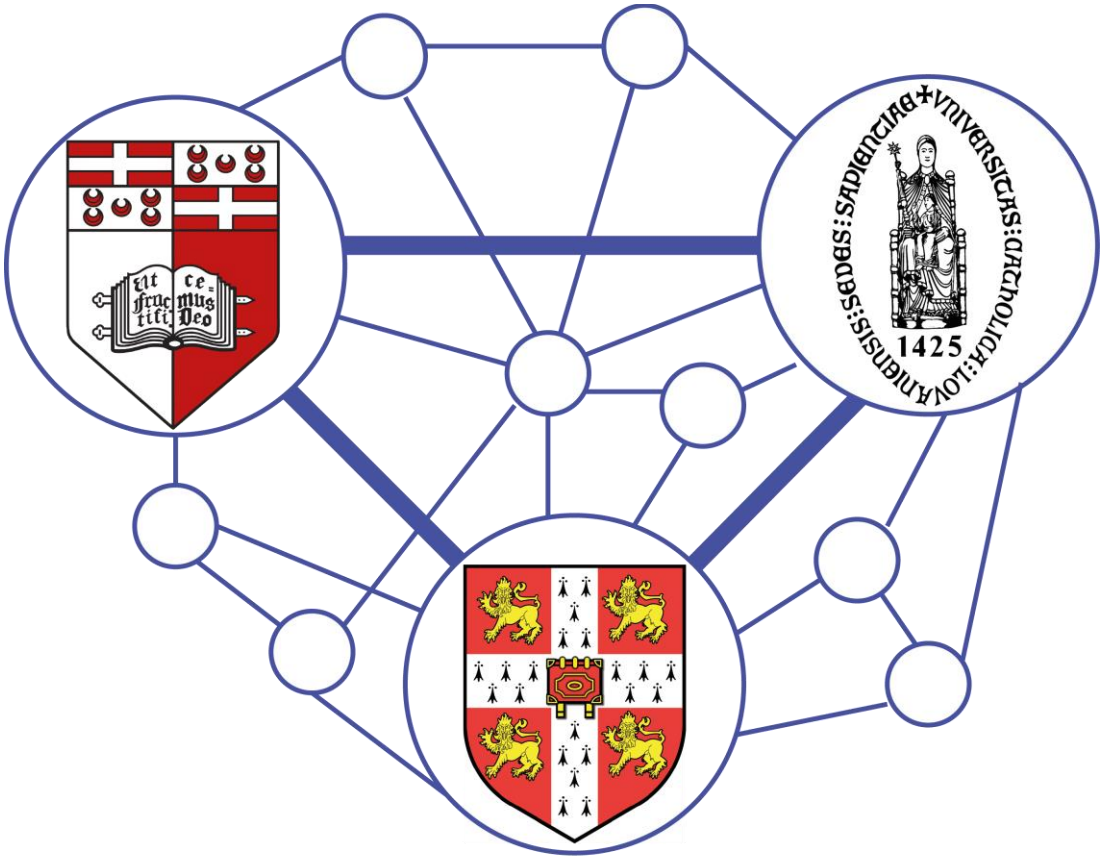

Networking event

Cambridge 01–02/06/2017

TrainMALTA Partners:

University of Malta
University of Cambridge
Katholieke Universiteit Leuven

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Day 1 - Thursday, 01 June 2017
Moller Centre

