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LabTech Training
Diagnosis of iron deficiency and anaemia of chronic disease
Julie R Fielding
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Abstract
Aberration in iron homeostasis can be clinically significant and extreme effects manifest as either iron deficiency anaemia or iron toxicity. Many patients suffering from iron deficiency (ID) or anaemia of chronic disease (ACD) will present with complex medical conditions including inflammation, infection or malignancy. Iron status in disease may be classified as early ID, iron deficiency anaemia (IDA), functional iron deficiency (FID), ACD or iron deficiency anaemia combined with ACD (IDA/ACD). It is important to differentiate between these clinical entities as the treatment regime will vary. A failure to diagnose anaemia or inappropriate treatment may lead to poorer clinical outcomes particularly in the newborn. The bone marrow iron stain was the initial standard for diagnosing iron deficiency but is impractical as a screening test. This test was superseded by the serum ferritin which is a very good indicator of iron status when inflammation is absent. Alternative or supplementary tests proposed to improve the diagnosis of IDA and ACD include serum transferrin receptor, reticulocyte haemoglobin content/equivalent, C-reactive protein and the Ferritin Index. Cut-off points for these tests may vary with different clinical presentations and a single reference range for such tests reduces their diagnostic effectiveness. The recently identified iron regulatory proteins like hepcidin are still research subjects but offer the potential for novel therapeutic agents in addition to better diagnosis of iron status. Those who are most at risk of IDA and/or ACD often reside in less developed or remote locations and would benefit from a more clinically effective diagnostic test such as a point-of-care (POC) test.

Keywords: Anaemia chronic disease, functional iron deficiency, iron deficiency, iron deficiency anaemia

Introduction
Iron homeostasis is critical to an individual’s well-being because iron is central to oxygen delivery, energy production and cellular metabolic processes. Iron is a key component in DNA synthesis. Extremes of iron levels manifest clinically as hypoxia (ID) versus iron toxicity (iron excess). Detrimental consequences include ID causing anaemia or iron excess causing free radical formation and potential organ dysfunction.

Anaemia affects at least two billion people worldwide (WHO 2004). ID and/or chronic inflammation are the most common causes with almost a billion people having IDA (Breyman et al 2002, Sheftel et al 2011). Populations in less developed nations or in tropical locations in developed nations like northern Australia have a higher incidence of IDA and ACD and it is particularly prevalent in the indigenous paediatric population. In Australia it has been estimated that the incidence of anaemia is 8% in preschool children, 12% in pregnant women and 15% in non-pregnant women. Indigenous communities have been shown to have a much higher prevalence of anaemia i.e. 55% in women and 18% in men (Pasricha et al 2010). These high risk populations usually have limited access to diagnostic technology and are often in composite clinical settings. In such situations a test for assessing iron status needs to be diagnostically robust and unambiguous. For maximum clinical effectiveness, these tests should have universal availability, a rapid test result turn-around-time (TAT) and low cost.

Ideally a diagnostic test for iron status should be able to diagnose the presence of ID with or without anaemia and also to differentiate early ID from IDA, ACD, FID and IDA/ACD; five different clinical presentations. This would provide clinical staff with a tool to enact early intervention, choose appropriate treatment options and significantly improve world-wide health morbidity and mortality. Australians still die of IDA (ABS 2006) and quality of life is significantly reduced in those with ACD.

This aim of this paper is to review the tests currently available for diagnosing IDA and ACD and their cited ability to differentiate between ID, IDA, ACD, FID and IDA/ACD.

Current understanding of iron metabolism
Traditionally the focus has been on those proteins involved in the storage (ferritin) and transport (transferrin) of iron. Additional proteins have recently been identified which appear to play an important regulatory role in cellular and mitochondrial iron haemostasis. Assays for these new iron regulatory proteins such as the liver hormone hepcidin are still mostly at the research level. Translation to
interferon gamma (IFN-γ), interleukin-1 (IL-1), tumour necrosis factor (TNF-β), bone morphogenic proteins 2, 4, 5, 6, 7, 9 (BMPs), growth differentiating factor 15 (GDF15) and matriptase-2 (TMPRSS6) (Huang et al. 2011).

Ferroportin has been identified as the main cellular iron export protein in the enterocytes of the gastro-intestinal tract and placenta and also in the macrophages of the reticulo-endothelial system (RES).

Hepcidin is now recognized as the major systemic (negative) regulator of iron through its ability to degrade ferroportin and subsequently to inhibit extracellular iron transit (Theurl et al 2009). An increase in hepcidin levels leads to reduced dietary iron absorption in intestinal enterocytes and also a blockade of iron export from the RES. Hepcidin has been shown to respond rapidly to changes in haematological status with anaemia, hypoxia and erythropoiesis all suppressing hepcidin expression (Thomas et al 2011). Synthesis of hepcidin is stimulated by iron overload, an increase in BMPs or the presence of inflammatory cytokines such as interleukin-6 (IL-6), interleukin-1 (IL-1), tumour necrosis factor (TNFβ) or interferon gamma (IFγ).

Laboratory testing

Due to the lack of clear cut diagnostic criteria for determining iron status it is not uncommon for medical personnel to forgo laboratory testing and prescribe a treatment of oral iron when presented with clinical signs and symptoms that suggest anaemia. For those patients who do not have ID/IDA such a regime has the potential to produce iron toxicity and a further deterioration in the patients’ health. Because of the high incidence of anaemia and ID there has been a drive to improve existing testing and to identify new biomarkers for iron.

1. Bone marrow iron stain

Initially the standard for determining the level of storage iron present in an individual was a bone marrow aspirate stained by Perl's method to demonstrate the presence of hemosiderin and ferritin (Dacie et al 2006).

In IDA neither macrophage nor erythroblast iron is present (Hoffbrand et al 2005), whereas in ACD or FID iron is usually present but not able to be utilised.

The bone marrow iron stain is not infallible: one study reported incorrect results in 30% of cases reviewed (Cook 2005). A bone marrow iron stain is unsuitable as a screening test because of the invasive nature of the test, the expense and often the lack of clinical expertise required to perform this procedure. Since the advent of biochemical blood tests like the serum ferritin the use of a bone marrow iron stain to assess iron status has declined.

2. Biochemical markers of iron status and inflammation

The most commonly used biomarkers for iron status all have advantages and disadvantages in regard to their ability to accurately and quickly differentiate between ID, IDA, ACD, FID and ID/ACD.

Serum ferritin

Serum ferritin is an early marker of ID with levels falling below normal limits well before morphological changes appear in the mature red cell. Values of <15µg/L are diagnostic of iron deficiency (Lewis et al 2006). In childhood lower ranges of serum ferritin correspond to the higher demands for iron required for growth.

IDA can develop within three months in premature infants in contrast to, four years for an adult male to deplete body iron stores and develop IDA due to dietary insufficiency or malabsorption.

The major limitation of the diagnostic ability of serum ferritin to detect ID is the sensitivity of serum ferritin to inflammation. Patients with ACD who have serum ferritin values within the reference range may still be iron deficient. Patients with chronic renal disease (CRD) on regular haemodialysis and undergoing treatment with recombinant erythropoietin have been shown to respond to iron therapy even with serum ferritin levels above 200µg/L. Serum ferritin levels are also known to be increased independent of iron status in various clinical conditions and cannot be used as a reliable indicator of iron storage. Such clinical situations include hyperthyroidism, liver disease, some malignancies, alcohol consumption and oral contraceptive pill therapy (Brugnara 2003, Cook 2005). If serum ferritin is to be used to diagnose ID in the presence of inflammation then diagnostic cut-off points need to be carefully evaluated to reflect the incidence of inflammation and reference ranges need to be tailored to the local patient demographic.

Serum iron

Serum iron lacks specificity as values are low in IDA, ACD and FID. The concentration of serum iron is variable and changes with recent iron ingestion and diurnal variation; iron levels are highest in the morning and lowest at midnight. Individuals may show a physiological variation of up to 50%.

Serum transferrin and total iron binding capacity (TIBC)

Transferrin is a glycoprotein which binds iron in the circulation and then transports it to transferrin receptors located on the surface of cells needing iron. It is synthesized in the liver in response to cellular demand and levels will be elevated in ID and reduced in iron overload. The life
and reference ranges vary considerably because of the lack of standardization between methods and this is a major analytical limitation of the sTfR in detecting ID.

### Zinc protoporphyrin (ZPP)

Zinc protoporphyrin is a product of abnormal heme synthesis. When iron supply for incorporation into the heme moiety of haemoglobin is restricted, there is an accumulation of the immediate precursor protoporphyrin IX. In such instances zinc is readily substituted in the absence of iron to form ZPP (Hoffbrand et al 2005).

Elevated levels of ZPP may detect the presence and severity of IDA but ZPP is not suitable for early detection of ID as the associated rise in protoporphyrin may take weeks to become evident. An increase in ZPP is not specific to ID as increased levels are also seen in lead poisoning and ACD. False elevation in ZPP values may be seen in patients on haemodialysis or with elevated serum bilirubin levels (Cook et al 2005).

The upper limit of the reference range for ZPP has been quoted by various authors as 40 (Hastka et al 1993), 60 (Lynch et al 1998) or 80 umol/mol heme (Lasocki et al 2011). IDA is said to present at levels of >80 umol/mol heme and severe IDA is associated with ZPP levels of >200 umol/mol heme, however, ACD is also associated with elevated ZPP levels (>100umol/mol of heme) (Hastka et al 1993).

### 3. Haematological markers of iron status

Automated haematology analysers provide accurate, rapid and reproducible cell measurements. The haemoglobin, red cell count and associated indices may be used to screen for the presence of ID and IDA. Many of these parameters lack sensitivity and/or specificity as they are based on erythrocytes which have a life span of up to 120 days.

Reticulocytes mature in two to four days and are a subset of erythrocytes. The haemoglobin content of reticulocytes will fall within days of the onset of ID and provide an indication of the most recent iron uptake in the bone marrow.

### Haemoglobin (Hgb)

Haemoglobin carries the largest component of iron in the body. There are 400 million molecules of haemoglobin in a single erythrocyte and thus any alteration in haemoglobin synthesis, erythrocyte production or lifespan will influence the body’s iron levels. A decreased level of haemoglobin generally indicates the severity of ID after anaemia has developed. But haemoglobin synthesis is also affected by a number of other clinical disorders and lacks specificity for the diagnosis of IDA. In the early stages of ID before erythropoiesis is affected, the haemoglobin level may still be normal.
Mean cell haemoglobin (MCH)

The MCH is a product of the mean erythrocyte cell volume (MCV) and the mean cell haemoglobin concentration (MCHC) and is an average of the haemoglobin content per red cell. An MCH <26pg is a more sensitive indicator of ID than an MCV <80fL (Bain 2006) unless the patient has large erythrocytes which are hypochromic; then the MCH value may fall within the reference range (Jandl 1996).

Mean cell haemoglobin concentration (MCHC)

The MCHC is the average concentration of haemoglobin in circulating erythrocytes. Values of <320g/l have been used to screen for ID but reference ranges are specific to the analysis technique. Also normal MCHC values may be seen in patients who have hypochromic erythrocytes but a proportionally low red cell mass (Jandl 1996). In vitro influences that affect the erythrocyte volume due to hypo-osmolar states may produce incorrect MCV, MCH and MCHC measurements (Bain 2006).

In a study of Malawian children with severe anaemia, it was proposed that “the MCHC may be an acceptable alternative screening test (for IDA) in resource-poor settings” (Phiri et al 2009).

Erythrocyte mean cell volume (MCV)

Haemoglobin contributes to over 90% of the erythrocyte and reticulocyte content. The cell size is considered to be an indirect estimate of the amount of haemoglobin present and therefore an indication of iron supplied for erythropoiesis (Urrechaga et al 2011). Microcytosis develops when the haemoglobin content in mature erythroblasts falls below 20pg/cell and an extra cell division occurs (Jandl 1996).

In the manufacture of haemoglobin, globin synthesis is coordinated with the synthesis of porphyrin, the precursor to haem. Microcytosis and hypochromia are both caused by a decreased concentration of haem. Haem regulates eIF2α (HRI), a key step in the synthesis of alpha and beta globin chains in erythrocytes. When intracellular haem is decreased the translation initiation factor eIF2α is inhibited and globin synthesis is reduced (Brugnara 2003).

The MCV lacks specificity for the diagnosis of ID as low MCV values are also seen in ACD, thalassaemia, and sideroblastic anaemia. Conversely renal dialysis patients may show a higher MCV compared to their base line values pre renal disease. Combined haematinic deficiencies of ID and megaloblastosis may give a normal MCV albeit with a raised RDW.

The MCV is subject to in vitro instability due to values increasing with specimen age and ambient temperature and therefore the MCV will not detect early ID and may not identify ID in a complicated and/or remote clinical setting.

Red cell distribution width (RDW)

The red cell distribution width (RDW) indicates the variation in size of the erythrocyte. It is measured as the standard deviation (SD) and coefficient of variation (CV) of the erythrocyte histogram from which the erythrocyte count and MCV are derived. The reference range for RDW CV is approximately 11–15% but will vary slightly depending on the technology used. An increase in the RDW is a very sensitive indicator for iron deficiency as erythrocyte anisocytosis precedes anaemia and the RDW is usually the first FBC parameter to become abnormal in ID. However, the RDW lacks specificity as raised RDW values are also associated with an increased reticulocyte count, other haematinic deficiencies and numerous haematological disorders.

Reticulocyte and hypochromic/microcytic red cell indices

The most immediate consequence of reduced iron supply is an inhibition in the haemoglobin formation in reticulocytes. The reticulocyte haemoglobin which measures the haemoglobin content of newly produced erythrocytes has been proposed as an indicator of the most recent iron availability in the bone marrow.

