 SD on the plus side of the mean	Westgard rules are widely used in clinical laboratories to assess the QC results. Its use is not restricted to Chemical Pathology but wherever numerical data is generated, these rules should be applied for QC.
217.	c. Inter-laboratory comparison	External Quality Control is also called External Quality Assessment (EQA) or Proficiency Testing (PT). Mostly EQA programmes are used internationally, though a national programme is also available in Pakistan by the name of NEQAPP (some details in the text). The hallmark of EQA programmes is 'Inter-laboratory Comparison' of results of QC material sent to these labs by the EQA agency. These programmes are also commercially available with reasonable cost.
218.	b. Delta Check	Per-analytical errors cannot be rectified by any internal or external QC programme. These errors should be suspected if a patient result is unusually high or low. The Pathologist predetermines the values of each analyte for creating alert called 'delta value'. Whenever the results of an analyte cross this delta value

		compared to previous results of the same analyte, delta failure occurs and results are not reported. The technologist or resident should verify this result by counterchecking from the previous record of the patient or from the ward or patient himself/herself. This procedure is called 'Delta Check'. This is an important component of 'Patient Safety' in clinical labs, too.
219.	e. Six Sigma	'Six Sigma' simply means minimum errors in any industrial or commercial system. Started in Japan, the concept was borrowed by Medical Field and then came in Clinical Laboratories. Some details are given in the text with an easy example from a clinical laboratory.
220.	b. Lean Management	This is a management concept but very useful application in clinical laboratories. The purpose is to save time and manpower for fruitful activities rather than wasting in un-necessary details, e.g. a technologist working alone in a lab should not waste time in searching the specimens from the racks in different refrigerators but an SOP should guide him to find the specimens quickly.

Summary
Chapter 19
Quality Management

- Quality Control (QC) is product checking in a manufacturing industry, while Quality Assurance (QA) encompasses the whole process of manufacturing, so that the product does not fail the QC checks and financial loss to the industry is avoided.
- The same concept has been borrowed in medical laboratories, QC is checking the final lab results and QA is ensuring quality at every step of the laboratory tests e.g. specimen collection, equipment maintenance, reagent storage, lab temperature monitoring etc.
- QC programmes helps in detection of analytical errors. QC programmes are of two types:
    - Internal QC: Control material of known values is run with every batch and results are interpreted after plotting on a graph paper.
    - External QC or Proficiency Testing Programmes are usually run by international organizations. Control material is sent to the lab and results of all participating labs are compared with one another. So, these programmes are mainly inter-laboratory comparisons
- Analytical lab errors can be random or systematic. Comprehension of these errors helps Pathologists and Technologists to find the cause and rectify it, a procedure called 'trouble shooting'.
- The pre-analytical lab errors are quite common and cannot be detected by QC programmes. A method of detection of these errors is 'Delta Check', meaning double-checking the abnormal lab results before dishing out. Delta Check is also a useful procedure to timely inform the clinician about a real change in the patient`s result due to illness or treatment.
- Six sigma is another industrial concept borrowed by Medical Field. It is maintaining a log of the errors in any system to minimize them e.g. 3.4 defects per million opportunities (DPMO) or 3.4 errors in one million events in an organization is taken as virtual error-free.
- Lean Management is an interesting management concept; time and energy should not be wasted in counter-productive events. In medical laboratories SOP should be developed to avoid wasteful activities.

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Chapter 20
Laboratory Management (Important Aspects)

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MCQs

(Please find the key at the end of the chapter)

221. A laboratory has reported Glucose as 102 mg/dl. In Scientific terms which of the following ***units of measurement*** this lab has adopted?
    a. British Units
    b. Field Units
    c. Metric Units
    d. Old Units
    e. SI Units

222. The most appropriate ***term*** for the values used for comparison of patients' results for the diagnosis or monitoring is?
    a. Normal Limits
    b. Normal Values
    c. Reference Limits
    d. Reference Ranges
    e. Reference Values

223. The ***values*** established on the basis of analysis of specimen from healthy and diseased persons are called:
    a. Clinical Decision Limits
    b. Observed Reference Limits
    c. Population Reference Values
    d. Reference Limits
    e. Subject Reference Values

224. Mrs Farrukh is an experienced Lab Manager. She wants to install new equipment in her lab to obtain Reagent Grade Water. For purification of water she wants to use an instrument based on a process in which water is forced to pass through a semipermeable membrane. In your opinion, which ***type of equipment*** she should procure:
    a. Distillation
    b. Double Distillation
    c. Ion Exchange
    d. Reverse Osmosis
    e. Ultraviolet Oxidation

225. Dr Rabia is working on her MPhil project. She wants to evaluate ***diagnostic accuracy*** of serum hyaluronic acid as a marker of liver fibrosis. The most important pre-requisite for her project is:
    a. Convenience sampling
    b. Double blinding
    c. Gender matching
    d. Randomization of samples
    e. Using a reference standard

226. Dr Sara wants to use ***'TSH only regime'*** for the screening of thyroid disorders. The most important requirement in the TSH assay is:
    a. High accuracy
    b. High cut-off value
    c. High positive predictive value
    d. High sensitivity
    e. High specificity

227. Dr Zia Ul Islam is heading a large hospital lab network spread all over the country. He wants to get his laboratory accredited in Pakistan. The ***International Standard*** he should adopt for preparation of this accreditation is (*Options are not in alphabetical order*):
    a. ISO 22870:2006
    b. ISO 9001:2000
    c. ISO/TR 22869:2005
    d. ISO/TS 22367:2008
    e. ISO 15189:2012

228. A firm has installed a new Laboratory Information Management System with state-of-the-art software and hardware in a hospital lab. The most important feature to ***prevent post-analytical transcriptional errors*** in this system will be:
    a. Automated analyser interface
    b. Bar-coding
    c. Electronic authorization
    d. Specimen tracking
    e. Work list creation

229. A laboratory director has made an agreement with manufacturers for the procurement of sophisticated closed system equipment. Vendor has agreed to provide the equipment free of cost on the condition that all the consumables will be purchased from the same company. The ***system of procurement*** used the above-mentioned scenario is:
    a. Instrument donation system

b. Instrument mortgage system
c. Per test cost contract system
d. Private-public partner system
e. Reagent rental system

230. Mr Tahir Jamal is an efficient laboratory manager. While ***calculating requirement*** of laboratory regent kits, he has added some extra kits for one test, on the pretext that these tests are frequently repeated in dilution to determine the exact level in the patients.
Which of the following tests most probably falls in this category?
    a. B_{12}
    b. Bilirubin
    c. Glucose
    d. Beta HCG
    e. TSH

Chapter 20
Laboratory Management (Important Aspects)

Pathologists have to carry out certain administrative job in the laboratory. In this chapter, we will present a brief overview of some important aspects of laboratory management i.e.

