DeRiMe - Deciphering Richter's syndrome Metabolism to identify novel therapeutic opportunities

❖ **Abstract**

Richter's syndrome (RS) is the transformation of chronic lymphocytic leukemia (CLL) into an aggressive lymphoma, typically a diffuse large B cell lymphoma. The clinical outcome of RS is generally poor, with a median survival of few months. Despite the tremendous advancement in CLL biology and therapy, limited knowledge and therapeutic options are available for RS, even because of different conditions as lack of cell lines and murine models that have hampered the understanding of genetic and biological mechanisms driving or contributing to the pathogenesis of this disease. For all these reasons, RS is a clinical need.

This project aims at exploring and defining the main metabolic pathways RS cells rely on to produce energy and the "building blocks" necessary to support the high proliferative rate. This feature will be investigated *per se* and as a result of the convergence on metabolism of different critical signaling pathways of B cells, such as the BCR cascade. Analyses will be performed taking advantage of RS-patient-derived xenograft models, established by the Proponent, and comparing RS cells with CLL samples to understand whether transformation into an aggressive lymphoma is reflected also by a metabolic reprogramming of these cells. Gaining insights in the metabolic profile of RS cells is relevant in a scientific perspective, but it is critical also by a translational standpoint to define potential therapeutic targets and design novel strategies, even based on the combination of targeted inhibitors or repurposed drugs.

The inference of the project will be the identification of novel or "old, but with new use" potential "bullets" in a bench-to-bedside translational perspective.

❖ **Riassunto (Italiano)**

La sindrome di Richter (RS) è la trasformazione della leucemia linfatica cronica (LLC) in un linfoma aggressivo, tipicamente un linfoma diffuso a grandi cellule B. La RS ha una prognosi infausta con una sopravvivenza di pochi mesi. Nonostante gli avanzamenti nella comprensione della patogenesi e nel trattamento della LLC, le conoscenze della RS sono limitate con poche opportunità terapeutiche, anche a causa della mancanza di linee cellulari e di modelli murini, che ha ostacolato la comprensione dei meccanismi genetici e biologici che guidano o contribuiscono alla patogenesi della malattia. Per queste ragioni, la RS è una necessità clinica.

Il progetto ha lo scopo di esplorare e definire le principali vie metaboliche da cui le cellule di RS dipendono per produrre energia e per la sintesi delle molecole necessarie a supportare l'elevato tasso di proliferazione. Il metabolismo della RS sarà indagato per sé, ma verrà studiata anche la sua modulazione da parte di specifiche vie di segnale, come la cascata del BCR. Le analisi verranno condotte sfruttando dei modelli di xenotrapianto di RS, stabilizzati dal Proponente, e confrontando la RS con la LLC per capire se la trasformazione in un linfoma aggressivo si riflette anche in un cambiamento metabolico di queste cellule. Fare luce sul profilo metabolico delle cellule di RS è importante sia dal punto di vista scientifico che dal punto di vista traslazionale per definire dei possibili bersagli terapeutici e disegnare nuove terapie, basate sia sulla combinazione di inibitori mirati al bersaglio che sul riposizionamento di farmaci. I risultati del progetto sono rappresentati dalla identificazione di nuove strategie terapeutiche o dall'uso di "vecchi, ma con nuove applicazioni" farmaci in una prospettiva traslazionale che arrivi al paziente.

❖ **Background**

Richter's syndrome (RS) is the acute transformation of chronic lymphocytic leukemia (CLL) into an aggressive lymphoma. It is a relatively rare condition occurring in the natural history of CLL with a cumulative incidence of approximately 10%[1]. The clinical outcome of RS is generally poor, with a median survival of few months[2]. Previous research has increased our knowledge on evolutionary patterns of RS, providing insights on risk factors predisposing to transformation[3].

