Detail projects from PIs for are as below:-

1. DR JYOTI KODE, KODE LAB

Project Title:

Elucidating role of small molecule inhibitors of NLRP3 inflammasome pathway using *in vitro* knockdown models of oral cancer

Project Summary:

NLRP3 has emerged as a central regulator in the inflammatory process, and its activation directly correlates with the progression of head and neck tumors. However, very few studies have been done to explore the molecular mechanism behind cross talk between NLRP3 inflammasome and head and neck cancers. The involvement of the NLRP3 inflammasome in head and neck cancers makes it a highly attractive drug target. To date, many small molecules have been identified as NLPR3 inflammasome inhibitors, and several of them have been studied in mouse models of human diseases. But none of them have reached clinical trial level, in drug development pipeline. Our previously published work (Kode et al, BioOrganic Chemistry, 2020) revealed **9a** as an anti-oral cancer compound. **9a** was found to act through inhibiting tubulin polymerization at protein level, using models oral cancer cell line/ spheroid cells and in vivo animal oral cancer xenograft model. **9a** had also shown significant reduction in radiolabeled-glucose uptake in xenograft mice model. Recently conducted study (manuscript in submission) has provided leads that **9a** could be acting through regulating NLRP3 inflammasome pathway. In current study, we propose to investigate the role of NLRP3 inflammasome markers in cell inflammation process and oral cancer progression using RNA interference. NLRP3 expression would be stably inhibited in AW13516 oral cancer cells. NLRP3 knockdown cells would be assessed for effect on downstream targets. NLRP3 knockdown cells will be further evaluated for effect on tumorigenic potential of AW13516 cells in immunodeficient mice models.

Our lab is also involved in identifying phytoextracts and phytochemicals as NLRP3 pathway inhibitors for development of cancer therapeutics.

Post-doc willing to join lab is preferred to have necessary experience in basic and /or advanced techniques.

Reference:

Kode J, Kovvuri J, Nagaraju B, Jadhav S, Barkume M, Sen S, Kasinathan NK, Chaudhari P, Mohanty BS, Gour J, Sigalapalli DK, Ganesh Kumar C, Pradhan T, Banerjee M, Kamal A. Synthesis, biological evaluation, and molecular docking analysis of phenstatin based indole linked chalcones as anticancer agents and tubulin polymerization inhibitors. Bioorg Chem. 2020 Dec;105:104447. doi: 10.1016/j.bioorg.2020.104447. Epub 2020 Nov 1. PMID: 33207276.
https://doi.org/10.1016/j.bioorg.2020.104447 https://pubmed.ncbi.nlm.nih.gov/33207276/

NCBI PubChem registration of 9a is in process. https://pubchem.ncbi.nlm.nih.gov/upload/#substance-113543

2. DR KAKOLI BOSE, BOSE LAB

Project Title:
Understanding the molecular mechanisms of cancer-associated mutations in Procaspase-8 DEF assembly.

Project Summary:

Cancer in its various forms is a major health hazard leading to current annual mortality of ~0.6 million in India and 10 million globally. Despite the presence of multiple treatment modalities including surgery and chemotherapy, it suffers a major setback due to recurrence and therapy resistance, thus comprising a majority of the burden of deaths. One of the major features of malignant cells is apoptosis resistance, in particular, against conventional treatments such as cytotoxic drugs. Therefore, there is a dire need to identify new cancer therapeutics using novel/alternate approaches, a promising one being modulation of the functions of the molecules in the apoptotic pathway such as procaspase-8.

The extrinsic apoptotic pathway is activated by stimulation of the death receptor (DR) family members such as CD95/Fas and TRAIL-R1/2, which initiates the maturation of procaspase-8-the major regulator of DR signaling and hence defines the onset of the apoptotic cascade. Procaspase-8 comprises tandem death-effector domains (DEDA&B) at its N-terminus followed by two protease domains: p10 and p18 at the C-terminus. Autocatalytic activation occurs upon its dimerization which is followed by autoprocessing to active caspase-8 heterotetramer $p10_2$-$p18_2$. Triggering of DR with the cognate death ligand (DL) leads to the formation of a death-inducing signaling complex (DISC), which comprises CD95, FADD, procaspases-8,-10 and c-FLIPs. It has been demonstrated that interactions of procaspase-8 DED motifs at the DISC lead to the assembly of the death-effector filaments (DEFs), which presents the major step in apoptosis regulation through the extrinsic pathway.

Several cancer-associated DED mutations have been identified that preclude apoptotic induction in cancer cell lines and promote cancer progression and/or chemoresistance. Collectively, unraveling the role of these mutations in pro- and anti-apoptotic networks and in cancer signaling pathways will be the central question of this section of the project. This approach will provide highly relevant innuendos toward the development of *de-novo* molecular probes with the aim of rescuing these phenotypes. Furthermore, these comprehensive functional analyses of the protease will enhance our understanding of how regulation of procaspase-8 maturation decides the life or death of the cells under normal physiological conditions and in cancer.

- **Aim:** Characterization of the DEF-deficient mutants (from *cancer databases*) using functional approaches to understand the molecular mechanisms underlying their role in DEF formation, apoptosis induction, cancer progression, and chemoresistance-

Expertise needed: Mammalian cell culture, apoptosis assays western blotting, immunoprecipitation, co-localization studies, pull-down studies, protein biochemistry

3. DR MANOJ MAHIMKAR, MAHIMKAR LAB

Project Title:

Understanding of modulation of DNA methylation during development of experimental oral cancers and their modulation during chemoprevention.

Project Summary:

Oral cancer is one of the most prevalent cancer in India associated with tobacco use. Despite improved technology and therapeutic approaches, the 5-year survival rate has not improved over the last 4 to 5 decades. Even though it is the most accessible site, most oral cancers are diagnosed at an advanced stage with lymph node metastasis and are associated with poor clinical outcome. Many natural and synthetic compounds are being evaluated for their preventive and/or therapeutic efficacy or their ability to modulate the efficacy or toxicity of established chemotherapeutic agent(s). Based on the evidence from experimental and epidemiological research chemoprevention by dietary phytochemical intervention is emerging as an important modality for cancer prevention. Polyphenolic compounds in green tea are extensively explored in various cancers and have reached clinical trials. However, black tea, the most commonly consumed beverage after water, is unexplored. Polymeric black tea polyphenols (PBPs) contribute about 47% of the total polyphenols present in black tea. However, very few preclinical studies have been conducted so far using PBPs despite of their abundance in black tea. We have earlier demonstrated chemopreventive potential of PBPs in experimental lung, skin and colon carcinogenesis in different preclinical rodent models. There are few reports where polyphenon B is used for oral cancer prevention in black tea extract or commercial product. However, none of the studies have used PBPs alone or in combination. Oral carcinogenesis is the complex multistep and multifactorial process involving the dysregulation of multiple molecular pathways. Oxidative stress is an important event in cell transformation. Transcription factor NF-E2-related factor 2 (Nrf2) regulates a battery of antioxidant and cellular protective genes, primarily in response to oxidative stress. Nrf2 is one of the key modulators of the process of carcinogenesis and has dual role in tumor prevention and progression. Transient expression of NRF2 is observed in chemoprevention while constitutively increased expression of Nrf2 is associated with chemo-radiation resistance and poor treatment response. Hence, we will be using Nrf2 proficient and NRF2 knocked out (NRF2KO) mice to understand Nrf2 dependent and/ or independent chemopreventive effects of PBPs. DNA methylation is one of the crucial event in modulating the process of carcinogenesis and chemoprevention. Hence, we plan to analyze global DNA methylation and gene expression during different stages of oral carcinogenesis and their modulation during chemoprevention. We will also analyze relevant surrogate biomarkers in animal serum/ plasma, which may serve as surrogate endpoints for human clinical trials planned in future. Thus, our study will help in comprehensive insight into the molecular mechanisms of PBP mediated chemoprevention and develop biomarkers relevant for human studies

4. DR PRASANNA VENKATRAMAN, PRASANNA LAB

Project Title:

Dissecting the Assembly and Functional Status of Proteasome in Cancer Cells

Project Summary:

Proteasome and its analogous ATP dependent proteases are major degradation machineries in many biological systems. Central to the efficient degradation of proteins by the proteasome is its complex structure. The proteasome is organized into a central cylindrical proteolytic core capped at one or both ends by the 19S regulatory particle. The 19S regulatory particle accepts substrates for degradation and its individual subunits ensure that the globular protein is deubiquitinated (where necessary), unfolded, and threaded through to the 20S core particle where proteolysis takes place. Inhibitors of proteasome active sites are anti-cancer drugs used in the treatment of multiple myeloma and to a limited extent in mantle cell lymphoma. These inhibitors so far have not been successful in the treatment of solid tumors (for multiple reasons).

Our efforts over the years has established that the seemingly innocuous assembly chaperone, PSMD9, is central to the biology of some cancers. We find that PSMD9 impacts proteasome assembly status and subunit connectivity in unprecedented ways in cancer cells. Its loss by CRISPR knock out, leads to accumulation of polyubiquitinated proteins and retards growth. Furthermore, PSMD9 as a 'chaperone' seems to play an even wider role in cancer cell biology by influencing subcellular organelle structure and biogenesis. Based on these multiple evidences, we believe that PSMD9, the new kid on the oncology block is an Achilles' heel in (at least) Breast Cancer.

- **Aim**: To characterize the assembly status of proteasome in PSMD9 KO cells and monitor proteasome activity using live cell probes. To screen functionally active fragments that can bind to modulators or substrates of the Proteasome pathway and identify the targets.

Skills Required: Sound knowledge in Biochemistry, Structure and Functions of proteins; Molecular Biology and Cell biology tools and Techniques.