9.30 - 9.40	**Welcome and introduction**
9.40 - 10.00	**Rosalin Bonetta**: MnSOD proteins: structure, function and bioinformatics
10.00 - 10.20	**Chris Penkett**: Whole genome sequencing of 10,000 samples from the NIHR BioResource rare diseases cohorts
10.20 - 10.40	**Melissa Formosa**: The zebrafish animal model to study bone diseases
10.40 - 11.00	**Denis Seyres**: Extreme phenotypes define epigenetic and metabolic myeloid signatures in cardiovascular diseases
11.00 - 11.20	**Coffee break**
11.20 - 11.40	**Rosienne Farrugia**: High throughput sequencing identifies p.Y1435X a novel truncating *PKD1* mutation
11.40 - 12.00	**Jose Guerrero**: Platelet secretory granules and molecular interaction of DOCK7, VAC14 and SEC16A with NBEAL2
12.00 - 12.20	**Chanelle Cilia**: Identifying genetic factors underlying osteoporosis and fragility fractures in Malta
12.20 - 13.05	**Emmanouil Athanasiadis**: Exploring the mechanisms of haematopoietic lineage progression at the single-cell level
13.05 - 14.30	**Lunch**
14.30 - 14.50	**Jessica Heremans**: RNA sequencing of Roifman syndrome megakaryocytes reveals a role for a small nuclear RNA in platelet and granule biology
14.50 - 15.10	**Rita Tome**: SMIM1 is an important player during megakaryopoiesis and erythropoiesis
15.10 - 15.30	**Daniel Ortmann:** Inducible modulation of gene function in human pluripotent stem cells and their derivatives
15.30 - 16.30	**Coffee break**
16.30 - 16.50	**Lucienne Gatt**: Induction of leukaemia differentiation by phenolic compounds
16.50 - 17.10	**Kate Downes**: ThromboGenomics: genetic diagnosis for patients with rare inherited bleeding, clotting, thrombotic and platelet disorders
17.10 - 17.30	**Analisse Cassar**: Effects of low-dose combinations of chromatin-modifying agents with retinoic acid on the terminal differentiation of leukaemia
19.00	**BBQ**

Day 2 - Friday, 02 June 2017
IT room Craik Marshall Building, Downing Site

9.30 - 9.45	**Louisa Bellis**: Overview of bioinformatics training
9.45 - 10.05	**Luigi Grassi**: Exploring BLUEPRINT RNA-seq and epigenetic data
10.05 - 10.25	**Stefan Graf**: Introduction to The Human Genetic Variation Archive (HGVA)
10.25 - 10.45	**Daniel Greene**: ontologyX: a suite of R packages for working with ontological data
10.45 - 11.00	**Coffee break**
11.00 - 12.30	**Hands-on gene exploration session**
12.30 - 14.00	**Lunch break** (packed lunches will be provided)
14.00 - 18.00	**Social activity** **Meet at Downing Site Entrance**
19.00	**Dinner at The Eagle**

Abstracts

MnSOD Proteins: Structure, Function and Bioinformatics

Rosalin Bonetta[1], Chi H. Trinh[2], Rebeca Garcia Fandiño[3], Thomas Stockner[4], Gary J. Hunter[1], Thérèse Hunter[1]

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[4]*Medical University of Vienna, Austria*

Manganese superoxide dismutase (MnSOD) acts as the first line of defense against reactive oxidative species (ROS) by dismutating superoxide to hydrogen peroxide and molecular oxygen. The substrate and product of this reaction are essential signalling molecules of numerous pathways in normal and tumour cells.

The X-ray structures of MnSOD-3 reveal conformational mobility at the tetrameric interface, a feature which has not been observed in other MnSODs studied to date. This indicates flexibility of the α-helical N-terminal hairpin, which leads to the substrate funnel and may affect the accessibility of superoxide and removal of hydrogen peroxide to and from the active site. Besides, kinetic studies show that although both MnSOD-3 and human MnSOD convert superoxide to hydrogen peroxide by a similar catalytic mechanism, at high superoxide levels, only human MnSOD is product–inhibited. This renders MnSOD-3 the more efficient enzyme.

At the Laboratory of Biochemistry and Protein Science (University of Malta), in collaboration with University of Santiago de Compostela and the Medical University of Vienna, we are currently carrying out molecular dynamics (MD) simulations on *C. elegans* MnSOD proteins. MD simulations provide information on the molecule that is not observable in the data obtained via X-ray crystallography experiments alone. Such studies are being performed in order to analyse inner-sphere and second-sphere water structures that affect proton transfer and subsequently, the catalytic mechanism of the enzyme. Predicting hydrogen bonding in MnSOD through MD is also crucial for analysing the structure and function of this type of biological molecule, especially in terms of enzyme catalysis.