Some of the newer reticulocyte and red cell indices are unique to the model and/or individual brands of haematology instruments and reference ranges for these values are usually analyser specific.

The reticulocyte haemoglobin content (CHR), mean cell volume of reticulocyte (MCVr), percentage hypochromic red cells (%HYPO) and percentage microcytic red cells (%Micro) are produced by the Advia 120 & 2120i® analysers (Siemens Healthcare, Dublin Ireland).

The reticulocyte haemoglobin equivalent (Ret-He), hypochromic red cell equivalent (Hypo-He) and percentage microcytic red cells (%MicroR) are produced by the Sysmex XE5000® (Sysmex Corporation, Kobe, Japan).

The red cell size factor (RSF) and low haemoglobin density percentage (LHD%) are produced by the LH 750® (Beckman Coulter Inc. California USA).

Reticulocyte haemoglobin content (CHR)

The CHR is the mean value of haemoglobin mass of each reticulocyte in pictograms. It is determined by two independent flow cytometric measurements:

\[
CHR \text{(pg)} = MCVr \times CHCMr.
\]

The MCVr is the reticulocyte MCV and the CHCMr is the cellular haemoglobin content mean of reticulocytes.
An increase in hypochromic reticulocytes produces a low CHr value whereas replenishment of iron status stimulates an elevation in CHr levels.

The CHr has been shown to detect early ID in adults and healthy adolescents. In one study the CHr reference range in healthy adults was cited as 29-32pg with a cut-off value of <29pg used to detect ID/IDA. Slightly lower cut-off values were used in infants (27.2pg) and toddlers (28.4pg) (Stoffman et al 2005). Such cut-off values may vary depending on co-existing clinical conditions. Thalassaemia patients have been shown to demonstrate a CHr value which was significantly lower than those seen in ID. Patients with megaloblastic/macrocytic erythropoiesis who have macrocytic reticulocytes (e.g. some chemotherapy patients) will have an increased CHr (Brugnara 2003).

CHr values have also been used to reduce dosages in iron therapy for haemodialysis patients who are refractory to recombinant erythropoietin treatment. A combination of CHr <29pg and percentage hypochromic red cell (%HYPO) >6% was found to have the best discriminative ability for detecting a response to intra-venous iron therapy (Tessitore 2001).

Reticulocyte haemoglobin equivalent (Ret-He)

The Ret-He is analogous to the CHr and is measured in the same units but as the measurement is indirect it has been termed an ‘equivalent’. It is calculated from the RET-Y which is the mean value of forward light scatter by reticulocytes and is a relative measure of the reticulocyte size (Bain 2006). The RET-Y is combined with the optical refractive index of the reticulocyte in a regression plot to obtain an estimate of the haemoglobin concentration of the reticulocyte:

\[ \text{Ret-He (pg)} = 5.5569e0.001 \text{RET-Y} \]

Investigations into the clinical usefulness of the Ret-He based on biochemical iron markers and the CHr showed it to be similar to the CHr in terms of reference ranges and ability to diagnose ID. The reference range for Ret-He (28.6-36.3pg) was similar to that of the CHr (27.8-34.5pg). Receiver operator characteristics curve (ROC) analysis for the diagnosis of ID gave a Ret-He cut-off of 27.2pg with a sensitivity of 93.4% and specificity of 83.2% (AUC = 0.913) P<0.0001 (Brugnara et al 2006). The Ret-He was approved for clinical application in the USA by the FDA in 2005 (Jayaranee et al 2002). A recent study showed that when diagnosing ID in haemodialysis patients the CHr and the Ret-He gave similar diagnostic performances but with higher values than that seen in non-haemodialysis patients. ROC analysis gave diagnostic cut-off values of 31.2pg for the CHr and 30.9pg for the Ret-He. This was higher than the target of 29pg proposed by the American Kidney Foundation (KDOQI) Guidelines 2006 (Buttarello et al 2010).

The Ret-He has also been used in haemodialysis patients to detect a response to iron therapy within two weeks of treatment (Miwa et al 2010).

For patients in remote locations the Ret-He may be useful. A study by Brugnara and colleagues demonstrated a stability of at least 48 h with a change in values of <1SD (0.35pg) in samples stored at room temperature (Brugnara et al 2006).

Red cell size factor (RSf)

The Red cell size factor (RSf) uses the volume of both mature (MCV) and immature red cells (MRV):

\[ \text{RSf (fL)} = \sqrt{\text{MCV} \times \text{MRV}} \]

Changes in volume reflect changes in the cellular haemoglobin content. Correlation of the RSf with the CHr was observed to be 0.85 (Urrechaga 2009). In another study the RSf was found to correlate with the Ret-He (r2 = 0.8154). This was in both normal subjects and in patients with ID, beta thalassemia minor, or those undergoing haemodialysis with iron therapy and erythropoiesis stimulating agents (ESA). The mean RSf for iron deficient patients was found to be 82.1 fL, compared with 76.9 fL for beta thalassaemia trait patients. Both of these patient groups had significantly different values compared to the reference range mean of 100.9 fL. However there was some overlap in RSf values between healthy patients and those with IDA and also between chronic kidney disease (CKD) patients and those with ID. The RSf had a higher SD compared to the Ret-He in all four patient groups (Urrechaga et al 2011).

Percentage hypochromic red cells (%HYPO), hypochromic red cell equivalent (Hypo-He) and low haemoglobin density percentage (LHD%)

When iron supply for erythropoiesis diminishes hypochromic erythrocytes are produced (Hoffbrand et al 2005). Hypochromia manifests before the advent of microcytosis (Urrechaga et al 2009).

The %HYPO is exclusive to the Advia 120 and 2120i®. The Sysmex XE5000® uses a similar but indirect measurement called the Hypo-He, and the Beckman Coulter LH 750® generates the LHD%.

The %HYPO measures the percentage of red cells with a cellular haemoglobin content mean (CHCM) <280 g/l (David et al 2006). For a diagnosis of ID a %HYPO value of >6% was found to be comparable to a CHr cut-off <29pg (Wish 2006). The %HYPO is also considered to be a sensitive marker of FID if measured within six to eight hours of collection. Limitations of the %HYPO relate to inaccuracies in the measurement of the CHCM and MCV. A false increase in %HYPO will occur when the erythrocyte swells with specimen age and higher ambient
storage temperatures (Brugnara 2003). The %HYPO has been recommended for use in conjunction with biochemical iron markers in the American Kidney Foundation (KDOQI) Guidelines for monitoring erythropoietin therapy in haemodialysis patients.

The Hypo-He calculates the percentage of red cells with an Hgb of <17pg (Urrechaga et al 2010), and utilizes both the absolute amount of haemoglobin present in the mature red cell as well as the size of the cell (Wish 2006). A reference range for the Hypo-He has been established as 0–0.6% and a cut-off value of >1.8% was proposed for the diagnosis of ID (AUC = 0.963, a sensitivity of 98.3% and a specificity of 91.1%) (Urrechaga et al 2009).

In a study by Grillo et al 2006, the red cells of renal dialysis patients appeared to be recognized as hypochromic by the %HYPO but normochromic by the Hypo-He. The Hypo-He was deemed to be unreliable in detecting hypochromia in renal dialysis patients. Another study investigating the diagnosis of ID in renal haemodialysis patients and also for predicting response to iron therapy obtained cut-off values of >5.8% for %HYPO (AUC = 0.72) and >2.7% for Hypo-He (AUC = 0.68). Hypo-He values were found to decrease with specimen age and produce false negatives. It was concluded that the CHr and Ret-He were the best predictors of response to iron therapy followed by the %HYPO (Buttarello et al 2010).

The low haemoglobin density percentage (LHD%) is derived from the MCHC using the formula:

\[
\text{LHD}\% = 100 \sqrt{1 - \frac{1}{e^{1.8(30 - \text{MCHC})}}}
\]

and it correlated with the %HYPO (\(r^2 = 0.869\)).

Using a LHD cut-off value of >4% for the diagnosis of IDA, ROC curve analysis gave an AUC = 0.976, a sensitivity of 95.2% and specificity of 93.3%. For LDH% was found to be non-Gaussian. The LHD% of normal subjects and beta thalassaemia minor patients demonstrated a narrow standard deviation, whereas patients with IDA, CKD and ACD had large standard deviations (Urrechaga et al 2010).

**Percentage Microcytic Red Cells (%MicroR)**

The %MicroR is a research parameter available on the Sysmex XE5000® and has the potential to detect aberrations in iron metabolism and measure response to iron medication. Clinical and analytical limitations encountered with the MCV also apply to the %MicroR. A study performed by Urrechaga et al 2009 established a reference range of 0.2 – 1.9 % (median 1.1, SD 0.44).

**Percentage hypochromic reticulocyte (%HYPOr)**

The %HYPOr is considered to be an early marker of diminished iron supply due to the rapid maturation of reticulocytes. In a study comparing %HYPOr against a bone marrow iron stain, patients with stainable iron had mean values which were significantly different to those with depleted iron stores i.e. 25.0 +/- 19.1 compared to 54.9 +/- 27.9. Unfortunately the ranges were so wide that there was considerable overlap between the two groups (Ervasti et al 2004).

**4. Iron regulatory proteins (IRPs)**

**Hepcidin**

Initial methods to measure urine and plasma hepcidin have been cumbersome and lacked standardization. Some commercial assays have become available but further harmonization is required before a hepcidin assay becomes routine (Galesloot et al 2011).

Hepcidin has been found to be more responsive to erythropoietic demands for iron than to the stimulus of inflammation. Patients with IDA are able to absorb dietary iron and mobilize iron from macrophages even in the presence of inflammation. Patients with ACD/IDA have lower levels of circulating hepcidin than those with ACD alone. The most significant increase in hepcidin values are seen at ferritin concentrations >200ug/L, when iron overload begins (Theurl et al 2009).

Using regression analysis it was proposed that hepcidin levels below 0.23nmol/L confirmed ID and values above 4 nmol/L excluded ID (Thomas et al 2011). A recent study in critically ill patients with anaemia proposed a plasma hepcidin cut off value of 129.5ug/l for diagnosing ID. Plasma hepcidin was undetectable (< 0.05ug/L) in patients with pure IDA compared to patients with ACD where the median plasma hepcidin was 566ug/L (range 297–1040). Critically ill patients who had ID either alone or in combination with inflammation gave a range of 0.05–548.3ug/L with a median of 80.5 (Lasocki et al 2010).

**Other IRPs**

Duodenal iron absorption is also inhibited by down-regulation of DMT-1), a transporter of iron, lead, zinc and copper across the apical boarder of enterocytes in the proximal duodenum.

Newly discovered erythropoiesis driven regulators of iron homeostasis are GDF15 and TMPRSS6. The former is a hormone that inhibits hepcidin expression and appears to be induced by inflammation. Levels of GDF15 are higher in both ACD and ACD/IDA compared to those with IDA alone (Cullis 2011). TMPRSS6 is sensitive to iron and blocks hepatic hepcidin synthesis by clearing membrane-bound Hemojuvelin (Theurl et al 2009; Lasocki et al 2011).

Hypoxia-inducible transcription factor (HIF) is an oxygen sensitive nuclear transcription factor which has been shown to stimulate expression of duodenal cytochrome b
(DcytB) and DMT-1 resulting in an increase in intestinal iron uptake (Shah et al 2009).

5. Algorithms for diagnosis ID/IDA/ACD

Rather than use a single test to determine a patient’s iron status, a flow chart or algorithm combining a number of tests may be used to interpret a patient’s biochemical and haematological profile (Fig.1 and Table 1).

![Figure 1. Possible algorithm for the differential diagnosis of IDA, ACD and ACD/IDA (Adapted from Cullis et al 2011 modified from Weiss & Goodnough 2005)](image)

Table 1. Anaemia screening

<table>
<thead>
<tr>
<th>Serum ferritin</th>
<th>Iron deficiency anaemia (IDA)</th>
<th>IDA +ACD</th>
<th>Anaemia of chronic disease (ACD)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Low</td>
<td>Low</td>
<td>30–100</td>
<td>&gt;100</td>
</tr>
<tr>
<td>Normal</td>
<td>Normal</td>
<td>High</td>
<td>High</td>
</tr>
<tr>
<td>CRP</td>
<td>CHr / Ret-He</td>
<td>Low</td>
<td>Low</td>
</tr>
<tr>
<td>High</td>
<td>High</td>
<td>High</td>
<td>Normal</td>
</tr>
<tr>
<td>sTfR</td>
<td>Hepcidin (urine or plasma)</td>
<td>Low</td>
<td>Low/Normal</td>
</tr>
<tr>
<td>sTfR/log ferritin &gt;2</td>
<td></td>
<td>Raised</td>
<td></td>
</tr>
<tr>
<td>Ferritin normal / increased &gt;100ug/l</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Ferritin index (sTfR/log ferritin)

The use of the transferrin-ferritin index was first proposed by Punnonen et al in 1997 to improve the detection of ID in particular cases with a high incidence of infection: sTfR Index = sTfR / [log serum ferritin].

A ratio of <1 suggests ACD whereas a ratio of >2 suggest IDA+/-ACD.

The use of this index was found to ameliorate the effects of infection on serum ferritin and also haemolysis on sTfR when investigating severe anaemia in Malawian children (Phiri et al 2009).

Ferritin index vs. CHr (Thomas Plot 1)

In 2002 Thomas and Thomas proposed a graph of CHr against the Ferritin Index (sTfR/log Ferritin). A CHr cut-off of <28pg and a Ferritin Index cut-off of >0.8 (if the CRP >5), or a Ferritin Index cut-off of >1.5 (when no inflammation was present) was used to produce four quadrants:

1. Normal: iron replete (Ferritin Index <0.8/1.5) with normal erythropoiesis (CHr >28pg)
2. ID: reduced iron supply (Ferritin Index >0.8/1.5) but normal erythropoiesis (CHr >28pg)
3. IDA: depletion of iron (Ferritin Index >0.8/1.5) with ID erythropoiesis (CHr <28pg)
4. FID: iron replete (Ferritin Index <0.8/1.5) with reduced haemoglobinisation of red cells (CHr <28pg).