1. Units of Measurement
2. Reference Values and Clinical Decision Limits
3. Water Purification System
4. Clinical Utility of Laboratory Tests
5. Laboratory Accreditation
6. Laboratory Information Management Systems
7. Instrument Procurement Systems
8. Inventory Management

1.Units of Measurement[1]

- In medical laboratories, currently two systems of measurement are widely used[1]:
- *Gravimetric Units:* These are based on the mass of the substance i.e. gram, milligram and microgram
- *The Systeme Internationale (SI):* It is in use internationally since 1960. It should be noted that gravimetric system is also derived from metric system called Meter, Kilogram and Second while SI Units have been derived from 'Amount of Substance' expressed in moles (Table 20.1)
- In SI units, three classes of units are used:
  - **Base:** Eight base units are given in Table 20.1. These are the fundamental units.
  - **Derived:** It is mathematically derived from two or more base units. They are the most commonly used type of units used in the medical laboratory e.g. density is kg per cubic meter (kg/m^3), volume is cubic meters.
  - **Supplementary:** The units borrowed by SI from other systems due to their usefulness e.g. litre which is equal to *cubic decimeter* derived from meter.

Table: 20.1 Eight Base Units of SI.

S/No	Quantity	Unit	Symbol
1.	Length	Meter	m
2.	Mass	Kilogram	Kg
3.	Time	Second	s
4.	Electric current	Ampere	A
5.	Temperature	Kelvin	K
6.	Luminous	Candela	cd

	Intensity		
7.	Amount of substance	Mole	mol
8.	Catalytic Amount	Katal	Kat

- For proteins and amino acids gram and submultiple of gram are still in use (e.g. mg and µg are used with 'litre' instead of 'dl'. So, albumin is expressed in traditional unit as 4.0 g/dl while in SI Units it will be 40 g/L.
- Details of units of measurement and conversion factors are given in the 'Appendix' of this book along with the reference values of commonly used analytes in traditional and SI Units.

Standardized Pattern of Reporting of Laboratory Results:

- **Logical Observation Identifiers Names and Codes** (**LOINC**)[1] is a database and universal standard for identifying medical laboratory observations. First developed in 1994, LOINC was created in response to the demand for an electronic database for clinical care and management and is publicly available at no cost. It is endorsed by the American Clinical Laboratory Association and the College of American Pathologists.
- **Format:** A formal, distinct, and unique 6-part name is given to each term for test or observation identity. The database currently has over 71,000 observation terms that can be accessed and understood universally. Each database record includes ***six*** fields for the unique specification of each identified single test, observation, or measurement:

1. Component: what is measured, evaluated, or observed (example: urea)
2. Kind of property: characteristics of what is measured, such as length, mass, volume, time stamp and so on
3. Time aspect: interval of time over which the observation or measurement was made
4. System: context or specimen type within which the observation was made (example: blood, urine)
5. Type of scale: the scale of measurement. The scale may be quantitative, ordinal, nominal or narrative
6. Type of method: procedure used to make the measurement or observation
7. A unique code: (format: nnnnn-n) is assigned to each entry upon registration

2.Reference Values and Clinical Decision Limits

Proper Terminology[2]

- Reference values /reference intervals is the most appropriate term

- Range is a single value!!
- 'Normal' is a statistical term denoting symmetrical data, so this term 'normal values' is now considered obsolete and must be avoided.

Clinical Decision Limits[2]

- These are replacing reference values for many analytes
- Usually provided in Guidelines by international bodies
- Examples: Lipids by NCEP- ATPIII (National Cholesterol Education Program-Adult Treatment Panel III), Glucose and Glycosylated Haemoglobin by American Diabetic Association (ADA)
- Labs need NOT to establish their own or verify these limits
- Also called Cut-offs

Concept of Health[3]

- Persons free of Physical, Mental and Social illnesses…….
- So 'Free of Disease' is a better concept for Ref Values
- Three steps:
  - Anamnestic Interviews
  - Clinical Exam
  - Essential Lab Tests

Randomization

- Means all the population should be collected (will undergo examination and application of selection criteria) and then sample of subjects selected by balloting etc.
- Not possible or feasible in reference values studies
- So, most of the studies of reference values are done by non-random sampling

Methods of Determination of Reference Values[4]

- Non-Parametric Methods e.g. rank-based
- Parametric (Mean±2SD)
- Parametric-transformed data e.g. log transformed

Rank-Based Method (Bootstrap Method)

- This is a WHO recommended method
- Can be used for parametric as well as non-parametric data
- Place all the values in the ascending order
- Take value at lowest 2.5th Percentile (0.025xn+1)
- Take value at highest 97.5th Percentile (0.97.5xn+1)
- Details of these methods will be given in the next level of this book i.e. 'Advanced Chemical Pathology'

3.Water Purification System[5]

Clinical Laboratory Reagent Water (CLRW) Reagent Grade Water

- Water is a reagent; CLRW (Clinical Laboratory Reagent Water) is also called Reagent Grade Water or Reagent Water.

- Water is the most common and important reagent often produced right in the laboratory.
- The quality of water has an impact on the testing method.