Whole genome sequencing of 10,000 samples from the NIHR BioResource rare diseases cohorts

Chris Penkett[1,2,3]

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We have sequenced 10,000 patients from a variety of rare diseases such as those associated with blood, retinal, neurological and immune systems or tissues. Most of the samples come from probands although there are some trios and other family structures in the overall study and originate from many hospitals throughout the country. After alignment and SNV/INDEL variant calling, we merge the data into a merged VCF file and annotate this data with a variety of variant and genomic databases. In addition, CNVs and SVs are called using two algorithms. Most projects have a Chief Analyst who uses these resources together with HPO and other clinical information to identify causal variants associated with the rare disease being studied. Most of these cases where a variant can be identified are then taken through a Multi-Disciplinary Team meeting (MDT) which includes clinicians, clinical scientists and bioinformaticians. Typically, a causal variant/CNV can be identified in approximately 20% of samples, although some cohorts such as the retinal dystrophy set have higher identification rates. Although most of these variants overlap coding regions of the genome, we are also looking into non-coding regions to get information on variation structure in the human genome as well as attempting to identify variants in non-coding regions for undiagnosed cases.

The zebrafish animal model to study bone diseases

Melissa Formosa

The zebrafish animal model has been successfully used to study bone disorders related to bone and ectopic soft tissue mineralisation, skeletal growth, bone remodelling and bone regeneration. Key regulators controlling bone developmental processes and cartilage formation overlap between zebrafish and mammals. This enables their use in the study of bone and cartilage abnormalities for both rare and common bone diseases. An extended Maltese family having multiple affected members with osteoporosis was analysed using a genome-wide linkage scan followed by whole exome sequencing. This lead to the identification of new genes that are hypothesised to affect bone development and that are
being studied in the zebrafish model. Gene knockdown using splice-blocking morpholinos and gene knockout using CRISPR (Clustered Regularly Interspaced Short Palindromic Repeat) genome-editing techniques have been carried out. Examination of the effect of these genes on bone are currently being assessed by staining methods of the whole fish (Calcein and Alizarin red-Alcian blue double stains), quantification of gene expression, and micro-computed tomography to quantify bone mineral density and visualise any skeletal abnormalities. This will help determine whether the selected genes are involved in bone physiology.

Extreme phenotypes define epigenetic and metabolic myeloid signatures in cardiovascular diseases

D. Seyres, J.L. Lambourne, P. Kirk, A. Cabassi, F. Burden, R. Kreuzhuber, S. Farrow, C. Kempster, H. Mckinney, A. Park, D. Savage, J. Griffin, O. Stegle, S. Richardson, K. Downes, W.H. Ouwehand, M. Frontini

Almost 6 million people live with a cardiovascular disease (CVD) and 26% of deaths are attributed to CVD in the UK each year. Thus, early detection of CVD onset is critical to improve people's quality of life and to decrease the economic burden on healthcare providers. The identification of predictive biomarkers and interpretable disease signatures by combining data obtained with different high-throughput omics is the ultimate step towards reconstruction and analysis of complex multi-dimensional diseases, enabling deeper mechanistic and medical insight. To this end, we collected data on 169 blood donors and 22 patients with three different diseases with high CVD risk. For each individual, monocytes and neutrophils were isolated and underwent whole genome sequencing, ChIP-sequencing for histone modifications marking regulatory elements (H3K4me1, H3K27ac), RNA-sequencing and DNA methylation analysis. Additionally, plasma metabolites and lipids were quantified in all individuals. ChIP-sequencing data were enhanced by chromatin imputation to obtain a high-quality dataset. We are currently integrating the different data types, working to reduce complexity, to select relevant covariates and to compare Bayesian profile regression and other clustering methods in view of their ability to identify signatures associated with a high CVD risk factor. To discriminate between epigenetic mechanisms and genetic contribution to a high CVD risk factor, we are performing quantitative trait loci mapping using histone modifications, methylation, transcripts expression, lipidomic and metabolic data. Our aim is to identify metabolic and/or epigenetic signatures that could be applied for the detection of CVD from an early onset.