Hepcidin vs CHr (Thomas Plot 2)

The ‘Thomas plot’ was later updated by substituting the serum hepcidin concentration for the Ferritin index. The hepcidin cut-off used for ID was <4.0nmol/L (Thomas et al 2010).

6. Point-of-care (POC)

There are a number of POC devices that can detect anaemia by measuring haemoglobin but none that can indicate the cause of anaemia or differentiate between IDA and ACD.

HemoCue® Haemoglobin

HemoCue (Angelholm, Sweden) have a number of models including the Hb201+ (measurement range 20–250g/L). Ten microliters (uL) of capillary, venous or arterial blood is required. The haemoglobin is measured at two wavelengths in order to compensate for turbidity. The Hb201+ system is factory calibrated against the hemiglobincyanide method and needs no further calibration. Turnaround time is one minute and precision is <4%.

Pronto-7® Haemoglobin

The Pronto-7 (Masimo, Irvine, California, USA) is a palm-sized handheld device which measures the haemoglobin (SpHb), arterial oxygen saturation (SpO2), pulse rate and perfusion index, by pulse oximetry. The SpHb measurement range is 0–250g/L with a SD of <1.1g/L. SpHb comparison with reference laboratory haemoglobin readings were within 2g/L for more than 95% of measurements. The Pronto-7 is non-invasive, provides results within one minute and does not require any consumables.

Measurement of haemoglobin mass via carbon monoxide rebreathing

Direct measurement of haemoglobin mass (Hbmass) via carbon monoxide (CO) rebreathing has been used...
to monitor treatment for iron deficiency anaemia in an endurance runner (Garvican et al 2011). Carbon monoxide rebreathing is not affected by plasma volume shifts and may be sensitive to changes in total haemoglobin content. The technique involves rebreathing of a CO bolus of 0.8-1.2 mL/kg for two minutes after which the percentage of HbCO in the blood was measured via capillary finger-tip samples (200uL). These samples were collected prior to and seven minutes after the bolus was administered and analysed immediately using an OSM3 hexiometer (Radiometer, Copenhagen, Denmark). Rebreathing of CO is unlikely to be recommended in sick patients.

Clinical disorders

Many patients suffering from IDA and/or ACD will also present with inflammation, infection or malignancy. Diagnostic cut-offs for many of the preceding tests may vary depending on the different clinical presentations.

ACD

ACD is the most prevalent anaemia in hospitalized patients and the second most common anaemia worldwide. Patients’ erythrocytes are usually normochromic and normocytic but with increasing severity or duration may become hypochromic / microcytic. The MCV is higher in ACD compared with IDA (Markiovic et al 2007).

Anaemia in ACD is caused by immune mechanisms. Such mechanisms include: an impaired biological activity and production of erythropoietin (EPO)’ reduced erythroid responsiveness to EPO, repressed erythropoiesis, a reduced erythrocyte survival and an increased iron sequestration within the RES.

Other features of this hypoproliferative anaemia are: a low serum iron decreased TSAT, reduced numbers of bone marrow sideroblasts, increased RES iron, a normal or raised serum ferritin and an increase in inflammatory cytokines. Tumour necrosis factor alpha (TNF-α) induces erythroid apoptosis and limits the numbers of erythroid progenitors. Interleukin six (IL-6) stimulates production of hepcidin (Cullis 2011). Interferon gamma (IF-γ) has been shown in a rat model to be associated with enhanced expression of DMT-1 resulting in the uptake of transferrin bound iron in macrophages, and a reduction in ferroportin synthesis and macrophage iron export (Theurl et al 2009).

Many patients present with ACD combined with IDA particularly if there is chronic blood loss. In such cases the TIBC, TSAT, CHr and Ret-He levels will be reduced, the sTfR will be raised, and the serum ferritin normal. Newer tests such as the CHr, Ret-He and sTfR have been found to have a better sensitivity for diagnosing ID in the presence of inflammation than serum ferritin. Treatment of anaemia in ACD is aimed at remedying the underlying condition. Differentiating between ACD and IDA/ACD is important because inappropriate iron therapy may be toxic and may also progress the underlying disease (Theurl et al 2009).

Renal haemodialysis patients on ESA therapy - FID

Correction of anaemia in haemodialysis patients provides the patient with a better quality of life via a higher tolerance for exercise, an improvement in cognitive and cardiovascular functions, reduced hospitalization and lower mortality (Buttarello et al 2010). Renal failure is associated with anaemia due to an inability to produce erythropoietin. This anaemia is usually treated with exogenous ESAe.g. recombinant erythropoietin as an alternative to repeated erythrocyte transfusions. Such ESA therapy reduces the risk of iron overload, immunomodulation and other potential transfusion hazards.

A common outcome of ESA therapy is FID and ESA ‘resistance’ which occurs when erythropoiesis is stimulated in excess of the amount of endogenous iron available. Treatment with intravenous iron has been shown to improve the haemoglobin level on such occasions. FID has been shown to occur in haemodialysis patients with serum ferritin levels of between 200–1000μg/l (Brugnara 2003). FID may be compounded by the presence of absolute ID which can occur in patients on haemodialysis due to blood loss. The CHr and Ret-He has been used to predict ID in the setting of ESA resistance.

Anaemia of the critically ill patient

Anaemia develops in many patients admitted to the intensive care unit (ICU) and is associated with increased length of stay and increased mortality. ID may affect as many as 30-40% of anaemic ICU patients. The concentration of EPO in critically ill patients has been found to be lower than would be expected for the level of anaemia present and iron infusions and injections of ESAs have been proposed as alternatives to blood transfusion for the treatment of IDA (Lasocki et al 2011).

Anaemia in surgery

The use of iron and ESA therapy as an alternative to blood transfusions has also been applied in orthopaedic surgery. In a Netherlands study, the Ret-He was used to identify ID in pre-operative orthopaedic patients and also to tailor pre and post-operative infusions of iron combined with injections of erythropoietin. A Ret-He cut-off of <30.61pg was used to identify the presence of ID reticulocytes and initiate treatment. Over a five year period of more than 1000 hip and knee operations, the number of units transfused dropped from 98 to 16 bags of blood per annum (Muusze et al 2009).

Pregnancy and the newborn

There is a high requirement for iron in pregnancy due to an expansion of plasma volume, increased erythropoiesis and increased demands from the feto-placental unit. The World Health Organisation (WHO) has defined anaemia in pregnancy as haemoglobin <105g/L in the second and third
ID has been shown to cause foetal growth retardation, amnion rupture, infection and intrauterine death. As a result of ID, abnormal foeto-placental development may cause health problems in the newborn and in adult life (Breymann 2002).

In the mother ID can increase the risk of preeclampsia and trigger the premature delivery of a low birth weight baby (Cetin et al 2011). The amount of iron in a neonate is contingent on its blood volume and haemoglobin concentration, which is in turn determined by the newborn's birth weight. Neonatal iron reserves are used for growth in the first six months of life but in a premature baby these iron reserves may be exhausted by two to three months of age. It has been recommended that premature infants be tested for iron deficiency at three months and all children be tested at nine months in order to prevent impaired intellectual development and other sequelae. In a paediatric study in the USA a CHr of >29pg gave a 0% chance of ID and a CHr <20pg gave a 90% chance of ID (Brugnara 2003).

In pregnancy, a low maternal iron status is associated with a risk for an enlarged placenta and high blood pressure for the offspring in adulthood (Cetin et al 2011). The high incidence of ID in Australian indigenous antenatal patients may contribute to the high incidence of hypertension in the adult Australian indigenous population.

**Anaemia in Australian indigenous children**

A study conducted on paediatric admissions to Royal Darwin Hospital found that most were suffering from anaemia and that the majority were of Aboriginal ethnicity (76%). IDA with or without inflammation was present in more than 50% of these paediatric patients. Approximately one-third still had ID and/or inflammation even in those who were not anaemic. Diagnosis of IDA was based on CRP, haemoglobin, MCV, RDW and the presence of microcytic, hypochromic red cells in the blood film. In this demographic the incidence of infection or inflammation was high and the sTfR was more accurate at diagnosing IDA than serum ferritin. A range of 2.7-3.2mg/L for the sTfR was suggested as a window for detecting early ID. It was concluded that the sub-set of patients most likely to benefit from the measurement of sTfR were those with suspected ID and concurrent infection (Ritchie et al 2004).

**Discussion**

Adverse consequences of ID include anaemia, impaired exercise capacity, alteration of behaviour and cognitive performance, premature delivery in pregnancy and impaired development in children. Early recognition of iron deficiency even before the development of anaemia is useful to optimize health and well-being particularly in neonates, adolescents and antenatal patients (Stoffman et al 2005).

For the first 70 – 80 years of the 20th century the benchmark for determining iron status was the bone marrow iron stain, but significant drawbacks of this test include the invasive nature of the sample collection, the expense and the inability to diagnose ACD or FID.

Diagnostic tests currently used for assessing iron status include indicators of haemoglobin maturation (Hgb, CHr, Ret-He), mature red cell indices (MCV, MCH, MCHC, RDW) and biochemical markers of iron uptake (TIBC, sTfR), iron availability (TSAT) and iron storage (serum ferritin) (Urrechaga et al 2009).

The serum ferritin and TSAT remain the favoured markers for assessment of iron status in uncomplicated ID; however the serum ferritin lacks sensitivity in the presence of inflammation, infection or malignancy. The clinician faced with a patient who has anaemia with multiple medical problems and is failing to achieve their therapeutic haemoglobin target or is suspected of having FID requires additional diagnostic criteria to help determine their patient’s iron status (Wish 2006).

Erythropoietin levels are increased in IDA (with or without ACD) but are non-existent in ACD alone; however the measurement of endogenous EPO levels is considered to be impractical and inconclusive.

The sTfR may be of value in patients whose conventional iron studies are inconclusive or are thought to be influenced by inflammation. It is necessary however to exclude other causes of increased sTfR levels before ID can be diagnosed, e.g. haemolysis or megaloblastosis. The reference ranges of the sTfR have yet to be harmonised and there is no consensus regarding cut-off values (Wish 2006). Cut-off values will also vary depending on the laboratory kit used and the age, ethnicity and altitude of the patient. Availability of the sTfR is limited and test TATs particularly for non-metropolitan patients may be lengthy.

The Ferritin Index has not gained wide acceptance (Theurl et al 2009) and has several shortcomings including the limitations of the sTfR mentioned above and also the inability to differentiate between IDA and IDA/ACD.

Reticulocyte haemoglobin measurements (Chr or Ret-He) have been proposed as an early measurement of ID when changes in the mature red cell are not yet apparent or biochemical tests are inconclusive. A number of studies have been performed to assess the clinical usefulness of reticulocyte indices for the differential diagnosis of ID, ACD.
and FID. In young children, the CHr has been found to be the strongest predictor of ID/IDA when compared to biochemical tests for ID. The CHr does not appear to be influenced by acute phase reactants and has been found useful in assessing patients with end stage renal failure. The CHr has also been used as an early indicator of response to iron therapy. The USA Food and Drug Administration approved the use of the CHr for clinical application in 1997 and the Ret-He in 2005. The American Kidney Foundation Guidelines NKF-K/DOQI published in 2006 has recommended the use of the CHr for diagnosing FID in renal haemodialysis patients being treated with ESA and states that ID is present if CHr <29.0pg. The Japanese Society for Dialysis Patients recommended a cut-off value CHr <32.2pg for ID in 2004. This discrepancy may be related to racial differences in reference ranges for the CHr. A low CHr is not specific to ID and thalassaemia needs to be excluded (Stoffman et al 2005).

Some Ret-He diagnostic cut-off values for detecting ID can overlap reference ranges as Ret-He measurements are influenced by cell volume. False normal values may occur in patients where ID co-exists with clinical conditions which produce macrocytic erythrocytes (megaloblastosis, polychromasia or pregnancy) and on these occasions ID could be missed. Renal dialysis patients have been found to have a higher MCV compared to non-dialysis patients independent of EPO therapy, folate or B12 levels. Macrocytosis in renal dialysis patients is due to uremia and/or other metabolic by-products of renal failure. A correlation was found between the CHr and the RET-Y (from which the Ret-He is derived) in patients with iron deficiency ($r^2 = 0.9033$), but not in renal dialysis patients ($r^2 = 0.699$) Grillo et al 2006.

Wider utilization of the CHr/Ret-He in the screening of ID might occur if reference ranges could be validated up to 48 h for local populations and/or different clinical categories. This would allow remote medical services such as the Royal Flying Doctor Service to add these tests to a routine full blood count (FBC) request. Specimens would need to be kept refrigerated to minimize the expansion of erythrocytes with specimen age.

A urine or circulating hepcidin level has the potential to differentiate between IDA and FID but may not have the sensitivity to differentiate between ACD and ACD/IDA. There is a lack of widely available methods for measuring hepcidin. Mass spectrometry and ELISA methods have been used in research studies and release of commercial assays have been limited (Cullis 2011). Reference procedures and standardized calibrators are still in the development phase and reference ranges have yet to be synchronized between the different methods (Kroot et al 2009, Galesloot et al 2011, Beramaschi et al 2009).

The exciting aspect of IRPs is their potential to identify therapeutic agents. Monoclonal antibodies directed against BMP-2, BMP-6, IL-6 and TNF-α have been shown experimentally to counteract the reduced erythroid responsiveness that occurs in ACDand heparin has been shown to inhibit hepcidin (Cullis 2011). Despite the fact that IRPs like hepcidin, BMPs and GDF15 have been identified, standardized assays have not yet translated to routine laboratory testing.

