Investigations in Medicine
Haematology

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Reticulocyte counts

- Larger bluish (polychromatinc) red cells (RNA)
- Increased with regenerating marrow
  - blood loss
  - haemolysis
  - post chemotherapy
- Reduced in
  - anaemia of chronic disease
  - deficiencies -B\textsubscript{12}, folate, Fe
  - significant marrow disorders eg aplasia, MDS
Anaemia of chronic disease

- Low Hb usually >90g/l, low reticulocyte count
- Unable to utilise iron
- Normal or low MCV
- High ferritin (acute phase/not utilised)
- High CRP
- May have low EPO, but often normal, few respond
- Mediated by TNF, γIFN, hepcidin
- If iron deficient %sat low, ferritin low-normal
- Trial of oral iron often indicated - will need investigation
- Anaemia of chronic disease may respond to iron infusion
Infection
Inflammation
Malignancy

> IL-6

Liver

Hepcidin

- Decreased iron absorption
- Decreased release of iron from macrophages

Macrophage
Genetic hemochromatosis

Anemia of inflammation
Macrocytosis

- Liver disease
- Alcohol
- B\textsubscript{12}/folate deficiency
- Drugs affecting DNA eg MTX, HU
- Hypoxia
- Reticulocytosis (gross)
- Myelodysplasia
- Others - hypothyroidism, cold aggs, aplasia, EPO
Low $\text{B}_{12}$ levels

- 20% due to pernicious anaemia
- Elderly - atrophic gastritis, reduction in absorption of food bound $\text{B}_{12}$
- Gastrectomy
- Vegans - (some $\text{B}_{12}$ on mushrooms)
- Terminal ileal disease
- Metformin
- Erroneous
- Low levels, but normal tissue stores
Pernicious anaemia

- **Morphology**
  - PB - RBC oval macrocytes, hypersegmented neutrophils, cytopenias
  - Marrow - megaloblastosis
- **IF Abs** in 50% (specific cf parietal cell)
- High serum gastrin
- High homocysteine levels (not specific)
- Schilling’s test - rarely performed
- Urinary methylmalonic acid - not routine
- Holo TC II – recently introduced some labs
Holo-TC II

Active-B12 (holotranscobalamin) 10-30% (Biologically active)

B12 bound to haptocorrin 70-90% (Biologically inert)
Causes of low serum B\textsubscript{12} (but normal stores)

- Elderly
- Severe folate deficiency
- Pregnancy/OC
- Myeloma
- 10% of patients with carcinoma
- HIV
- Rare - TC 1 deficiency, Vitamin C overdose
- High CV of assay
Thalassaemias - globin chain production imbalance
ß thalassaemia trait

- ß thalassaemia - over 100 mutations, usually nucleotide substitutions (2 beta genes)
- Normal haemoglobin (lower limit)
- Low MCV (constant) – hypochromic, microcytic red cells, target cells, stippling
- High HbA$_2$ and HbF (check not iron deficient)
- Hb electrophoresis no abnormal bands unless haemoglobinopathies eg HbE
α thalassaemia

• α thalassaemia - usually large deletions of the alpha gene (4 alpha genes normally)
• Single gene mutation - low-normal MCV (α+/αα)
• Two gene deletions - low MCV, positive HbH bodies - on the same gene more significant (αo/αα compared to α+/α+)
• Three gene deletions - HbH disease - life long anaemia, previously managed without Tx, splenomegaly
• Four gene deletions - hydrops foetalis - Hb Bart’s (γ)
α thalassaemia

- Hb H preparation - ‘golf balls’ from precipitation of β chains, not reliable, therefore **genetic** studies
- Hb A₂, Hb F levels, Hb EPG normal in adults if one or two gene deletion
Tests that suggest haemolysis

• Fall in haemoglobin, increased bilirubin (uncong), urobilinogen on dipstick
• Increased reticulocytes, LDH, low haptoglobin
• Tests that suggest a cause of haemolysis
  – Blood film eg fragments, blister, sickle cells
  – DAT (Coomb’s)
  – Urinary haemosiderin (IV haemolysis)
  – Family history, male gender for G6PD
Patients cells coated with Antibody (IgG, IgA, IgM or C3) in vivo

Red = in vivo

Test cells get coated with antibody/C’ from the patient’s serum
Blood films in haemolysis