High throughput sequencing identifies p.Y1435X a novel truncating P*KD1*mutation

Adrian Pleven[1], Valerie Said Conti[2], Francesca Borg Carbott[1], Ritienne Attard[1], Karen Cassar[3], Stephanie Bezzina Wettinger[1], Rosienne Farrugia[1]

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[2] Department of Child and Adolescent Health, Mater Dei Hospital, Malta
[3] Faculty of Medicine and Surgery, University of Malta, Msida, Malta

Introduction: Autosomal dominant polycystic kidney disease (ADPKD), the commonest heritable renal disorder, is caused by mutations in *PKD1* or *PKD2*. *PKD1* mutations account for 85% of ADPKD cases and cause more severe disease with earlier onset of end-stage renal disease (ESRD). Maltese ADPKD patients reach ESRD at an earlier age, generally between 40 and 50 yrs.
Materials and Methods: Blood and saliva samples were obtained from a family with ADPKD. High throughput sequencing (HTS) was used to sequence the entire *PKD1* and *PKD2* coding regions extending up to 50bp into the introns in each direction. SureSelectXT Target Enrichment capture of 2.6Mb of the genome, including *PKD1* and *PKD2* was followed by sequencing on Illumina HiSeq4000. HTS data was mapped to GRCh37 as paired-end libraries using NextGENe software. A BED file was used to ensure mapping to *PKD1* and *PKD2* excluding their pseudogenes. To remove potential pseudogene contamination of the data, the mutation list was filtered against an in-house database of 90 control HTS datasets and by pairwise blast of *PKD1* and relevant pseudogenes.
Results: A heterozygous novel mutation in exon 15 of *PKD1*, c. 4305C>G, p.Y1435X, was identified in the proband and his affected offspring but in none of the control datasets. The novel stop codon is in the 6th consecutive extracellular PKD domain resulting in a truncated protein lacking a number of domains, including all the transmembrane domains.
Conclusions: We report a novel, pathogenic, nonsense mutation in *PKD1* in a Maltese family.

Platelet secretory granules and molecular interaction of DOCK7, VAC14 and SEC16A with NBEAL2

José Guerrero

Variants in NBEAL2 are causal of Grey Platelet Syndrome (GPS), a rare bleeding disorder characterized by absence of alpha- and specific- granules in platelets and neutrophils, respectively. The role of NBEAL2 in cell biology and granule homeostasis is unknown. We performed proteomics to identify NBEAL2's binding partners followed by further validation including biochemical, cellular and functional analysis in vitro and in vivo. HEK293T cells ectopically expressing the Pleckstrin homology (PH), BEACH and the WD40 repeat domains of NBEAL2 fused with a Tandem Affinity Purification (TAP) tag, or GFP-TAP tag used as control were used for immunoprecipitation and mass spectrometry. 130 proteins were identified and unobserved in control. Reverse immunoprecipitation confirmed the binding of DOCK7 and VAC14 to NBEAL2's BEACH domain, while SEC16A specifically binds to the WD40 domain. Similar patterns of interactions were replicated in the megakaryocytic cells (CHRF) overexpressing PH-BEACH-WD40-TAP. Proximity ligation assays on endogenous proteins carried out in stem cell derived megakaryocytes (MKs) revealed significant interactions of NBEAL2 with DOCK7 and VAC14. Insertion of GPS-causing variants in the BEACH domain significantly impacted on its interaction with DOCK7 (P2100L, R2172H, G2290W) and VAC14 (M2080K, P2100L, G2290W), respectively. DOCK7 was significantly reduced in platelets from GPS mice and molecular elements of this pathway (IQGAP1, P-cofilin) were dysregulated leading to defective platelet activation and shape change. Our study shows the first NBEAL2 protein interactions and places DOCK7, SEC16A and VAC14 as binding partners of this uncharacterized multidomain protein. Further validation of new proteins from the network will allow us to understand and define the biochemical and functional role of NBEAL2 in cell biology.