Water Purification

- Water purification is the transition of dirty harmful (contaminated) water into clean safe water

Water for Laboratory Analysers

- Cuvette washing
- Tubing and probe rinsing
- Reagent and buffer reconstitution
- Dilution
- Water baths

Consequences of Impure Water in the Lab

- Frequent calibrations
- High CV%
- Fluctuation in quality results over the day/week/month
- Difficulty with coagulation and Haematology analyses
- Interference with immunoassays
- Instrumentation problems
- Leaching of contaminants from improperly regenerated deionisation resins
- Difficulty with cell culture procedure

Contaminants and Water Testing

The five types of contaminants that may be found in water are:

- Particulates
- Dissolved inorganics (solids and gases)
- Dissolved organics
- Microorganisms
- Pyrogens

Purification Methods

Different methods are commonly used to purify water. These are:

- Distillation
- Deionization
- Reverse osmosis
- Activated carbon filtration
- Micro porous filtration
- Ultra filtration
- Ultraviolet oxidation
- Electro dialysis

Distillation

- Distillation is the process of vaporizing and condensing a liquid to purify or concentrate a substance or to separate a volatile substance from less volatile substances.
- Removes effectively bacteria, pyrogens, particulate material, dissolved ionised solids and to lesser extent dissolved organic contaminants.
- Not useful for elimination of dissolved ionised gases such as NH_3, CO_2 and CL or low boiling point organic compounds.

Reverse Osmosis

- Water is forced under pressure through a semi permeable membrane, leaving behind remnants of dissolved organic, ionic and suspected impurities including microbial and viral contaminants
- Does not remove dissolved gases effectively.

Ion Exchange (Deionization)

- Deionization is an ion-exchange process in which all charged species or ionizable organic and inorganic salts are removed from a solution.
- Resistivity and conductivity are used to assess the ionic content of purified water
- Higher the ionic concentration, higher the conductivity and lower the resistivity

Resistivity[6]

- Resistivity is used in the measurement of water with few ions.
- It is expressed in 10^6 Ohms (megohms-cm or MΩ/cm)
- Deionized pure water is a poor electrical conductor, having a resistivity of 18.2 million ohm-cm (18.2 megohm).

Conductivity:

- It is the amount of ionized substances (or salts) dissolved in the water, which determines water's ability to conduct electricity
- Microsiemens is unit of conductivity (opposite to resistivity). Pure water has conductivity of 0.055 microsiemens.

Limitations of Deionization

- Limited or no removal of particles, colloids, organics or microorganisms
- Capacity related to flow rate and water ionic content
- Regeneration needed using strong acid and base
- Prone to organic fouling
- Multiple regenerations can result in resin breakdown and water contamination
- Risk of organic contamination from previous uses

Ultra Filtration

- In ultra filtration water is passed through a semi permeable membranes of pore size ≤0.2 μm removing particulate matter, emulsified solids, most bacteria and pyrogens.
- 0.1μm post membrane filters are being used to achieve an improved bacteria-free and pyrogens-free product.
- Ultrafiltration does not effectively remove dissolved ionised solids, gases and most organic contaminants.

Ultraviolet Oxidation and Sterilization

- UV oxidation and sterilization are used after purification process to remove trace amounts of organic contaminants (oxidation) and bacteria (sterilization). 185 min for oxidation and 254 min for sterilization.
- UV treatment is limited by intensity, contact time and flow rate.

Types of CLRW[6]

- There are several grading systems for water, ASTM, USP, ACS, CAP, NCCLS.
- The most well-known is the ASTM systems i.e. Type I (most pure), Type II and Type III (least pure) (Table 20.2)

Type I CLRW

- For all quantitative and most qualitative lab procedures.
- Electrophoresis,
- Toxicology
- Atomic absorption
- Flame emission spectrometry
- Ligand assays
- Enzymatic procedures sensitive to trace metals
- High sensitivity chromatographic procedures
- In preparation of buffers, standards and controls
- For trace elements and heavy metals

Type II CLRW

- Test methods in which presence of bacteria is tolerated
- Reagents with preservatives
- General reagents without preservatives
- Reagents that are sterilized and in most stains and dyes
- Microbiology system (not sterilized)
- Microbiology system (to be sterilized)

TYPE III CLRW

- Is acceptable for washing and preliminary rinsing of glassware
- As source for further purification.
- As bacteriological media preparation

Table 20.2 Specifications of Type I and Type II

ASTM Type	I	II
Specific Conductance (microsiemens)	<0.06	<1.0
Specific Resistance (MΩ.cm)	>16.67	>10
Total Matter (max. mg/l)	Water Passed Through 0.2 µm filter	Water Passed Through 0.2 µm filter
Silicate (max. mg/l)	Not Detected	Not Detected
KMnO4 Reduction	>60.0	>60.0

Microbial testing

- Microorganisms constitute another group of contaminants found in water.
- Surface water may contain a wide variety of microorganisms, including bacteria, protozoa, algae, amoebae, rotifers, diatoms and others.
- Since most laboratory water comes from municipal water treatment plants, which is extensively treated to remove microorganisms, the chief microbes of concern for water purification systems are bacteria.
- A typical bacterial level for a potable laboratory water supply is one colony forming unit per millilitre (cfu/ml).

4. Clinical Utility of Laboratory Tests[7]

Evidence-Based Laboratory Medicine

- It is defined as the judicious and explicit use of best available evidence in the use of laboratory medicine investigations in making decisions about the care of individual patients.
- Before starting any laboratory test, its clinical utility must be determined by carrying out 'Diagnostic Accuracy Studies'

Diagnostic Accuracy Studies[8]

- In studies of diagnostic accuracy, results from the new test (Index Test) are compared with the results obtained from a gold-standard test (called the Reference Standard) on the same subjects.
- Such accuracy studies are a vital step in the evaluation of new test
- May be used for new employment of an existing diagnostic technology

'The Test' in Diagnostic Accuracy Studies

- Laboratory Test
- Radiological Test / Imaging
- History / Clinical Examination

- Function Tests e.g. Lung Function Test

Essentials of Diagnostic Accuracy (PIRT)[7]

- Patients. Selecting a group of patients (e.g. suspected cases)
- Index test: The test in question
- Reference Standard: It is best available test to diagnose the target condition
- Target condition: Which condition should be ruled in or ruled out
- Assigning status to each test result as per Table 20.3

Table 20.3: How Positives and Negatives can be True and False

	Reference (Gold) Standard **Positive**	**Reference (Gold) Standard** **Negative**
Index Test Positive	True Positive (TP)	False Positive (FP)
Index Test Negative	False Negative (FN)	True Negative (TN)

Attributes of a Test[7-11]

- **Sensitivity:** Sensitivity is the proportion of those people who really have the disease (TP+FN) and who are correctly identified as such (TP)

Sensitivity = TP / (TP+FN) or True positive/Total diseased

- **Specificity:** Specificity is the proportion of those people who really do not have the disease (TN+FP) who are correctly identified as such (TN)

