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**The Relationship Between Helicobacter Pylori Positivity and B12 Deficiency**

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One of the common causes of vitamin B<sub>12</sub> deficiency is failure of absorption. There have been several reports from researchers overseas that helicobacter pylori infection may prevent adequate B12 absorption and that triple therapy may restore it. We were interested to ascertain whether this was the case in our patients. An initial retrospective study of 132 patients in our databases was insufficiently powered (40%) to obtain statistically useful results. Accuracy was further marred by incomplete data, incompletely characterised population, and variable B12 determination methodology. We therefore undertook a prospective study to investigate the correlation between B12 levels and Clo-test positivity in our local patient populations, and whether there were significant interactions with other factors such as smoking, diet, and population group. Patients undergoing gastroscopy and Clo-test at Middlemore Hospital, and who had not received B12 injections, were tested for B12 levels, with consent. Power calculations reveal that at least 320 subjects are required to have an 80% chance of observing a medium-sized change in B<sub>12</sub> levels. So far no significant correlation has been found. Recruitment is ongoing however, and updated results will be presented.

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**‘Never again’ or ‘no worries’: Auditing the patient’s experience of bone marrow aspiration and trephine**

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**Aims:** Bone marrow aspiration and trephine (BMAT) is a relatively simple procedure which is generally regarded as painful and invasive. However some patients report very little discomfort. Our aims were to audit patients’ experiences of the bone marrow procedure at our centre, to make an assessment of which factors influence these experiences and to compare the reported experiences with the perceptions of staff performing the procedures. **Methods:** All consenting patients presenting to our centre for BMAT between January and May 2003 were administered a patient questionnaire for self reporting of pain using a visual numerical rating scale of pain experienced at consecutive stages of the BMAT procedure. Further questions enabled comparison of pain experienced with age, gender, number of BMATs, site of procedure, individual performing the BMAT, analgesics used, indication for the procedure and patients’ understanding of this, presence of anxiety, waiting time prior to the procedure, duration of BMAT and complications experienced. Medical and nursing staff were administered a separate questionnaire which enabled comparisons of pain reported with staffs’ perceptions. Data were analysed using the student’s T test. **Results:** 98% of patients consented to participation and 368 patients completed the questionnaire. Four patients, unable to read English, could not complete the form. 64% of patients reported only moderate or minor discomfort at all stages of the BMAT. The trephine biopsy was the most painful part of the process. Pain resolved rapidly at completion of the procedure. Patients returned to usual activities without delay. Younger patients more frequently experienced severe pain. Pain was not correlated with gender, number of BMATs,

doctor performing the procedure, site of BMAT or patients' understanding of what would be involved. Patients administered midazolam or entonox experienced least pain. Pain was positively correlated with anxiety before the procedure, waiting time, newly diagnosed diseases and duration of procedure. No complications were reported during the audit. Doctors and nurses consistently rated their impression of patients' pain as equivalent or higher than rated by patients. Conclusions: For the majority of patients undergoing BMAT at our centre minor or moderate discomfort is experienced. When waiting times and duration of procedure are minimised patients report less pain. Anxious and younger patients report more severe pain and may benefit from additional analgesia such as entonox or midazolam. The patients' pain is often less than doctors and nurses perceive it to be. Complications are rare and recovery is rapid.

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**The use of ARROW L.Port dual lumen detached catheter system in Therapeutic Plasma Exchange – The Liverpool Hospital experience.**

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Long term venous access in Apheresis patients can prove to be difficult especially if treatment is required more frequently than weekly. Multiple needling over time into peripheral veins can lead to poor access and flow rates. The Apheresis Arrow L. Port dual lumen was used in two patients who had both experienced previous peripheral and central venous access difficulties. The objective of using the dual lumen Port-A-Cath was to perform therapeutic plasma exchange in patients whilst maintaining adequate venous flow levels and access pressures. The insertion of the dual lumen Port-A-Caths were performed under General Anaesthetic, and used within two days of insertion. An Arrow single use 14 gauge 19mm length straight non-coring needle was used for access and a 19 gauge x 25mm 90° non-coring metal hub needle was used for return. Access was undertaken using aseptic technique. The access needle is manufactured with a metal introducer and metal sheath but no sealing device at the attachment end. After insertion of the needle and removal of the metal introducer, the risk of air embolus was of concern. The patients were requested to breath hold at the time the introducer was removed and a three way stopcock applied. Initially inadequate flow pressure was experienced on the return line. This was due to the small gauge plastic extension tubing (15cm length x .4ml volume) required for locking the Port-A-Cath under pressure at the completion of the procedure. A Three way stopcock was used for the 2<sup>nd</sup> procedure with the alleviation of return pressure problems. Patient 1 became febrile within two weeks of insertion, and the Port-A-Cath was considered possible source of infection, and was consequently removed. The Dual Lumen Port-A-Cath proved very successful for therapeutic plasma exchange in patient 2. It was successfully used for plasma exchange second weekly, blood and platelet transfusions weekly, intravenous antibiotics and fluids intermittently for approximately eight months. The dual lumen Port-A-Cath has been used by a variety of departments including Apheresis, the Haematology Ward, Cancer Therapy Centre, and Ambulatory Care within Liverpool Hospital.

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### **Reduction of Intravascular Catheter Exit Site Infections among Neutropenic Patients by Sustained Release Chlorhexidine Dressings: Results from a Prospective Randomized Controlled Trial**

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Exit site and tunnel infections of central intravascular catheters are a frequent source of morbidity among neutropenic patients and may necessitate catheter removal. They require antimicrobial therapy that increases health care costs and is associated with adverse drug reactions. A prospective randomized clinical trial was conducted among adult patients undergoing chemotherapy in a haematology unit. Intravascular catheters were randomized to receive the control of a standard dressing regimen as recommended by the British Committee for Standards in Haematology, or to receive the intervention of a sustained release chlorhexidine dressing. Follow up data were available in 112 of 114 intravascular catheters which were randomised. Exit site or combined exit site tunnel infections occurred in 23 (43%) of 54 catheters in the control group, and 5 (9%) of 58 catheters in the intervention group {OR for intervention group compared with control group = 0.13, 95% CI 95%, 0.04 - 0.37}  $p < 0.001$ . More intravascular catheters were prematurely removed from the control group than the intervention group for documented infections {20/54 (37%) v 6/58 (10%) OR = 0.20(CI, 95% 0.53-0.07)}. However there was no difference in the numbers of intravascular catheters removed for all proven and suspected intravascular catheter related infections {21/54 (39%) v 19/58 (33%)} or in the time to removal of catheters for any reason other than death or end of treatment for underlying disease. Thus chlorhexidine dressings reduced the incidence of exit/tunnel infections of indwelling intravascular catheters without prolonging catheter survival in neutropenic patients and could be considered as part of the routine management of indwelling intravascular catheters among neutropenic patients.

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### **Oral Mucositis After High Dose Chemotherapy: A Pilot Study of Lenograstim Mouthwash Added to Standard Therapy.**

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Oral mucositis is a common adverse effect of high-dose chemotherapy, occurring in up to 90% of patients. It results in pain, reduced oral nutrition and an increase in oral and systemic infections. The cause of oral mucositis includes the antiproliferative effect of chemotherapy on rapidly dividing mucosal cells, and loss of the oral mucosal protectant effect of neutrophils. The use of G-CSF or GM-CSF has been shown to hasten neutrophil recovery following myelosuppressive chemotherapy. The aim of trialing oral 'Granocyte' as a mouthwash in conjunction with standard therapy is to investigate the incidence and severity of oral mucositis after high-dose chemotherapy. Patients on 'BEAM' chemotherapy for autologous stem cell transplantation were studied. Patients were randomly chosen by nursing and medical staff for the double-blind study. Dental evaluation was performed by nursing staff prior to chemotherapy, then 2<sup>nd</sup> daily after oral 'Granocyte' was commenced. Oral 'Granocyte' was commenced on the same day as systemic 'Granocyte', and was given BD until systemic 'Granocyte' was ceased upon marrow recovery. The 'Granocyte' mouthwash contained carboxymethylcellulose sodium 0.1g, lenograstim 130mcg, oleum citrii gtt. I, and, purified water 5mls. The control mouthwash comprised of the

above ingredients without lenograstim. Patients were instructed to rinse with the mouthwash vigorously for 1 minute and swallow, then to avoid eating for 1 hour. Mucositis was assessed second daily and graded to the World Health Organisation scoring system. A subjective pain scale was completed by the patient, using a scoring system of 1 to 10. Diarrhoea was documented and medications taken were listed. Days of fever, hospitalisation and parenteral nutrition were documented. Second daily neutrophils were measured. So far 8 patients have been accrued to the study, 4 receiving 'Granocyte' mouthwash and 4 placebo. The four patients who used the 'Granocyte' mouthwash had nil oral pain throughout their treatment, and two of the four patients on the control had oral pain scores of two and three out of ten. Of four patients on oral 'Granocyte' only one had grade 1 mucositis, whereas two of the four patients on the control arm had oral mucositis grade 1 out of 4. This pilot study has indicated that oral 'Granocyte' may reduce the pain and severity of oral mucositis in patients receiving high-dose chemotherapy.

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**Efficacy of Recombinant Human Erythropoietin-Beta (rh-Epo-beta) in Anemia of Elderly**

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Background: Senile anemia is an anemia in elderly which no establish therapy was well documented.

Aim: We study an efficacy of recombinant human erythropoietin-beta in treatment of senile anemia. Definition of senile anemia: age >60 years old who had anemia without any following conditions: chronic infection, chronic inflammation, malignancy, chronic renal failure, chronic liver disease. The Hb was less than 13 g/dL in male and less than 12 g/dL in female, WBC greater than 4,000/mm<sup>3</sup>, platelets count greater than 140,000/mm<sup>3</sup>. Serum ferritin was greater than 45 ng/ml and/or positive iron stain in bone marrow. Mean corpuscular volume was less than 105 fL with normal kidney function (creatinine<124 mmol/L), liver function test and erythrocyte sedimentation rate (ESR) or C-reactive protein (CRP). Peripheral blood smear and bone marrow study showed no features of myelodysplastic syndrome (MDS) nor any malignancy or specific marrow disorder with normal karyotype. The patient has no occult blood in stool with normal chest x-ray.

Materials and Methods: rh-Epo- $\alpha$  10,000 IU subcutaneous 3 times a week for 4 weeks then evaluate the response every 4 weeks. If the hemoglobin increase less than 1 mg/dl, the dosage of rh-Epo- $\alpha$  is raised to 20,000 and then 30,000 IU if it still doesn't respond to rh-Epo- $\alpha$ .

Results: all the patients (9/9) respond to rh-Epo- $\alpha$  within 4 week after treatment without any adverse events.

# **Posterior Leukoencephalopathy complicating the Treatment of Haematological Malignancy in two patients**

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Posterior leukoencephalopathy (PL) is a rare but distinct clinicoradiological syndrome comprising headache, altered conscious state, visual disturbance, and seizures with symmetric occipital white matter changes on T2-weighted MRI. PL is a well recognised complication of malignant hypertension, eclampsia, renal failure, and cyclosporin/tacrolimus toxicity; its pathogenetic mechanism is poorly understood but may relate to failure of cerebral vascular autoregulation. PL is rarely described in the context of haematological malignancies. Between 12/02 and 3/03, 2 patients developed PL during treatment for haematologic malignancies. Patient one was a 65 year old man with multiple myeloma who underwent salvage therapy with DT-PACE (dexamethasone, thalidomide, cisplatin, adriamycin, cyclophosphamide and etoposide). On d8 post chemotherapy he developed severe headache, bilateral cortical blindness, oculoparesis and rapid decline in conscious state, progressing within 6 hours to a coma with recurrent focal seizures; he was moderately hypertensive (BP 160/80) and fluid overloaded. MRI confirmed the clinical diagnosis of PL and excluded cerebral venous thrombosis. With medical control of blood pressure, he recovered consciousness within 12 hours and made a full neurological recovery within one week. Repeat MRI two weeks later showed complete resolution of PL. Patient two was a 60 year old man with relapsed lymphoblastic lymphoma who received salvage therapy with high dose methotrexate and cytarabine, complicated by severe methotrexate toxicity and acute renal failure requiring treatment with carboxypeptidase, intravenous alkalinisation and hydration, and prolonged folinic acid rescue. On d8 he developed severe right sided headache and visual blurring, progressing to confusion, gait unsteadiness and dysarthria. 36 hours later, he suffered a generalised clonic-tonic seizure. BP was 150/80. MRI revealed occipital changes characteristic of PL, and a CSF examination was unremarkable. The patient made a full neurological recovery, and repeat MRI three months later show near complete resolution. Discussion: Both patients received multi-agent chemotherapy, and were significantly fluid overloaded with moderate hypertension at development of PL. Little is known about PL in patients with haematological malignancies but no specific agent is reproducibly implicated, and hypertension and fluid overload are common factors. The cause of preferential posterior involvement is unknown but may relate to decreased sympathetic innervation relative to the anterior circulation, resulting in decreased ability to autoregulate sudden shifts in blood pressure and/or fluid states. In conclusion, PL is an important diagnosis to consider in patients with haematological malignancies who develop unexplained posterior cerebral symptoms. Diagnosis is clinicoradiological and supportive therapy with blood pressure control usually leads to full neurological recovery.

