PROTOCOL

Protocol Title:	Collection of Peripheral Blood from Patients with Myelodysplastic Syndrome, Acute Myeloid Leukemia, or Chronic Myelomonocytic Leukemia for Measuring Baseline Ascorbic Acid Levels and Future Epigenetic Cancer Research
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1. BACKGROUND AND RATIONALE

Research over the past two decades has shown that epigenetic changes are essential in the initiation and development of hematological cancer, and that DNA methylation are among the earliest and most common events in carcinogenesis (Baylin and Jones, 2011). De novo methylation of the promoters and enhancers of tumor suppressors is an alternative to DNA mutation in gene silencing in cancer. An additional layer of epigenetic regulation of gene expression relies on the methylation of lysine residues on the histone tails.

Recent investigations have shown that mutations in epigenetic regulators are common, both in the apparently normal hematopoiesis of the elderly (Busque et al., 2016; Genovese et al., 2014; Jaiswal et al., 2014), and in patients with myeloid cancers (Shih et al., 2012).

It was long anticipated that DNA methylation was a permanent silencing mark, but with the discovery of the TET enzymes (Tahiliani et al., 2009) it became clear that active demethylation occurs. The initial steps in this process are catalyzed by TET enzymes (Pastor et al., 2013), which are however frequently mutated (Shih et al., 2012) and methylated (Cimmino et al., 2015) in hematological cancers. The Jumonji enzymes, which catalyze histone demethylation, are also aberrantly regulated in hematological cancers (Monfort and Wutz, 2013).

Vitamin C was identified in the 1930'ies as the necessary micronutrient in the prevention of scurvy (Grzybowski and Pietrzak, 2013). Unlike plants and most animals, humans are unable to synthesize vitamin C from glucose due to lack of the required enzyme, L-gulonolactone oxidase (Nishikimi et al., 1994). Therefore, vitamin C must be provided through the diet. The role of vitamin C in cancer has remained controversial. Initial studies of high dose vitamin C supplement showed increased survival time and palliative effects (Cameron and Campbell, 1974; Cameron and Pauling, 1978). However, later two randomized, placebo-controlled trials were unable to confirm the findings by Cameron and coworkers. In these trials cancer patients were given oral vitamin C (10 g/day) or placebo, but no significant differences were found with regards to symptoms or survival time (Creagan et al., 1979; Moertel et al., 1985). More recently, several groups suggested the use of vitamin C in combination with chemotherapy, the rationale being that vitamin C may both enhance the effects of the chemotherapy and/or reduce side effects (Du et al., 2013; Jacobs et al., 2015). Mechanistic studies showed that vitamin C is capable of killing *KRAS* and *BRAF* mutated colon cancer cells by elevating the levels of endogenous reactive oxygen species (ROS), which inhibit GAPDH leading to energy depletion and cell death (Yun et al., 2015). Importantly, recent investigations from our group show that hematological cancer patients are severely vitamin C deficient (Liu, Ørskov et al., submitted).

In addition to the functions of vitamin C described above, recent studies recognize vitamin C as an important cofactor for the Fe(II)- and 2-oxoglutarate dioxygenase family. These include the TET enzymes, which are involved in the conversion of 5-methylcytosine (5-mC)to its oxidized derivatives 5-hydroxymethylsytosine (5-hmC), 5-carboxyl cytosine (5-caC) and 5-formylcytosine (5-fC) (Blaschke et al., 2014), and the Jumonji enzymes that are involved in histone demethylation (Monfort and Wutz, 2013) (Figure 1). Accordingly, vitamin C may potentially play an important role in the regulation of DNA and histone demethylation.

It has been shown that the formation of 5-hmC and its derivatives may be compromised in healthy individuals (Busque et al., 2016) and patients with TET mutations (Ko et al., 2010). However, since many of these mutations are heterozygous, and since the three TET enzymes (TET1, TET2 and TET3) may have some redundancy, we speculate whether restoration of vitamin C to physiological levels may have an impact on the level of 5-hmC/5-mC in individuals with TET mutant clonal hematopoiesis or hematological cancer.

