Semester-IV
Unit 1
Haemostatsis & Coagulation tests

Haemostasis : (Stoppage of bleeding) When any blood vessel is injured then the bleeding is stop by a natural process called haemostasis. It is the process by which the blood is retained within the vascular system. It is very complex process & platelets are also involves.

Mechanisms of Haemostasis/Theory of Blood Coagulation: It include three steps :

1. Vascular response
2. Platelet plug formation
3. Coagulation of blood (clot formation)

1. Vascular Response: The vascular response (vasoconstriction) is a immediate response during injury of blood vessels. It reduce the blood flow which assists in the process of platelets plug formation (Next step). Muscular contraction & release of serotonin by the damaged platelets are contributory factors in the formation of platelets plug.

2. Platelet plug formation : The blood vessels are internally lined by a single layer of epithelial cells, this is known as entothelium. If the endothelium is disrupted due to injury, the underlying basement membrane containing collagen fibers is exposed. When platelets circulating in the blood come in contact with the collagen, they immediately adhere to the wall the site of trauma. The platelets then release Adenosine diphosphate (ADP) which promotes the adhesion & aggregation of platelets.

3. Coagulation of Blood : The circulating plasma & tissues surrounding the blood vessels contain a total 12 factors. These coagulation factors are various organic compounds, like proteins & co-enzyme & the inorganic element calcium. All these coagulation factors are present in the inactive form. These are active after injury of blood vessels. These clotting factors participate in the process of coagulation in the three sequential stages :

(i) Intrinsic Pathway
(ii) Extrinsic Pathway
(iii) Common Pathway

Name & Properties of Clotting Factors:

1. I Fibrinogen: The fibrinogen is synthesised in liver. Its molecular weight is 340,000 dalton. Its synthesis is not depend on the vitamin K. It is participated at 56^0C.

2. II Prothrombin : It is also a protein. Its molecular weight is about 69,000 daltons. it is also synthesised in liver & its synthesis depends on the action of vitamin K. It is heat stable. It is consumed in coagulation process.

3. III Tissue Thromboplastin: It is a lipoprotein. Its molecular weight is very high (45000-1 million). It is present in all tissue of body but in lungs, present in very large amount.

4. IV Calcium: The factor IV is ionic calcium (Ca^{++}). It acts as bridge between the platelets & other clotting factors.

5. V Proacceler (Labile Factor) : It is globulin in nature. It is synthesised in liver. Its molecular weight is 330,000 daltons. It is a labile factor. It is rapidly deteroites in oxalated plasma. It is completely consumed in coagulation.

6. VI does not exist.

7. VII Proconvertin (Stable Factor) : It is b-globulin in nature. It is also synthesised in liver & depend on the vitamin K. Its molecular weight is about 48000 daltons. It is a stable factore therefore it neither consumed nor destroyed.

8. VIII Antihaemophilic Factor : It is b-globulin in nature. The site of its synthesis is not known. In circulation its two functional subunits :

(i) Factor VIII: C:- It is a coagulation factor.

(ii) Factor VIII: VWF (Von Willebrand's factor) :- It is not necessary for coagulation mechanisms but it facilitates adhesion of platelets.

9. Factor IX Plasma Thromboplastin component (Christmas factor) :

It is also B-globulin in nature. Its molecular weight is 57,000 daltons. It is also synthesized in liver & require vitamin K. It is not consumed during clotting. It is stable at 4^0C for upto 2 weeks.

10. Factor X (Staurt Power Factor) : It is a-globulin. its molecular weight is 59000 daltons. It is synthesised in liver & require vitamin K. It is a stable factor. It is partially consumed during clotting process.

11. XI Plasma Thromboplastin antecedent : It is b-globulin. Its molecular weight is 160,000 to 200,000 daltons. Its synthesis in the liver & its synthesis dependent on the vitamin K. It is stable at room temperature.

12. XII Hagemen Factor : It is Gamma globulin. Its molecular weight is about 80000 daltons. It is synthesised in liver & does not depend on vitamin K. It can be stored at 4^0C for about 3 months in oxalated plasma. It is heat resistant upto 60^0C for 30 minutes.

13. XIII Fibrinase or Fibrin Stabilizing factor : It is alpha globulin. It is a high molecular weight clotting factor. The site of its synthesis is not known. It circulate in blood as two pairs of subunits of dissociable complex (a2b2). It also helps in growth & repair of tissue.

Clotting Factor categories vise :

1. Fibrinogen Group : This group include factor I, V, VIII, XIII. All this factors are interacts with them all.

2. Prothrombin Group : This group include factors II, VII, IX, X.

3. Contact Group : This include factors XI, XII, prekallikrein.

Proteins involved in Blood Coagulation

Plasma Protein	**Synonym**	**Conc. (mg/dl)**	**Action**
Fibrinogen	Factor I	200-400	Converted to fibrin along with platelets
Factor II	Prothrombin (Prethrombin)	10-15	Is converted to thrombin (IIa) which splits fibrinogen into fibrin
Factor V	Proaccelerin, Labile factor	0.5-1.0	Supports Xa activation of II to IIa
Factor VII	Stable factor Proconvertin	0.2	Activates X
Factor VIII:C	Antihemophilic factor (AHF) Platelet co-factor I	1.0-2.0	Supports IXa activation of X
Factor IX	Christmas factor Plasma thromboplastin component (PTC)	0.3-0.4	Activates X
Factor X	Staurt-Power factor (AVTD	0.6-0.8	Activates II

	prothrombin III)		
Factor XI	Plasma thrombo-plastin antecedent (Antihemophilic	0.4 factor C)	Activate XII and Prekallikrein
Factor XII	Hagemen factor	2.9	Activates XI and prekallikrein
Factor XIII	Fibrin-stabilizing factor Laki-Lorand factor	2.5	Crosslinks fibrin & other proteins
Von Willebra-nd's factor	Factor VIII-related antigen VIII : VWD	1.0	Stabilizes VIII,med--iates platelet adhesion
Prekallikrein	Fletcher factor	5.0	Activates XII and prekallikrein, cleaves HMWK
High molecular weight, kinin-ogen (HMWK)	Fitzgerald factor	4.7-12.2	Supports reciprocal activation of XII, XI and prekallikrein

Pathways for Coagulation Process : During the process of coagulation the fibrin (clot) is the end product. It is formed due to activation of prothrombin to thrombin & fibrinogen to fibrin. The activation of prothrombin is required two pathway, one is the intrinsic pathway & second is the extrinsic pathway. Now both these pathway are activate the common pathway (Activation of Prothrombin)

Explanation on Coagulation Pathways :

1. Intrinsic pathway : The necessary component of intrinsic pathway are present in circulating blood. When the blood is contact with a foreign surface, a series of reactions mediated by enzymes are start. The prekallikrein & high molecular weight kininogen participates in the activation of factor XII to XIIa. Now the XIIa in turn activates factor XI to XIa. This process continues further and involving factors IX, VIII & X factor X is converted to Xa by the help of calcium ions and phospholipid on the platelets.

2. Extrinsic Pathway : The extrinsic pathway is stimulated after injury of blood vessel. The damaged tissue releases thromboplastin which is normally absent in blood. Thromboplastin along with factor VII activates factor X to produce Xa in the presence of calcium. This pathway is quicker and shorter than Intrinsic pathway.

Common Pathway : The combination of Intrinsic pathway and extrinsic pathway activates factor X to Xa. Now this factor convert factor V into Va. After this the factor Va & Ca^{++} converted prothrombin into thrombin. Now this thrombin convert the fibrinogen into fibrin with help of Ca^{++}.Thus the blood is clotted.

Role of Platelets in Haemostasis : The platelets perform three important function :

1. During platelets plug formation the platelets collect at the injury site & form a plug to create obstruction to blood loss. After adhesion of platelets to the exposed collagen fibres of the blood vessels starts activation of platelets. This activated platelets change their form & become spherical with long projections. Then the surface area of platelets is increased & enhances interaction with other platelets & coagulation factors.
2. The platelets helps in activation of coagulation factors and participate in the process of coagulation. Platelets produce PF3 which is necessary for the activation of certain coagulation factors.
3. The thrombocytes also secrete certain products which help to maintain & repair the endothelium of blood vessels.

Fibrinolysis : (Fibrin-clot, Lysis-To Break).

The process of removing unwanted, insoluble deposits formed as result of coagulation is known as fibrinolysis. This is a physiological process in which a fibrin clot is broken down with the help of enzymes into soluble fragments. This mechanism involves conversion of plasma proenzyme plasminogen into plasmin. Now the plasmin digests fibrin & breaks it into soluble fragments.

Activators

Plasminogen Plasmin

Plasmin

Fibrin Soluble fragments

(Insoluble)

The plasminogen activators are present in largest amount in the endothelial cells of capillaries. The endothelial cells also secrete another important plasminogen activator called tissue-type plasminogen activator (t-PA). The high level of t-PA is related with bleeding disorder.

Increased level of Plasminogen : This is observed in acute bacterial infection, inflammatory conditions, AMI, Pregnancy, sepsis.

COAGULATION DEFECTS (DISORDERS)

There are two types of coaglation disorders:

1. Inherited Disorders : It include

(a) Hemophilia A
(b) Hemophilia B
(c) Hemophilia C
(d) Von Willebrand syndrom
(e) Coagulation Disorders due to deficiency of Coaglulation factors.

2. Acquired Disorders: It include

(a) Vitamin K Deficiency.
(b) Heparin Therapy.
(c) Diffuse intravascular coagulation (DIC)
(d) Lupus - type anticoagulants.

1(a) Hemophilia A :

This is known as Classical Hemophilia also. This is due to lack of factor VIII antigen (viii:Ag). This is carried on the X-Chromosomes. It occurs in males mostly.

Symptoms:

The main symptoms of this disease is hemorrhagic conditions eg; Bleeding in joints, muscles, bleeding after surgery, major injury, bleeding may also occur through the nose, mouth,eyes & G.I.T.

Lab. Findings :

1. Abnormal PTT.
2. Thombin generation test abnormal.
3. Prothrombin Consumption test.
4. Factor with is deficient.

But PLTs count, Platelets function test, thrombin times are normal.

1(b) Hemophilia B:

This disease resultant of deficiency of factor IX: It is also X chromosome linked disorder. The differentiation between Hemophilia A & B is necessary because the treatment for both is not same.

Laboratory Findings:

1. PTT is prolonged.
2. Factor viii is normal.
3. Factor ixth is Diminshed.
4. Thrombin time & B.T is normal.

1(c) Hemophilia C :

The severity of this disorder is not related with factor XIth deficiency. The symptoms of this disease is just like other hemophilic disorders. The laboratory findings of this disease is similar to other hemophilia except essay of factor XI.

1(d) von Willebrand Syndrom :

This disease also related with deficiency of factor VIII. The factor viiith is a complex of two molecules. VIII :C & VIII : Ag.

The small active molecule is the factor viii coagulent-antigen (VIII : Ag). itss deficiency leads to hemophilia A.

A larger fragment is known as von-Willebrand factor(vWF). The vWF factor circulating in blood which is bind with factor VIII:Ag. In other words we can say that the factor VIII:C does not function in the absence of WF factor & bleeding does not stop.

Laboratory Findings:

1. Prolonged Bleeding Time.
2. Factor viii activity is reduced.
3. factor viii R:Ag is reduced.
4. The PTT is abnormal.
5. The Prothrombin consumption test is abnormal.
6. PLTs adhesion test & Aggregation tests are also abnormal.

1(d) Coagulation Disorders due to deficiency of various clotting factors :

(i) Deficiency of Factor vii : (Lab. Findings) :

1. Severe Bleeding.
2. Prolonged Prothombin time.
3. Normal Thrombin time.
4. Factor vii level is reduced.

(ii) Deficiency of Factor xii or HMWK : (Lab. Findings) :

1. Mild Bleeding Tendencies.
2. Prolonged APTT.
3. Normal Thrombin & Prothrombin time.
4. Factor xii level is reduced.

(iii) Deficiency of Factor Xth : (Lab. Findings) :

1. The degree of bleeding is vary (Depend on severrity.
2. Prolonged APTT & Prothrombin time.
3. Stypven time in the presence of prolonged prothrombin time.