### **Efficacy and Toxicity of Amphotericin B Colloidal Dispersion for Suspected Fungal Infections in Haematology Inpatients**

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**Objective:** Invasive fungal disease continues to present management problems in patients treated for haematological malignancies. The key therapeutic tool for 30 years has been Amphotericin B, but its usage has been complicated by renal and hepatic dysfunction and infusion reactions. Newer formulations including liposomal and colloidal amphotericin seek to avoid these problems. We present the results of a 4 month usage of colloidal amphotericin in Haematology patients with suspected fungal disease. **Methods:** Twenty-two patients commenced on amphotericin B colloidal dispersion (Amphocil™) between March and end of June 2003 were submitted to retrospective analysis – ten of these patients had undergone autologous or allogeneic stem cell transplantation. Case notes and pathology results of these patients were analysed for documented evidence of drug efficacy and side effects including infusion reactions, electrolyte imbalance, rashes, renal and hepatic dysfunction. **Results:** Of the 22 patients who received one or more doses of Amphocil™, only two received treatment with prophylactic intent (neither of whom developed infection). The remaining twenty were treated for suspected fungal disease, seven of whom had suggestive radiological changes, and two of whom had biopsy proven disease. The median duration of treatment was 8 days, with the majority receiving a dose of 3.5mg/kg/day or above. At least partial resolution of infection was demonstrable in four out of nine patients with radiological or biopsy evidence of infection, while seven other patients defervesced during treatment (often coinciding with granulocyte recovery). The notable toxicity was infusional, with 15 patients (68%) developing marked rigors, 13 (59%) developing fevers, and four cases of hypoxia. Consequently treatment was ceased prematurely in seven patients (after 5 doses or less). The only patient who developed renal failure had several contributing comorbidities. Nine patients had increased potassium requirements, and six patients had asymptomatic doubling of liver enzymes, four of whom were also on Total Parenteral Nutrition. **Conclusions:** Drug efficacy was demonstrated in almost 50% of patients with evidence of fungal infection. There were no cases of renal impairment solely attributable to Amphocil™. There was a high rate of infusional toxicity, which limited the ability of several patients to complete a therapeutic course.



**Normal Cytokine Production by T cells in Patients with Breast Cancer**Trickett AE<sup>1,3</sup>, de Souza P<sup>2,3</sup>, Hicks C<sup>1,3</sup>, Case J<sup>1,3</sup>, Kwan YL<sup>1,3</sup><sup>1</sup>Haematology and, <sup>2</sup>Oncology, St George Hospital, Sydney, NSW; <sup>3</sup> University of New South Wales, Sydney

**Background:** It is often stated that patients with cancer have reductions in T lymphocyte number and/or function. The studies that gave rise to such statements were generally performed over 20 years ago. Following recent improvements in techniques for assessment of T cell function, it is now possible to determine cytokine production by individual cells in response to specific antigens and super-antigens. **Aims:** This study aimed to assess T cell cytokine production in response to the super-antigen staphylococcus enterotoxin B (SEB) and cytomegalovirus (CMV) specific antigen in patients with breast cancer before and after systemic treatment. **Methods:** T cells in whole blood were stimulated by incubation with SEB or CMV, combined with antibodies to the co-stimulatory molecules CD28 and CD49d, and brefeldin A. Cells were fixed and permeabilised, then stained for intracellular expression of interferon (IFN) $\gamma$ , interleukin (IL)-4, IL-2, and tumour necrosis factor (TNF) $\alpha$ . Samples were analysed by four-colour flow cytometry. Post-surgical patients with breast cancer were tested before systemic treatment (Pre), one month after chemotherapy (CT) with doxorubicin and cyclophosphamide, and six months after radiotherapy (RT), and compared to 11 healthy age and sex-matched control subjects. **Results:** The mean  $\pm$  1SD number of CD4<sup>+</sup> cells per  $\mu$ l blood that produced cytokines in response to SEB is shown in the table. Despite a reduction in the number of CD4<sup>+</sup> T cells in patients following CT, the number of these cells responding to SEB was similar at all time points to the healthy controls. Likewise, there was no significant difference in the response of CD4<sup>+</sup> lymphocytes to CMV between controls and patients at any time point (data not shown).

Cell / cytokine	Controls (n=11)	Pre (n=13)	CT (n=9)	RT (n=4)
CD4 <sup>+</sup>	927 $\pm$ 411	887 $\pm$ 380	676 $\pm$ 272*	755 $\pm$ 400
CD4 <sup>+</sup> / IFN $\gamma$	45 $\pm$ 32	61 $\pm$ 55	63 $\pm$ 47	69 $\pm$ 54
CD4 <sup>+</sup> / IL-4	4 $\pm$ 2	6 $\pm$ 4	6 $\pm$ 4	4 $\pm$ 2
CD4 <sup>+</sup> / IL-2	77 $\pm$ 51	93 $\pm$ 76	87 $\pm$ 59	110 $\pm$ 86
CD4 <sup>+</sup> / TNF $\alpha$	120 $\pm$ 81	144 $\pm$ 101	132 $\pm$ 82	168 $\pm$ 124

\* p &lt; 0.01 compared to pre-treatment (paired t test)

**Conclusion:** T cell function, as assessed by intracellular cytokine production in response to SEB or CMV, is normal in patients with breast cancer following surgery, chemotherapy and radiotherapy.

**The First Reported Chinese Family with Haemoglobin Ube-4**

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Hb Ube-4, an alpha globin chain variant (116 GAG  $\rightarrow$  GCG; Glu a Ala), has been described in two reports in a Japanese and a Korean family. We encountered the third case of Hb Ube-4 in a Chinese family. A 5-year old boy presented with features of thalassaemia intermedia. A family study was done and the results are shown in the table below.



	Patient	Father	Mother
<b>Sex</b>	M	M	F
<b>Age (yrs)</b>	5	35	37
<b>Hb (g/L)</b>	5.6	13.6	12.9
<b>MCV (fL)</b>	63.4	63.3	77.0
<b>HbA %</b>	13.7	55	68.4
<b>HbF %</b>	69	0.9	8.6
<b>HbA2 %</b>	3.1	3.9	3.3
<b>Hb Variant %</b>	9.1	Neg	17.7
<b>Hb H bodies</b>	Neg	Neg	Neg

The patient has markedly elevated Hb F (69%), normal Hb A<sub>2</sub> level and a Hb variant band in the alkaline gel electrophoresis, which moves between Hb S and Hb A<sub>2</sub>. Globin chain electrophoresis suggested that the Hb variant is an alpha chain variant. His HPLC tracing showed three abnormal peaks, one between Hb F and Hb A, the other two smaller bands in the D-window. The father's findings are consistent with beta thalassemia trait. Interestingly the mother showed a Hb variant, which moves in a different position in alkaline gel electrophoresis when compared with his son's variant band. It moves between Hb S and Hb F, which is faster than the child's variant band. In her HPLC tracing, the bands are at the D/S windows, which are at similar positions as her son's two minor bands. Her globin chain electrophoresis also suggested that the Hb variant is an alpha chain variant.  $\beta$ -globin genotyping showed that the father is heterozygous for codon 17 (A->T)  $\beta^0$ -mutation while the mother is heterozygous for nt-29 (A->G)  $\beta^+$ -mutation. The child was shown to be compound heterozygous for codon 17 and nt-29 mutations ( $\beta^0 / \beta^+$  configuration), which explains the thalassaemia intermedia presentation with markedly elevated Hb F level.  $\alpha$ -globin genotyping showed that both the mother and child have a point mutation in the  $\alpha 1$  gene (116 GAG  $\rightarrow$  GCG, Glu  $\rightarrow$  Ala), which confirms the alpha haemoglobinopathy to be Hb Ube-4. The different electrophoretic mobilities of the Hb variant in alkaline gel can be explained by the binding of the  $\alpha$ -globin variant with the abundant  $\gamma$  globin chain in the child ( $\alpha^{\text{variant}}\gamma$ ) while the  $\alpha$ -globin variant binds with the  $\beta$ -globin chain in the mother ( $\alpha^{\text{variant}}\beta$ ).

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### Sickle Cell Disease in Pregnancy

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Pregnancy has previously been associated with poor maternal and fetal outcomes in women with sickle cell disease (SCD comprising Haemoglobin SS, Haemoglobin SC and Haemoglobin S/beta-thalassaemia). Despite improvements in the general obstetric and medical management of this disorder over the last twenty years we lack large trials concerning beneficial interventions. Management of the pregnant patient with SCD in Australia is compounded by the relative rarity of the condition here. We present our experience of six pregnancies in four women with HbS/beta-thalassaemia between 1999 and 2003. There was one stillbirth and four live births, with another baby due 28/7/03. Intrauterine growth retardation was not noted, and all deliveries were induced at between 38-39 weeks. All babies were conceived naturally, and

haemoglobinopathy was not detected in the fathers. There was no maternal mortality; maternal morbidity consisted of one case of pre-eclampsia, and one episode of a painful crisis. The women varied in their antenatal sickling severity, ranging from largely asymptomatic to more than five painful crises per year, including chest syndrome. No patient was on a transfusion program antenatally or iron chelation therapy. Challenges in the management of these pregnancies included conception while on hydroxyurea, late presentation to the tertiary referral centre (one at 28 weeks gestation, at term in the case of the stillbirth), and multiple red cell antibodies precluding automated exchange. Transfusion management strategies varied from simple transfusion at twenty weeks and simple transfusion throughout pregnancy, to automated red cell exchange from the third trimester, and manual red cell exchange prior to delivery. The majority of cases maintained Hb>90g/L and HbS < 30%. Our experience is that pregnancy in SCD is best managed in a team approach between the haematology and high-risk obstetric units, and that this should ideally begin pre-conception.

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### **Antenatal Haemoglobinopathy Screening – improving the process**

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“Pregnancy represents one of the great unknowns in life. Everyone’s at least a little worried about how it will turn out” Lawrence Kutner

Haemoglobinopathies are a significant public health problem in Australasia and worldwide. The aim of antenatal screening is to identify inherited haemoglobinopathies and thalassaemias in parents and prospective parents that may lead to clinically significant disease in their children. Accurate and timely diagnosis enables a multidisciplinary team to counsel parents regarding potential outcomes and available options. Where there is an election to continue with the pregnancy this screening also allows better planning of the peri-partum care. The Melbourne Health Shared Pathology Service (MHSPS) screens approximately 2,000 prospective mothers per annum, of whom 49% are born overseas. In our catchment there is a high proportion of mothers and partners from S.E. Asia and other regions where haemoglobinopathies, including the thalassaemias occur commonly. The British Committee for Standards in Haematology (BSCH) 1998<sup>1</sup> and 1994<sup>2,3</sup> Guidelines utilize red cell indices (MCV <80 fL and MCH <27 pg) and ethnic origin as screening tools. In our experience this approach failed to detect all potential clinically significant haemoglobinopathies. In consideration of this, the logistic difficulties of such selected screening, and the diverse ethnic background of the prospective parents presenting to our Service, our approach now includes performing a Full blood count and film and High performance liquid chromatography (HPLC) at the first antenatal visit on every woman, regardless of red cell indices or apparent ethnicity. We have set the maternal red cell indices that trigger early partner screening and consideration of molecular testing at MCV < 80 fL, or MCH < 28 pg. Follow up on abnormal screens, with early partner and molecular testing as indicated, requires a pro-active approach and good co-ordination between the laboratory and the obstetric team. We believe these screening process changes assist this.

<sup>1</sup> Guidelines on the laboratory diagnosis of haemoglobinopathies. British Journal of Haematology 1998; 101, 783-792

<sup>2</sup> Guidelines for the fetal diagnosis of globin gene disorders. Journal of Clinical Pathology 1994; 47, 199-204

<sup>3</sup> Guidelines for investigation of the alpha and beta thalassaemia traits. Journal of Clinical Pathology 1994; 47, 289-295

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### **Investigation of Rare Haemoglobin Variants**

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Southern Cross Pathology at Monash Medical Centre (MMC) is Victoria's reference centre for haemoglobinopathies. We currently assess approximately 1500 patients per annum with the majority of patients being referred for antenatal screening and the remainder for investigation of various red cell disorders. Screening is performed using the Bio-Rad Variant HPLC system with alkaline electrophoresis (Titan III cellulose acetate) and acid electrophoresis (Helena acid agar gel) performed on abnormal variant samples. The diagnosis of rare haemoglobin variants is confirmed by DNA sequencing of alpha, beta or both globin genes in the Clinical Genetics Department. Twenty eight rare haemoglobin variants have been diagnosed over the past three years. A library of the chromatograms, haematological data (full blood examination, haemoglobin electrophoresis) and genetic testing results has been compiled. It is proving to be an important tool in such investigations. Most rare haemoglobin variants described are of no clinical significance, however their identification is necessary particularly in the antenatal context. HPLC has revolutionised haemoglobinopathy testing providing rapid qualitative and quantitative information in the chromatogram. Together with other haematological data, it can provide important information as to the likely identity of a variant haemoglobin or act as a guide for genetic testing.

