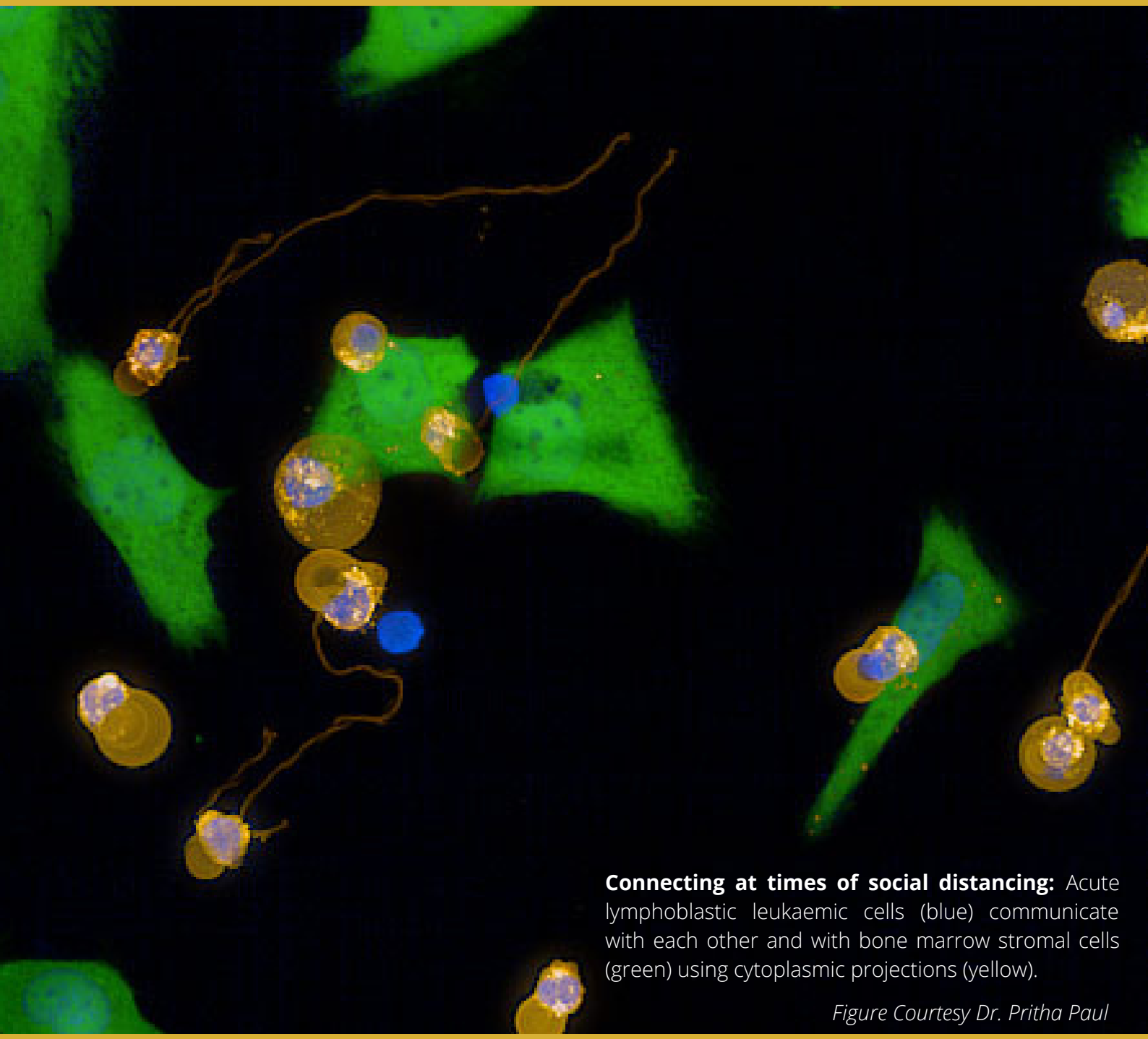
2020

ANNUAL SCIENTIFIC REPORT



Connecting at times of social distancing: Acute lymphoblastic leukaemic cells (blue) communicate with each other and with bone marrow stromal cells (green) using cytoplasmic projections (yellow).

Figure Courtesy Dr. Pritha Paul

TATA TRANSLATIONAL CANCER RESEARCH CENTRE

WWW.TTCRC.ORG

Contents

From the Directors Desk

Vaskar Saha

Head - Paediatric Hematology
Director -TTCRC

What a year this has been! In early January, when we heard about the virus outbreak in Wuhan, we treated it as normal news, as we had done with SARS and MERS. We then went deep into the unknown as a global pandemic ensued. Over time, clarity emerged as to what the virus did, how it spread and more importantly how to treat those affected. Vaccines came rolling in – a triumph of medical science and technology. Mixed with a bit of hyperbole. As with all scientific knowledge, the more we learn, the more there is left to know. Above all that has come human resilience and the ability to work together to face the odds. We are not out of the woods yet, but a path has appeared.

Along with everyone else, TMC learnt to sift the good facts from the bad, set up the SARS2-COVID screening, arrange for PPE and develop protocols to keep patients and staff safe. Patient load dropped for a while, mostly due to the enforced lockdown. We are operational again running at almost full capacity – a testimony to the fortitude, courage, and ingenuity of all those involved. Despite that, all of us have been directly or indirectly affected by illness and death.

In March, just prior to the lockdown, TTCRC moved to shut operations. First to reduce the risk to staff and second to decrease the chance of bringing the infection into the hospital. For a couple of weeks, Shekhar was the last man standing, supporting both the clinical team as well as babysitting TTCRC. Pritha, on secondment to Manchester, managed to get the on the last inbound flight back to Kolkata. Skype gave away to zoom and we began working out how to work from home. As things settled down, staff found ways to get back to coming into work. Some, and here given what little we knew back then, had the courage and fortitude to help with the virus testing program. Others came in regularly to maintain equipment and essential activities. Even

Barun, my driver, stepped into ferry people who had no means of transport. By May, we had gradually begun work again, though biobank remained shut. The bank premise was the only site suitable to set up a secure area for the Covid PCR test and sample collection was deemed to be too risky. By August, the biobank team has set up a temporary facility within TTCRC and the labs were up and running. More staff began to come into work, some now travelling together, overcoming the sum of all their fears.

In late February, I had gone on what was to be a short visit to Germany and UK. I was only able to return on the second flight allowed from London to India in September. The fact that TTCRC was fully active by then speaks volumes of Shekhar's calm influence, the commitment of the senior TTCRC staff and the willingness of everyone to get back to work. For this I am humbled and truly grateful. We have had some scares, but then this is the new normal.

As you will see from the annual report, lockdown may have stymied, but it has not stopped the groups from making progress. CRU continues to recruit patients to the frontline trial and has expanded into exploring designs for early phase trials. Biobank is regrouped and reorganised, waiting to move back to its own area. The genomics lab has completed the analyses of High Hyperdiploid ALL and the MRD lab is moving to a NGS platform. Leukaemia Biology has developed new tools and techniques for analyses, generating new hypotheses on the way. Drug Discovery has come of age allowing us to identify alternative therapies for patients with a suboptimal response to frontline therapies. The organoid team has setup a multidisciplinary team to elucidate the pathogenesis of gallbladder cancer and successfully established a organoid laboratory. The mass spec has finally begun to sing. Ankita (Organoid Laboratory) became our first doctoral student, joint with IIT-Kharagpur. Jaydeep, who worked with biomaterials in the Organoid Laboratory, has joined for a PhD program working on biomaterials in CAR-T (IIT-Bombay with Monash University). Meanwhile Tushar and Kankana were awarded well deserved PhD's.

Bindhu, Chumki, Dipshikha, Shivani, Jaydeep, Ruma, Akash, Madhuparna, Parna, Piyali, Sayan, Soumita, and Susri left for fresh opportunities. I wish them every success. Abhirupa, Amrita, Ananya, Anushka, Aparajita, Arnav, Asama, Bishwaranjan, Dwijit, Manimaran, Pravin,

Saheli, Samik, Sayantani, Shreyasree, Shinjini, Srijita, and Subhoshree have joined.

We have a busy year ahead. All the best everyone, work hard and work together. Stay safe, keep others safe – we are not quite out of the woods yet.

I leave you with a quote from a lady who is an inspiration to all of us in the field of cancer "Nothing in life is to be feared. It is only to be understood. Now is the time to understand more, so we may remain fearless." What Marie Curie said a 100 years ago, resonates today.

Clinical Research Unit

Background

The Clinical Research Unit (CRU) at TTCRC leads and participates in the design, development, management, analysis and reporting of investigator-initiated and investigator-led clinical studies in cancer. These studies provide the platform for the translational research programme at TTCRC.

Research programmes

The CRU is involved in the following research programmes

A. Acute lymphoblastic leukaemia
B. Gallbladder cancer

Studies

A. Acute lymphoblastic leukaemia (ALL)

1. The Indian Paediatric Oncology Group's Indian Collaborative Childhood Leukaemia ALL-14 multicentre randomised clinical trial in children and young people with newly-diagnosed ALL (InPOG-ALL-15-01-ICiCLe-ALL-14; CTRI/2015/12/006434)
   a. Pre-trial phase: from March 2013
   b. Trial: opened in late October 2016, continuing enrolment

c. Investigators: Shekhar Krishnan (principal); Vaskar Saha (chief)
d. Funding: National Cancer Grid and Indian Council of Medical Research

2. The Indian Paediatric Oncology Group collaborative multicentre treatment protocol for children and adolescents with relapsed acute lymphoblastic leukaemia (InPOG-ALL-19-02-TMC-ALL-R1; CTRI/2019/10/021758)
   a. Pilot: from August 2016
   b. Study: opened in June 2019, continuing enrolment
   c. Investigators: Niharendu Ghara (principal); Vaskar Saha (chief)
3. Summary of activities in 2020
   a. Enrolment in the ICiCLe-ALL-14 clinical trial slowed substantially or was paused for 4-6 months between March and August 2020 on account of the SARS-CoV-2 pandemic, affecting the projected timeline for completion of target enrolment. A submission was made to the National Cancer Grid (March 2020) for extended funding for a fourth year. Trial observations demonstrate improvements in risk stratification diagnostics, a sustained lower rate of treatment-related mortality compared with the pre-trial phase and the pronounced adverse effect of anthracycline treatment in younger patients (<10 years).
   b. Analysis of outcomes of patients (N=~2500) treated in the pre-trial phase is underway. Preliminary observations confirm improvements in survival with risk stratified therapy but the outcomes do not match those reported in the west. The findings suggest opportunities to refine risk stratification diagnostics, investigate distinct biologic determinants of poor treatment response, improve the quality of generic cytotoxic drugs (specifically the biopharmaceutical L-asparaginase) and strengthen the outpatient supervision of the 96-week maintenance treatment phase.
   c. The remote data capture system for the ALL-R1 study is complete and awaits incorporation of a few corrections before going live. At least nine centres have indicated interest in participating in the study. The study protocol was reviewed at the NCG's CReDO 2020 workshop (Dr Ghara). Study enrolment is underway at the Tata Medical Center and increasingly, families opt for intensive

treatments aimed at cure or good quality remission.

d. An ICiCLe-ALL study website was designed and developed in early 2020 (ND, SP) and is accessed through the TTCRC homepage (https://icicle.ttcrc.org/)

4. Key proposed activities in 2021
   a. Interim analysis of the ICiCLe-ALL-14 trial is scheduled in mid-2021, four years from start of enrolment. Findings from the analysis will inform decisions on trial randomisations and enrolment.
   b. Rollout of ALL-R1 to participating centres is expected to begin in the second half of 2021.

B. Gallbladder Cancer Research Programme

Investigators: Dr Manas Roy; Dr Anindita Dutta

Collaborations: Medical Oncology; Digestive diseases; Diagnostic imaging; Histopathology

Gallbladder cancer is a malignancy of high endemicity and poor outcomes. The multidisciplinary translational research programme in gallbladder cancer aims to investigate the pathogenesis of this disease and discover new therapies, using suitable pre-clinical disease models. The role of the CRU in this programme is evolving. In 2020, the focus was on strengthening the tissue collection pathways required for development of the pre-clinical models. In 2021, the focus of the CRU would be on extending nascent efforts to create a clinical registry and consolidate the same with tissue banking and research laboratory records to develop an integrated study database.

Sub-studies / Projects

These projects and sub-studies are spin-offs of the CRU's clinical research programmes. The CRU is involved in two principal projects:

A. L-Asparaginase in ALL
B. Maintenance therapy in ALL

A. L-asparaginase in ALL

Investigators: Jasmeet Sidhu (principal); Shekhar Krishnan

CRU: MG; SS; ND

Laboratory: Arko Bhowal (from Nov 2019); Priyanka Bose (Jan 2018 – Sep 2019); Pritha Paul

Tissue banking: Ritam Siddhanta

Study 1: Investigating *E. coli* asparaginase (EcASNase) biogenerics in India

Study 2: Optimising dose and scheduling of an EcASNase biogeneric

Study 3: Clinical monitoring of PEG-EcASNase biogenerics (PEG, polyethylene glycol)

Study 4: Clinical trial to determine safety and efficacy of recombinant EcASNases

Study 1 highlighted the unsatisfactory quality and therapeutic activity of EcASNase biogenerics available in India (**Figure 1**). A draft manuscript has been prepared for submission.

Study 2 was halted on account of the SARS-CoV-2 pandemic (March 2020) but the observations confirm that switch to an alternate-day schedule of Leucoginase EcASNase (VHB Life Sciences; 10,000 IU/m^2/dose every 48 hours) provides adequate asparaginase activity in a majority of patients, importantly during the critical induction phase of ALL treatment. Analysis is ongoing and the findings will be communicated as part of the report on outcomes in the ICiCLe-ALL-14 pre-trial phase, as exemplar of strategies to optimise use of generic cytotoxic agents. Study 2 is supported by a grant from VHB Pharma.

Study 3 has been initiated and will be implemented in 2021. This will involve administration of PEG-conjugated EcASNase biogenerics marketed in India as part of a monitored study to determine drug activity, optimum dose, dosing schedule, toxicity and immunogenicity.

Study 4 is part of a collaboration with Gennova Biopharma Limited (Gennova) to develop affordable high-quality asparaginases. With part-funding from DBT's Biotechnology Industry Research Assistance Council, Gennova has developed recombinant EcASNase products (unconjugated and lyophilised PEG-conjugated formulations) that are likely to be approved soon for clinical testing. The proposed clinical study (target, second half of 2021) will investigate the safety and therapeutic activity of the recombinant products and determine the dose and schedule suitable for clinical use. This study will require additional personnel (coordination, lab studies), substantial preparatory work (protocol preparation, ethics approvals, data plans, laboratory studies) and dedicated funding.

B. Maintenance therapy in ALL

CRU: TM; MG; SP

Collaborations: Prof Sangeeta Das (IIT Kharagpur); Prof Kiranmoy Das (ISI Kolkata)

The CRU supports the clinical monitoring of the maintenance treatment phase in first-presentation and relapsed ALL. In parallel, research studies carried out by Tushar Mungle as part of his doctoral thesis highlighted the problem of suboptimal antimetabolite drug dosing in the majority of patients and identified potential strategies to address this

shortcoming. Three strategies have been proposed:

(a) Incorporation of visualisation tools (i) to monitor blood count trends and drug dose decisions in real-time and, (ii) to review weighted mean leucocyte counts against antimetabolite dose intensities at the completion of each maintenance cycle (12 weeks) as measures of optimal dosing practice (**Figure 2**)
(b) Development, testing and validation of an automated dose decision advice system based on protocol-based dose rules for initiation, continuation, interruption and escalation of antimetabolite drug doses based on longitudinal information from blood counts, dose tolerance and time elapsed from last dose changes
(c) Exploring computational modelling approaches to predict antimetabolite drug doses, based on traditional statistical modelling or through development of intelligent systems.

The SARS-CoV-2 pandemic has provided impetus for this work. As a result of pandemic-related travel restrictions, the proportion of ALL patients requiring remote supervision during the maintenance phase has increased sharply. An estimated two-thirds of patients now have their maintenance treatment supervised largely remotely, up from 25% last year. This transition has required development of data systems and strategies to record information longitudinally during the maintenance phase and reorganisation within the CRU to support remote dose advice clinics ('e-mail clinics'). The focus in 2021 will be to establish these data systems to support remote management of ALL maintenance, evaluate the use of visualisation and automated dose advice tools in maintenance management and publish the findings from TM's doctoral work. Findings from Prof Kiranmoy Das' joint modelling analyses highlighting the adverse impact of suboptimal 6-mercaptopurine dosing on the risk of ALL treatment failure will be communicated as part of the proposed publication on outcomes in the pre-trial phase, serving as exemplar of potential strategies to optimise treatment practice to improve ALL outcomes in India.

Other sub-studies undertaken in 2020 include

(a) Evaluation of impact of risk stratified therapy on direct treatment costs in patients with ALL treated at the Tata Medical Center: This work is complete and a draft manuscript is being prepared (ND; TM; SP)

(b) Evaluation of treatment toxicity in ALL in patients treated at the Tata Medical Center, including evaluating the frequency, nature and severity of infection and non-infection treatment toxicities and the influence of patient, disease and treatment variables on toxicity characteristics (ND; PD). Data collection is underway, analysis will begin in the second half of 2021, and a draft manuscript is targeted in early 2022.

(c) Observational study of the value of therapeutic drug monitoring with high-dose methotrexate schedules in first-presentation ALL (investigator, Dr S Chitturi, Fellow, Paediatric Haematology-Oncology). Contrary to other reports, findings from this work (76 patients, 290 infusions) emphasise the necessity of monitoring blood methotrexate levels in patients, especially in patients with T-ALL administered higher doses of intravenous methotrexate. Drug monitoring enabled timely intervention with augmented alkaline hyperhydration in nearly a quarter of infusions and extended leucovorin rescue in a further ~10% patients. Levels of serum creatinine were useful chiefly as negative predictors of supratherapeutic blood methotrexate levels. A draft manuscript is proposed (from CRU: ND, SC)

Sub-studies proposed in 2021 include the following

(a) Evaluation of the safety and efficacy of newer agents and therapies in ALL (SS, PD, ND)

The availability of generic targeted agents (dasatinib, venetoclax) and antibody therapies (blinatumomab, accessed as part of a donation programme in partnership with St Jude Global) and experience with newer agents (e.g. bortezomib in relapsed ALL) expands the therapeutic options for patients at high-risk of treatment failure including patients with high risk genetic subtypes and/or poor treatment response. These interventions will be introduced to deepen disease remission prior to allogeneic haematopoietic stem cell transplantation or where transplant treatment is not feasible, evaluated as potential strategies to extend disease-free remission. The use of these agents also provides an opportunity to prospectively evaluate their cost-effectiveness.

Others

1. The ICiCLe-ALL-14 (non-randomised) treatment protocol for risk-stratified management of children and young people with newly-diagnosed ALL at the NRS Medical College Hospital (Department of Haematology; Dr Rajib De, investigator)

   The CRU works with NRSMCH to support with coordination and funding of laboratory studies outsourced to the Tata Medical Center and advise on data management and analysis. CanKids India has committed to extend funding support for laboratory studies at TMC.
2. The CRU works closely with the tissue banking and laboratory groups to support laboratory-based research at TTCRC.

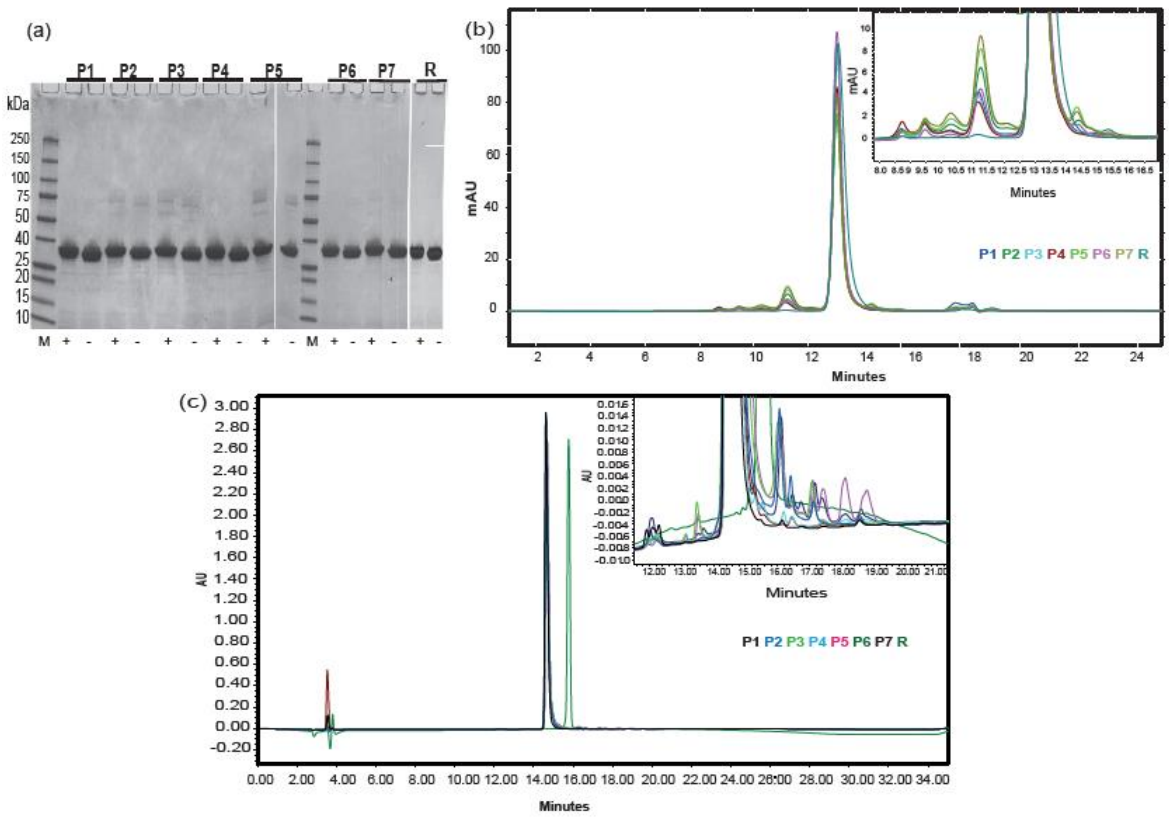


Figure 1: Analysis of EcASNase biogenerics P1-P7 and the reference EcASNAse (R) indicates impurities in the biogeneric products. (a) Coomassie-stained image of SDS-PAGE analysis of EcASNase products examined in reducing ('+') and non-reducing ('-') conditions confirms presence of the ~36kDa EcASNase subunit in all products, and shows additional higher molecular weight bands in the biogenerics suggesting presence of protein impurities (M, molecular weight marker); (b) SEC analyses of EcASNase products indicates that in addition to the primary peak of EcASNase, biogeneric products are characterised by additional smaller peaks with shorter retention times (inset, magnified view), suggesting the presence of higher molecular weight impurities, likely multimer aggregates of EcASNase (mAU, milli-absorbance unit); (c) RP-HPLC analyses of EcASNase products indicates difference in retention times (~1 minute) between EcASNase biogenerics and the reference product,

likely related to differences in hydropathicity owing to amino acid sequence variations in the biogeneric EcASNases. Additional smaller peaks with varying retention times are also observed in the biogeneric products, suggesting impurities (inset, magnified view). (AU, absorbance unit)