Specificity = TN / (TN+FP) or True negative/Total non-diseased

- **Positive Predictive Value (PPV)**: PPV is the proportion of the people who test positive (TP+FP) to those who truly have the disease (TP)

Positive predictive value = TP / (TP+FP) or True positive/Total positives

- **Negative Predictive Value (NPV):** NPV is the proportion of the people who test negative (TN+FN) who truly do not have the disease (TN)

Negative predictive value = TN / (TN+FN) or True Negative/Total negatives

- **Receiver operating characteristic (ROC) plot:** It is a statistical tool used to determine the diagnostic validity (Area Under Curve) of a test and to determine the optimum 'cut-off' value of a numerical test data.
- **Likelihood Ratio of Positive Test:** (For a qualitative test): *Probability of occurrence of a specific test value when the disease is present (True Positive*

Rate) divided by the probability of the same test value if the disease was absent (False Positive Rate). A helpful test will have a large LR positive:

LR (Positive) = Sensitivity / 1- Specificity

- **Likelihood Ratio of Negative Test**: *Probability of occurrence of a specific test value when the disease is absent (specificity) divided by the probability of the same test value if the disease was present.*
  A helpful test will have a small LR negative
  LR (Negative) = Specificity / 1-Sensitivity)
- **Diagnostic accuracy**: Overall efficiency of the test is determined by:

TP + TN / TP + TN +FP +FN or All True / Total Screened

Problems with Predictive Values

- Predictive values are dependent on the prevalence of the disease
- Our population is often quite different from the study population
- They are influenced by the prevalence of disease in the population that is being tested (Table 20.4)

An Example

Table 20.4 Effect of Prevalence on Predictive Values

• Prevalence: 2%	• Prevalence: 98%
• n = 1,000	• n = 1,000
• Sensitivity: 92%	• Sensitivity: 92%
• Specificity: 90%	• Specificity: 90%
• TP: 21(18)	• TP: 901
• TN: 882	• TN: 18
• FP: 98	• FP: 2
• FN: 2	• FN: 78(79)
• PPV= 17.6% (15.5%)	• PPV = 99.8
• NPV: 99.8	• NPV= 18.7 (18.6%)

Sensitive Test –To Rule Out (SnOut)

- A sensitive test can pick up affected persons
- Used to rule out diagnoses
- Used when there is a penalty in missing a case
- Diagnosis of a dangerous but treatable condition (e.g. Tuberculosis), Blood screening for HIV

- Used at an early stage of a diagnosis work-up
- A sensitive test is most useful when negative
- Interpretation according to the prevalence

Specific test –To Rule In(SpIn)

- A specific test can pick up non-affected persons
- Used to rule in diagnoses
- Used when a false positive can harm a patient
- HIV test for individual counselling
- Cancer diagnosis before chemotherapy
- Used to confirm a diagnosis suspected because of other data
- A specific test is most useful when positive
- Interpretation according to the prevalence

5.Laboratory Accreditation

Certification Vs Accreditation[12]

Certification is used for verifying that personnel have adequate credentials to practice certain disciplines, as well as for verifying that products meet certain requirements.

Accreditation is used to verify that laboratories have an appropriate quality management system and can properly perform certain test methods and calibration parameters according to their scopes of accreditation. Any laboratory conducting medical testing is eligible to apply for the accreditation program

Accreditation Initiative at National Level

A laboratory accreditation initiative within a country requires at least 3 elements:

a. A laboratory policy framework that makes accreditation a requirement for laboratories.
b. Designated quality standards against which laboratories can be assessed.
c. Accrediting bodies (local or international) authorized to assess laboratories and certify their performance against the designated quality standards

Accreditation Bodies[13]

Pakistan National Accreditation Council (PNAC): This council works under the auspices of Ministry of Science and Technology, Government of Pakistan. PNAC has accredited a few laboratories in Pakistan e.g. Armed Forces Institute of Pathology (AFIP) Rawalpindi, Chughtai Lab Lahore, Islamabad Diagnostic Lab.

Joint Commission International (JCI) and College of American Pathologists (CAP): These are international bodies, which carry out inspection for grant of accreditation. Two laboratories in Pakistan have got JCI accreditation e.g. AKU Hospital Karachi and Shifa International Hospital lab Islamabad.

Accreditation Standards[13,14]

a. ISO 15189 is a globally recognized standard that has been specifically created for medical laboratories to help them develop their quality management systems and assess their competence.
b. ISO 17025 is for labs not involved in clinical work e.g. calibration labs or forensic sciences labs.
c. ISO 15190:2003 for lab safety
d. ISO 22870:2016 for POCT

Proficiency Testing (PT) is a Must for Lab Accreditation[15,16]

- Participation in PT programs (please see Chapter 18), a key component of accreditation, leads to more accurate test results. PT failures among laboratories were noted to decrease with successive PT challenges
- Adherence to such quality standards and participation in accreditation programs that certify this adherence can improve operational efficiency and customer service and reduce rates of laboratory errors.
- The variability of test results and the frequency of errors can be reduced by implementing and monitoring a comprehensive laboratory quality management system.
- Accreditation provides verification that laboratories are adhering to established quality and competence standards deemed necessary for accurate and reliable patient testing and the safety of staff and the environment

Accreditation is Recognition[15]

- Laboratories that achieve accreditation are recognized for superior
    - Test reliability
    - Operational performance
    - Quality management and competence
- Accredited laboratories can become more accountable and less dependent on external support.
- Efforts made to achieve accreditation may also lead to improvement in the management of laboratory networks by focusing attention on areas of greatest need and accelerating improvement in areas such as supply chain, training, and instrument maintenance.
- Adherence to such quality standards and participation in accreditation programs that certify this adherence can improve operational efficiency and customer service and reduce rates of laboratory errors.

Initiative for Accreditation[16]

- Laboratory policy updates
- Long-term strategic planning
- Improved procurement and supply chain systems
- Laboratory networking

- Human resources management and training
- Instrument service maintenance
- Data and quality management.