Identifying genetic factors underlying osteoporosis and fragility fractures in Malta

Chanelle Cilia

Osteoporosis is a complex metabolic bone disease characterised by low bone mass and micro-architectural deterioration increasing fracture susceptibility, especially those of the hip, spine, and wrist. In Malta, approximately 20% of women and 6% of men are affected with osteoporosis. Bone mineral density (BMD) is used to diagnose osteoporosis and predict fracture risk. BMD heritability ranges from 50% to 85% implying that a number of underlying genetic factors contribute to disease. Different study approaches have been used to identify the underlying genetic factors contributing to osteoporosis, particularly population-based and family-based studies. An extended Maltese family having three or more affected members with osteoporosis and/or fragility fractures has been recruited. BMD measurements by Dual energy X-ray absorptiometry (DXA) and blood biochemical investigations were used to diagnose osteoporosis and exclude the presence of any comorbidities. Whole genome sequencing (WGS) will be performed using the BGISEQ-500 platform available at the Beijing Genomics Institute (BGI). A number of filtering steps will be performed to narrow down the list of variants and these will subsequently be assessed using a number of *in silico* tools. Shortlisted novel variants will be tested in a collection of Maltese cord blood samples to determine the allele frequency. In addition, potential causal gene variants will be replicated in a case-control collection of 1045 Maltese post-menopausal women to determine whether where is an association with BMD and fracture risk.

Exploring the mechanisms of haematopoietic lineage progression at the single-cell level

Emmanouil Athanasiadis

The success of marker-based approaches for dissecting haematopoiesis in mouse and human is reliant on the presence of well-defined cell-surface markers specific for diverse progenitor populations. An inherent problem with this approach is that the presence of specific cell surface markers does not directly reflect the transcriptional state of a cell. Here we used a marker-free approach to computationally reconstruct the blood lineage tree in zebrafish and order cells along their differentiation trajectory, based on their global transcriptional differences. Within the population of transcriptionally similar stem and progenitor cells our analysis revealed considerable cell-to-cell differences in their probability to transition to another, committed state. Once fate decision was executed, the suppression of transcription of ribosomal genes and upregulation of lineage specific factors co-ordinately controlled lineage differentiation. Evolutionary analysis further demonstrated that this haematopoietic program was highly conserved between zebrafish and higher vertebrates.

RNA sequencing of Roifman syndrome megakaryocytes reveals a role for a small nuclear RNA in platelet and granule biology

Jessica Heremans

Background: Roifman syndrome is a rare inherited multisystem disorder characterized by spondyloepiphyseal dysplasia, growth retardation, cognitive delay and antibody deficiency. In some cases, immune thrombocytopenia has been reported. Compound heterozygous variants in *RNU4ATAC*, a nuclear RNA essential for minor intron splicing, were recently identified to cause this disorder.
Aims: Investigate platelet defects in 3 patients with Roifman-like phenotypes from 2 unrelated pedigrees. All cases had low platelet counts with abnormal alpha- and dense-granules but no bleeding. The patients underwent whole genome sequencing in the BRIDGE-BPD study.
Results: Phenotype similarity regression identified a significant association between two rare alleles in *RNU4ATAC* and a Roifman-like phenotype. CD34+ hematopoietic stem cells of 2 patients and controls were differentiated to megakaryocytes (MKs) for RNA sequencing. Minor intron retention was assessed by DEXseq, adapted for differential intron usage. Immuno-staining and Western blot were done on MKs and platelets to study cytoskeleton and granules.
Results: We found novel pathogenic variants in *RNU4ATAC*. Relative to controls, Roifman MKs reach similar ploidy levels, but are smaller and generate less proplatelets with abnormal cytoskeletal organization. Roifman platelets are also rounder, show elevated tubulin and actin levels and an increase in granule markers CD63 and vWF. RNA-seq of Roifman MKs revealed minor intron retention in 354 genes of which the top 50 were mainly involved in vesicular transport, cytoskeleton organization or cognitive function. One of the most disrupted genes (P = 1.49x10-127) was *DIAPH1*, which is linked to cytoskeleton and macrothrombocytopenia.
Conclusions: Roifman syndrome is the second disorder after TAR syndrome implicating RNA processing in megakaryopoiesis, but the first in which variants in a non-coding RNA are pathogenic.