In addition to diagnostic accuracy and reproducibility, the clinical utility of a test encompasses the cost, the test TAT and Quality Assurance Program (QAP) availability. The test TAT will vary between laboratories and may also be affected by the proximity of the patient to the testing laboratory. The majority of the newer iron markers which have progressed to the pathology laboratory such as the sTfR, CHr and Ret-He do not have Quality Assurance Program (QAP) assays with which to perform peer review.

**Conclusion**

Iron metabolism is a dynamic process and depending on the clinical presentation may require more than one diagnostic test to evaluate the iron status of the patient and even then a clear cut diagnosis may not be evident.

For a more accurate diagnosis of ID in a variety of disorders and clinical situations, it is recommended that reference ranges and cut-off values for serum ferritin, sTfR, reticulocyte haemoglobin (CHr, Ret-He), HYPO%, MCV, MCH and MCHC be established to reflect the local patient population based on clinical outcomes, rather than a gold standard. Environmental exposure to inflammation or infection as well as differences in age, gender, race, pregnancy status, habitat and altitude are influences which need to be accounted for. The accuracy, sensitivity and specificity of an algorithm, or single test's ability to diagnose ID in various clinical situations might then be improved.

Until diagnostic cut-off values and reference ranges can be tailored to the patient population the newer tests are less likely to be credible as screening tests for ID and will remain as supplementary aids for interpreting the more challenging cases.

Often those at highest risk of ID are the least likely to be serviced promptly by advanced medical technology due to their remote location. Ideally a POC test would best suit these patients’ needs. Such an assay would need to be widespread, robust, informative and readily available to clinical staff for the purpose of diagnosing ID in patients with uncharacteristic markers of iron metabolism.

Until a better POC test can be designed and proven, a clinician may obtain a reasonably reliable and prompt answer to assessing iron status using the serum ferritin when inflammation is not present. For patients where inflammation and/or malignancy are likely, the CRP, RDW, CHr, Ret-He, MCH and MCHC could assist in
determining the patients iron status. Further diagnostic refinement could be attained if the diagnostic cut-off values and reference ranges for these supplementary tests can be tailored to the resident population and their prevalent diseases. The newer IRPs are still research subjects and the sTfR lacks standardization and often has a test TAT that reduces clinical effectiveness. The recent Australian Health Survey 2010-11 measured the biomarkers haemoglobin, serum ferritin and soluble transferrin receptor to estimate the prevalence and severity of ID nationally (ABS Anaemia D5-D64). This emphasizes the importance of identifying ID early and accurately so that the human and financial toll of treating the complications of IDA can be reduced.

References


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Low temperature, heat-induced epitope retrieval on free-floating/whole-mount archival human gynaecological pathology paraffin sections

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Abstract

The detection of nerve fibres in the endometrium of women with endometriosis has led to an interest in studying their relationship with infertility and pain. All studies so far involved in the description of neuronal structures have relied on conventional histological tissue sections. However neuronal networks have rarely been studied in three-dimensions. Biological systems exist and operate in three-dimensional surroundings, therefore it makes good sense to gain access to three-dimensional quantitative information based upon observations made on three-dimensional projections. This study was designed to utilise thick (≥ 50µm) formalin fixed sections and low temperature heat-induced epitope retrieval to reveal by immunofluorescence a pan neuronal marker (Protein Gene Product 9.5) and cytokeratin marker using laser scanning confocal microscopy. This will allow the quantitative assessment of the three-dimensional relationships of endometrial neuronal structures.

Keywords: Three-dimensional, computer reconstruction, low temperature heat-induced epitope retrieval, endometrium, immunofluorescence, nerve fibres, laser scanning confocal microscopy.

Introduction

Endometriosis is a benign gynaecological disease defined by the presence of endometrial-like tissue, including glands and stroma, ectopically outside the uterus (Meibody et al 2011). It is calculated that nearly 80 million women worldwide are affected by the disease. It is assumed in the medical literature that the prevalence of endometriosis is 5-15% in all women of reproductive age (Haas et al 2012). Laparoscopy is still the gold standard for diagnosis although it has some limitations in terms of false negative findings (Meibody et al 2011, Fraser 2008).

Recently, small unmyelinated sensory nerve fibres have been described in the functional layer of eutopic endometrium of women with endometriosis (Al-Jefout et al 2009, Tokushige et al 2006). These nerve fibres are not present in the functional layer of endometrium in women without endometriosis.

These findings are so consistent that detection of nerve fibres in the functional layer of the endometrium is now a promising and novel method for diagnosing endometriosis, through use of an endometrial biopsy (Al-Jefout et al 2007, Al-Jefout et al 2009, Bokor et al 2009, Meibody et al 2011). Currently, early diagnosis involves a high index of clinical suspicion of symptoms, followed by appropriate investigations.

The above studies have relied on conventional histological tissue sections. In these types of study the detection of neuronal structures has been greatly aided by the development of immunohistochemical staining using a specific pan neuronal marker, most commonly Protein Gene Product 9.5 (PGP9.5) (Al-Jefout et al 2007, Al-Jefout et al 2009, Bokor et al 2009, Meibody et al 2011, Tokushige et al 2006). Pre-treatment of tissue sections with heat-induced epitope retrieval (HIER) was required for optimal demonstration of PGP9.5.

Shi (1991) conducted the initial HIER study method utilising a microwave oven. Since this inception HIER methods are widely used in many areas of diagnostic and research immunohistochemistry. The utilization of material from surgical pathology archives or tissue databanks offers a large supply of formalin fixed biomedical material.
Numerous other methods for inducing heat have been used, such as autoclave (Bankfalvi et al 1994, Shi et al 1991), pressure cooker (Brassil 1997, Miller et al 1995, Norton et al 1994), and water bath heating (Igarashi et al., 1994, Suurmeijer and Boon, 1993).

The variant in the effectiveness of HIER lies with the type of epitope that is being unmasked. There are a number of factors that influence the heat-induced antigen retrieval procedures as originally discussed by Shi et al (1993). The type of heating (such as microwave oven, autoclave, pressure cooker, water bath) and time of heating (in minutes) are probably the most important factors determining the efficacy of heat-induced antigen retrieval.

The pH of the antigen retrieval solution has also been shown to have a marked influence on the efficacy of retrieving the antigens. In one of the first reports, Shi (1997) stated that most antibodies fell into three different patterns of staining based on the pH of the antigen retrieval solution (a) the stable type which only changed slightly with pH, (b) V-form type gave the best results at extremes (high and low) of pH and (c) ascending type improved with increasing pH.

Initial reports indicated that the chemical composition of the antigen retrieval solutions seems to have an effect on the unmasking of the epitope sites. Morgan (1994) postulated on the likely functions of the chemical components of the antigen retrieval solution. Studies also demonstrated that when using certain buffers the concentration of the buffer could have an effect on the antigen retrieval process. A number of solutions used in HIER have been reported e.g. sodium citrate (Brown and Chirala, 1995, Kanai et al 1998, Pellicer and Sundblad, 1994, Pileri et al 1997, Shi et al 1997), ethylenediaminetetra-acetic acid (EDTA) (Pellicer and Sundblad, 1994, Pileri et al 1997, Taylor et al 1996), tris base buffer (Balaton et al 1995) and tris-EDTA (Evers and Uylings, 1994, Pileri et al 1997, Taylor et al 1996).

What many HIER methods have had in common is they utilised boiling point temperatures across a short period of time (20-30 mins). However, performing this technique with thick / free floating (≥ 50 µm) sections alters their morphology significantly, whereas a lower temperature of 60 °C combined with a longer time period (18-24 h) has brought about an effective retrieval method for rabbit anti-PGP9.5 and rabbit anti-goat tetramethylrhodamine (TRITC) that were used to target and visualise the neuronal structures, and the mouse anti-human cytokeratin and mouse anti-goat fluorescein (FITC) that were employed to label and visualise epithelial tissue.

The aim of this study was to assess the ability of an available target retrieval solution to bring about effective antigen retrieval and preserve tissue morphology on thick cut, unstained free floating/wholemount (≥50 µm) archival sections using low temperature 60 °C water bath for 18 – 24 h.

### Material and methods

#### Tissue collection

This study was approved by the Human Ethics Committees of the Central Sydney Area Health Service and The University of Sydney.

Paraffin embedded full-thickness hysterectomy samples (containing both endometrium and myometrium) were obtained from Tissue Pathology and Diagnostic Oncology archives, Royal Prince Alfred Hospital, Sydney, Australia. Each case met the following criteria: aged between 25-45 yr, no usage of exogenous contraceptives in the three months prior to hysterectomy, undergoing hysterectomy for prolapse or pre-invasive cervical disease, no history of local radiotherapy, no clinical evidence of endometrial dysfunction and no uterine pathology on hysteroscopic examination.

#### Endometrial dating

Serial sections were stained with haematoxylin and eosin (H&E) and the endometrium was dated from the women’s menstrual history and blindly assessed by a specialist gynaecological pathologist using the criteria of Noyes, Hertig and Rock (Noyes et al 1950).

#### Micrometry

Initially the tissue was sectioned at 4 µm and routinely stained with H&E. Following the H&E assessment the tissue was further thickly sectioned at 50 µm and collected into a 15 mL glass vial, in preparation for deparaffinisation in xylene. The free-floating sections were then transferred into 15 mL polystyrene vials (to minimise protein binding) and rehydrated via descending concentrations of alcohol (100-70%) to water.

#### Pretreatment

All sections were pre-treated with 6x15 min changes of sodium borohydride (Sigma-Aldrich, St Louis, MO, USA) 1 mg/mL followed by a 10 min incubation at 37 °C of 500 U/mL collagenase (Sigma-Aldrich, St Louis, MO, USA) in phosphate buffered saline (PBS) (Sigma-Aldrich, St Louis, MO, USA) pH 7.4. Sections were then washed in 4 x 5 min changes of tris buffered saline (TBS) (Sigma-Aldrich, St Louis, MO, USA) pH 7.4 and agitated using a magnetic stirrer.

The pre-treatment of the thick sections served two purposes; it allowed for the antibodies (PGP 9.5 and cytokeratin) and fluorochromes (fluorescein isothiocyanate (FITC) and tetramethyl rhodamine isothiocyanate (TRITC)) to penetrate deeply and evenly into the thick section and it also reduced the autofluorescence (Lee et al., 1997).
Heat-induced epitope retrieval

The preparation of tris-EDTA buffer pH 8.0 antigen retrieval solution is as follows weigh out 5 g of EDTA disodium salt (BDH Chemicals Pty Ltd, Victoria, Australia); 3.2 g of tri-sodium citrate (dehydrate) (Bacto Laboratories Pty Ltd, Liverpool, NSW, Australia) and 2.5 g of Trizma base (Sigma-Aldrich, St Louis, MO, USA). Mix to dissolve and pH to 8.0, and make up to 1000mls with distilled water. To use: dilute 1:10 with distilled water and this can be kept for up to a week at 4 ºC. The sections were then treated with the heat-induced epitope retrieval solution in a 60 ºC water bath for between 18–24 h. Post retrieval the sections were allowed to cool at room temperature for 2 h.

Immunofluorescence

The following sequence was applied to the sections, with non-specific (background) staining reduced by pre-incubating for 24 h at 4 ºC with 2% BSA + 10% glycerine. The monoclonal mouse anti-human cytokeratin, (M0821, DAKO, Corporation, Carpinteria, USA), was diluted 1:400 and was then incubated for 48 h at 4 ºC. The FITC labelled goat anti-mouse (F-2761, Molecular Probes, Inc, Eugene, OR, USA) was diluted 1:1000 and incubated for 24h at 4°C. The polyclonal rabbit anti-PGP 9.5 (Z5116, DAKO, Corporation, Carpinteria, USA), was diluted 1:500 and incubated for 48 h at 4ºC. The etramethyl TRITC labelled goat anti-rabbit (T-2769, Molecular Probes, Inc, Eugene, OR, USA), was diluted 1:200 and then incubated for 24 h at 4 ºC. Each antibody incubation step was followed by 4 x 5 min changes of TBSpH 7.4 and agitated using a magnetic stirrer.

All slides were mounted using fluorescent mounting medium (DAKO, Corporation, Carpinteria, USA) and nail polish was used to seal the coverslip to the slide to prevent the evaporation of the mounting media and drying out of the specimen during storage.

Laser scanning confocal microscopy (LSCM)

A total of 10 confocal volumetric fields of view (ranging between 40–50 µm depending on endometrial thickness) were selected. Uterine nerve fibres and glandular epithelium were visualized using immunofluorescence, Nikon 800 microscope (Nikon, Shinagawa-Ku, Tokyo, Japan) equipped with a Radiance 2000 confocal scanning laser attachment (Bio-Rad Microscience, Hemel Hempstead, UK) with HQ 500LP or E570LP filter sets that are normally used for either FITC or TRITC detection respectively. Using the HQ500 LP filter set the specimens were illuminated with the 488 nm excitation line of a 25 mW argon ion laser and any fluorescence emitted at wavelengths greater than 500 nm then passed to a photomultiplier detector. With the E 570LP filter set, the specimens were illuminated with the laser’s 543 nm excitation line and fluorescence emitted at wavelengths greater than 570 nm passed to the photomultiplier. Using the ability of confocal microscopy to optically section intact or thick specimens, sequential confocal fluorescence images of approximately 0.5 µm optical section thickness were collected with the x20 objective through the full depth of each 50 µm section.