(iv) Deficiency of Factor v : (Lab. Findings) : It is found rarely

1. Prolonged Prothombin time..
2. Prolonged PTT.

3. Prolonged TGT.

(iv) Deficiency of Factor XIII : Mostly laboratory tests are normal. The diagnostic test is based on solubility of the unstablised fibrin clot in a five molar urea solution or 1% mono-chloroacetic acid.

(v) Deficiency of Fibrinogen : It is found very rarely. It is lead with the severe bleeding. Laboratory result ahow abnormality in all the tests which have a fibrin clot as the end point

mality in all the tests which have a fibrin clot as the end point e.g' prothrombin time, thrombin time and PTT. The PCT and TGT are normal except in severe cases.

(vi) Deficiency of Prothrombin : It is quite rare. The laboratory findings show prolonged prothrmbin time and PTT, bleeding time,PLTs count,clot retraction and thrombin time are normal. Diagnosis is made by the specific assay for prothrombin.

2. Acquired Disorders :

2(a) Vitamin K deficiency : As we know Vit. K is must for synthesis of some clotting fators. Therefore deficiency of vitamin K leads to defects in the synthesis of coagulation factors VII, IX, X and prothrombin, Vitamin K is normally obtained in two ways : through the dietary intake of the vitamin produced by plants, and the vitamin synthesised by the intestinal bacteria. Vitamin K deficiency may result from inadequate diet, intestinal malabsorption, biliary obstruction or gut sterilisation. Sometime Vitamin K deficiency may be intentionally induced by the administration of anticoagulant drugs e.g. Coumarin.

(b) Heparin Therapy : The Heparin is a fast-acting mucopolysaccharide which interfers with coagulation by inhibiting the action of factors IIa, XIa, XIIa and XIII by blocking the convesion of fibrinogen to fibrin. It also reduce platelet aggregation. Heparin therapy may be used in the treatment of thrombosis so that the coagulation is extended with minimum of bleeding. The anticoagulant activity of the heparin treated plasma is best monitored by the help of activated partial thromboplastin time (APTT).

(c) Diffuse intravascular coagulation (DIC) : DIC syndrome due to a wide variety of pathological processes which lead to activation of the coagulation system. In this syndrome, Intravascular fibrin deposit may be produced with excessive consumption of coagulation factors. Reduction in the level of coagulation factors is related with imbalance in the haemostatic mechanism and uncontrolled bleeding may start. This process is often accompanied by fibrinolysis which increase the bleeding tendency.

The aetiological agents of DIC are sepsis, neoplasms, autoimmune diseases, haematologial diseases, obsteric problems and intravascular haemolysis.

Laboratory findings :

1. DIC is very widely (Depend on the cause and its severity).
2. Prothrombin time, thrombin time and PTT are increased.
3. In most cases of DIC, there is thrombocytopenia with leucocytosis.
4. Schistocytes may be seen in the peripheral blood smear.

Platelet Disorder

1. Inherited Disorders of Platelet Function

(i) Glanzmann's thrombasthenia

(ii) Bernard's-Soulier syndrome

(iii) Storage granule abnormalities

2. Acquired Disorders of Platelet Function

(i) Drug induced disorders

(ii) Other factors

3. Quantitative Platelet Disorders

(i) Thrombocytopenia

(ii) Thrombocytosis

1. Inherited Disorders of Platelet Function

(i) Glanzmann's disease (Thrombasthenia) : In this disease, the platelet count and morphology is normal, but they show a characteristic tendency to resist clumping together. The bleeding can be very severe.

Lab. Diagnosis : The laboratory tests show abnormal bleeding time, prothrombin consumption test and clot retraction.

(ii) Bernard's-Soulier Syndrome : This is an inherited disorder related with absence of some membrane glycoprotein which are essential for platelet adhesion.

Lab. Diagnosis : The laboratory findings include abnormally large (70 to 75 □m) platelet forms, prolonged bleeding time and reduced platelet adhesion to glass. The clot retraction is normal.

2. Acquired Disorders of Platelet Function :

(i) Drug Induced disorders : A large number of drugs influence platelet function. Aspirin and its derivative are the principle causes of such disorders. Other examples include antibiotics such as penicillins and cephalosporins; analgesics and anti-inflammatory agents like ibprofen; and other miscellaneous drugs such as heparin. The platelet function usually returns to normal when administration of causative agent is discontinued.

Aspirin :

- Asprin is used in prevention of thrombosis in case of coronary artery disease or stroke. Aspirin blocks cyclo-oxygenase enzyme inhbiting Thromboxane A_2, PGE_2□ synthesis and thus platelet aggregation is affected.
- Defective aggregation lasts for 3-4 days even at a low dose of 60 mg aspirin.

(ii) Other Factors : Due to some disease platelets function and counts are adversaly affected e.g. in AML, CML, haemorrhagic thrombocythaemia, myelofibrosis, autoimmune diseases, systemic lupus erythematosus and anaemia.

3. Quantitative Platelet Disorders :

(i) Thrombocytopenia : A reduced number of circulating platelets (thrombocytopenia) can result from :

(a) Decreased production of platelets : Due to decreased production of megakaryocytes or ineffective platelet production due to vitamin B_{12} deficiency can lead to thrombocytopenia.

(b) Disorders of distribution : Due to hypothermia or transfusion of stored blood.

(c) Platelet destruction : Some conditions can lead to platelet destruction such as Infection, tissue injury, snake bite, thrombotic thrombocytopenic purpura, autoimmune diseases, liver disease and malignancies are some of the conditions that can cause platelet destruction.

(ii) Thrombocytosis (Increased platelet count) : This is associated with benign, reactive process or may be associated with a myeloproliferative disorder. In majority of cases, platelet stimulation and aggregation is affected.

Bleeding Time

Introduction : The time required for the blood to stop flowing after a standardised capillary puncture. This depends on the number and function of platelets and capillary integrity.

Clinical Significance :

(i) Thrombocytopenia

(ii) Vascular abnormalities

(iii) Inherited or acquired platelet function defects.

(iv) Inherited plasma defects (e.g. von-Willebrand's disease, factor V deficiency)

(v) Drugs e.g. aspirin and antihistamine.

There are two methods used for measuring bleeding time :

1. Ivy Method :

Normal Range : 2-6 minutes. Values between 6 and 10 minutes are considered borderline and the test should be repeated.

Requirement :

(i) BP Cuff

(ii) Spirit or 70% alcohol as antiseptic

(iii) Blotting paper or filter paper.
(iv) Sterile lancet
(v) Stopwatch

Procedure:

(i) Place a blood pressure cuff on the upper arm . Inflate the cuff to 40 mm Hg and hold this exact pressure for the entire period.
(ii) Clean the area of the forearm below the anticubital fossa with antiseptic.
(iii) Hold the skin tightly by grasping the underside of the arm firmly and make two separate punctures (3 mm deep) 5 to 10 cm apart, in rapid succession using disposable lancet.
(iv) Start the stop watch when bleeding starts.
(v) Blot the blood from each incision site on a separate piece of circular filter paper every 30 seconds. The filter paper should not touch the incision point at any time.
(vi) After bleeding stop, record the bleeding time of the two punctures and determine the mean time. Remove the blood pressure cuff and cover the puncture site with plaster.

Duke Method :

Normal Range : 1-6 minutes.

Requirement :

(i) Spirit or 70% alcohol as antiseptic
(iii) Blotting paper or filter paper.
(iv) Sterile lancet
(v) Stopwatch

Procedure :

(i) Clean an ear lobe or a finger tip with antiseptic.
(ii) Make a deep puncture using a disposable lancet.
(iii) Start the stop-watch when bleeding starts.
(iv) Using filter paper blot the drop of blood coming out of the incision every 30 seconds. Do not allow the filter paper to touch the bleeding spot.
(v) When bleeding stop then stop the stopwatch.
(vi) Count the number of drops on the filter paper and multiply by 30 seconds.
(vii) Report to the closest minute.

Note : Ivy method is more reliable than Duke Method.

Clotting Time

This method screens for coagulation defects. This test is a qualitative measurement of factors involved in the intrinsic pathway. Deficiency in the factors of intrinsic pathway i.e. I, II, V, VIII, IX, X, XI, XII i.e. the method is not very sensitive.

Clinical Significance : Only severe clotting factor deficiency is recognized by this method. Therefore, the test is of little value as a screening procedures. In case of prolonged clotting time the patient should be subjected to more detailed tests for the identification of missing coagulation factors.

By Capillary Method :

Normal Range : 4 to 9 minutes.

Reuirements :

(i) Sterile lancet
(ii) Capillary tubes
(iii) Cotton
(iv) Spirit or 70% alcohol
(v) Stop watch

Procedure :

(i) Apply spirit or 70% alcohol to the patient's fingertip and dry the finger after applying the antiseptic.

(ii) Make a deep incision with a sterile lancet and start the stopwatch.
(iii) Wipe off the first drop of blood and allow the ensuing drop of blood to flow into two capillary tubings held side by side.
(iv) After 2 minutes, break off the capillary tubing, 1 to 2 cm from the end, with the help of a glass file.
(v) Repeat this break off procedure after every 30 seconds.
(vi) When a thin string of fibrin can be visible between the broken ends of the capillary tubes, stop the stop watch and note the time.

Lee-White or Venipuncture Method

Normal Range : 4-9 minutes.

Requirement :

(i) Sterile syringe and needle.
(ii) Cotton and spirit
(iii) Test-tubes
(iv) Stopwatch
(v) Water bath

Procedure :

(i) Dispense 1 ml of blood in two test tubes and start the stopwatch.
(ii) Plug the tubes with non-absorbent cotton wool and place them in the waterbath at 37^0C.
(iii) After every 30 seconds, by tilting the tube, find out whether the blood has clotted.
(iv) When the blood has clotted in one tube, note the time and confirm clotting in the second tube.

Note : Lee & White method is considered more reliable than Capillary method.

Clot Retraction & Lysis time

Introduction : A clot forms as the end product of blood coagultion. Normal blood clots completely in 5-10 minutes. The clot under normal condition undergoes contraction,where serum is expressed from the clot, and the clot become more denser. Thrombosthenin, a substance produced by platelets is responsible for clot retraction.

Clinical Significance : Poor clot retraction is noted in thrombocytopenia and in poor platelet functions (thromboasthenia)

Normal Value :

Clot Retraction : Clot retraction is reported when less than 50% of clot retraction occurs at the end of one hour at 37^0C.

Lysis Time: Fibrinolysis is considered abnormal when lysis at 37^0C is seen within 24 hours. Normal clot lysis time is about 72 hours.

Requirement :

(i) Sterile Syringe and needle.
(ii) Cotton and spirit
(iii) Test-tubes
(iv) Stopwatch
(v) Water bath

Procedure :

(i) First determine the clotting time by Lee-white method. After the determination of clotting time by Lee-White method, continue to incubate the tubes in the waterbath at 37^0C.
(ii) Examine the clot after 1 hour and after 24 hours.
(iii) Note the size and consistency of the clot and nature of retraction (normal, poor). If the clot has partly left the side wall of the tube, and is about 50% retracted, the retraction is normal.
(iv) After reporting clot morphology and retraction, examine the tube for any lysis. Lysis is recognized by dissolving of the clot, the red cells sinks to the bottom of the tube.

(v) Continue the observation of the clot until 72 hours.
(vi) Report the clot lysis time. Shortened clot lysis time indicates increased fibrinolytic activity.