Metabolic rewiring is a hallmark of cancer which allows cells to modify their metabolism and fulfill the requirements needed to sustain survival and proliferation. Changes in metabolic phenotypes can contribute both to transformation and tumor progression[4,5] and each type of cancer has distinct features and modifies its metabolism in response to diverse stimuli[6,7]. CLL lymphocytes display heightened mitochondrial respiration, elevated levels of reactive oxygen species and enhanced antioxidant capacity[8-10] compared to normal B cells. Metabolic based approaches have been tested in the clinic for CLL, mainly based on preclinical results targeting mitochondrial respiration and glucose uptake (www.clinicaltrials.gov)[11].

A point to be taken in mind when focusing on metabolic aspects of neoplastic B cells is the role of the BCR, a driving forces of these cells. Indeed, several components of this pathway are known to modulate and promote

metabolic responses. Several evidence suggests a role for Btk, NF-kB and Myc in influencing glucose and glutamine uptake, as well as fatty acid metabolism[11-14] and ample literature supports the role of PI3K-Akt pathway in cancer cells metabolic reprogramming through the modulation of substrate transporters and enzymes[15]. Several metabolic inhibitors have been developed with the intent to kill or sensitize cancer cells to chemotherapies[16]. So far, more than 100 clinical trials using metabolic inhibitors are ongoing in the field of cancer[17].

❖ **Preliminary data**

While significant efforts have been dedicated to CLL to study its genetic and biological features, little is known on RS. Our group is interested in studying this lymphoma from different complementary perspectives to gain insights with both scientific and translational implications.

This project arises from these two pieces of evidence.

1) A preliminary comparison between CLL and RS cells, looking at the transcriptomic data, indicates clear distinct profiles, suggesting that RS evolves from CLL being a distinct disease entity and adapting its metabolic demand and substrate usage to sustain a more aggressive behavior. Preliminary gene ontology analyses on RS upregulated genes revealed "mitochondrial organization and functions" as well as "nucleotides biosynthetic processes" as the most enriched terms.
2) Evidence from literature and from our previous results in CLL indicate that elements of the BCR signaling cascade can modulate several metabolic aspects. Indeed, we have shown that inhibition of NF-kB in CLL cells results in a compromised mitochondrial respiration and in the induction of reactive oxygen species[14]. Moreover, we have recently reported that duvelisib, a PI3K inhibitor, alone or in association with venetoclax, a drug targeting Bcl-2, is capable of inducing apoptosis in RS cells, maybe as consequence of the modulation of the metabolic demand of these neoplastic cells[18].

❖ **Experimental plan**

The main aims of this project are i) to determine the metabolic profile and dependency of RS capable of sustaining its aggressive behavior; ii) to analyze the potential modulation of metabolic features of RS by elements of the BCR signaling cascade or of the anti-apoptotic machinery; iii) to test the impact of drugs specifically targeting or inhibiting metabolic pathways. The project relies on the availability of primary samples as well as on RS cells purified from unique RS-patient derived xenograft (PDX) models[19].

Task 1. Transcriptomic analysis of the metabolic profile of RS versus CLL. Timing: 0-8 months

This part of the project will be dedicated to the analyses of the transcriptomic profile of RS and CLL cells, pointing the attention specifically on genes related to metabolism with the aim of identifying both similarities and dissimilarities. Data referring to CLL are freely available in several databases (e.g., TCGA)[20], while data for RS are in part already available in the lab and partly will be obtained during the first months of this project. The former ones were obtained in the past months by sequencing primary samples together with the derived-RS-PDX models. The latter ones will be obtained by sequencing a cohort of 16 RS primary samples obtained through a collaboration with different hematologists and pathologists. Expression of key genes involved in the main metabolic pathways will be validated by RT-PCR and western blot.