- Oxidant drugs and G-6PD deficiency - blister, bite cells
- Microangiopathy, valve haemolysis, TTP - fragments
- Hereditary spherocytosis, warm AIHA, burns - spherocytes
- Hb S - sickle cells
- Pyruvate kinase deficiency - spiny cells
- Cold AIHA - agglutinated cells
Further diagnostic tests for haemolysis

- G6PD deficiency and oxidative haemolysis
- Hereditary spherocytosis
- PNH
- PCH
- Cold agglutinins (titre)
- Hb S - sickling test, Hb EPG
Hereditary spherocytosis

- Anaemia, high MCHC, spherocytes >2%
- DAT negative
- Family history
- Increased osmotic fragility (no longer performed)
- Increased autohaemolysis, improved with glc
- Specialised - spectrin analysis, flow cytometry - eosin-5- maleimide
**HS: vertical**
- Ankyrin: 50%  ANK1
- Spectrin: 30%  SPTB
- Band 3: 20%  EPB3
- Pallidin / 4.2

**HE: horizontal**
- α or β spectrin dimer formation abn
- α or β spectrin-ankryin assoc abn
- Protein 4.1 def/abn & Band 3 abn
- SE Asian ovalocyte band 3 del
Warm autoimmune haemolytic anaemia

- **Blood film**, spherocytes, assoc with CLL/NHL
- **DAT** - most likely positive in warm h’lysis
  - Ig G+/−C3d
- Otherwise associated with ANA positivity, drugs or idiopathic
Cold autoimmune haemolytic anaemia

- **Blood film** - spherocytes, *cold agglutinins*, high MCV, high MCHC
  - may see atypical or malignant lymphocytes

- Cold haemolysis due to *IgM* (to I or i)

- **DAT** - if positive - **C3d only**

- **EBV** & **Mycoplasma** serology

- **PCH** - *cold reactive IgG anti-P*, Donath-Landsteiner test (rare)
Oxidative haemolysis eg G6PD def
Glucose-6-phosphate dehydrogenase deficiency

- Pentose phosphate pathway, X linked
- No haemolysis between episodes
- Precipitated by fava (broad) beans, sepsis and oxidant drugs e.g. dapsone, salazopyrine, cotrimoxazole, glibenclamide, primaquine, chloroquine, Fansidar, Maloprim
- Screen those at risk e.g. malaria, neonatal jaundice
- Reticulocytosis, helmet (arrow) and blister cells
- Heinz body preparation - denatured haemoglobin — few labs performing now
- Fluorescent spot screening or G6PD enzyme assay
Paroxysmal Nocturnal Haemoglobinuria

- Cytopenias, iron deficiency, thromboses (unusual sites) and intermittent dark urine (IV haemolysis)
- Marrow may be aplastic, Hb in urine
- Obsolete tests
  - sucrose lysis, Ham’s test
PNH – flow cytometry

- Reduced glycososphosphatidyl-inositol (GPI) anchor on haemopoietic cells, due to mutations in the PIG-A gene
- Less inhibition of the complement cascade-lysis
  - DAF - CD55
  - MIRL - CD59* - flow cytometry of red/white cells
Polycythaemia

- **Primary** - itch, splenomegaly, panmyelosis
  - Marrow - hypercellular with inc reticulum
  - 50% splenomegaly on ultrasound
- **Secondary** -
  - lung disease ?sleep apnoea
  - congenital heart disease
  - Renal – PCKs & carcinoma
- pO$_2$ helpful, EPO not as helpful
- JAK2 mutation in > 95% PRV
- Other MPDs – CALR mutn~ 30%
  - MPL <10%
  
  EPO receptor – Jak-2 mutation
Causes of a long PT

• Liver disease
• Vitamin K deficiency
• Warfarin (INR allows laboratory comparison)
• Erroneous specimen
• (specific factor deficiency – rare)

• With prolonged APTT
  – Excess heparin
  – DIC, severe hypofibrinogenaemia
  – Lupus inhibitor (rarely effects PT)
  – Extreme of the above
Causes of a prolonged APTT

- Unfractionated heparin (line contamination)
- Lupus anticoagulant (inhibitor)
- Coagulation deficiency
  - Haemophilia A & B, von Willebrand disease
  - Acquired haemophilia (inhibitor – rare), but important not to miss
- Factors affecting both or common pathways
  - e.g low fibrinogen, DIC
- Incorrectly collected specimen
Coagulation tests and Novel ACs