5. DR SANJAY GUPTA, GUPTA LAB

Project Title:

Exploring the potential of class 1 histone deacetylase inhibitor in combination with standard chemotherapeutic drug in gastric adenocarcinoma patient samples

Project Summary:

Epigenetic mechanisms play a crucial role in controlling DNA-mediated biological processes, and chromatin alterations may lead to the onset and progression of many diseases like cancer. Acetylation of histone proteins is regulated by the balance between histone deacetylases (HDAC) and histone acetyltransferases (HAT). HDAC inhibitors are emerging as a promising epi-drug, particularly with other anti-cancer drugs. Earlier studies from the lab have shown that HDAC inhibitor valproic acid increases the efficacy of DNA-binding chemotherapeutic drugs by reversing the acetylation status and opening of chromatin in i*n vitro* and pre-clinical *in vivo* models. Moreover, their use in the pre-clinical model showed a significant therapeutic window, with reduced effects on normal cells. Further, the frequency of HDAC levels and acetylation status varies significantly in different cancer patient samples, suggesting a potential alteration of the enzyme function.

In light of the above, we propose that the patients with a higher level/activity of class 1 HDACs may be poor responders to chemotherapeutic drugs like cisplatin, epirubicin, etc. The proposed study includes an analysis of histone acetylation, class 1 HDAC levels, and activity for HDAC inhibitor therapy. The project's objective is to establish the association between the level and activity of class 1 HDACs with the response rate measured according to response evaluation criteria in solid tumors for gastric adenocarcinoma. The secondary objective will be to understand the relationship between the level/activity of HDACs with loco-regional and metastatic recurrence, and survival. The study will help patients stratification for HDAC inhibitor therapy and execute a phase 1 clinical trial in gastric cancer.

6. DR SORAB DALAL, SORAB LAB

Project Title:

Identifying the LCN2 interactome to identify pathways required for therapy resistance and tumor progression.

Background:

Lipocalin 2 (LCN2), also known as NGAL (neutrophil gelatinase associated lipocalin), is a secreted glycoprotein (1,2) and is required to maintain the integrity of the gastro-intestinal mucosa (3). LCN2 forms a complex with bacterial and human siderophores (reviewed in (1)) thereby inhibiting bacterial growth and regulating iron homeostasis in mammalian cells. LCN2 bound to iron is imported into cells by complex formation with the LCN2 receptors, SLC22A17, MCR4 and LRP2 or Megalin (reviewed in (1) and (4)). The import of iron into cells by LCN2 is required for LCN2 functions in maintaining kidney homeostasis (5,6) and in regulating apoptotic cell death (4,7). LCN2 has also been shown to prevent apoptosis in thyroid cancer cells (8) suggesting that LCN2 might have context dependent functions. It binds to and protects the matrix-metalloprotease MMP9 from auto-degradation with a concomitant increase in MMP9 activity (9). This is consistent with the observation that LCN2 can promote invasion and angiogenesis and is associated with metastasis in multiple tumour types (10-14). In addition, LCN2 has been reported to increase the expression of enzymes required for clearing reactive oxygen species (ROS) such as superoxide dismutase 1 and 2 and hemoxygenase 1 (15). However, under conditions of inflammation in cardiac muscle LCN2 stimulates the production of mitochondrial ROS and inhibits oxidative phosphorylation (16). LCN2 inhibits autophagy in chronic conditions such as diabetes (17) or ischemic heart disease (18,19), however, no effect on autophagy has been reported in tumour cells.

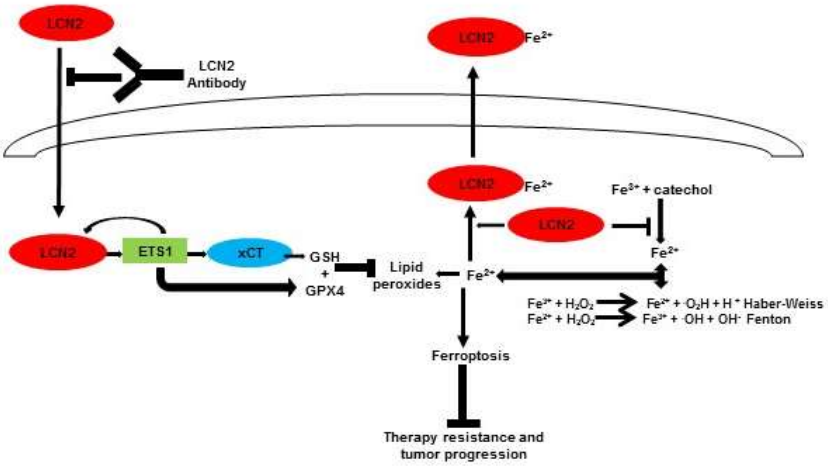
LCN2 is over-expressed in many tumour types (reviewed in (1,20). Other reports suggest that LCN2 expression is increased in breast cancer (21-23) and loss of LCN2 expression leads to decreased tumour progression in mouse models of human breast cancer (24,25). In addition, agents that inhibit LCN2 expression can inhibit transformation and neoplastic progression in breast cancer cell lines (11,26). Further, LCN2 expression is associated with angiogenesis in both breast and pancreatic cancer (14,27) and inhibition of LCN2 expression with siRNA inhibits angiogenesis (11). Other reports suggest that LCN2 might support metastatic progression in both breast (13) and cervical cancer (10). LCN2 expression was increased in colorectal carcinomas in multiple clinical studies and is an indicator of colon cancer progression from adenoma to carcinoma (28-30). LCN2 over-expression leads to increased tumour formation in xenograft models of colon cancer suggesting that LCN2 over-expression drives tumour progression in the colon (29).

We have previously demonstrated that loss of the desmosomal plaque protein plakophilin3 (PKP3), led to increased tumour formation in multiple cell lines (31). The increase in tumour formation and invasion upon PKP3 loss is dependent on LCN2 expression (32). In addition, our recent publication demonstrated that LCN2 levels are increased in colon tumour samples as compared to adjacent normal tissue. Further, we demonstrated that LCN2 expression correlated with resistance to 5FU in vitro and in vivo due to the ability of LCN2 to inhibit ferroptosis. Finally, we demonstrated that inhibiting LCN2 function with a monoclonal antibody led to an increase in sensitivity to 5FU and tumour regression (figure 1) (33). Therefore, this antibody that targets LCN2 and inhibits LCN2 mediated tumour progression and reverses the LCN2

mediated resistance to chemo and radiotherapy ((33) and figure 1) could serve as a potent therapeutic agent in multiple tumour types.

Ferroptosis is an iron dependent cell death mechanism that is distinct from other forms of cell death such as apoptosis and necroptosis (reviewed in (34)). The induction of ferroptosis is dependent on the presence of reactive iron radicals (35), which promotes the formation reactive oxygen species (ROS), including lipid peroxides, by a pair of cyclic reactions termed the Fenton and Haber-Weiss reactions (36). Lipid peroxides are incorporated into cell membranes leading to toxicity and cell death. Cells undergoing ferroptosis show an inability to reduce peroxidated phospholipids either due to a decrease in the levels of the enzyme glutathione peroxidase (GPX4) (37,38) or due to a decrease in glutathione (GSH) production due to a decrease in cystine import by the cystine glutamate transporter, system x_c^-(39). Consistent with these observations, compounds that inhibit the accumulation of lipid peroxides such as liproxstatin (40) and ferrostatin (35) inhibit ferroptosis while compounds that inhibit the activity of GPX4 (RSL3) or system x_c^- component xCT (erastin) (41-43) promote ferroptotic cell death. Notably, the inhibition of ferroptosis using either drugs or genetic manipulation (44,45) confers chemo-resistance in cancer cells suggesting that this pathway could serve as an important target in tumours that are resistant to cytotoxic therapeutics. Our work has demonstrated that LCN2 inhibits ferroptosis in two ways: by regulating ferrous iron levels in cells and by stimulating the expression of the ETS1 transcription factor which activates the expression of GPX4 and xCT (33). As far as we are aware this is the first example of a gene that affects both arms of the ferroptosis pathway. The antibody to LCN2 described above can inhibit the ability of LCN2 to regulate ferrous iron levels and promote the expression of ETS1 and subsequently GPX4 and xCT (33). Thus, inhibiting LCN2 function is a novel mechanism for reversing ferroptosis.

Figure 1. Mechanism by which LCN2 regulates therapy resistance.



Autophagy is a catabolic process that is required for the degradation of damaged proteins and organelles (reviewed in (46)). Autophagy can both promote and inhibit tumour progression (reviewed in (47-49)). For example, loss of autophagy genes can result in a decreased tumour progression (50,51) and decreased metastasis (52) in multiple tumour models. However, tumour formation can be inhibited in multiple tumour types by inhibiting autophagy (53-63). These divergent outcomes can depend on several factors such as p53 status, the oncogenic pathways activated and the status of mitochondrial dysfunction. Previous results have demonstrated that autophagy might inhibit ferroptosis (64,65) and can promote invasion by regulating

turnover of focal adhesions (66-68). LCN2 has been shown to both promote (69) and inhibit (17,19) autophagy depending on the cell type and the disease context being studied. Autophagy also contributes to therapy resistance (reviewed in (70)). Given the role played by LCN2 in regulating therapy resistance, invasion, and tumour progression it is worth determining how these LCN2 functions are mediated by autophagy and the cross-talk between these various pathways.

Results from our laboratory suggest that LCN2 is over expressed upon PKP3 knockdown in cell lines derived from the colon (HCT116) (figure 2A) suggesting that PKP3 loss results in an increase in LCN2 expression (32). We have also generated LCN2 over-expressing cell lines in HCT116 cells (figure 2B) and have demonstrated that LCN2 expression varies among colon cancer cell lines (figure 2C). LCN2 levels correlate with resistance to chemotherapeutic drugs such as 5-fluorouracil (5-FU) in vitro and in vivo (33). Inhibiting LCN2 function with a monoclonal antibody that we have developed in collaboration with Mazumdar Shaw Medical Foundation and Beyond Antibody (patent application submitted) results in a reversal of resistance to 5FU in vitro and in vivo (33). All of this data suggests that LCN2 expression drives tumour progression and therapy resistance in colon cancer.