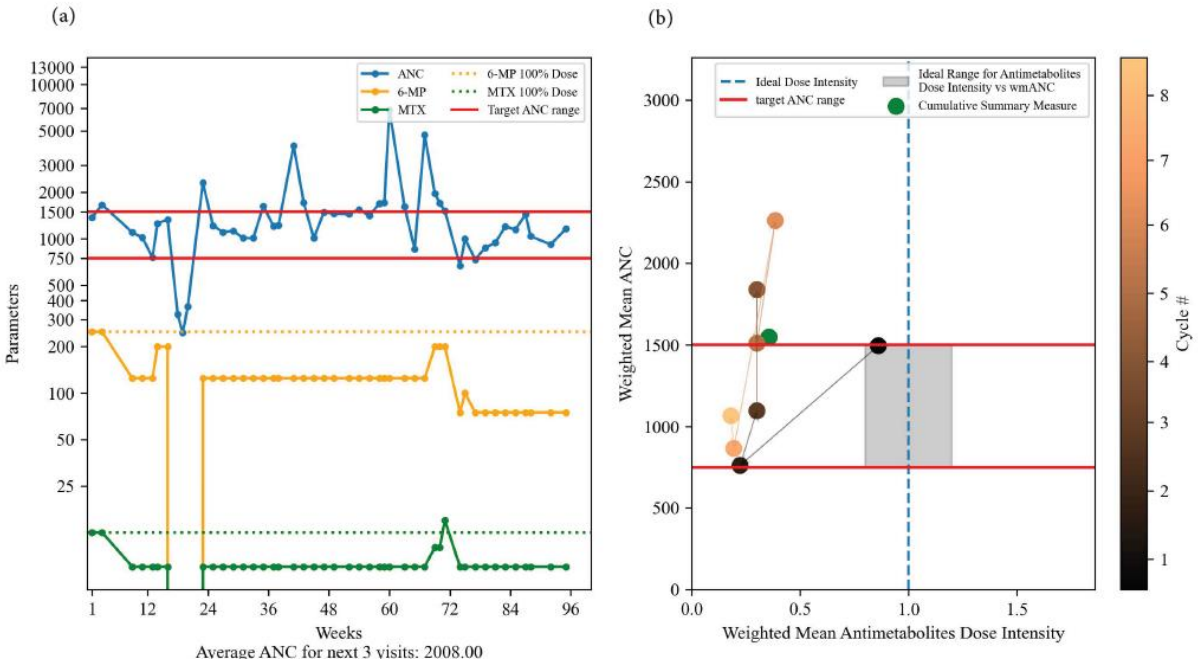


Figure 2: (a) Line graph showing longitudinal information of maintenance therapy over 96 weeks; (b) summary measure graph showing weighted means absolute neutrophil counts and antimetabolite dose intensity over 8 cycles

Biobank

Abhirupa Kar

Biobank Manager

Ritam Siddhanta

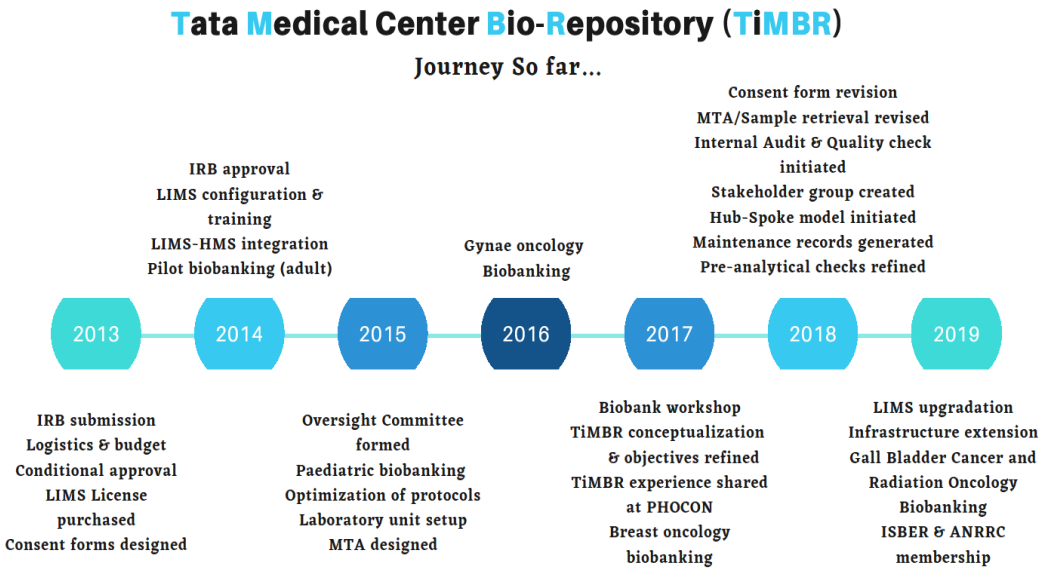
Biobank Technologist

Kankana Das

Research Assistant

Cancer Research Centre. TiMBR aims to facilitate access to high quality bio-specimens for translational cancer research leading to precision oncology, following international, national and institutional regulations involving operational, legal and ethical aspects of a biorepository.

TiMBR has come a long way since its inception. Being a single, organized, common resource, TiMBR provides samples to various research domains namely Genomics, Proteomics and Cell Biology for downstream experimental



TiMBR – Tata Medical Center Biorepository is a research infrastructure providing critical fuel to research activities performed at Tata Translational

procedures.

The year 2020-21 was full of ups and downs for TiMBR. The core team got reshuffled with new members joining force. The biobank oversight committee

was dissolved paving the path for introspection on the governance and operations of TiMBR. The current team is comprised of members from diverse backgrounds with expertise that not only makes our journey dynamic but also opens up avenues for branching out and evolve from routine biobanking processes. Mr. Ritam Siddhanta has joined TiMBR in 2018 and has been responsible for optimisation of biobanking protocols pertaining to paediatric leukaemia. He is overseeing the activities related to the quality of biospecimens. Dr. Kankana Das got introduced to TiMBR in 2019 starting off with paediatric leukaemia biobanking and later got involved in Gallbladder cancer biobanking as well as downstream research activities. Recently, Kankana received her doctoral degree from University of Calcutta. She is now responsible for quality assessment of extracted RNA and managing retrieval process of cryo preserved cells. For further validating the quality of the cryopreserved cells Dr. Kankana has undertaken post retrieval cell viability assays. Ms. Abhirupa Kar has recently joined TiMBR in August 2020 and is in-charge of managing the operational and structural aspects of the biobank.

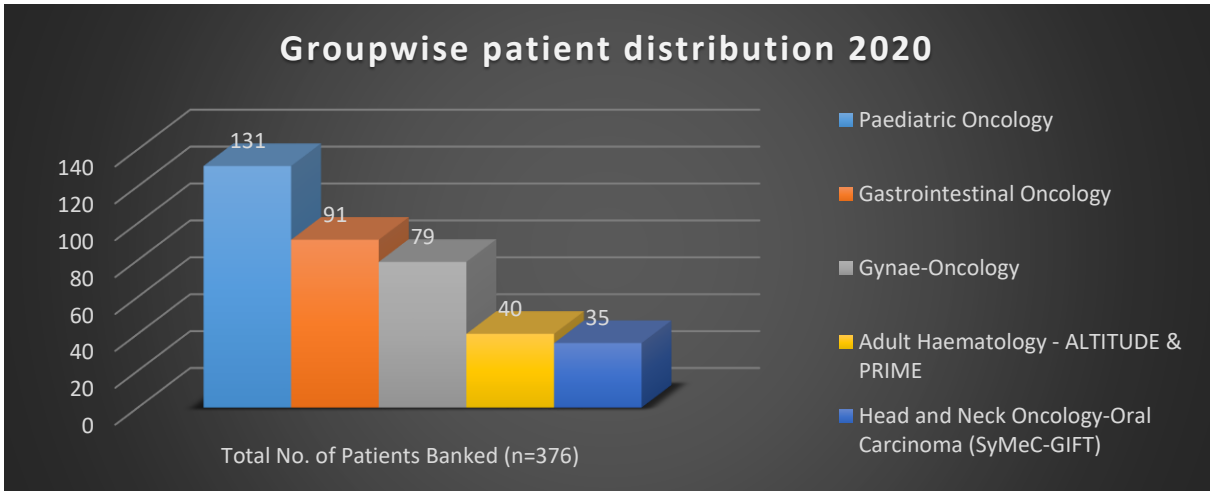
With the COVID-19 outbreak forcing the world to come to a halt, TiMBR extended its support to the institutional COVID-19 response strategy. As a unique facility built following international standards, TiMBR could be repurposed to meet regulatory guidelines to become a COVID-19 testing facility. In April 2020, TiMBR's processing facility was opened up for SARS CoV 2 RNA extraction and continues to do so. TiMBR resumed its activity after a month's gap with paediatric leukaemia group reinitiating banking in May 2020. With COVID-19 response taking centre-stage, biobanking was relocated to various TTCRC specialised laboratory units and to the diagnostic facility of TMC hospital unit. Despite the pandemic, TiMBR continues its banking services overcoming obstacles at multiple levels, from facility to personnel.

This report comprises of the following:

- Summary of the projects banking with TiMBR
- Information on samples collected and released
- Governance and audits
- Event and news (upcoming plans)

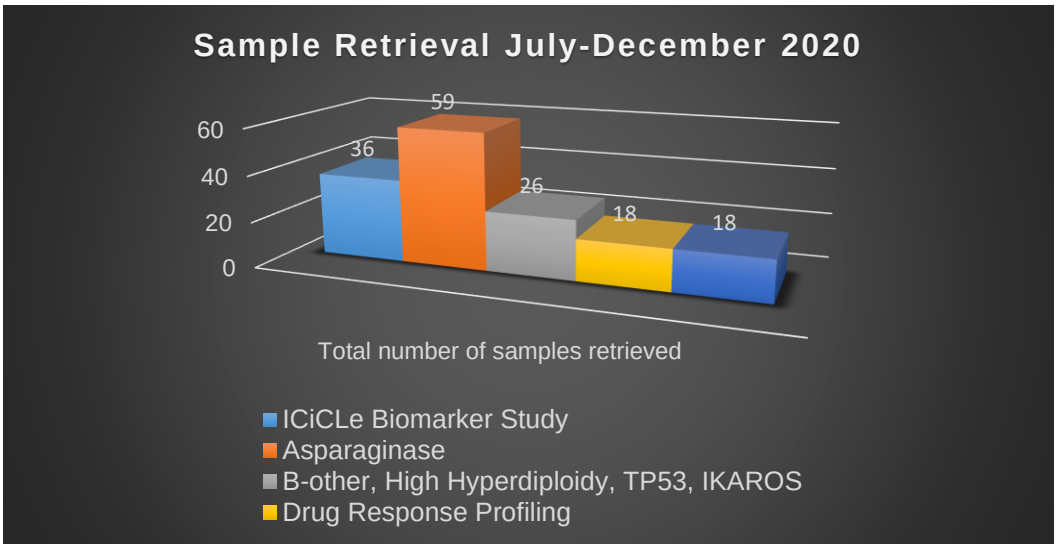
In 2020, TiMBR has actively banked for five departments at Tata Medical Center providing samples to 12 projects groups (details given below).

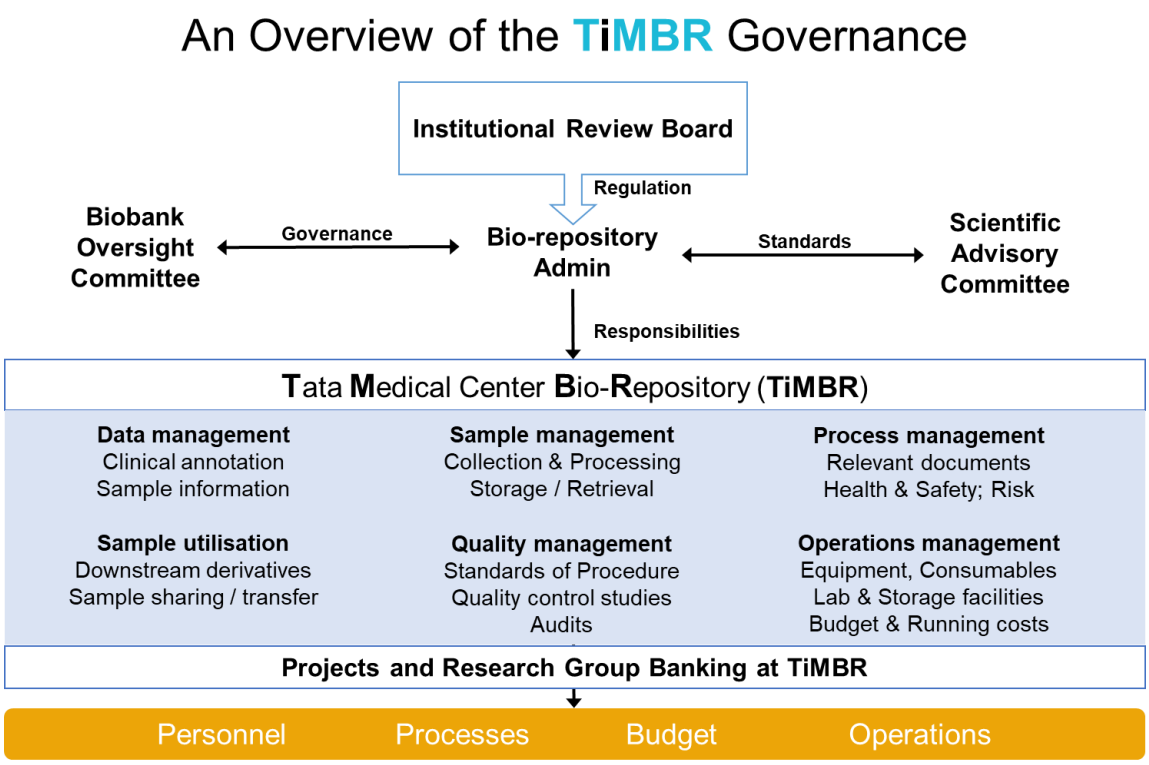
S. No.	Groups Banking with TiMBR	Project	Types of samples banked	Status
1.	Paediatric Oncology	ICiCLe Biomarker Study, Asparaginase	Bone marrow aspirate, Peripheral Blood, Cerebrospinal Fluid, Tissue	Active
		B-other, High Hyperdiploidy		Active
		Drug Response Profiling		Active
		TP53		Active
		IKAROS		Active
2.	Gastrointestinal Oncology	Gallbladder Carcinoma Biomarker Study	Tissue, Peripheral Blood, Hair, Nail, Gall Bladder stone, Bile	Active
3.	Gynae-Oncology	Cervix (SyMeC)	Cervical Scrape, Tissue, Peripheral Blood	Active
		Ovary (PROVAT)	-	Tenure complete
4.	Adult Haematology	ALTITUDE	Bone marrow aspirate, Peripheral Blood	Active
		PRIME		Active
5.	Head and Neck Oncology	Oral Carcinoma (SyMeC-GIFT)	Tissue, Peripheral Blood	Active
		miRNA Study	-	Tenure complete
6.	Breast Oncology	BREXO	-	Inactive
7.	Radiation Oncology	INTELHOPE HYPORT	-	Inactive



Along with sample storage, TiMBR has also witnessed high amount of retrieval in the last half of the year. In case of Gall Bladder cancer group, samples collected through TiMBR are directly used up for downstream processing and only excess samples are stored for retrospective analysis.

S. No.	Project	Type of derivative retrieved	Purpose of Retrieval
1.	ICiCLe Biomarker Study	DNA	Minimal residual disease tracking
2.	Asparaginase	Plasma	Therapeutic drug monitoring
3.	B-Other, High Hyperdiploidy, *TP53*, *IKAROS*	Cryo preserved mono nuclear cells (MNCs), DNA & RNA	RNA sequencing, SWATH-MS, SNP Array, TES
4.	Drug Response Profiling	Cryo preserved MNCs	Drug Response Profiling
5.	Oral Carcinoma (SyMeC-GIFT)	Tissue, Peripheral Blood	Transfer to NIBMG





At TiMBR, audits are undertaken internally every year by each project groups as well as by TiMBR admin. Last year, in order to overcome infrastructural shortcomings triggered due to the pandemic, quality check is adopted as a routine activity by the TiMBR core team. All retrieval carried out for in-house downstream research undergo quality checks. Two types of checkpoints have been set up for maintaining value of bio specimens.

- Direct quality assessment through estimation of DNA yield from mononuclear cells, RNA yield from cells in TRIzol, cell viability checks by flow cytometry.
- User group feedback on cryo-retrieved cells.

Events and news in TiMBR:

On 3rd December 2020, a hands-on-training of LabVantage was organised for user groups of Tata Medical Center. Professor Usha Menon graced the occasion as the keynote speaker followed by a technical session by Mr. Mayookh Sengupta from LabVantage.

TiMBR participated in a virtual symposium organised by International Society for Biological and Environmental Repositories (ISBER) in collaboration with UHN Biospecimen Services on 22nd -23rd October 2020.

Upcoming activities:

Operational:

1. Optimisation of bone marrow stromal cell culture as a research activity complimenting Paediatric Leukaemia group.
2. Exploring the possibility of organoid biobanking in collaboration with Gallbladder Cancer group.

Structural:

1. Refurbishment of the sample processing facility.
2. Activation of TiMBR oversight committee and scientific advisory or user committee.
3. Introduction of e-forms for efficient project management.

Flow Cytometry Facility

Arunima Maiti
Proteomics Technologist

The flow cytometry facility provides investigators with technological resources and assistance for high quality, multi-parametric flow cytometry acquisiton and analysis. The facility is equipped with a benchtop cell analyser, BD Accuri C6 Plus and a cell sorter, *BD FACSAria*. BD Accuri C6 Plus can simultaneously measure and analyse multiple physical characteristics, like, relative size, internal complexity, and fluorescence intensity. *BD FACSAria* is capable of cell sorting ensuring sterility, from heterogenous cell population based on their relative size, granularity and antigen expression using upto eighteen fluorochromes, sorting upto four separate populations simultaneously.

We support investigators in creating efficient and cost-effective experimental designs, through optimizing cytometry-specific reagent and fluorochrome selection, and provide assistance in operating both instruments, as well as in data analysis.

In 2020, we optimised standard operating procedures (SOPs) for some of the common flow cytometry experiments, for ready reference of users. We created equipment booking forms along with instrument setup guidelines for smooth running of the facility. Between the period of Jun 2020 - Dec 2020, BD Acurri C6 Plus was used to perform about 74 experiments assessing cell cycle, apoptosis, mitochondrial health, immunophenotyping of primary cancer cells. BD Aria Fusion was used for 35 experiments. Upto 7 parameters (2 scatter + 5 colours) immunophenotyping was performed in BD Aria Fusion. Drug resistant viable ALL cells were bulk sorted, while GFP+ cells were single sorted for clonal expansion. Data generated from both Accuri C6Plus and Aria Fusion were analysed using FCS Express 6.

Some of the data generated in-house are shown below:

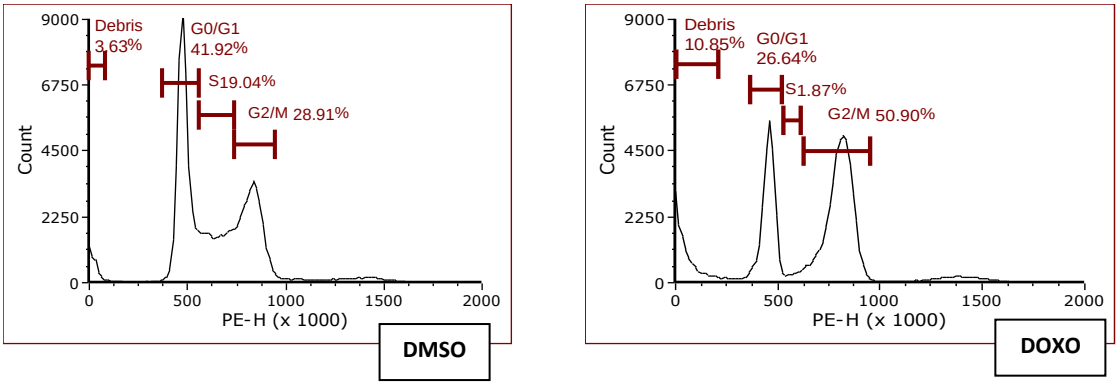


Figure 1: Cell cycle profiling of leukaemia cell line NALM-6 showing G2 arrest upon treatment with 100nM Doxorubicin (DOXO).

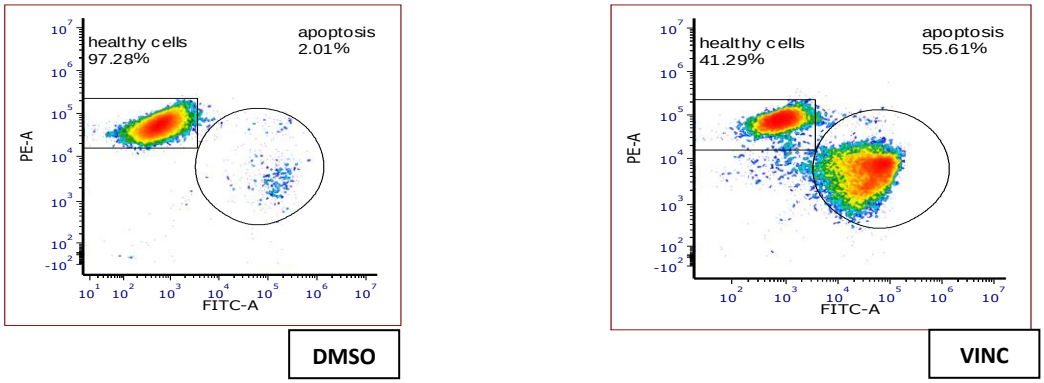


Figure 2: Mitotracker assay showing 97.28% cells containing healthy mitochondria in control REH cells. Upon treatment with 1µM Vincristine (VINC) for 48 hrs, proportion of healthy cells decreased to 41% with increment in cell apoptosis to 55.6%.

Proteomics biomarker discovery laboratory

Arunabha Chakraborty
IA Early Career Fellow

Arnav Bhattacharya
Research Assistant

Sangramjit Basu
Bioinformatics technologist

Development of high-throughput Mass Spectrometry for Discovery of Cancer Biomarkers

Aims and objectives:

- ✓ To develop high-throughput proteomics technologies for rapid identification of biomarkers in cancer.
- ✓ Detection of specific biomarker(s) in Biological samples for patient monitoring and development of targeted therapy.
- ✓ To complement genomic data for better functional implication of genetic abnormalities in cancer patients.
- ✓ Proteomics and metabolomics profiling of specific subtypes of cancer for therapeutic intervention.
- ✓ Qualitative and quantitative analyses of biological samples (both targeted and discovery) for small- and macromolecular expression in a specific time point or condition.
- ✓ **Types of samples:** Examples of types of samples which will be used for study using our mass spectrometer are provided below.