SMIM1 is an important player during megakaryopoiesis and erythropoiesis

Rita Tome

Vel is a universal antigen present on red blood cells (RBCs) membrane, which defines the Vel-blood group. Population studies have shown a frequency of ~1/4000 Vel-negative individual in Europe. *SMIM1* is a recently identified gene, underlying the Vel-antigen. It is a novel regulator of erythropoiesis and megakaryopoiesis and it is likely part of a membrane multi-protein complex. Previous observations in Zebrafish *knockdown* for *smim1* and gene expression data of different blood cell progenitors and precursors highlighted that *SMIM1* is not only important in RBCs biology but also in the specification of the cell fate at megakaryocyte erythrocyte progenitor (MEP). Moreover, a genome wide
association study in human RBCs described a SNP (rs1175550) in the SMIM1 gene as regulator of mean cell haemoglobin concentration, which suggests a possible implication of *SMIM1* in iron homeostasis. My PhD project aims to identify possible partners of SMIM1 multi-protein complex and to obtain a detailed understanding of the mechanism(s) by which *SMIM1* plays a role in fate commitment at MEP stage and in iron uptake. These questions will be addressed using induced pluripotent stem cells (iPSCs) derived from Vel-negative donors; *SMIM1* knockout iPSCs generated using CRISPR/Cas9n; *SMIM1* knockout mice and analysis of blood parameters of Vel-negative donors. Preliminary results demonstrated that Vel-negative blood donors do not have a striking phenotype in RBCs and platelets formation, and iron metabolism. Furthermore, *SMIM1* is expressed at protein level in RBCs, megakaryocytes (MKs) and platelets but remarkably SMIM1 membrane-expression is not detected in MKs and platelets.

Inducible modulation of gene function in human pluripotent stem cells and their derivatives

Daniel Ortmann

To realise the full potential of human pluripotent stem cells (hPSCs), robust ways to modulate gene expression are paramount. Here we present efficient inducible systems to knock-down, knock-out or over-express genes based on targeting of genomic safe harbours.
For knock-down and knock-out applications, we combined an improved tetracycline-inducible system with either shRNA or CRISPR/Cas9 technology respectively. Importantly, the system can deliver individual or multiplexed gene knockdown or knockout in both hPSCs and multiple differentiated cell types. To showcase its functionality in various contexts, we employed this system to investigate the function of transcription factors (OCT4 and T), cell cycle regulators and epigenetic modifiers during pluripotency or differentiation of hPSCs.
In order to obtain an efficient over-expression platform, we optimised inducible gene expression in hPSCs using a dual safe genomic harbour targeting strategy. We then utilised this system to over-express crucial transcription factors to reprogram hPSCs directly to various mature cell types. As proof of principle, we demonstrated rapid, robust and deterministic reprogramming of neurons (iNGN2) and skeletal myocytes (iMYOD1). Finally, we developed a forward programming strategy to efficiently and expeditiously generate human oligodendrocytes.
Taken together, this system enables controlled, efficient and reproducible modulation of gene expression in hPSCs and their derivatives. Employed for forward programming, new cell types can be swiftly obtained.