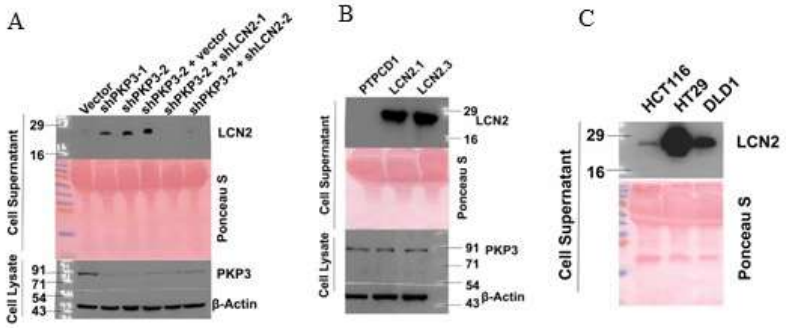


Figure 2. LCN2 levels in colon cancer cell lines. (A-C) 200µg of acetone precipitated cell supernatants, or 100µg of whole cell extracts (WCE) were prepared from HCT116 derived vector control clone (vec), PKP3 knockdown clones (shpkp3-1 and shpkp3-2), the LCN2+PKP3 double knockdown clones (shpkp3-2+shlcn2-1 andshpkp3-2+shlcn2-3) and the shpkp3-2 derived vector control clone (shpkp3-2-vec) (A) or the HCT116 derived LCN2 overexpressing clones (LCN2.1 and LCN2.3) and the vector control (PTPCD1) (B) or the indicated colon cancer cell lines (C) were resolved on SDS PAGE gels followed by Western blotting with the indicated antibodies. A Ponceau-S stain of the blot served as a loading control for LCN2.

Our results have also demonstrated that an increase in LCN2 levels correlates with an increase in autophagy as measured by measuring LC3B foci over time post treatment with either radiation (figure 3A-F) or 5FU (figure 4A-D). The increase in autophagy seems to be required for resistance to radiation (figure 5A) and ROS clearance (figure 5B). Similarly, inhibiting autophagy leads to a decrease in survival and the clearance of ROS upon treatment with 5FU (figure 5C-D). Further, LCN2 expression is required for invasion (32) and inhibiting LCN2 function with a monoclonal antibody leads to a decrease in invasion in multiple cell lines (figure 6A-C). Therefore, in addition to regulating the response to 5FU, LCN2 might also stimulate invasion leading to tumour progression.

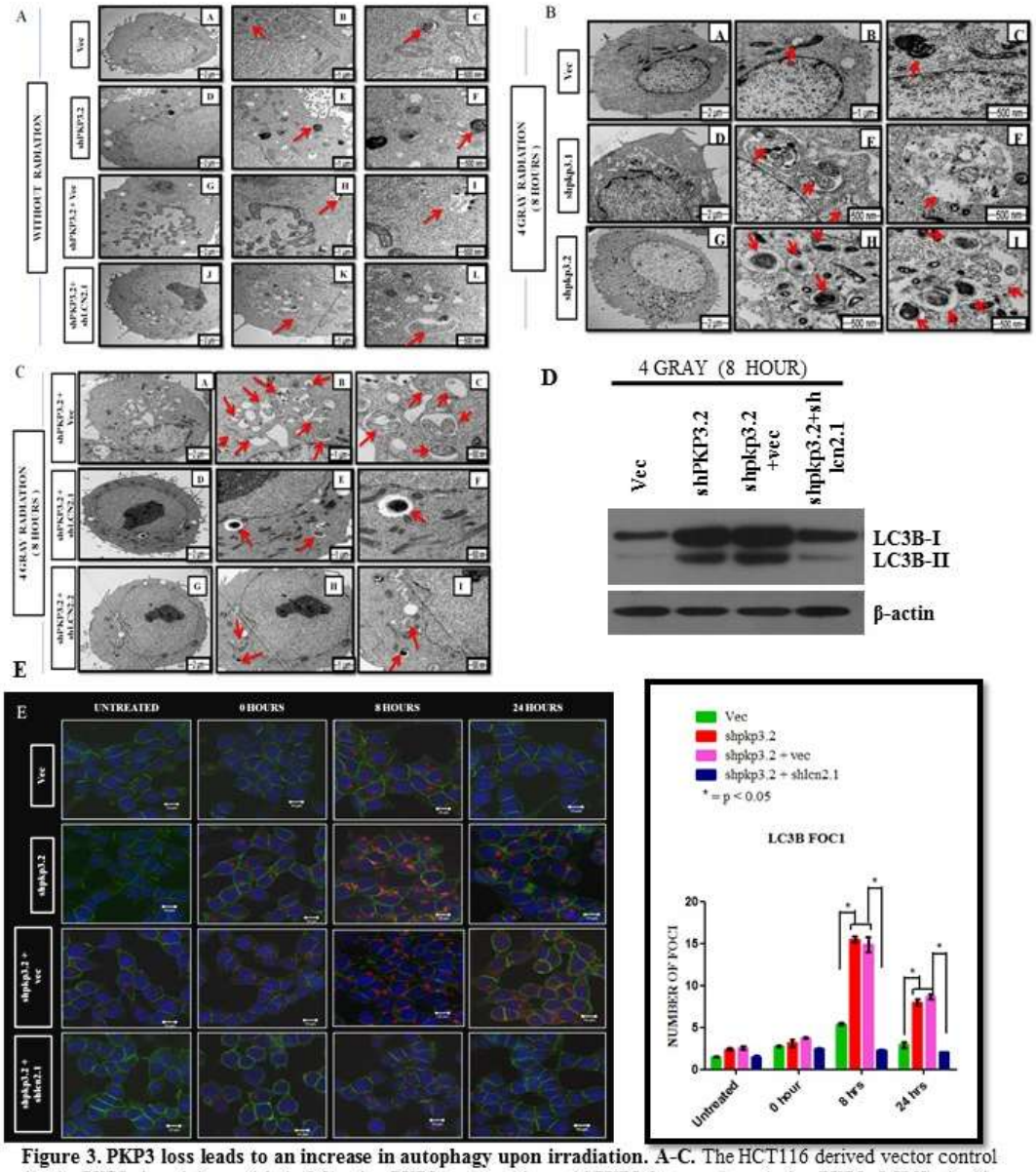


Figure 3. PKP3 loss leads to an increase in autophagy upon irradiation. A-C. The HCT116 derived vector control (vec), PKP3 knockdown (shpkp3.2), the PKP3 vector clone (shPKP3.2 + vec) and the PKP3 LCN2 double knockdown (shpkp3.2 + shlcn2.1) cells were unirradiated (A) or irradiated (B-C) followed by transmission electron microscopy to detect autophagosomes. Note that the number of autophagosomes (red arrows) increases post radiation in the cells with PKP3 knockdown (shpkp3.2 and shpkp3.2 + vec) in comparison to the vector control and the double knockdown cells. **D**. The indicated cell types were irradiated and Western blots performed using the indicated antibodies. Actin served as a loading control. **E**. Unirradiated or irradiated cells at various time points post radiation were stained with antibodies to LC3B followed by immunofluorescence analysis and the number of LC3B foci (mean and standard deviation) plotted on the Y-axis at different time points post radiation. Where indicated p values were determined using a students t-test.