Types of samples	Examples
Cell	Cell lysates from: 1. Cell lines 2. Primary cells
Tissue	Solid tissue lysates from different cancer types e.g. Gall bladder cancer
Organoids	Gallbladder organoids from patients
Body fluids	Blood, CSF, Plasma

We started to develop our proteomics pipelines with analysis of cell lysates prepared from cell lines. Initial experiments were performed with leukaemia cell line NALM-6. Spectral library was generated using NALM-6 wild type cell line. We have created NALM-6 cell lines with different gene knock-outs (e.g. *IKZF1* and *P53* genes) as part of different projects. These knock-out cell lines were used to study differential proteomic expression compared to the wild type cell line. These initial data acquisition (both data dependent acquisition and data independent acquisition) are being used to develop data analysis pipelines to generate differential proteome data. Once streamlined, this pipeline can be used further to analyse DDA and SWATH data generated from different source of samples for both identification and quantitation purpose.

Experimental approaches:

Two types of experimental approaches we are currently following for both identification and quantification of proteins present in a particular sample- Data Dependent Acquisition (DDA) or Information Dependent Acquisition (IDA) and Data Independent Acquisition (DIA) or SWATH.

DDA/IDA

In this type of data acquisition, MS spectrum is collected on a broad m/z range (e.g. 200 Da-1400 Da) and The analyte peaks are detected and sorted by descending intensity. This procedure is repeated over and over again across the LC gradient. A narrow Q1 isolation window is used to send only that analyte through for MS/MS analysis.

In this procedure there is a high risk of lower level analytes either not being detected in the original MS spectrum or the mass spec running out of time (too slow relative to the complexity of the sample) to capture MS/MS spectra for everything detected in MS mode. This creates gaps in the data.

DDA is usually performed after fractionation of the complex samples and combine all the MS and MS/MS run from all the fractions to generate spectral library for identification of all the proteins expressed in that sample. Fractionation of the samples can be done by different means for example, by offline HPLC or by running a gel and excise several bands from the gel as different fractions before tryptic digestion of the protein samples.

DIA/SWATH

In this type of data acquisition, the mass spec uses a wider Q1 isolation window and steps it across the entire m/z mass detection range. This way, the mass spec collects full MS/MS spectra on every detectable analyte that passes through each Q1 window. The full mass range is interrogated in an LC time frame (short cycle times). This creates a complete MS and MS/MS picture of everything detectable in your sample, without the need for repeated runs or re-analysis. This method of acquisition requires a critical inter-relationship in technology performance: Q1 acquisition window control and MS/MS acquisition resolution at high speed.

Data quality control

The instrument functionality and the data generated in the mass spec are always quality checked before analysis. Instrument quality check is performed by running tuning solution directly to the mass spec. Data quality check is performed by running β-Galactosidase digest run as a calibration standard (Figure 2). In Analyst software, presence of specific peaks with higher than a cut-off intensity is considered to be acceptable for further acquisition of samples. If cell lysate is used as sample, we also run K562 cell lysate digested with trypsin protease and check for TIC intensity and pattern as a function of time to be sure about instrument performance. Then only we can run samples of interest.

The data generated from the Sciex Triple TOF 6600 comes out from the detector as .wiff files. Initial quality check of the data is performed in Peakview software which represents the Total Ion Current (TIC) of the sample run as a function of time. Also, in Peakview, qualitative and quantitative visualisation of the generated DDA and SWATH data are performed (Figure 3).

Data analysis pipelines

After the initial quality check, the raw data files from sample acquisition can undergo data analysis. We are in the process of developing the pipelines for analysing DDA data for spectral library generation and SWATH data for quantitative analyses. Detailed data analysis pipeline is provided as a flowchart in Figure 1 (*Nature Protocols* volume 10, pages426–441; 2015).

Mass spec facility as a support to different projects

The following supports are expected to be provided by our mass spec facility-

- ✓ Experimental design for qualitative and quantitative analysis of samples
- ✓ Generation of spectral library most appropriate and relevant samples in a project
- ✓ Proteomic characterisation of cell models created in different projects. Characterisation of tissue and organoids obtained from solid tumors
- ✓ Patient specific SWATH data acquisition and analysis for differential proteomics in two or more different conditions
- ✓ Rapid identification of prognostic and therapeutic biomarker in patient samples (e.g. Plasma/serum, CSF, Tissue, Bone marrow derived MNCs etc.)

Discussions/ Future plan

- ✓ Trans Proteomic Pipeline (TPP) for analysis of SWATH data is being developed for differential proteomics studies with cell models as well as with patients.

- ✓ Spectral libraries are generated by DDA/IDA run from relevant samples or can be downloaded from previously generated proteome data; DDA spectral libraries are used for quantitative analysis of SWATH data. We are generating most relevant spectral library to represent our samples and generating SWATH data with different project needs for differential proteomics analysis.

- ✓ SWATH data, once generated, will be stored in server re-analysed anytime in future. Depending on the research questions we are asking, we can access the data from server and analysis pipeline will be run depending on the research objectives.

- ✓ Patient-specific SWATH data would be generated and analysed for the purpose of developing personalized targeted therapy and identification of rapid prognostic and therapeutic biomarker.

- ✓ All the pipelines are being standardised based on the research objectives of two types of cancers- Acute Lymphoblastic Leukaemia (ALL) and Gallbladder cancer (GBC).

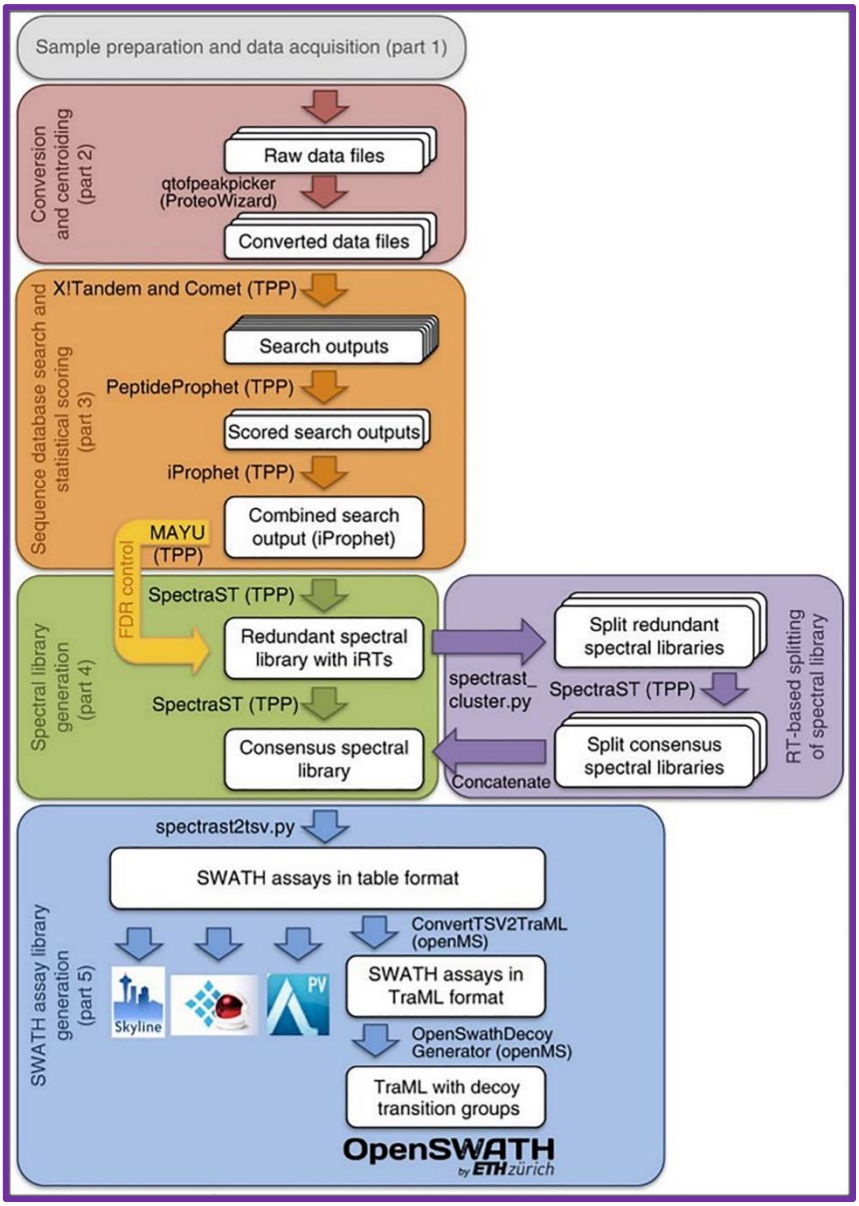
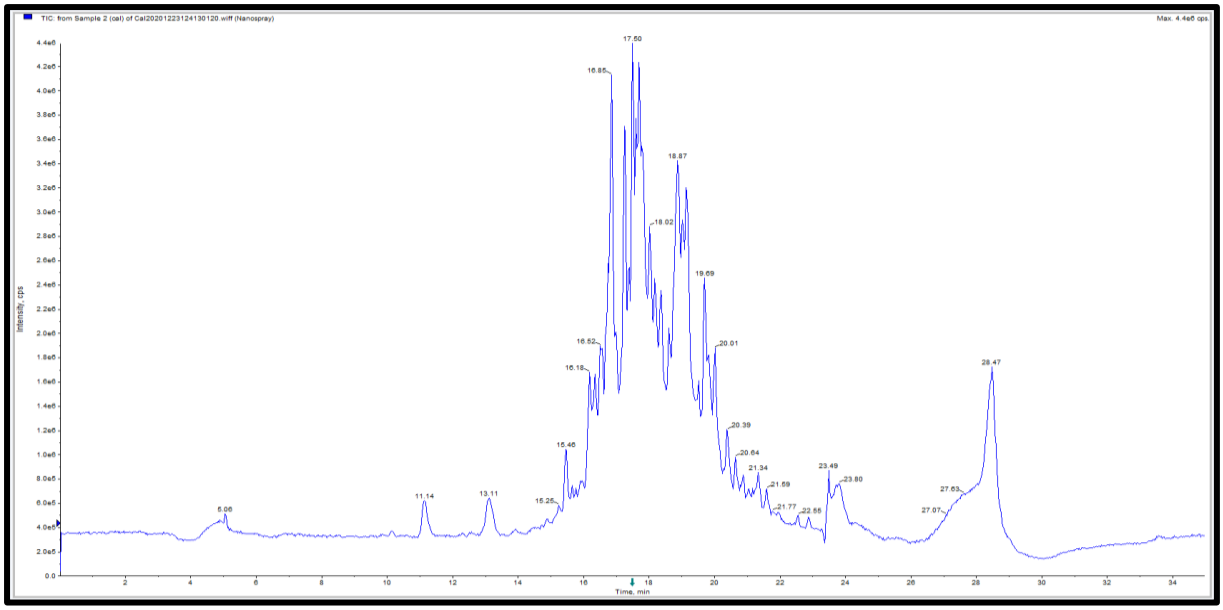


Figure 1 Data analysis pipelines for DDA and SWATH acquisition for preparation of spectral library and quantitation of proteins



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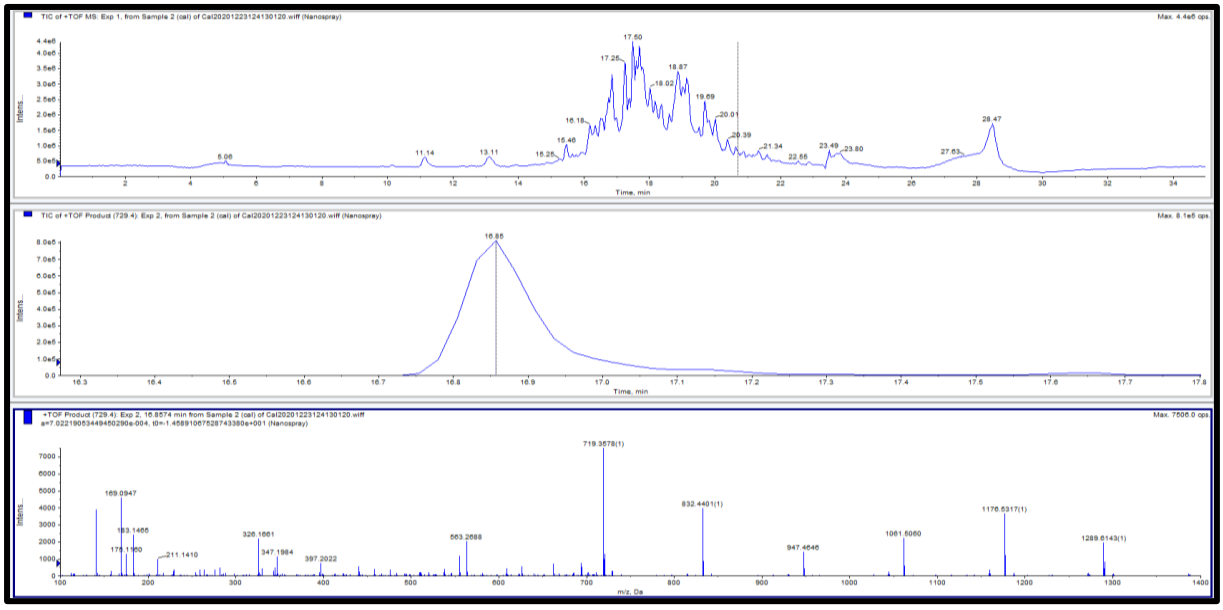
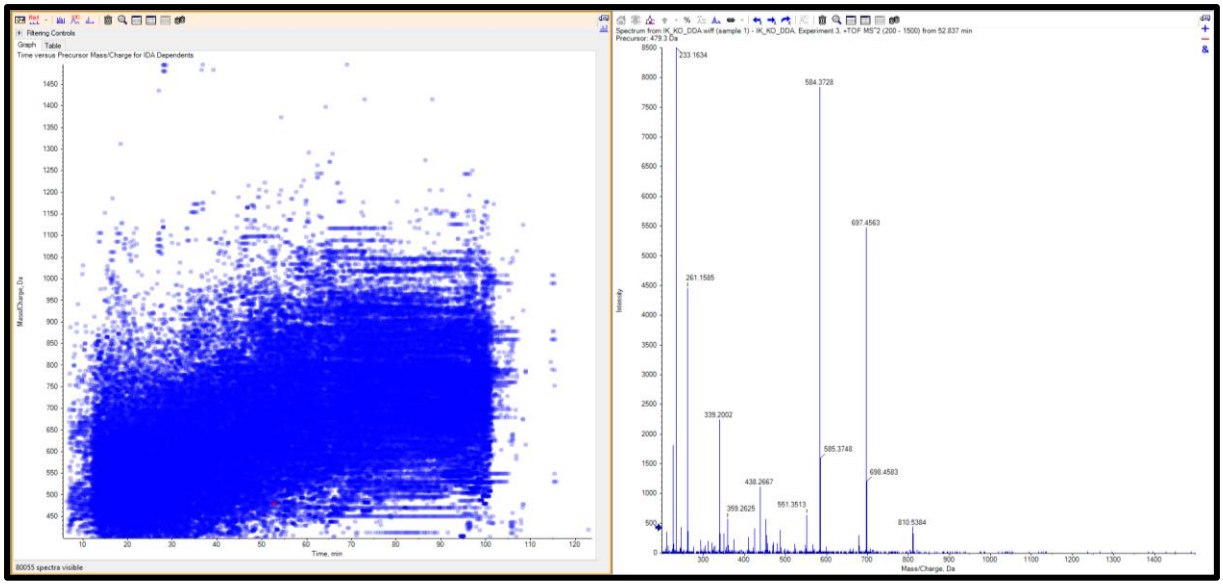


B

Figure 2 A. TIC distribution of DDA run of β-galactosidase digest. **B**. MS spectral distribution and MS/MS fragment spectra of precursor ion 729.4 from β-galactosidase digest



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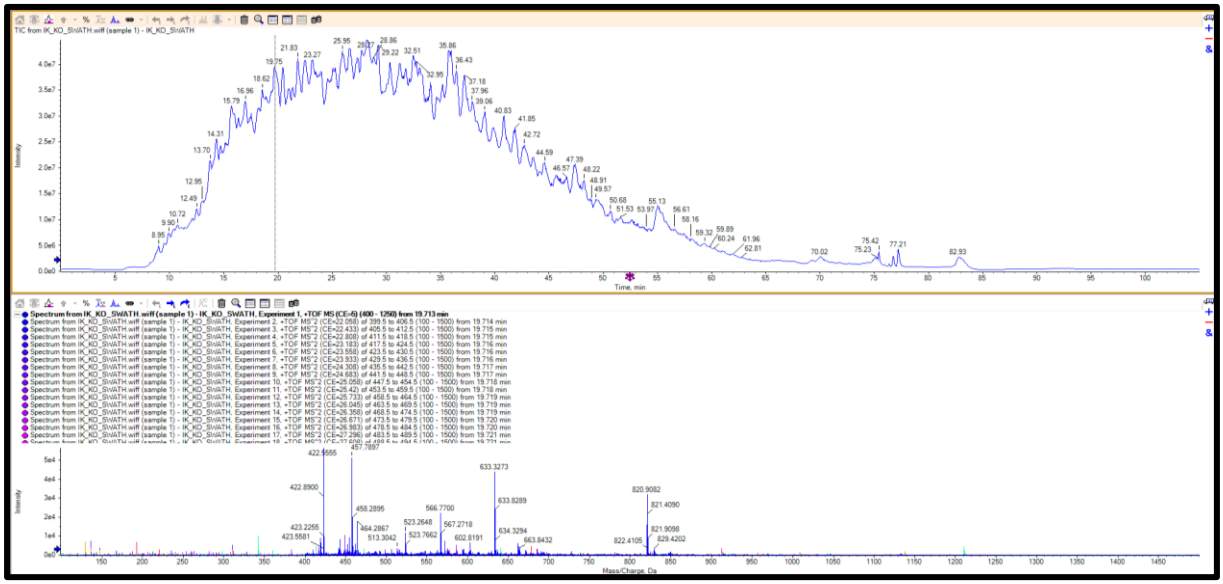

B

Figure 3 A. Visualisation of number of spectra generated and spectral distribution generated from a sample (*IKZF1* knock-out NALM6). **B**. TIC and MS/MS spectral distribution in all time points of the SWATH run for the sample (*IKZF1* knock-out NALM6) distributed throughout the mass range

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Team Composition

Team TTCRC:

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Team TMC:

Piyali Biswas, Dr Chumki Bhattacharya and Dr Mayur Parihar

At TTCRC, the Cancer Genomics team is multidisciplinary consisting of highly motivated and well-trained individuals in the wetlab who set up project specific experimental workflows; a team of enthusiastic and skilled bioinformaticians and a supportive bio-banking team. The Clinical Research Unit (CRU) and Cytogenetics department serve as the backbone of this team and working together to provide better care for cancer patients.

Minimal Residual Disease monitoring in childhood ALL- Success, Challenges and Future

The Minimal Residual Disease (MRD) laboratory at TTCRC was established with an aim to offer more sensitive and advanced tools for detecting residual clones in childhood ALL. Though flow-MRD (FCM) is available across all ICiCLe centres offering a rapid turnaround time, interpretation is subjective and the technique requires viable cells for analysis. So, clonotypic Ig/TCR based genomic PCR-MRD testing was established at our centre to address the problems of reproducibility, sensitivity and sample portability. This has enabled clinicians to take decisions on appropriate therapy for patients based on the depth of response to therapy.

Our experience suggests PCR-MRD complements FCM in identifying high-risk patients more precisely. PCR-MRD has a greater sensitivity of 10^{-5}, which is a log higher than that of FCM. Comparing the sensitivities of FCM vs PCR-MRD for the discordant cases where FCM reported negative at the level of 10^{-4}, and PCR-MRD was either positive or positive but not quantifiable, about 20% were in the range between 10^{-3} and $5*10^{-4}$ and 80% lies beyond the range of 10^{-4}, which is the assay limiting sensitivity of FCM. Therefore, PCR-MRD has been able to successfully identify low MRD positivity in 45% patients, where FCM reported negativity at $<10^{-4}$.

Here, we report an exemplar of discrepancy between PCR and FCM-MRD. Figure 3, shows the MRD kinetics of a relapsed patient at longitudinal time-points. As depicted, MRD by PCR was high at end of induction (FU1) and consolidation (FU2), but undetectable by FCM. The patient was further treated using a Blinatumomab-based modified therapy which resulted in achieving molecular remission at subsequent time-points by both PCR and FC-MRD.

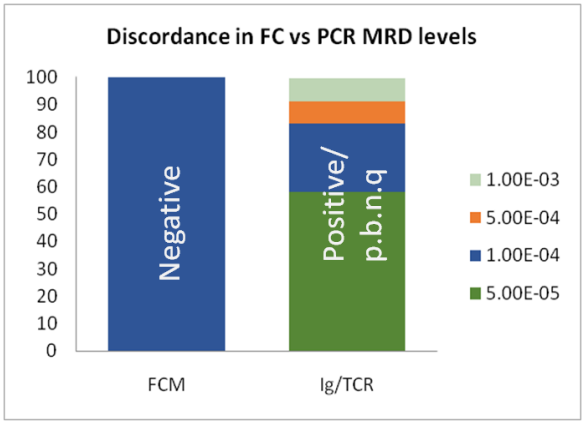


Figure 2. Bar plot showing discordance between FCM negativity and PCR-MRD positivity at levels ranging from 10^{-3} to 10^{-5}.

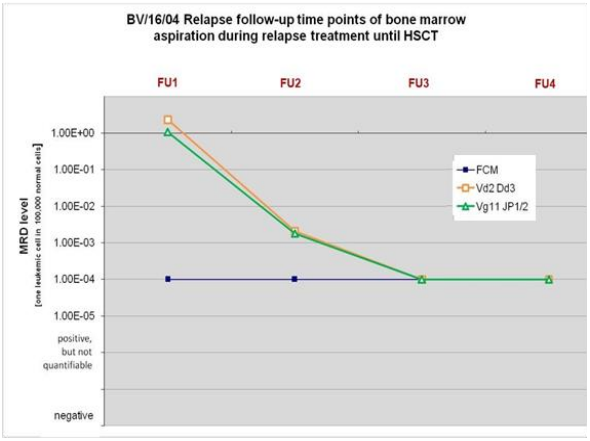


Figure 3. MRD Kinetics of relapsed patient showing levels assessed by FCM and PCR-MRD at serial follow-up time points during treatment.

Though PCR-MRD is sensitive, robust and reproducible, the current workflow is labour-intensive and time-consuming. With the available manpower and resources, we are able to offer testing in 5 patients per month at our hospital, which is clearly insufficient for our needs. Moreover, there are technical challenges with samples having borderline leukemic blasts <30%, resulting in loss of a significant proportion of patients with non-informative markers (~30%), who are lost to follow-up tracking.

Transitioning to NGS-based MRD clonal marker identification

To overcome the present challenges, we intend to make a transition to high-throughput sequencing based clonal marker identification, which would allow for multiplexing patient samples at lower turnaround times. Currently, we are in the process of standardizing the NGS-based MRD workflow, which is adapted from the protocol developed by EuroClonality-NGS Consortium. As illustrated in Figure 4A, it involves a two-step library preparation. In the first step, the clonal targets (TRB, TRG, TRD, IgK, IGH) are amplified using different multiplex PCR reactions, followed by a second round of PCR, which allows for dual indexing of patient samples for multiplexing in the sequencing run. We have successfully been able to standardise the first round of PCRs using positive controls like cell-lines and patient samples. (Figure 4B).

By end of 2021, we propose to optimise the library and sequencing protocols for marker identification using Illumina-based platform. We are hoping that this would increase our throughput for screening to atl east 4 times more than our current monthly output with standard PCR-based workflow.