Task 2. Functional analysis of metabolic pathways active in RS. Timing: 2-12 months

The aim of this task is to sketch a picture of the main metabolic pathways RS cells rely on. To address this point, different experimental approaches will be adopted, analyzing PDXs-derived RS cells. Specifically:

i) Real-time evaluation of the cellular energetic status by checking the a) ATP/AMP ratio, b) the oxygen consumption rate (OCR), c) the extra-cellular acidification rate (EACR), and d) the ATP synthesis through the Fo-F1 ATP synthase as markers of oxidative or glycolytic metabolism;
ii) Evaluation of metabolic dependency looking at the three primary substrates that fuel the mitochondria, long chain fatty acids, glucose/pyruvate, and/or glutamine, to investigate how RS cells shift oxidation of specific substrates. Analyses described in i) and ii) will be performed with the Seahorse platform (available at the Dept. of Medical Sciences);
iii) *Ad-hoc* functional experiments based on the administration of fluorescent-labelled substrates or probes to trace the active metabolic pathways by confocal microscopy or flow cytometry analysis (e.g., 2-NBDG for glucose uptake and BODIPY for fatty acid/lipid uptake and storage inside the cells);
iv) Evaluation of the production of oxidative stress as consequence of aerobic metabolism by the analysis of reactive oxygen species (ROS) production, malondialdehyde levels as a marker of lipid peroxidation, activity of antioxidant enzymes (e.g., catalase superoxide dismutase and glutathione peroxidase).

To shed light on potential similarities/differences between RS and CLL, read-outs will be performed also on CLL cells obtained from patients characterized by an indolent or aggressive disease.

Task 3. Metabolic rewiring of RS following BCR and apoptotic signaling cascades interference. Timing: 10-18.

Cellular metabolism is controlled at different levels by several molecular pathways within the cells, including the PI3K-Akt network, NF-kB and anti-apoptotic proteins (e.g., Bcl-2)[15,21,22], which have been shown to regulate nutrient transporters, metabolic enzymes as well as to control transcription factors that regulate the expression of key metabolic components. Moreover, we have recently showed that RS cells are sensitive to the duvelisib (PI3K inhibitor) and venetoclax (Bcl-2 inhibitor) treatment through the modulation of Akt, Myc and Mcl-1[18].

This task is aimed at understanding whether interfering with PI3K, Bcl-2 and NF-kB may impact on the metabolic demand and reliance of RS cells. To this purpose, RS cells will be treated with selective inhibitors and transcriptional/post-translational regulation of metabolic enzymes and downstream process will be assayed by:

i) Real-time evaluation of the cellular energetic status by checking the a) ATP/AMP ratio, b) the oxygen consumption rate (OCR), c) the extra-cellular acidification rate (EACR), as in Task 2;
ii) Evaluation of potential metabolic shifts to use different source of energy (long chain fatty acids, glucose/pyruvate, and/or glutamine) as consequence of drug exposure;
iii) Evaluation of reactive oxygen species production and mitochondrial membrane potential;
iv) Evaluation of the transcriptional levels of genes encoding for metabolic enzymes, genes regulated by transcription factors (e.g., *FOXO, MYC, ATF4*) under the control of Akt/NF-kB/Bcl-2/Mcl-1.

Task 4. Impact of metabolic targeting by drug repurposing. Timing: 18-24

Mounting evidence shows that many metabolic features of cancer cells, such as dysregulated Warburg-like glucose metabolism, increased fatty acid synthesis, glutaminolysis, increased mitochondrial metabolism at least, in part, drive chemoresistance in cancer, thus having crucial implications for targeted and efficacious therapies. Therefore, targeting metabolic pathways that import, catabolize, and synthesize essential cellular components, can be effectively in re-sensitizing drug-resistant cancer cells to apoptosis.

This task is complementary to Task 3 and will explore the effects of a couple of drugs, Metformin and Ritonavir, recently reconsidered for the treatment of selective neoplasia. Metformin is in a Phase II clinical trial for patients affected by diffuse large B cell lymphoma and CLL and it acts through the inhibition of the PI3K-Akt pathway, interfering with the OXPHOS metabolism. Ritonavir is a glucose transporter inhibitor acting on GLUT4 thus preventing the availability of this substrate for glycolytic consumption by neoplastic cells. After exposure to these drugs, RS cells will be assayed as indicated in Task 3.

❖ Expected results and contingency plan

This project aims at investigating the metabolic profile and dependency of RS cells, a critical aspect in cancer biology. Results are expected to be of relevance both for i) the scientific community, to gain insight into a disease with fragmentary knowledge of pathogenetic mechanisms, and ii) patients in a translational perspective. Putting together the pieces of the puzzle and understanding how these neoplastic cells function is essential to design effective therapeutic strategies to improve RS patients' outcome.