6. Laboratory Information Management Systems[17,18]

A Laboratory Information Management System (LIMS), sometimes referred to as a Laboratory Information System (LIS), is a software based laboratory and information management system that offers a set of key features to support a modern laboratory's operations:

- Computerized information management system designed for laboratories
- Manages lab data from sample log-in to reporting
- Interfaces with analytical instruments
- Sorts and organizes data into various report formats
- Stores data for future reference and use

Pillars of LIMS

- Hardware
- Software
- People
- Procedures
- Data

Role of LIMS

To manage

- Data
- Work flow
- Changing business needs/processes
- Existing systems and improving where required
- Resources
- Quality assurance / Quality control

Functions of LIMS

- Track specimens from receipt, processing, testing, reporting to storage
- Electronically capture results from lab diagnostic equipment and store with specimen details
- Protocols and algorithms for testing and final result determination
- Patient focus report generation
- Enable determination of patient outcomes
- Integrate patient and specimen information
- Support patient management and care/treatment

Decision about Installation of LIMS

- Volume of specimens
- Types and number of tests
- Size of staff/users

- Existing system
- Requirements and expectations

Advantages of LIMS Use

- Improves data management in lab to increase lab potential
- Enables centralization of information
- Supports and enhances business processes of the lab
- Takes advantage of new lab information technology
- Provides easy access to data
- Reduces transcription errors
- Faster processing with direct instrument uploads
- Real time control of data quality with built in QC criteria
- Direct report generation meeting specific client requirements
- Direct electronic reporting to clients or direct client access to data
- Improves the efficiency hence productivity.
- Improves data quality (all instrument are integrated).
- Saves time because the information is obtained at the snap of the button
- Improves level of data access for all stakeholders of any project.
- Automated customer reports (TAT, Work Load)

Disadvantages/Concerns

- Customization of LIMS/interfaces required for specific lab/client needs
- Adequate validation to ensure data quality
- Data integrity and confidentiality, especially when clients have direct access to data
- Limited interface between lab and field computer systems

Format

- Reports
- Information available on LIMS
- Project, samples, tests documentation
- Sample tracking history within the Lab
- Reporting results (hardcopy, electronic file)
- Financial information by test, client, dates
- Information on productivity

Responsibilities of the Laboratory

- Evaluate LIMS capabilities
- System validation/maintenance
- Interfacing capabilities
- Confidentiality/data integrity protection
- Regulatory compliance/accreditation.

New Trends

- Web based programmes
- No requirement of servers
- Mobile apps connected with LIMS
- Literature available for patient education

7.Instrument Procurement Systems

Types of Procurement[19]

Procurement on reagent rental basis

- Lease agreement between diagnostic companies and laboratories
- Lease cost is built into the reagents i.e.an analyzer will be placed in a laboratory in exchange for the guaranteed purchase of reagents over a period.

Procurement on cost-per-test contracts

- Analyzer would be placed in the laboratory with the agreement that the laboratory would pay a specified amount per test run on the analyzer, based on the laboratory's estimated test volume by type.

Purchase of new instrument / Total Lab Automation

Reagent Rental and Cost Per Test

Advantages:

- Prevents the initial high cost of the instrument used from capital equipment budget rather it will appear in operating budget
- Continuous supply of reagents for the contract period will be ensured
- Some contracts also include service/maintenance expenses in the cost, providing additional savings for the laboratory
- When a contract has expired, laboratories can simply replace older analyzers with new instruments that offer them the most extensive, cutting-edge test menus.
- Laboratories can update technology much more readily as they are not absorbing the cost of the analyzer.
- Provides guaranteed income for diagnostic manufacturers as well, so a true win-win situation in diagnostics markets that has typically seen modest growth at best in recent years

Disadvantages:

- Consumer will be bound to purchase reagent kits from that vendor only
- Reagent cost may be 10-15% higher (but not necessarily) for the consumer on reagent rental basis than when purchased directly from the market.
- Not very profitable for smaller laboratories and physician office laboratory settings

8. Inventory Management[20]

- A well-managed laboratory should have a system for inventory maintenance and purchasing inventory of the equipment.
- The system will require planning and monitoring, analyzing needs, developing forms and procedures, and maintaining an inventory.
- The laboratory will maintain an inventory system for all reagents and supplies used in the laboratory.

Salient Features of Inventory Management in the Lab

Procurement and Inventory Management:

- Efficient and cost-effective laboratory operations need an uninterrupted availability of reagents, supplies, and services. Establishing a purchasing and inventory management program will ensure that:
  - Supplies and reagents are always available when needed
  - High quality reagents are obtained at an appropriate cost
  - Reagents and supplies are not lost to improper storage or kept and used beyond expiration.

Purchasing Considerations

Factors to consider when setting up procedures for purchasing are as follows:

- Understand any local or national government requirements that need to be accommodated in the contracts
- Negotiate for the best price without undermining quality
- Carefully review all contracts to make sure the laboratory's requirements are being met
- Contracts should clearly address payment mechanisms and provisions to assure reliable availability and delivery of reagents and supplies.
- Ask if there are penalties for ending a contract
- Determine how payments will be made, and how the vendor will assure reliable availability and delivery of supplies and reagents

Inventory Management Challenges

- The challenge of inventory management is balancing the availability of supplies and reagents in stock with their expiration dates.
- The life-span of reagents can vary from a few weeks to several years. It is important to continuously monitor the expiration dates to make sure needed reagents are always on hand and have not expired. It is too costly and wasteful, however, to overstock and it must be avoided
- Equipment and supplies received or accepted from donors must meet the clients' and operational needs of the laboratory

Stock Logbook

- Name and signature

- Date of receipt
- Quantity
- Date of expiry
- Minimum stock
- Stock balance
- Other information:
  - Shelf number
  - Destination

Calculating Annual demand of the Reagent Kits

While calculating demand of any reagent kit, following considerations should be kept in mind (Table 20.5):

- Workload of the test in the previous year (financial or calendar year)
- Likely increase in the workload. It can be calculated by seeing the workload of the previous 2-3 years.
- Allowance for quality control, calibration, and repeat tests
- Special allowance for the analytes which are frequently repeated to determine their exact level e.g. beta HCG (re: Scenario in MCQ No 230) and Ferritin
- Volume of the kit (ml)
- No of tests per ml of the reagents / No of tests per kit as per manufacturer`s claim