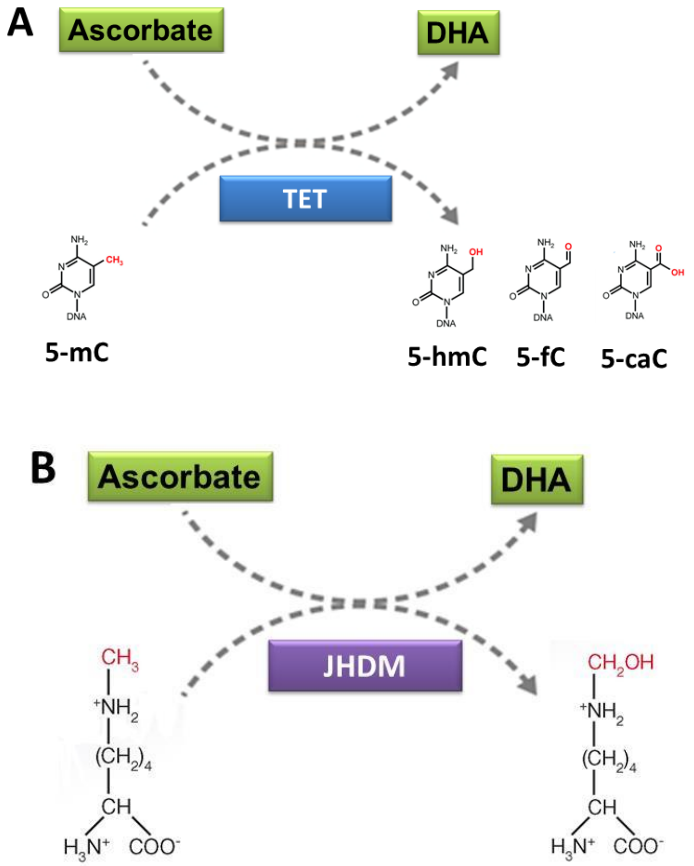


Figure 1: Vitamin C as a cofactor: Ascorbate (the dominant form of vitamin C at physiological pH) is an essential cofactor for **A)** the TET-mediated oxidation of 5-mC to 5-hmC, 5-fC and 5-caC and **B)** the Jumonji-domain-containing histone demethylases which catalyze the demethylation of lysine residues of histone proteins. In both processes ascorbate is reduced to dehydroascorbic acid (DHA).

Because of its dynamic nature, DNA methylation is attractive as a therapeutic target of EMA- and FDA-approved DNA methyltransferase inhibitors (DNMTi's) (5-aza-2′-deoxycytidine (5-aza-CdR) or 5-azacytidine (5-aza-CR)), which are currently the drugs of choice in the treatment of higher risk myelodysplastic syndrome (Fenaux et al., 2009) and certain subtypes of AML (Fenaux et al., 2010; Treppendahl et al., 2014). 5-Aza-CdR is incorporated into the DNA of proliferating cells during S phase and inhibits methylation of the newly synthesized DNA strand by trapping DNA methyltransferases (DNMTs) onto the DNA, leading to their proteolysis (Egger et al., 2004). Thus, as opposed to the active demethylation by the TET enzymes, DNMTi dependent demethylation occurs via a passive dilution during DNA replication.

While it has long been suggested that DNMTi's reactivate tumor suppressor genes, our collaborators recently observed that inhibiting DNA methylation by DNMTi leads to upregulation of endogenous retroviruses (ERVs) and formation of double stranded RNA that mimics a viral infection and induces the

viral defense pathway. This in turn elicit an interferon response, which may be responsible for apoptosis and sensitization of the immune system (Chiappinelli et al., 2015; Roulois et al., 2015).

Only about 50% of patients with MDS or AML respond to DNMTi, and most patients become resistant over time. Recent in vitro studies from our group suggest that physiological levels of vitamin C potentiate the effects of DNMTi's in upregulation of ERVs and in induction of the viral defense pathway. We specifically show a synergistic effect of the combination treatment with increased amounts of 5-hmC at the loci that control ERV expression and upregulation of double stranded ERV RNA, and the genes in the viral defense pathway (Liu, Ørskov et al., submitted). In addition, ERV proteins may be expressed and recognized by T-cells of the adaptive immune system. Thus, we suggest that combination treatment with vitamin C and DNMTi may potentially optimize the outcome of treatment in cancer patients (Figure 2).