Blood Collection for coagulation studies : A clean venipuncture is made and 4.5 ml of blood is collected in 0.5 ml of 3.2% Trisodium citrate and the two are mixed (1:9 ratio of anticoagulant and blood). After collection, blood should be placed in refrigerator at 40C till it is centrifuged to collect plasma. Centrifugation should be carried out in a refrigerated centrifuge.

Precautions

1. Venous blood which is collected that should be free of tissue fluid.
2. Sample should not be hemolyzed while collection of blood and while transferring of blood through the needle into the vial.
3. An exact 9:1 ratio should be employed while collecting 4.5 ml of blood in 0.5 ml of sodium citrate solution. Any invariability in ratio of blood and anticoagulant will affect the results of coagulation studies.
4. Glassware should be clean.
5. Siliconized syringes are useful when there is sample collection for research studies and contemplated.

Determination of Prothrombin Time :

Clinical Significance : Prolonged PT suggests the possibility of deficiencies of factor II, V, VII and X. Since excess of coumarin drugs may lead to haemorrhagic conditions, prothrombin time determination is also used to monitor the drug therapy.

Normal Value : 14 $\pm$2 seconds

Requirement :

(i) Water bath
(ii) Stop watch
(iii) Brain thromboplastin
(iv) 0.15 g/dl calcium chloride

Principle : Thrombokinase preparation by Rabit plasma containing calcium ions is added to citrated plasma. In the presence of factor VII, stage II of coagulation mechanism triggered and clot is observed after addition of thrombokinase with calcium ions. The time is recorded as prothrombin time. Thus factors XII, XI, IX and VIII and platelets are bypassed, and test depend on the activity of factor VII, V, X, II and I. Deficiency of factor II, V, VII, X and I may cause prolongation of clot formation in this test.

Procedure :

(i) Place a test tube containing about 2 ml of calcium chloride at 37^0C.
(ii) Dispense 0.1 ml of plasma in a small test tube.
(iii) Add 0.1 ml of brain thromboplastin and mix & wait for 2 minutes.
(iv) Add 0.1 ml of pre-warmed calcium chloride solution, mix and start the stop watch.
(v) Hold the tube in front of a source of light and keep tilting the tube gently. At the first appearance of a fibrin clot, stop the stopwatch and note the time.
(vi) Repeat the steps 1 to 5 twice to check the reliability of results.
(vii) Repeat the procedure by using normal plasma as control.
(viii) Report prothrombin time in seconds.

Calculation for Prothrombin time Index (PTI)

Control clotting time X 100
Pt. clotting time

FNR :

As per WHO each thromboplastin should have ISI (International sensitivity India) value.
Pt. obtained by using different thromboplastin are then compared by calculating INR.
The ratio of the patient P.T. compared to mean PT (normal) raised to the power of ISI is called INR.
Calculation :
P.T. of patient
Mean PT of normal control

Note : INR is used to monitor the effectiveness of oral anticoagulant.
Normal range with Anticoagulant : 2-3 seconds.

Determination of Partial Thromboplastin Time (PTT) :
Introduction: In Partial Thromboplastin Time a phospholipid emulsion is used instead of plasma with varying amount of platelets. Abnormality in PTT indicates defect in stage 1 of clotting mechanism.
Clinical Significance : PTT is prolonged in deficiency of factors I, II, V, VIII, IX, X, XI, XII. It also prolonged in presence of coagulation inhibitors.

Normal Range : 60 to 80 seconds.
Requirement :
(i) Test tubes
(ii) Calcium chloride : 0.025 M
(iii) Waterbath and stop watch
(iv) Chloroform extract of brain (Phospholipids) or Soyabean phosphatide
(v) Control citrated plasma
Procedure :
(i) Dispense 0.2 ml of brain extract in a small test tube.
(ii) Mix 0.2 ml of test plasma. Mix and keep it for 30 seconds in the waterbath.
(iii) Add 0.1 ml of pre-warmed calcium chloride and start stop watch.
(iv) Mix and leave undisturbed for 40 seconds.
(v) Tilt the tube back and forth till fibrin clot appears.
(vi) Note the time.
(vii) Replicate the procedure for control serum.
Determination of Activated Partial Thromboplastin Time with Kaolin :
Introduction : The test reagent used in APTT is the same as PTT. In this testm factor XII which is known as Contact factor is added through some activator e.g. Kaolin.
Clinical Significance : Value of APTT test is increased in deficiencies of factor VIII, IX, XI, V, X and XII. This test is useful in the diagnosis of Hemophilia. This test is performed for the diagnosis of hemophilias that involve the deficiencies of the factors such as VIII, IX, XI, V, X and XII. APTT is increased in the presence of inhibitors of coagulation and in disseminated intravascular coagulation (DIC). It is widely used as the test of choice for the control of heparin therapy. APTT is more reliable than PTT.
Normal Range :35 to 40 seconds
Requirement :
(i) Test tubes
(ii) Water bath and stop watch
(iii) Calcium chloride
(iv) Brain extract in chloroform
(v) Kaolin reagent : 2.0 g/dl in normal saline

Principle : Partial thromboplastin (brain extract in chloroform) is incubated with kaolin. The Kaolin act as control factor. After the addition of calcium ions, clotting time of plasma is observed & this is known as Activated Partial Thromboplastin Time (APTT).

Procedure :

(i) Transfer 0.1 ml of brain extract, 0.1 ml of kaolin suspensin in a glass test tube which is placed in a waterbath (37^0C).

(ii) Add 0.2 ml of test plasma.

(iii) Incubate at 37^0C for one minute.

(iv) Add 0.1 ml of calcium chloride and start the stop watch.

(v) Mix and leave the tube undisturbed for 20 seconds.

(vi) After 20 seconds observe the formation of clot by tilting the tube.

(vii) At the appearance of the strands, stop the stopwatch and note the time.

(viii) Repeat the procedure by using a normal control plasma.

Hess test (Capillary resistance test)

Introduction : In this test, we observe capillary resistance with certain pressure. It may positive in thrombocytopenia, defective PLTs function, thromboasthenia, vascular purpura and scurvy.

Procedure :

(i) Apply sphygmomanometer cuff at upper arm to 80 mm Hg for 5 minutes.

(ii) Release the inflation after 5 mintues. Patient is asked to lift the arm to shoulder height.

(iii) Gently tap the area below antecubital fossa with hand.

(iv) Examine the flexor aspect of forearm (below the elbow) in an area of 5 cm in diameter.

(v) There are less than 5 petechiae present in a normal individual.

Unit 2

Bone Marrow Examination

Defintion :

Bone marrow is a cellulovascualr tissue occurring the space between the trabeculae of bone in the marrow cavity, which because it contains mostly blood, haematopoietic cells, and fat, is a soft tissue. It is one of largest organs in the body, the principle site of all blood forming cells.

After birth, haematopietic cels fill all cavities of bone of newborn but with increased age it becomes localized in cavities of upper shafts of femur, humerus and pelvis, spine and bones of thorax.

The marrow lies entirely within bone and its ability to expand is limited. The total volume of bone marrow or haemopoietic tissue in an adult is 1-2 litre.

Distribution (in adults)

Principle site	Marrow weight

	1 g of Red marrow	% of total
1. Cranium & mandible	137	13
2. Humeri; scapulae; clavicle	87	8
3. Sternum & ribs	106	10
4. Vertebrae	300	28
5. Pelvic bones	379	34
6. Femur	40	4

Types : Bone marrow are of two types.

a) Red bone marrow
b) Yellow bone marrow

However, white marrow may occur as a manifestation of extreme atrophy or starvation, its dominant elements being stromal cells with a gelatinous intercelluarl materials.

a) Red bone marrow : It is an active marrow producing blood cells (i.e. myeloid, erythroid and megakaryotic series cells). That's why it is also called blood forming/ haemopoietic marrow. The rich circulation and erythropoiesis imparts a red colour to it.
   During the first few years of life, all bones have red bone marrow and are celluar. By the forth year, significant no. of fat cells in teh diaphysis of the long bones starts slowly to replace the haemopoietic elements and expand centripetally until at about the age of 18, haemopoietic marrow is found only in the vertebrae, ribs, skull, pelvis and proximal epiphysis of the femur and humeri. This persists throughout life. Red bone marrow contains an abundance of haemopoietic cells along with fat cells and connective tissues.
b) Yellow bone marrow : It is an inactive marrow and prepondencae of fat cells give some areas of the marrow a yellow colour. It is made up of adipose tissue, a little no of capillaries and reticulum cells. By the age of 18 in human beings, virtually all the marrow of the limbs is yellow. Though they are not capable of forming cells, this marrow can be converted into active form in cells of abnormal demand.

Importance of Bone Marrow Examination :

1. Bone marrow examination is done for teh diagnostic purposes and for studying the progression of a disease & the effects of therapy.
2. Bone marrow should be examined when teh clinical history, routine laboraotyr test results or blood films suggests the possibility of a primary or secondary haematological disorders for which morphologic analysis or special studies of the marrow aid in the diagnosis.
3. If the findings on routine blood film do not give the expected result to match the clinical history then bone marrow examination may be the only way to wake correct diagnosis.
4. The marrow examination provides a semi-quantitative and qualitative assessment of the state of haematopoiesis and the normalacy of the blood cell precursors of all lineages.
5. It can provide the diagnosis of several hereditary and acquired benign and malignant disease.
6. It is an important test to assess teh response to treatemtn of the leukemias and some lymphomas.
7. It can be useful in assessing the state of iron stores and of metabolic disease that affect macrophages, such as Gaucher disease.
8. It is a quick adn safe procedure in which teh marrow may be obtained without significant risk and with only minor discomfort and is quickly and easily processed for examination.
9. The primary diagnosis of hematologic malignancies and many non neoplastic hematologic disorders relies on visual examinations of the marrow.

History

Arinkin first proposed the use of needle aspiration of the bone marrow in 1929 and since that time, neelde aspiration have gained preference over open surgical biopsy.

Clinical Significance and Indications

Diagnosis of

- Anemia e.g. Aplastic, Sideroblastic anemia.
- Leukemia e.g. AML, ALL
- Thrombocytopenia
- Immunoglobulin disorders
- Prior to splenectomy to determine functional state of bone marrow.
- Following progress of therapy in anemias, leukeias etc.
- Miscellaneous conditions e.g.
    - Gaucher's diseae
    - Kala azar adn some time in MP
    - Infections mononucleosis (Little significance)
    - Polycythemia vera

Advantages	Disadvantages
- It is simple and safe. - It is relatively painless. - It can be repeated a number of times. - It seems to be safe even in the case of ITP. - It is free of hazards.	- The arrangements of cells with in the marrow and the relationship between one cell and another are more or less destroyed by the process of aspiration. - In fibrotic marrows, little blood is also aspirated.

Structure and Composition of Bone Marrow

Bone marrow consists of

1. Reticulum cells and reticulin fibres : Reticulum cells along with their numerous cell processes and argentophilic fibres forms a meshwork of supporting framework for other marrow elements. They make up teh scaffolding in which haematopoietic cells and arranged in teh marrow cords and forms a transport system for haematopoietic cells.
   Reticulum cells plays an important role in the delivery of cells to the circulation.
   They can accumulate fat to become teh adipocytes, predominant, constituents of yellow marrow. They contain iron and alkaline phosphatise. The normal bone marrow comprises with about 1% reticulum cells but are increased in certain pathological conditions such as myelosclerosis.
2. Vessels and Nerves : Marrow arterioles empty into complex network of venous sinusoids which in turn drain into a central vein. Venous sinusoids are lined by endothelial cells. There are gaps in the basement membrane through which cells can pass. There are inter-endothelial gaps through which haemopoietic cells pass from haemopoietic islands into the sinusoids and thence into the circulation. Sensory nerves are also present in marrow whose presence has been shown during suction pain on marrow aspiration.
3. Haemopoietic Islands : The haematopoietic tissue in marrow lies between the venous sinuses as a branching system of cords. They give rise to teh precursors of the RBCs, WBCs adn platelets which lies freely between the reticulin fibres.
4. Fat cells : The fat cells are considered to be reticulum cells with stored fat. The fat of the marrow is amongst the most labile tissues of the body and in case of sudden need it can be replaced by active haemopoietic tissue in a short time. Fat also acts as a sort of buffer.