Table 20.5 An Example of Calculating Glucose Reagent Kit Demand

Total Glucose Tests during 2017:	120,000
Total Glucose Tests during 2016:	110,000
Volume of Glucose kit:	500 ml
Volume of Reagents per test used by Autoanalyser in your lab	250 µl
Tests done per kit:	2000
Allowance for QC, calibration, and repeat tests (20%) per kit	400
Net tests done per kit:	1600
Expected workload during 2018:	133,000
No of kits Required during 2018:	84

Chapter 20
Laboratory Management (Important Aspects)

MCQs with Key and Explanation

MCQ No	Best Option	Explanation
221.	c. Metric Unit	The so-called 'Traditional Units' used most of the laboratories in Pakistan are actually from metric system of measurement (Meter, Kilogram and Second-MKS). On the contrary, the SI units are relatively newer system of measurement derived from 'Amount of Substance' expressed in moles. Some details of SI are given in the text of the chapter.
222.	e. Reference Values	The term 'Normal' should not be used as it is a term used to describe a certain type of data; similarly, the term 'range' should also be discouraged as in statistics 'range' is a single value, for example Interquartile Range is a single value.
223.	a. Clinical Decision Limits (CDL)	For many tests reference values are not used now, instead CDL are used. For example, reporting cholesterol and other lipid parameters with reference values have been abandoned because the upper limit of cholesterol was found to be too high (250 mg/dl or 6.50 mmol/L). On the other hand, CDL of cholesterol was found to be i.e. 200 mg/dl (5.1 mmol/L). CDLs are determined by International Organizations and every laboratory is NOT required to establish their own CDLs.
224.	d. Reverse Osmosis (RO)	This is a very effective method of purification in which energy is used to pass the water through a semipermeable membrane. The size of the pores in the membrane through which water is passed is important. For example, reagent grade water is required to be produced using a membrane of 0.2 µm. Please note RO does not remove ionisable material, which must be removed using a deionizer technique like ion exchange resin. The monitoring systems used with water purification systems are based on electrochemical technique (conductimetry) and give status of ionisable material only. For other impurities pore size of the membrane is the criteria and for microorganisms, colony count is used.

225.	e. Using a reference standard	Establishing clinical utility of tests is one of the most important aspects of test evaluation. This is done by carrying out diagnostic accuracy studies; in these studies, the test to be evaluated (Index Test) is compared with a 'Gold Standard Test' now called 'Reference Standard Test'. By carrying out these studies, attributes of the 'Index Test' are determined e.g. specificity and sensitivity.
226.	d. High sensitivity	When a test is being used for screening of a disease the test should be very sensitive. In this MCQ, TSH should be used as the only screening test for thyroid dysfunction, so highly sensitive TSH is used. The logic behind is to minimize 'False Negative' as a negative screening test is not further investigated.
227.	e. ISO 15189:2012	ISO standard for Laboratory Accreditation is different for Clinical Laboratories (ISO 15189) and non-clinical laboratories e.g. calibration labs or Forensic labs (ISO 17025). Both these standards 'Accreditation Standards' differ from 'Certification Standards' like ISO 9001 the management standards etc.
228.	a. Automated analyser interface	Laboratory Information Management Systems (LIMS) are now quite commonly used in clinical laboratories in Pakistan. One of the most useful features of LIMS is direct transfer of patient results generated by autoanalysers to the LIMS. This greatly reduces possible human errors in manual entries (called post-analytical transcriptional errors).
229.	e. Reagent rental system (RRS)	Almost all the medium and large sized labs in our country procure laboratory equipment by RRS now. This saves a lot of initial cost; the only requirement is that the consumables have to be purchased from the same firm, which has provided the equipment with a certain volume. A major advantage of RRS is that the maintenance of the equipment is the responsibility of firm. Reagent price and other details are agreed between the laboratory (or institute/hospital) and the firm.
230.	d. Beta HCG	Calculation of reagent kits and other consumables for the whole year involves very careful prediction of increase in the workload and other factors. In case of beta HCG and ferritin the results may vary from as low as <1.0 to one million and may require tests to be repeated in multiple dilutions.

Summary

Chapter 20

Laboratory Management (Important Aspects)

- Two different units of measurements are used in the world. In UK and other European countries, Canada, Australia and New Zealand, SI is used while in USA and most of the Asian countries traditional metric system is used. Pathologists and Technologists should be well-versed with both.
- Reference values are the numerical values determined for the native population, to compare the lab results; while clinical decision limits are issued from time to time by certain International Organizations for comparison of lab results
- Water purification system is of paramount importance for smooth sailing of a laboratory especially for smooth functioning of the autoanalysers. Reverse osmosis and deionization are the two major procedures used for water purification. Measurement of conductivity of water is an important control mechanism (ideally it should be near to zero but <10 is acceptable).
- Clinical utility of a test should be determined before starting the test facility by comparing the result of this test with a well-reputed lab or radiological test. It includes specificity, sensitivity, predictive values and probability values.
- Laboratory Information Management System (LIMS) or any other IT system is now an essential component of a good clinical lab. By using LIMS labs get rid of papers and human errors are also minimized.
- Lab accreditation is recognition of a lab as a good diagnostic and prognostic service provider. In Pakistan, this accreditation is carried out based on ISO 15189:2012 by Pakistan National Accreditation Council (PNAC).
- Lab instruments can be procured, free of cost, from a vendor without paying its cost on an MOU that the consumables will be purchased from the same vendor. This system of procurement is call 'Reagent Rental System'
- Maintenance of reagents inventory is one of the most important managerial skills a lab director and manger should learn. Demanding a justified stock of kits at the start of the year is a crucial step in inventory management.

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Appendix
Reference Values of Common Biochemical Analytes

In the following table, reference values and clinical decision limits of some commonly used analytes along with the conversion factors are given[1-3]. Reference values of other analytes may be obtained from Teitz Textbook of Chemical Pathology[1].