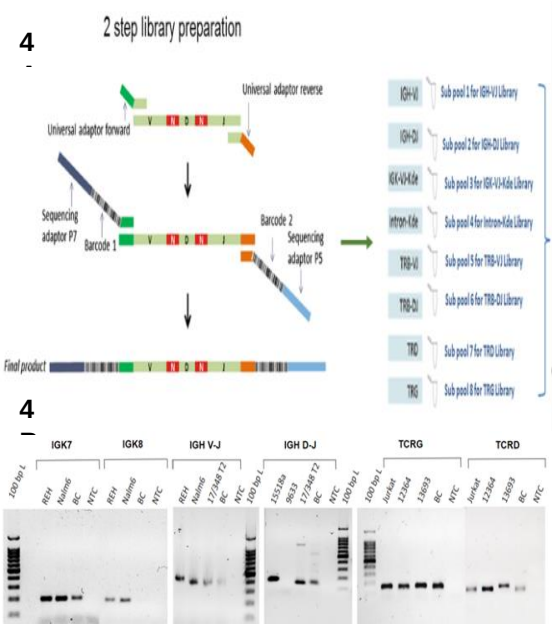


Figure 4. **A** Schematic workflow of NGS-based MRD marker identification developed by EuroClonality-NGS group, showing the 'two-step' library preparation approach. **B** 'First round PCRs' in Ig/TCR loci (TRG, TRD, IgK, IGH) showing amplification with positive controls and buffy coat (BC) DNA. No amplification seen in 'No template control' (NTC).

In addition to this, we have collaborated with DKMS who are developing an alternative approach for marker identification and MRD quantification using unique molecular identifiers (UMI) (Figure 5). Sequencing is initially being standardized using Illumina and would later be done using the Oxford Nanopore technology, MinION.

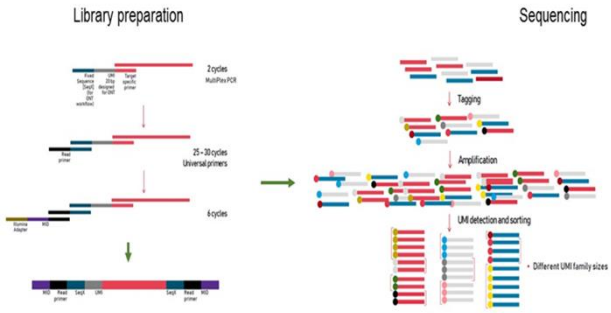


Figure 5. Schematic workflow of the UMI-based NGS assay developed by DKMS for MRD marker identification and MRD quantification in ALL.

Going forward, we wish to adapt the EuroClonality-NGS workflow on MiNION using the dual-pore R10 flow cells and compare our findings with that of Illumina for both the approaches. MinION-based sequencing will not only lower the costs of sequencing significantly but also provide greater flexibility in terms of multiplexing samples at reduced run-times. We are optimistic to develop this in collaboration with DKMS, which would allow for wider access to smaller centres, who lack expertise and resources to set up a sequencing facility.

Genetic Characterisation of ALL and Gall Bladder Cancer

We have studied a cohort of total 57 high-hyperdiploid (HeH) BCP-ALL patients included from the ongoing multi-centric clinical trial (InPOG-ALL-15-ICiCLe-ALL-14; CTRI 2015/12/006434) between 2015 to 2019 of ALL patients in Tata Medical Center (TMC), Kolkata. As HeH subtype is characterized by gain of chromosomes, a high-density SNP array analysis (using CytoScan HD platform, Affymetrix) was also performed to get additional information on the copy number alterations (CNA) in these patients which further revealed the

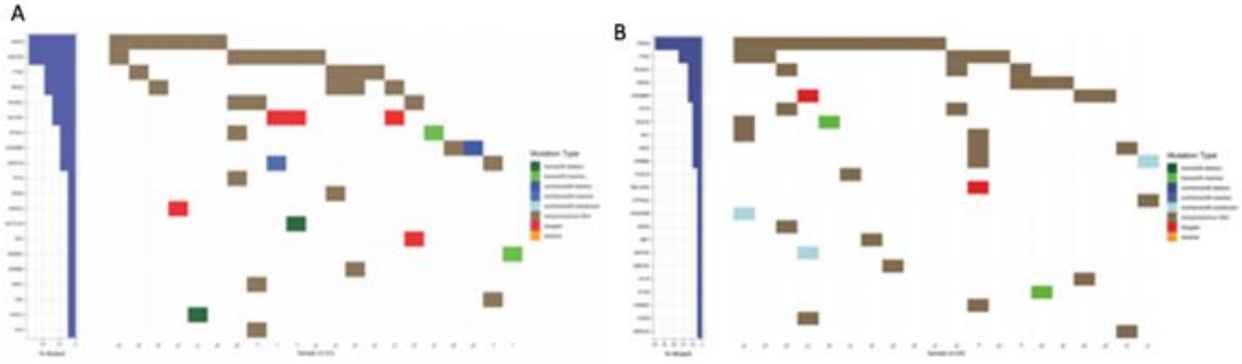


Figure 6. Waterfall plots of UPD (A) and non-UPD (B) sample cohort.

presence of whole chromosomal uniparental disomies (wUPDs) within a subgroup of patients (44%). We previously reported our mutational findings in HeH patients based on the Targeted Sequencing Panel. Genes involved in RAS signalling pathway (*KRAS*, *NRAS* and *TYK2*) as well as genes which act as chromatin modifiers (*KMT2D* and *CREBBP*) were mutated in almost 69% of samples. Further analysis on the mutation signatures between the UPD and non-UPD patients revealed that mutations in *KRAS*, *TYK2* and *ERBB2* genes were more frequent in nUPD patients whereas genes like *KMT2D*, *NRAS* and *ARID1A* harbor more mutations in UPD patients.

We have also performed the whole transcriptomic profiling of 28 HeH patients to identify the differentially expressed genes in UPD (n=14) vs nUPD (n=14) cohort. A tSNE analysis showed four distinctive clusters in patients based on presence or absence of UPD samples.

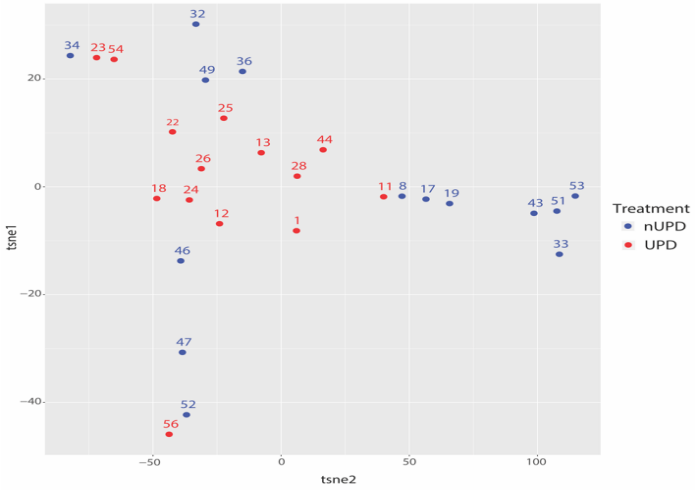


Figure 7. The tSNE plot of 1000 most variable genes derived transcriptomic data of 14 UPD and 14 non-UPD.

Different combinatorial approaches were considered to identify the differentially expressed genes within our sample cohort. Consensus predictive methods determined 40 upregulated and 11 down-regulated genes in UPD and nUPD patients. The identified genes were further considered for their functional analysis. The pathway enrichment analysis shows their probable involvement in signal transduction, transcription regulation, protein metabolism, transport mechanism and disease related pathways. However, cellular response to stress, cell cycle regulation, apoptosis, programmed cell death and regulation of immune systems were found to be upregulated only in UPD patients.

In the coming year, we are also embarking into the genetic characterisation of B-Other ALL which represents one of the most heterogeneous and complex molecular subtype of BCP-ALL. Lack of reliable prognostic markers often interfere with risk stratification and therapeutic interventions in these patients which

further leads to poor treatment response and relapses. Hence we aimed to characterize the genomic landscape of “B-others” using whole genome SNP array (CytoSNP 850K) and mRNA sequencing approach to improve prognosis and disease management.

Integration of genetic information like copy number alterations (CNAs) and single nucleotide polymorphisms (SNPs) with treatment response in patients and exploring the novel fusions and gene-expression through transcriptome sequencing may improve the clinical outcome in these patients.

We are also establishing a genomic pipeline in TTCRC to better characterize gall bladder cancer (GBC) based on two high-throughput approaches – 1) whole exome sequencing (100X) of GBC tumour samples to explore the somatic mutational profile and 2) m-RNA sequencing of GBC tumour and tumour-derived organoid samples.

Computational Biology

Anindyajit Banerjee

Lead - Bioinformatics

Tushar Mungle

Clinical Informatician

Sangramjit Basu

Bioinformatics technologist

The computational biology group at TTCRC investigates the complexity in cancer biology by integrating large data sets generated by different experimental conditions. These include identification of genomic variants, e.g somatic copy number variations, single nucleotide variations, differential gene expression patterns, protein expression and the putative impact on cancer. The department is currently equipped with high-end computational facilities which include sophisticated tools and techniques in-terms of computational power and storage.

The TTCRC computational biology group works on multi-omics data. This includes identification of patient-specific biological markers using genomic and transcriptomic profiling of sample cohorts. solid and liquid tumour studies. High throughput sequencing data derived from Illumina and Oxford Nanopore platforms provide data on genomics and transcriptomic data. Proteomics data is generated from samples analyse on a Triple TOF and Imaging data from a high throughput confocal microscope.

Structural Variance and Single Nucleotide Variation Detection

Computational approach provides an in-depth understanding in determining the chromosomal structural variance from whole genome analyses. Sequencing data derived from a 95 gene panel across the sample cohort provided a better understanding in detecting the mutational burden across the sample cohort. To minimize manual intervention, the data were processed through a standardized multi-layered workflow. Each step accounts for processing of data through rigorous filtration criteria, leading to identification of pathogenic variants.

Transcriptomic analysis and Fusion gene identification

Transcriptomic data processed through in-house multi-layered automated analysis

pipeline respectively. The process ensures prediction of high-quality reads derived from protein coding region. Differential gene expression analysis is done from normalized reads counts and multiple statistical programs. This provides a basic understanding in the change in gene expression patterns among the different experimental groups. RNAseq data are processed to determine the probable fusion transcripts within the sample cohort using our multi-layered robust in-house pipeline. Fusion transcripts are identified with high confidence with experimental validation. Additionally, mutation detection derived from RNAseq data provides an addition layer of filters in validating the somatic variants determined from the gene panel sequencing data.

Functional characterization of differentially expressed gene sets

Downstream analyses lie in investigating the potential biological effects of genes expressed differentially in the sample cohorts. Differentially expressed genes were considered for pathway discovery. The approach used key differentially expressed genes, likely to be involved in disease processes. This process is known as Functional Gene Set (FGS) analysis.

High-throughput microscopy image analysis

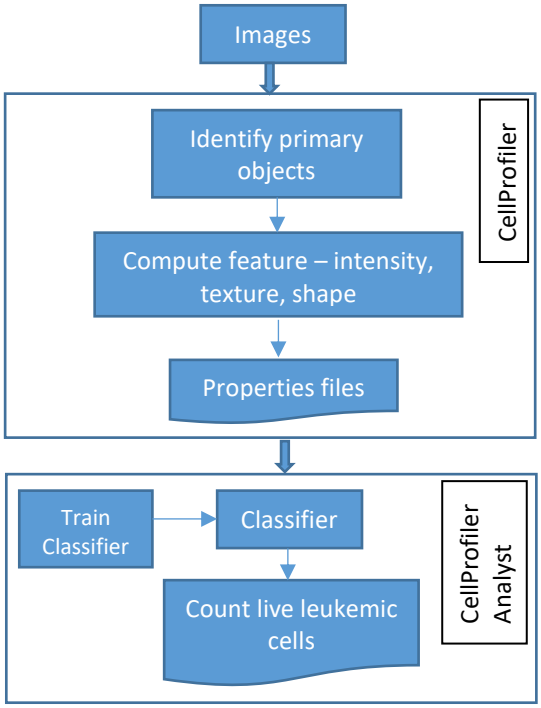


Figure 1: Overview of image analysis pipeline for live leukemic cells quantification.

Imaging based drugs screening process involves treating primary ALL cells in co-culture with several drugs in different log concentrations. Images are then acquired and analysed for population of live leukemic cells. CellProfiler and CellProfiler Analyst software is used to quantify the live leukemic cells from the images. Primarily, all the objects are identified from the images. Different features related to cells such as intensity, shape, and texture are computed that is followed by training the classifier based on the features for live leukemic cells as positives and the rest as negatives. The trained classifier classifies the cells from the set of images to give live population of leukemic cells for respective drug treatments. Figure 1 provides an

overview image analysis workflow developed for the work.

High throughput Proteomics analysis

Proteomics analysis rely on data acquisition process in terms of both Data Dependent (DDA) and Data independent (DIA) acquisition mode. The DDA analysis is a multi-step process which includes combination of multiple tools and PeptideShaker. Initially file formats are converted and most intense 250 peaks are obtained for downstream analysis. The selected peaks are searched against human proteome (Uniprot database) using multiple algorithms like Xtandem and MSGF+. Results are merged at 0.01 FDR with PeptideShaker. Obtained results are used to create a sample group specific spectral library which is in turn used to quantify the elucidated proteins from the SWATH (DIA run) traces. This analysis is executed in Skyline. Both PeptideShaker and Skyline are application with intuitive graphical user interface that helps to both visualize the data as well perform data Quality Check.

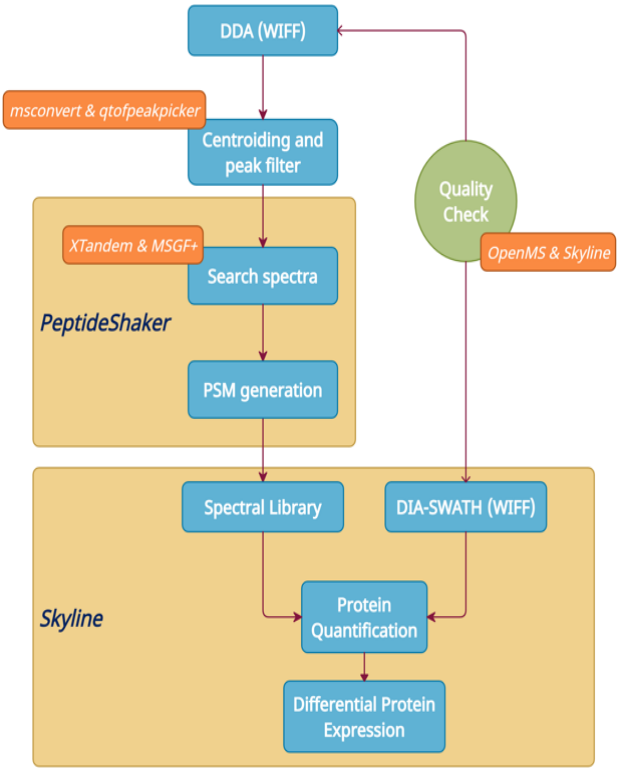


Figure 2: Schematic representation of proteomics analysis pipeline used for data acquisition and interpretation respectively.

India Alliance Fellows

Arunabha Chakraborty

IA Early Career Fellow

The role of the IK6 isoform of IKZF1 in childhood acute lymphoblastic leukaemia

My research goal is to investigate the mechanisms by which the imbalances in *IKZF1* isoform expression promotes leukaemic cell survival under cytotoxic stress. Understanding the impact of *IKZF1* deletion isoforms, particularly IK6, on cell survival after chemotherapy and the mechanisms of drug resistance in leukemic clones with the deletion.

Hypothesis:

IKZF1, in association with other transcription factors (e.g. *PAX5*) restricts the supply of glucose and energy to pre-B cells to initiate differentiation. Wild-type *IKZF1* (IK1) is transported to the nucleus and functions as a dimer. It is a transcription factor and acts both as an activator and a repressor. The IK6 (Δ4-7) isoform lacks the DNA binding domain and nuclear localising signal. It does not enter the nucleus and cannot function as a transcription factor. It retains the carboxyl zinc finger interactive domain, which could

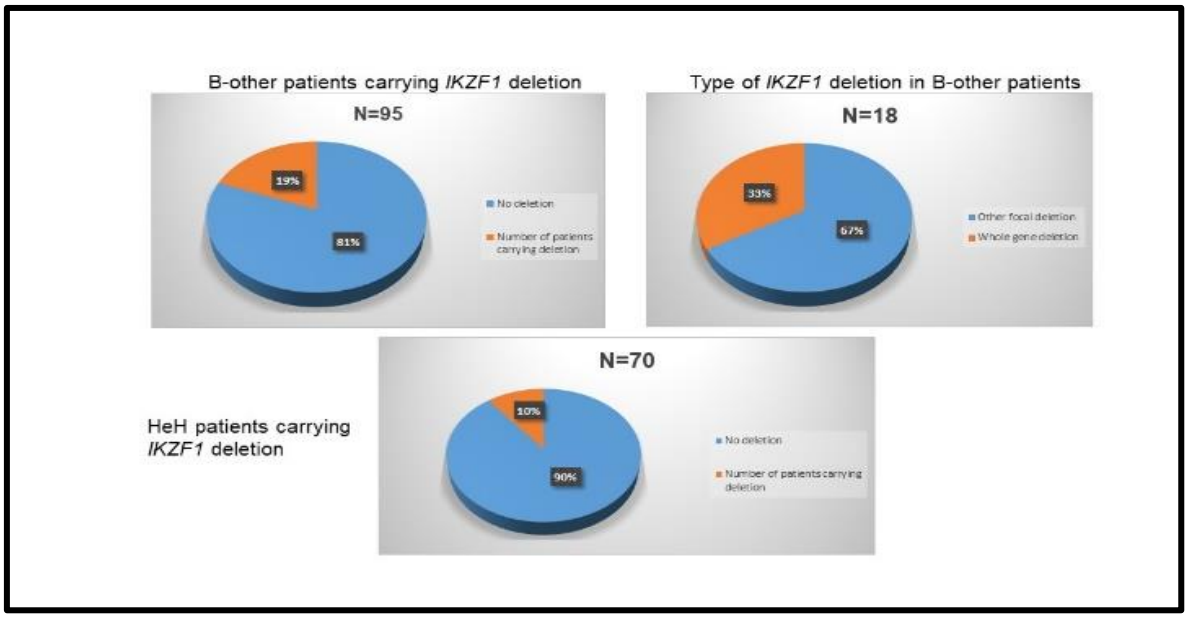


Figure 1. Screening of BCP-ALL patients for *IKZF1* deletion revealed the highest propensity of deletions found in B-other BCP-ALL (19%). 10% of the HeH patients were found to carry a deletion in *IKZF1*. Deletions were either a whole gene deletion or focal deletions. Δ4-7 was found to be the most prevalent among all the focal deletions in our patients

impede IK1 entry into the nucleus due to heterodimerisation. in the cytoplasm to prevent its tumor suppressor function. I hypothesize that, IK6, at least in part, is responsible for the metabolic adaptation of the leukemic cells with its microenvironment. This promotes survival. I also hypothesize that clones carrying an Ikaros deletion, are more adhesive and invasive in nature. This allows these cells to migrate from the bone marrow to other organs.

Why is this study important?

A. This study will establish the pathways through which IK6, the deletion isoform of Ikaros, implements its role towards leukaemic cell survival and resistance of the leukaemic clones against chemotherapeutic drugs.
B. Novel functions of IK6 will be identified.

Current findings/expected outcome:

Patient screening for *IKZF1* deletion

Screening of BCP-ALL patients at Tata Medical Center to identify presence of *IKZF1* deletions in different cytogenetic subtypes. This has been done using multiplex PCR based approach specific for *IKZF1* gene (Fluorescent PCR); followed by fragment analysis, Multiplexed Ligand-dependent Probe Amplification (MLPA) which analyses 9 genes including *IKZF1* and Cytoscan HD (Affymetrix) which a genome wide SNP array analysis.

Patient screening data suggested that propensity of *IKZF1* deletion is maximum in the B-other cytogenetic subtype of BCP-ALL (19%) (Figure 1) and it is seen in 10% of paients with High Hyperdiploid (HeH) (Figure 1). In our patients, deletion of *IKZF1* in exon 4-7 (Δ4-7), which expresses the IK6 isoform is the most prevalent of all the focal deletions found in *IKZF1*.

CRISPR-Cas9 based knock-out of *IKZF1* in leukaemia cell line NALM6

A CRISPR-Cas9 mediated knock-out of *IKZF1* (IK-KO) has been performed in leukaemia cell lines NALM6. IK-KO clones were sorted using GFP expression and propagated in several passages. Absence of IKAROS expression was confirmed by western blot analysis. Five different IK-KO clones were selected and propagated

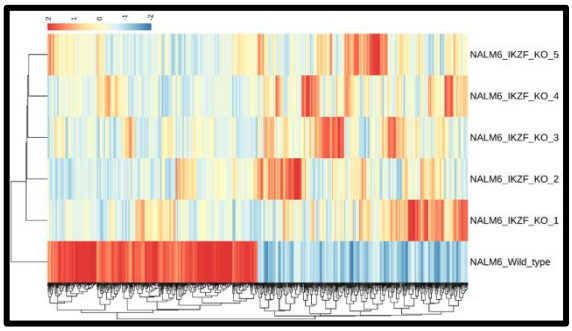


Figure 2. Heat-map of differential gene expression in five *IKZF1*-ko NALM6 cell lines compared to the NALM6-wt cell lines

in culture. These IK-KO cell lines were used further for differential gene expression and compared to that of the NALM6-wt cells.

Transcriptomic analysis of the *IKZF1*-KO NALM6 cell line compared to the NALM6 wild type (NALM-wt) cell line

We performed RNA-sequencing (RNA-seq) to compare the transcriptome profile of the *IKZF1*-KO NALM6 cells (five different single cell clones) as well as NALM6-wt cell line. Gene expression profile (GEP) of both the cell lines revealed that there are distinct GEP of the knock-out cells compared to that of the wild type cells (Figure 2).

Pathway analysis of the top 350 up- or down-regulated genes in the *IKZF1*-ko NALM6 cells revealed that genes involved in important pathways related to leukaemia were found to be differentially expressed in the *IKZF1*-ko NALM6 cells compared to the NALM6-wt cells (Figure 3).

Gene set enrichment analysis (GSEA) with the differentially expressed genes in *IKZF1*-ko NALM6 cells showed number of genes enriched in the gene sets ALL cell proliferation and glucose metabolism.

Further analysis of the *IKZF1*-KO NALM6 cells using SWATH proteomics is ongoing for a better understanding of pathways and proteins altered in the *IKZF1*-ko NALM6 cells compared to the NALM6-wt cells. After the proteogenomic analysis is established and candidates are identified to be involved in the disease process, validation will be performed with samples from BCP-ALL patients.