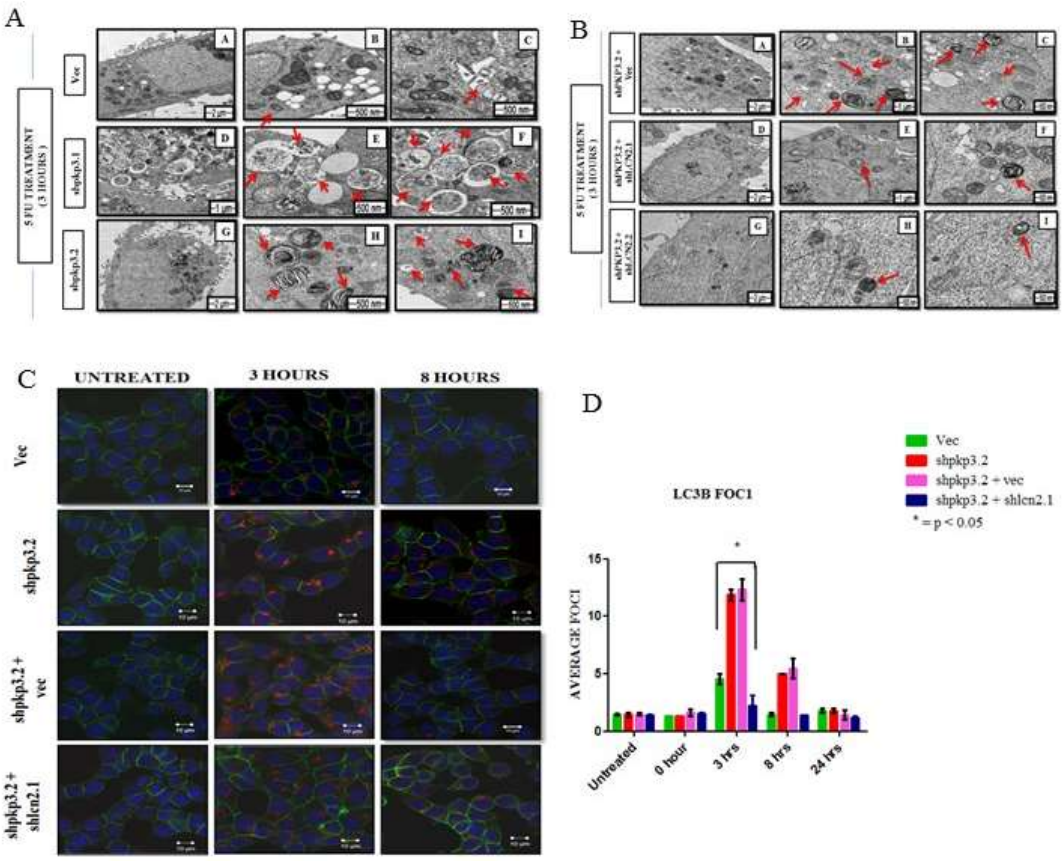


Fig 4. PKP3 loss leads to increase in autophagy and LCN2 is required for this phenotype. A-B. The HCT116 derived vector control (vec), PKP3 knockdown (shpkp3.2), the PKP3 vector clone (shPKP3.2 + vec) and the PKP3 LCN2 double knockdown (shpkp3.2 + shlcn2.1) cells were untreated (A) or treated with 5FU (B) followed by transmission electron microscopy to detect autophagosomes. Note that the number of autophagosomes (red arrows) increases post treatment in the cells with PKP3 knockdown (shpkp3.2 and shpkp3.2 + vec) in comparison to the vector control and the double knockdown cells. **C-D**. Untreated or cells treated with 5FU cells at various time points post treatmentwere stained with antibodies to LC3B followed by immunofluorescence analysis (C) and the number of LC3B foci (mean and standard deviation) plotted on the Y-axis at different time points post treatment (D). Where indicated p values were determined using a students t-test.

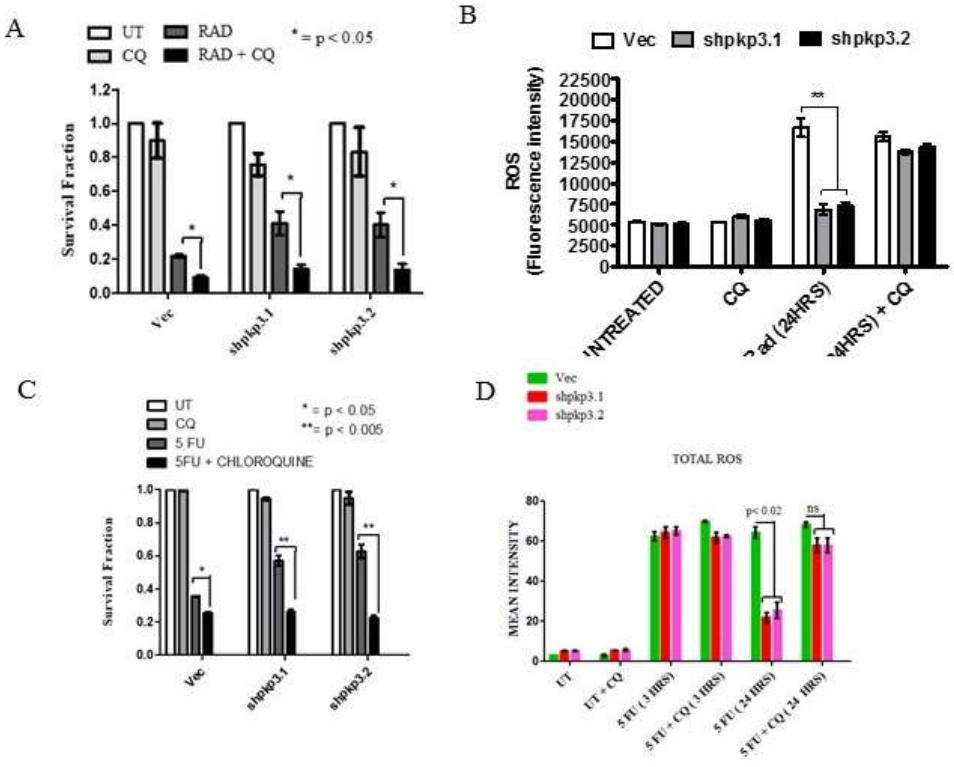


Fig 5. Inhibiting autophagy leads to decreased survival and ROS clearance. A-B. The indicated cell lines were treated with radiation in the presence or absence of chloroquine (CQ) and clonogenic assays performed (A) and ROS levels measured (B). The mean and standard deviation of three independent experiments is plotted. C-D. The indicated cell lines were treated with 5FU in the presence or absence of CQ and clonogenic assays performed (C) and ROS levels measured (D). The mean and standard deviation of three independent experiments is plotted. p values were generated using a students t test.

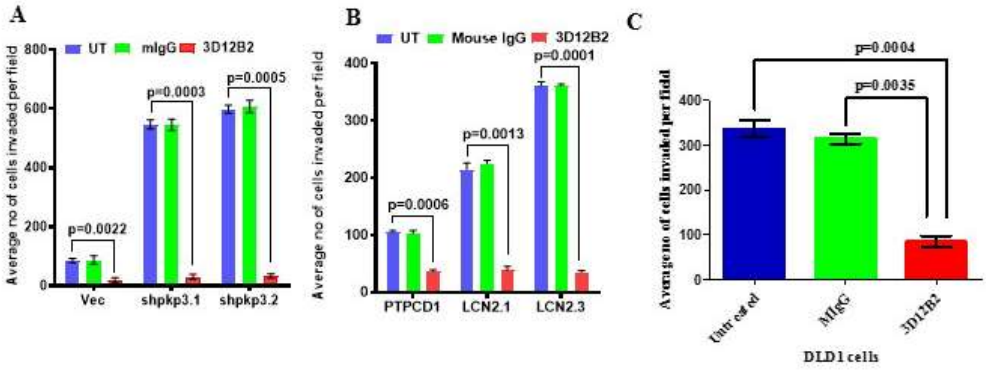


Figure 6. LCN2 expression leads to an increase in invasion. A-C. Invasion assays using matrigel as the substrate were performed on the indicated cell lines. Prior to the assay, the cells were treated with either vehicle control, non-specific mouse IgG or the LCN2 monoclonal antibody 3D12B2. Note that the LCN2 antibody inhibits invasion in all cell lines tested. In all cases the mean and standard deviation of three independent experiments is plotted and p values were generated using a students t-test.

Rationale

LCN2 expression leads to therapy resistance, increased autophagy, increased invasion and tumour progression (figures 2-6 and (32,71). Inhibiting LCN2 either by vector driven RNAi or by treating cells with a monoclonal antibody to LCN2 results in a reversal of all these phenotypes. Therefore, it is likely that in addition to regulating iron homeostasis, LCN2 performs these functions by binding to various proteins and modulating their function. Hence, performing an unbiased screen to identify proteins that form a complex with LCN2 or whose expression is altered upon LCN2 expression or the loss of LCN2 expression could result in identifying an LCN2 dependent network that regulates the processes of therapy resistance, invasion and tumour progression and how these processes are linked. This will not only provide information on how LCN2 regulates tumour progression but will also identify surrogate markers downstream of LCN2 that can be used for diagnosis as well as further molecular validation of the monoclonal antibody to LCN2 so that it can be used as a potential therapeutic.

Aims and objectives

1. Identification of proteins that bind to LCN2.

2. To determine the contribution of these interactions to invasion, autophagy and therapy resistance.

Experimental Plan

1. Identification of proteins that bind to LCN2. Our collaborator Manjula Das from the Mazumdar Shaw Medical Foundation has generated two monoclonal antibodies to LCN2. The first, 3D12B2, inhibits LCN2 function in multiple assays resulting in increased sensitivity to 5FU and decreased tumour formation in immunocompromised mice (71). The other (NNAB) does not inhibit LCN2 function but has increased affinity for LCN2 and can immunoprecipitate (IP) LCN2 from cell extracts (figure 7). Therefore, this second antibody can be used as an affinity agent in co-immunoprecipitation (COIP) assays to identify proteins that form a complex with LCN2.

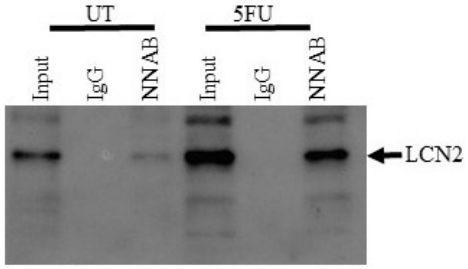


Figure 7. Identification of an antibody that can immunoprecipitate LCN2. Protein extracts prepared from DLD1 cells that were either untreated (UT) or treated with 5FU were incubated with either non-specific IgG or the LCN2 antibody (NNAB). Post IP the reactions were resolved on SDS-PAGE gels and Western blots performed with antibodies to LCN2. Note that the LCN2 signal is only observed in the NNAB lane.

To identify proteins that form a complex with LCN2 we will make protein extracts from DLD1 cells and perform IPs for LCN2 as shown in figure 7. After immunoprecipitation, to identify proteins that bind differently to LCN2, the bound proteins will be subjected to trypsin digestion and the peptides released will be labelled using iTRAQ reagent (figure 8). The labelled peptides will be separated on a liquid chromatography column followed by inline identification by Mass spectrometry as previously described (72). The mass spectrum will be searched against Human Proteome to identify and quantify the relative abundance of interacting partners using andromeda, a peptide centric search engine integrated to MaxQuant environment as previously described (73,74). Novel interactors will then be subjected to a KEGG pathway and STRING analysis to identify potential protein networks that might be regulated by LCN2. As controls, we will perform IP experiments with a non-specific IgG to identify proteins that specifically COIP with

LCN2. As LCN2 is a secreted protein, we will also perform similar experiments on LCN2 that is secreted into the media. This will permit identification of proteins that bind to LCN2 inside the cell and in the extracellular milieu.