• Monitoring not routine

• **Dabigatran** – inc aPPT, PT but not linear, inc TCT but too sensitive.
  – Hemoclot or dilute TCT

• **Rivaroxaban** – inc PT, but not reliable for monitoring
  – Specific anti-Xa assay

• **Apixaban** – only mildly elevated PT
  • Specific anti-Xa assay
von Willebrand Factor
Diagnosis of von Willebrand disorder

- **Family history** of mucosal bleeding - autosomal dominant (common forms)
- Prolonged **APTT** (may require repeat test, often N)
- vWF:Ag or Glycoprotein 1b receptor binding assay
- **Factor VIII:C** level
- **Functional assay**
  - ristocetin cofactor (vWF:RCO)
  - and/or collagen binding assay (vWF:CBA)
- Diagnosis can be difficult, mutational analysis not routine
Thrombocytopenia

• ?decreased production or increased destruction
  – with other cytopenias - as for neutropenia
• Severe sepsis +/- DIC
• ITP
• Viral infections (usually mild e.g hep C, rarely IM is associated with severe ITP, also HIV)
• Drugs
  – immune - heparin, quinine, thiazides
  – suppressive - cytotoxics, alcohol
Diagnosis of ITP

- Low platelet count confirmed on blood film and rebleed, blood film otherwise NAD
- Marrow showing inc megakaryocytes
  - now not routine in young adults
- ANA, B<sub>12</sub>, folate, HIV, hepatitis B and C, TFTs (helicobacter)
- Platelet antibodies - not specific
- Normal spleen size
- Exclude drug cause and associated lymphoproliferative disorder
Thrombosis - consideration for further investigation

- Patient < 45 years and unprovoked
- Second or subsequent thrombosis
- Positive family history
- Unusual site
  - Consider whether will alter mgt or adversely affect family members
  - 50% of inherited thrombophiliyas have a thrombosis secondary to immobility or pregnancy
Acquired thrombotic tendency

• Pregnancy, oral contraceptives
• Antiphospholipid antibodies
  – lupus AC, anticardiolipin, anti-β-2-glycoprotein Abs
• Malignancy - breast, mucin secreting
• Rarer
  – Myeloproliferative disorders
  – Nephrotic syndrome
  – Chronic haemolysis
  – PNH (very rare)
Diagnosis of antiphospholipid syndrome

Thrombosis and/or Recurrent pregnancy loss

Persistently positive test for LAC and/or aCL and/or anti-\(\beta_2\)GPI

Consequences for treatment & prognosis

Diagnosis of lupus inhibitor

- Prolonged APTT (normal thrombin time)
- **Mixing experiments** consistent with an inhibitor
  - normal plasma does not correct APTT time
- **Specific lupus AC testing**
  - Kaolin Clotting Time
  - Dilute Russell Viper Venom Time
  - Correction of APTT with platelet phospholipid
- **Associated antiphospholipid antibodies**
  - e.g. ACL, antiβ2GPI
Figure 4

A. True Anticardiolipin Antibodies

Cardiolipin

B. Anti-β2-GPI (conformational epitope) Domain I antibodies

Cardiolipin + β2GPI

C. Anti-β2-GPI (conformational epitope) Non-Domain I antibodies

Cardiolipin + β2GPI

D. Does not detect anti-prothrombin antibodies

Cardiolipin + Protein

Coagulation is initiated by tissue factor and other coagulation-factor complexes on the surface of endothelial cells and monocytes. The activated factor X that is consequently generated requires activated cofactors V and VIII to produce thrombin, which in turn forms a complex with thrombomodulin. Protein C activation takes place by way of interaction between the thrombomodulin–thrombin complex and the endothelial protein C receptor. Activated protein C, together with its cofactor, protein S, inactivates factors V and VIII to provide negative feedback to the generation of thrombin. Complex 1 comprises tissue factor and coagulation factors VII, IX, and X; complex 2 comprises factors IX and X and cofactor VIII; and complex 3 comprises factor X, prothrombin, and cofactor V.
Inherited thrombotic tendency

- Factor V Leiden/ APCR - 20-50%
- Prothrombin 20210A - 3-5%
- Protein C deficiency - 5%
- Protein S deficiency - 5%
- Antithrombin III deficiency - <5%
- ? Hyperhomocysteine deficiency (MTHFR)
- Rare - dysfibrinogenaemia
- Not-defined (50%) or ill defined
  - eg plasminogen activator deficiency
Factor V Leiden

- Activated protein C resistance (APCR)
  - screening test, PCR for genetic testing
- 1-7% of the Caucasian population
- Glu to Arg mutation in FV at nucleotide 1691
- Site where APC cleaves and inactivates FVα

Thrombotic risk - 10X for heterozygotes
  - 80X for homozygotes

Compound heterozygotes with other thrombophilias
What tests should I do?