One of the main challenges that can be envisaged is the impact of the targeting described. Indeed, duvelisib and venetoclax long-term exposure results in apoptosis of RS cells, but nothing is known about their impact on metabolism. A similar observation can be made for metformin and ritonavir, whose effects on RS cells are unknown. However, several pieces of evidence obtained in CLL indicate that they are effective in interfering with leukemic cells metabolic demand. These potential limitations may be overcome by alternative strategies adopted based on the results obtained in the first tasks of the proposal. Indeed, they are designed to obtain a snapshot of the metabolic profile of RS cells that can be of help in tracing novel targeting approaches in addition to the ones already indicated in Tasks 3-4.

❖ CV: Tiziana Vaisitti, Associate Professor of Medical Genetics

- **Positions and Honors**

2002-2003: Internal student, Lab of Analytical Chemistry, Dept. of Chemistry, Univ. of Torino, Italy.

2003-2006: PhD student, Lab of Immunogenetics, Dept. of Genetics, Biology and Biochemistry, Univ. of Torino, Italy.

2004: Visiting scientist, Dept. of Evolutionary Biology, Univ. of Siena, Italy.

2007: Visiting scientist, The Feinstein Institute for Medical Research, North Shore-Long Island Jewish, Manhasset, NY.

2009: Visiting scientist, Dept. of Medical Biochemistry and Immunology, Cardiff University.

2007-2009: AIRC/FIRC Fellowship, Lab. of Immunogenetics, Dept. of Genetics, Biology and Biochemistry, Univ. of Torino, Italy.

2009-2014: Senior Post-Doc, Dept. of Medical Sciences and Human Genetics Foundation, Univ. of Torino.

2014-2016: Visiting Fellow, Dept. of Pathology and Laboratory Medicine, Weill Cornell Medicine, New York.

2017-2018: Assistant Professor of Medical Genetics (RTD A), Dept. of Medical Sciences, Univ. of Torino.

2018-2021: Assistant Professor of Medical Genetics (RTD B), Dept. of Medical Sciences, Univ. of Torino.

2021-: Associate Professor of Medical Genetics, Dept. of Medical Sciences, Univ. of Torino.

2011: AACR-SIC Scholar in Training award from American Association for Cancer Research

2017: Best abstract award for abstract presented at the XVII International Workshop on Chronic Lymphocytic Leukemia, New York.

- **Contribution to Science**

1. Identification and functional characterization of novel genetic lesions in chronic lymphoproliferative diseases. Dr. Vaisitti is a part of a net of researchers working on the discovery and functional validation of genetic lesions characterizing patients with selected hematological malignancies.
2. Set-up and genetic/transcriptomic analysis of patient-derived xenograft models of Richter's syndrome. Dr. Vaisitti is the PI of several projects that aim at genetically and functionally characterize Richter's syndrome cells in a translational perspective.
3. Role of nucleotides and nucleotide-metabolizing enzymes in shaping the tumor niche. Dr. Vaisitti is part of a group of researchers dedicated to the functional analysis of these enzymes in CLL.
4. Identification by clinical exome sequencing of mutations relevant for the diagnosis of genetic diseases responsible for organ failure (kidney, liver, heart). Dr. Vaisitti is responsible for the sequencing and bioinformatics data analysis.
5. Identification of early markers of organ rejection by combining liquid biopsy (cell free DNA) and droplet digital PCR. Dr. Vaisitti is part of a net of researchers working on the set up and validation of protocols based on cfDNA analysis to detect organ rejection.