Once we have identified proteins that bind to LCN2, we will confirm the interaction by performing IP experiments as described above and the reactions will be resolved on SDS-PAGE gels followed by Western blotting with antibodies to the proteins or LCN2. As controls IPs will be performed with a non-specific IgG and similar experiments will be performed in DLD1 cells with a knockdown of LCN2. The proteins identified in the experiments in the paragraph above should COIP with LCN2 only in DLD1 cells and not in DLD1 cells with an LCN2 knockdown. Only the proteins that fulfil these requirements will be assessed in the experiments described below.

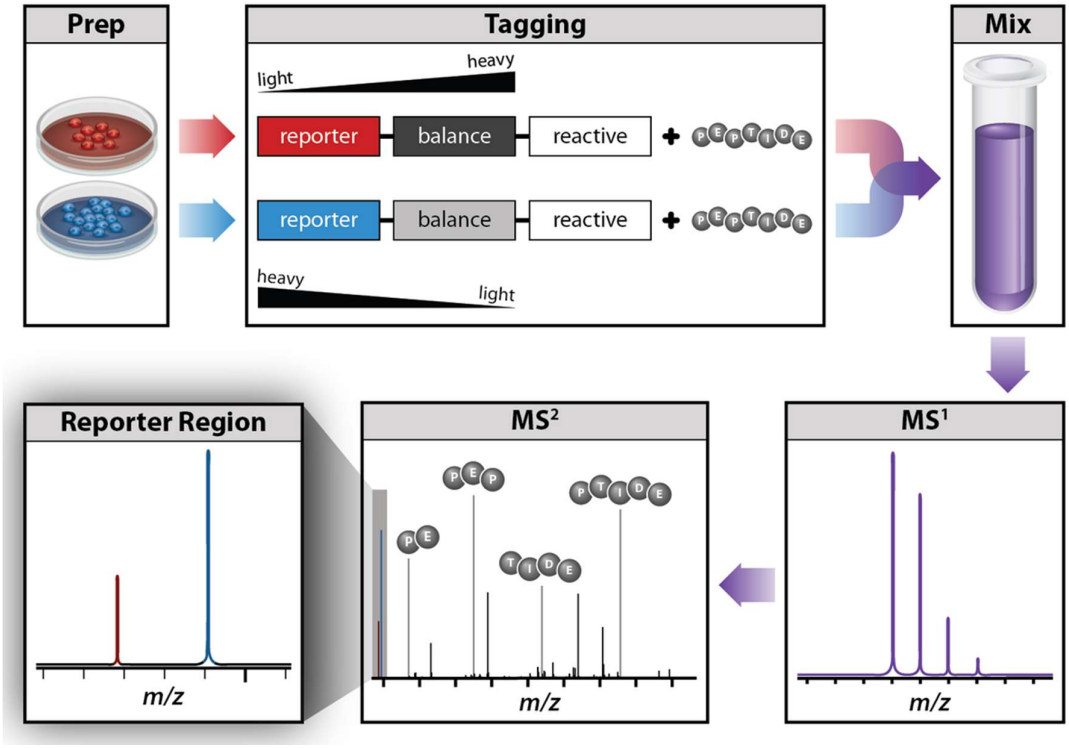


Figure 8. Schematic protocol for labeling of bound proteins using iTRAQ. Taken from https://upload.wikimedia.org/wikipedia/en/thumb/5/52/Isobaric_labeling.png/1200px-Isobaric_labeling.png

Our previous work suggests that the ability of LCN2 to bind iron is required for the ability of LCN2 to regulate invasion and sensitize cells to 5FU (71). Similarly, while LCN2 is required for iron secretion, LCN2 also regulates the intracellular trafficking of EGFR and this doesn't require LCN2 secretion as secretion defective mutants still mediate EGFR trafficking to the cell surface by recycling endosomes (75). We have generated an iron binding mutant of LCN2 (K125AK134A) that doesn't form a complex with iron and are in the process of generating a secretion defective mutant of LCN2. These mutants and WT LCN2 will be tagged with the HA epitope tag at the C-terminus. These constructs and the vector control will be transfected into DLD1 cells. 48 hours post transfection, protein extracts from these cells will be

prepared and IPs performed with antibodies to the HA epitope tag. The reactions will be resolved on SDS-PAGE cells followed by Western blots with antibodies to HA and to the proteins. These experiments will identify proteins that bind differentially to the iron binding mutant and to the secretion mutant as compared to WT LCN2 and will provide further insights into how LCN2 regulates these pathways.

2. To determine the contribution of these interactions to invasion, autophagy and therapy resistance. Our previous results have demonstrated that LCN2 expression leads to increases in therapy resistance, invasion, autophagy, transformation and tumour progression (figures 3-6 and (32,71). The experiments in the previous objective will identify a set of proteins that bind to LCN2 and whether they bind to the secreted form of LCN2 and to the iron binding deficient mutant of LCN2. A prediction of how these proteins might affect invasion autophagy and therapy resistance will be obtained from the KEGG and String analyses described in the first objective. As a first step we will determine the levels of these proteins in cells with high and low levels of LCN2. If the protein levels are elevated in cells with high levels of LCN2, we will inhibit the expression of these proteins using vector driven RNAi and perform invasion assays as described (32,71). If a loss of these proteins leads to changes in LCN2 mediated invasion, we will over-express these genes in cells with low levels of LCN2 to determine if exogenous expression of these proteins is sufficient to promote invasion. Similar experiments will be performed to determine whether these gene products regulate autophagy in response to treatment with 5FU as shown in figures 3-5 and whether they can promote resistance to 5FU in clonogenic assays as previously described (71).

Another possibility is that these proteins are negatively regulated by association with LCN2 and that they are required to inhibit invasion, autophagy and might sensitize cells to therapy. In this case, over-expression of the proteins in cells with high levels of LCN2 might lead to a sensitization of these cells to therapy, prevent the induction of autophagy and prevent invasion. If this is the case, then inhibition of the expression of these genes in cells with low levels of LCN2 should lead to a corresponding increase in invasion, autophagy and therapy resistance in assays similar to the ones described above. This set of experiments should enhance our knowledge of how LCN2 regulates these important pathways and provide further insights into how tumour cells acquire resistance to therapy.

Budget

S. No	Item	Amount
1	iTRAQ labeling kits	4,00,000
2	Cell culture reagents	2,00,000
3	Antibodies	2,00,000
4	Protein and Western blot reagents	2,00,000
	Total	10,00,000

7. DR SYED HASAN, HASAN LAB

Project Title:

Development of CRISPR based highly sensitive and point-of-care diagnostic assay for acute promyelocytic leukemia

Project Summary:

Acute promyelocytic leukemia (APL) is an aggressive subtype of acute myeloid leukemia (AML) (~10–15% cases in AML) characterized by presence of too many promyelocytes (immature blood-forming cells) in the blood and bone marrow. APL is majorly caused by a reciprocal translocation, t(15;17)(q24.1;q21.2) between promyelocytic leukemia (*PML*) and retinoic acid receptor alpha (*RARα*) genes on chromosomes 15 and 17, respectively in 90-95% of patients. APL is a medical emergency and therefore necessitates the prompt diagnosis, initiation of therapy and immediate referral of the patient to specialized cancer centres. The leukaemia is usually diagnosed with patient's vital signs, complete blood count (CBC) and blood cell morphology and later with morphology of bone marrow aspirate. Following the standard diagnostic procedures in patients with acute leukemia the patient suspected for APL is further confirmed using specific qRT-PCR and fluorescence in-situ hybridization (FISH) or Immunofluorescence analysis. All these tests are time taking and required sophisticated instruments as well as experts for analysing the results. Also, multiple methods for APL testing further increase the cost of the diagnosis. Therefore, a rapid and point-of-care detection method is highly warranted for APL diagnosis.

The major causative reciprocal translocations in APL (bcr1 and bcr3) are very specific and can be detected with almost absolute accuracy using nucleic acid detection methods. CRISPR–Cas systems (particularly Cas13 and Cas12) have been employed for the specific, sensitive, and portable diagnosis of nucleic acids. The approach further utilizes the recognition of target sequence using specific guide RNA and after binding to a specific target nonspecific endonuclease activity (collateral activity) of activated Cas13/Cas12 enzymes leading to *trans* cleavage of bystander RNA molecules (aka reporters). The collateral activity of activated Cas that cleaves the reporter complex molecules provide a specific and sensitive indication of the presence of nucleic acid targets in sample.

Here, we aim to develop a CRISPR based highly sensitive and point-of-care diagnostic assay that will provide an alternative and cost-effective method for the detection of APL.

Details study protocol:

A) Background:

Acute promyelocytic leukemia (APL) is an aggressive subtype of acute myeloid leukemia (AML) (~10–15% cases in AML) characterized by presence of too many promyelocytes (immature blood-forming cells) in the blood and bone marrow (Grimwade et al., 2010). The failure in differentiation of promyelocytes into mature blood cells leads to the accumulation of immature and highly proliferative promyelocytes in the body (Golomb, Rowley, Vardiman, Testa, & Butler, 1980). APL is majorly caused by a reciprocal translocation, t(15;17)(q24.1;q21.2) between promyelocytic leukemia (*PML*) and retinoic acid receptor alpha (*RARα*) genes on chromosomes 15 and 17, respectively in 90-95% of patients (Grignani et al., 1994; Rowley, Golomb, & Dougherty, 1977). The disease is highly curable with the treatment of differentiation agent all-trans retinoic acid (ATRA) and arsenic trioxide (ATO) (Ablain et al., 2013; Coombs, Tavakkoli, & Tallman, 2015; Iland, 2019). However, late diagnosis and delayed treatment initiation along with the elevated WBC counts, are significantly associated with severe bleeding and early deaths in APL (Breccia et al., 2010; Micol et al., 2014; Rego et al., 2013). The early death rate in developed countries is approximately 5–10% in newly diagnosed patients, while in less privileged regions it may go up to 20–30% (Breccia et al., 2010; Lehmann et al., 2011). APL is a medical emergency and therefore necessitates

the prompt diagnosis, initiation of therapy and immediate referral of the patient to specialized cancer centres.