Addition of colour to the dataset

Images that were collected from the LSCM are typically 8-bit greyscale format. False colour was then added to these images prior to three-dimensional reconstruction to highlight the regions of interest or to selectively differentiate between the different areas of the endometrium (e.g. neuronal networks and tubular glandular epithelial structures and surface epithelium). The false colours were assigned in either the LSCM integrated software LaserSharp (Bio-Rad Microscience, Hemel Hempstead, UK) via a Look Up Table (LUT) or the third party volume rendering software ImarisBasic 4.1 visualization suite (Bitplane, AG, Scientific Solutions, Zurich, Switzerland).

Three-dimensional reconstruction

The collected data sets were processed with methods appropriate for the type of information to be visually transformed. A LSCM data set could either be processed as a series of two-dimensional slices (optical sections) or the complete volume could be rendered as a whole using either the integrated LSCM software LaserSharp (Bio-Rad Microscience, Hemel Hempstead, UK) or third party software ImarisBasic 4.1 visualization suite (Bitplane, AG, Scientific Solutions, Zurich, Switzerland). An orthogonal (also known as orthographic) projection was a way of providing a two-dimensional graphic view of an object in which the projecting lines are drawn at right angles to the plan of projection. It has a couple of advantages: it was quicker, and allowed a region to remain a constant size in different views.

Results

Biological systems exist and operate in three-dimensional surroundings; therefore it makes good sense to gain access to the three-dimensional qualitative information, based upon observations made on three-dimensional projections.

Figure 1 shows computationally reassembled image stacks utilising ImarisBasic 4.1 visualization suite (Bitplane, AG, Scientific Solutions, Zurich, Switzerland) to provide a three dimensional reconstruction of the neuronal network architecture - ‘false’ red and for the glandular epithelial structures and surface epithelium - ‘false’ green… It also demonstrated that the utilisation of a lower temperature 60 ºC combined with a longer time period (18–24 h) has bought about an effective retrieval method for two antibodies requiring heat-induced epitope retrieval — i.e. cytokeratin and PGP 9.5 — without altering morphology.
Discussion

The use of HIER and LSCM has provided for the first time a three-dimensional visualisation of well-developed endometrial neuronal networks and their relationship with endometrial glandular structures.

The mode of HIER chosen by our laboratory was the water bath as the 60 °C temperature could be controlled via a thermostat, and section morphology was retained after a 24 h duration. Previous HIER methods have utilised boiling point temperatures across a short period of time. In performing this technique with thick/free floating sections it was found to have altered the morphology significantly, whereas a lower temperature combined with a longer time period bought about an effective retrieval for the labelling of the pan neuronal marker PGP9.5 and cytokeratin. Previous studies have also reported heat-induced epitope retrieval methods on 40 µm free floating / thick sections or on paraffin-embedded slide-mounted sections (Jiao et al 1999, Manconi et al 2001). The tris-EDTA buffer pH 8.0 antigen retrieval protocol coupled with decreased temperature and an increased incubation time allowed the unmasking of the formalin epitopes in thick (≤50 µm) sections of archival formalin fixed, paraffin embedded tissue of longitudinal sections. Correct shapes can be retrieved without resorting to external sources of alignment information (Manconi et al 2003). The non-invasive nature of optical sectioning enables the observation of living cells (Simbar et al 2003) as well as fixed cells to be made with greater clarity. Scanning in the z direction, as well as in the x and y direction, gives an overall effect of viewing the focal plane from the side. Confocal microscopes have a shallow depth of field of between 0.5–1.5 µm and this enables the information to be collected from a well-defined optical section.


The future direction is to study the three dimensional co-localization of blood vessels and nerve fibres in the female reproductive tract. Previous studies have indicated that blood vessels and nerve fibres course throughout the body in an orderly pattern, often alongside one another (Carmeliet and Tessier-Lavigne 2005). Tokushige (2006) demonstrated that the eutopic endometrium from women with endometriosis contains small, unmyelinated nerve fibres in the functional layer and these nerve fibres are not observed in women without endometriosis. The presence

Figure 1. An orthogonal projection of a small volume of endometrial tissue [0.017mm3/mm3] from a woman with endometriosis. The tissue was taken from the uterine fundus during the secretory phase, and immunofluorescently illustrates PGP 9.5 ‘false’ red which are individual longitudinal segment lengths of neuronal networks (white arrows) and endometrial epithelium ‘false’ green labeled as tubular glandular epithelial structures (G) and surface epithelium (SE).
of these nerve fibres in women with pain symptoms strongly suggests that in women with endometriosis, the eutopic endometrium is involved in the generation of these symptoms.

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References


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Evaluation of an agar cell block method to improve cell yield in non-gynaecological cytology specimens

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Abstract

Achieving optimal cellularity in cell block preparations from cytology specimens is increasingly important as the demand for adjunct testing grows. This study aimed to determine if modifications to our agar cell block (CB) method improved cell yield for fine needle aspiration (FNA) and for body fluid specimens. The modifications were designed to concentrate the cells on a single plane and provide a visual marker to indicate the plane for optimal sectioning. This was achieved by the use of a small flat bottom 5 mL tube for the final centrifugation step and a visual marker to indicate the correct level for sectioning. The new method (marker method) was used prospectively for 210 consecutive FNA specimens (fluid collected or washed out of the needle) from a wide range of sites and 133 fluid specimens. Haematoxylin & eosin (H&E) stained CB slides were evaluated to determine if sufficient material was present for diagnostic interpretation and/or immunohistochemistry. Results were compared with historical data for 250 consecutive specimens (158 FNAs and 92 fluids) prepared using our old agar CB method (agar method). The marker method was simple to perform and resulted in a higher proportion of adequate CBs. Adequacy rates for the agar versus marker method were: total FNA 35% versus 51%; FNA non-attended 32% versus 48%; scientist attended FNA 41% versus 59%; fluids 79% versus 86.5%. The improvement was statistically significant for all FNAs (P=0.003), non-attended FNAs (P=0.017) and for FNAs with scientist in attendance (p=0.05), but not for fluid specimens (P=0.2). The modifications therefore significantly improved the proportion of CB preparations with adequate cell numbers, especially for poorly cellular samples (most FNA washouts). The visual marker ensures specimens are not over- or under-cut and examination of the H&E slide quickly allows assessment of whether the correct level has been sampled.

Keywords: Cell block, cytology, fine needle aspiration, serous effusion

Introduction

Optimising cell yield in CB preparations is becoming increasingly important as the demand grows for adjunct testing of cytology specimens. Additional testing may provide valuable information relating to tumour typing, primary sites and response to therapeutics and enhance the cost-effectiveness and efficacy of these minimally invasive tests. Cell block preparations provide a useful platform for the performance of histochemical, immunohistochemical (IHC) stains and molecular techniques, as well as providing additional morphological information. However cytological specimens, especially FNA rinse samples, are often poorly cellular and may lead to frustratingly high rates of inadequate cell block preparations.

We recently modified our agar method to incorporate two steps used by Varsegi and Shidham (2009) in a method described for making CBs from ThinPrep™ (Hologic) specimens. The modifications were designed to concentrate the cells more effectively on a single plane and provide a visual marker to indicate the plane for optimal sectioning.

After an initial trial period the marker method was used prospectively for all non-gynaecological cytology cases and the results obtained over a three month period were compared with results obtained when our agar method had been used.

Methods

Cell block method

Both methods involved concentration of cells by centrifugation and suspension of the cell pellet in liquefied agar followed by further centrifugation. The resulting agar pellet was cooled to solidify prior to fixation in formalin, processing, embedding and microtomy. The modifications included the use of a small flat bottom 5 mL tube for the final centrifugation step and a visual marker to indicate the correct level for sectioning. The latter involves the addition of a 2 x 2 mm (approx) piece of banana skin that is spun with the cell pellet and comes to lie with the cells on the bottom of the flat bottomed tube. The marker method was readily adapted to routine practice (Table 1). The marker could be clearly recognised in the cell block and in stained sections (Fig.1).
Markers were prepared by drying a piece of banana skin (either at room temperature or in a drying oven). A 2 mm strip was then cut with a scalpel and diced into squares. The markers were then placed in a petri dish on a filter paper with desiccating beads (Fig. 2). When dry and brown/black the markers were stored in formalin in order to preserve them and to prevent the markers from floating to the top when agar is added.

Table 1. Marker cell block method

<table>
<thead>
<tr>
<th>Step</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>1.</td>
<td>Decant fluid into 10 mL centrifuge tube(s). For large volume specimens at least 400 mL should be spun in 50 mL tubes with the sediment transferred to a 10 mL tube.</td>
</tr>
<tr>
<td>2.</td>
<td>Centrifuge at 1200 rpm for 5 min.</td>
</tr>
<tr>
<td>3.</td>
<td>Prepare a flat bottomed 5 mL tube by labelling it and placing a marker inside at the bottom. Markers are made from dried banana peel cut into 2 x 2 mm pieces and stored in formalin.</td>
</tr>
</tbody>
</table>
| 4.   | After centrifugation of the specimen inspect the pellet:  
| a)   | If a large homogeneous pellet (>0.5 mL) is visible: pipette off 0.5 mL of the pellet and add to the flat bottomed tube. |
| b)   | If a large heterogeneous pellet (>0.5 mL) and with clots or variably coloured layers) sample all layers and make multiple blocks. |
| c)   | If a large bloodstained pellet (>0.5 mL) drain off the supernatant and haemolyse, then pipette off 0.5 mL of the pellet and add to the flat bottomed tube. Note: Do not treat bloodstained pellets if they are <0.5 mL in volume. |
| d)   | If small or no pellet (<0.5mL) pipette the pellet and bottom 0.5 mL of fluid into the flat bottomed tube. |
| 5.   | Heat Mueller-Hinton agar (obtained from microbiology) in a glass beaker in the microwave until liquid (about 10 sec). |
| 6.   | Add agar to the material transferred to the flat bottomed tube and mix. Ensure centrifuge is available for immediate use. |
| 7.   | Centrifuge at 1200 rpm for 5 min. This must be done quickly while the agar is still liquid. |
| 8.   | After centrifugation check that the marker is resting on the bottom of the tube. If not, reheat the tube briefly in the microwave and repeat step 7. |
| 9.   | Place tube in a refrigerator at 4 °C for approximately 15 min to ensure the agar is set. |
| 10.  | Dislodge the agar button by carefully pushing a glass pipette down the side of the tube until it touches the bottom. Gently rotate the pipette around the circumference of the tube while pipetting in 10% neutral buffered formalin. The disc of agar then will float up the tube in the formalin. Once dislodged, continue to fill the tube with formalin. |
| 11.  | Transfer to a cassette, ensuring the marker is placed face down in the base of the mould, gently tamper. Following processing and embedding, blocks should be faced until the marker is exposed and becomes clearly visible. |

Specimens

CBs were prepared from residual body fluid specimens or, in the case of FNAs, from material either aspirated directly from the lesion, washed from the needle with saline or, in most cases, rinsed into a 10 mL tube with medium supplied by the laboratory (HAM’s media with heparin and antibiotics). In our laboratory CBs are selectively performed on cases to resolve differential diagnoses, sample large volume fluids or perform additional stains. A total of 210 consecutive FNA specimens were selected for CB processing (about 7% of FNAs reported in the period) from a wide range of sites and 133 fluid specimens (around 30% of serous effusions and peritoneal washings reported) were prepared with the marker method (Table 2). The FNA samples
included those prepared and assessed for adequacy on-site by a cytology scientist (attended) and those where a cytologist was not in attendance at the collection (non-attended). H&E stained CB slides were evaluated to determine if sufficient material was present for diagnostic interpretation and /or IHC. Results were compared, to those from historical data for 250 consecutive specimens from a similar range of sites (158 FNAs and 92 fluids) prepared using the agar method using Fisher's exact test.

For CBs made using both methods the volume of body fluid received varied from 5-2000 mL and were of similar median volume (agar method = 50 mL; marker method = 60 mL). Samples available for CB preparations from FNA specimens were comparable for both methods, with 84% and 86% respectively of FNAs prepared by the agar method and the marker method consisting of 7 mL of washout fluid.

### Table 2. Specimens prepared using the two cell block methods

<table>
<thead>
<tr>
<th>Specimen types</th>
<th>Marker</th>
<th>Agar</th>
</tr>
</thead>
<tbody>
<tr>
<td>Abdomen/pelvis</td>
<td>8</td>
<td>8</td>
</tr>
<tr>
<td>Adrenal gland</td>
<td>2</td>
<td>1</td>
</tr>
<tr>
<td>Arm</td>
<td>2</td>
<td>1</td>
</tr>
<tr>
<td>Axilla</td>
<td>20</td>
<td>21</td>
</tr>
<tr>
<td>Back</td>
<td>1</td>
<td>1</td>
</tr>
<tr>
<td>Bone</td>
<td>6</td>
<td>3</td>
</tr>
<tr>
<td>Breast</td>
<td>8</td>
<td>4</td>
</tr>
<tr>
<td>Chest wall</td>
<td>5</td>
<td>3</td>
</tr>
<tr>
<td>Head/face</td>
<td>3</td>
<td>5</td>
</tr>
<tr>
<td>Kidney</td>
<td>0</td>
<td>3</td>
</tr>
<tr>
<td>Leg</td>
<td>6</td>
<td>1</td>
</tr>
<tr>
<td>Liver</td>
<td>9</td>
<td>6</td>
</tr>
<tr>
<td>Lung</td>
<td>8</td>
<td>19</td>
</tr>
<tr>
<td>Mediastinum</td>
<td>4</td>
<td>4</td>
</tr>
<tr>
<td>Neck</td>
<td>83</td>
<td>51</td>
</tr>
<tr>
<td>Pancreas</td>
<td>6</td>
<td>3</td>
</tr>
<tr>
<td>Salivary gland</td>
<td>18</td>
<td>9</td>
</tr>
<tr>
<td>Thyroid</td>
<td>6</td>
<td>6</td>
</tr>
<tr>
<td>Total FNA</td>
<td>210</td>
<td>158</td>
</tr>
<tr>
<td>Ascitic/peritoneal fluid</td>
<td>42</td>
<td>28</td>
</tr>
<tr>
<td>Peritoneal washings</td>
<td>12</td>
<td>2</td>
</tr>
<tr>
<td>Pericardial fluid</td>
<td>0</td>
<td>4</td>
</tr>
<tr>
<td>Pleural fluid</td>
<td>77</td>
<td>58</td>
</tr>
<tr>
<td>Urine</td>
<td>2</td>
<td>0</td>
</tr>
<tr>
<td>Total Fluids</td>
<td>133</td>
<td>92</td>
</tr>
<tr>
<td>Total specimens</td>
<td>311</td>
<td>250</td>
</tr>
</tbody>
</table>

### Results

Modifications required for the marker method were simple to perform and resulted in a higher proportion of adequate CBs (Table 3). Adequacy rates for the agar versus marker method were: total FNA 35% versus 51%; FNA non-attended 32% versus 48%; attended FNA 41% versus 59%; fluids 79% versus 86.5%. The improvement was statistically significant for all FNAs (p=0.003), non-attended FNAs (p=0.017) and for FNAs with scientist in attendance (p=0.05), but not for fluid specimens (p=0.2). No artefactual alteration to staining reactions, cellular morphology or diagnostic features was observed in the marker CBs (Figs 3 to5).