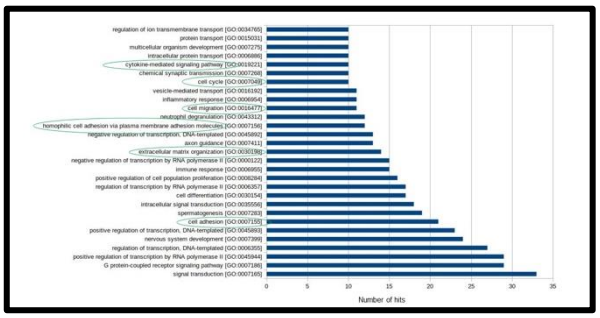


Figure 3 Major pathways represented by top 350 up- and down-regulated genes in IK-KO NALM6 cell line compared to NALM6-wt

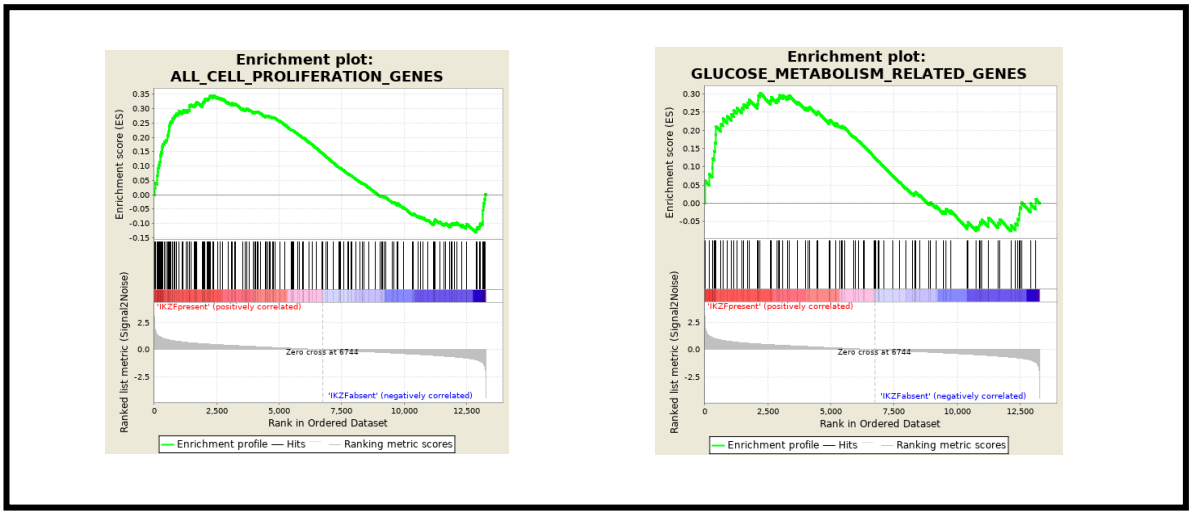


Figure 4. Gene set enrichment analysis for genes involved in ALL cell proliferation and glucose metabolism

in progress:

Characterisation of the cells with different Ikaros status are being performed using several functional studies under different types of stress (e.g. chemo stress, glucose deprivation, serum deprivation) in hypoxia and normoxia; with respect to cell viability/proliferation, cell cycle, apoptosis. A IK-KO is being created in the RS 4;11 ALL cell line as well to validate the findings from NALM-6 cell line models. Lentiviral transduction of IK6 into the IK-ko cell lines has already been done to generate IK6 overexpressed NALM6 cell lines. Re-introduction of IK1 to study whether cells are going through rescue from IK6 effects is the next plan in near future.

Proteogenomic characterization of cell lines as well as patient samples with different Ikaros status are ongoing for development of rapid identification of biomarkers.

Jasmeet Sidhu
IA Early Career Clinical Fellow

Developing strategies for acute lymphoblastic leukaemia patients with poor therapeutic response

Summary: Therapeutic options for patients with poor treatment response or patients with relapse/refractory acute lymphoblastic leukaemia (ALL) are limited. *Ex-vivo* drug screening has a positive correlation with clinical patient response. High-throughput phenotypic drug screening can identify alternate sensitive agents for patients with difficult-to-treat or poor response ALL and can help design personalised effective therapies for these patients.

Background: Childhood ALL is characterised by prognostically significant genetic subtypes. Genotype based risk stratified chemotherapy cures >85% of patients in the west [1,2]. Within all prognostic subcategories, the most significant determinant of outcome is the minimal residual disease (MRD) burden after induction therapy. The current understanding is that chemo-resistant subclones survive induction and cause MRD and ultimately, lead to relapse [3]. In India, a risk-adapted MRD strategy is used in the national clinical trial for childhood ALL (ICiCLe-ALL-14; CTRI/2015/12/006434). Post-induction intensification of therapy in those with MRD levels $\geq 10^{-4}$ improves outcome[4]. But still, ~20% of patients relapse and have limited therapeutic options. A better understanding of the factors that contribute to MRD in children on the ICiCLe-ALL-14 protocol is required for further optimisation of therapy to improve outcomes.

Hypothesis: Automated agnostic phenotypic high-throughput drug screening can identify alternate therapeutic agents for patients with difficult-to-treat ALL (MRD ≥0.01% or relapsed refractory disease).

Aims: Decreasing the MRD burden can be achieved in ICiCLe-ALL-14 by identifying alternative induction agents in genetic subgroups prevalent in Indian children.

Research objectives:

1. High-throughput imaging-based *ex vivo* drug screening to identify anti-tumour drug combinations in real time
2. Transcriptomic studies to identify drug survival mechanisms using an *ex vivo* model of MRD

Methodology:

Cell culture and reagents: Immortalised human bone marrow cell line, hTERT, was cultured in DMEM (Invitrogen) with 10% foetal bovine serum (FBS).

Cytotoxicity and viability assays: Drug responses were assessed in primary ALL cell cocultures on hTERT-immortalized primary bone marrow mesenchymal stromal cells (MSCs) (**Figure 1**). MSC (2.5x10^3 cells/well) were plated in 384-well plates (Greiner) in 30µL serum free medium (AIM-V., Life Technologies). After 24h incubation at 37°C, 5% CO2, 2.5 x 10^4 viable leukaemia cells suspended in 30µL medium were added and incubated for an additional 24h followed by addition of drug solution. Drug stock solutions were prepared in dimethyl sulfoxide (DMSO; stored at -20°C). Six serial log dilutions (10 µL per well) were used in triplicates for screening. Optimal concentration rages were calculated based on the frequency distribution of IC50 values per each drug and as reported previously. After 72h of incubation with compounds, live cell numbers were evaluated using CyQUANT (Life Technologies) live cell staining (7µL per well) and incubated for 1 hour at 37°C, 5% CO2. Automated imaging was performed using the ImageXpress microscope (Molecular Devices) with 10x Plan Fluor objective (Nikon) (covering 70% of the well surface). Images were processed and analysed using Metaexpress (Molecular Devices). Drug response quantification was performed using curve-fitting non-linear regression on

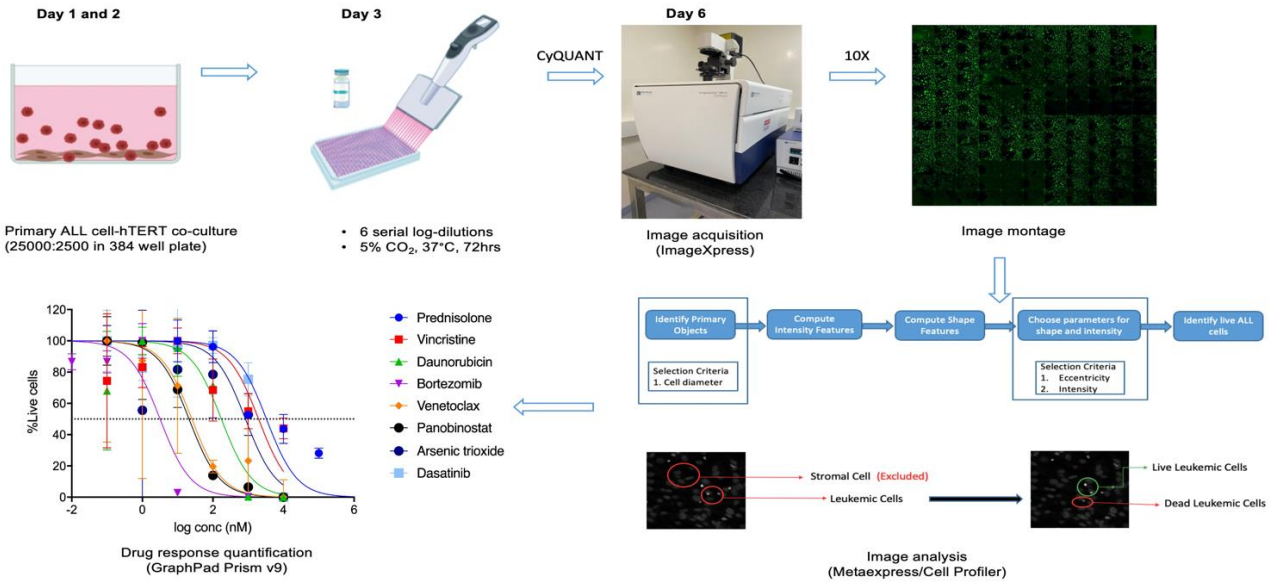


Figure 1: Schematic representation of high-throughput drug screening pipeline

ALL, acute lymphoblastic leukaemia, CO_2, carbon-dioxide

Table 1: Patient characteristics

	N
Age (years)	
Mean	8
Range	3 - 18
Sex	
Male	11
Female	6
Immunophenotype	
Precursor B	11
T	6
Risk (frontline patients)	
SR	3
IR	2
HR	9
Cytogenetics	
ETV6-RUNX1	2
HeH	3
BCR-ABL1	0
KMT2Ar	1
TCF3-PBX1	3
B-others	1
TAF15-ZNF384	1
Unknown/T	6
<0·01%	8
≥0·01%	4
Pending	2

data normalized against untreated samples (GraphPad Prism version 9).

Drug resistance profile: A drug resistance profile was made for each patient by combining the results in *ex-vivo* sensitivity to induction drugs (prednisolone, vincristine, daunorubicin and asparaginase). For each of 4 induction drugs except prednisolone, patients were classified into 3 groups as sensitive (33% lowest IC50 values), intermediately sensitive (33% intermediate IC50 values), or resistant (33% highest IC50 values) [5]. For prednisolone, these 3 groups were defined using cut-off values of 1µM and 64µM as previously reported [6]. Sensitivity toward a drug was scored as 1, intermediate sensitivity was scored as 2, and resistance was scored as 3 for each individual drug. Combining the separate scores of 4 induction drugs of each patient resulted in an individual PVAD score that varied between 4 (sensitive to all three drugs) and 12 (resistant to all three drugs).

Results: Total of 17 ALL (11 BCP ALL and 6 T ALL) patients were profiled with *ex-vivo* drug screening using cryopreserved pre-treatment diagnostic samples (ICiCLe-ALL-14 study cohort). Patient characteristics are in **Table 1**. The mean viability of the recovered ALL cells was 69% after thawing (determined using Trypan

blue exclusion assay) with mean blast percentage 78%. Median PVAD score in patients with MRD <0.01% and MRD ≥0.01% was 6 and 8 respectively. But there was no statistically significant correlation of PVAD score with MRD (Spearman correlation coefficient: r=0.6, P=0.057). This could be due to a smaller number of patients screened till now.

Drug screening was performed for an average of 7 drugs (range 4-9) per sample (**Figure 3**) and identifies different ex-vivo drug sensitivity response pattern in patient with low and high MRD. In samples of patients with MRD≥0.01% or relapsed/refractory patients, a group of drugs was found to be sensitive in drug screening (**Figure 4a**). These include venetoclax (*BCL-2* inhibitor), bortezomib (proteosome inhibitor) and panobinostat (histone deacetylase inhibitor). Dasatinib was found to be additionally sensitive in a subset of T-ALL patients (**Figure 4b**). It has been previously reported by our group that patients with T ALL having high *SRC* expression are sensitive to dasatinib[7].

Future plan:

<u>Short-term goals:</u>

- Refining and launching cell profiler-based image analysis pipeline

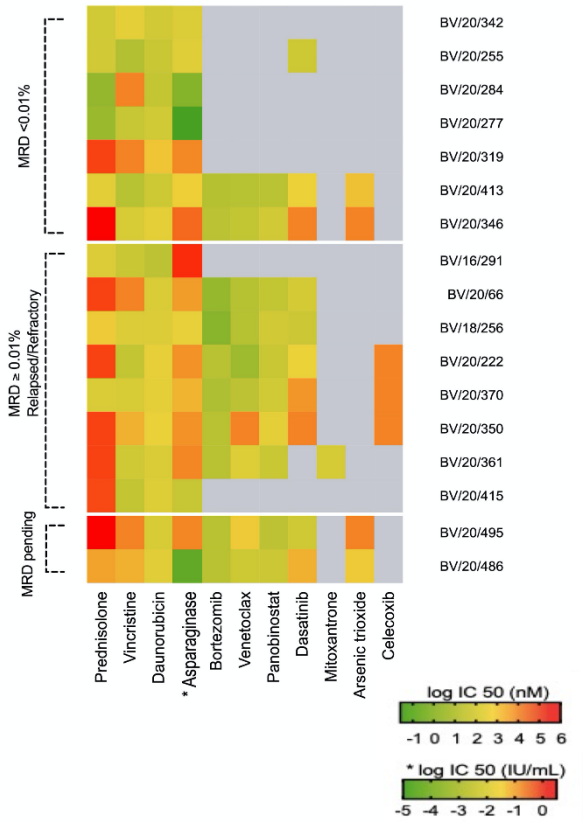
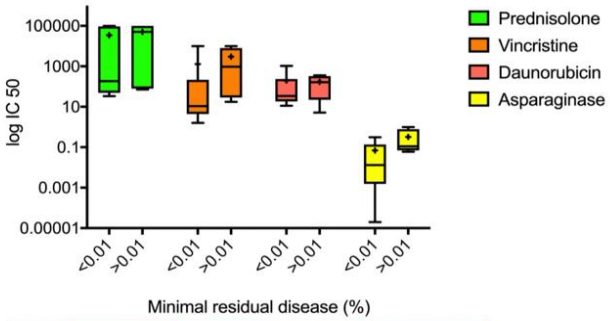


Figure 2: Heatmap showing relative ex-vivo drug response (log IC50) to 9 drugs screened across primary ALL cells of varying genomic subtypes and MRD response (N = 17). Grey boxes represent drug(s) not tested for patient. log, logarithm, MRD, minimal residual disease, IC50, half-maximal inhibitory concentration, nM, nanomolar

- Merging the image analysis pipeline with drug response quantification
- Widen the panel of drugs used for screening (use automated liquid handling system)
- Use of synergy-finder tools to identify sensitive drug combinations
- Use of multi-drug screening to identify & isolate "MRD" cells – understand mechanism of drug resistance

<u>Long-term goals</u>

- Systematic evaluation of DRP as potential strategy for HR patients in frontline therapy



	Prednisolone		Vincristine		Daunorubicin		Asparaginase	
MRD (%)	<0.01	>0.01	<0.01	>0.01	<0.01	>0.01	<0.01	>0.01
Median	0.2 µM	50 µM	11 nM	955 nM	34 nM	163 nM	0.01301 IU/mL	0.1117 IU/mL
IQR	0.05 – 93 µM	0.08 – 100 µM	4 – 216 nM	29 – 7962 nM	18 – 236 nM	22 – 329 nM	0.0015 – 0.07 IU/mL	0.07 – 0.78 IU/mL

Figure 3: Positive association of *ex-vivo* drug response to induction therapy drugs and end-of-induction MRD.
(**A**) Boxplot representation of log IC50 values for prednisolone, vincristine, daunorubicin and asparaginase against MRD categories (<0.01% and >0.01%) (N = 11). Boxes represent interquartile ranges, whiskers encompass values between the 5th and 95th percentile, horizontal bars and '+' within boxes indicate median and mean values respectively, (**B**) Inset table denotes IC50 values (median and IQR) in MRD <0.01% and >0.01% groups [Log, logarithm, MRD, minimal residual disease, IC50, half-maximal inhibitory concentration, IQR, inter-quartile range, µM, micromolar, nM, nanomolar, U/ml, units per millilitre]

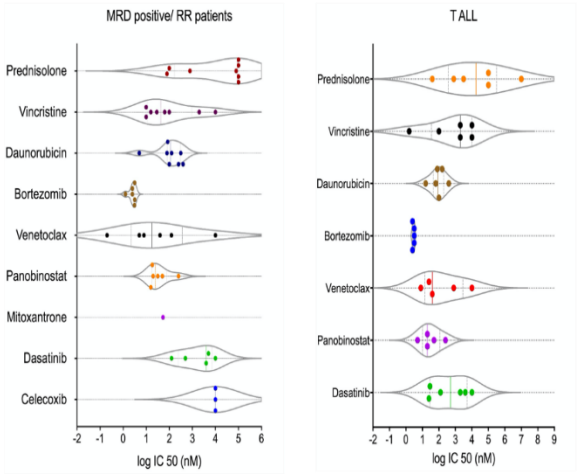


Figure 4: Distinct drug activity patterns detected for individual patients and sub-groups of interest.
(**A**) Violin plot represents drug response as log IC50 (nM) of primary relapsed/refractory and MRD >0.01% ALL patients (n = 8) tested using banked diagnostic bone marrow samples. Data represents persistent resistance to standard chemotherapy and individual sensitivity to alternate drugs. Each dot represents a patient. The vertical bars inside each violin plot denotes median (solid line) and inter-quartile range (dotted line). (**B**) Scatter plot representation of drug response of T ALL patients to alternate therapies [log, logarithm, MRD, minimal residual disease, IC50, half-maximal inhibitory concentration, nM, nanomolar, RR,

- Potential pre-treatment drug screen platform to develop phenotypic personalized therapy

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Amrita Roy
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Leukaemia Cell Biology

Welcomed 2020 on a high-spirited note. I had identified that ALL cells with *TP53* deletion were resistant to anthracyclines, most likely by downregulating expression of p53 direct targets involved in apoptosis (e.g. *FAS*, *TNFRSF10B*) and cellular stress (*TP53INP1*, *ZMAT3*) (Annual Report 2019). Excitement of returning to TTCRC and an increased vigour to maximise my academic time at Alderley Park marked my final months at University of Manchester. And then the world came to a standstill (literally!).

Self-isolation, post my return to Kolkata, was followed by an urgency to help set up the COVID-19 testing centre at Tata Medical Center. The p53 project seemed like a fanciful thought. While most of the team members were working from home, a few of us trickled into the offices and lab to work out the transition from "work-from-home" to "work-on-site" for the rest. Those were difficult 4 months, with more to learn than any experimental failure or success could have ever taught us.

Come August, with the team members back on site (some of who were new faces for me), we resumed lab work. Much to my delight, the p53 project became a reality again. The project welcomed a new member, Arko Bhowal. Two young interns, Ananya Mahadevan and Amrita Roy, courageous souls with a desire to expand their technical prowess joined the p53 bandwagon. While Amrita focused on validating results (Figure 1A) from the transcriptomic profiling of p53WT (wild-type) and p53KO (knockout) clones performed in University of Manchester in 2019, Ananya got busy establishing wild-type

TP53 in a lentiviral backbone (Figure 1B) to rescue the effects of p53 loss on downstream signaling.

While at Alderley Park, I had the opportunity to use the Agilent Seahorse extracellular flux analyser and assess the mitochondrial activity of p53WT and p53KO clones under steady state conditions (Figure 2A). We hypothesised that chemoresistance results from an altered metabolic state; a shift from glycolysis to oxidative metabolism. Evidence is mounting in favour of this hypothesis [1-5]. Exposure to sub-lethal doses of mitoxantrone revealed a small population of viable p53WT cells with enhanced mitochondrial activity (Figure 2B). The data indicates that p53KO cells are driven by oxidative phosphorylation without or with stress, whereas, some p53WT cells can switch from glycolysis to oxidative metabolism under conditions of cytotoxic stress. This metabolic plasticity implores further investigation.

Much awaits 2021. Ananya is optimising her protocol for site-directed mutagenesis to establish p53 mutants (p.G245S/R and p.R248Q/W) and introduce these in p53KO cells. Amrita plans to cross-examine the transcriptomic profile with the proteomic profile of unstressed and stressed p53WT versus p53KO and/or p53 mutated cells. Our aim is to identify pathways enriched in cells lacking functional p53 that can explain the chemoresistant phenotype of p53 altered cells. Arko faces an uphill task. He has to demonstrate that increased

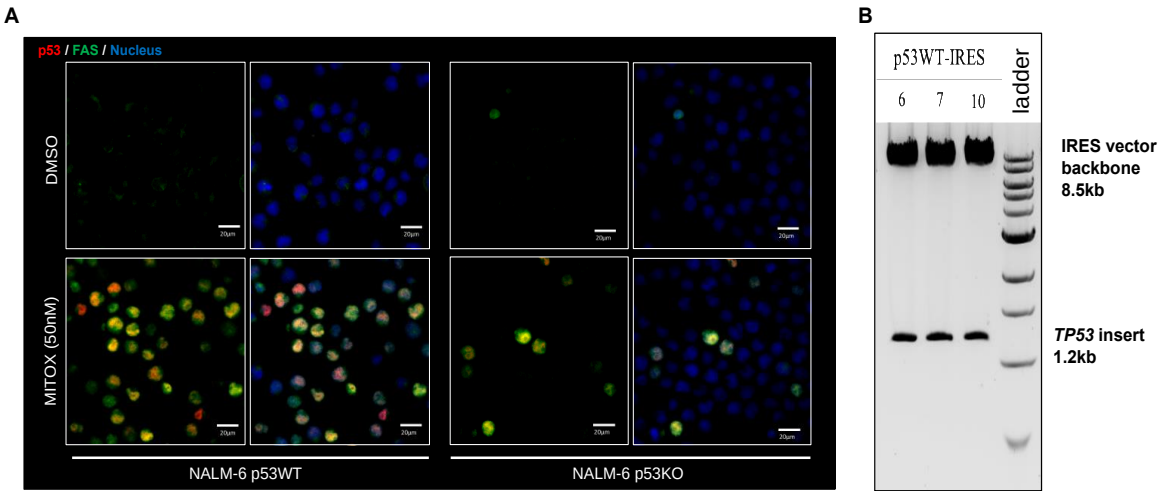

Figure 1: Establishing tools to study p53's role on stress adaptation.
A: p53 was deleted in ALL cells with wild-type p53 (NALM-6) using CRISPR-Cas9. Mitoxantrone-treated p53WT cells co-expressed FAS and p53. p53KO cells failed to elicit expression of both FAS and p53, validating the loss of *TP53,*
B: Wild-type *TP53* was cloned into IRES lentiviral backbone. Clone #7 will be used to rescue the effect of p53 functions. WT, wild-type; KO, knockout; MITOX, Mitoxantrone.

mitochondrial activity marks chemoresistant cells. His aim is to identify a common mechanism of stress adaptation involving mitochondrial respiration. Overall, we aim to establish a p53-regulated expression profile in conjunction with the ability to adapt to stress to identify patients likely to fail conventional chemotherapeutic protocols before starting therapy. We expect our work to identify novel processes of drug resistance with the potential of developing cheaper and less toxic therapies.