The leukaemia is usually diagnosed with patient's vital signs, complete blood count (CBC) and blood cell morphology and later with morphology of bone marrow aspirate (Stein & Tallman, 2012). Following the standard diagnostic procedures in patients with acute leukemia the patient suspected for APL is further confirmed using specific qRT-PCR and fluorescence in-situ hybridization (FISH) or Immunofluorescence analysis (Albano et al., 2015; Borrow et al., 1992; Diverio, Riccioni, Mandelli, & Lo Coco, 1995; Lo-Coco, Ammatuna, Montesinos, & Sanz, 2008). The FISH and immunofluorescence are used to detect the specific translocations whereas determination of the *PML*/*RARα* isoform (bcr1, bcr2, bcr3) require qRT-PCR analysis during diagnosis as well as monitoring of minimal residual disease (MRD) (De Angelis & Breccia, 2015; Dyck, Warrell, Evans, & Miller, 1995; Falini et al., 1997; Grimwade et al., 1996; Reiter, Lengfelder, & Grimwade, 2004). All these tests are time taking and required sophisticated instruments as well as experts for analysing the results. Also, multiple methods for APL testing further increase the cost of the diagnosis. Furthermore, major improvements in cure and prevention of early death rates will depend not only on new drugs but also on the prompt and accurate diagnosis of the disease. Therefore, a rapid and point-of-care detection method is highly warranted for APL diagnosis.

The locations of reciprocal translocations [t(15;17)(q24.1;q21.2)] within the exon/introns of *PML* and *RARα* genes has been studied extensively. Majority of APL patients comprised three major breakpoint clusters in *PML* gene located in intron 3 (bcr3), exon 6 (bcr2) and intron 6 (bcr1) (Cancer Genome Atlas Research et al., 2013; Falini et al., 1997). While there is one breakpoint in *RARα* genomic sequence lying upstream of exon 4 that encodes the B-region of the RAR and N-terminal part of the DNA binding domain. The bcr3 and bcr1 isoforms of *PML* fused with B-region of *RARα* accounts for ~90% of APL patients (Zelent, Guidez, Melnick, Waxman, & Licht, 2001). These reciprocal translocations (bcr1 and bcr3) are very specific and can be detected with almost absolute accuracy using nucleic acid detection methods (Choppa et al., 2003; Huang et al., 1993; Kane et al., 1996).

Recently, CRISPR–Cas systems (particularly Cas13 and Cas12) have been adapted for the specific, sensitive, and portable diagnosis of nucleic acids (Kellner, Koob, Gootenberg, Abudayyeh, & Zhang, 2019). Clustered regularly interspaced short palindromic repeats (CRISPR) and CRISPR-associated (CRISPR-Cas) based adaptive immune systems of microbes comprises programmable endonucleases that can be alternatively used for CRISPR based diagnostics (CRISPR-Dx). Initially discovered Cas enzymes mainly target DNA (Shmakov et al., 2015; Zetsche et al., 2015), while recently discovered single-effector RNA-guided ribonucleases (RNases), such as Cas13a (Shmakov et al., 2015), can be reprogrammed with CRISPR RNAs (crRNAs) to specifically diagnose the target RNA sequence (Abudayyeh et al., 2016; East-Seletsky et al., 2016; Shmakov et al., 2015; Smargon et al., 2017). The approach further utilizes the nonspecific endonuclease activity (collateral activity) of activated Cas13 and Cas12 enzymes leading to *trans* cleavage of bystander RNA molecules (reporters) after binding to a specific target (Gootenberg et al., 2018; Gootenberg et al., 2017). This collateral activity of activated Cas enzyme is used to cleave the reporter complex molecules that provide a specific and sensitive indication of the presence of desired nucleic acid targets using lateral flow assay (Kellner et al., 2019).

B) Rationale:

The FC examination used for APL diagnosis is not conclusive because of heterogeneity of cell surface markers whereas PG-M3 antibody used for IF is not available commercially. The qRT-PCR uses probes, consumables and sophisticated instruments for analysis and generally require about 3-4 days for completion. Multiple methods (FISH+qRT-PCR) for APL testing also increases the cost of the diagnosis. Therefore, a rapid and point-of-care detection method is highly warranted for APL diagnosis. The *PML-RARα* fusions are very specific and can be detected with almost absolute accuracy using CRISPR based method. The proposed CRISPR based multiplexing will facilitate specific and highly sensitive detection of *PML-RARα* fusion at a very affordable cost.

C) Objectives:

- **i.** Development of CRISPR based rapid diagnostic tool for the detection of acute promyelocytic leukemia.
- **ii.** Validation of specificity and sensitivity of diagnostic assay using samples of acute promyelocytic leukemia patients.

D) Novelty/Innovation of Proposed work:

The bcr3 & bcr1 isoforms of *PML* fused with 3rd exon of *RARα* accounts for ~95% of APL patients. These reciprocal translocations are very specific and can be detected with almost absolute accuracy using CRISPR based method, whereas, for bcr2 (~3-6% of APL), we will be using a novel approach (refer methodology). Considering the high specificity and sensitivity of Cas enzymes in a diagnostic assay, we predict that the technique can be used in APL diagnosis with high accuracy.

Keywords: **Acute Promyelocytic Leukemia; CRISPR-Cas; Point-of-care diagnosis; Molecular diagnosis**

Expected output:

In view of low cost associated with CRISPR based reagents, the deliverables of the proposed project can be completed with the research grant of the NPDF scheme. The CRISPR based strategy for the detection of PML-RARα has not been explored yet, however, this technique has been successfully applied for the detection of virus specific sequences in infectious diseases. After standardization and validation of specificity and sensitivity of the assay, we are expecting this technique to be widely available for APL diagnosis at low cost and rapid diagnosis of APL (less than 2 hours). The present assay will provide a point-of-care diagnosis of APL without the requirement of any sophisticated instruments.

E) Plan of work:

Essential consumables: Cas Proteins, TwistAmp Basic RPA kit, RNAse alert, T7/rNTPs, RNAse inhibitors, Plasmids Addgene, PureLink™ HiPure Plasmid Maxiprep Kit, Benzonase nuclease, Strep-Tactin Superflow Plus resin, SUMO protease, HiScribe T7 Quick High Yield RNA Synthesis Kit, Beckman Coulter Agencourt RNAClean XP Kit, SYBR Gold nucleic acid gel stain, NxGen T7 RNA polymerase, RNaseAlert Lab Test Kit v2, Milenia HybriDetect 1, Cas specific antibodies, Other miscellaneous chemicals, Lipofectamine, RPMI media, Pen-Sterp-Neo, Fetal Bovine Serum, cDNA preparation kit, SYBR Green qPCR kit, CTG assay kit.

RNA samples from APL patients: RNA samples from 50 *de novo* APL patients diagnosed with RT-PCR will be enrolled in this study as per the inclusion and exclusion criteria.

Cells and plasmids- NB4, HEK293T, pRSV-Rev, pMDLg/pRRE, pMD2.G; LwaCas13a, PsmCas13b, CcaCas13b, and AsCas12a

In this project, we will use two Cas13 and one Cas12 orthogonal CRISPR enzymes to detect the *PML*/*RARα* fusion transcripts. This assay will use loop-mediated isothermal amplification (LAMP) that can amplify DNA/cDNA and introduce a T7 RNA polymerase promoter, allowing RNA transcription, multiplexing with CRISPR enzymes and a visual readout on lateral flow strips. Detection of nucleic acid targets by Cas enzymes (LwaCas13a, PsmCas13b, and AsCas12a) utilizes specific preferences of dinucleotide motifs (rAU, rAAAAA and AT) connecting the reporter (FAM, HEX, and Cy5 fluorescein) and biotin/quencher and gives a detectable signal after cleavage of these motifs.

Milestone 1 (M1): Development of CRISPR based rapid diagnostic tool for the detection of acute promyelocytic leukemia.

Task 1. (T1) Recombinant expression and purification of Cas Enzymes

The plasmid constructs containing different Cas enzymes (two cas13 and one Cs12) will be procured from Addgene; (plasmid id: 90097, 115211, 113430, and 115208). The plasmid DNA will be transfected into Rosetta 2 cells for protein expression. Induce the expression of Cas enzymes using IPTG and incubate the culture of biological shaker. Harvest the cells by centrifugation at 5,200xg and lyse using 4× (wt/vol) lysis buffer. Centrifuge and add Strep-Tactin Superflow Plus resin to the supernatant and keep it for 2 h with gentle shaking at 4°C. Pour the resin–sample suspension over the prepared column and collect the flow-through. Then wash the collected resin and add 15 mL of SUMO protease cleavage solution to the resin and incubate overnight at 4°C under gentle shaking. The next day, drain the column and collect the cleavage solution into a separate 50-mL centrifuge tube. Wash the remaining sample to ensure complete transfer of the cleaved protein. Using an FPLC system purify the Cas protein with a standardized protocol. Apply the sample and start size-exclusion chromatography. Pool and concentrate the fractions containing Cas protein by centrifugation at 4,000xg for 15 min at 4°C. Add protein storage buffer (SB) to the same centrifugal filter to bring the volume to 15 mL and repeat the centrifugation step. Measure the final protein concentration, dilute to 2 mg/mL in protein SB, and store as 5 µL aliquots at -80°C until use (**Figure 1**).