- **Scientific output**

Publications with Impact Factor (2005-2021): **55;** H-Index (Scopus): **26;**

Publications in the last 5-years (2016-2021): **25;** Impact-factor in the last 5-years (2016-2021): **8,495**

1) Vaisitti T. et al., "Targeting metabolism and survival in chronic lymphocytic leukemia and Richter syndrome cells by a novel NF-kB inhibitor." Haematologica. 2017 Nov;102(11):1878-1889. *(I.F. 7.702)*
2) Vaisitti T. et al., "Novel Richter's syndrome xenograft models to study genetic architecture, biology and therapy responses" Cancer Res. 2018 Jul 1;78(13):3413-3420. (*I.F. 9.122)*
3) Vaisitti T. et al, "ROR1 targeting with the antibody drug-conjugate VLS-101 is effective in Richter syndrome patient-derived xenograft mouse models." Blood. 2021 Jan 14:blood.2020008404. *(I.F. 22.113)*
4) Iannello A. et al., **"**Synergistic efficacy of the dual PI3K-δ/γ inhibitor duvelisib with the Bcl-2 inhibitor venetoclax in Richter syndrome PDX models." Blood. 2021 Jun 17;137(24):3378-3389. *(I.F. 22.113)*
5) Chakraborty S. et al., "B Cell Receptor signaling and genetic lesions in TP53 and CDKN2A/CDKN2B cooperate in Richter Transformation" Blood. 2021 Sep 23;138(12):1053-1066. *(I.F. 22.113)*

- **Research Support**

2018-2021: Italian Ministry of Health, Young Investigator Grant #GR-2016-02364298 "Highlighting the tumorigenic role of long non coding RNA in patients with Anaplastic Large cell Lymphoma". (PI of one Unit).

2019-2021: Ricerca Locale ex-60%, University of Torino "Next Generation sequencing (NGS) to screen for inherited cardiac conditions leading to organ failure".

2020-2025: Italian Association for Cancer Research (AIRC) - My First AIRC Grant #23107 "Probing Richter's syndrome by multiple "omics" approaches to find its Achille's heel".

2021-2023: Fondazione CRT "Erogazioni ordinarie" - "Analisi della risposta terapeutica di cellule neoplastiche di sindrome di Richter: colpire nuovi bersagli molecolari".

- **Selected lectures and seminars**

2016: Speaker at the 58th American Society of Hematology (ASH) Annual meeting, San Diego (CA).

2017: Speaker at the 17th International Workshop on Chronic Lymphocytic Leukemia, New York (NY).
2018: Invited Seminar: "Richter's syndrome: an orphan disease in search of identity" University of Genoa, Genoa, Italy.
2018: Speaker at the 60th American Society of Hematology (ASH) Annual meeting, San Diego (CA).
2019: Invited Seminar: "Richter's syndrome: looking for culprit(s)" Azienda USL-IRCCS di Reggio Emilia, Italy
2021: Speaker at the 63rd American Society of Hematology (ASH) Annual meeting, Atlanta (GE).

- **Previous experience in collaborative research**

In the last 10 years, I've been working in several collaborative projects, involving both italian and foreign partecipants, on different research topics: i) identification and functional characterization of recurrently mutated genes in chronic lymphoproliferative syndromes. These studies led to the recognition of mutations in *NOTCH1, SF3B1* and *BIRC3* in CLL patients and of *NOTCH2* in SMZL patients; ii) set-up and genetic characterization of xenograft models of Richter's syndrome; iii) identification and characterization of genetic lesions contributing to Richter's syndrome transformation. All these collaborations led to scientific papers published in leading journals in the field of haematology.