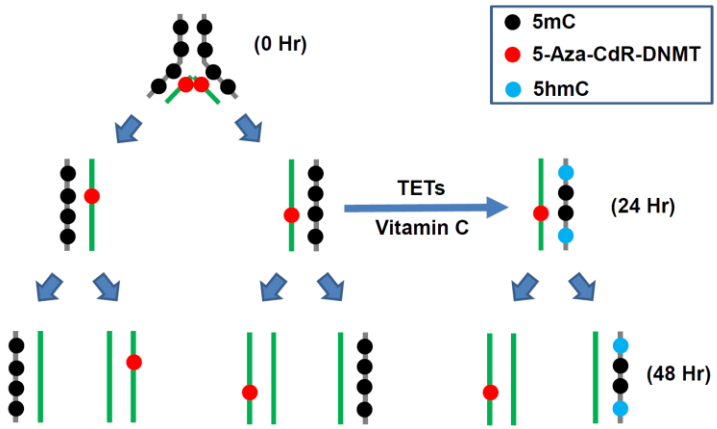


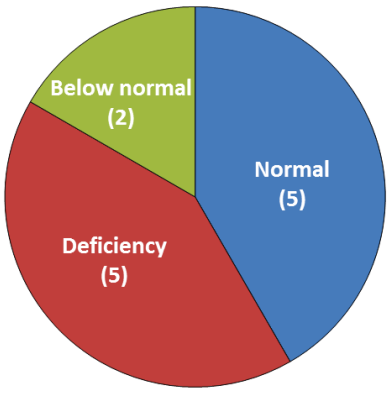
Figure 2: Proposed mechanism of action of the combination treatment: 5-aza-CdR becomes incorporated into DNA, and traps the DNMTs to DNA so that the methylation pattern is no longer copied in subsequent cell divisions. TETs and its co factor vitamin C actively convert 5-mC to 5-hmC on the old stand, thereby enhancing DNA demethylation. This specifically happens at the regulatory long terminal repeats (LTRs) of ERVs and will lead to upregulation of ERVs, formation of dsRNA and activation of the viral defense pathway.

Interestingly our collaborators in Copenhagen recently conducted a study which shows that 89% of a randomly picked group of hematological cancer patients were below the minimum value of the normal range of vitamin C, while patients taking recommended doses of vitamin C supplement restore normal levels (Liu, Ørskov et al., submitted). We find the similar deficiency reflected in the preliminary results of our (same) collaborator's ongoing trial randomizing AML/MDS patients naïve to Vit C supplements are treated with 500mg Vit C supplement or placebo. The patients baseline vitamin C levels thus far are shown in *Figure 3*.

Figure 3. Baseline Plasma Vitamin C Concentration

- the first twelve Evita patients

(Normal range: 26-84 uM; deficiency: <11.4uM)



Note: Three of the five patients in normal range were taking oral viamin C

It is critical now, prior to expanding clinical trials, that we study vitamin C levels in another population of AML, MDS, and CMML patients from an area outside of Copenhagen to confirm the described deficiencies are not limited to the Nordic population (perhaps from dietary or cultural factors).

Hypothesis: AML, MDS, and CMML patients on active anti-cancer therapy have severely deficient plasma vitamin C levels.

2. STUDY OBJECTIVES

To collect peripheral blood from AML, MDS, and CMML patients for use in exploration of epigenetic basic biology, including:

Primary objective:

- Evaluation of peripheral blood ascorbic acid levels.

Secondary objective:

- Evaluation of the expression of endogenous retroviruses(ERVs) and levels of 5-methylcitosine (5-mC) and 5-hydroxymethylcytosine(5-hmC) in cancer patients.
- Bio-bank plasma and buffy coat specimens for future cancer research.

3. STUDY DESIGN

This study is a non-interventional, specimen collection and laboratory study. There is no intention to return research results to patients.

The Clinical Investigators will recruit patients from their practice to collect peripheral blood and limited clinical information.

This study will seek to enroll no more than 50 subjects.

Coded specimens and data will be sent from the Clinical Site to VARI and may be secondarily shared with VARI-SU2C Epigenetics Dream Team collaborators.

The blood specimens will be initially used to measure ascorbic acid and DHA levels, but may also be used for additional genetic, epigenetic, and other analyses as exploratory research dictates.

4. STUDY POPULATION

4.1. Inclusion Criteria

- Patients actively receiving treatment for Acute Myeloid Leukemia (AML), Myelodysplastic Syndrome (MDS), or Chronic Myelomonocytic Leukemia (CMML).
- Patients diagnosed with AML, MDS, or CMML and are treatment naïve.
- Patients who are 18 years old or older.