![Figure 3](image3.png) Sparsely cellular marker cell block with rare groups of cells (arrows). Note marker top left. (H&E; x4obj).

![Figure 4](image4.png) Marker cell block from Parotid FNA demonstrating features of a Warthin’s tumour (H&E; x10 Obj).

![Figure 5](image5.png) Marker cell block from pleural fluid: mesothelioma (EMA; (x40 Obj)).
Table 3. Comparison of cell block adequacy for the agar and marker cell block methods for FNA and body fluid specimens.

<table>
<thead>
<tr>
<th>Specimen type</th>
<th>Method</th>
<th>Total</th>
<th>Adequate</th>
<th>In-adequate</th>
<th>% Adequate</th>
<th>p value</th>
</tr>
</thead>
<tbody>
<tr>
<td>FNA non-attended</td>
<td>Agar</td>
<td>99</td>
<td>32</td>
<td>67</td>
<td>32.3</td>
<td>0.017</td>
</tr>
<tr>
<td></td>
<td>Marker</td>
<td>142</td>
<td>68</td>
<td>74</td>
<td>47.8</td>
<td></td>
</tr>
<tr>
<td>FNA attended</td>
<td>Agar</td>
<td>122</td>
<td>95</td>
<td>27</td>
<td>40.7</td>
<td>0.05</td>
</tr>
<tr>
<td></td>
<td>Marker</td>
<td>141</td>
<td>96</td>
<td>45</td>
<td>45.8</td>
<td></td>
</tr>
<tr>
<td>Total FNAs</td>
<td>Agar</td>
<td>219</td>
<td>154</td>
<td>65</td>
<td>70.1</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Marker</td>
<td>283</td>
<td>226</td>
<td>57</td>
<td>80.0</td>
<td></td>
</tr>
<tr>
<td>Fluids</td>
<td>Agar</td>
<td>133</td>
<td>115</td>
<td>18</td>
<td>86.5</td>
<td>0.20</td>
</tr>
<tr>
<td></td>
<td>Marker</td>
<td>178</td>
<td>156</td>
<td>22</td>
<td>87.4</td>
<td></td>
</tr>
</tbody>
</table>

Discussion

Cell blocks of limited cellularity were difficult to face to the correct level with the agar method and it was not possible when examining H&E stained slides to determine if the optimal level had been sampled. The marker method improved CB adequacy rates for fluid cases with 7% more cases considered adequate, however the difference was not statistically significant. Fluids with inadequate CBs were more likely to be small volume (72% were < 50 mL versus 29% of cases with adequate CBs), however cellularity of the specimen rather than volume is the important factor. Seventeen cases had adequate marker CBs made when less than 20 mL was received, whereas four cases had inadequate CBs when >500 mL was received and processed. The latter were either poorly cellular transudates and/or contained very sparse suspicious cells in the smears. The markers were easily prepared and did not interfere with cellular morphology or staining.

FNAs rinse specimens are generally of much lower volume and cellularity than fluid specimens and it was in these cases that the marker method showed most improvement in adequacy. Overall, CB adequacy was improved by 16% for FNAs in the marker method group. Most FNA samples in our laboratory are received as prepared slides accompanied by an FNA washout tube that may be used for additional cytological preparations, CBs or flow cytometry for cell surface markers. CBs are prepared selectively on FNA cases, following evaluation of the smears. Most FNA specimens are collected without a scientist or pathologist in attendance and, although our collection advice recommends rinsing the needle preferably after collection of a dedicated pass, the cellularity of this fluid is often low. Several factors contribute to this including the nature of the lesion, technique of the collector and whether or not a dedicated pass is collected. Cases attended by a scientist are more likely to have additional material collected into the washout at the request of the attendee, and hence are more likely to have an adequate CB. Almost 20% more marker FNA CBs were adequate when a scientist attended the collection. This has significant implications for patient management and the cost effectiveness of the procedure, given the improvements in diagnostic precision that can often be achieved when adequate CB material is available. However, even though the marker method improved adequacy rates, about 40% of attended FNAs were still considered inadequate. Harvesting cells from prepared smears for IHC or molecular techniques may offer a way of providing additional material in some cases (Nga et al 2005, Hookim et al 2012).

Several methods have been reported for cell block manufacture including plasma-thrombin clot (Kulkarni et al 2009), tissue coagulum clot (Yung et al 2012), sedimentation (Nigro et al 2007) and Cellient™ (Hologic) (Wagner et al 2007). All aim to produce a concentrated pellet of cells in a sufficiently solid form to process as a histological specimen, embed in paraffin wax and section. One of the more commonly used methods relies on agar as a means of producing a semi-solid pellet for processing. The use of a visual marker and flat bottomed tube in the current study significantly improved the proportion of CBs from FNA cases with adequate numbers of cells in sections and may be applicable to other methods that utilise different means of capturing the cell sample, such as the plasma thrombin clot method.

Acknowledgements

The authors are grateful to Jo Cosier, Alan Sutton and the Sullivan Nicolaides histology technical staff for their assistance.

References


A one-day-old infant born with Down syndrome (DS) at the Royal Hospital for Women had a full blood count performed. The results were as follows:

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<tr>
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<tbody>
<tr>
<td>Hb</td>
<td>186</td>
<td>RR</td>
<td>121-191 g/L</td>
<td></td>
</tr>
<tr>
<td>HCT</td>
<td>0.522</td>
<td>RR</td>
<td>0.37-0.60</td>
<td></td>
</tr>
<tr>
<td>MCV</td>
<td>106.5</td>
<td>RR</td>
<td>101-117 fL</td>
<td></td>
</tr>
<tr>
<td>MCH</td>
<td>38.0</td>
<td>RR</td>
<td>33.0-38.0pg</td>
<td></td>
</tr>
<tr>
<td>WBC</td>
<td>33.6</td>
<td>RR</td>
<td>9.6-30.4 x 10^9/L</td>
<td></td>
</tr>
<tr>
<td>PLT</td>
<td>302</td>
<td>RR</td>
<td>195-434 x 10^9/L</td>
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</tbody>
</table>

Morphologically, the white blood cells showed a left shift with myelocytes, metamyelocytes and band forms. There were 42% blasts and 68 NRBC/100 WBC also present on the blood film.

An initial diagnosis of transient myeloproliferative disorder (TAM) was made on this one-day-old infant. The differential diagnosis for this infant was congenital leukaemia.

The blasts were medium to large in size with an open chromatin pattern and multiple nucleoli. The cytoplasm was abundant, basophilic and agranular, demonstrating cytoplasmic budding or pseudopod formation. The blasts resembled megakaryoblasts.

Flow cytometry was performed on the peripheral blood with the following results:

CD45+/HLADR+/4+/7+/33+/34+/71+/117+/cytoCD61+/cytoCD41a+/cytoCD42a/MPO-

The most commonly occurring markers in TAM include CD45, CD34 and CD33 along with the megakaryocytic markers CD41 and CD61 and aberrant expression of CD7.

Cytogenetic studies on the peripheral blood failed to grow on this infant. In addition to the trisomy 21 of DS, no specific chromosomal abnormalities are associated with transient myeloproliferative disorder of DS.

Repeated blood counts demonstrated a gradual drop in the number of white blood cells as well as the number of blasts. By three months of age the white cell count was within the normal range and there were no blast cells seen in the peripheral blood. Thus the initial diagnosis of TAM was confirmed.

TAM occurs in approximately 10% of neonates with DS. The peripheral blood shows a variable number of blast cells with an associated thrombocytopenia; other cytopenias are uncommon. The blasts typically show megakaryocytic markers with flow cytometry. Approximately 20% to 30% of patients with DS and TAM develop acute megakaryoblastic leukaemia by three years of age.

Recently, somatic mutations of the gene encoding the hematopoietic transcription factor GATA1, found on the X chromosome, have been found in the megakaryoblasts of patients with DS, suggesting a significant role for these mutations in the development of megakaryoblastic leukaemia. The mutations of the GATA1 gene have been detected in foetal liver haemopoietic cells.

Three years later the above patient presented at the Sydney Children’s Hospital with general malaise and bone pain. A full blood count was performed with the following results:
Table 2: Patient blood counts in 2012

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<thead>
<tr>
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<tbody>
<tr>
<td></td>
<td>RR</td>
<td>107-136 g/L</td>
</tr>
<tr>
<td></td>
<td>RR</td>
<td>0.1-0.38</td>
</tr>
<tr>
<td></td>
<td>RR</td>
<td>73-85 fL</td>
</tr>
<tr>
<td></td>
<td>RR</td>
<td>24.8-29.9 pg</td>
</tr>
<tr>
<td></td>
<td>RR</td>
<td>4.9-12.8 x 10^9/L</td>
</tr>
<tr>
<td></td>
<td>RR</td>
<td>214-483 x 10^9/L</td>
</tr>
</tbody>
</table>

The white blood cell differential included 39% blast cells. The blasts had the appearance of megakaryoblasts; they were large with an open chromatin pattern, prominent nucleoli and basophilic cytoplasm with prominent budding. A bone marrow biopsy was performed. The marrow was hypercellular with 73% blasts.

Flow cytometry markers on the bone marrow were as follows: CD45+/HLA-DR-/7+/33+/38+/61+/34-/117-/MPO-/cytoCD61+/cytoCD41a+/cytoCD42a+

Cytogenetic studies showed multiple abnormalities:

46,XX,del(1)(q21q25),der(10)t(10;12)(q22;q13)add(10)(p11.2),del(11)(q23),der(12)t(10;12)(20)

A diagnosis of acute megakaryoblastic leukaemia was made.

This child is now awaiting a cord blood transplant.
Three scholarships of $1200 each will be offered in the categories below to financial members of AIMS to attend the AIMS NSM 2013.

This year one Remote Attendee Scholarship, one First Time Presenter Scholarship and one Early Career Scientist Scholarship are available.

Applicants must be current members of AIMS and must have held membership for at least six months at the time of application.

Affiliate, Retained or Student members are not eligible to apply.

For full details and application forms see the AIMS website www.aims.org.au or contact the National Office.

APPLICATIONS CLOSE
4 pm AEST MONDAY 29th APRIL 2013
I attended the NSM 2012 with the support of the AIMS first time presenter award. It was held at the convention centre on Darwin's waterfront, quite a picturesque location for the delegates. The weather was simply perfect all day, every day.

For me, the NSM started off well with attendance at the workshop on histology artifacts, faults and failures presented by my past university lecturer Geoff Rolls. In fact, all the presentations that I attended during the week were of a very high quality and in very diverse areas of medical science. Looking back, the real eye openers were being told about the “pathology laboratories” in remote and developing areas of Papua New Guinea and the Solomon Islands. It goes to show how we often take things for granted in our laboratories with regards to suitable facilities, modern equipment and adequate supplies of good quality reagents. In addition, the poster presentations relating to histopathology were excellent. I gained many useful technical tips and tricks to try as a result of speaking to the authors directly.

In keeping with the theme of the conference, there were many presentations on tropical diseases. Up there, it’s not just the crocodiles or stingers that can cause you grief so it was useful to see specific case studies and hear first-hand accounts of melioidosis, scrub typhus, dengue and filariasis. As for my own presentation, it went well considering it was the last one just before the conference dinner. Speaking of the dinner, I will always have fond memories of it being held outdoors at the sailing club, in perfect weather with good food, music, a splendid sunset and a cold beer in my hands.

Socially, not only did I get to see my past mentors and colleagues from university and previous employment, I also met fellow scientists for the first time. It was interesting to hear them talk about their work. Some are totally engrossed in their research and diagnostics while others are fed up and due for a career change or mid-life crisis. This is not such a bad thing as Wayne Melrose will testify to when his “moment” took him to Antarctica. It is somewhat fascinating listening to an individual’s unique journey in life to get to where they are today.

My thanks go to AIMS for the opportunity to attend and present at the NSM held in Darwin 2012. It was a good opportunity to compare notes and listen to how the art and science of histology could be done in a way that is different to your own. This experience has definitely helped me in my career as a medical scientist. I will now leave you with a good tip provided by an elder of the Larrakia people, the traditional inhabitants of the Darwin area: that is to not worry if you miss out on a barramundi feast in the Northern Territory because there is always an opportunity for a barra-Tuesday and to follow that up with a barra-Wednesday….