- Difficult! Not many! Will they alter management? (Choosing Wisely)
  - FBE
  - Lupus AC
  - Congenital haemophilias – perhaps ATIII is the only one where a result may influence mgt
  - JAK2 mutation for MPD if unusual site e.g. Budd Chiari
Flow cytometry

• Used for
  – lymphocyte analysis eg CD4 number
  – classification, diagnosis and detection of residual disease of lymphoproliferative disorders, acute leukaemias and myeloma
  – PNH, platelet Abs, foetomaternal haemorrhage
  – Stem cell enumeration of stem cell transplantation.

• PB, nodes and BM
• Aberrant or immature markers or
• Monoclonality of B cells (not T) by kappa/lamba ratio
Flow cytometry (2)

- T cell markers - CD3
  - CD4 (T helpers) - low in HIV
  - CD8 (T suppressor/cytotoxic)
- B cell markers - CD19
  - kappa/lambda light chain ratio 2:1
- CLL - CD19/CD5 dual markers
Algorithm for B lymphocytosis

Persisting Lymphocytosis

Flow Cytometry

Monoclonal B Lymphocytes ≥ 5,000 / g / l

CLL Immunophenotype
- CLL
  - Staging Prognostic Factors

Non CLL Immunophenotype
- NHL
  - NHL Subtype Classification (Histology, Cytogenetics)

Monoclonal B Lymphocytes < 5,000 / g / l

No Lymphadenopathy and no Organomegaly and no NHL-Typical Symptoms
- MBL

Lymphadenopathy or Organomegaly or NHL-Typical Symptoms
- NHL / SLL
  - NHL Subtype Classification (Histology, Cytogenetics)
Cytogenetics/FISH

- Can be diagnostic, prognostic or direct treatment.
- Conventional cytogenetics in metaphase.
- Florescent in situ hybridization (FISH). Can be used in interphase, often used for lymphoid malignancies as often hard to induce cell division.
Cytogenetics/FISH

- Examples of **diagnostic** are myelodysplasia in conjunction with morphology and subtypes of AML, e.g. APML, inv16
- **Prognostic** examples are numerous, but e.g. poor risk cytogenetics in AML may lead to allografting or treatment is not given in patients with high toxicity risk.
- **Treatment** altering e.g. ATRA for APML, 17p- in CLL (poor prognosis, respond to Bruton kinase inhibitors such as ibrutinib).
Examples of molecular tests other than for MPN fairly specific for diagnosis of haem malignancy

- **bcr-abl** - CML (Ph chromosome, t9:21)*
- **PML-RARA** - APML (t15:17)*
- **c-myc** - Burkitt and other high grade NHL
- **cyclin D1** - mantle cell lymphoma (t11:14)
- **bcl-2** - follicular lymphoma (t14:18)
- **MYD88** - Waldenstrom’s macroglobulinaemia (LPL)
- **BRAF** - hairy cell leukaemia
  - *Some molecular tests are quantitative and can be used to monitor disease
Molecular testing and next generation sequencing (NGS)

- Tests a suite of genes quickly e.g. myeloid panel.
- Depending on institution can be very expensive.
- Can be used to test for JAK2/CALR/MPL at once rather than sequentially - in some institutions can be cost equivalent and efficient.
- Can’t always diagnose myelodysplasia with certainty as clonal haematopoiesis of indeterminate potential (CHIP) is very common.
- Requires lots of expert interpretation.
Minimal Residual Disease (MRD) evaluation and monitoring

• Associated with increased incidence of relapse in ALL and AML.

• Gives prognostic information in these and other disorders, often incurable such as CLL, myeloma and hopefully informs treatment decisions e.g. more intensive therapy, allografting.

• Performed by
  – flow cytometry (as discussed above)
  – immunoglobulin rearrangements
  – quantitative PCR e.g PML-RARa (APML), NPM1 (AML).