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### **A Simplified Endogenous Erythroid Colony Assay for the Investigation of Polycythaemia**

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The *in vitro* growth of erythroid colonies in the absence of erythropoietin, known as endogenous erythroid colonies (EEC), is a diagnostic criterion for polycythaemia vera (PV). The availability of EEC cultures in routine clinical setting is limited as culture methods are laborious, technically demanding, difficult to standardise and expensive. We assessed the performance characteristics of a simplified method using ammonium chloride red cell lysis followed by culture in commercially available, batch-tested methylcellulose media. Methods: Patients who had EEC cultures performed between April 1998 and October 2002 were screened for inclusion and classified into three diagnostic groups: (1) PV (independent of EEC results), (2) Non-PV (secondary polycythaemia and apparent polycythaemia) (3) Idiopathic erythrocytosis. Cultures were performed on peripheral blood and/or bone marrow following ammonium chloride red cell lysis. Methylcellulose culture media that had been batch-tested and specified not to contain erythropoietin (Methocult H4531, Stemcell Technologies) was used. Cells were plated into two

petri dishes and incubated for 14 days. The presence of any erythroid colony of any size was considered positive. Sensitivity and specificity were calculated from groups 1 and 2 only. Results: Seventy-six patients were included; four were secondarily excluded due to culture failure. Of the 14 patients with PV, 13 (93%) were positive for EEC on at least one occasion: 90% (9 out of 10) of bone marrow and 67% (6 out of 9) of peripheral blood specimens were positive. All 30 patients with secondary polycythaemia (n=12) or apparent polycythaemia (n=18) were negative for EEC. The incidence of positive EEC in idiopathic erythrocytosis was 40% (8 out of 28): 50% (5 out of 10) in those who also met one minor criterion for PV, 17% (3 out of 18) in those who did not (p=0.09). The sensitivity and specificity of EEC for PV was 93% and 100% respectively. Patients with idiopathic erythrocytosis were excluded from analysis as they were pathologically heterogeneous and would require prolonged follow-up for definitive diagnosis. We utilised red cell lysis in place of density centrifugation and adherent cell depletion to isolate the mononuclear cell fraction. Parallel studies with added erythropoietin were omitted. The use of batch-tested media could have contributed to the lack of non-specific EEC growths in non-PV subjects. This allowed a simple positive / negative scoring system to be applied successfully. This method required one hour of labour and AUD\$22 in consumables. The results were comparable to those achieved by more elaborate methods.

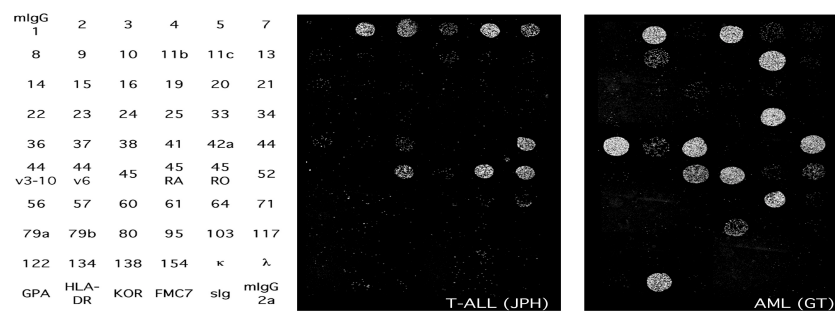
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### **Immunophenotypic Analysis of Acute Leukaemias Using a Novel CD Antibody Microarray**

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We have developed a microarray of 88 CD antibodies (Leukaemia Diagnostic Assay, LDA) which captures leukocytes expressing the corresponding surface molecule. The LDA has been used to determine extensive immunophenotypes for more than 50 acute leukaemias. Standard nomenclature describes myeloid and lymphoid sub-types of acute leukaemia with some overlap ('biphenotypic leukaemias') using clinical features, morphology, limited immunophenotype from flow cytometry, cytochemistry, cytogenetics and molecular studies. The FAB classification recognises 8 sub-types of Acute Myeloid Leukaemias (AML, M0-M7), which are often poorly correlated with the immunophenotype, and 3 Acute Lymphoblastic Leukaemias (ALL, L1-L3) where the immunophenotype improves recognition. The recent WHO classification describes 19 AML types and multiple precursor B-cell and T-cell neoplasms, many with significant prognostic impact.



When a patient presents with acute leukaemia, a rapid and precise diagnosis is often important due to clinical urgency, but full phenotypic, cytogenetic and molecular analysis can be time-consuming. The LDA provides an extensive immunophenotype for pan-leukocyte, myeloid, NK, T- and B-lymphoid, stem cell, adhesion and other non-lineage antigens (see figure) using a rapid and technically simple procedure. Significant differences were seen in the immunophenotypes of acute leukaemias sampled from peripheral blood and bone marrow. Cell morphology, absolute cell number and homogeneity of the leukaemic clone should be considered with the immunophenotype from the LDA in evaluating patients. The LDA should significantly enhance the immunophenotypic evaluation of acute leukaemias.

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### Reciprocal Translocation (X;20) as the Sole Chromosomal Abnormality in a Patient with Myeloproliferative Disorder.

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**Introduction:** We describe a case of myeloproliferative disorder in the setting of an uncommon cytogenetic abnormality involving the long arms of chromosomes X & 20. **Case summary:** A 64 year-old woman presented to our hospital with a six-month history of generalised fatigue. The patient's past medical history was of a T1N0M0 infiltrating ductal breast carcinoma diagnosed in 1998, managed with breast-conserving surgery & adjuvant radiotherapy without chemotherapy or hormonal treatment. Physical examination revealed no organomegaly, lymphadenopathy or focus of infection. The full blood picture was leucoerythroblastic with a normocytic anaemia, marked granulocytic leukocytosis (white cell count  $105.9 \times 10^9/l$ , with differential neutrophils/ band forms 77%, immature myeloid forms 18%, lymphocytes 5%, no basophilia or eosinophilia) & moderate thrombocytopenia. Bone marrow biopsy was hypercellular with markedly elevated myeloid: erythroid ratio & predominantly mature myeloid forms. The final diagnosis was myeloproliferative disorder, unclassifiable. The patient was commenced on hydroxyurea therapy. She had a rapid response with normalisation of the peripheral blood leukocytosis. Cytogenetic analysis demonstrated a mosaic karyotype with eleven out of 20 metaphase cells containing the t(X;20)(q13,q13.3). **Discussion:** Acquired translocations involving either the long arm of chromosome 20 or chromosome X have rarely been reported. When reported, however, translocations involving X cluster to q13 & the haematological diseases are invariably of myeloid

origin. This suggests a role for genes in this region in the neoplastic process. Alternatively, spreading of X inactivation into the derivative 20q is a possible mechanism for the loss of function of tumour suppressor genes on chromosome 20q as opposed to the deletion of chromosome 20q commonly seen in myeloid malignancies. Conclusion: The finding in this patient of t(X;20) together with three others reported in the literature indicates that this may represent a primary non-random abnormality associated with myeloid malignancy and the specific molecular events involved in tumorigenesis.

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### **Molecular Remission following ATRA and Idarubicin, Imatinib Mesylate and Autologous Stem Cell Transplantation in a Patient with Chronic Myeloid Leukaemia in Promyelocytic Blast Crisis**

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The presence of the Philadelphia chromosome and the t (15; 17) translocation in acute myeloid leukaemia is a rare event, with nine cases reported in the literature over the last twenty years. The majority of these have been documented in patients with known chronic myeloid leukaemia (CML) entering promyelocytic blast crisis, with the prognosis poor. Responses to ATRA have been reported, although all cases have remained Philadelphia chromosome positive. We report here the achievement of a stable molecular remission, following treatment that included ATRA and Idarubicin, Imatinib mesylate, and autologous stem cell transplantation, in a patient presenting with acute myeloid leukaemia with the simultaneous occurrence of the t (9; 22) and t (15; 17) translocations. The patient, a 62 year old woman, presented in July 2000 with acute promyelocytic leukaemia. Bone marrow cytogenetics revealed the (9; 22) and (15; 17) translocations in all twenty metaphases examined. She obtained a morphological remission following treatment with ATRA and Idarubicin as per the PETHEMA protocol, and was negative by RT-PCR for the PML-RAR $\alpha$  fusion gene transcript, however 7/50 cells remained positive for the t (9; 22) translocation. She remained Philadelphia chromosome positive but negative for PML-RAR $\alpha$  by RT-PCR despite ongoing consolidation and maintenance therapy, which included 6 mercaptopurine, methotrexate,  $\alpha$ -interferon, arsenic trioxide and ATRA, however she became Philadelphia chromosome negative after three months therapy with Imatinib mesylate 400mg daily, although the bcr-abl fusion gene transcript was still detected by RT-PCR. In September 2002 she underwent an autologous stem cell transplant, with Busulphan and Cyclophosphamide conditioning, followed by maintenance therapy with Imatinib mesylate 400mg daily. Although she was still positive by RT-PCR for bcr-abl at six weeks post transplant, since April 2003 she has been in ongoing molecular remission for both the PML-RAR $\alpha$  and bcr-abl fusion gene transcripts, with the latter confirmed on two occasions three months apart. Although this patient presented with acute promyelocytic leukaemia with coexistence of the Philadelphia chromosome we speculate that this represented promyelocytic blast crisis of chronic myeloid leukaemia. This case differs from those previously reported and documents the achievement of molecular remission for both translocations following targeted therapy which included both ATRA and imatinib mesylate.



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**Treatment of AML with continuous combined granulocyte colony stimulating factor (G-CSF) and oral chemotherapeutic agents.**

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Background: Older age is a poor prognostic factor in acute myeloid leukaemia (AML). Continuous low-dose oral chemotherapy agents (OCA) can achieve partial remission in AML and may be suitable as palliative therapy in elderly patients. It is however complicated by drug-related neutropenia and thrombocytopenia. G-CSF may lessen the associated neutropenia and some anecdotal reports suggest G-CSF alone may induce remission in AML. Hence we have combined G-CSF plus continuous low-dose OCA in elderly patients. Methods: We describe a retrospective audit of 12 patients with AML, diagnosed between 2000 and 2002, who were either unfit for induction chemotherapy or who had had treatment failure with intravenous chemotherapy. Patients were started on an OCA with either 6-mercaptopurine 50-100mg/d, thioguanine 40-80 mg/d, or hydroxyurea 500-1000 mg/d with concomitant G-CSF 283µg or 300µg three to seven days/week. The median age at diagnosis was 80 years (range 70-89 years). Eight (67%) had a preceding myelodysplastic syndrome. Results: *Neutrophil Response*: All patients responded with an increase in neutrophil count  $> 0.5 \times 10^9/L$ . Neutrophil recovery was achieved within 13 days of starting treatment. From the onset of treatment patients had neutrophils greater than  $0.5 \times 10^9/L$  for 71% of the time, despite being on continuous cytotoxic treatment. *Platelet response*: Five patients (42) had a platelet response with a rise in count above  $100 \times 10^9/L$ . *Blast response*: Data for blast count was available for only 10 of the 12 patients. Of these, nine (90%) had a response to treatment, with blast disappearance obtained within 17 days of starting treatment. *Survival*: Eleven of the twelve patients have died. In the first year after the diagnosis, 8 (73%) patients died, and all 11 died within 2 years. Median survival was 9.5 months (3.5-18.8). G-CSF treatment was well tolerated and no patient ceased due to side effects. Conclusion: The analysis of this small cohort suggests that G-CSF administered in combination with one of the above OCAs, may be a novel way of providing treatment to elderly patients with AML which is both tolerable and of potential survival benefit. The results show that the combination therapy is associated with disappearance of peripheral blood blasts, neutrophil recovery and resolution of thrombocytopenia. Median survival was 9.5 months, which compares favourably to historical data considering the mean age of this cohort at 80 years. Despite the limitations of this retrospective audit, the results suggest that a comparative study with current alternatives is warranted.

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**Low Dose Melphalan Treatment for High Risk Myelodysplastic Syndromes**

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The myelodysplastic syndromes (MDS) constitute a heterogenous group of clonal haematopoietic disorders which predominantly affect elderly patients. The majority of MDS patients can not tolerate intensive chemotherapy and are managed by supportive care. The "high risk" patients have a median survival time of less than 6 months. There has been some interest in using low dose chemotherapy to see if there is any survival advantage. Two previous small cohort studies using low dose melphalan have reported clinically meaningful responses in a few selected patients. The best results appear to be in hypocellular refractory anaemia in transformation or in secondary AML. Method: We treated 9 patients (6 men, 3 women) with intermediate to high risk



MDS or secondary acute myeloid leukaemia (sAML). The international prognostic score system values ranged from 1.5 – 3.0 with a median of 2.5. Patients were aged between 56 – 78 (median age = 71 years). They had RAEB (n = 2), RAEBt (n = 2), and 5 cases of sAML. Patients were treated with melphalan 2 mg orally once a day and treatment was discontinued when a complete peripheral response was obtained or if there was evidence of treatment failure after a minimum of 4 weeks. Patients who belonged to the first category could be offered treatment when they relapsed. Results: We observed 3 (33%) complete responses which occurred within 4 – 10 weeks of commencing treatment. Responses included one sAML that lasted 18 months and two other sAML patients with ongoing responses at 13 months and 3 months respectively. There was one partial response and the other patients had no response or disease progression. The 3 patients that responded had normal or hypocellular marrows and a normal karyotype. Conclusions: Our results support the results from two previous studies that low dose melphalan can produce durable remissions in selected patients with high risk MDS. Treatment was well tolerated and convenient for the patients, avoiding hospitalisation. It would be of interest to see whether new agents such as 5-azacytidine can produce similar results in MDS patients.