Trung Nguyen
Australian Institute of Medical Scientists
Immuno haematology Quality Assurance Program

- Runs bi-monthly starting in June at the beginning of each financial year
- Level of difficulty to suit both small & large laboratories
- Includes blood grouping, antibody screening/identification & compatibility testing
- Ability to send multiple returns on the one subscription

- The reports provide participants’ own results along with graphical representation of the results of their peers allowing for easy comparison and analysis by supervising staff
- Absolute confidentiality of results is assured

For enrolment enquiries contact
Steve Mackay  E-mail: aimsqap@dspl.com.au
AIMS has initiated a scholarship program for the AIMS/RCPA QAP Morphology Workshop.

In 2013 there will be a scholarship available for all financial members of the Institute for the second workshop held on the 25th and 26th July 2013, at the new RCPA QAP Offices in Herbert Street, St Leonards (Sydney), NSW.

This workshop has been held at least twice a year for the last 22 years. It is organised through the RCPA QAP in Haematology office by a group of AIMS members and Haematologists. The workshop is now acknowledged as the premier workshop of its type, not only in Australia but also in the Asia Pacific Rim. It is a ‘wet’ workshop held over two full days. This year there is an optional extra half day for malaria morphology.

CONDITIONS
Applicants would be expected to have some basic knowledge and the scholarship is particularly suitable for members who either do not have resources for continuing education available to them or have a need for retraining due to rationalisation or multiskilling in their workplace. Previously unsuccessful applicants are encouraged to apply.

VALUE
The value of each scholarship will not exceed $1000.

DEADLINE
Friday 30th March 2013

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Application Form

AIMS/RCPA QAP Blood Cell Morphology Workshop
AIMS Scholarship

Name: ..................................................................................................................

Address: ..................................................................................................................

.................................................................................................................. Postcode: ..........................

Telephone: ...........................................................................................................

Email: ..................................................................................................................

☐ My resume is enclosed which details my place of work, qualifications and employment history.

In 50 words or less please explain why you believe you should receive the scholarship
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SEND APPLICATION TO
AIMS National Office
PO Box 1911
MILTON QLD 4064
Phone: 07 3876 2988
Fax: 07 3876 2999
Email: aimsnat@aims.org.au
Questions relating to ‘Diagnosis of iron deficiency and anaemia of chronic disease’, page 2 of this issue.

1. Serum ferritin is an early marker of ID with levels falling below normal limits well before morphological changes appear in the mature red cell. Values of <15ug/L are diagnostic of iron deficiency.

2. Serum ferritin is an acute phase reactant and will be raised in cases of inflammation thus limiting its usefulness in anemia of chronic disease.

3. As the sTfR is affected by the presence of inflammation, it has no potential to be a useful marker for ID or FID when the serum ferritin is no longer suitable. In a diverse hospital population sTfR levels will not be up-regulated for reasons other than iron depletion.

4. Initial methods to measure urine and plasma hepcidin have been cumbersome and lacked standardization.

5. Populations in less developed nations or in tropical locations in developed nations like northern Australia have a higher incidence of IDA and ACD and it is particularly prevalent in the indigenous paediatric population.

6. Hypoxia-inducible transcription factor (HIF) is an oxygen sensitive nuclear transcription factor which has been shown to stimulate expression of duodenal cytochrome b (DcytB) and DMT-1 resulting in an increase in intestinal iron uptake.

7. With regards to the ferritin index a ratio of <1 suggests ACD whereas a ratio of >2 suggest IDA+/-ACD.

8. ACD is the least prevalent anaemia in hospitalized patients and the second least common anaemia world-wide.

9. Due to the lack of clear cut diagnostic criteria for determining iron status it is not uncommon for medical personnel to forgo laboratory testing and prescribe a treatment of oral iron when presented with clinical signs and symptoms that suggest anaemia.

10. Assays for these new iron regulatory proteins such as the liver hormone hepcidin are found in most routine laboratories.
Journal-based CPD No.35

Questions relating to ‘Low temperature, heat-induced epitope retrieval on free-floating/whole-mount archival human gynaecological pathology paraffin sections., page 14 of this issue.

1. Biological systems exist and operate in two dimensional surroundings; therefore it makes good sense to gain access to the three-dimensional qualitative information, based upon observations made on three-dimensional projections. True/False

2. In Figure 1 False Green indicates the individual longitudinal segment lengths of neuronal networks (white arrows). True/False

3. The sections were then treated with the heat induced epitope retrieval solution in a 60°C water bath for between 48-72 h. True/False

4. Serial sections were stained with haematoxylin and eosin (H&E) and the endometrium was dated from the women’s menstrual history and blindly assessed by a specialist gynaecological pathologist using the criteria of Noyes, Hertig and Rock. True/False

5. The case definition for inclusion into the study included 25-45 yr, no usage of exogenous contraceptives in the three months prior to hysterectomy. True/False

6. The pH of the antigen retrieval solution has also been shown to have a marked influence on the efficacy of retrieving the antigens. True/False

7. The presence of these nerve fibres in women with pain symptoms strongly suggests that in women with endometriosis, the eutopic endometrium is involved in the generation of these symptoms. True/False

8. Pre-treatment of tissue sections with heat-induced epitope retrieval (HIER) was not required for optimal demonstration of PGP9.5. True/False

9. All studies so far involved in the description of neuronal structures have relied on electron microscopic histological tissue sections. True/False

10. Uterine nerve fibres and glandular epithelium were visualized using immunofluorescence. True/False

Name: _________________________________________________________________________

Email: _________________________________________________________________________

Please photocopy this page or print it from the electronic AJMS which is stored in the AIMS 'Member centre' under the heading 'Journal' at www.aims.org.au. Circle your answers then post, fax or scan and email to us by 31st June 2013 to:

AJMS APACE Questions, AIMS National Office, PO Box 1911, Milton Qld 4064. Facsimile: 61 7 3876 2999
Environment and Lifestyle - Effects on Disorders of the Digestive Tract  

Falk Symposium 176 October 2010  
H.E. Blum, R.H. Hunt & J. Scholmerich  
Karger  
Soft cover  114 page  
ISBN: 978-3-8055-9809-5  
USD $108.00

This is the first edition of a collection of articles based around not only the genetics of digestive disease but focuses on the effects of environment and lifestyle as contributors to the disorder obtained by the patient. It is accessible on line www.karger.com

The work is presented as soft cover text of 268 pages. It is well set out with logical sequence of chapters grouped into related topics. These are Genetic Basis of Digestive Diseases, Mechanisms of Environmental Effects I, Mechanisms of Environmental Effects II, The Liver as a Target, Herbert Falk Award Lecture which is “From Aphthous Ulcer to Full-Blown Crohn’s Disease, The Gut as a Target, Upper Gastrointestinal Tract and Pancreas as a Target, Implications for Treatment.

Gastrointestinal disorders plague a high percentage of the population and whilst many are lifestyle related illness can be debilitating. This text aims to analyse these causes together with genetic, environmental causes, viral such as hepatitis related illness, drug toxicity and bacterial underlying issues. Each organ of the digestive tract affected is also analysed, summing up with an outline of treatment and prevention options.

Treatment and new therapies are also explored.

The text is required reading for gastrointestinal specialists, and scientists with an interests in this area. It is not suitable for the “man on the street” wanting to know about a personal inherent health disorder.

The final sentence of the preface sums up the intentions of the text “The scientific organizers of the conference hope that the proceedings of the symposium presented in this publication will be of interest to those who attended and also to those who were unable to participate.”

Noelene Wilson MAIMS  
Hospital Scientist  
Hunter Area Pathology Service

Case Studies in Clinical Biochemistry  

M.J. Murphy, R. Srivastava & A. Gaw  
SA Press  
Soft cover + 132 pages  
GBP £7.99

This book is a pocket sized relative to the text Clinical Biochemistry: An Illustrated Colour Text (4th edn). This book covers many areas including acid-base disorders, diabetes, renal function, calcium and bone disease, adrenal function, thyroid function, paediatrics, lipids, toxicology and pituitary function.

The authors have stated this text is targeted to readers with existing knowledge of clinical biochemistry and as a student resource for those studying health sciences.

The book is presented to test existing knowledge by providing a clinical case study containing a brief patient history, diagnostic results and then pose a set of questions for the scenario. By turning the page the authors explain and discuss the important findings of the diagnostic results including differential diagnoses and further testing.

The real point of difference of this text was that it focuses on patient case histories providing a realistic situation for all laboratory scientists; I personally find that this helps to bring the human aspect back to the science we perform.

Keeping in mind this text was written by three authors with a medical background, the text tends to sway towards a clinical viewpoint. The authors clearly state that whilst laboratory and testing quality are integral this “need not be the concern of most clinicians requesting biochemical services”.

At the end of the book the authors provide a comprehensive list of texts for further reading to advance your knowledge. This list is comprised of books and website addresses which provide further academic articles and case studies.

Uniquely the authors have established a Facebook page as another medium to access additional information and to provide direct communication with the authors. This page posts up links to academic articles and other current affairs in the world of science.
I feel Case Studies in Clinical Biochemistry would be a good resource particularly for those undertaking postgraduate studies in the field and as a text for students in the latter parts of their undergraduate education.

Sophie Wylde MAIMS
Senior Scientific Officer (Biochemistry)
Curtin University

“Now In Remission - A Surgical Life”
Ken Clezy
Wakefield Press

279 pages
ISBN: 978 1 74305 014 9
AUD $27.95

“NOW IN REMISSION - A Surgical Life” is the autobiography of Ken Clezy. An Australian surgeon and missionary whose career took him down the path much less travelled.

The book commences with Clezy recounting the events of 30th December 2002. The day when three of his colleagues were gunned down in the hospital in which he worked in Yemen. He too would have surely been killed but was home eating breakfast with his wife at the time. Clezy, a devout Christian believes that it was God's will for him not to be at the hospital on that morning, just as it was God's will for him to find himself in strange places around the world helping others most in need.

Subsequent chapters detail his early life in rural South Australia, his family's history in Australia, his time at medical school and then his career which mainly saw him in Papua New Guinea, the United Kingdom, India, Australia and finally in Yemen.

Clezy gives an honest account of his professional and personal life in some of the world's more exotic (some would say more dangerous) locations. The book mainly examines Clezy's professional life. However he also revisits events, both fantastic and horrific which have punctuated his and his family's lives. Through thick and thin, Clezy and his wife stayed on, driven by their faith and loyalty to those they knew were most in need.

Clezy operated in places where he was able to do things his way. His practices were based on what was available at hand and on maximising patient outcomes. His leprosy work in Papua New Guinea was pioneering. He wasn't bound by modern day bureaucracy and he flourished in environments where even simple surgeries made a huge difference to people's quality of life.

Doctors, nurses and others who have worked or volunteered in developing countries will be able to closely relate with many of Clezy's experiences, particularly the obstacles and barriers to providing health care in these places and the trims and trappings of an ex-pat's existence. Clezy does delve into detail when he talks about cases but always attempts to explain things clearly to the lay reader.

This book illustrates the life of an amazing Australian who has committed his life to helping those far less fortunate.

Anthony Draper MAIMS
Medical Scientist
Dili TIMOR-LESTE
Following is a list of books available for review by resource consultants and members of the Institute with particular expertise in the field. The reviewer is invited to retain the complimentary copy of the book once the review is received.

As per our agreement with the book publishing companies, complimentary books are submitted to the Institute provided that all reviews are published in the Australian Journal of Medical Science. These reviews must be of a high quality as buying decisions and the reputation of the book and author are important considerations.

Books not requested will be allocated at discretion of the Editors for the Australian Journal of Medical Science. Reviews should be 300 to 700 words depending on the volume of the book. Time limit for return of review is six weeks.

Unfortunately AIMS is unable to send books overseas.

Please send your request to: Australian Institute of Medical Scientists PO Box 1911 Milton Qld 4064
Tel: (07) 3876 2988 Fax: (07) 3876 2999 Email: aimsnat@aims.org.au

Latest additions:

- Life’s Logic

1. Alzheimer’s Disease - Modernizing Concept, Biological Diagnosis and Therapy edited by H. Hampel & M.C. Carrillo. Karger. vi+194 pages


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<th>Title</th>
<th>Authors/Editors</th>
<th>Pages/Volume</th>
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<tr>
<td>22.</td>
<td>Hepatitis C: Antiviral Drug Discovery &amp; Development</td>
<td>edited by S Tan &amp; Y He, Caister Academic Press.</td>
<td>x + 390</td>
</tr>
<tr>
<td>24.</td>
<td>Knowing One's Medical Fate Challenges for Diagnosis and Treatment, Philosophy, Thics and Religion</td>
<td>edited by G. Pfleiderer, M. Battegay &amp; K. Lindpaintner.</td>
<td>vi + 122</td>
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<tr>
<td>25.</td>
<td>Lactobacillus: Molecular Biology – From Genomics to Probiotics</td>
<td>edited by Asa Ljungh &amp; Torkel Wadstrom.</td>
<td>206</td>
</tr>
<tr>
<td>27.</td>
<td>Life’s Logic</td>
<td>by Juliet Flesch. Australian Scholarly Publishing.</td>
<td>280</td>
</tr>
<tr>
<td>40.</td>
<td>Retroviruses: Molecular Biology, Genomics &amp; Pathogenesis</td>
<td>edited by Reinhard Kurth &amp; Norbert Bannert.</td>
<td>xviii+ 454</td>
</tr>
<tr>
<td>41.</td>
<td>Rhabdoviruses Molecular Taxonomy, Evolution, Genomics, Host-Vector Interactions, Cytopathology and Control</td>
<td>edited by Ralf G. Dietzgen &amp; Ivan V. Kuzmin. Caister Academic Press.</td>
<td>276</td>
</tr>
<tr>
<td>42.</td>
<td>Stress Response in Microbiology</td>
<td>edited by Jose M. Requena. Caister Academic Press.</td>
<td>436</td>
</tr>
<tr>
<td>43.</td>
<td>The Regulatory Genome: Gene Regulatory Networks in Development &amp; Evolution</td>
<td>author EH Davidson. Academic Press.</td>
<td>289</td>
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YEAR 2013