4.2. Exclusion Criteria

- Patients deemed as too ill to participate as determined by the Clinical Investigator.
- Non-English speaking Patients
- Patients unable to provide informed consent.

5. STUDY PROCEDURES

Potential subjects will be identified by the Clinical Investigators or the Study Coordinator will screen the schedules of the Study Physicians. Potential subjects who meet criteria for the study will be approached by a member of the study team and offered the option to participate, and, if interested, the study staff member will proceed with the informed consent process. Informed consent will be sought using institutional procedures of the clinical site.

Subjects who satisfy inclusion/exclusion criteria, who are interested in participating, and who provide informed consent will be considered enrolled in the study and assigned a unique Study ID Number by the Study Coordinator.

After enrollment in the study, the Study Coordinator will then arrange for peripheral blood specimen collection. The Study Coordinator will advise the individual collecting the blood to follow the directions in section 5.1.

5.1. Peripheral Blood Collection

Prior to specimen collection, the Study Coordinator will ensure the processing laboratory staff is ready to receive the specimens. This may be also done by the Study Coordinator.

For 12 hours prior to blood collection, patients must fast from vitamin C (supplements containing vitamin C, foods or beverages with vitamin C).

Peripheral blood will be collected from all subjects by venipuncture and for which institutional precautions and procedures of the clinical site will be followed. If feasible, blood will be collected during

the time of a subject's routine care blood collection or IV start. Blood will be collected prior to disease (e.g., MDS, AML, CMML) treatment infusions or injections (on the same day).

Blood tubes will be labeled according to clinical institutional standard procedures, or at minimum, with the Study Subject's ID Number.

Up to 6 mL of blood will be collected into one 6 mL BD vacutainer with K2 EDTA (catalog no. BD 368661). Blood will be drawn carefully to avoid hemolysis.

Following collection, blood specimens will be transported immediately to the laboratory for processing. Date and time of collection will be recorded on the *Specimen Data Collection Form* (appendix 2).

5.2. Specimen Processing and Handling

Blood specimens should be processed according to the *Laboratory Manual* (Appendix 1).

The resulting products, frozen aliquots, will be coded by the Clinical Site using VARI-provided study specimen labels. Specimens will not be marked with any identifying information.

Aliquots will be batch shipped to VARI on dry ice by local courier. At VARI, the specimens are received, inventoried, and kept in freezers until time of use.

6. DATA HANDLING AND SHARING

Data points about the Subjects to be captured by the Clinical Site will include:

- Age
- Gender
- Diagnosis (MDS, AML, or CMML)
- Date of diagnosis
- Disease treatment history (medication, start/stop date or number of cycles)
- Current therapies (medication, cycle number, treatment start date)
- Vitamin C supplementation status (asked at time of blood collection)

Coded subject data will be shared from the Clinical Site to VARI.

The identification code—a list of Subject Study ID Numbers and the identity of the Subjects—will be generated and maintained by the Clinical Site. VARI Investigator(s) will not have access to the identification code nor attempt to identify the subjects or specimens.

Study data will be kept at the Clinical Site on password-protected, encrypted computers and/or servers with access limited to study personnel. Paper study documents will be kept in access-restricted, locked rooms in locked file cabinets.

Subject clinical data will be shared by the Clinical Site to VARI in an electronic excel spreadsheet format or case report form. Electronic excel spreadsheets will be password-protected and shared via encrypted email to VARI. Case Report Forms will be sent to VARI with specimens batch shipments.

Specimen data will be shared by the Clinical Site to VARI on the *Specimen Data Collection Form* that will be included with the specimen shipments to VARI.

VARI will store data in computer databases on either VARI encrypted, password-protected computers or VARI servers, on which multiple levels of security (firewalls) exist. Study data access at VARI is limited to the individuals listed on the VARI IRB application. Paper study documents will be kept in secure offices with limited access to study team members.

Results from the research of VARI and Collaborators will be shared with the Clinical Site Investigators, however, the research results generated from this research are not anticipated to be clinically actionable.

7. REGULATORY

IRB/EC approval will be sought for this protocol by the Clinical Site, VARI, and the institutions of any Collaborating Investigators, as applicable.

8. APPENDICES

- Appendix 1 - Specimen Data Collection Form
- Appendix 2 - Laboratory Manual