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### **Results of a Pilot Study Using Anti-Apoptotic and Differentiating Agents in Patients with Myelodysplastic Syndromes (MDS)**

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Myelodysplastic syndromes are clonal haematological disorders characterised by ineffective haemopoiesis. One of the important pathogenetic features is the increased apoptosis. The aim of our study was to explore the use of antiapoptotic agents oxpentifyllin, ciprofloxacin and dexamethasone along with the differentiation promoting vitamin D3 in patients with myelodysplastic syndromes. Material and methods: Patients with proven MDS and IPSS > 0 were eligible into the study. After three months of observation, patients started on oxpentifyllin 400 mg tds, ciprofloxacin 500 mg b.d. and dexamethasone 4 mg daily for the first two weeks after which the dose of oxpentifyllin was increased to 800 mg tds. Vitamin D3 was introduced on week 9 of treatment in addition to the previous drugs at weekly incrementing dose, starting at 0.5 µg/day and up to 2.25 µg/day until the end of planned 16 weeks of treatment. All drugs were administered orally. During the treatment with vitamin D3 patients were educated to keep low their calcium intake. At the end of 16 weeks, treatment was ceased and patients were followed for another 6 months. Blood and platelet transfusion was given as needed. Results: 18 patients were referred for enrolment since April 2001, but only 15 were eligible. Amongst the eligible patients 5 completed the full 16 weeks of treatment, 5 stopped earlier due to progressive disease (n=3) or significant side effects (oedema and thrombocytopenia)(n=2), 1 died the day after consenting with study (not assessable) and 4 refused treatment. Of 5 patients who completed the treatment, 4 (all Intermediate-1 risk group) had significant improvement in their neutrophil count, which remained sustained after secession of treatment in 2 patients for a median of 8 months. One of these (RAEB and ring sideroblasts) showed in addition a reduction in the bone marrow blast cells to 4% and of ring sideroblasts from 20% to 10%. The treatment had no effect on haemoglobin and platelet count across the study. Most common side effects in all patients included insomnia and irritability. Conclusion: Oxpentifyllin, ciprofloxacin, dexamethasone and vitamin D3 might be a useful treatment for patients with MDS, especially Inter-1 risk group. In the next phase of the study treatment will start as soon as the diagnosis was made, Vitamin D3

will be administered from the beginning and in higher dose and pulse dexamethasone will substitute for continuous administration of the drug.

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### ***de novo* Acute Basophilic Leukaemia**

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*de novo* acute basophilic leukaemia is a very rare subtype of acute myeloid leukaemia, comprising of <1% of all cases of AML and usually associated with a poor prognosis and resistance to treatment. A 20-year-old female presented to our institution with a clinical history of joint pain, minor skin itch (no rash) and basophilia on a previous FBC. Full blood count showed a WCC = 8.9x10<sup>9</sup>/L, Hb = 110g/L, platelets = 328x10<sup>9</sup>/L. The differential revealed 35% basophils with abnormal/immature morphology and 5% blasts. A bone marrow biopsy showed abnormal granulopoiesis with 44% immature/blastic basophils with abnormal basophilic granulation, and 21% blasts. No mast cells were present. Cytochemistry was toluidine blue negative, weakly MPO positive, exhibiting coarse staining, and ANAE/CAE negative. Immunophenotyping was positive for early myeloid and aberrant markers: CD45 weakly +; CD34/HLD-DR weakly +; CD38/CD2 +; and CD33/CD13/CD11b weakly +. Cytogenetics showed 47,XX,+mar (not classified) and molecular analysis did not detect any BCR/ABL fusion transcript, nor any genetic mutations in *c-kit* exon 17 (codon 876). A diagnosis of Acute Basophilic Leukaemia (ABL) was made on these findings. The patient received induction therapy with standard cytarabine/idarubicin, attained complete remission, and then received two courses of high dose Ara-C based consolidation. ABL is associated with a myriad of clinical and haematological features including hyperhistaminaemia, urticaria, peptic ulceration or anaphylaxis. Diagnostic FBC may show a pancytopenia or be normal, with a mild to severe basophilia. It is important to distinguish ABL from other basophilic proliferations frequently seen in CML, AML (M2) and hypersensitivity reactions. Blasts cells and immature basophils are often present, with a variable number of blasts cells containing coarse basophilic granules. Granules may be both toluidine blue and MPO positive or negative. Frequently in cases lacking basophilic granules, ultrastructural demonstration of blasts granule peroxidase reaction is required for diagnosis. Immature myeloid markers are usually present. ABL has no recurring cytogenetic or molecular abnormalities, although involvement of 12p or t(6;9) has been associated with basophilia. This presentation of ABL is characteristic of the heterogeneous nature of this rare leukaemia.

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### **SDF-1/IL-7/IL-3 Enhance the Proliferation of Pre-B Acute Lymphoblastic Leukemia Cells**

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Although acute lymphoblastic leukemic (ALL) is responsive to chemotherapy, a significant proportion of patients relapse following treatment. Clearly a greater understanding of pre-B ALL biology is required to improve current therapies and to develop new treatment protocols. Stromal derived factor-1 (SDF-1) is a key regulator of normal and leukemic B cell

progenitors and it binds the G protein coupled receptor CXCR4. CXCR4/SDF-1 interactions drive the chemotaxis and migration as well as the survival and proliferation of numerous hematopoietic cells including leukemic pre-B ALL cells. Recently we demonstrated that inhibition of the SDF-1/CXCR4 axis with the CXCR4 antagonists AMD3100 and T140NH2 has a negative effect on the proliferation pre-B ALL cells grown in complex dexter stroma without any substantial effects on their viability. SDF-1 has been shown previously to enhance the proliferative response of pre-B cells and myeloid progenitors to cytokines such as IL-7. In this study we examined the ability of SDF-1 to support the viability and enhance the proliferation of 11 different patient pre-B ALL cells in conjunction with the cytokines IL-7, and IL-3. under serum and stroma free culture conditions. In 4 of 11 cases studied addition of SDF-1 (200ng) in combination with IL-7 (50U) and IL-3 (20U) did not result in greater viability of ALL with all cells dying in the 4 day period. In 4 of the 7 remaining cases, however, some increase in viability was observed with SDF-1/cytokine treatment compared with the media only control. When proliferation was examined using PKH labeling and thymidine incorporation, 3 of the 7 cases displayed enhanced proliferation with SDF-1/cytokine treatment compared with media alone, 2 showed SDF-1/cytokine independent proliferation and 2 had no proliferation. These results suggest that similar to normal pre-B cells SDF-1 acts as a catalyst enhancing the proliferation of pre-B ALL cells to growth factors such as IL-7 and IL-3. This again highlights the importance of the SDF-1/CXCR4 axis as a potential therapeutic target in acute pre-B leukemias.

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### **The Usefulness of Anti-CD68 in the Flow cytometric Analysis of Acute Leukaemia**

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Anti CD68 has been recommended for inclusion as part of a primary acute leukaemia typing panel. We assessed the diagnostic relevance of this antibody by retrospectively reviewing acute leukaemia cases typed in our laboratory over the past two years. CD68 is an acidic highly glycosylated lysosomal associated membrane protein. It is expressed primarily intracellularly in association with lysosomes but is reported to be detected to a lesser extent on the surface of cells of myeloid and macrophage lineage. It is reported to be weakly expressed on 50% of B-ALL cases. The routine detection by flowcytometry entails intracellular detection using a cell permeabilisation step. 57 consecutive patients with acute leukaemia were immunophenotyped. Our primary immunophenotyping panel consisted of antibodies to CD3, CD7, CD10, CD13, CD19, CD20, CD33, CD34, CD117, HLA-DR cytoplasmic myeloperoxidase and cytoplasmic CD68. Additional markers were tested when required. These included CD2, CD9, CD38, CD56, CD61, CD64, CD71 cytoplasmic CD3, IgM, CD79a and nuclear TdT. Not all markers were tested in all cases. Intracellular antigen testing was performed using Intraprep permeabilization reagent (Beckman Coulter). Mean channel fluorescence (MCF) was recorded for CD68 stained cell populations and interpretation of staining reactions was related back to the MCF of the lymphocyte population. Cases assessed included AML n=50, B-ALL n=6, T-ALL n=1. The sensitivity and specificity for CD68 in determining myeloid lineage was 92% and 100% respectively. All four myeloid leukaemia cases negative for CD68 expression were strongly positive for CD34. Indicating a more immature blast cell phenotype. Interpretation of positivity or negativity was straightforward in the majority of cases. Staining intensity relative to the staining patterns of lymphocytes and monocytes allowed discrimination of blasts showing monocytic differentiation. CD68 is a useful marker, with high sensitivity and specificity, to

include in an acute leukaemia typing panel. Much information can be gleaned from the careful interpretation of the staining pattern and its use in a primary immunophenotyping panel is recommended.

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**Hodgkin's Lymphoma Cell Lines Express A Fusion Protein Encoded By Intergenically Spliced mRNA for the Multilectin Receptor DEC-205 (CD205) and a Novel C-type Lectin Receptor DCL-1**

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Classical Hodgkin's lymphoma (HL) tissue contains a small population of morphologically distinct malignant cells called Hodgkin and Reed-Sternberg (HRS) cells, associated with the development of HL. Using 3'-RACE we identified an alternative mRNA for the DEC-205 multilectin receptor in the HRS cell line L428. Sequence analysis revealed that the mRNA encodes a fusion protein between DEC-205 and a novel C-type lectin DCL-1. Whilst the 7.5 kb DEC-205 and 4.2 kb DCL-1 mRNA were expressed independently in myeloid and B lymphoid cell lines, the DEC-205/DCL-1 fusion mRNA (9.5 kb) predominated in the HRS cell lines (L428, KM-H2 and HDLM-2). Further molecular analysis demonstrated that the fusion mRNA is generated by cotranscription and intergenic splicing of juxtaposed DEC-205 (*LY75*) and DCL-1 (*KIAA0022*) genes. The resulting reading frames encode the DEC-205 ectodomain plus DCL-1 ectodomain, transmembrane and cytoplasmic domain. Using DCL-1 cytoplasmic domain specific polyclonal and DEC-205 monoclonal antibodies for immunoprecipitation /western blot analysis, we showed that the fusion mRNA is translated into a DEC-205/DCL-1 fusion protein, expressed in the HRS cell lines. These results imply an unusual transcriptional control mechanism in HRS cells, which co-transcribe an mRNA containing DEC-205 and DCL-1 prior to generating the intergenically spliced mRNA to produce a DEC-205/DCL-1 fusion protein. We are currently investigating the expression of DEC-205/DCL-1 fusion mRNA and/or protein *in situ* to clarify its diagnostic value.

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**Polymorphism in the Interleukin 13 (IL-13) Gene Promoter is Associated with Varying Levels of IL-13 Production and Susceptibility to High Grade Non Hodgkin's Lymphoma but not Hodgkin's Disease**

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Introduction: IL-13 is a pleiotropic cytokine produced predominantly by activated T cells. Like IL10, it acts on several cell types including B cells to stimulate proliferation and enhance cell survival. IL-13 is expressed by HD cell lines & HD Reed-Sternberg cells and can act as autocrine growth factor. A novel single base substitution polymorphism in the IL-13 gene promoter, at position -1055 relative to the transcriptional start site has been described. The functional significance of this polymorphism is unclear. Our aim was to determine whether this polymorphism influences the amount of IL-13 produced by cells and whether it predisposes individuals to lymphoma. Methods: The IL-13 polymorphism at -1055 was determined using the mutagenically separated polymerase chain reaction (MS-PCR). Its influence on levels of IL-13 production was determined using an IL-13 specific

ELISA to measure levels in the culture supernatant of ConA stimulated lymphocytes obtained from patients of known IL-13 genotype. Results: In lymphocyte cultures stimulated with ConA, the CC genotype produced significantly higher levels of IL-13 relative to the TC genotype in healthy controls ( $p < 0.02$ ). Similar, but not significant, trends were observed in both HD and NHL patients. In NHL patients, both the CC and TC genotypes produced significantly higher levels of IL-13 than both the CC and TC genotypes in healthy controls ( $p < 0.005$ ,  $p < 0.004$ ). No significant differences were observed between HD patients and healthy controls. The TT genotype was not analysed in this study because of its low incidence (approx 1%). We analysed frequencies of the single base substitution in the IL-13 promoter in patients with high grades of lymphoma (B-cell DLCL and other aggressive histologies  $n=57$ ), low/ intermediate grade lymphoma ( $n=69$ ) and Hodgkin's disease ( $n=42$ ) and compared them to healthy controls ( $n=70$ ). The frequency of the high IL-13 producing CC genotype was significantly lower in patients with high grades of NHL lymphoma compared to patients with either low or intermediate grades of NHL and that of healthy controls ( $p=0.002$ , odds ratio=0.2828, 95% C.I 0.1291 to 0.6195;  $p=0.0498$ , odds ratio=0.4993, 95% C.I 0.2467 to 1.051). No association was found between IL-13 genotype and HD or less aggressive forms of lymphoma. Conclusions: The CC genotype in the IL-13 gene promoter is associated with a higher level of IL-13 production compared to the TC genotype. The CC genotype has a significantly lower incidence in high grade forms of NHL. No associations were found in low/intermediate grades of NHL or in HD. Particular genotypes of the IL-13 gene promoter may influence susceptibility to aggressive forms of NHL, or may contribute to the pathogenesis of this disease. Further investigation is required.