APRIL 13 - 14
AIMS SA/NT Branch Multidisciplinary Weekend Seminar
Clare Country Club
Clare   SA   AUSTRALIA
www.aims.org.au/events/category/sa

APRIL 22 - 25
19th Annual ISCT Meeting
Auckland Convention Centre
Auckland   NEW ZEALAND

APRIL 30 - MAY 1
Technology Enhanced Learning in Health
Rydges
Melbourne   VIC   AUSTRALIA
www.techlearninghealth.com

MAY 9
RCPA-ISBER: Pathology at the Core of Biobanking and Translational Research
Sydney Convention Centre
Sydney   NSW   AUSTRALIA
http://www.isber.org/mtgs/2013/pathologysession.cfm

MAY 17 - 19
AIMS NSW South West Division Annual Conference
Rydges Capital Hill
Canberra   ACT   AUSTRALIA

MAY 19 - 23
Euromedlab
Milano Convention Centre
Milano   ITALY
http://www.milan2013.org/

MAY 30 - JUNE 1 & JULY 25 - 27
AIMS/RCPAQAP Haematology Morphology Workshops
Two haematology workshops combining Malarial Parasite and Morphology are held each year. Applications to attend the workshops are now open.
St Leonards   NSW   AUSTRALIA

JUNE 7 - 9
Capricornia Medical Science Association
Mercure Capricorn Resort
Yeppoon   QLD   AUSTRALIA
http://www.cmsa2013.com

AUGUST 22
15th International Congress of Immunology
Rome   ITALY

AUGUST 25 - 28
International Council on Alcohol Drugs and Traffic Safety Conference
Brisbane Convention and Exhibition Centre
Brisbane   QLD   AUSTRALIA

SEPTEMBER 2 - 4
AIMS National Scientific Meeting
Grand Hyatt
Melbourne   VIC   AUSTRALIA

SEPTEMBER 16 - 19
AACB 51st Annual Scientific Conference
Evidence Based Laboratory Medicine
Gold Coast Convention & Exhibition Centre
Gold Coast   QLD   AUSTRALIA

OCTOBER 6 - 9
Asia-Pacific Federation for Clinical Biochemistry and Laboratory Medicine Congress
Bali Nusa Due Convention Centre
Bali   INDONESIA

OCTOBER 20 - 23
HAA Meeting
Gold Coast   QLD   AUSTRALIA

DECEMBER 2 - 5
43rd Annual Scientific Meeting
Australasian Society for Immunology
The Michael Fowler Centre
Wellington   NEW ZEALAND
http://cmnzl05.eventcms.co.nz/page/63/home

DECEMBER 8 - 11
54th ASH Annual Meeting and Exposition
Atlanta   GA   USA
http://www.hematology.org/
The Australian Institute of Medical Scientists is proud to host and invite you to the 2013 AIMS National Scientific Meeting (NSM), 2-4 September 2013.

The Meeting will bring to the Grand Hyatt, Melbourne, a top level forum of leading national and international speakers to address topical issues in medical science.

Please refer to the AIMS NSM website for information regarding formatting requirements and submission guidelines.  

KEY TIME FRAMES
- Deadline for submission of abstracts: 3 May 2013
- Notification to authors, including accepting or rejecting abstracts: 24 May 2013
- Early bird registration date closes: 19 July 2013

FOR FURTHER INFORMATION:
Contact: Maree Overall of ASN Events
E-mail mo@asnevents.net.au
Instructions to authors

The following instructions are based on the “Uniform Requirements for Manuscripts Submitted to Biomedical Journals”, also known as the Declaration of Vancouver, and on the Australian Government Style manual: for authors, editors and printers, 6th edition, 2002. URLs were correct on September 29th, 2008.

Manuscripts that do not fully comply with the following 'Instructions to Authors’ may be returned for revision before they are considered for publication.

The Australian Journal of Medical Science (AJMS) will consider for publication any paper relevant to the field of Medical Science. Disciplines include blood banking, clinical biochemistry, haematology, histopathology, immunology, microbiology and molecular biology. Areas of general interest to medical laboratory scientists, including toxicology, epidemiology, public and community health, and professional and management issues will also be considered.

Papers published in the AJMS are in the form of:
- Review Articles
- Original Articles
- Brief Communications
- Technical Notes
- Case Studies
- Letters to the Editor
- Book Reviews

Articles submitted for publication are understood to be offered only to the AJMS and those accepted become the property of the AJMS.

All individuals listed as authors must have made a substantial contribution to the conception and design of the study, the acquisition of data or the analysis and interpretation of data; the drafting of the article or revising it critically for important intellectual content; and final approval of the version to be published. The corresponding author must take responsibility for obtaining permission from all the authors for the submission of any version of the manuscript and for any changes in authorship.

When the manuscript is submitted the authors must disclose any potential conflict of interest and/or commercial support.

Requirements & preparation of manuscripts

General

Articles should be submitted in electronic format to ajms@aims.org.au. If an article is too large to be submitted by email, it should be submitted on a CD.

Number pages consecutively commencing with the title page.

Arrange the article in the following sequence:
- Title page
- Abstract and key words
- Main Text

- Acknowledgements
- References
- Tables - each table, complete with title and footnotes, on a separate page
- Legends for illustrations.

Authors should ensure that their manuscript communicates their ideas and concepts simply and clearly so that the article is easily read and understood. Authors are strongly recommended to refer to the recommendations on reporting standards as outlined in the statements and checklists of the CONSORT group (see: http://www.consort-statement.org/) and similar groups such as STARD (see: http://www.stard-statement.org/). The principles outlined in these standards may be used as general guidelines and not just as applied to clinical trials and diagnostic studies.

Title page

The title of the article should not exceed three lines (40 characters per line), including punctuation and spacing. All authors must be identified on the title page (e.g., William Smith, Susan Yeo, …”). Where applicable, the title page should also include the name of the institution with which each author is affiliated and to which the work should be attributed. In the case of multiple authors, the name, postal address, email address, telephone and facsimile number of the author responsible for correspondence relating to the manuscript should be indicated.

Abstract & keywords

The abstract should be approximately 150 words and should make sense when read alone or in conjunction with the article. The abstract should be a concise overview that describes the important details of the article including the purpose of the study/investigation, basic procedures (study subjects/experimental animals/observational and analytic methods) and the results and principal conclusions. New and important aspects of the work and its implications may also be included. References should not be included.

Three to ten keywords may be listed. Authors are advised to comply with the terms from the Medical Subject Headings (MeSH) list from Index Medicus (see http://www.nlm.nih.gov/mesh/). Keywords should be given below the Abstract.

Text

The style of writing should conform to acceptable English usage. Do not use slang, medical jargon or unnecessary abbreviations. Accepted spelling is the first choice given in the latest edition of the Macquarie Dictionary.

Wherever possible observational or experimental articles should be divided into sections headed:
- Introduction
- Materials and methods
- Results
• Discussion
• References

For other types of articles such as commentaries, reports and reviews, use an appropriate format or consult the Editors for guidance.

Introduction
Clearly state the purpose of the article leading the reader from the known to the unknown. Summarise the rationale for the study and state the question to be answered as appropriate. Give only strictly pertinent references, and do not review the subject extensively.

Materials & methods
Present the materials and methods in a logical sequence. Describe the selection of the observational or experimental subjects (patients or experimental animals, including controls) clearly. Notification of ethics approval must be given where relevant. Identify the methods, apparatus and procedures in sufficient detail to allow other workers to reproduce the results. Give references to established methods, including statistical methods. Adequately describe new or substantially modified methods. Identify precisely all drugs and chemicals used, including generic name(s), dosage(s), and route(s) of administration. Do not identify patients or hospitals without consent.

Results
Present the results in the same sequence as given in the Materials and methods; use tables and illustrations where these will help the reader understand the work being presented. Do not repeat in the text all the data in the tables or illustrations.

Discussion
Indicate the new and important aspects of the study and emphasise the conclusions that follow. Do not repeat in detail data given in the Results section and do not add new data. Include in the Discussion the implications of the findings and their limitations and compare the observations to other relevant studies. Recommendations may be included if appropriate. Link the conclusions with the goals of the study and answer the experimental question stated in the Introduction. However, avoid unqualified statements and conclusions not completely supported by your data. Avoid claiming priority and alluding to work that has not been completed. State new hypotheses when warranted, but clearly label them as such.

Acknowledgements
Acknowledge individuals who have made substantial contributions to the study including technical work and financial support. Authors are responsible for obtaining consent from all the individuals acknowledged by name as inclusion may be interpreted as an endorsement of the article's contents.

References
The AJMS uses a modified Harvard System (author-date system).

Throughout the body of the manuscript cite the author/s name and the publication year in parentheses as in the following examples:

(i)  Research in this area (Jones 1999) …

(ii)  It has been successfully demonstrated that (Smith and Brown 1981; Auteur 1995; Scienziato et al 2007).

(iii)  Following further investigation, Wetenschapper (2002) highlighted the difficulties inherent in…

Where there are three or more authors, acknowledge only the first author, e.g., (Smith et al 2007). For two authors the following style should be used: (Smith and Brown 2007).

The reference list should be in the format described below. Journal titles should be abbreviated in Index Medicus format (see: ftp://nlmpubs.nlm.nih.gov/online/journals/ljiweb.pdf) using standard abbreviations from the ISSN List of Title Word Abbreviations (see: http://www.isssn.org/en/node/344) All authors should be given in the reference list.

Do not use abstracts as references. “Unpublished observations” and “personal communications” may not be used as references, although references to written, not verbal, communications may be cited (in parentheses) in the text. Include in the references manuscripts accepted but not yet published, designate the journal followed by “in press” (in parentheses). Information from manuscripts submitted but not yet accepted should be cited in the text as “unpublished observations” (in parentheses).

Examples of the correct form for references are given below:

i) Journal Reference:

ii) Personal Author(s) of a book:

iii) Editor, Compiler, Chairman as Author:

iv) Chapter in Book:

v) Online documents:

Tables
Number tables consecutively with Arabic numerals and supply a brief title for each. Give each column a short or abbreviated heading. Place explanatory matter in footnotes, not in headings. Explain in footnotes all non-standard abbreviations used in each table.

For footnotes, use the following symbols in this sequence:

* † ‡ § ¶ ** ††
In preparing tables, consideration should be given to the page width of the Australian Journal of Medical Science. All tables should be prepared for publication vertically. In the text, cite each table in consecutive order, and mark in the margin of the text its approximate location.

If data from another published or unpublished source is used, written permission must be obtained and a copy must accompany the manuscript.

Illustrations

Colour illustrations may be submitted on a CD. Images should be scanned at a minimum of 300 dpi.

When plotting points, the following symbols are preferred:

- ○ ● △ □ ■

In most instances, figures will be reduced to one column in width. All letters and numbers should be drawn to be at least 1.5 mm high after reduction, symbols at least 1.0 mm. Titles for illustrations belong in the legends for illustrations and not on the illustrations themselves.

Photomicrographs must have internal scale markers and the magnification must be stated. Symbols, arrows, or letters used in the photomicrographs should contrast with the background.

Cite each figure in the text in consecutive order, e.g., “Figure 1 illustrates …” or “… as shown (fig. 2)”. If a figure has been published, acknowledge the original source and submit with the manuscript written permission from the copyright holder to reproduce the material. Permission is required, regardless of authorship or publisher, except for documents in the public domain.

Legends for illustrations

When symbols, arrows, numbers, or letters are used to identify parts of illustrations, identify and explain each one in the legends. The figure legend must contain a boldface (a) name (“Figure” + arabic figure number) and (b) substantive title.

Avoid abbreviations in the title. The full term for which an abbreviation stands must precede its first use in the text unless it is a standard abbreviation for a unit of measurement.

Report measurements in the units in which the measurements were made. In most countries the International System of Units (SI) is standard.

Commonly used abbreviations

<table>
<thead>
<tr>
<th>Abbreviation or Symbol</th>
<th>Standard Units of Measurement</th>
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Additional information

The following are useful sources of information. The first two publications are used by the AJMS as standard references.


AIMS NSM 2013
"Tackling Science, Kicking Goals"
2nd - 4th SEPTEMBER 2013 | Grand Hyatt, Melbourne Victoria

The Australian Institute of Medical Scientists is proud to host and invite you to the 2013 AIMS National Scientific Meeting (NSM), 2-4 September 2013. The Meeting will bring to the Grand Hyatt, Melbourne, a top level forum of leading national and international speakers to address topical issues in medical science.

SUBMITTING ABSTRACTS
This information is to be submitted electronically through the AIMS NSM website. Please refer to the AIMS NSM website for information regarding formatting requirements and submission guidelines.

POSTER PRESENTATIONS
You are welcome to send an abstract that will describe your poster submission. Please refer to the AIMS NSM website for information regarding formatting requirements and submission guidelines.

KEY TIME FRAMES
- Deadline for submission of abstracts: 30th April 2013
- Notification to authors, including accepting or rejecting abstracts: 24th May 2013
- Early bird registration date closes: 19th July 2013

www.aims.org.au

This document contains information on products which is targeted to a wide range of audiences and could contain product details or information otherwise not accessible or valid in your country.
HAEMATOLOGY UPDATE

Transformation from a transient myeloproliferative disorder of Down syndrome to acute megakaryoblastic leukaemia

ORIGINAL ARTICLE

Diagnosis of iron deficiency and anaemia of chronic disease

ORIGINAL ARTICLE

Low temperature, heat-induced epitope retrieval on free-floating/whole-mount archival human gynaecological pathology paraffin sections