5. Stromal cells : Stromal cells and accessory cells are needed for optimum haematopoietic cells development.
6. Mast cells : Mast cells are readily recognized by their content of dark-blue granules, which usually completely fill the cytoplasm and may obscure the nucleus. These cells are round or spindle shaped and are often located deep in the particles, frequently lying along blood vessels. The nucleus is often not visible, but when seen, it is round or oval with a vesicular chromatin pattern.
7. Bone cells : Osteoblasts are usually oval cells up to 30 □m in the longest diameter. They often occur in groups. The nucleus is usually eccentric and is relatively small. The chromatin pattern is uniform and there are 1-3 nucleoli. The cytoplasm is light blue and may contain a few red granules.
   Osteoclasts are large cells and may be more than 100 □m in diameter. They superficially resemble megakaryocytes but contain multiple separated nuclei which have a moderately fine chromatin pattern with nucleoli. The cytoplasm varies from slightly basophilic to intensely eosinophilic due to the content of eosinophilic granules. Osteoclasts may contain coarse basophilic debris.

Function of Bone Marrow

a) Formation of blood cells : Functionally the marrow is a most active organ, constantly producing an enormous number of RBCs, WBCs adn platelets to maintain the demand of these cells by the body. It has been estimated that to maintain the normal red cells population of the peripheral blood, the marrow produces about 2.1 X 10^{11} red cells per day.
   Bone marrow produces about 6 billions cells per kg per day.
b) Marrow as reservoir : Not only is the normal marrow able to constantly maintain the number of cells, but it has a large reserve capacity which enables it to significantly increase output in response to increased demands.
   May be able to increase as much as 13 times normal.
c) Destruction of old and degenerated RBC by virtue of R.E. cells present in the bone marrow.
d) R.E. system : Bone marrow is rich in R.E. cells and serves all other important functions of R.E. system such as mainly defence, destruction mechanism and also removal of unwanted particles.
e) Immune system : Production of plasma cells (for the production of antibody), lymphocytes, macrophages by bone marrow help in immune response of body.
f) Osteogenic function : The cellular elements which take part in the formation of bone are formed in the marrow. The osteoclast, osteoblast, osteocyte, endosteum blood vessels are found within the marrow.
g) Connective tissue function : Due to its different connective tissue contents, the bone marrow performs several functions associated with the connective tissues.

Aspiration of Bone Marrow

Requirements :

- Bone marrow puncture needle : There are a number of needles, each marginally different, designed for marrow puncture. They include :

Salah and Klime Needles	Island Needle	Modified Islam	A Jamshidi, modified Illinois sternal-iliac bone marrow aspirated needle
- Stout needle - Made up of hard stainless steel - Length of about 7-8 cm - Provided with well-	- Slightly larger needle. - T-bar handle at the proximal end. - Provide a better grip. - More manoeuverable	- Has multiple holes in the distal portion of the shaft in addition to opening at the tip, in order to overcome sampling errow when	- Satisfactory for use in percutaneous aspiration of the marrow.

fitting stylete. - Provided with adjustable guard. - Bore of 18-20 gauze.	- More successful for excessively hard (e.g. osteosclerosis) or soft (profound of bone osteoporosis)	the marrow is not uniformly involved in a pathological lesion.	

- Disinfectants :
  (a) 70% alchol (e.g. ethanol) or
  (b) 0.5% chlorhexidine (5% diluted 1 in 10 in ether)
  (c) Tincture of Iodine.
- Local Anaesthesia :
  (a) 2% Xylocaine or
  (b) 2% Lignocaine
- Clean glass slides and anticoagulated vials. (Trisodium citrate)
- Fixative – Absolute methanol.

Sites of Bone marrow aspiration

1) In adults
   a) Sternum
   b) Spines of lumbar vertebrae.
   c) Iliac crest
2) In infants
   a) Tibial tubercle

Methods of Bone Marrow Aspiration

Sternum Puncture :

Site : It is the maubrium or the first or second pieces of the body of the sternum (second intercostals space). Lower third of sternum is unsatisfactory because congenital abnormalities in this region are common.

The site for puncture of the manubrium should be about 1 cm. above the sternomanubrial angle & slightly to one side of the mid-line, if the body of the bone is to punctured, this should be done opposite the second intercostals space slightly to one side of the mid-line.

Procedure :

1. The procedure is performed under sterile condition after washing and shaving the skin to be punctured.
2. Ask the patient to lie on his back.
3. Disinfect the area with the disinfectant.
4. Infiltrate the skin, subcutaneous tissue and periosterum overlying the selected site with local anaesthesia.
5. Apply sterile cotton or gauze piece closely around the selected site of aspiration.
6. Puncture the site with appropriate needle, salan and Klima mostly preferred piercing the skin and subcutaneous tissues by rotation.
7. When the needle point reaches the periosteum, adjust the guard on a needle to allow it to penetrate for about 5 mm further. Then push the needle with a boring motion into the cavity of the bone.
8. Remove the stiletto, attach a 2 or 5 ml syringe and aspirate 0.2-0.5 ml of bone marrow by employing a negative pressure. If no manual is obtained the needle should be rotaed to adjusted slightly and suction again should be applied.
9. Make films from some of the aspirate without delay. Any excess, blood on the slide can be removed by careful blotting with filter paper without touching the particles.
10. Some material can be kept in anticoagulated vial or fixed in methanol according to the necessity.

11. Sterile gauze is kept over the puncture site after the withdrawal of needle and patient is asked to aply sustained pressure over the area and to take enough rest.

Iliac Puncture :

Site : The usual site for puncture is the posterior iliac crest.

Procedure :

1. The patient is asked to lie down in prone position or sidewise as for a lumbar puncture (left or right lateral decubitus position)
2. Clean the area by washing and shaving.
3. Disinfect the area with disinfectant.
4. Infiltrate the skin, subcutaneous tissue and periosteum overlying the selected site with local anaesthetic (not more than 20 ml of 1% lidocaine)
5. Insert the marrow needle through the skin, subcutaneous tissue and cortex of the bone with a slight twisting motion.
   When the superior iliac crest is punctured the needle is introduced 1-2 inches posterior to the anterior superior iliac spine, and the needle is directed into the plane of the iliac spine, entering the trabecular bone. The posterior superior iliac spine is entered vertically into this ovoid protuberance.
6. Penetration of the cortex can be sensed by a slight, rapid forward movement accompanied by a sudden increase in the case of advancing the needle.
7. The stylet of the needle is removed promptly. Attach a 10-20 ml syringe and aspirate 0.2-0.5 ml of fluid by employing a negative pressure.
8. When aspirate is complete, the stylet is reinserted and the needle is removed from the bone immediately.
9. Pressure is applied to the skin over the aspiration site for at least 5 mins to minimise bruising at the site. In thrombocytopenia patient, firm pressure should be applied for at least 10-15 min.

Spine of Lumbar Vertebrae puncture :

Site : The spinous process of the vertebrae, the ribs, other marrow containing bones. Good samples of marrow may be obtained from adults by puncturing the spines of lumber vertebrae.

Procedure :

1. The procedure is performed under sterile condition after washing and shaving the skin to be punctured.
2. The patient is asked to lie on the side as for a lumbar puncture (left or right lateral decubitus position) or sitting up.
3. Disinfect the area with the disinfectant.
4. Infiltrate the skin, subcutaneous tissue & periosteium overlying the selected site with local anesthetic.
5. Insert the marrow needle through the skin, subcutaneous tissue and cortex of the bone with a slight twisting motion.
   Pass the needle into the spine of a lumbar vertebrae slightly lateral to the mid line in a direction at eight angles to the skin surface.
6. The stylet of the needle is removed promptly. Attach a 10-20 ml syringe and aspirate 0.2-0.5 ml of fluid by employing a negative pressure.
7. When aspirate is complete, the stylet is reinserted and the needle is removed from the bone immediately.
8. Pressure is applied to the skin over the aspiration site for at least 5 mins to minimise bruising at the site. In thrombocytopenia patient, firm pressure should be applied for at least 10-15 min.

Tibial Puncture :

Site : In small babies (< 2 years) the medial aspect of the upper end of the tibia just below the level of tibial tubercle is the active site for puncture.
In older children, the tibial cortical bone is usually too dense and marrow within is normally less active.
Appearance of aspirated materials : The bloody fluid that is aspirated contains light coloured particles of marrow containing bony or fatty pieces (spicules) from 0.5-1 mm in diameter. They are often readily visible in the syringe but may not be detected until the syringe contents are discharged on a glass slide to prepare films.

Appearance of Aspirated Material
The blood fluid that is aspirated contains light coloured particles of marrow containing bony or fatty pieces (spicules) from 0.1-1 mm in diameter. They are often readily visible in the syringe but may not be detected until the syringe contents are discharged on a glass slide to prepare films.

Preparation of Smears :
Several types of preparation can be made from the marrow aspirate to make maximal use of the diagnostic material. The important ones are :

1. Direct smears
2. Concentrate films
3. Imprint smears
4. Aspirated bone marrow smears

1. Direct smears : They are made immediately from the aspirate. This is the best preparation for evaluation of cellular morphology and differential counts of the marrw. This particular film is best for estimation of marrow cellularity and megakaryocytes abundance, but morphology is observed in the thicker parts of the films. Directs smeas can be
   a. Thin marrow film
   b. Particle film
   a. Thin marrow film :
   (i) Deliver a drop of unanticoagulated marrow pool aspirate on to a clean glass slide about 1 cm from one end.
   (ii) Quickly suck off most of the blood with a fine pateur pipette applied to the edge of the each drops or with the help of blotting paper.
   b. Particle Film : They provide a large no. of particles from which cellularity of the marrow may be estimated and which are useful for estimation of the amount of hemosiderin present.

2. Concentrate films : They are prepared from the buffy coat of the marrow.

Procedure :

a) An aliquot of the EDTA-anticoagulated sample is centrifuged at 1500 g for 10 min in a wintrobe tube to concentrate the cellular elements of the marrow.
b) After centrifugation, the fatty layer & plasma are removed and is buffy coat is mixed with an equal volume of plasma.
c) Multiple film are made of this preparation.

3. Imprint Smear : It is useful to select one or two particles of marrow from the aspirated materials and to make several rows of imprints on clean slides.

Procedure :

a) One or two glass slides are gently touched in several places by the exposed marrow at the surface of the specimen.
b) Dry the imprint smear in air.
c) Fix the smear in methanol.

d) Stain as per required.

4. Aspirated bone marrow sections

Fixative used :

- 50% ethanol in 15% formalin.
- Absolute ethanol – 50 ml
- 15% formalin (!5ml/l of 40% HcHO) – 50 ml
- Specific gravity = 0.93 (similar to that of body fats(

When a marrow aspirate is added to this fixative, the blood remains in suspension while the marrow particles rapidly sediments even fatty marrow settles down in a few seconds.

Procedure :

a) Add 0.25 ml of bone marrow aspirate to 20 ml of fixative taken in a tube and mix thoroughly.
b) Allow it to fix overnight at room temperature.
c) Resuspend the sediment by several inversion.
d) Allow them to settle at the bottom.
e) With a Pasteur pipette, pick out the coarses marrow fragments.
f) Transfer them to a round bottom test tube containing 70% ethanol.
g) Leave it as it is for at least 15 mins.
h) Dehydrate with two changes of absolute ethanol leaving the peptidase for 1 hour in each.
i) Drain off the ethanol and clear with toluene for 1 hour.
j) Decant off the toluene and replace it by a toluene-paraffin wax mixture and then impregnate embedded by two changes of paraffin wax.
k) Free the block, by breaking the tube when the wax has cooled and hardened.
l) The marrow fragments will have settled as a small mass at the bottom of the block.
m) Little or no trimming will be required.
n) Cut sections of 4-5 □m thickness, the thinner the better.
o) Stain the smear as required.