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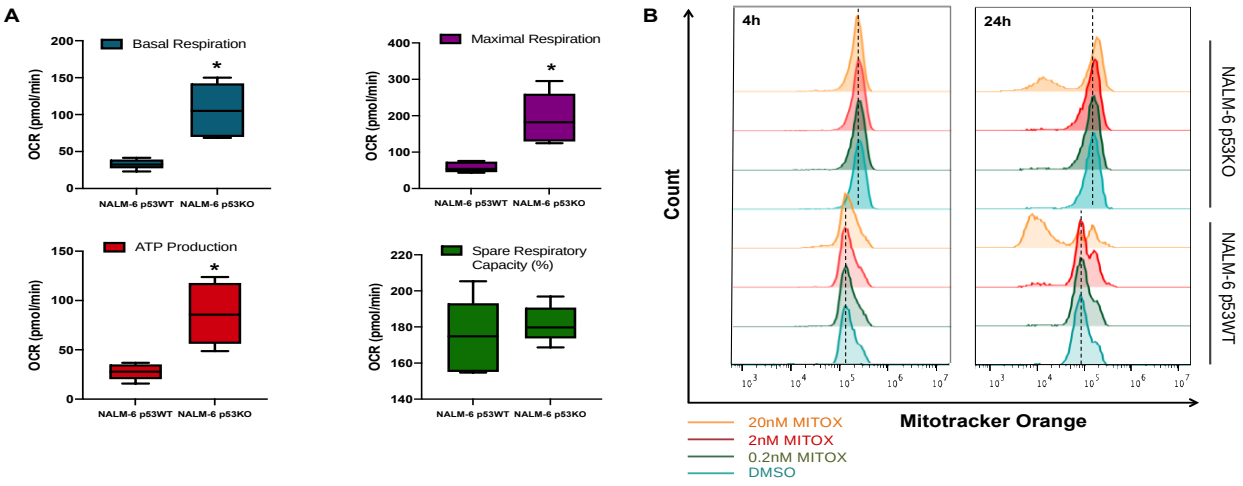


Figure 2: Loss of wild-type p53 increases mitochondrial activity in ALL cells *in vitro*.
A: Under steady-state conditions, NALM-6 p53KO cells exhibit high mitochondrial activity in terms of basal respiration, maximal respiration and ATP production in comparison to p53WT cells,
B: p53KO cells without or with Mitoxantrone treatment demonstrate higher mitochondrial activity (dotted line) in comparison to p53WT cells. Mitochondrial membrane potential (MMP) as a measure of mitochondrial activity was assessed by Mitotracker Orange staining (n = 2, in triplicates). OCR, Oxygen consumption rate; WT, wild-type; KO, knockout; MITOX, Mitoxantrone. * denotes p = 0.05.

TCS at TTCRC

Amit Saxena
Head
Genomics and Translational Research

Anju Goel
Head
Translational Research Platform

Binuja Verma
Principal Investigator
Genomics and Translational Research

TCS is contributing to accelerate research initiatives by deploying platform and solutions for Translational Research. TCS is also collaborating with TTCRC on projects related to Knowledge Graph based insight generation and Imaging Analytics. TCS solutions also support multi-center clinical trials and analytics of data from EMR and other systems.

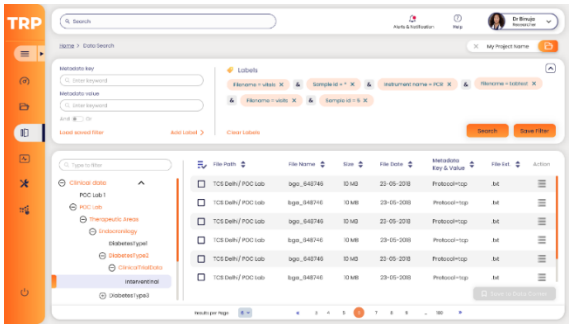
The Translational Research Platform has undergone a technology refresh. It is now cloud based with new and improved features. The UI is more intuitive to the needs of scientists and researchers. Scalability of the platform is enhanced for large volumes of data storage and processing. Some of the key features include Project management, Metadata based Data Search, Data Corner, Tools Repository and Analytics Corner.

Translational Research Platform Features:

Data Search

Users will be able to search for files based on metadata key value pairs e.g., file type, name, owner, Ext. etc. using the platform feature. Users can access files based on hierarchy/folder structure in data search.

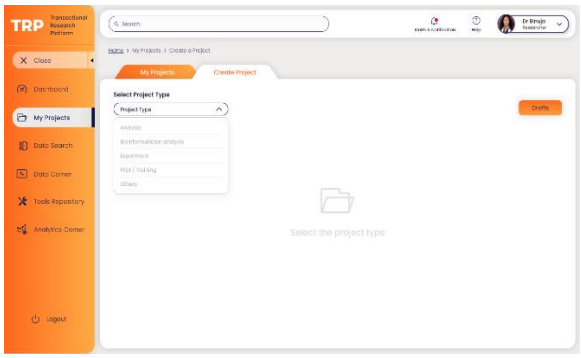
Data Corner



Users will be able to view & select platform files and save them into Saved Data tab to be used further. Users can upload his/her own files in My data for processing and further analytics.

My Projects

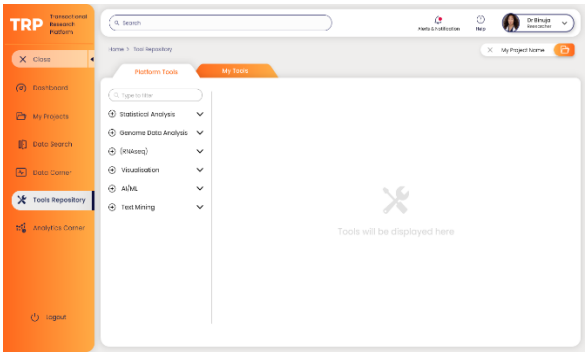
Users can define projects and capture project related information. Users will be



able to access My Projects in the platform to organize and manage the data files and perform related analysis within a workspace. Each workspace can have multiple projects in it.

Tool Repository

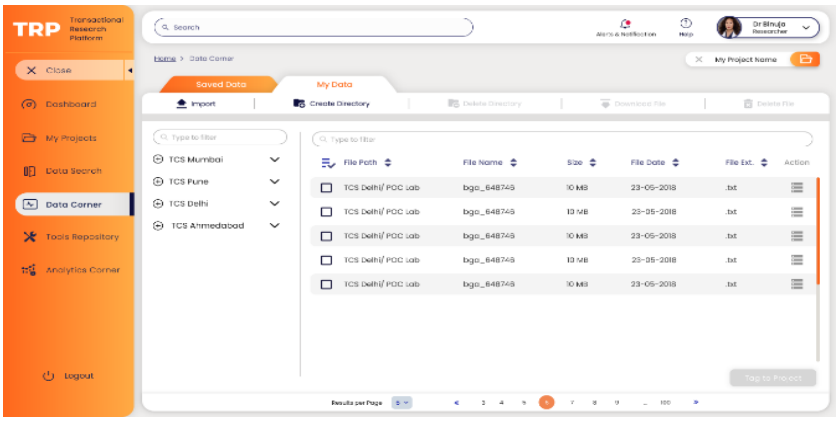
Users will be able to view and select tools for Analysis based on roles/permissions.



Analytics Corner

Users will be able to create workflow/pipelines in Analytics Corner in the platform to perform analysis using CLI (Command Line Interface) tools. Users can launch Notebook in Analytics Corner for coding in R/ Python.

Fig: Translational Research Platform – Data Corner



Projects and Initiatives:

High-throughput imaging-based ex vivo drug screening to identify anti-tumour drug combinations in real time

We have initiated an Imaging Data Analytics project with the Researchers at TTCRC. The project aims to develop Imaging Data Analytics workflows and algorithms to analyze high throughput images generated for ex vivo drug screening to identify anti-tumour drug combinations in real time in ALL patients. Aim is to develop an image processing application which is able to detect the live cells present post drug infusion into the culture. These cultures are imaged on different days to understand the efficacy of various drugs on the cancer cells. The amount of data which gets generated per patient is quite huge (around 20GB per patient) as different drugs are tried on the culture in high-throughput drug screening machine. Current image analysis software in use does not have the necessary accuracy and is not robust as it is using intensity and shape features only.

Use of Knowledge Graphs for Exploratory Analysis

Assess comparative effect of chemotherapy on patients with and without UPD in high Hyper- diploid ALL patient's Initial analysis dataset has 25 UPD patients and 33 non-

UPD patients. Out of this, RNA-sequencing has been performed for 28 patients (13 UPD and 15 non-UPD patients). Top 97 upregulated and 80 downregulated genes in patients with UPD were identified by PI. Our primary objective is to use knowledge-graph based approach to identify significant gene-disease relationships in these patients for prioritizing expressed proteins as probable targets. Some of the public databases used for creation of the knowledge graph include data from UCSC, Uniprot and Reactome among others.

ICiCLe Trial website development:

The ICiCLe ALL-14 Trial Website was developed to reach patients and their families, health care professionals, researchers, and the public on a wider scale so that they can have easy access to information on the study.

The website focuses on all the aspects of the treatment protocol being used to treat the patients with ALL and therefore helps us to understand the benefits of this multicenter randomized trial. This study website also answers many of the queries which are being frequently raised by the patients and other people and moreover gives us a glimpse about the collaborating centers as well as the funding bodies associated with this trial.

The website can be accessed via the TTCRC homepage at https://icicle.ttcrc.org/

Pretrial Cost Analysis:

Evaluation of impact of risk stratified therapy on direct treatment costs in patients with ALL treated at the hospital. Calculation and Analysis of Direct Medical Cost of Treatment on ICiCLe Protocol - Assessment in the Pre-trial cohort of Tata Medical Center Kolkata. Some of the data extracted include:

1. IP Cost, OP Cost, Overall Cost as per various categories of billing.
2. Length of stay, ICU Admissions, IP Admissions, Emergency ward admissions data.
3. Discharge summary data, First admission and discharge dates for patients, Bifurcation of each category and subcategories of billing.

Integrated Data Management for ICICLE trial:

ICICLE is the first clinical trial created on IDM for TTCRC. This is a multicenter trial to create treatment strategy for Acute Lymphoblastic Leukaemia (ALL), which is the most common cancer of childhood. This clinical study will help to improve outcome of clinical treatment. Clinical DB is set up as per complex requirements to collect, review and analysis of data for patients enrolled for study. We have supported to resolve technical challenges faced by users and to improve user experience. There is a data for 2000+ patients entered in IDM and DB is capable

to handle data with stability. We will support to meet upcoming clinical study milestones.

The Consolidated dataset (consisting of more than 300 columns) was created by combining data related to Registration, Risk Stratification, Study Consent forms along with details of End of Induction, End of Consolidation, Randomization 1, Eligibility for SR, Randomization 2 as well as Death, Relapse and Trial Withdrawal forms from CTMS. Since this complex report consists of information of multiple forms combined, it makes easy for clinicians to analyze the data in a consolidated manner rather than looking across data separately for each of those forms.

r-ALL1 on IDM

This is the second clinical study built on IDM. This study will assess the feasibility of a uniform strategy to manage first relapse ALL and evaluate event free survival with this protocol. This study is built on IDM. There were additional complex requirements added by the study team after study build to reduce errors and to generate quality data for patients enrolled. We have collaborated extensively with programming team to explain, plan, and implement changes in the database. These approaches will help to get clinical data generated with high accuracy and reduce cycle time for review and analysis of data.

Translational Study in Breast Cancer: Markers and tools for the prediction of response to radiation therapy /or sensitivity

Collaborators: Dr. Sanjoy Chatterjee, Dr. Rosina Ahmed

The ongoing project is a part of **HYPORT Trial** which aims to develop markers and tools for the prediction of response to radiation therapy /or sensitivity in breast cancer patients undergoing Hypo-Fractionated Radiotherapy Schedule of 35GY in 10 Fractions in advanced incurable Breast Cancer

Summary

As radiation therapy is a key modality in the treatment of cancer, it is of tremendous importance to increase our understanding of the molecular pathogenesis of radiotherapy toxicity. This will lead to find ways of predicting those patients likely to suffer with long term side effects and to develop new approaches for their management. The field of radio genomics is expanding with evidence of genetic polymorphisms underlying inter-individual differences in radiotherapy responses. The effective response to ionizing radiation (IR) exposure is complicated by biological heterogeneity, as certain patient tumors may be inherently more insensitive to a given dose of IR. We are working in developing markers and tools for the prediction of response to radiation

therapy/or sensitivity in breast cancer patients undergoing a specific protocol of Radiation therapy. This is a retrospective study design using bio banked Fresh Frozen and FFPE samples, with the aim to identify the genetic markers of clinical toxicity and investigate the clinical response of breast cancers to hypo fractionated course of radiation therapy. Currently exome sequencing data is being generated in a pilot set of samples (pre and post treatment) which would be analyzed for potential genetic alteration that could be correlated to clinical toxicity and outcome of therapy. We would also attempt to build a pan cancer analysis of possible molecular and cellular mechanisms contributing to radiation toxicity and response. This is to gain a deeper understanding of mechanisms of radiation response and thereby the clinical outcome as it is hypothesized based on the contribution of different factors including genetic aberrations, epigenetic alterations, changes in the response to cellular signaling, metabolic alterations, and beyond. This can be done using publicly available data like from TCGA, IGCA etc. integrating multi-omics data comprising of whole exome sequencing, transcriptome, epigenome and proteomics.

Current Status

We have initiated the genome sequencing project with the objective to analyze and identify the genetic variations associated with clinical toxicity and treatment response. The pilot phase of the study is being completed. In this phase we have standardized and established the experiment and analysis pipeline for exome sequencing using DNA extracted from a subset of FFPE study samples (N=12/fine needle biopsy samples). The SOPs and protocols for the wet lab methods have been developed. Currently bioinformatics pipelines for analyzing WES data is being standardized.

NGS data Analysis

A. Variant Calling

NGS analysis for variant calling (SNVs/indels/CNVs) have been completed.

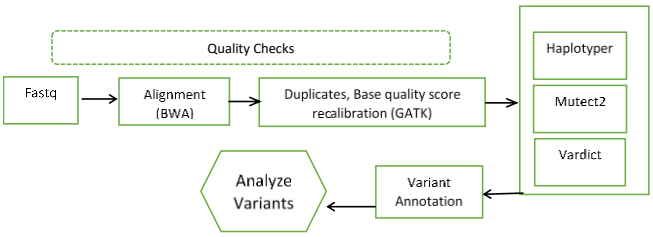


Fig1: Overview of Variant calling pipeline

B. Variant Annotation and filtering

Method is being finalized.

Next Steps

To complete the data Analysis

Correlate genetic variation with clinical data (clinical outcomes)

System Medicine Cluster (SyMeC)

Geetashree Mukherjee
Senior Consultant
Histopathology

ORAL CANCER RESEARCH

The project is in collaboration with the National Institute of Biomedical Genomics (NIBMG), Kalyani, and funded by the Department of Biotechnology, Govt of India, New-Delhi.

PI Geetashree Mukherjee, CO-PI Vivek Radhakrishnan, Co-Investigators: Deepak Mishra, P. Arun and Joydeep Ghosh.

The study investigates the relationship between genomic alterations and tumor immune microenvironment in oral squamous cell carcinoma – gingivo buccal (OSCC-GB) with the aim to identify prognostic and predictive biomarkers. The Primary Objective is to determine whether the burden of genomic/epigenomic alterations in the tumor correlates with immunological diversity in treatment naïve, OSCC-GB patients.

Methodologies being used at Tata Medical Center (TMC) are a) Immunohistochemistry (IHC) and b) Flow Cytometry (FACS).

IHC: On 124 cases have been performed for 40 markers.

FACS: 40 samples (Tissue) & 40 samples (Blood) have been performed till date. Rest of the samples could not be processed or were incomplete due to inadequate number of cells. Experiments are ongoing with more samples.

RESULTS TILL DATE - LIKELY TO CHANGE AS THE STUDY IS ONGOING. SEQUENCING IS BEING DONE AT NIBMG, THE RESULTS OF WHICH WILL BE INTEGRATED.

Summary till date :

- SCC-GB - Hot or Altered Immune (98.38%)
- Consistent expression of PDL-1 (87.096%)
- Over all picture is that of “Exhausted T lymphocytes”.

Probable mechanism of immune escape of tumor cells is predominantly by expressing PDL-1.

Other possible mechanisms of immune escape:

- IDO & COX2 } High IDO-1 and COX-2 expression in the tumour cells are thought to facilitate T cell anergy and immune escape.
- Hypoxia - The HIF 1 α expression in tumour cells was consistent in each sub-group of CD8. (both IM & CT); provide a motivation to observe the HIF 1α association with PDL-1 expression at metabolomics and RNA Seq level.

There is a good level of concordance betweee Flow data and IHC data.

Publications:

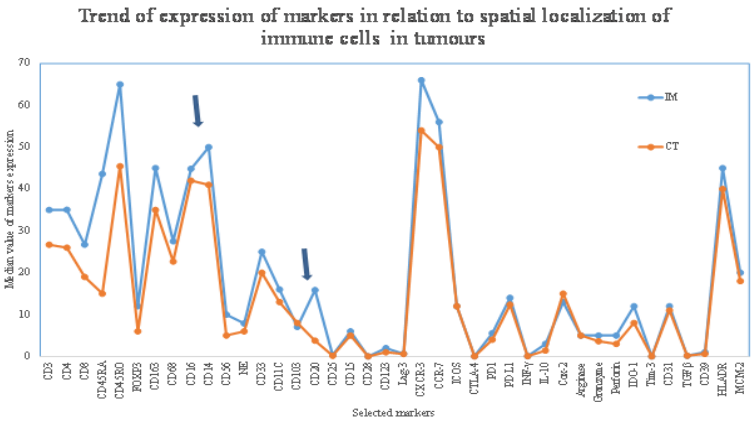
1. Mukherjee G, Bag S, Chakraborty P, Dey D, Roy S, Jain P, roy P, Soong R, Majumder PP, Dutt S. Density of CD3+ and CD8+ cells in gingivo-buccal oral squamous cell carcinoma is associated with lymph node metastases and survival. *PLoS ONE.* 2020;15(11): e0242058. DOI: 10.1371/journal.pone.0242058
2. Chaudhary A, Bag S, Arora N, Radhakrishnan VS, Mishra D, Mukherjee G. Hypoxic Transformation of Immune Cell Metabolism within the Microenvironment of Oral Cancers. *Frontiers in Oral Health.* 2020; 1:585710. doi: 10.3389/froh.2020.585710.
3. Chaudhary A, Bag S, Mukherjee G. MALDI-MS Molecular Imaging for Cancer Metabolomics: An Emerging field for Research and Personalized Medicine. *European Journal of Pharmaceutics and Biopharmaceutics*. In Review.

Clinical and translation study on cervical cancer

Tata Medical Center is the clinical nodal center of this consortium with NIBMG, IICB, IISER, Bose Institute and ISI and is funded by Department of Biotechnology (DBT) from 2017 till 2021.

SyMeC cervical cancer screening study (CSS)

Aims and Objectives: Identify biomarkers for HPV persistence in screened positive women and help develop a triage strategy for colposcopy and treatment for precancerous lesions.



A screening cohort of 2502 women was recruited from Jan 2018 - Sept 2019 by primary HPV testing and Qiagen HC2 Hybrid Capture II test detected 253(10%) women positive for HPV infection. These women were further evaluated by colposcopy to detect precancerous lesions caused by HPV. In women (89%) who underwent colposcopy and directed biopsy, we identified 46% women with no HPV related lesions, 44% women with low grade HPV infection – koilocytosis and CIN 1, and 9.2% women with high grade infection – CIN 2 & 3. To help formulate a triaging strategy after primary HPV screening, women with low grade lesions were followed after 1 year with a repeat HPV test; women with CIN2 were followed for 6 months before undergoing treatment and women with CIN3 underwent treatment immediately. This protocol would identify women with persistent HPV infection and those with progression of CIN2 lesions. These clinical and biomarkers parameters will provide information that will help stratify colposcopy referrals in order to improve detection of high-grade lesion and prevent overtreatment of low-grade lesions. The clinical parameters with regards to cervical biopsy and high-risk HPV genotypes of the HPV positive women have been generated (Table 1&2). The follow-up of these patients is ongoing to identify women who have "persistent" HPV infection and those who have "cleared" the HPV infection on subsequent visits. We intend to analyze further the cervical scrapes and blood of these women to identify the reason for persistence and clearance of HPV infection which plays an important role in the development of cervical cancer.

Screen and treat approach	No. of colposcopies	Normal	K'cytosis	CIN1	CIN 2&3	PPV HSIL	PPV for all lesions
HPV primary screening	253	95	28	73	21	8.3%	48.2%
HPV primary screening + HPV 16/18 triage	86	31	11	25	10	11.6%	53.4%
HPV primary screening + HPV 16/18/31/33/45/52/58 triage	129	47	17	38	12	9.3%	51.9%

Table 1: Stratifying colposcopy referrals

HPV genotype	Number of women with HPV subtypes	Normal	Koliocytosis	CIN1	CIN2	CIN3	CIN2& CIN3	PPV CIN2/3
ANY HPV	253	95	28	73	17	4	21	8.30%
HPV 16	54 (21.3%)	20	6	15	5	1	6	11.10%
HPV 18	37 (14.6%)	13	5	11	4	1	5	13.51%
HPV 31	11 (4.3%)	5	2	2	1	0	1	9.09%
HPV 33	7 (2.7%)	0	2	3	0	0	0	0
HPV 35	4 (1.5%)	1	0	2	0	0	0	0
HPV 39	4 (1.5%)	2	1	1	0	0	0	0
HPV 45	10 (3.9%)	5	1	3	0	1	1	10%
HPV 51	22 (8.6%)	8	0	4	2	2	4	18.18%
HPV 52	14 (5.5%)	3	1	7	0	0	0	0
HPV 56	20 (7.9%)	11	1	2	4	0	4	20%
HPV 58	27 (10.6%)	9	4	8	2	1	3	11.10%
HPV 59	9 (3.5%)	5	0	1	3	0	3	33.33%
HPV 68	39 (15.4%)	12	4	17	0	0	0	0
HPV 16 & OTHERS	22 (8.6%)	10	0	8	2	0	2	9%
HPV 18 & OTHERS	11 (4.3%)	2	3	4	1	1	2	18.20%
Non 16/18 HPV TYPE	21 (8.3%)	9	3	3	2	0	2	9.50%
UNDETERMINED	67 (26.4%)	25	8	18	4	0	4	5.90%

Table 2: HPV genotypes and cervical biopsies

SyMeC translational cervical cancer study (TCS)

Aims and Objectives: Identify biomarkers of treatment failure to standard chemo radiation therapy, identify possible biomarkers and radio sensitizers that can reduce this failure and recurrence of cervical cancer.

A cohort of 190 women with cervical cancer were recruited to facilitate translational work at NIBMG and IICB to identify genomic and immune signatures/biomarkers that would be correlated to their outcomes to treatment and survival status. The analysis is ongoing and will be available next year. A subset of these cancer patients will have their functional imaging by MRI and PET CT to analyze the parameters such as ADC texture, diffusion and metabolic parameters. This is likely to help with improved diagnostic performance for assessing high- grade cervical carcinomas, parametrial invasion, lymph node metastasis, stages III–IV, and recurrence relative to the performance of using ADC values. This analysis and follow-up of patients is ongoing till 2021-22.