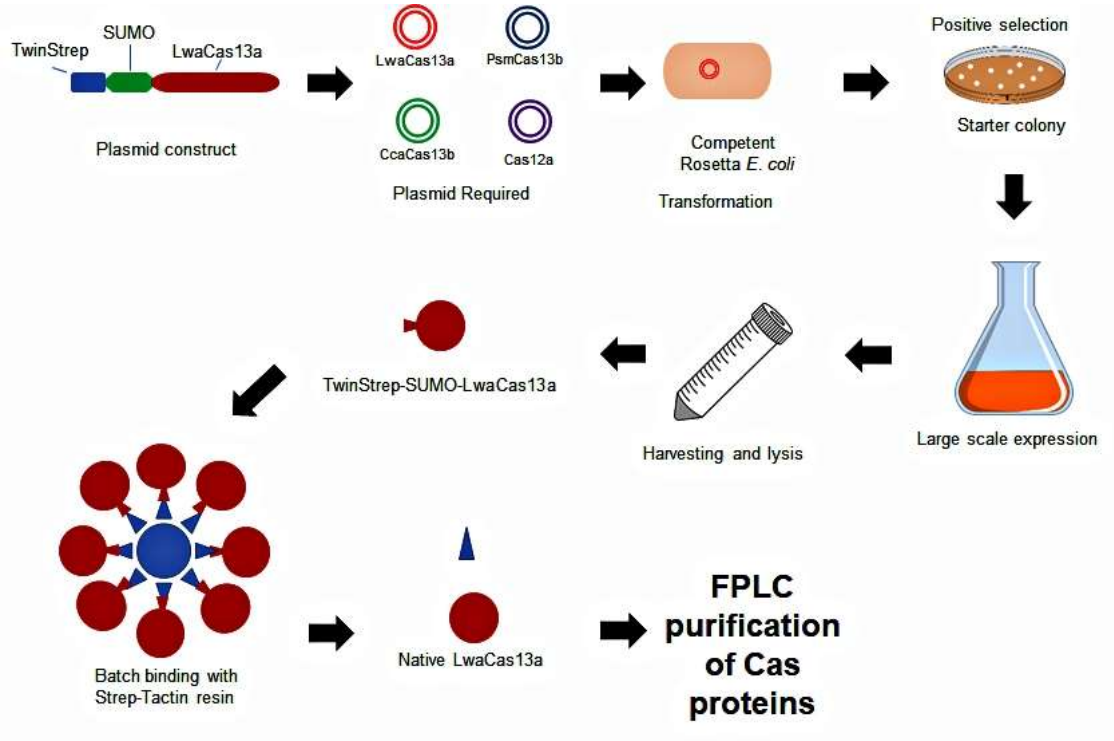


Figure 1: Recombinant expression and purification of Cas proteins.

Task 2. (T2) Preparation of standard plasmids for bcr1, bcr2 and bcr3 variants of PML-RARα fusion

For the initial standardization of the assay, reference plasmids will be prepared by cloning the bcr1, bcr2, and bcr3 variants. For the cloning of PML- RARα fusion transcripts, we will follow the protocol of Dongen et al. (van Dongen et al., 1999). PCR products will be cloned into the PCR II TOPO vector (Invitrogen, The Netherlands). The reference plasmid clones will be sequenced for the confirmation of their inserts using Sanger sequencing.

Task 3. (T3) Standardization of Loop-mediated isothermal amplification of nucleic acid

The LAMP reaction proceeds at a constant temperature using a strand displacement reaction. Four sets of primers are designed (designated as F1-F3, B1-B3, F1c-F3c, and B1c-B3c) on the target region. F2 region of FIP hybridizes to F2c region of the target DNA and initiates complementary strand synthesis (2i-ii). Outer primer F3 hybridizes to the F3c region of the target DNA and extends, displacing the FIP linked complementary strand (2iii). This displaced strand forms a loop at the 5'- end (2iv). This single-stranded DNA with a loop at the 5' end serves as a template for BIP. B2 of BIP hybridizes to B2c region of the template DNA (2iv). DNA synthesis is now initiated, leading to the formation of a complementary strand and opening of the 5' end loop (2v). Now, the outer primer B3 hybridizes to the B3c region of the target DNA and extends, displacing the BIP linked complementary strand (2vi). This results in the formation of a dumbbell-shaped DNA (2vii). The nucleotides are added to the 3' end of F1 by DNA polymerase, which extends and opens up the loop at the 5' end (2viii). The dumbbell-shaped DNA now gets converted to a stem-loop structure. This structure serves as an initiator for LAMP cycling, which is the second stage of the LAMP reaction. To initiate LAMP cycling, the FIP hybridizes to the loop of the stem-loop DNA structure (2ix), and strand synthesis is initiated. As the FIP hybridizes to the loop, the F1 strand is displaced and forms a new loop at the 3' end (2x). Now nucleotides are added to the 3' end of B1. The extension takes place, displacing the FIP strand. This displaced strand again forms a dumbbell-shaped DNA. Subsequent self-primed strand displacement DNA synthesis yields one complementary structure of the original stem-loop DNA and one gap repaired stem-loop DNA. Both these products then serve as a template for a BIP primed strand displacement reaction in the subsequent cycles. Thus, a LAMP target sequence is amplified 13-fold every half cycle (**Figure 2**).

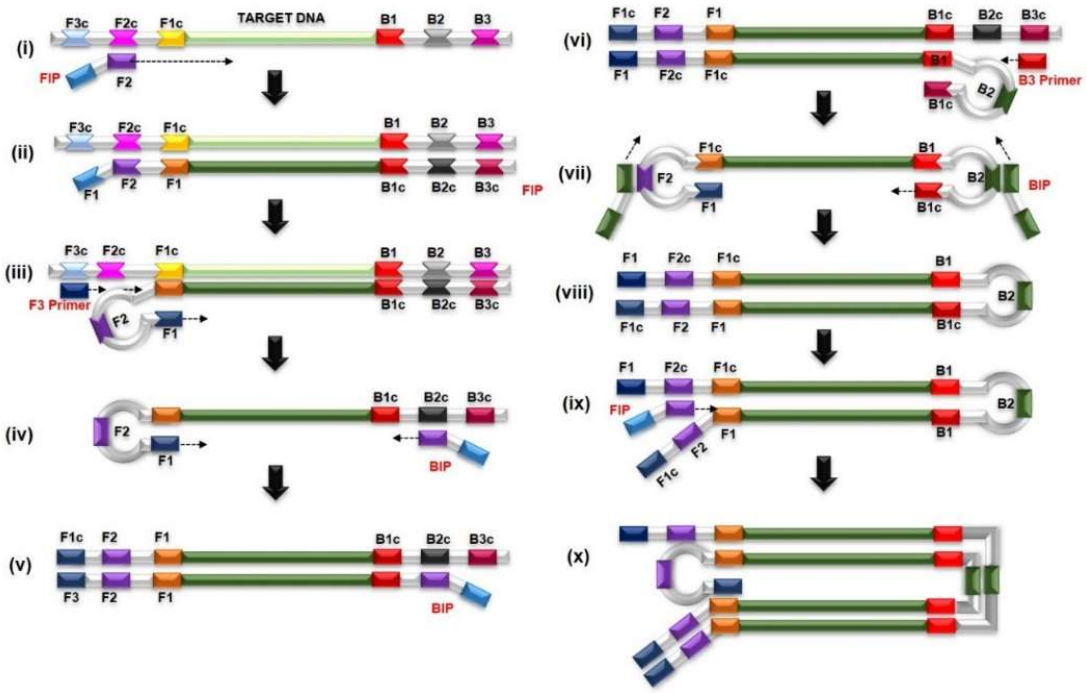


Figure 2: Loop-Mediated Isothermal Amplification (LAMP) technology.

Task 4. (T4) Standardization of the colorimetric-based lateral-flow detection assay

The Cas12 and Cas13 enzymes comprise target-dependent promiscuous DNase and RNase activity, respectively leading to the trans cleavage of adjacent flow reporter molecules. Upon target recognition (i.e., *PML-RARα* fusion sequence), Cas enzymes get activated, cuts its target sequence on fusion transcript, and produce multiple cleavages in adjacent single-stranded DNA/RNA target fluorescein-biotin complex

(linked with RNA/DNA oligos). Anti-fluorescein antibodies labeled with gold nanoparticles (NPs) on sample pad of lateral flow strip (commercially available from TwistDx, UK) will bind to the fluorescein end of the reporter. On the strips, a line of streptavidin will bind to biotin, capturing all the intact reporter in case of a negative sample for *PML-RARα* fusion transcript. When RNA reporters are cleaved (in case of positive samples for the *PML-RARα* fusion) gold NP–labeled antibodies will flow over to the second line of the anti-fluorescein secondary antibody, capturing all the antibodies and forming a dark purple color at the second line that indicates the presence of the target.

The novel approach for detection of PML-RARα fusions: The bcr3 and bcr1 isoforms of *PML* fused with 3rd exon of *RARα* accounts for ~90-95% of APL patients. These reciprocal translocations are very specific and can be detected with almost absolute accuracy using the crRNAs designed from the junctions of *PML* and *RARα* (*PML*:exon3-*RARα*:Exon3 for bcr3 and *PML*:Exon6-*RARα*:Exon3 for bcr1). For bcr2 variants, we have planned a novel method where instead of identifying the *PML*:*RARα* fusion, we will use the indirect approach to identify successful LAMP reaction. For the LAMP reaction, the forward primers [F2(bcr1.2) and FIP(bcr1.2)] are designed from the junctional region of exon5 and exon6 of *PML* and reverse primers from exon3 of *RARα* (BIP and B3) (**Figure 3**).