Staining of Bone marrow smear

May-Grunwald-Giemsa's stain : It stains well films of bone marrow and aspirated material. Its principal is based on Romanowsky stain.

Composition :

May-Grunwald's stain :

May-Grunwald powder : 0.3 g

Acetone-free methyl alcohol : 100 ml

Dissolve the dye in 100 ml methanol warming at 50^0C for 10 minutes, shaking time to time. Filter after 24 hours.

Procedrue :

1. Firstly, fix the smear in methanol for 1-2 minute.
2. Now stain the film with dilute dye solution (1:10) for 5 minutes.
3. Now the stain the film with dilute Giemsa's stain for 10-15 minutes.
4. Wash with buffered water and dry in the air.

Examination of Smear : Marrow smears and screened first under 10^x objective (scanner) to look for the presence of marrow particles. Majority of marrow particle are present in the tail of smear and trails made by these particles provide enough cells to study their morphology.

1. Cellularity :

- Children marrow is highly cellular with less than 25% fat.
- In adults fat constitute 40-50%.

- Marrow is reported hypercellular, normocellular or hypocellular or assessed by 10^x objective.

Normal range for DLC in Bone Marrow Smear :

Undifferentiated blast cells	0.1-2%
Myeloblast	0.1-1%
Promyelocytes	0.5-5%
Myelocytes (a) Neutrophil (b) Eosinophil (c) Basophil	 5-20% 0.1-3% 0-0.5%
Metamyelocytes	10-30%
Granulocytes (a) Neutrophil (b) Eosinophil (c) Basophil	 7-25% 0.2-3% 0-0.5%
Lymphocytes	5-20%
Monocytes	0-0.2%
Megakaryocytes	0.1-0.5%
Plasma cells	0.1-3.5%
Proerythroblasts	0.5-5%
Normoblast	2-20%
Myeloid/erythroid ratio	2.5-5.0%

2. M.E. ratio : At least, 500 marrow cells are counted and percentage of different cells calculated. Normal M.E. ratio is 3:1 to 15:1.
3. Erythropoiesis : In bone marrow examination, erythroid cells are examined for maturation, dyserythropoiesis and type of reaction – normoblastic/ megaloblastic/ micronormoblastic.
4. Myelopoiesis : Myeloid series cells are evaluated for any maturation arrest, granulation – lack of it/ hypergranulation, number of blast cells, degenerative changes like vacuolations in cytoplasm of myeloid cells.
5. Megakaryopoiesis : During bone marrow examination, megakaryocyes are examined in the marrow particles and in the cell trails. Apart from number of megakaryocyes, it is important to assess budding of platelets from megakaryocytes and also relative number of promegakaryocytes to megakaryocyte.
6. Other cells and Abnormal cells : Smears should be examined for other than haemopoietic cells i.e. reticulum cells, plasma cells, metastatic tumor cells, any parasite and fungus.
7. Iron store : Iron store can be assessed by Perl's stain.

Perl's Staining reaction (Prussian blue reaction)

Introduction : After absorption from small intestine iron is carried to the site of iron storage. The main site is bone marrow. In bone marrow, iron is present in its ferric form, as Hemosiderin, in the R.E. cells and free in the marrw.

Clinical Significance : Iron store are increased in

- Haemolytic anaemias
- Anaemia of chronic renal failure
- Multitransfused patients e.g. in thalassemia, aplastic anaemia (haemosiderosis)
- Megaloblastic anaemia
- Sideroblastic anaemia

Principle : The ferritin or Haemosiderin (Iron containing compounds) reacts with potassium ferrocyanide in acid solution and yield a Prussian blue color.

Reagent :

1. 4% HCl w/v
2. 4% Potassium ferrocynide.
3. Basic Fuchsin solution as counter stain

- Stock solution

  Basic fuchsin – 1gm
  Absolute alcohol – 10 ml
  5% Ag. Phenol solution – 90 ml
  Working solution - 3 ml stock solution in 100 ml D/w.

4. Formalin
5. Absolute alcohol

Requirements :

- Staining jars
- Microscope
- Clean slides with coverslips.

Procedure :

1. Firstly dry the bone marrow film with marrow particles.
2. Now fix it for 10 minutes in formalin.
3. Now mix equal amount of 4% hydrochloric acid and 4% potassium ferrocyanide solution in a staining jar.
4. Now dispense the smear into the freshly prepared solution and wait for 10 minutes.
5. Rinse well in tap water.
6. Immerse in dilute fuchsin for 5 minutes to counterstain.
7. Wash with tap water, rinse in absolute ethyl alcohol and wash in tap water.
8. Now air dry.
9. Examine under high dry objectis and select the portio of the film where the marrow cells are well separated. After this change to oil-immersion.

Examination :

1. Examine the Sideroblast which are nucleated RBC – contain one or more small blue granules.
2. Macrophage : Spherical, irregular shaped finely granular particles.
3. Reticulin cells – Haemosiderin granules are present in reticulin cells in marrow fragments.

Grading of Iron Stores on Bone marrow aspiration staining smear :

	Grading of Iron stores on the BMA	Interpretation
0	No iron granules seen	Iron deficiency
1	Small granules in reticulin cells only under oil-immersion	Diminished iron stores.
2	Few small granules visible with low power lens	Normal iron stores
3	Numerous small granules in all marrow particles	Normal iron stores
4	Large granules in small clumps	Increased iron stores
5	Dense large clumps of granules	Increased iron stores
6	Very large deposits obscuring the	Increased iron stores

	marrow cells	

Dry Tap : When only blood and no marrow material are obtained on aspiration, then it is called dry tap. Conditions in which dry tap is frequent are :

- Aplastic anaemia
- Myelofibrosis
- Very hypercellular marrow in leukaemia
- Faulty technique

Bone Marrow Biopsy (Trephine Biopsy)

When apriation of the bone marrow does not provide adequate material (dry tap) or in those clinical situations in which a biopsy is expected to provide additional information, a biopsy is performed.

A special biopsy called the Trephine biopsy (Trephine = A surgical instrument used to remove a circular area of tissue usually from bone)

Indications

1. Red cell disorders : Aplastic anemia
2. Myelofibrosis
3. White cell disorders : Hypoplastic acute leukemia.
4. Metastasis in cancer cases : Marrow positive for metastatic deposit indicates disseminated malignancy i.e. stage IV.
5. Involvement of bone marrow by lymphoma.
6. Study of bone morphology in chronic renal failure, osteoporosis and osteomalacia.
7. Myeloproliferative disorders for studying reticulin and the proliferative cell lines for typing.
8. Pre and post bone marrow transplantation in acute leukemia, CML and multiple myeloma.
9. PUO – for studying tuberculous granuloma.
10. Myelodysplastic syndrome.

Site of trephining

1. Posterior superior iliac spine is the preferable site.
2. Anterior superior iliac spine.
3. Spinous process of vertebra.

Jamshidi needle, wester man-Jensen needle and Islam needle is used for this procedure. The core of bone marrow is obtained in this technique. Smears made by imprint smear method. Biopsy material can be processed by paraffin section technique in histopathology after decalcification. Following staining technique can be used as per requirement

- H and E stain
- Reticulin stain
- Masson's trichrome stain
- Immunocytochemical stain for acute leukemia.

Unit 3
Leukemia

The name Leukemia was proposed and diagnosed as a distinguished & a separate clinical entity by CRAIGE, BENNET a Dvirchow (1845).
It can be defined as abnormal, uncontrolled proliferation of haemopoietic cells causing progressive infiltration of bone marrow, although in certain forms the lymphatic tissues are particularly affected.

Leukaemia is generally considered to be neoplastic disorder originating in a haemopoietic cell which has undergone an intrinsic change, causing it to escape from the normal restraints imposed on proliferative activity.
Still today, despite all our advancements in the medial field no theory of etiology of leukemia has so far received general recognition. The most commonly proposed cause are any chemical reaction, virus, ionizing radiation theory, inheritance etc.

Classification of Leukaemia

Leukemia

Acute Leukaemia Chronic Leukaemia

AML ALL CML CLL

Presenting Manifestation of Acute Leukemia

Common	Less common	Occasional
Anaemia Fever, malaise Haemorrhage, bruising, petechiae	Infection of the mouth and pharynx Pains in bones and joints (childhood especially) Upper respiratory tract infection (childhood especially) Superficial lymph node enlargement (Childhood especially) (More common in ALL)	Abdominal pain Mediastinal obstruction (childhood especially) Nervous system abnormalities Skin rash Gum hypertrophy

Acute Myeloid Leukaemia
This is found in children & teenagers. Patient got complication i.e. fever, body pain, weakness, loss of weight, bleeding from nose and gums, tenderness on sternum etc. There is hepatomegaly and Spleenomegaly on examination. AML is usually more dangerous than CML.

Laboratory Diagnosis :

1. Haematological Investigation

- RBC count decreased.
- Platelets is decreased.
- TLC is increased. (App. 1 lac/cumm^3)
- ESR is increased.
- Blood picture : PBF shows Anisocytosis, Piokilocytosis with mild polychromasia, blast cells seen (Myeloblast) is 50-80%. Other cells of Myeloblastic series i.e. Myelocyte, Metamyelocyte, Promyelocyte etc. Are seen. Myeloblast contains auer rods and blast cells and 3-5 nucleoli. Other cells are mature polymorphs and few lymphocytes are seen.

2. Cytochemical reaction :

Peroxidase reaction	-	Positive
Suddan black reaction	-	Positive
Acid phosphatise reaction	-	Negative

3. Markers

CD 19 (Pan B)	-	Negative
CD 7 (Pan T)	-	Negative
CD 13	-	Positive
CD 33	-	Positive

4. Bone marrow changes :

- Hypercellularity in some cases there is hypocellularity.
- Majority of Myeloid series cells are Myeloblasts.
- Immature granulocytes, erythroblasts, moderately increase in plasma cells and megaloblastic erythroblasts, ringed sideroblasts are present.
- Megakaryocytes are reduced in number.

5. Cytochemistry

Myeloperoxidase reaction	-	Positive
Suddan Black reaction	-	Positive
PAS reaction	-	Positive

Acute Lymphoid Leukemia

This is found mostly in children. Patient got complication i.e. pain in body, fever, weakness, bleeding etc. There is hepatomegaly and spleenomegaly on examination. Lymphnodes are enlarged.

Lab. Diagnosis of Acute lymphoid leukemia

1. Haematological Investigation

- RBC count is decreased
- Platelets are decreased.
- ESR is increased.
- WBCs count is increased (50000-100,000/cumm^3)
- Some time WBC count decreased
- Blood picture – Anisocytosis, Piokilocytosis, More than 50% lymphoblast are seen. Lymphoblast are round, small cells with scanty homogenouse cytoplasm. Granules are present in cytoplasm. Nucleus show one or two nucleoli. Granulocytic series cell are few only. Mature lymphocytes are also seen.

2. Cytochemcial Reaction

PAS reaction	-	Negative
Suddan Black reaction	-	Negative
Acid Phosphatase reaction	-	Positive

3. Markers

CD 19	-	Positive

CD 7 - Positive
CD 13 - Negative
CD 33 - Negative

4. Bone marrow Changes :
   (a) Erythropoietic cells are reduced. Dyserythropoiesis, megaloblastic features and ring sideroblasts are commonly present.
   (b) Megakaryocytes are reduced or absent.
   (c) The blast cells are lymphoblasts which are earliest identifiable precursor of lymphoid cells. These are large cells 10-18 micrometers in diameter, containing large round or oval or indented nuclei. The nuclei are slightly clumped or stippled with nuclear chromatin. The nuclear membrane is denser and the numbers of nucleoli are fewer (1-2 only). The cytoplasm is scanty, basophilic non-granular. There should be at least 30% blast cell in the bone marrow for diagnosis of leukaemia, as per FAB classification. According to WHO, 20% blast cell in marrow is diagnostic.
   (d) Blast cell predominance : If the blasts are present in adequate numbers in the peripheral blood then the marrow study is superfluous. But in evaluating the course of therapy, marrow examination is necessary as the blasts disappear from the peripheral blood before the marrow in response to treatment.