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#### **Detection of Gain of Chromosome 2 Short Arm in Hodgkin Lymphoma Using FISH.**

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Cytogenetic studies of Hodgkin lymphoma (HL) are problematic due to the low number of malignant cells, low mitotic rate and complexity of karyotypes. Recent studies using comparative genomic hybridisation have shown gain of chromosome 2 short arm (2p) occurs in approximately 50% of cases of classical HL (cHL), being particularly common in the nodular-sclerosing (cHL-NS) subtype. We investigated whether chromosome 2p gain could be detected using fluorescence in situ hybridisation (FISH) with commercially available probes on touch imprints from lymph node biopsies, and compared this to conventional cytogenetics. Probes utilised included ALK localised to 2p23 and chromosome 2 centromere (Vysis Inc.). In 5 cases studied, gain of 2p was observed in 3 cases (1 nodular-sclerosing HL, 1 mixed-cellularity HL and 1 lymphocyte-rich HL) with 5-6 copies present. In one of these cases there was gain of ALK without concomitant gain of chromosome 2 centromere, indicating partial 2p gain. These represent relative gain of 1-2 copies of chromosome 2 in the typically pseudotetraploid tumours. Two cases (1 mixed-cellularity HL and 1 nodular-sclerosing HL), showed 4 copies, representing no gain. Cytogenetic studies indicated 2p gain in only one case, with the remaining 4 cases showing only normal metaphases or incomplete complex karyotypes in 2-3 cells. To directly correlate 2p gain with identified Hodgkin-Reed-Sternberg (HRS) cells, touch imprints were stained for morphological identification of HRS cells with subsequent FISH analysis. The identified cells were relocated and scored for 2p copy number. The results indicated that a common cytogenetic abnormality in cHL (gain of 2p) can be detected in morphologically identified cells. This will enable a larger series of HL to be tested using relatively routine methods to further characterise the frequency and specificity of this abnormality in cHL subtypes.



### Comparison of Flow Cytometry, Immunohistology and PCR for the Detection of Bcl-2 Rearrangement/Overexpression in Follicular Lymphoma

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**Introduction:** Immunohistology (IH) for aberrant expression of Bcl-2 protein in lymph node germinal centres is an established method for distinguishing follicular lymphoma (FL) from reactive lymph node hyperplasia (RLH). *Bcl-2/IgH* rearrangements can be identified using PCR methods. Flow cytometry (FC) has also been used to detect cytoplasmic Bcl-2 overexpression in FL. The frequency of Bcl-2 overexpression in FL, non-follicular lymphoma and non-malignant cases using FC is unknown. Furthermore, a comparison between IH, PCR and FC methods for detecting Bcl-2 overexpression/rearrangements in FL is lacking.

**Methods:** Cytoplasmic Bcl-2 was analysed in specimens from 12 FL, 29 non-follicular lymphoma and 19 patients without malignancy. Samples included bone marrow aspirate (25), peripheral blood (7) and lymph node biopsies (28). For FC, cell suspensions were stained with Bcl-2-100 antibody (Walter and Eliza Hall Institute, Melbourne) followed by goat anti-mouse FITC (Southern Biotech, USA). For PCR, DNA was extracted from biopsy shavings. Two rounds of nested PCR amplification using *MBR/mcr* primers were performed using standard methods. Amplification of the *HGH* gene was used as an internal control. Immunohistology was performed with Bcl-2 (clone 124) antibody using the DAKO LSAB II system.

**Results:** FC detected cytoplasmic Bcl-2 in (9/12) 75% FL, (16/29) 55% non-follicular lymphoma and (6/19) 32% non-malignant cases. In the 12 FL cases, germinal centre expression of Bcl-2 by IH was found in 92% cases (Table 1). Amplification of the control *HGH* gene by PCR failed in 3/11 FL samples. Of the 8 cases with *HGH* amplified, 4 (50%) had an *MBR* rearrangement. No cases of *IgH/Bcl-2* rearrangement in the *mcr* were identified. In the one case where IH was negative, FC was positive for Bcl-2 and an *MBR* rearrangement was detected.

Table 1. Comparison of FC, IH and PCR methods for detecting abnormal Bcl-2 expression/rearrangements in follicular lymphoma

Diagnosis	Cytoplasmic Bcl-2 detection by FC	Germinal centre Bcl-2 expression using IH	<i>Bcl-2/IgH</i> rearrangement using PCR
Follicular lymphoma	9/12 (75%)	11/12 (92%)	4/8 (50%)

**Discussion:** Flow cytometry compared favorably with IH and PCR methods in detecting abnormal Bcl-2 expression in FL. Abnormal Bcl-2 expression is reported in 80-93% using IH, in agreement with our study. Southern blotting for *Bcl-2/IgH* rearrangements and FISH analysis for t(14;18) has 88% sensitivity in FL but is not widely available. Standard PCR methods detect *MBR/mcr* rearrangements in 30-60% but is confounded by the presence of *Bcl-2/IgH* transcripts in the blood of normal people. Bcl-2 overexpression alone is not able to distinguish FL from other forms of lymphoma and those without malignancy. In summary, FC is an alternative to IH for identifying Bcl-2 in NHL in tissue, bone marrow and blood samples and has potential

diagnostic, prognostic and therapeutic utility, particularly with the emergence of targeted novel therapies such as Bcl-2 antisense.

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### **A Rare Case of Pyrexia of Unknown Origin (PUO)**

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A 45 year old man was referred with PUO, mild night sweats, and weight loss. His skin was pale but otherwise unremarkable, and his spleen was palpable 3 cm below the left costal margin. Blood examination showed anemia (Haemoglobin 87g/L), and Ferritin, CRP, and ESR were markedly elevated. Ultrasound and CT scan of the abdomen confirmed splenomegaly of 15 cm, craniocaudally. Hepatomegaly, lymphadenopathy, and focal splenic lesions were not seen. The patient underwent a Gallium scan showing diffuse enrichment in both lungs. Subsequent high resolution CT imaging proved diffuse patchy ground glass opacities in the lung. Lung function tests were normal except that the DLCO was markedly reduced to 40% of the expected value. This prompted a bronchoscopy when 4 transbronchial biopsies were taken. Histopathology showed a cellular infiltrate confined to intraseptal capillaries, arterioles and arteries. The cells were large with a high nuclear to cytoplasmic ratio, nuclear hyperchromasia and small nucleoli, and stained strongly positive for the B-lymphocyte marker CD20. Counterstaining with endothelial markers CD31 and 34 confirmed the intravascular localization of the infiltrate. The diagnosis of Intravascular Large B-cell lymphoma (ILBCL) was made because of the high specificity of these findings. Subsequently, the patient underwent MRI of the brain demonstrating a focal lesion and multiple small areas of white matter infarction. ILBCL is a rare variant of Extranodal Diffuse Large B-Cell Lymphoma that has been reported with increasing frequency in recent years<sup>1</sup>. It displays a predilection for the CNS<sup>2</sup>, skin<sup>3</sup> and the lung<sup>1</sup>, is often disseminated at diagnosis, and takes a rapidly progressive course<sup>4,5</sup>. PUO appears to be the most common clinical presentation and may precede dyspnea, skin lesions and overt neurological manifestations<sup>1,4</sup>. Screening for interstitial lung disorder and CNS disease should be undertaken once the suspicion of ILBCL has been raised. Lung function studies may prove to be the most sensitive respiratory investigation<sup>1</sup> while MRI of the brain in the absence of neurological symptoms may yield clues to the diagnosis. Combined chemotherapy and radiotherapy is warranted for this complex disease. Our patient commenced eight cycles of CHOP given every 14 days, intrathecal methotrexate (8 doses), and radiotherapy to the brain and medulla. Although he is asymptomatic after first cycle of CHOP it remains to be seen whether or not he will achieve long term remission.



## **Maintenance Therapy with Weekly Chlorambucil in Patients with Symptomatic or Progressive Follicular Lymphoma**

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Although follicular lymphoma (FL) remains as an incurable disease, the current trend is to aim for a complete remission with initial treatment (to improve survival and to minimise the risk of histological transformation) and to consider a non-toxic form of maintenance therapy. Clinical trials for the latter have largely comprised  $\alpha$ -interferon or rituximab. Chlorambucil (Chl) has a proven and well-accepted track record in the initial treatment of patients with FL. However, there is little information on its role as maintenance therapy. This report describes 12 patients with symptomatic, bulky or progressive FL who received long-term maintenance therapy with weekly Chl.

Patients: male 7; female 5; age 52-85 yrs (mean 70.3 yrs). Indication for treatment [A) Symptomatic disease; B) Bulky disease; C) Progressive disease]: A=5, A+B=4, C=2, A+C=1. Nine of the 12 patients had Stage III or IV, low-grade FL (small cleaved cell) exclusively, whilst three had co-existing aggressive lymphoma as indicated by divergent histology(1) and/or sites of gallium avidity.

The three patients with co-existing aggressive lymphoma were initially treated with radiotherapy for localised disease(1) or combination chemotherapy (LOPP-1; CHOP-1). All three were subsequently treated with Chl 0.06mg-0.08mg/kg/d for their FL, as did the other nine patients with exclusive FL. The duration of daily Chl therapy ranged from 2mo to 7mo (mean 4.3mo). In patients achieving complete remission (CR:resolution of symptoms/signs, normal blood counts and normal thoraco-abdominal CT scan examination) the frequency was reduced to two consecutive days each week (mean duration 10.4mo) and then to once weekly. In patients achieving a good partial remission (PR) with stable disease (SD), only the first step of maintenance therapy was instituted.

Results: Ten patients achieved CR; two patients achieved PR. All 12 are alive. Nine of the 10 CR patients are still in CR at 12-72mo (mean 41.4mo); the tenth patient has suffered a localised transformation to diffuse large cell lymphoma at 33mo. The two PR patients remain in PR with SD at 13mo and 21mo respectively. None of the patients developed any treatment-related complications.

Conclusion: Weekly maintenance therapy with Chl appears to sustain remission status in most patients with FL. In view of its simplicity, convenience and low cost, this approach deserves further evaluation in randomised studies including  $\alpha$ -interferon and/or rituximab given as maintenance therapy.

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### **Leukaemic Subtype of Marginal Zone Lymphoma: a rare variant**

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There are three currently recognised subtypes of marginal zone lymphomas (MZL) : mucosa-associated lymphoid tissue MZL (MALT MZL), splenic MZL (SMZL) and nodal MZL (NMZL). As a group, MZL share morphological and immunophenotypic features with marginal zone B-cells in secondary follicles, the postulated common cell of origin. We have identified three patients whose disease does not fit the currently recognised categories, and are best categorised in the putative category of MZL, leukaemic subtype (LMZL). Patients 1 and 2 were male aged 78 and 83; patient 3 was female aged 45. All 3 patients presented for evaluation of incidentally noted atypical lymphocytes in peripheral blood (total lymphocyte count range  $2.4 - 5.8 \times 10^9/L$ ); flow cytometric analyses in all cases were consistent with MZL (CD5-, CD10-, CD19+, CD20+, CD22+, CD23- and sIg+ with light chain restriction). Complete evaluation including computerised tomography (CT; 3 patients), <sup>18</sup>FDG-Positron Emission Tomography (PET; 2 patients) and gastroscopy (1 patient) failed to identify a focus of mucosal, splenic or nodal disease. Other investigations included normal cytogenetics (2 patients) and negative bcl-2/IgH rearrangement by PCR (2 patients). One patient had elevated  $\beta 2$ -microglobulin, but none had elevated LDH. Patient 3 had active hepatitis C (HCV). Her HCV was successfully treated with interferon and ribavirin, attaining persistent HCV-PCR negativity; however her lymphoma remained unchanged following antiviral therapy, in contrast to published reports of SMZL improving following treatment of HCV. Patients 1 and 2 have been observed without therapy. At follow up periods of 42, 11 and 27 months, all 3 patients remained asymptomatic with no evidence of disease progression. The finding of circulating cells with immunophenotype consistent with MZL mandates a careful search for evidence of MALT, splenic or nodal disease; in those patients lacking any such focus, a provisional diagnosis of LMZL is appropriate. Little is known about LMZL except that it tends to affect older patients, rarely results in symptoms or cytopenias, and maintains prolonged disease stability without active treatment. Whether LMZL is a truly separate disease or an early stage of one of the three currently recognised types of MZL is unknown. No distinctive immunophenotypic, cytogenetic or molecular feature of LMZL have been identified to date. Our knowledge of LMZL is limited at present and we hope that with further recognition of this disease we can better define its pathogenesis, clinical behaviour and optimal management strategy.