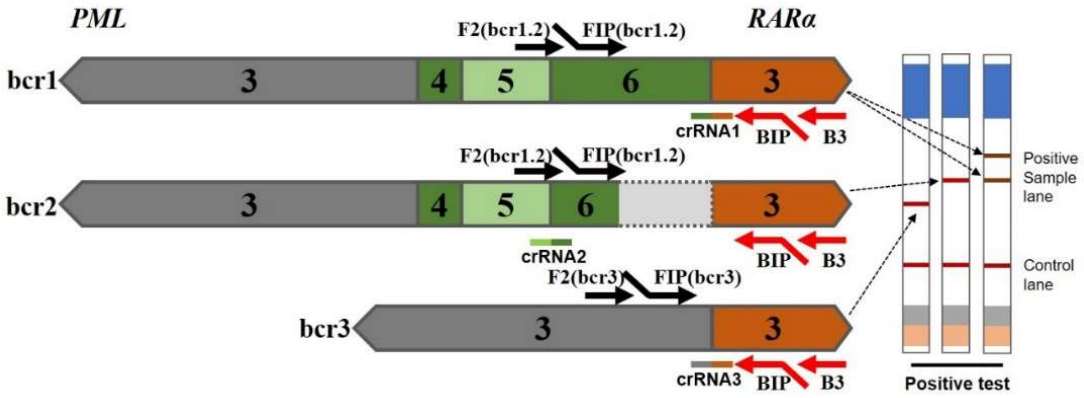


Figure 3: PML-RARα fusions, their amplification, and detection strategy.

The first step will be the conversion of *PML-RARα* fusion transcript into cDNA for LAMP mediated amplification, where BIP or B3 primers will serve as gene specific primers (GSP) for the conversion of *RARα* mRNA as well *RARα* and PML fusion mRNA into cDNA. The LAMP reaction using the primers mentioned above will only amplify the *PML-RARα* fusions (both bcr1 and bcr2 types) and therefore results in the accumulation of concatemers. After the recognition of their targets, each Cas enzyme (Cas13 and Cas12) will also cut the reporter molecules that will be ultimately detected by lateral flow assay.

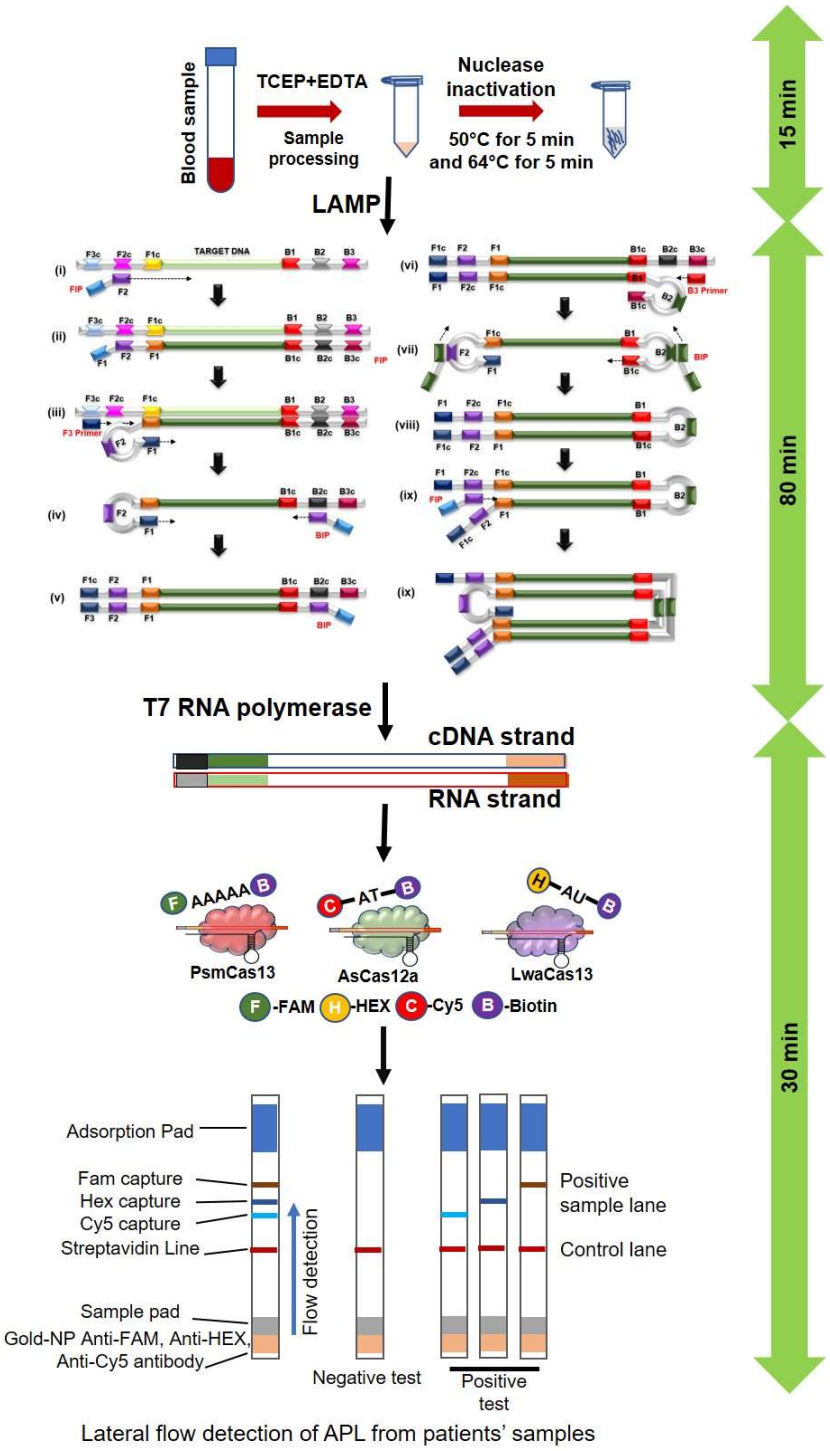


Figure 4: Schematic of multiplexed lateral flow assay using Cas enzymes.

The bcr3 will provide one band for a positive reaction, bcr2 will results in 1 band (due to the detection of exon5 and exon6 of *PML*) while bcr1 will give two bands (one from specific detection of *PML*:exon6-*RARα*:exon3 fusion target and second from the detection of exon 5-6 of *PML*). For avoiding any false positive detection of bcr2 variants (due to the presence of wild type *PML* mRNA), we will use two approaches. (1) The collateral activity of Cas enzymes requires optimum levels of target sequences, and therefore in case of bcr2 indirect detection, the wild type *PML* mRNA may not results in a false-positive reaction; however, the final assay development will require careful standardization. (2) We will use a DNA dependent Cas12 enzyme for bcr2 variant detection. The RNA of the bcr2 variant will only be converted

into cDNA by BIP and B3 designed from *RARα* followed by amplification using LAMP reaction. Therefore, the RNA from wildtype *PML* will not interfere with the specificity of the reaction.

Milestone 2 (M2): Validation of specificity and sensitivity of diagnostic assay using samples of acute promyelocytic leukemia patients.

Task 5. (T5) Patients' sample collection, standardization of RNA extraction protocol and validation of specificity and sensitivity of the diagnostic assay

APL patients' samples will be collected from the Hematopathology Laboratory after the informed consent as per the institute's ethical guidelines. The Hematopathology laboratory of ACTREC carries out the routine diagnosis of APL using the qPCR method. The standardization of assay will be initially carried out using reference plasmids, and further validation on APL patients' samples will be carried out at the Hematopathology laboratory of ACTREC.

For sample processing, we will also standardize the HUDSON (heating unextracted diagnostic samples to obliterate Nucleases) protocol of Myhrvold et al. (Myhrvold et al., 2018) or protocol of Ladha et al. (Ladha, Joung, Abudayyeh, Gootenberg, & Zhang, 2020) that does not require any RNA extraction step. The protocol has been standardized for qRT-PCR based detection of nucleic acid targets from the blood sample. The protocol requires 5 min for sample extraction and very cheap compared to RNA extraction protocols. After standardization and validation, the protocol for RNA extraction may be adapted along with the proposed diagnostic method for point-of-care detection. Alternatively, the RNA sample extraction will be carried out using the RNeasy Plus Mini Kit (Qiagen, DE) as per the recommended protocol for comparison. The specificity of the assay will be checked using 50 retrospective patient samples (analyzed using qRT-PCR) obtained from our hematopathology laboratory. Also, the assay will be used to diagnose 25 prospective samples. The bcr variant type detected in these 25 prospective patients using multiplex lateral flow assay will also be confirmed using Sanger sequencing. The sensitivity of diagnostic assay will be carried out using serial dilutions of 25 patient samples (the copy number of the undiluted samples will be initially analyzed by qRT-PCR).

Timeline of the project activities.

Activities	**Timeline (Months)**			
Development of CRISPR based highly sensitive and point-of-care diagnostic assay for acute promyelocytic leukemia	**06**	**12**	**18**	**24**
M 1: Development of CRISPR based rapid diagnostic tool for the detection of acute promyelocytic leukemia				
T1. Recombinant expression and purification of Cas enzymes				
T2. Preparation of standard plasmids for bcr1, bcr2 and bcr3 variants of *PML-RARα* fusion				
T3. Standardization of Loop-mediated isothermal amplification of nucleic acid				
T4. Standardization of the colorimetric-based lateral-flow detection assay				
M 2: Validation of specificity and sensitivity of diagnostic assay using samples of acute promyelocytic leukemia patients.				
T5. Patients' sample collection, standardization of RNA extraction protocol and validation of specificity and sensitivity of the diagnostic assay				
T6. Report preparation, publication				
M # - Milestone; T # - Task				