S/No	Analyte	Conventional Unit	Conversion Factor*	SI Unit
1.	17-Hydroxy-progesterone (17 OHP)	• Newborn: < 630 ng/dl • Prepubertal: < 100 ng/dL	SI to Conventional: x 33 Conventional to SI: x 0.03	• Newborn: < 19 nmol/L • Prepubertal: < 3 nmol/L
2.	ABG (Arterial Blood Gases) Partial pressure of carbon dioxide (PCO_2)	35 - 45 mmHg	SI to Conventional: x7.5 Conventional to SI: x0.133	4.6 – 5.9 kPa
3.	ABGs Partial pressure of oxygen (PO_2)	75 - 100 mmHg	SI to Conventional: x7.5 Conventional to SI: x0.133	10 – 13.3 kPa
4.	ABGs pH:	7.35 - 7.45	-	-
5.	ABGs Hydrogen [H+]:	-	-	36 - 44 nmol/L
6.	ABGs Base excess:	(-3) - (+3)	-	-
7.	ABGs Bicarbonate (HCO_3):	23 - 28 mmol/L	1.0	23 - 28 mmol/L
8.	ABGs O_2 saturation	96% - 100%	-	96% - 100%
9.	Adrenocorticotropic hormone (ACTH) (0800 hours)	< 120 pg/ml	SI to Conventional: x 4.5 Conventional to SI: x 0.22	< 26 pmol/L
10	Alanine aminotransferase (ALT)	M: < 45 U/L F: < 34 U/L	x 0.017	M: < 0.77 µKat/L F: < 0.58 µKat/L
11	Albumin	3.5 – 5.2 g/dL	x 10	35-52 g/L
12	Alkaline phosphatase (ALP) (IFCC 37^0C)	M: 53 -128 U/L F: 42 - 98 U/L	x 0.017	M: 0.90 - 2.18 µKat/L F: 0.71- 1.67 µKat/L
13	Alpha 1_antitrypsin	90-200 mg/dL	x 0.01	0.9 - 2.0 g/L
14	Alpha fetoprotein (AFP)	< 15 mg/L	1.0	< 15 g/L
15	Ammonia	15 to 45 µ/dL	SI to Conventional: x 1.4 Conventional to SI: x 0.71	11 to 32 µmol/L
16	Amylase	28 - 100 U/L	x 0.017	0.48 - 1.70 µKat/L
17	Angiotensin-converting enzyme (ACE)	23 - 57 U/L	-	-

18	Aspartate aminotransferase (AST):	M: < 35 U/L F: < 31 U/L	x 0.017	M: <0.60 µKat/L F: <0.53 µKat/L
19	Bilirubin (Direct)	0.0 – 0.2 mg/dl	SI to Conventional: x 0.058 Conventional to SI: x 17.1	0 – 3.4 µmol/L
20	Bilirubin (Total)	0.0 - 2.0 mg/dl	SI to Conventional: x 0.058 Conventional to SI: x 17.1	0 - 20 µmol/L
21	C-reactive protein	< 0.5 mg/dl	X10	< 5 mg/L
22	CA 19.9	< 37 U/ml	1.0	< 37 kU/L
23	Calcium (Ionized, free)	4.6 - 5.3 mg/dL	SI to Conventional: x 4.0 Conventional to SI: x 0.25	1.16 – 1.32 mmol/L
24	Calcium (Total)	8.5 - 10.2 mg/dL	SI to Conventional: x 4.0 Conventional to SI: X 0.25	2.1 - 2.6 mmol/L
25	Carcinoembryonic antigen (CEA)	< 3 ng/ml	1.0	< 3 µg/L
26	Ceruloplasmin	20 - 60 mg/dL	x 0.01	0.2 – 0.6 g/L
27	Chloride	98 - 107 mmol/L[@]	1.0	98 - 107 mmol/L
28	Cholesterol (Total)**	• Desirable: <200 mg/dL • Borderline: 200-240 mg/dL • High Risk: >240 mg/dL	SI to Conventional: x 38.6 Conventional to SI: x 0.026	• Desirable: < 5.2 mmol/L • Borderline: 5.2-6.2 mmol/L • High Risk: > 6.2 mmol/L
29	Cholesterol High-density lipoprotein (HDL-C)**	Desirable: > 44 mg/dl	SI to Conventional: x 38.6 Conventional to SI: x 0.026	Desirable: > 1.1 mmol/L
30	Cholesterol Low density lipoprotein (LDL-C)**	Primary Prevention: < 130 mg/dL Secondary Prevention: < 100 mg/dL	SI to Conventional: x 38.6 Conventional to SI: x 0.026	Primary Prevention: < 3.36 mmol/L Secondary Prevention: < 2.26 mmol/L
31	Cholesterol Very Low density lipoprotein (VLDL-C)**	Desirable: < 23 mg/dl	SI to Conventional: x 38.6 Conventional to SI: x 0.026	Desirable: < 0.60 mmol/L
32	CK-MB	< 25 U/L		-
33	Copper	M: 70 - 140 µg/dL F: 80 – 155 µg/dL	x 0.16	M: 11 – 22 µmol/L F: 12 – 24 µmol/L

34	Cortisol (Serum)	• Morning (8 am): 6-23 µg/dL • Evening (11pm): 3-15 µg/dL	SI to Conventional: x 0.036 Conventional to SI: x 27.6	• Morning (8 am) 170 - 635 nmol/L • Evening (11 pm) 82 - 413 nmol/L
35	Creatine kinase (CK) (IFCC 37^0C)	< 171 U/L	x 0.17	< 2.90 µKat/L
36	Creatinine	M: 0.9 -1.3 mg/dL F: 0.6 -1.1 mg/dL	SI to Conventional: x 0.11 Conventional to SI: X88	M: 80 - 115 µmol/L M: 53 - 97 µmol/L
37	Ferritin	• Male: 20 - 250 ng/mL • Female: 10-120 ng/mL	1.0	• Male: 12 – 250 µg/L • Female: 12 - 120 µg/L
38	Folate (serum)	2.6 – 12.2 µg/L	x 2.26	6 - 28 nmol/L
39	Follicle stimulating hormone (FSH)	• Male: 1 - 15 mIU/mL • Female (follicular/luteal) 1 - 10 mIU/mL • Postmenopausal: 19 - 100 mIU/mL	1.0	• Male:1 - 15 IU/L • Female (follicular/luteal): 1 -10 IU/L • Postmenopausal : 19 - 110 IU/L
40	Gamma glutamyl transferase	M: < 55 U/L F: < 38 U/L	x 0.017	M: < 0.94 µKat/L F: < 0.65 µKat/L
41	Glucose (Fasting)**	Normoglycaemia: < 100 mg/dl (Please see Chapter 1 for further details)	SI to Conventional: x 18 Conventional to SI: x 0.055	Normoglycaemia: < 5.6 mmol/L (Please see Chapter 1 for further details)
42	Growth hormone (Basal) (No significance without stimulation. Please see Chapter 13)	2 - 5 ng/mL	1.0	2 – 5 µg/L
43	Human Chorionic Gonadotrophic Hormone (Beta)	M: < 1 mIU/mL F: (non-pregnant): < 3 mIU/ml	1.0	M: < 1 IU/L F: (non-pregnant): < 3 IU/l
44	Iron (serum)	M: 75-175 µg/dL F: 28-162 µg/dL	SI to Conventional: x 5.6 Conventional to SI: x 0.17	M: 13 - 31 µmol/L F: 5 - 29 µmol/L
45	Iron-binding capacity (TIBC)	250-410 µg/dL	SI to Conventional: x 5.6 Conventional to SI: x 0.17	45 - 73 µmol/L
46	Lactate dehydrogenase (LD):	125 - 220 U/L	x 0.02	2.1 – 3.7 µKat/L
47	Lead	< 25 µg/dL	x 0.048	< 1.21 µmol/L
48	Lipase	< 38 U/L	x 0.017	< 0.65 µKat/L
49	Luteinizing hormone (LH) (serum)	• Male: 1 - 10 mIU/mL • Female	1.0	• Male: 1 - 10 IU/L • Female Follicular:

		Follicular: 2 - 10 mIU/mL Mid-cycle: 15-65 mIU/mL • Postmenopausal: 12 - 65 mIU/mL		2 - 10 IU/L Mid-cycle: 15-65 IU/L • Postmenopausal: 12 - 65 IU/L
50	Magnesium	1.6 – 2.6 mg/dl	SI to Conventional: x 2.43 Conventional to SI: x 0.411	0.66 – 1.07 mmol/L
51	Oestradiol	• Male: 10 - 50 pg/mL • Female (follicular): 20 - 150 pg/ml • Female (luteal): 30 -450 pg/ml • Postmenopausal: < 21 pg/ml	x 3.69	• Male: 37 – 184 pmol/L • Female (follicular): 73 – 550 pmol/L • Female (luteal): 110 – 1652 pmol/L • Postmenopausal: < 74 pmol/L
52	Osmolality (serum)	285 - 295 mOsm/kg	1.0	285-295 mmol/kg
53	Osmolality (urine)	38-1400 mOsm/kg	1.0	38-1400 mmol/kg
54	Parathyroid hormone (PTH)	10-65 pg/mL	1.0	10-65 ng/L
55	Phosphorous (inorganic)	2.5 – 4.5 mg/dL	SI to Conventional: x 3.1 Conventional to SI: x 0.323	0.81 - 1.45 mmol/L
56	Potassium	3.5 – 5.0 mmol/L[@]	1.0	3.5 – 5.0 mmol/L
57	Progesterone (ovulatory cycle)	70 - 280 ng/dL	x 3.18	223 – 890 nmol/L
58	Prolactin	M: < 14 ng/mL F: < 23 ng/mL	1.0	M: < 14 µg/L F: < 23 µg/L
59	Prostate - specific antigen (PSA)	< 4 ng/ml	1.0	< 4 µg/L
60	Pyruvic acid	0.30 - 0.9 mg/dL	x 0.114	0.03 – 0.10 µmol/L
61	Sodium	136 -145 mmol/L[@]	1.0	136 - 145 mmol/L
62	T3 (Free)	210 – 440 pg/dL	x 0.015	3.1 – 6.6 pmol/L
63	T3 (Total)	70 -204 ng/dL	x 0.015	1.05 – 3.06 nmol/L
64	T4 (Free)	0.8 – 2.7 ng/dL	x 12.9	10 – 35 pmol/L
65	T4 (Total)	4.6 - 10.5 µg/dL	x 12.9	59 – 135 nmol/L
66	Testosterone	M: 260 –1000 ng/dl F: 15 - 70 ng/dl	0.035	M: 9 - 35 nmol/L F: 0.5- 2.43 nmol/L
67	Thyroid stimulating hormone (TSH)	0.4 – 4.2 µIU/mL	1.0	0.4 – 4.2 mIU/L
68	Thyroxine-binding	1.2 – 2.5 mg/dL	10	12 - 25 mg/L

	globulin (TBG)			
69	Total protein	6.0 – 8.0 g/dL	SI to Conventional: x 0.1 Conventional to SI: x 10	60 - 80 g/L
70	Transferrin	200 - 360 mg/dL	0.01	2 -3.6 g/L
71	Triglycerides*	Desirable: < 150 mg/dL	SI to Conventional: x 88 Conventional to SI: X0.011	Desirable: < 1.70 mmol/L
72	Troponin:	Please see Chapter 10		
73	Urea	6 - 20 mg/dl	SI to Conventional: x 6 Conventional to SI: x 0.357	2.1 -7.1 mmol/L
74	Uric Acid	M: 2.54 – 6.9 mg/dl F: 2.54 – 5.9 mg/dl	SI to Conventional: x 59 Conventional to SI: 0.357	M: 150 - 410 µmol/L F: 150 - 350 µmol/L
75	Vitamin A	30 - 80 µg/dL	x 0.035	1.05 – 2.8 µmol/L
76	Vitamin B_{12}	> 206 - 678 ng/L	x 0.733	151 – 497 pmol/L
77	Vitamin C	0.4 – 1.5 mg/dl	x 56.8	23 – 85 µmol/L
78	Vitamin D (25 Hydroxy D)	• Deficiency: < 20 ng/mL • Insufficiency: <30 ng/mL • Normal: 30-150 ng/mL • Hypervitaminosis: > 300 ng/mL	SI to Conventional: x 0.4 Conventional to SI: x 2.5	• Deficiency: <50 nmol/L • Insufficiency: <75 nmol/L • Normal: 75-375 nmol/L • Hypervitamin: 750 nmol/L
79	Zinc:	80 – 120 µg/dL	0.153	12 - 18 µmol/L

* For some of the common tests both conversion factors are given i.e. SI to Convention units and Conventional Units to SI Units.

** In these tests, Clinical Decision Limits (CDLs) are given instead of Reference Values. Please see chapter 20 to see the difference between CDLs and Reference Values.

@ Conventional unit of potassium and sodium is mEq/L but it is not in vogue these day.

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