Team Composition:

PI: Dr Mammen Chandy ; Co-PI: Dr Jaydip Bhaumik, Dr Sanjay Bhattacharya, Dr Geethashree Mukherjee, Dr Divya Midha, Dr Santam Chakraborty , Dr Saugata Sen, Dr Aditi Chandra, Dr Sumit Mukhopadhyay, Dr Soumendranath Ray, Dr Jayanta Das; Project Consultant: Dr Sonia Mathai;Project Staff: Shrabanti Sarkar Ghosh, Barnali Ghosh, Anuradha Biswas, Manali Mukherjee, Ajit Mukhopadyay, Anamika Palit, Kasturi Das, Sona Chowdhary, Thumpa Das, Priya Hati, Rama Gupta, Shyamali Mondal, and Dr Ratnaprabha Maji.

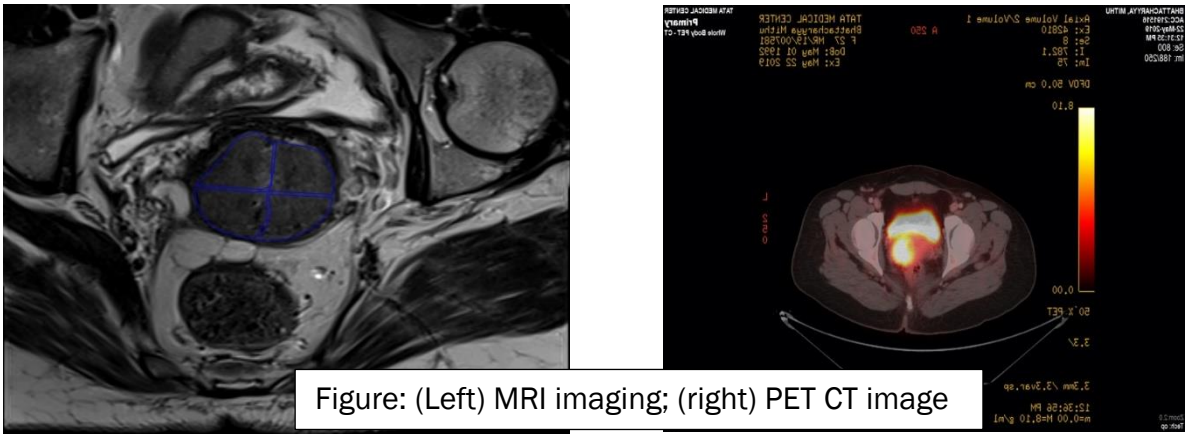


Figure: (Left) MRI imaging; (right) PET CT image

Clinical Hematology Oncology and HCT

Vivek Radhakrishnan

Senior Consultant

Clinical Hematology and BMT

FACULTY

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Dr. Rizwan Javed *MD PDF MSc* [Apheresis & Cryopreservation]

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Ms. Disha Pyen

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Ms. Pranita Mishra

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Ms. Sutapa Chatterjee

Ms. Arna Das

Ms. Sainee Roy

Prof. Dr Reena Nair *MD*

Dr. Vivek S. Radhakrishnan *MD, DM, PDF, MSc*

Dr. Saurabh Bhave *MD, PDF*

Dr. Jeevan Kumar Garg *MD, DrNB, PDF*

Dr. Arijit Nag *MD MRCP(UK) DM PDF*

Ongoing Investigator Initiated Translational Projects

1. Acute Myeloid Leukemia, ALTITUDE Study

- Peer Reviewed Grant funding by Tata Education and Development Trust
- Joint Institutional Collaborators: Laboratory Hematology, Molecular Pathology, Cytogenetics and Microbiology
- PI: Dr. Vivek Radhakrishnan
- To establish a Precision oncology work platform at Tata Medical Center in patients with Acute Myeloid Leukemia (AML) and Myelodysplastic Syndrome with excess blasts (MDS-EB). Multimodal-omics based comprehensive genomic Characterization of a uniformly treated cohort of AML patients,

accompanied by MRD assessments.

- and SOPs. The enrollment of the patients is ongoing, and as for now 30 AML patients were screened, out of which 19 were recruited in the study. Biobanking is being carried out for Bone marrow aspirate (mutational profile and MRD) and faecal-oral microbiome samples at different time points (as per the study protocol).
- For the gene mutation profiling and MRD assessment, targeted sequencing panel and cancer database are being developed. Standard Bioinformatics pipeline for data analysis has been developed and is undergoing validation. With the in-principle approval of a related grant, the immune cell profile at defined timepoints will be assessed by two distinct methods of estimating the immunoscore viz., immunohistochemistry (IHC) with Digital Image Analysis and Nanostring Digital Spatial Profiling using Tissue Microarray.

2. **CLARION Project: Academia Industry Collaboration to establish a Cellular Immunotherapy Program for Cancer patients.**

- **We have established the** infrastructure, manpower, protocols
- Peer-reviewed Grant funding approved in-principle: Department of Biotechnology, BIRAC
- Industry partner: Intas Pharmaceuticals, Ahmedabad
- Study Chair: Prof. Mammen Chandy
- Principal Investigators: Dr. Vivek Radhakrishnan (TMC) and Dr. Lakshmikanth Gandikota (Intas)
- The goal of this study is to establish a clinical grade Cellular Therapy (specifically CAR-T Cell manufacturing) and a clinical administration facility at Tata Medical Center Kolkata, and further facilitate the clinical development of indigenous CAR-T cell and other cellular immunotherapies.
- Infrastructure development ongoing: This will establish a and commission a cGMP facility is ongoing.
- After full funding received equipment and clinical trial/regulatory submissions will begin

3. Immuno-Oncology: GIFT Study

- Peer-reviewed Grant funding through Department of Biotechnology Systems Medicine Cluster project for cancers of Cervix

and Oral cavity (Gingivo-buccal sulcus)

- PI: Dr. Geetashree Mukherjee
- Co-PI: Dr. Vivek S Radhakrishnan [Immuno-oncology component]
- Genomic Immune profile of Tumour-Excavating the relationship between genomic alterations and tumor immune microenvironment in oral squamous cell carcinoma – gingivo buccal (OSCC-GB) to inform immunotherapy.

Ongoing Investigator Initiated Prospective Clinical Trials

1. **PRIME STUDY:** Effect of Pomalidomide Bortezomib- Dexamethasone induction on MRD status in patients with newly diagnosed Multiple Myeloma.

- PI: Dr. Vivek Radhakrishnan/ Dr. Jeevan Kumar
- To determine the activity of a combination of Pomalidomide-bortezomib dexamethasone as initial therapy in NDMM, by assessing response using MRD assessment.
- Patient recruitment ongoing.

2. **R-BED Study**- Phase II study of Bortezomib, Etoposide, Dexamethasone combination therapy, with or without Rituximab, in Adult Relapsed or Refractory, B-cell Acute Lymphoblastic Leukemia who are transplant ineligible.

- PI: Dr. Vivek Radhakrishnan/ Dr. Arijit Nag
- To determine the safety and activity of a combination therapy containing Bortezomib, Etoposide and Dexamethasone, with or without Rituximab, in relapsed refractory adult patients with B-ALL.
- Patient recruitment and analysis ongoing

3. **RIC-FT10***:* Reduced Toxicity and Reduced Intensity conditioning regimen using Fludarabine and Treosulfan for high-risk hematological malignancies undergoing allogenic hematopoietic cell transplantation.

- PI: Dr.Vivek S Radhakrishnan/ Dr. Saurabh Bhave
- To evaluate the toxicity profile of Fludarabine and Treosulfan conditioning regimen as reduced intensity and reduced toxicity conditioning therapy in high risk hematological malignancies. To determine the 100 day regimen related toxicity.
- Patient recruitment ongoing.

Ongoing Investigator Initiated Registry Studies and Projects

1. National Lymphoma Registry Project- Database formation of details of lymphoma patients in ONCOCOLLECT software. [PI: Prof. Reena Nair]

2. National CML Registry Project- Database formation of details of CML patients in ONCOCOLLECT software. [PI: Dr. Vivek Radhakrishnan]

3. Institutional Acute Myeloid Leukemia and MDS Clinical database and cost-analysis outcomes project [PI: Dr. Vivek S Radhakrishnan]

4. International T-cell Lymphoma Project v2.0 [PI: Prof. Reena Nair]

5. CRIMSON Project- Database formation of details of cancer patients receiving immunotherapy and precision medicine in Tata Medical Center [PI: Dr. Vivek S Radhakrishnan]

6. CIBMTR Registry [CIBMTR (Center for International Blood & Marrow Transplant Research)], Hematopoietic Cell Transplantation and Cellular Therapies) [PI: Dr. Vivek S Radhakrishnan]

7. National IMAGE Study- Multiple Myeloma [PI: Dr. Jeevan Kumar].

Ongoing Pharma Sponsored Registry Studies:

1. Lymphoma: RITUXIMAB generic (Reditux):- Promise Registry to compare Effectiveness, Safety, and Resource Utilization of Reditux (Rituximab) vs. the reference Medicinal product to treat Diffuse Large B –Cell lymphoma and Chronic Lymphatic Leukemia in Routine Clinical Practice . The enrolment of patient for this study is completed and follow-up ongoing. [PI: Prof. Reena Nair]

2. Lymphoma: RITUXIMAB generic (Mabtas) :- A multi-center, observational, data collection registry study to monitor the routine clinical use of MABTAS in Indian patients". The enrolment of patients for this study is ongoing. [PI: Dr. Saurabh Bhave]

Ongoing Pharma Sponsored Clinical Trials: Ongoing/ Approved

1. A Phase 1 Study(SPARC): to Determine Safety, Tolerability, Pharmacokinetics, and Activity of K0706, a Novel Tyrosine Kinase Inhibitor (TKI), in Subjects with Chronic Myeloid Leukemia (CML) or Philadelphia Chromosome Positive Acute Lymphoblastic Leukemia (Ph+ALL) Protocol No.: CLR_15_03 (Dr. Vivek S Radhakrishnan).

2. A phase 3(BOSTON): randomized, controlled, open label study of selinexor, bortezomib, and dexamethasone (svd) versus bortezomib and dexamethasone (vd) in patients with relapsed or refractory multiple myeloma (rrmm) karyopharm. Protocol No.: KCP-330- 023 (Dr. Jeevan Kumar).

3. Clinical Outcomes of CLL and MCL patients treated with Ibrutinib: An Observational retrospective medical chart review from India that may require exchange of certain information that is confidential and proprietary in nature. (Dr. Vivek S Radhakrishnan).

4. DARZALEX: A Prospective, Single-Arm, Multicenter, Pragmatic Phase-IV Trial Investigating Safety and Effectiveness of

DARZALEX (Daratumumab)In Indian Subjects With Relapsed and Refractory Multiple Myeloma, Whose Prior Therapy Included a Proteasome Inhibitor and an Immuno modulatory Agent. (Dr. Vivek S Radhakrishnan).

5. A randomized, double-blind, placebo controlled phase III multi-center study of azacitidine with or without MBG453 for the treatment of patients with intermediate, high or very high risk myelodysplastic syndrome (MDS) as per IPSS-R, or Chronic Myelomonocytic Leukemia-2 (CMML-2).

6. Clinical Outcomes of CLL and MCL patients treated with Ibrutinib: An Observational retrospective medical chart review from India that may require exchange of certain information that is confidential and proprietary in nature. (Dr. Vivek S Radhakrishnan).

Closed/Completed Studies:

1. A Phase 2, Open-Label Randomized Trial Evaluating the Efficacy and Safety of Two Dosages of Once Daily Oral CA-170 in Patients with Selected Relapsed Advanced Tumors (ASIAD). Protocol No.: CA-170-201. (Dr. Vivek S Radhakrishnan).
2. A Randomized, Double-blind, Multicenter, Multi-national Trial to Evaluate the Efficacy, Safety, and Immunogenicity of SAIT101 Versus Rituximab as a First-line Immunotherapy Treatment in Patients with Low Tumor Burden Follicular Lymphoma Protocol No.: AGB002. (Dr. Vivek S Radhakrishnan).
3. A Phase 2b Open-Label Study of Selinexor (KPT-330) in Patients with Relapsed/Refractory Diffuse Large B-Cell Lymphoma. Protocol No: KCP-330-009. (Dr. Vivek S Radhakrishnan).
4. Safety and efficacy study of Azadine® (Azacitidine): in treatment of myelodysplatic syndrome in indian patients. Protocol No.: 484-14. (Dr. Vivek Radhakrishnan).

Ex vivo models to dissect pathogenesis and improve outcomes in gallbladder cancer

Anindita Dutta
Lead – Cell Biology

Dwijit Guha Sarkar
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Post Doctoral Fellow

Ankita Dutta
PhD Student

Shinjini Chandra
Research Assistant

Srijita Sen
Intern

Aparajita Das
Intern

This project was conceptualised in April, 2019. The aim is to understand why certain patients get gallbladder cancer and identify alternative therapeutic modalities. Due to its rarity in most part of the world, there is a lack of a representative model to study gallbladder cancer pathogenesis. In 2019, the team trained at the University of Cambridge in developing *ex vivo* organoid models. Subsequently, a dedicated research facility at TTCRC has developed patient derived organoid models of gallbladder and gallbladder cancer. The focus is on developing robust, representative, reproducible *ex vivo* model systems for fundamental research and high throughput drug discovery.

TMC Team Members

Manas Roy	(Surgery)
Sudeep Banerjee	(Surgery)
Saugata Sen	(Radiology)
Paromita Roy	(Pathology)
Mohandas Mallath	(GI)
Joydeep Ghosh	(Medicine)
Shrikant Atreya	(Palliative)

Past Member

Jaydeep Das

The Team: This is a multidisciplinary collaborative programme of research. At

the Tata Medical Centre, the clinical team includes the Hepato-biliary Surgeons, Digestive Disease, Medical Oncology, Palliative Care Unit, Radiology and Pathology. CRU works with the clinical and research team to follow up and track patients reporting to the clinic and undergoing treatment. TiMBR collects and processes samples from patients consented for the study. At TTCRC, DGS, SC and AkD are involved in processing clinical materials and developing *ex vivo* models. MP is engaged in creating resources to understand the molecular mechanisms of disease pathogenesis. AkD is in a PhD programme, jointly supervised by TTCRC and IIT-KGP, focusing on organoids models of gallbladder cancer. Analytical processes include integrated cell biology, genomics and proteomics analysis available at TTCRC. Jaydeep Das, involved in developing biomaterial-based scaffold for GBCa cells, has moved to pursue a PhD with IIT-Bombay and Monash University focusing on biomaterial-based culture of CAR-T cells.

Background:

While gallbladder cancer (GBCa) is rare in most parts of the world, North, East and North-East India with Pakistan have among the highest incidences internationally. It is uncommon in other parts of India. At Tata Medical Center, ~300 patients are diagnosed with GBCa per year. Due to its rare presentation in the high-income countries, limited chemotherapeutic interventions have been tested for GBCa. Outcomes are poor worldwide with a median survival of 6 months after diagnosis.

GBCa is more common in women and obese individuals. Geographical locations suggest a genetic predisposition. Gallbladder (GB) stones are implicated in the pathogenesis, although <3% of patients with GB stones develop GBCa. *Salmonella* spp. colonise the GB, are cytotoxic to human gallbladder cells and have been identified in GBCa samples.

Table 1

Reagents	Purpose
Williams E media without phenol red	Keep tissue moist
Rock inhibitors	Prevent cell death
EGF	Prevent cell death
b-mercaptoethanol	Maintain RNA integrity
Fluconazole	Antifungal
Amphotericin B	Antifungal
Penstrep	Antibiotic
Primocin	Antibiotic

Other risk factors associated with GBCa include type 2 diabetes, deficiency of zinc and selenium, exposure to copper, cadmium and other heavy metals, such as, arsenic.

The majority (>90%) of GBCa is adenocarcinoma. Of the ~1200 mutated genes identified, mutations of *TP53*

(*mutTP53*) are the most frequent. Activating *KRAS* mutations (e.g. KRAS G12D), are regularly (18%) found in Japanese patients, however, less frequent (7%) in Indian patients. Recently mutations in the *ELF3* gene have been identified in Indian patients with GBCa.

Aims:

(i) Develop preclinical models of gallbladder cancer pathogenesis.

(ii) Identify potential prognostic and therapeutic biomarkers.

(iii) Assess novel therapeutic modalities.

Hypothesis:

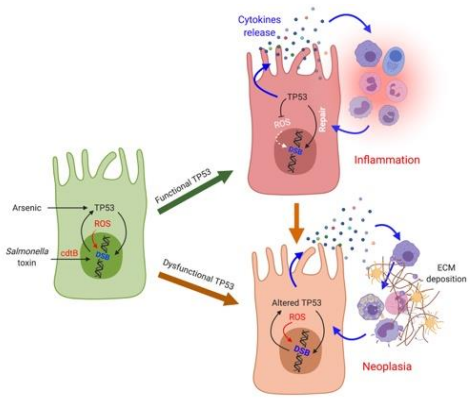


Figure 1. Schematic diagram of proposed model of GBCa pathogenesis.

We postulate that chronic tissue injury from combinations of exposure to biliary cholesterol, trivalent arsenic and salmonella toxin results in generation of oxidative stress in gallbladder epithelial cells leading to DNA damage of the GB epithelium. Stress-induced DNA damage with dysfunctional TP53 promotes neoplastic transformation (Figure 1). Aberrant epithelial cells interactions with stromal and infiltrating immune cells in the microenvironment further favour progression of the disease.

1.1. Establishment of an annotated tissue and organoid biorepository for GBCa

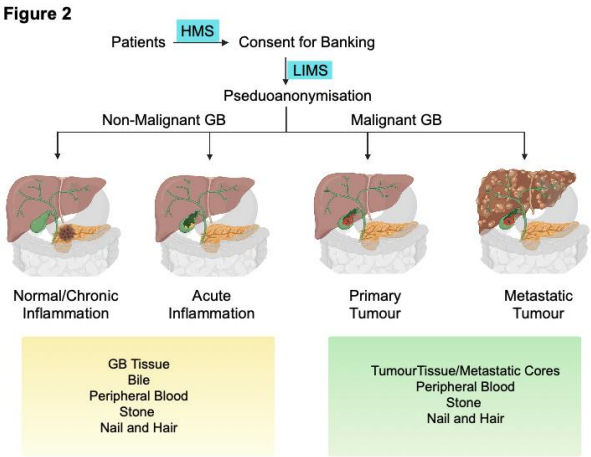


Figure 2. Schematic flow of GB/GBCa samples in the biobank. Non-malignant tissues are categorised as clinically normal or with chronic inflammation and acute inflammation. Malignant tissues are grouped either as primary tumour or tumour with metastasis. Tissue, peripheral blood, bile, stone, nail and hair are collected from respective patients.

Institutional Review Board approval for biobanking and use of biobanked samples was obtained in 2019. Samples are obtained from patients undergoing surgery or image guided biopsy at the Tata Medical Center. Tissues are categorised as normal GB, inflammatory GB (chronic or acute) and tumour GB (primary or metastatic). Transport processes for collecting and banking of high-quality tissue derivatives for downstream studies has been established (Table 1). Samples collected from each patient include primary tissue, peripheral blood, bile, nail and hair for nucleic acid, protein characterisation. Fresh samples are processed for *ex vivo* organoid model generation (described below in 3.1). The customised workflow is shown in Figure 2. Going forward, we aim to improve the conditions for banking of cryopreserved organoids.

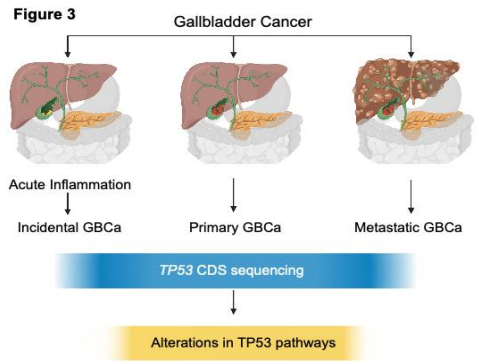


Figure 3. TP53 alterations in GBCa. Formalin fixed paraffin embedded blocks for tumour gallbladder obtained from patients with incidental GBCa, primary GBCa or Metastatic GBCa. Coding region of *TP53* gene sequenced and direct targets of TP53 studied in the corresponding section of blocks with immunohistochemistry.

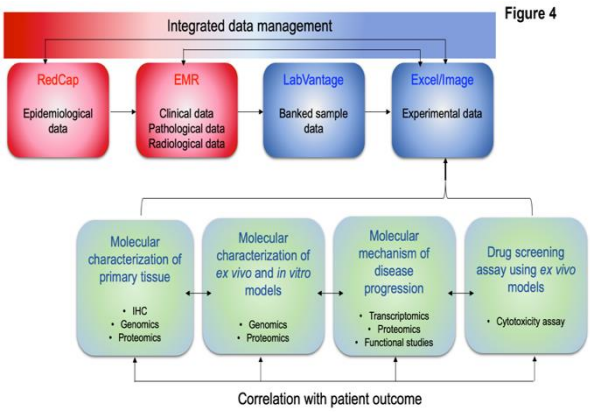


Figure 4. Overview of data integration for the GBCa project

1.2. Molecular and pathological characterisation of GBCa

Our hypothesis is that inflammation and TP53 alterations (genetic and/or epigenetic) contribute to tumour behaviour. To test this hypothesis, we aim to investigate the *TP53* mutation status in GB samples with inflammation (acute or chronic) and early detected (primary GBCa) or metastatic GBCa (Figure 3) along with the alterations in expression of TP53 target genes which regulate cell cycle, proliferation, apoptosis and angiogenesis pathways. Genetically modified cell lines and/or *ex vivo* models are being created to investigate the role of identified*TP53* mutants in disease progression and therapy response.

- TP53 CDS sequencing has been established (see section 4)

- TP53 sequencing of DNA extracted from buffy coats and organoids has been optimised
- Optimisation of DNA extraction and sequencing of *TP53* are ongoing
- All histopathology slides are jointly evaluated with the pathologists to identify degree of inflammation and phenotypic changes in the GB epithelium.

Prospectively we will test for correlations between the different types of TP53 alterations and the degree of inflammation and tumour behaviour in culture.

1.3. Integrated data management

Clinical data linked to each sample are recorded by the surgical team. Pathological and imaging features of the samples are evaluated by the pathologists and radiologists. All data are captured through the hospital EMR. Epidemiological information (including demography, family history, life-style) are entered into RedCap. Pre-analytical variables linked to each sample and downstream analyses of the samples are recorded in Excel. A comprehensive data management system (Figure 4) within the Translational Research Platform (TRP) is being created to integrate and analyse the clinical, epidemiological and research data linked to each sample.

2.1. Establishing a reproducible primary 3D organoid system

Organoids, representing the same basic intrinsic patterning events i.e. organ-like organization, are valuable tools for disease modelling. Development of tissue specific organoids relies on the pluripotent nature of tissue resident adult stem cells (ASCs) or embryonic stem cells. At TTCRC, three approaches have been established to derive organoids from malignant or non-malignant primary gallbladder tissues. Tissue is collected in media containing factors to preserve cell viability and tissue integrity. In specific culture conditions, mature cholangiocytes (COs) (left panels, Figure 5) or ASCs (middle panels, Figure 5) are enriched to generate the organoids from normal gallbladder. Organoids appear as a sphere with a clear lumen at the centre. Mature COs maintain planar cell polarity and are slow growing compared to ASC derived organoids. In a third approach, both ASCs and mature COs are extracted using enzymatic processing and maintained to enrich mature COs to develop organoids (right panels, Figure 5).