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### **Serum MUC1 as a Marker of disease status in Multiple Myeloma (MM) Patients Receiving Thalidomide $\pm$ Interferon- $\alpha$ -2b**

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Objective: MUC-1 is a glycosylated transmembrane protein normally found on the luminal surface of secretory glands. Serum MUC-1 (measured by serum CA15-3 assay) has also been

detected on the surface of plasma cells in MM. The aim of this study was to prospectively assess the role of CA15-3 as a marker of response.

**Methods:** We took serial measurements of MUC-1 from pts as part of a phase-II trial of thalidomide (T)  $\pm$  interferon- $\alpha$ -2b (INF) in advanced MM pts. Pts commenced T at 200 mg/d po, increasing by 200 mg q14d, to 800 mg/d. After 12 weeks pts were planned to continue T and start concurrent INF (1.5–3.0 MU, SC, TIW), continuing T  $\pm$  INF until progressive disease (PD) or intolerance. CA 15-3 levels were measured at baseline and then monthly by automated mouse monoclonal B27.29 antibody immunoassay (ULN of 31U/mL). Bone marrow trephines of 36 pts were stained for MUC1 by immunohistochemistry (IHC) and correlated with the serum CA15-3 level.

**Results:** Analysis of 75 enrolled pts was performed. Overall response rate (CR+PR) was 28%. 62 of 75 pts had baseline CA15-3 levels recorded. 22/62 (35%) had CA15-3 levels >ULN. Median level was 46 U/mL (range 32-158). Of those with elevated levels, in 19% the level was 32-50U/mL, in 11% >50-100U/mL and in 5% >100U/mL. There was no correlation between the baseline CA15-3 and baseline levels of the majority of other conventional markers (CRP, LDH, Creatinine, Hemoglobin, Platelet count, %Bone marrow infiltrate, Serum paraprotein, Bence Jones protein). There was a weak correlation with baseline levels of Beta-2-microglobulin: correlation coefficient 0.25 ( $P = 0.0471$ ). The serum level of CA 15-3 was correlated with the IHC score (% positive plasma cells  $\times$  intensity): correlation coefficient 0.43 ( $P = 0.047$ ). Of responding pts, baseline levels were >ULN in 14/18 pts (78%) and  $\leq$ ULN in 4/18 (22%). There was no significant difference in percentage of pts with baseline CA15-3 >ULN who achieved SD or PD. In univariate analysis there was a trend to CA15-3  $\leq$  ULN being predictive of response to thalidomide: RR 35% cf. 14% ( $P = 0.084$ ). Among the 20 responders, the median baseline CA 153 was 28 U/ml (range 5-80), and fell to a significantly lower median of 19U/ml (range 6-42) at first response ( $p=0.03$ ). Among all 29 pts who developed PD on thalidomide, including 9 who initially responded, there was no significant difference between the median baseline level and the median at PD.

Of the 9 pts who developed PD after initial response/SD to thalidomide, there was a significant rise in CA15-3 at PD: median at response = 13(6-34) cf median at PD = 25(8-52);  $P=0.037$ . 18/62 pts (29%) had clinically significant changes in CA153, meaning the serum level changed by >25% and fell or rose in conjunction with other recognised markers of disease.

**Conclusions:** Our results suggest that elevated serum CA 15-3 levels are a useful and novel marker in high-risk MM pts that may be predictive of response to thalidomide and may also be of value in monitoring disease.

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### **Acquired Myeloma-Associated Type III Hyperlipidaemia Responsive to Myeloma Treatment in a Patient with Apo E2/E4 Genotype**

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A 48 year old man presented with Stage III myeloma in October 2001. Immunoglobulin(Ig) G level was 72 g/L with monoclonal IgG paraprotein and free monoclonal lambda light chains in serum and urine. Bone marrow examination revealed 58% plasma cells but a skeletal survey was normal. At presentation, he had marked dyslipidaemia with total cholesterol (TC) levels of 16.8, triglycerides (TG) 9.5 and high-density lipoproteins (HDL) <0.50 mmol/L. Lipoprotein

electrophoresis confirmed Type III hyperlipidaemia with raised intermediate-density lipoproteins (IDL) and very low density lipoproteins (VLDL). He had no family history of ischaemic heart disease; haemoglobin A<sub>1c</sub>, renal, liver and thyroid function tests were all normal. Of note, his Apo E genotype was E2/E4 and not E2/E2 which is typical of Type III hyperlipidaemia. Chemotherapy with vincristine, adriamycin and dexamethasone (VAD) was commenced and he achieved an IgG plateau of 29 g/L in March 2002 following six courses. He then proceeded to high dose melphalan conditioning with an autologous peripheral blood stem cell transplant in May 2002. Maintenance treatment with prednisone was then commenced whilst awaiting a HLA-identical sibling non-myeloablative allogeneic transplant (NMAT). His IgG continued to fall to a nadir of 5.6 g/L in December 2002 when he proceeded with NMAT following total body irradiation and fludarabine conditioning. Following myeloma treatment, his lipid levels also improved with TC ranging from 5.5 – 6.0 and TG 3.7 – 5.7 mmol/L. His Ig levels remained stable post NMAT (5.6 – 6.2 g/L) until February 2003 when they progressively rose from 9 to a peak of 17.1 g/L in March 2003. In February 2003, predating the peak IgG level, his TG level rose to 13.7 mmol/L. A bone marrow trephine confirmed relapse with a focal collection of plasmablasts. Tacrolimus was withdrawn and he was commenced on thalidomide. Donor lymphocyte infusions were not given due to prior history of acute graft-versus-host disease. His IgG fell to 4.7 g/L and as predicted, the TG levels subsequently progressively decreased. A bone marrow examination done 2 months after commencement of thalidomide (6 months post NMAT) confirmed subsequent remission. Hyperlipidaemia with myeloma is a rare but documented association. There have been 7 case reports of myeloma-associated Type III hyperlipidaemia; 4 had IgA, 2 IgG and 1 IgM paraproteinaemia with two having Apo E2/E2 genotype. The parallel reduction in lipids in conjunction with reduced IgG levels suggests that the IgG paraprotein in this case complexed with lipoprotein particles preventing their clearance.

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### **Longitudinal Analysis of Dendritic Cell Subset Following Allogeneic Stem Cell Transplantation: A Potential Predictor of Graft vs Host Disease**

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**Aim:** We predicted that Dendritic cells (DC) would play a key role as antigen presenting cells in Graft vs Host Disease (GVHD) after haematopoietic stem cell transplantation (HSCT). This study is undertaking a prospective evaluation of the number and activation status of DC in the blood following myeloablative and non-myeloablative allogeneic stem cell transplantation (alloSCT) in a longitudinal manner. **Methods:** Peripheral blood was taken from patients undergoing alloSCT including 5 myeloablative (3 matched siblings, 2 matched unrelated donor recipients) and 5 non-myeloablative sibling transplant patients prior to and after transplantation (up to 100 days). DC subsets (CD11c<sup>hi</sup> DC and CD123<sup>hi</sup> DC) were enumerated using a novel 3<sup>rd</sup> generation method (Vuckovic *et al*) and the activation status was assessed by the expression of activation antigen (CD83, CD86 and CMRF44), by four- colour flow cytometry in whole peripheral blood samples. The data were then analysed for possible correlation between DC number and their activation status and the development of GVHD. **Results:** In both myeloablative and non-myeloablative transplant patients groups, absolute numbers of DC

decreased rapidly following conditioning, from 115.0 cells/ $\mu$ l (range 336.6-2.05 cells/ $\mu$ l, two patients had excessively high DC numbers possibly due to their condition) to 0 cells/ $\mu$ l), and increased gradually around 2 days post transplant, with a peak at day 25, mean of 4.1 cells/ $\mu$ l (range 0.23-12.9 cells/ $\mu$ l) in six out of the ten patients. Thereafter a gradual decline in DC numbers was observed from 5 weeks post transplant, from 4.1 cells/ $\mu$ l (range 0.23-12.9 cells/ $\mu$ l) at day 25 to 0.2 (range 0.21-2.4 cells/ $\mu$ l) at day 55. CD11c<sup>+</sup> DC expressed CD86 in all patients with about 90% of CD11c<sup>+</sup> DC from day 0 to day 100 (range 50%-100% of CD11c<sup>+</sup>) positive. However, the CD83 and CMRF44 antigens were expressed only intermittently and at lower levels than CD86 in 8 out of 10 patients at various time points. CD11c<sup>+</sup> DC from 2 matched unrelated donor recipients expressed little or no CD86. Conclusion: This systematic longitudinal study of DC subset numbers, included, for the first time, non-myeloablative along with myeloablative alloSCT patients. Consistent fluctuations in CD11c<sup>+</sup> DC numbers, absence of CD123<sup>hi</sup> DC and expression of CD86 on CD11c<sup>+</sup> DC were observed in both groups after alloSCT. The changes in DC counts are likely to have biological relevance relationship of this data to clinical events, especially GVHD, continues to be evaluated.

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#### **Adhesion Receptor Expression on Haemopoietic Stem Cell Mobilisation**

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In haemopoietic stem cell (HSC) transplantation, adhesion receptors (AR) have drawn significant interest due to their involvement in cell mobilisation from bone marrow (BM) and transplanted cell homing and engraftment. In a number of studies, AR have been analysed in patients and normal donors undergoing HSC mobilisation. However, the data accumulated so far is rather controversial. Both increased and decreased levels of AR have been reported in similar mobilisation procedures. In different studies, BM or peripheral blood (PB) samples were analysed. The cells were processed differently: whole blood, mononuclear cell fraction or CD34<sup>+</sup> sorted cells were used. Selected adhesion molecules were studied, therefore their different dynamics could be attributed to peculiarities of particular AR. We undertook a complex investigation of AR expression during HSC mobilisation in patients and allogeneic HSC donors using a panel of monoclonal antibodies against AR: beta-1 group integrins (CD49d, CD49e, CD29), beta-2 integrins (CD11a, CD11b), selectin receptors and ligands (CD62L, CD162), mucin like (CD164) and chemokine receptors (CD184) and CD44 molecule. Patient and donor blood samples were taken before and in the course of G-CSF induced HSC mobilisation. AR expression was estimated in flow cytometry using mean specific fluorescence of CD34 positive cell cluster. Compared to pre-mobilisation PB HSC phenotype, mobilised cells had generally lower AR expression. Longer mobilisation lead to further loss of surface AR. The highest changes were observed for integrins: CD29 – up to 59% maximum specific fluorescence reduction in our study, CD49d-46% and CD49e-39%, and for CD184 – up to 45%. However, some patients did not down-regulate or even up-regulate AR in response to mobilisation. Noticeably, this was characteristic for poor mobilisers, in whom circulating CD34 cell level was low and unresponsive to mobilisation. Moreover, correlation of AR expression with CD34 percentage and absolute contents in PB was observed in mobilising donors when absence of further down-regulation of AR on subsequent mobilisation days was followed by decreased HSC yield. In contrast to other AR analysed, CD44 was always highly expressed and in all individuals increased steadily on mobilisation with the highest gain of 79%. Our complex approach and



correlative analysis of AR expression in conjunction with HSC mobilisation can bring better understanding of the regularities of AR dynamics and their role in HSC mobilisation. It can also be suggested that HSC collection depends, apart from cell release from BM, on circulating cell marginalisation and peripheralisation, which is functionally linked to AR expression on HSC.

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### **Does Respiratory Syncytial Virus Infection Affect the Engraftment Kinetics after Stem Cell Transplantation?**

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There are few reports regarding the effect of respiratory syncytial virus (RSV) on the pre-engraftment period after stem cell transplantation. One report claimed that RSV infection during the pre-engraftment period might lead to graft failure in allogeneic stem cell transplantation (SCT). To date there are no reports to confirm this finding in patients who have undergone autologous transplantation. We studied the engraftment kinetics of 3 patients undergoing autologous (2) and allogeneic (1) SCT. All of these patients had RSV infection during the immediate post-transplant period. No other viruses or bacteria could be identified in these 3 patients at the time of RSV infection. One autografted patient received a stem cell dose of  $3.12 \times 10^6/\text{kg}$  and had neutrophil engraftment  $>0.5 \times 10^9/\text{l}$  on day 14, while the second received a stem cell dose of  $3.47 \times 10^6/\text{kg}$  and had neutrophil engraftment  $>0.5 \times 10^9/\text{l}$  on day 21. Both autografted patients showed delayed platelet engraftment with platelet count  $>25/\text{nl}$  on day 18 and day 15 and had reached platelet levels  $>50/\text{nl}$  on day 21 and day 20, respectively. The median time to platelet engraftment  $>50/\text{nl}$  following autograft was reported by To *et al.* (1997) to be 11 days for the patients who received stem cell doses of  $1.5\text{--}4.9 \times 10^6/\text{kg}$ . The median time to neutrophil engraftment following autograft was reported by the same reference to be 11 days for the patients who received stem cell doses of  $1.5\text{--}4.9 \times 10^6/\text{kg}$ . The autografted patients have sustained long-term platelet and neutrophil engraftment. The allografted patient received a stem cell dose of  $6.54 \times 10^6/\text{kg}$ . This patient showed early signs of engraftment on day 13 post-transplantation with a white cell count of  $500/\mu\text{l}$  and a neutrophil count of  $400/\mu\text{l}$ . Both counts declined very rapidly after the onset of RSV infection. The patient remained pancytopenic and died 64 days post-transplantation following a massive cerebral haemorrhage.