Difference between AML and ALL

	Acute Lymphoid Leukaemia (ALL)	Acute Myeloid Leukaemia (AML)
Cell morphology	- Scanty cytoplasm - No granules in the cytoplasm - Auer rods in the cytoplasm not seen. - Chromatin of nucleus rather compact and indistinct nucleoli only 2 or < 2	- There is moderate amount of the cytoplasm - Few granules in the cytoplasm - Auer rods in the cytoplasm seen. - Chromatin in nucleus is faint, sieve-like appearance, nucleoli are > 2, usually 3-5
Cytochemically	Myeloperoxidase – negative Sudan Black – negative PAS –positive Non-specific esterase – negative Acid phosphatase – positive	Myeloperoxidase – positive Sudan black – positive PAS – Negative Non-specific esterase – Positive Acid phosphatase – Negative
Enzyme test	TdT- Positive	TdT- Negative
Speical marker	CD2, CD5, CD7, CD 10- positive	CD13, CD14, CD15, CD33-positive
Surface immunoglobulin	Positive	Negative
Serum lysozyme	Negative	Positive

Chronic Leukaemia

Chronic Myeloid Leukaemia

This is found in people in between 20-50 years age. Patient got complication i.e. weakness, fever, tenderness on sternum, loss of weight & bleeding. There is hepatomegaly and spleenomegaly on examination.

Presenting manifestation of CML

Common	Moderately common	Occasional
Anaemia Splenomegaly Fatigue Weight loss	Night sweats Minor bruising	Joint pain Bone pain Amenorrhoea

Laboratory Diagnosis

1. Haematological Investigation

- Hb is decreased.
- RBC is decreased.
- ESR is increased.
- Platelets is in normal range or high
- WBC count is high (50,000 $cumm^2$) after some time App. 30,000 $cumm^3$
- Blood picture : There is marked increase in the granulocytes with a left shift in differentiation including increase in the myeloid series cells like promyelocytes, myelocytes and metamyelocytes.
    - Granulocytes = 20-50% or metamyelocytes = 20-505
    - Promyelocytes = 4% may be 20% if the leucocyte count is high.
    - Myeloblast = 2% may be 12% if the leucocyte count is high.

2. Chromosomal findings :
   Philadelphia chromosome abnormality present in – 90% cases of CML.
3. Bone marrow changes :

- Marrow is like a packed marrow with little fat space left, expansion of parenchymal marrow beyond the normal extent.
- Myeloid cells predominate, increased myeloid-erythroid ratio. The differential blood cell count shows predominance of myelocytes and type of cells that is predominantly present in the peripheral blood smear.
- Megakaryocytes are smaller in size.
- The reduction of erythropoietic cells with relative increase of normoblasts.
- Neutrophil alkaline phosphatise (NAP) scores are reduced.
- Cytogenetics of bone marrow- The presence of Ph chromosomes, t (9;22) in 70-80% of cases of CML.
- The proportion of immature cells to mature neutrophil is higher in the bone marrow than the peripheral blood picture.

Chronic Lymphocytic Leukaemia

This is found in people in between 50-60 years age. Patient feels weakness. Fever and recurrent infection can occur. There is tenderness on sternum. There is hepatomegaly and spleenomegaly on examination. Lymphnodes are enlarged.

Classification of CLL

- Common B cell type
- Rare T cell type
- Hairy Cell leukaemia (B-type)
- Prolymphocytic leukaemia

Presenting Manifestation of CLL

Common	Occasional
Lymph nodes enlargement Anaemia	Predisposition to infection Haemorrhagic manifestation

Accidental discovery on clinical or haematological examination	Acquired haemolytic anaemia Splenomegaly Gastrointestinal symptoms Skin infiltration Nervoys system manifestations Bone or joint pains Mediastinal pressure or obstruction

Laboratory Diagnosis of Common B cell type CLL

1. Haematological investigation

- TLC is increased – 20000 to 40000/cumm^3
- RBC is decreased
- Hb is decreased
- ESR is increased
- Platelets is 1to 1.5 lacs/cumm^3
- Blood picture : PBF shows 85-90% mature lymphocytes, lymphoblast are present rarely. Granulocytes are few. There may be presence of some larger cells with detectable nucleolus (Pro lymphocyte). Smudge cells are numersous in PBF.

2. Chromosomal abnormality

Trisomy 12 is teh most common chromosomal abnormality associated with CLL.

3. Bone Marrow changes :

- Cellularity – hypercellular : Cellularity is increased because of infiltration of marrow by mature lymphocytes.
- Myelopoiesis : On bone marrow examination, myeloid cells are normal in number and their maturation is in initial stages.
- Erythropoiesis : Erythropoiesis is normal and their reaction is normoblastic.
- Megakaryopoiesis : Megakaryopoiesis is normal in initial phases.
- Lymphocytic infiltrate : > 30% of the nucleated cells of marrow are mature lymphocytes and is the diagnostic feature of CLL marrow.

These cells are positive for Pan B-cell markers i.e. CD 19, CD20, CD21, CD5 and CD23 positivity are distinctive for CLL.

Lab. Diagnosis of Prolymphocytic Leukaemia

1. Haematological investigation

- TLC is increased
- HB is decreased
- Platelets coutn is decreased
- Blood picture : More than 55% is promyelocytes.

2. Bone Marrow : Marros is hypercellular with infiltration by prolymphocytes.

Lab. Diagnosis of Hairy cell Leukaemia

Haematological Investigations

- TLC range is 3000 to 4000 /cumm^3
- Total RBC count is decreased
- Platelets count is decreased.
- HB is decreased.
- Blood picture : PBF shows 10-50% abnormal hairy cell, 30-505 neutrophil, lymphocytes – 35-55%, few are other cells. Monocytes and eosinophils usually in normal range.

Hairy cell :

- Medium sized cell -10-20 □m
- Round to oval nucleus though many are notched or dumb bell shaped.
- Chromatin pattern is uniformly reticular.
- Chromatin part is more condensed in some cells resembling the lymphocytes.
- Cytoplasm is moderately stains grey blue.
- Cytoplasm exhibit irregular villi resulting in an irregular scarred hairy edge and also pseudopondial extension.
- Cytochemically acid phosphate present in hairy cell.

Bone marrow Changes

- Dry tap is common because of myelofibrosis, hypocellular marrow and biopsy is often needed to study the marrow picture.
- The chromosomal mononuclear cells show hairy cytoplasmic projections (Best recognized under phase contrast microscope)
- Tartrate-resistant acid phosphatase (TRAP) reaction is positive for these cells.
- These cells may show cell surface immunoglobulins and positivity for sheep erythrocyte rosette test indicating their B cell origin.
- Pan B cell markers CD 19 and CD 20 and the monocyte-associated antigen CD11c and plasma cell-associated antigen is positive but normal B cells are not positive for this cluster of antigens.

FAB Classification (French American British Classification)

Type	Characteristics	Percentage	Typical Proein Marker
MI AML without maturation Cell type : Myeloblast	Myeloblasts with non-granular cytoplasm or rare azurophil granules/Auer rods; more mature myeloid cells not present; some blast cells myeloperoxidase positive	20%	CD 34, 33, 13
M2 AML with maturation Cell type : Myeloblasts, promyelocytes	Promyelocytes and more mature myeloid cells in addition to myeloblasts; myeloid cells myeloperoxidase positive.	25%	CD 34, 33, 15, 13
M3 Acute promyelocytic leukaemia Cell type : Hypergranular promyelocytes	Most cells myeloperoxidase-positive promyelocytes with heavy cytoplastic granulation and reniform nuclei; multiple auer rods, often in parallel bundles (‘faggots’); also rare hypogranular type.	10%	CD 33, 13 (HLA-Dr-)
M4 Acute myelomonocytic leukaemia Cell type : Monocytoid cells	Immature and mature cells of both myeloid (myeloperoxidase positive) and monocytic (non-specific esterase positive) lineage.	20%	CD 34, 33, 15, 14, 13
M5 Acute monocytic leukaemia Cell type : M5a : Monoblasts	Poorly differentiated type (M5a) : non-specific esterase-positive monoblasts with non-granular cytoplasm or rare azuorphili granules/auer bodies. Differentiated type (M5b) : promonocytes and monocytes in	20%	CD 33, 15, 14, 13

M5b : Monoblasts, Promonocytes, Monocytes	addition to monoblast		
M6 Acute monocytic leukaemia Cell type : Erythroblasts	Erythroblasts > 50 percent of marrow nucleated cells; myeloblasts and promyelocytes increased. Erythroblast (usually present in peripheral blood) often strongly Periodic-Acid Shiff stain positive and possess morphological abnormalities	5%	CD 33, glycoprotein
M7 Acute megakaryocytic leukaemia Cell type : Megakaryocytes	Megakaryoblasts, some with cytoplasmic budding; positive platelet peroxidase reaction on electron microscopy and reactivity to monoclonal antibodies specific for platelet-specific surface antigens.	5%	CD 33, 41
Acute lymphoblastic leukaemia (ALL)			
L1 Acute lymphoblastic Leukaemia Cell type : Microlymphoblasts	Homogeneous small lymphoblasts; scanty cytoplasm, regular round nuclei, inconspicuous nucleoli	75%	CD 10, 19, 35, T dt
L2 ALL Cell type : Large undifferentiated lymphoblasts	Heterogeneous lymphoblasts; variable amount of cytoplasm, irregular or cleft nuclei, large nucleoli	20%	CD 10, 19, 34, T dt
L3 ALL Cell type : Burkitt type	Large homogeneous lymphoblasts; basophilic cytoplasm, round nuclei, prominent nucleoli, cytoplasmic vacuolation	5%	CD 19, 20 sIg

Leukomoid Reaction

This is a non-leukaemic disease but PBF of patient resemble with PBF of a leukaemic patient. In this reaction, total leucocyte count is increased & some immature cells are present in PBF. Leukomoid reaction is of two types.

1. Myeloid leukomoid reaction
2. Lymphoid leukomoid reaction

1. Myeloid Leukomoid Reaction : PBF of a patient resemble with PBF of chronic myeloid leukemia. In PBF immature cells of myeloid series are present e.g. promyelocytes, myelocytes, metamyelocytes etc. TLC is increasd to 50,000/cumm.
   Causes :
   (i) Acute pyogenic infection
   (ii) Burns
   (iii) Vascular thrombosis

2. Lymphoid Leukomoid Reaction : PBF of a patient resemble with PBF of lymphatic leukaemic patient.In DLC 80-90% cells are lymphocytes. TLC is 25000-5000/cumm.
   Causes :
   (i) Tuberculosis
   (ii) Pertusis (Whooping cough)
   (iii) Some viral infection

Difference between Leukaemia and Leukomoid Reaction

	Leukomoid reactions	Leukaemia
Clinical features	Clinical features of the causative disorder often obvious	Splenomegaly, lymph node enlargement, and haemorrhage more common than with leukaemoid reactions
Haematological examination		
Total white cell count	Increase usually only moderate; seldom exceeds 100 X 10^9/1	Can exceed 100 X 10^9/1
Proportion of immature cells	Usually small or moderate. Myelocytes seldom exceed 5-15 percent, and 'blasts' 5 per cent	Usually numerous
White cell morphology	Toxic changes may be seen in infective cases	Cells often atypical as well as immature. Toxic changes uncommon
Anaemia	May occur, but often slight or absent	Usually present and progressive
Nucleated red cells	Frequent in leuco-erythroblastic anaemia due to marrow infiltration	Less frequent
Platelets	Mainly normal or increased, but reduced in leuco-erythroblastic anaemia and intravascular coagulation	Decreased, except in chronic granulocytic leukaemia
Bone marrow	White cell hyperplasia may be present but seldom to same degree as in leukaemia	Hyperplastic with potentially large proportion of immature cells
Autopsy	Infiltration of organs and tissues absent	Leukaemic infiltration of organs and tissues

Unit 4
Body Fluid Examination

Serous Fluid analysis
(Pleural, Pericardial and Peritoneal , Ascitic Fluids)

Serous fluids are the fluids contained within the closed cavities of the body. These cavities are lined by a contiguous membrane that forms a double layer of mesothelial cells, called the serous membrane.