In xanthogranulomatous cholecystitis an enrichment of the disease phenotype was observed when mature COs were enriched (left panels, Figure 6). Diseased organoids were morphologically characterised by an irregular shape and darker lumen when compared to normal organoids. Only normal phenotypes were observed when organoids were derived by enriching ASCs (middle panels, Figure 6). A mix of both normal and diseased phenotypes were observed when a mix of ASCs and mature COs were cultured (right panels, Figure 6).

Comparative qPCR data shows enrichment of mature cell markers, *CFTR*, in the mature CO enriched organoids compared to the ASC enriched organoids (p=0.003). Expression of transient amplifying cell markers (*CK19, CK7, SOX9*) are consistently expressed in all

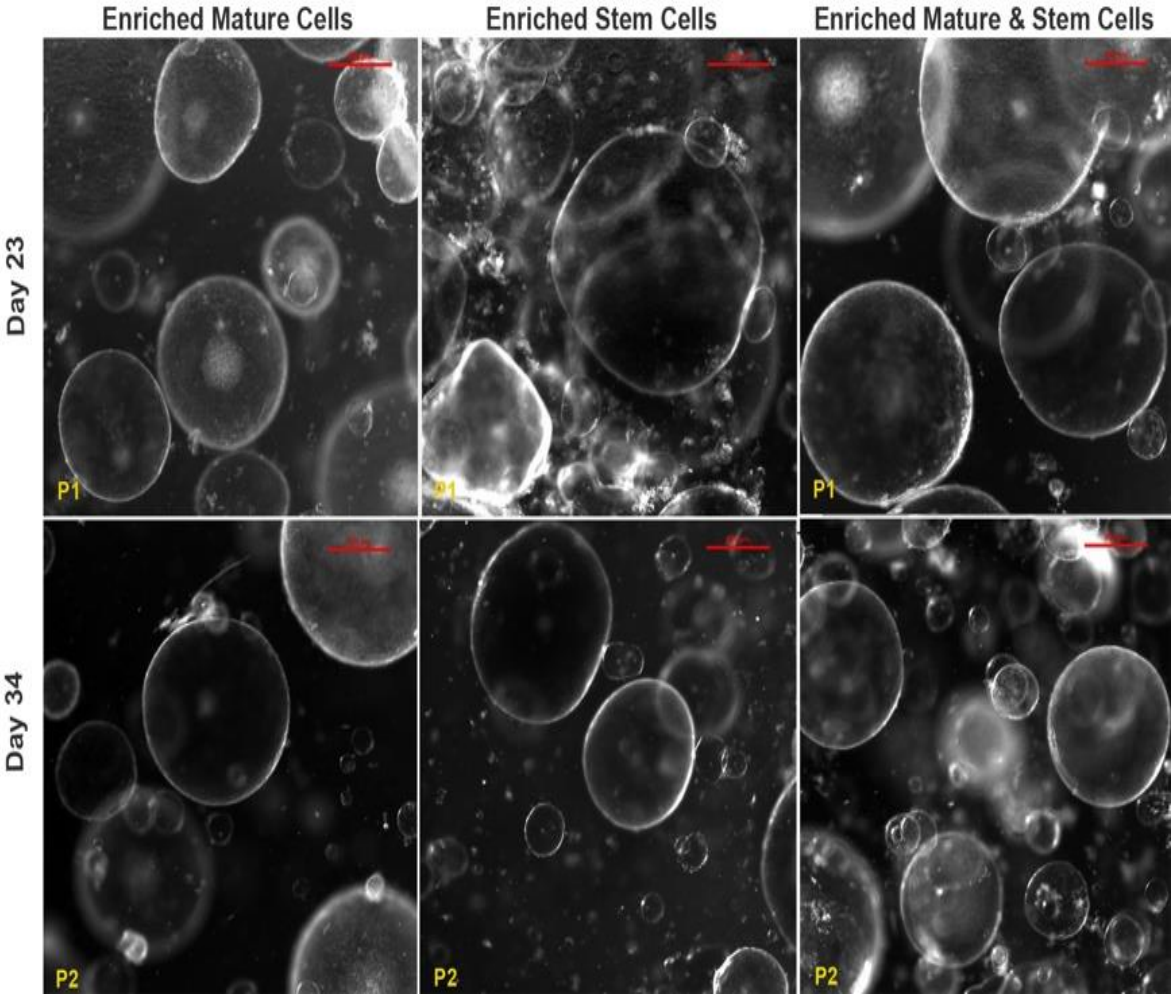


Figure 5. Generation of organoids from normal gallbladder tissue. Representative bright field images of mature cholangiocyte enriched organoids (left panels), stem cells enriched organoids (middle panels) and both mature cholangiocyte and stem cells containing organoids (right panels). Top panels show representative images from 1st passage of the organoids and bottom panels represents the morphology at 2nd passage. Scale bar: 500□m.

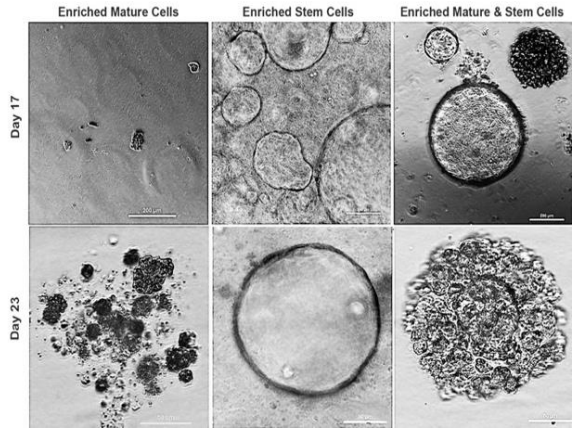


Figure 6. Generation of organoids from inflammatory gallbladder tissue. Representative bright field images of mature cholangiocyte enriched organoids (left panels), stem cells enriched organoids (middle panels) and both mature cholangiocyte and stem cell enriched organoids (right panels) from xanthogranulomatous cholecystitis. Growth and morphology of organoids with different approaches at day 17 (top panels) and at day 23 (bottom panels) after seeding. Scale bar: 200m (top panels); 50m (bottom panels).

three types of organoids (Figure 7). This data

suggests that the two different approaches result in either stemness or planar cell polarity respectively. Expression of *LGR5* (p=0.05) is not significantly elevated in stem cell population. To confirm stem cell enrichment, we will compare the expression of other pluripotent stem cell markers *OCT4*, *NANOG* and *PDX1*. Next steps include proteogenomic characterisation of the different types of organoids and introduction of stromal cells into the current organoid model to more closely mimic the microenvironment.

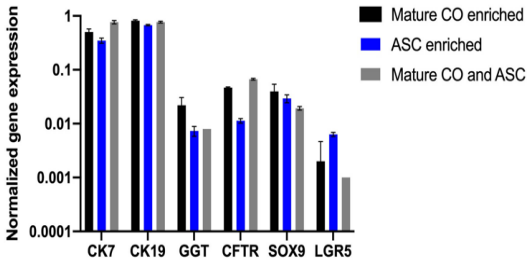


Figure 7. Comparative gene expression of key biliary markers for mature and stem cells. qPCR is performed for all three types of the organoids (mature cholangiocyte enriched: black bars; stem cell enriched: blue bars; both stem and mature cells enriched: grey bars). *CFTR* is a mature cholangiocyte marker. *LGR5* represents stem cells. *CK7, CK19, SOX9 and GGT* are transiently amplified biliary cell markers. Values are normalized against the housekeeping gene *GAPDH* and represented in $\log_{10}$ scale. Error bar: Mean±SD.

2.2 Molecular and functional characterisation of 3D organoid model

Organoids mimic the organ-specific expression of molecular markers and their functional characteristics. We have confirmed expression of CO-specific markers in the organoids with immunofluorescence imaging (Figure 8A). Organoids express Cytokeratin-19 in the cell membrane (left panel). Cytoplasmic expression of Muc5B (middle panel) and Claudin-2 (right panel) in the organoids confirm the characteristics of cholangiocytes, lining the gallbladder epithelium. As gallbladder organoids are derived from ciliated columnar epithelial cells, they are expected to conserve functional epithelial tight junction. Entry of FITC coupled dextran was prevented by the epithelial

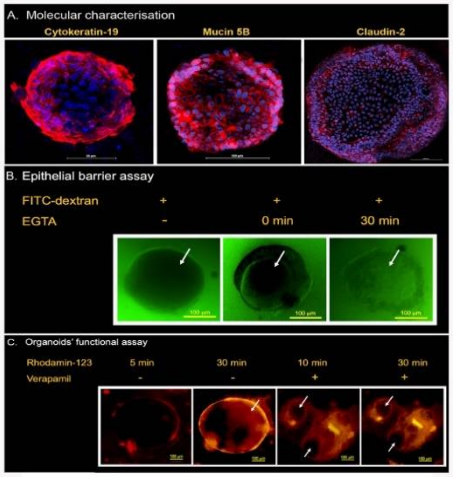


Figure 8. Characterization of the organoids generated from a normal gallbladder tissue. (A) Immunofluorescence images of organoids showing the expression (red) of key biliary markers Cytokeratin-19 (left, scale bar: 50□m), Mucin 5B (middle, scale bar: 100□m) and Claudin-2 (right, scale bar: 100□m). Hoechst-33342 is used to stain the nuclei (blue). (B) Fluorescence images showing tight junction activity of organoids. FITC labelled dextran (green) is used as substrate taken up by the epithelial cells of organoids in the lumen in absence (left) and presence (middle and right panels) of EGTA. Middle and right panels show effect of EGTA at 0 min and 30 min respectively. The arrow indicates the lumen of the organoid. Scale bar: 100□m.(C) MDR pump activity of the organoid cells is measured using rhodamine labelled substrate. Left and right panels show rhodamine uptake at 5, 10 or 30 min in absence (left two panels) or presence (right two panels) of verapamil. The arrow indicates the lumen of the organoid. Scale bar: 100µm.

tight junction of the organoids (Figure 8B, left and middle panels). Disruption of the tight junction by the calcium chelator EDTA, allowed FITC-dextran to enter into the lumen of the organoids (Figure 8B, right panel), confirming the functional epithelial barrier of the organoids. The functional characteristic of the gallbladder cholangiocytes in the organoids were confirmed by evaluating MDR pump activity (Figure 8C). The substrate for MDR pump is labelled with Rhodamine and pumped within the lumen of the organoids (left panel). Blocking the activity of the pump by Verapamil, inhibited the entry of the Rhodamine labelled substrate within the lumen of the organoids (middle and right panels), suggesting active MDR pumps of the organoid forming cholangiocytes.

3. Developing biomimetic platforms for *ex vivo* organoid models

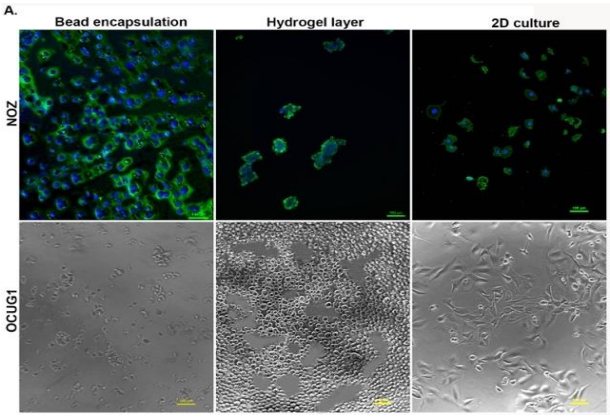


Figure 9A. Morphology of GBCa cell lines grown in different extracellular matrix. GBCa cell lines, NOZ (top panels) and OCUG1 (bottom panels) are grown encapsulated in hydrogel (left panels), on hydrogel layer (middle panels) or on plastic cell culture plates (right panels). NOZ cells are stained with wheat-germ agglutinin (WGA) to visualise the cellular architecture. Hoechst-33342 is used to stain the

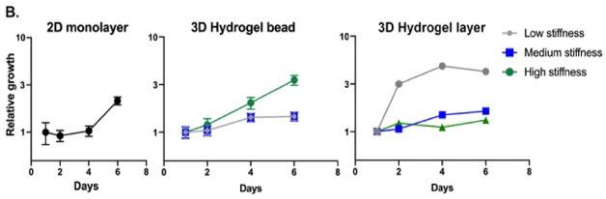


Figure 9B. GBCa cell line growth on matrix with different stiffness. OCUG1 cells were grown in cell culture plastic plate (left panel; stiffness = 2 GPa) or in hydrogels of varying stiffness (low stiffness = 0.4 kPa; medium stiffness = 0.8 kPa; High stiffness = 1.4 kPa). Cells were grown either in hydrogel bead (middle panel) or in hydrogel layer (right panel). Growth of OCUG1 cells were measured on day1, 2, 4 and 6 using WST-1 metabolic assay. All values are normalized by the growth observed on day1 and relative growth is plotted. Error bar: Mean±SD.

Further optimisation is required to create microenvironments which most closely mimic the tissue microenvironment. In collaboration with University of Manchester, we have been developing composite biomaterials to create culture supporting matrices of different stiffnesses and porosity. Alginate-gelatin based biomaterials were synthesised and used to study the growth and survival of GBCa cells. GBCa cells grew in alginate-gelatin hydrogels either encapsulated (left panels, Figure 9A) or used as extracellular matrix (middle panels, Figure 9A). On decreasing the stiffness of the hydrogel, proliferation of GBCa cell line OCUG1 is enhanced (Figure 9B). We will next evaluate the suitability of these hydrogels to grow primary gallbladder cells.

4. Generation of *wt* and *mut TP53* cell lines of gallbladder cancer

We hypothesise that altered TP53 transactivation contributes to the transformation of GB cells. Restoration of wild type TP53 activity/signalling could hinder progression of oncogenesis in stressed GB cells. To test this hypothesis, GBCa cell lines are being generated to express either *wt* or *mut TP53*. The cell line, OCUG-1 (JCRB) has a R267W gain-of-function mutation. TP53 is active and localised within the nucleus of resting cells (Figure 10A). *TP53* will be deleted in this cell line and the *wt TP53* reintroduced. In the GBCa cell line NOZ, there is a Q331V missense mutation in TP53 leading to a truncated TP53. In NOZ cells, the truncated TP53 is expressed at

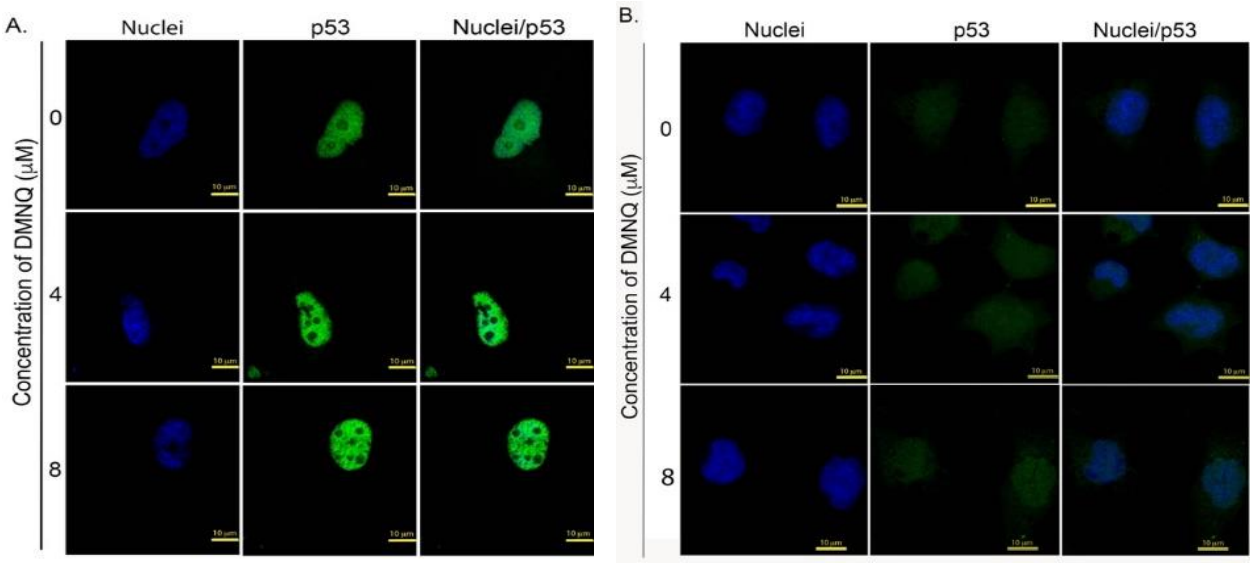
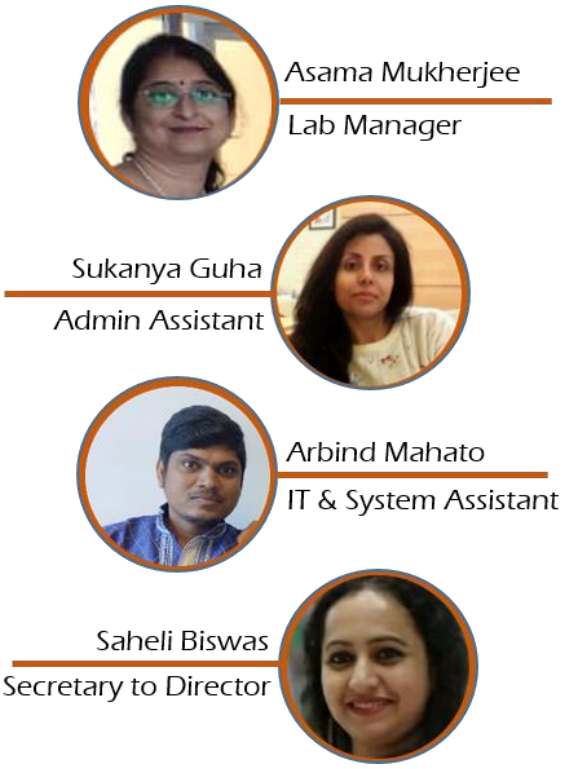


Figure 10 A. Immunofluorescence images of TP53 localisation in OCUG-1 cell line. OCUG-1 cells treated with 0 (top row), 4□M (middle row) and 8□M (bottom row) □M DMNQ for 48 hours. Localisation of TP53 is probed with the Alexa-488 tagged antibody (green). DAPI is used to stain the nuclei (blue). Scale bar:10µm. **B.** Immunofluorescence images of TP53 in NOZ cell lines. NOZ cells treated with 0 (top row), 4 (middle row) and 8 (bottom row) □M DMNQ for 24 hours. Expression and localisation of TP53 is observed using Alexa-488 tagged antibody. DAPI is used as counterstain. Scale bar:10µm.

low levels and localised to the cytoplasm in resting cells (top panel, Figure 10B). There was no change in TP53 expression upon exposure to stress (ROS) (bottom panels, Figure 10B). Ongoing analyses will investigate the role of these different TP53 mutations to disease progression and therapy.

Future Plans: In the coming year, histopathology and proteo-genomic characteristics of the non-malignant and malignant organoids will be correlated with their respective primary tissue counterparts. Organoids derived from normal or inflamed GB will be exposed to environmental stresses to study the pathogenesis of gall disease. Culture conditions of inflammatory gallbladders will further be optimised by co-culturing the immune cells with the organoids.

Administration



Team Members:

The year 2020 brought a wave of disruption in the way of our normal functioning. In spite of several challenges we had been very adaptive with plans for work from home while continuing to support essential services in CRU, biobank and other lab activities. We have used virtual platforms for internal meetings, external talks as well as for candidate interviewing process. We have adopted safe practices and implemented guidelines as the team started to move back to work with the relaxation of lockdown.

Currently TTCRC has over 40 members including PhD student and interns. Tushar Mungle has completed his degree through our collaborative PhD program with IIT Kharagpur while Ankita Dutta was enrolled in this year. We have also started a well-structured internship program for MSc/M.Tech students. The administration extends their support to this growing team to enable them perform their day to day tasks by liaising effectively with the TMC departments of HR, Finance, IT, Materials and Estates. We manage the general administrative responsibilities along with lab operations by working in tandem with the research laboratories and the core facilities.

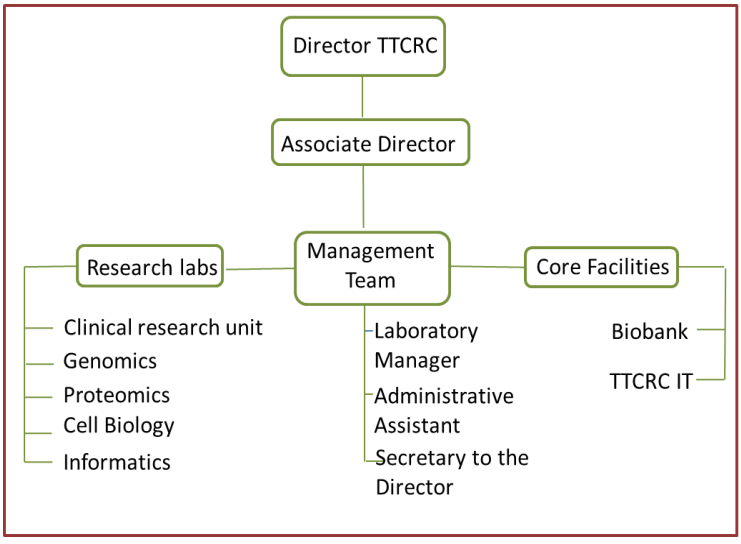


Figure 1. Organizational structure of TTCRC

To increase the visibility of our research in this year we have taken a step towards

launching the TTCRC website https://ttcrc.org. Email services has migrated from the tmckolkata.com address to ttcrc.tmckolkata.org to allow improved file sharing, increased mailbox capacity, security filtering and device compatibility. A 100TB storage server has been installed for data storage. TTCRC has also achieved email migration from. We continue to work to support the laboratories operate to the highest standards of academic and translational research practice.

The team participated in The Annual Review (2020) process conducted between January 15-16, 2021, with great enthusiasm. We have observed oral and poster presentation sessions by our team members along with participation from TCS. We are grateful to Prof. Anindya Dutta for his insightful presentation on this occasion.

Invited Presentations

1. 'In vitro models or avant-garde molecular cuisine' by Dr Annalisa Tirella, University of Manchester (9th May 2020).
2. 'Lessons from developmental disorders: Specificity of KMT2D mutations determine the resultant phenotype' by Dr Siddhartha Banka, University of Manchester (3rd June 2020)
3. 'The tumour suppressor P53; the story unfolds' by Dr Patricia Muller, Cancer Research UK, Manchester Institute (12thJune 2020).
4. 'Development of integrated platform for biomarker and drug target discovery using proteomics' by Prof Anthony Whetton, University of Manchester (17th June 2020)
5. 'Architecture of phosphhoinosidine signalling systems' by Dr Raghu Padinjat, NCBS Bangalore (July 29th).
6. 'Improved restratification in childhood ALL' by Dr. Anthony V. Moorman, Newcastle University (3rd September 2020).
7. 'Experiences with Primary Cell Culture' by Dr Cornelia Eckert - Charité, University Medicine Berlin (17th and 28th September 2020).
8. 'Cholangiocyte organoids for clinical applications' by Dr Fotios Sampaziotis, University of Cambridge (6th November 2020).
9. 'Developmental origins of infant ALL' by Dr. Anindita Roy, Department of Paediatrics University of Oxford (18th November 2020).
10. 'Novel aspects of genomic instability as they impact on cancers: extrachromosomal circles of DNA and DNA repair gene mutations' by Prof. Anindya Dutta (15th January 2021)