These preliminary data suggest that RSV infection during the pre-engraftment period affect the engraftment kinetics after autologous SCT and might even lead to graft failure in allogeneic SCT. Further studies to clarify the mechanism of delayed engraftment and to confirm these results are warranted.

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### **The Impact of Stem Cell Dose and Lymphocyte Subpopulations on Allogeneic Peripheral Blood Stem Cell Transplantation Outcome.**

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The impact of infused CD34+ cell dose has been extensively studied and it is well known that the CD 34+dose is an important predictor of outcome in allogeneic transplantation. However it is not understood if the relationship between CD34+cell dose and outcome might be influenced by other cells within the graft. Furthermore, it is unclear if there is a relationship between an upper limit to the number of infused T or NK cells and transplant outcome. In this study we evaluated 102 consecutive allogeneic sibling matched stem cell transplants and correlated their outcome with CD4+, CD8+, CD3+, NK and CD34+ cell dose in the graft. For each cell dose, the patients were classed into "high" or "low" dose groups based on whether they received above the 75<sup>th</sup> percentile or below the 25<sup>th</sup> percentile dose of cells. Parameters evaluated included neutrophil engraftment, platelet engraftment, transplant related mortality, acute GVHD, chronic GVHD, 1 year DFS, and number of infection days. The high CD34+ group was associated with statistically shorter platelet and neutrophil engraftment compared with the low CD 34+ group (P = 0.02 & P= 0.002 respectively). There was a trend to less acute GVHD amongst the high CD34+ group (51.4% vs 72.7%, P=0.07). No other outcomes were significantly affected by the level of CD34+ dose. The dose of other cell subtypes (CD4, CD8, NK cell, CD3) did not significantly affect the reported outcomes. This study confirms that CD34+ count is an independent predictor of rate of engraftment and may be an important factor in GVHD outcome. We could find no upper limit on the dose of infused T or NK cell numbers on GVHD or other transplant outcomes.

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### **Cytomegalovirus retinitis following unrelated peripheral blood stem cell transplantation: The first case report of an immune restoration disease.**

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**Background:** Cytomegalovirus (CMV) retinitis is a rare complication of bone marrow transplantation. It is much more common in HIV-induced immunodeficiency where there are two patterns of presentation: (1) associated with severe immunodeficiency (CD4 T-cell count <50/□L), and (2) during immune restoration after the use of highly active antiretroviral therapy (HAART). CMV and other herpesvirus diseases after HAART are usually associated with increased CD4 T-cell counts and with the HLA-A2 -B44 -DR4 haplotype and IL12B-3'UTR\*1 polymorphism of the IL-12 gene, highlighting the importance of the immune response in the disease process. The concept of immune restoration disease (IRD) has thus been recognised in the setting of treatment of HIV infection. Bone marrow transplantation with subsequent immune reconstitution provides a potential background upon which IRD may also occur. **Case report:** A partially matched unrelated CMV negative PBSC transplant was performed in CR2 as treatment for relapsed T ALL in a 27 year old CMV seropositive woman. The graft was homozygous for the HLA B4402 allele mismatched at HLA DRB1 and DRQB1. The recipient carried alleles 1 and 2 at IL12B-3'UTR. Total body irradiation and melphalan 140mg/m<sup>2</sup> conditioning with



GVHD prophylaxis with Campath 1H, methotrexate and cyclosporin was used. CMV prophylaxis was undertaken with valaciclovir 2g QID. There was mild GVHD involving the skin, hypogammaglobulinaemia and lymphopaenia during immune reconstitution. CMV viraemia in the absence of CMV disease was diagnosed by surveillance CMV PCR at day 117. Treatment with intravenous ganciclovir and CMV hyperimmune globulin cleared the viraemia at day 147. At this point, ganciclovir doses were gradually weaned with cessation at day 213. Throughout this period, CD4 T-cell counts remained below 30/ $\mu$ L and CMV PCR remained negative. CMV retinitis involving the right eye was diagnosed at day 227. The CD4 T-cell count had risen to remain between 50 and 200/ $\mu$ L throughout the course of the illness. PCR did not demonstrate CMV viraemia ( $<4 \times 10^2$  copies / ml) and remained negative throughout the course of the illness. Treatment with intravitreal injections of ganciclovir in addition to systemic valganciclovir was commenced. Visual acuity improved from 6/18 to 6/9 following treatment. Conclusion: This case demonstrates the occurrence of CMV retinitis at the time of immune reconstitution and in the absence of CMV viraemia, and illustrates that IRD occurs in the setting of bone marrow transplantation.

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### **Nonmyeloablative HLA-identical sibling allogeneic stem cell transplant using donor with essential thrombocythaemia (ET): evidence of engraftment without manifestation of ET in recipient**

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A 48 year old man presented with Stage III myeloma in October 2001. Immunoglobulin(Ig) G level was 72 g/L with monoclonal IgG paraprotein and free monoclonal lambda light chains in serum and urine. Bone marrow examination revealed 58% plasma cells. Skeletal survey was normal but  $\beta_2$ -microglobulin was raised at 5.97 mg/L. He received chemotherapy with 6 courses of vincristine, adriamycin and dexamethasone (VAD) and then proceeded to high dose melphalan conditioning with an autologous peripheral blood stem cell transplant in May 2002 when his IgG was 27 g/L. Maintenance treatment with prednisone was then commenced whilst awaiting an HLA-identical sibling non-myeloablative allogeneic transplant (NMAT). However, his donor, a 52 year old sister, had been diagnosed with essential thrombocythaemia(ET) in April 2002 when she presented with a platelet count of  $777 \times 10^9$ /L. She was commenced on hydroxyurea (HU) with improvement in platelet count to  $382 \times 10^9$ /L by October 2002. Ethical issues arose with regards to her suitability as a donor as well as donor safety during withdrawal of drug treatment at the time of the harvest. The Ethics Committee was consulted and the recommendations were that, if the transplant was deemed beneficial; if both donor and recipient were fully informed of the risks and were willing to proceed, the transplant could be performed. A literature search and personal correspondence with the International Bone Marrow Transplant Registry revealed no prior cases of NMATs using donors with myeloproliferative diseases. A change from HU to anagrelide was suggested prior to harvest; however, she could not tolerate anagrelide and HU was recommenced. This was tailed off over 2 weeks prior to the non-primed bone marrow harvest which yielded with  $5 \times 10^6$  CD34<sup>+</sup> cells/kg. Her platelet counts remained stable during this period ranging from  $350-408 \times 10^9$ /L. NMAT proceeded in December 2002(when IgG was 5.6 g/L), following total body irradiation and fludarabine conditioning and graft-versus-host disease(GVHD) prophylaxis with tacrolimus and mycophenolate mofetil. Donor T-cell and neutrophil engraftments are documented in the table below. BMAT on Day 55 post-NMAT

confirmed remission. However, BMAT on day 87 revealed myeloma relapse with a focal collection of plasmablasts seen. Tacrolimus was withdrawn and he was commenced on Thalidomide on Day 137 post NMAT. Donor lymphocyte infusions were not given due to a prior history of acute GVHD. BMAT on Day 178 post NMAT confirmed subsequent remission. Platelet numbers have remained normal with no evidence of ET on BMAT.

#### **Donor Chimerism Analyses post NMAT**

Days post NMAT	T-cell (%)	Neutrophil (%)
53	68	99.9
91	76.5	97
118	69	-
178	88	100

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#### **Autologous Stem Cell Transplantation for B-Cell Non-Hodgkin's Lymphoma using Conditioning with Beam & Standard Dose I-131 Iodine Labelled Rituximab.**

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Failure of long term disease control remains a major problem in many cases of non-Hodgkin's lymphoma (NHL). With standard combination chemotherapy, the 3 year disease free survival for intermediate/ high grade NHL is 44%, and autologous bone marrow transplanting provides encouraging results for chemosensitive relapse with 48% long term disease free survival. Success with autologous transplantation for relapsed or refractory high grade NHL have been limited. Thus, the use of alternative strategies warrants investigation. Autologous transplantation is established effective treatment in both advanced disease and transformed low grade disease. Residual lymphoma not eradicated by the conditioning regimen plays a significant part in disease recurrence post autologous transplant. The addition of radioimmunotherapy provides a different modality to eradicate lymphoma cells, and may provide improved long term disease free survival. While total body irradiation has significant toxicities to vital organs including lung and liver, radioimmunotherapy provides targeted therapy, limiting the radiation exposure to such organs, while providing increased dosage to target tumour sites. B-cell monoclonal disease accounts for at least 80% of NHLs. The addition of radioactive isomer to rituximab provides additional activity against malignant B-lymphoid cells. The I-131 rituximab has been used at this institution with encouraging results, appearing safe and effective. Other combinations of radioimmunotherapy and chemotherapy have been used with promise. We have embarked on this phase II study combining BEAM with radioimmunotherapy in autologous transplantation. We report the successful transplantation of the first two cases using this preparative regimen. There were no unexpected or additional early toxicities, and engraftment was prompt. One patient had an aggressive MALT-type lymphoma, while the other had Mantle Cell lymphoma. At the time of transplant they were in their second and fourth complete remission. One patient had evidence of hypothyroidism prior to receiving the I-131 rituximab (this had previously been used with excellent response as single agent therapy). Both cases developed late neutropenia at approximately Day +50, one requiring G-CSF with prompt

response. There have been no significant infections, and the lymphocyte subsets have revealed low B-cells in the peripheral blood, but fairly prompt T-cell recovery. In summary, combined conditioning with I-131 Rituximab & BEAM is feasible, and promises to provide improved disease control in patients with B-cell NHL. We will continue to monitor these and future patients with great interest.

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### **Graft-Versus-Lymphoma Effect In Refractory Cutaneous T Cell Lymphoma**

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Cutaneous T cell lymphomas (CTCL) are rare, and the management of patients with advanced stage-disease is made difficult by their short duration of response to conventional and high-dose treatments. In the more common low-grade lymphomas, durable remissions can be achieved following allogeneic haematopoietic stem cell transplantation (allo-SCT), even in patients with chemotherapy-refractory disease, due to a presumed graft-versus-lymphoma (GVL) effect. This finding has led to the investigation of allo-SCT in poor-risk CTCL. To date, only eleven cases of allo-SCT have been reported in patients with CTCL, five of these being non-myeloablative allo-SCT. We report three patients with CTCL who received fludarabine 25mg/m<sup>2</sup> and melphalan 120-140mg/m<sup>2</sup> (flu-mel) HLA-matched sibling allografts. Case 1: A 35 year-old male with MF, refractory to multiple therapies including alemtuzumab. He underwent a flu-mel allograft from an HLA-matched sibling donor. He achieved disease remission by day 32, but in the context of intensive immune suppression for gut GVHD with steroids he had a cutaneous relapse of MF at 4 months. This relapse and another at 13 months responded well to reduction of steroids and cyclosporine. A further MF relapse at 24 months was treated with 4 escalating doses of donor lymphocyte infusions. Interferon was added to enhance the allogeneic reaction. This induced only very mild GVHD and brief improvements in MF, but new lesions would soon appear. After the fourth DLI (1 x 10<sup>8</sup>/kgCD3+ cells), he still had MF with minimal GVHD so immunosuppression is currently being weaned. Case 2: A 49-year old male with refractory Sezary Syndrome, with bone marrow and lymph node involvement underwent a flu-mel HLA-matched sibling allograft which induced brisk necrosis of his skin lesions. He had a biopsy-proven relapse on day 43 post-allograft, which regressed after reduction of cyclosporine. A further relapse at 4 months was treated with DLI with disease regression, but with significant GVHD. Whilst this achieved an objective disease remission, he subsequently died 11 months post allograft of progressive chronic GVHD complicated by severe pneumonitis. Case 3: A 48 year-old woman with refractory, transformed MF. A flu-mel HLA-matched sibling allograft was performed in January 2003 and achieved a good initial disease response. Biopsy-proven limited cutaneous relapse of her low-grade disease occurred at 4 months, immunosuppression was reduced, and at 6 months the lesions have objectively improved in the context of significant GVHD. Conclusion: Whilst these cases demonstrate a substantial GVL effect in CTCL, this effect appears to be restricted to the context of active GVHD. Further improvements

in optimising GVL whilst minimising morbidity due to GVHD underpin the future management of these diseases.