Induction of leukaemia differentiation by phenolic compounds

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Though chemotherapy is widely used in the treatment of a variety of cancers including leukaemia, it is broadly cytotoxic to normal tissues, and many patients suffer a relapse. This is due to haematopoetic stem cells which are not affected by chemotherapeutic drugs and regenerate. Moreover, for acute myeloid leukaemia (AML) five year survival rates are estimated to be around 30% (Tallman, Gilliland, and Rowe, 2005; Kohrt and Coutre, 2008). For these reasons, efforts have been made to develop alternate methods of treatment, one of which is *differentiation therapy*, whereby following administration of a bioactive compound, a decline in proliferative capacity is induced, followed by apoptosis or terminal differentiation initiation. This method highly contrasts with chemotherapy which is generally nonspecific and is often accompanied by highly toxic side effects (Leszczyniecka, Roberts, Dent, Grant, and Fisher, 2001).
In this study, a variety of phenolic compounds were tested for potential differentiation-inducing activity on four different leukaemia cell lines: a high passage HL-60 that is unresponsive to all *trans* retinoic acid (ATRA), K562, KG1a and NB4R2. Differentiation capacity was tested using the NBT (Nitroblue Tetrazolium) and MTT (Thiazolyl Blue Tetrazolium Bromide) assays for both monocytic and granulocytic differentiation and positive results were confirmed at day 3 and day 5 by morphological analysis using Leishman's staining. Tyrosol at a concentration of 9.23 ppm was selected to study cell cycle influence as well as analysis of cell surface markers associated with late stage differentiation using Flow Cytometry. For observation of granulocytic and monocytic differentiation of HL-60 cells, CD 11b and CD 14 antibodies were used respectively. In this presentation, results from these experiments are presented and discussed.

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ThromboGenomics: genetic diagnosis for patients with rare inherited bleeding, clotting, thrombotic and platelet disorders

Kate Downes[1,2]

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The next generation sequencing (NGS) era has seen a reduction of sequencing costs by orders of magnitude; this in combination with the consistent development of the technologies to selectively capture sequences, has allowed for high throughput sequencing of large and specific genomic regions. The ThromboGenomics platform is a single and affordable DNA-based test for 74 genes involved in 79 coagulation, platelet and thrombotic disorders. As well as simultaneously screening multiple genes, the ThromboGenomics platform test will scan most of the possible mutations in those regions, from just a single blood sample.

Effects of low-dose combinations of chromatin-modifying agents with retinoic acid on the terminal differentiation of leukaemia

Analisse Cassar

Leukaemia is the most common type of cancer that occurs in children worldwide. Despite the intense research carried out in several facilities, acute myeloid leukaemia is still considered the highest cause of cancer death in children to young adults with a long-term survival rate of only approximately 10%. The successful use of all *trans* retinoic acid (ATRA) in the treatment of acute promyelocytic leukaemia has prompted further research of differentiation therapy to include other leukaemia sub-types. The rationale is to force leukaemia cells into entering the apoptotic pathway by undergoing terminal differentiation rather than use cytotoxic drugs to treat this cancer.

The use of chromatin-modifying agents (CMA) to overcome treatment resistances has been long tested and applied clinically. In this study four histone deacetylase inhibitors and a DNA demethylating agent were used on five leukaemia cell lines to alter the chromosomal conformation and promote binding of ATRA.

Differentiation was assessed through reduction of nitroblue tetrazolium and results were normalized using the MTT assay. Positive results were further analysed with differentiation markers on Flow cytometry.

Differentiation was achieved using a combination of CMA at low concentration, together with ATRA. The combination of low doses with multiple CMA proved more efficient than higher doses of a single CMA probably allowing for alteration of the DNA at multiple sites and decreasing the chances of acquired drug resistances. Differentiation of leukaemia patient primary cells was also successful with ATRA and CMA combinations. Combinations of CMA with ATRA proved also effective on leukaemia patient primary cells indicating new possible combinations which can be used to treat leukaemia.