❖ **TEAM**

1) **Tiziana Vaisitti, Ph.D.**, the Proponent, will be the coordinator of the project. She will take an active role in designing the experimental plan, discussing the results with the rest of the team and preparing the relevant manuscripts. She will be actively involved in the analysis of the transcriptomic profile of RS cells (Task 1) and provide her skills to set up and run metabolic experiments.
2) **Andrea Iannello, Ph.D., Post-doc,** graduated from the Biology School in 2013, he obtained his PhD in Complex system for life sciences, University of Torino, in 2019. He is trained in cellular and molecular biology, and sequencing data analysis. Since joining the PI's lab in December 2018, he's worked with in vivo models and has been involved in the functional characterization of specific signaling pathways in CLL and RS cells. He will take care of metabolic experiments, data analyses, and drugs screening (Tasks 2-4).
3) **Lorenzo Brandimarte, B.S.,** is a PhD student at the University of Turin (PhD curricula: Biomedical Sciences and Oncology) who joined the PI's lab in Autumn 2020, after obtaining his Master Degree in Biotechnology. He is trained in cellular/molecular biology and in in vivo working. He will take care of the maintenance of the available RS-PDX models to obtain RS cells, confocal microscopy analyses and will coordinately work with Dr. Iannello in performing ex-vivo treatment of RS cells with selective drugs, prior to analyze their metabolic profile (Tasks 2-4).
4) **Matilde Micillo,** is a undergraduate student (Biotechnology) with experience in biochemical and molecular biology analyses. She will take care of RT-PCR and western blotting analyses to profile the expression of enzymes involved in the main metabolic pathways (Tasks 1-4) and cytofluorimetric analyses (Tasks 2-4).

1 Rossi, D. & Gaidano, G. *Semin Oncol* **43**, 311-319, doi:10.1053/j.seminoncol.2016.02.012 (2016).
2 Khan, M., *et al. Ann Hematol* **97**, 1-15, doi:10.1007/s00277-017-3149-9 (2018).
3 Parikh, S. A. & Shanafelt, T. D. *Curr Hematol Malig Rep* **9**, 294-299, doi:10.1007/s11899-014-0223-4 (2014).
4 Cluntun, A. A., *et al. Trends Cancer* **3**, 169-180, doi:10.1016/j.trecan.2017.01.005 (2017).
5 DeBerardinis, R. J. & Chandel, N. S. *Sci Adv* **2**, e1600200, doi:10.1126/sciadv.1600200 (2016).
6 Gentric, G., *et al. Antioxid Redox Signal* **26**, 462-485, doi:10.1089/ars.2016.6750 (2017).
7 Wolpaw, A. J. & Dang, C. V. *Trends Cell Biol* **28**, 201-212, doi:10.1016/j.tcb.2017.11.006 (2018).
8 Jitschin, R. *et al. Blood* **123**, 2663-2672, doi:10.1182/blood-2013-10-532200 (2014).
9 Mayer, R. L. *et al. Mol Cell Proteomics* **17**, 290-303, doi:10.1074/mcp.RA117.000425 (2018).
10 Tili, E. *et al. Blood* **120**, 2631-2638, doi:10.1182/blood-2012-03-415737 (2012).
11 Galicia-Vazquez, G., *et al.. Blood Cancer J* **8**, 13, doi:10.1038/s41408-017-0039-2 (2018).
12 Galicia-Vazquez, G. & Aloyz, R. *Front Oncol* **8**, 411, doi:10.3389/fonc.2018.00411 (2018).
13 Rozovski, U. *et al. Leuk Lymphoma* **59**, 2686-2691, doi:10.1080/10428194.2018.1439167 (2018).
14 Vaisitti, T. *et al. Haematologica* **102**, 1878-1889, doi:10.3324/haematol.2017.173419 (2017).
15 Hoxhaj, G. & Manning, B. D. *Nat Rev Cancer* **20**, 74-88, doi:10.1038/s41568-019-0216-7 (2020).
16 Galluzzi, L., *et al. Nat Rev Drug Discov* **12**, 829-846, doi:10.1038/nrd4145 (2013).
17 Meynet, O. & Ricci, J. E. *Trends Mol Med* **20**, 419-427, doi:10.1016/j.molmed.2014.05.001 (2014).
18 Iannello, A., *et al. Blood* (2021).
19 Vaisitti, T. *et al. Cancer Res* **78**, 3413-3420, doi:10.1158/0008-5472.CAN-17-4004 (2018).
20 Beekman, R. *et al. Nat Med* **24**, 868-880, doi:10.1038/s41591-018-0028-4 (2018).
21 Kapoor, I., *et al. Cell Death Dis* **11**, 941, doi:10.1038/s41419-020-03144-y (2020).
22 Roca-Portoles, A. *et al. Cell Death Dis* **11**, 616, doi:10.1038/s41419-020-02867-2 (2020).