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**Treatment of Sclerodermatous Graft Versus Host Disease with Extra-corporeal Photophoresis: early subjective response predicts for sustained benefit in cutaneous manifestations**

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Background: Extracorporeal photophoresis (ECP) is increasingly reported in the literature as an effective therapy for steroid refractory chronic graft versus host disease (cGVHD). However, factors predictive of response to ECP are currently poorly defined. Aims: To determine clinical factors predictive of response to ECP when used in combination with other immunosuppressive therapies as treatment for steroid refractory sclerodermatous cGVHD. Methods: All patients with steroid refractory sclerodermatous cGVHD treated with ECP at our institutions were retrospectively reviewed. ECP was initially performed weekly until stabilization and / or improvement in GVHD was noted, after which time treatment frequency was progressively reduced to fortnightly then monthly if tolerated. Reduction in other immunosuppressive therapy was attempted once sustained objective improvement was achieved. Response to ECP was graded both subjectively, based upon patients reporting of symptomatic improvement, and objectively via physician's assessment of skin thickness and erythema. FBC and liver function tests (LFTs) were also performed at each treatment. Results: In total 7 patients were treated (see Table). In 4 cases, patients reported subjective improvement in skin symptoms within 6 weeks of commencing ECP; objective improvement was subsequently noted within 3 months in each case. All 4 of these patients continue on monthly ECP (median 9.5 months of therapy, range 6-15 months) with ongoing improvement in cutaneous symptoms and signs (all partial (PR) or minor (MR) responses). In 2 cases, subjective improvement in skin symptoms occurred >6 weeks after commencing ECP (at 5 and 6 months respectively). In both cases progression of cutaneous cGVHD occurred on attempted reduction in ECP frequency; re-increasing ECP frequency subsequently re-controlled cutaneous symptoms in one case (MR), but had no effect in the other. The seventh patient suffered relapsed leukaemia within weeks of commencing ECP and was discontinued on all treatment. A >50% improvement in LFTs occurred in 2 of 4 patients with presumed hepatic GVHD after 3 and 12 months of therapy respectively. Sicca symptoms failed to respond in any of 4 patients with this complication. Reduction in concomitant immunosuppression has been tolerated in 4 (57%) patients to date. Conclusion: When added to other immunosuppressive regimens, ECP is potentially an effective therapy for the cutaneous manifestations of steroid refractory sclerodermatous cGVHD. Early subjective response (within 6 weeks) appears to predict for a sustained benefit in treating cutaneous manifestations of this disease. The role of ECP in the prevention and treatment of GVHD will be further assessed in phase III trials currently underway in Australia.

Age / sex	Total months of ECP	Cutaneous response	>50% Response in LFTs	Response in sicca symptoms	Reduction in other immunosuppression during ECP
52F	7	PR; weaned to monthly treatments	Yes	No	Yes
36 M	15	PR; weaned to monthly treatments	Yes	No	Yes
54F	12	MR; weaned to monthly treatments	No	No	No
28 M	6	PR; weaned to monthly treatments			Yes
31 M	9	Cutaneous flare month 8; no response to increased ECP frequency		No	No
43F	17	Cutaneous flare month 11; MR to increased ECP frequency q2wkly			Yes
21F	1.5	No response; died relapsed leukaemia	No		No

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### Comparison of PCR v Galactomannan for the Diagnosis of Invasive Aspergillosis

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Invasive aspergillosis (IA) is of major importance in immunocompromised patients due to its increasing incidence and high morbidity and mortality. Reasons include the unreliability of current diagnostic methods and the lack of efficient strategies for the use of drugs for IA which are potentially toxic and/or expensive. Between 1998-1999 we prospectively collected serial blood samples from patients at risk of IA, managed them in a standard fashion and analysed their samples retrospectively for galactomannan antigen (GM) using the Platelia test and for fungal DNA using a PCR-ELISA method adapted from J Clin Microbiol 1997; 35: 11 353-60. Samples were collected from 66 patients but matched GM and PCR analyses were performed on 263 samples from 25 patients. Patients were classified for potential IA according to EORTC/MSG consensus criteria with 5 patients analysed as positive (4 proven; 1 probable) and 20 analysed as negative (7 possible; 13 no evidence IA). All 5 patients with IA were positive by PCR with positive results in 24/82 samples whereas 3/5 patients were positive by GM with 4/82 samples being positive. 3/20 patients without IA were positive by PCR in 18/181 samples whereas results for GM detection were 1/20 and 1/181 respectively. Adjustment of ELISA cut-off values and/or the requirement for 2 consecutive samples to be positive could generate different results for assay sensitivity, specificity, positive predictive value (PPV) and negative predictive value (NPV), however, lowering the positivity index for GM detection from 1.5 to 0.5 did not improve the assay's sensitivity. Optimal results for PCR detection were 100% sensitivity, 85% specificity, PPV = 0.625 and NPV = 1 with a false positive sample rate (FPR) of 8%, whereas results for GM detection were 60% sensitivity, 95% specificity, PPV = 0.75 and NPV = 0.80 with FPR of 0.4%. We conclude: a) that this PCR method is very sensitive for the diagnosis of IA and is associated with a moderate rate of false positives whereas the GM assay exhibited poor sensitivity but high specificity and b) that further evaluation of PCR assays for the diagnosis of IA and other invasive fungal infections is warranted. We have since refined our PCR methodology for IA to make it



more robust, cheaper and quicker such that it would be suitable for routine screening and or diagnosis of high risk patients.

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### **Achievement and Maintenance of Good Manufacturing Practice for Production of Cellular Therapies**

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**Introduction:** The development and delivery of novel cellular therapeutics requires dedicated facilities for cell isolation, propagation and manipulation. These cellular products must be manufactured according to the Code for Good Manufacturing Practice (cGMP) – Human Blood and Tissue as outlined by the Therapeutic Goods Administration (TGA) as well as meeting the requirements from the Office of the Gene Technology Regulator (OGTR). The Peter Mac has taken a leading position in delivery of cellular therapies by constructing and operating a GMP facility and developing a GMP compliant quality systems (QS) program. In 1996 we undertook an exciting journey to provide cellular therapies and an AS1386 Class 350/PC3 GMP facility was built and officially opened in 1999. The acronym “Q.U.A.C.K.E.R.S.” has been a tool to provide direction for continued compliance to GMP to manufacture a reproducible, safe product for cellular therapies. **Methods:** The GMP facility consists of two clean-room suites, Quality Assurance laboratory and service room, with pass-through hatches connected to each suite. The clean-room suites can be run in either positive or negative pressures, with HEPA filtered supply and exhaust air. Lengthy testing of the air supply, air pressures and particle counts took place prior to commissioning. A priority was given to a high level of Quality Management, through collaboration with the Australian Red Cross Blood Services (ARCBS), to meet the demands of a TGA licence to manufacture. **Results:** The facility has a permanent core staff consisting of Director, Production Manager and Deputy, and Supervisor, with two ARCBS QS Officers. Each project has a Project Scientist and team of cell therapy technicians, who are either scientists or pharmacy technicians. The GMP activities have been regularly audited by the TGA and OGTR has conducted its own inspections, with twice-yearly internal audits conducted by the ARCBS. Day to day running of the facility and training of technicians and clean-room assistants is carried out by the Supervisor who oversees the operation according to the QUACKERS:

Quality, Questions

Understanding

Application

Cost, Compatibility, Confidential, Communication

K.I.S.S., Keys

Equipment

Regulations

Safety, Security

Production undertaken to date includes ex-vivo expansion of CD34+ cells, dendritic cells, macrophage-activated killer cells and chondrocytes. **Conclusion/Discussion:** We recommend the use of QUACKERS as a guideline for the ongoing maintenance of Quality Systems in this environment.

### Optimising the *ex vivo* Expansion of Megakaryocyte Precursors with CFU-Mk Potential of CD34+ Enriched PBSC.

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**Background;** The long-term goal of this study is to produce *ex-vivo* expanded megakaryocytes (Mk) from peripheral blood stem cell (PBSC) harvests. In the first stage of the study, we aimed to determine the optimum combination of cytokines together with MDGF in serum-free culture, to give greatest expansion of functional CD 34+ Mk progenitor cells from PBSC. Unexpectedly, Flt-3L added to 4 growth factors (GF) - MGDF, IL-3, SCF and GM-CSF - significantly reduced the fold expansion of primitive (CD61/CD38/CD34) and committed (CD41a/CD71/CD33) Mk progenitor cells. In the current study we aim to investigate this further by examining the clonogenic potential of these expanded progenitor cells cultured in 4GF+- Flt3-L (5GF combination). **Methods;** PBSC from patients were monocyte depleted then CD34+ enriched using the MACS system. Cells were cultured for 7 days in serum-free media with MGDF and an extensive combination of growth factors added in factorial assays including; IL-3, IL-6, IL-11, Epo, SCF, GM-CSF and Flt-3L (10ng/ml). Cells were harvested; cell counts and viability/phenotypic analysis performed, or expanded cells were assessed for specific Mk clonality potential ie. CFU-Mk. **Results;** 1. The MACS separation consistently gave >95% purity of CD34+ cells (data not shown).

2. While stem cell expansion was increased with the addition of Flt-3L, however the total fold expansion in the CD61+ and CD41+ cells was reduced as follows ; (GF combination; surface antigen; fold expansion)

4GF; CD61+;	20.3.	5GF; CD61+;	13.1.
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4GF; CD41a+;	36.6	5GF; CD41a+;	28.6
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3. CFU-Mk assays produced large colonies (>21 cells) formed from primitive Mk precursors and small colonies (3-21 cells), from more differentiated precursors. Total number of Mk colonies was 20% less with the addition of Flt-3L (5GF) compared with 4GF. Further, 36% of large colonies and 100% of small colonies from stem cells cultured in 4GF were Mk, compared with 26% of the large colonies 70% of the small colonies from the 5GF combination identified as Mk. 4. Co-culture of CD34+ enriched stem cells with bone marrow endothelial cells (BMEC) in the same 4GF and 5GF conditions resulted in a 10 and 16 fold expansion of total cell numbers respectively. CFU-Mk results however, indicated that CD34+ cells co-cultured with BMEC + 4GF gives the greatest expansion in Mk progenitor cells and produced 60% more Mk colonies than stem cells cultured in 4GF alone. **Conclusions;** The addition of Flt3-L to the optimal Mk expansion cocktail containing MGDF, IL-3, GM-CSF and SCF, increased the expansion of stem cells, although it reduced Mk progenitor expansion and the Mk colony forming potential of the expanded cells. Co-culture of CD34+ stem cells with BMEC further amplified the expansion of stem cells, and combined with 4GF produced significantly more primitive and committed Mk progenitors with clonogenic function.



# **Bone Morphogenic Protein 4 Promotes Ex Vivo Expansion of Cord Blood Primitive Haematopoietic Stem Cells**

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Haematopoietic stem cells (HSC) are capable of self renewal and have the ability to reconstitute the haematopoietic system of lethally irradiated recipients. Umbilical cord blood (UCB) is increasingly being utilized as a readily available HSC source, though an inherent disadvantage is limited cell dose which may result in delayed engraftment and subsequent graft failure. Therefore UCB HSC expansion is needed to increase the cell numbers required for adult recipients. The murine fetal liver AFT024 cell line has been used to support human long-term repopulating cells in culture. AFT024 are thought to provide a distinct environment for the stem cells by producing growth factors specific for primitive cell expansion. Bone morphogenic proteins (BMP) are involved in blood cell formation in embryonic development and studies show that BMP can induce UCB HSC self-renewal and expansion in ex vivo cultures. We investigated the expression of BMP-2, BMP-4 and BMP-7 by AFT024 and UCB CD34+CD38-lin- cells. UCB cells were cultured with or without cytokines in the presence of AFT024 for 6 or 12 hours and BMP RNA levels were compared by real-time PCR. AFT024 produced low basal levels of BMP-2, -4 and -7 mRNA which were all upregulated in the presence of cytokines. Conversely, UCB cells had a high basal BMP-4 level which further increases to up to 10 times after 12 hours culture with cytokines. In further experiments, UCB CD34+ cells were co-cultured with AFT024 using transwells in the presence of cytokines (Flt-3L, SCF and TPO at 10ng/mL) and exogenous recombinant human BMP-4 at different concentrations. Cells were assessed after 14 days by immunophenotyping, CFU-GM and LTC-IC assays. Cell analysis showed an increase total cell expansion by BMP-4 at an optimum concentration of 5ng/mL. Expansion of primitive cell populations (CD34+CD38) were higher compared to control and BMP-4 at higher concentrations. LTC-IC assay results confirmed that exogenous BMP-4 increases early progenitor cell frequency at 5ng/mL but not at higher concentrations. The opposite effect on CFU-GM was observed which may be explained by decreased committed progenitors in BMP-4 treated cultures. These experiments show that BMP-4 which is produced by AFT024 stroma and UCB HSC is capable of promoting haematopoietic progenitor expansion while maintaining their primitive phenotype and functional capacity. The optimum concentration of exogenous BMP-4 was 5ng/mL. These findings may have significant implications for expansion of HSC for transplantation.