Pleural Fluid : It is the fluid of pleural space. Normally, about 1-10 ml of pleural fluid will be present around the lungs. It is derived by ultrafiltration of plasma.Normal quantity of proteins in this fluid is 1-2 g% with no fibrinogen. Abnormal fluid accumulation is caused by increased capillary permeability, decreased plasma colloid osmotic pressure, increased hydrostatic pressure, decreased lymphatic drainage.

Pericardial fluid : It is the fluid of pericardial sac. Under normal conditions, 20-50 ml of clear straw coloured fluid will be present. It may be bloody, milky, or similar to gold paint.

Peritoneal fluid : It is straw coloured, clear fluid present in peritoneal cavity. Its normal value is less than 100 ml.

Transudate : Increased venous pressure or decreased oncotic pressure, usually from a disturbance of cardiac function, leads to a greater exit of fluid from the vascular system than is reabsorbed. The aspirated fluid is low in protein (less than 2 gms%), low specific gravity (1.005 to 1.015) and low cell count (scattered mesothelial cells of serosal origin). A few histiocyte or lymphocyte are also present. It has a little tendency to clot.

Exudates : Exudates are fluid accumulation that results from the break-down of the normal formation-resorption mechanism due to inflammation or toxic damage to the pleural, peritoneal or pericardial capillaries. Irritation of the serosal mucosa caused by various agents, such as cancer, inflammation, a foreign body or haemorrhage is associated with damage to the underlying wall of capillaries and venules of the mesothelial membrane. The difference between transudate and exudate are given below**.**

Physical Examination : During physical examination, amount, color, pH, specific gravity is noted.

Difference between Transudate and exudates

Different characteristics of effusions		
Properties	Transudate	Exudates
Appearance	Clear and watery	Cloudy, may be clotted
Sp. Gr.	< 1.015	>1.015

Protein	Low	High
Fibrin	Poor or absent	Rich
Sugar	High	Low
Cell count	Few	Abundant
pH	7.0	More than 7.2

Chemical Examination

1. Estimation of Protein

(i) Screening test done by refractometer. In this test, small amount of specimen is required.

(ii) Dipstick method : As done in urine specimen.

(iii) Biuret Method for Quantitative test : Same as given in Biochem. But sample quantity is double because protein concentration is low in fluids.
Consequently, the serum protein standard should also be diluted two folds.

Normal Range of Trandudate fluid : < 3 gm/dl

2. Test of Glucose/Sugar

- Glucose estimation in body fluid is done from the supernatant after centrifuge. (at 3000 rpm for 5-10 minutes)
- Estimation is done by GOD-POD Method as described in Biochem.

Normal Range of Tranudate Fluid : Same as in Blood.

Microscopic Examination

Microscopic examination of serous fluid, like other body fluids consists of total cell count by haemocytometry and differentiatl count of white cells from the stained smear. Anticoagulated specimen is required for the cell count. A predominance of neutrophils suggest Bacterial inflammation with a predominance of lymphocytes indicates the possibility of viral infection, tuberculosis, lymphoma or carcinoma.

Total Cell Count

(i) Use well-mixed serous fluid for the cell count.

(ii) The cell count of normal serous fluid is low and hence dilution is not necessary.

(iii) Filed with a drop of undiluted specimens by means of a blood diluting pipette or by a capillary pipette.

(iv) To count white cells, first rinse the pipette with glacial acetic acid and then draw the specimen into it to fill the chamber.

(v) The method of counting red cells and white cells is the same as described in the study of blood.

Differential Count

The differential count of white cells is done with the help of stained smear of the sediment which is obtained from anticoagulated serous fluid. While taking differential count, if any malignant tumor cells are seen, the smear must be referred to a pathologist.

Microbiological study :

Smear Examination : Prepare two smears. Dry in air, heat fix and stain with gram stain and Ziehl Neelsen acid-fast stain. Record the preliminary report and inform the physician in case of any abnormal finding.

Culture : Send the Reference Laboratory for culture as per requirement Gram stain and Z.N. stain.

Synovial Fluid

Synovial fluid is found arount the joints such as knee, ankle, hip, elbow, wrist and shoulder. The chemical composition of synovial fluid resembles with that of other body fluids such as serous fluids and spinal fluids. In addition it contains a mucopolysaccharide, hyaluronic acid, which acts as a binding and protective agent for the connective tissues.

Composition of Normal Synovial Fluid :

Volume in a joint	0.1 to 2.0 ml, transparent straw coloured droplets.
Content per 100 ml	Protein 1-3 gm% Hyaluronate 0.3-0.4 gm% Glucose 70-110 mg% Uric acide 2-8 mg% Lactate 10-20 mg%
Cytology	Leucocyte : 20-50/cumm, upper limit of normal, usually 1-2.0 cells/cu mm; these are mainly lymphocytes/histiocytes.

Clinical Significance : The laboratory examination of this fluid helps to assist in the diagnosis of joint arthritis, gout or infection of the joint.

Specimen collection : The specimen is collected in the following sterile tubes :

1) EDTA tube : For cell count and microscopic examination.

2) Plain tube (without anticoagulant) : For gross examination, mucin clot test, evaluation of viscosity and for microbiological and serological tests.

3) Fluoride-oxalate tube for glucose determination.

Physical Examination : The various observation are as follows :

a) Normal synovial fluid is clear, straw coloured and viscous. It does not clot.

b) Turbid appearance :Synovial fluid is turbid in Inflammatory and infected conditions. It may be due to presence of crystals, amyloid and cartilage fragments.

Viscosity Test : Synovial fluid is viscous and the viscosity is due to the presence

of hayluronic acid. The viscosity decreases due to the breakdown of hyaluronic acid by the enzyme hyaluronidase in inflammatory disorders.

Procedure : Drop the fluid from a syringe and note the length of the string formed. Use a scale to measure the length.

The normal fluid forms a string of at least 4 cm long. If the string breaks before reaching 3 cm lenght, the viscosity is lower than normal.

Microscopic examination :

(a) Total Leucocyte count : (i) Use saline containing methylene blue as diluent for turbid specimen (ii) normal clear specimen should be used undiluted.

Normal WBC count =20-50 cells/cumm^3.

(b) Differential WBC count : Prepare a thin smear of the sediment and stain. Normal fluid contains about 25% of polymorphonuclear cells. Increase in these cells (above 70%) indicate bacterial arthritis. Moderate increase in neutrophils (40 to 60%) is observed in rheumatic fever, gout, tuberculous arthritis and in rheumatic arthritis.

Microscopic examination

Polarised Microscopy	
- Needle shaped crystal of Monosodium urate	Gouty arthritis
- Rhomboid shaped crystals	Chondrocalcinosis and pseudogout.

Gram Film : Gonococcus or other bacteria may be detected in deposit.
Culture : SF is to be cultured, particularly when gonococcal arthritis is suspected.

Chemical Examination :
(a) Determine glucose by any conventional method.
(b) Determine proteins by biuret method.

Difference between Inflammatory and Non Inflammatory Synovial Fluid

Inflammatory Fluid	Non inflammatory Fluid
- This is turbid, yellow in color. - Leucocyte count < 300 cells/cumm with a mononuclear cell predominance. - Glucose level is normal	- Clear, viscous, amber-coloured. - Cell count is 3,000 to 5,000/cu mm - Glucose level is normal or low

Cerebrospinal Fluid (CSF)
Cerebrospinal fluid (CSF) is found in the space known as the subarachnoid space between the arachnoid mater and the pia mater - two of the three membranes comprising the meninges covering the brain and spinal cord (From the outside in the dura mater, the arachnoid mater, and the pia mater). The CSF is made continuously by small masses of blood vessels which line the ventricles of the brain. An adult person produces 450-750ml of the fluid daily. From these, 120-150ml of the fluid is required to fill the arachnoid space between the brain and the spinal cord. The CSF is reabsorbed by the small blood vessels in the arachnoid called the arachnoid villi. The CSF has composition similar to the plasma with the exception that it contains less protein, less glucose and more chloride ions. Cerebrospinal fluid serves to protect the underlying tissue of the central nervous system. It acts as a mechanical buffer to prevent trauma, to regulate the volume of intracranial pressure, to circulate nutrients, to remove metabolic waste products from the central nervous system, and to generally act as a lubricant for the system.

Collection, Transportation and Processing of CSF specimen

Collection

- Collection of CSF must be done by an experienced medical officer.
- It is performed under strict aseptic conditions.
- It is done by lumbar puncture needle (a stylet inside the LP present)
- Make the patient lie on one side with legs and neck flexed, with knee and chin approximated.
- Back of patient should be at the edge of the bed.
- Feel for the iliac crest.
- Now mark the area between L_3 and L_4 lumber spines.
- Clean the area around L_2 to L_5 with iodine followed by alcohol twice.
- Now inject 1% lignocaine in to skin over L_3 and L_4, taking all aseptic precautions.
- Then thrust the needle towards the centre of intervertebral space, injecting the solution as needle goes deeper. Wait for 3-5 minutes for the area to be anaesthetized.

- After anaesthetized the area, introduce the sterile L.P. needle with stylet firmly through the skin in the middle line between L_3 and L_4 spines.
- When the needle enters the spinal cavity, the resistance gives way. Withdraw the stylet.
- CSF starts flowing from the needle.
- Now collect CSF in the three sterile bottles.
- First specimen is for Biochemical examination and 2^{nd} is for cell counts and 3^{rd} is for culture.

Transportation

- Should be transported immediately to the laboratory without delay.
- If delay is expected, especially in case of CSF subjected to microbiological investigations, it should be incubated or left at room temperature.
- If delay is unavoidable, CSF should be collected in a transport medium such as 1% glucose broth, Stuart's transport medium or Amies medium.

Physical examination :

Condition	Pressure	Color	Clot/coagulum of protein
Normal	50-180 mmH_2O	Crystal clear colourless	Nil
Subarachnoid haemorrhage	Increased	Blood stained xanthochromatic	Nil
Pyogenic meningitis	Highly increased	Cloudy	Big protein clot develop within a few mintues
Tuberculous meningitis	Moderately increased	Clear/slightly turbid	Fine, thread-like spider web coagulum develops after a few hours.
Viral meningitis	Normal/slightly increased	Clear	Nil
Multiple sclerosis	Normal	Clear	Nil

Routine Processing :

1. Gram's Stain :

- Centrifuge the Ist bottle's CSF at 1500 rpm for 10-15 minutes.
- Now transfer the supernatant fluid in other tube for Biochemical investigations.
- Now make two smears from centrifugal deposit on a clean glass slide.
- If some sediment is remaining in tube that can be used for India Ink preparation.
- Dry it and fix by gently heating on spirit lamp.
- Now do Gram's Stain as mentioned in microbiology.

Note : If unevently stained irregular in size yeast cells (showing budding), suggestive of Cryptococcus neoformans, then process other specimen for India Ink preparation.

2. India Ink Preparation :

- India ink preparation is used for detection of cryptococcal neoformans.
- Mix the remaining sediment of Ist bottle and transfers a drop of the sediment to a slide, and add a drop of India Ink, mix and cover with a cover slip.

- Examine the preparation using High power objective.

Examination

Look for oval or round cells, some showing budding irregular in size measuring 2-10 □m in diameter and surrounded by a large unstained capsule.

Note : Serological test should be done for confirmation (Test kits commercially available in market).

Z.N. Stain : Do Z.N. stain for AFB if N. meningitis and look for AFB in Z.N. stained smear.

Condition	Gram stain	Acid-fast stain
Normal	Nil	Nil
Subarachnoid haemorrhage	-	-
Pyogenic meningitis	Gram negative or +ve cocci or bacilli found	-
Tuberculous meningitis	-	Acid-fast bacilli often found
Viral meningitis	-	-
Multiple sclerosis	-	-

Haematological examination

Cell count :

Requirement :

1. WBC diluting fluid (Turk's fluid)
2. Neubauer counting chamber
3. WBC pipette (Thoma pipette)
4. Microscope
5. Test tube
6. 0.2 ml pipette

Procedure

1. Take 0.2 ml of CSF in a small test tube (from 2nd specimen)
2. Add 0.2 ml of WBC diluting fluid.
3. Mix it and leave for 10 minutes.
4. Remix and charge the Neubauer counting chamber.
5. Now calculate the cells in four corner squares which is recommended for TLC.

Calculation :

=N X Dilution factor

Area X Depth

= N X 2

4 x 0.1

= N x 2 x 4 x 10

= N x 80 cells counted in per cumm^3

Note : If cells are more in numbers then a Thoma pipette is used for Dilution (1:20) and calculate as per formula mentioned in above calculation.

DLC

Smear is prepared from centrifuged deposits and fix it in acetone free methanol. Stain it with Leishman stain and examine under 100^X objective.

Condition	Cell count (Total cell per cumm)	Type of cell
Normal	1-4	Lymphocytes
Subarachnoid haemorrhage	Increased red blood cells	Red cells
Pyogenic meningitis	1,000-20,000	Neutrophils – 98% Lymphocytes – 2%
Tuberculous meningitis	50-2,000	Lymphocytes – 90% Neutrophils – 10%
Viral meningitis	20-200	Lymphocytes
Multiple sclerosis	1-100	Lymphocytes + Plasma cells

Biochemical Tests :

1. Determination of Protein in CSF

Trichloroacetic acid method

Requirement ;

(i) Colorimeter
(ii) Trichloro acetic acid 5%
(iii) Std. Reagent 50 mg%
(iv) Test tubes
(v) w/w

Procedure

(i) Make the test tubes, test and std. and dispense the reagents and specimens as follows :

	Test	Std.
1. Std. protein	-	2 ml
2. CSF	1.2 ml	-
3. D/w	0.8 ml	-
4. TCA reagent	2 Ml	3 ml

(ii) Mix well by inversion and allowed to stand for 5 minutes.
(iii) The solution turns turbid.
(iv) Read the O.D. at 530 nm in colorimeter.

Calculation :

$$\frac{\text{O.D. of test}}{\text{O.D. of std.}} \times 50 = \text{CSF protein/dl}$$

2. Pandy' test : (Globulin test, Qualitative test)

Principle : Globulin are precipitated by saturated solution of phenol in water.

- The screening test detects rise in C.S.F. glucose. It is qualitative test.

Clinical Significance : The test provides an approximate comparison between □-globulin increase and that of total protein of CSF. The □-globulin is disproportionately elevated in multiple sclerosis and neurosyphilis.

Reagent : Saturated solution of Phenol in water.

Procedure :

(i) Pipette about 1 ml of phenol solution in a test tube.
(ii) Now put 1 drop of CSF specimen in phenol solution. Do not mix.
(iii) Look for immediate cloudiness around the deep of CSF against dark background.
(iv) If immediately cloudiness appears then the test is positive and it indicates presence of excess globulin in CSF specimen.

3. Determination of sugar in CSF

Method : Orthotoludine method

Requirement ;

(i) Orthotoludine reagent - oth
(ii) Std glucose 100 mg/dl
(iii) Colorimeter
(iv) Test tubes

Procedure :

(i) Mark the test tubes, test and std. dispense the reagents and specimen as follows :

	Test	Std.
1. Std. reagent	-	0.1 ml
2. CSF	0.1 ml	-
3. O.T. reagent	4 ml	4 ml

(ii) Keep it in boiling water bath for 10 to 15 minutes.
(iii) Blue green color is formed, cool and read the O.D. at 620 nm.

Calculation :

C.S.F glucose in mg/dl = O.D. of test X 100

O.D. of std.

Condition	Protein	Glucose	Chloride
Normal	30-50 mg%	40-80 mg%	720-760 mg%
Subarachnoid haemorrhage	Increased	Normal	Normal/slightly increased
Pyogenic meningitis	Highly increased (100-600 mg%)	Decreased (10-20 mg%)	Decreased
Tuberculous meningitis	Slightly increased (80-120 mg%)	Decreased (30-50 mg%)	Extremely diminished
Viral meningitis	Slightly increased (60-80 mg%)	Normal/increased	Decreased

Multiple sclerosis	Normal/slightly increased	Normal/increased	Normal/decreased

Unit5

Semen Examination

Semen: - Semen consist of spermatozoa which is suspended in Seminal plasma. Freshly ejaculated semen is a highly viscid, opaque, white or greyish white coagulam. After 10-20 minutes these coagulam liquefy and convert into a translucent, turbid, viscid fluid which is mildly alkaline.

Indications of Semen Examination

(i) Routine examination of semen is often advised in relation to infertility.
(ii) It is also necessary at times to examine vaginal secretions or clothing stains for the presence of semen for alleged or suspected rape.
(iii) Semen analysis may sometimes be necessary to check the effectiveness of vasectomy.

Note : In relationship to sterility the defect in the male partner constitutes about 50% cases. The cause of male infertility may be either in the quality or in the quantity of spermatozoa.

Collection of Semen :-

Semen sample is collected in clean and dry container. There is abstinence of five days before sample collection. Sample is collected by masturbation process. Sample must be transferred to lab. in 30 minutes after collection.

Physical Examination :-

1) Viscosity :- We can check its viscosity by dropping it. If semen falls drop by drop then its viscosity is normal. Freshly ejaculated specimen is a highly viscid, opaque.
2) Liquefaction :- Semen Liquefaction must be complete in 30 minutes. The absence of liquefaction may inhibit the movement of spermatozoa in the vaginal canal and interfere with fertilization.
3) Volume :- Normal Semen Volume is 1.5 ml to 5.0 ml. Volumes less than 1.5 ml is considered below normal. Greatly reduced semen volume can result in poor penetration of the cervical mucous by sperm.

Microscopic Examination:-

1) Sperm Count :- After Semen liquifaction Total sperm counting is done.

Reagent used :- Semen diluting fluid

Composition :-

a)	Sodium Bicarbonate	5 gm
b)	Formalin or Phenol	1 ml
c)	Distilled water	100 ml

Procedure :- Collect 0.38 ml Semen diluting fluid in a penicillin bottle. Now pour 0.02 ml semen with the help of Sahli pipette and mix it. Charge the Neubauer counting chamber. Keep it for 5 minutes and examine in 4 corner squares of Neubauer Counting chamber.

Calculation :- $\frac{N \times 10 \times 20 \times 1000}{4}$ = Sperms/ml

Normal Value :- 60-150 million/ ml

2) Sperm motility :- Take a clean and non-greasy glass slide and pour one drop of liquified semen on the slide. Now keep coverslip on the slide. Examine in 45x objective and observe for motility. Minimum 100 sperms are counted and represent in the following way :

Active motile	%
Sluggish Motile	%
Dead	%

In Normal Semen 70% motile sperms are noted. Observe RBC, Pus cells , Epithelial cells and immature sperm cells in semen.

Azoospermia :- No dead or alive sperm seen
Oligozoospermia :- when the total sperm count is less than 60 million/ml
Necrozoospermia :- Spermatozoa are present but immobile
3)Sperm Morphology :- After Liquefaction pour a drop of semen on a slide and make a thin film. Fix it by heating and stain with Field's stain and examine normal and abnormal types of sperm by using oil immersion objective of microscope. Report normal and abnormal forms in percentage. In semen of a normal person 70% normal spermatozoa is present.

Normal Form of Sperm :-

Head is dark coloured, rounded white tails and middle piece is pink.

Abnormalities

- Abnormally shaped heads,
- Abnormally sized heads- giants and minute.
- Double heads,
- Middle piece or neck may be absent, bifurcated or swollen,
- Tail may be double, curled, rudimentary or absent.

Unit 6

Lupus Erythematosus (LE) Cell

Introduction to LE Factor

- L.E. (Lupus Eruthromatosus) is called systemic lupus erythromatosus (SLE).
- It is a connective tissue disorder and more frequent in females (child baring age).
- In SLE, degenerating connective tissue relieves a protein in to blood stream which acts as an antigen.
- As we know, antigen induce antibody production. So, this antigen stimulate the antibody production. In this way, anti antibody produced against his own antigen. (Auto antibodies)
- This auto antibodies is known as L.E. factor which is anti nuclear.

Clinical Significance/ Importance

LE cella re positive in

- SLE
- Lupoid hepatitis
- Penicillin sensitivity
- Rheumatoid arthritis
- Collagen disorder.

Although L.E. cell demonstration is prognostic test. Its positivity or disease should be confirmed by other test also e.g. ANA.

Principle : Serum of SLE patient's contain L.E. factor (Gamma-globulin fraction). L.E. factor does not attack healthy neutrophil. The leucocytes traumatize with the help of glass beads. When nuclear membrane of neutrophil is damaged then the nucleoprotein is exposed and it interact with L.E. factor and combination of both (L.E. Factor + Nucleoproteion of traumatized neutrophil) is known as L.E. body. Now this L.E. body is ingested by a healthy neutrophil. This neutrophil is known as L.E. cell. It is visible under microscope after

Leishman's stain. After traumatization the neutrophil, blood is centrifuged and a smear of the 'buffy coat' is made. Stain it and examination is done under oil immersion lens after Leishman's stain.

Specimen : EDTA anticoagulated or clotted blood.

Requirements :

(i) Centrifuge tubes, glass beads, rubber bungs
(ii) Microscope glass slides, test tubes
(iii) Leishman's stain
(iv) Centrifuge, microscope, blood mixing rotor or vortex mixer

Procedure :

1. Traumatize Leucocytes

(i) Take about 5 ml of EDTA blood in a test tube.
(ii) Add five glass beads and stopper the tube.
(iii) Rotate on a blood mixing rotor at 50 rpm for 30 minutes.
(iv) Place the tube at 37^0C for 10 minutes.

2. Buffy coat smear preparation

(v) Transfer the blood to a Wintrobe tube.
(vi) Centrifuge Wintrobe tube at 3000 RPM for 30 minutes.
(vii) Remove hemolyzed serum and then remove carefully the buffy coat.
(viii) Make smears of the buffy coat on clean and dry glass slides.

3. Staining and Examination

(ix) Fix the air dried smear in methanol.
(x) Stain by using Leishman's staining solution. (As mentioned in DLC section)
(xi) Screen under high power objective (40x) and observe specific areas under oil immersion objective.

L.E. cell is characteristics of

- L.E. cell appears as a neutrophil which contains a large spherical body in its cytoplasm.
- The Neutrophil's nucleus is pushed aside and the ingested material (L.E. body) show no nuclear structure.
- It stains a pale purple homogenous mass.

Tart Cell : This can be found in normal patient. Some times during preparation of LE cell Lymphocytes are engulfed by Monocytes. This cell resemble LE cell and this is called tart cell.

Difference between L.E. Cell & Tart Cell

L.E. Cell	Tart cell
1. This is found in patient of L.E. factor. 2. This cell is made from Neutrophil. 3. In cytoplas of L.E. cell large spherical body is seen.	1. This is found in normal individual. 2. This cell is made from Monocytes. 3. In cytoplasm of Tart cell, large structure is seen.