Understanding and targeting epigenetic dysregulation, aberrant metabolism, and immune evasion in cancer with ascorbic acid

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for the award of the degree of Doctor of Philosophy of the
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I. Declarations

Whilst registered as a candidate for the above degree, I have not been registered for any other research degree award. The results and conclusions embodied in this thesis are the work of the named candidate and have not been submitted for any other academic degree award.

13 May 2020

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Date

II. Acknowledgments

“All that I am or ever hope to be, I owe to my angel mother”- Abraham Lincoln

These words could not be truer in my case. My mother instilled in me both the thirst for knowledge and pursuit of academic excellence at a very young age. While her love was unconditional, words of praise were mostly limited to academic excellence. Soon after I started medical school aged seventeen, she had initial symptoms of what was later diagnosed as an early onset, sporadic, progressive fronto-temporal dementia. This has had a strong bearing on much of my adult life. While the decision to pursue a career in a medical specialty with the most unmet research need felt natural, the choice of oncology over neurosciences was a difficult one, and only made because cancer reduces quality life-years far more than dementia worldwide. The helplessness, desperation, and grief- for patients and families- are similar in both diseases.

I thank the rest of my immediate family (father, brother, grandparents), extended family, and close friends for their unwavering support and encouragement over the years. I thank my former girlfriend (a physician), and current girlfriend (a scientist), for their support and understanding, as I juggled between the all-consuming tasks of both advanced clinical training and simultaneous research pursuits over much of the last decade.

My patients, books, and the internet (with NCBI *PubMed* and Google search engines), have been my greatest teachers in the profession. I thank my former mentors, and (then) post-doctoral researchers who taught me basic laboratory techniques when I started bench research nine years ago. I thank my current lab members who have significantly helped my transition to, and initial journey as, a principal investigator.

My sincere gratitude to Dr. Sassan Hafizi for his truly selfless efforts over the last year, as the Director of Studies for this thesis. His expert guidance and input throughout the process of my selection and enrollment in the program, navigation of administrative/ visa issues, preparation of this thesis, and planning for the viva defense, are highly appreciated.

Finally, I want to thank the University of Portsmouth for this excellent opportunity.

III. Abstract

Understanding the comprehensive interactions between genetic changes, epigenetic dysregulation, aberrant metabolism and immune evasion in cancer provides a deeper insight into its pathogenesis and progression, and helps identify vulnerabilities for therapeutic exploration. This Commentary highlights a series of published studies uncovering the following: molecular mechanisms and prognostic impact of aberrant DNA methylation/hydroxymethylation in clear cell renal cell carcinoma (ccRCC) which accounts for 80% of all kidney cancers; the potential of ascorbic acid (AA) in reversing aberrant methylation in cancers (ccRCC and lymphoma) via activation of the Ten-Eleven Translocation (TET) enzymes *in vitro* and *in vivo*; AA-induced demethylation and re-expression of tumor-suppressors and endogenous retroviral transcripts in lymphoma cells; mechanistic and anti-tumor synergy between high-dose AA and anti-PD1 immunotherapy in a lymphoma mouse model; and important considerations while exploring the potential of AA as an anti-cancer agent *in vitro* and *in vivo* to enhance translation of pre-clinical findings.

These findings have led to two actively accruing multi-center phase 2 randomized trials combining IV ascorbic acid with standard of care treatment in ccRCC and diffuse large B cell lymphoma (DLBCL). They also provide a compelling rationale for testing combinations of high-dose AA and anti-PD1 agents in patients with aggressive B cell lymphoma and in preclinical models of other malignancies.

IV. Table of contents

V. Dissemination

1. **Shenoy N***, Pagliaro L: *Sequential pathogenesis of metastatic VHL mutant clear cell renal cell carcinoma: putting it together with a translational perspective*. **Ann Oncol**, 2016 Sep;27(9):168595. PMID: 27329246. *corresponding

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VI. Introduction

The eight recognized hallmarks of cancer (1) constitute fundamental building blocks in the complexity of cancer biology, and are as follows:

- Sustained proliferative signaling
- Replicative immortality
- Evasion of tumor suppressors
- Resisting cell death
- Induction of angiogenesis
- Activation of invasion and metastasis
- Deregulation of cellular energetics
- Avoiding immune destruction

From a translational standpoint, understanding the molecular mechanisms governing each of these hallmarks, and how each hallmark interacts with and enables the other through the various stages of cancer pathogenesis and progression, could help delineate targetable vulnerabilities. Equally important is understanding the mechanisms of resistance to conventional therapeutic approaches, such as chemotherapy, radiotherapy, targeted therapy, and immunotherapy. This aids the pursuit of combinatorial strategies targeting resistance mechanisms, and enhancing the anti-tumor effects of conventional strategies.

In keeping with the concepts above, this Introduction consists of 5 sub-sections that also, in part, form the basis of the original scientific investigation described in this thesis: (i) epigenetic dysregulation in cancer, (ii) aberrant metabolism in cancer, (iii) mechanisms of immune evasion in cancer; (iv) clear cell renal cell carcinoma as a model for resistant cancers; and (v) preliminary work leading to the aims of the PhD investigation.

VI.1 Epigenetic dysregulation in cancer

A brief introduction to epigenetics

Epigenetics is any process that alters the expression of a gene, without a change in the genetic code, which plays a role not only in normal cellular development and differentiation, but also in disease. Nuclear chromatin is composed of condensed nucleosome subunits, each of which consists of approximately 147 base pairs of genomic DNA wrapped around a histone octamer core consisting of 2 copies each of the core histones H2A, H2B, H3, and H4 (Figure 1). The H1 histone associates with linker DNA connecting nucleosomes (considered

by some as a part of the nucleosome complex) (2). Epigenetic processes such as DNA methylation and histone modifications at regulatory regions alter the three-dimensional structure of chromatin and accessibility of DNA, thereby influencing the binding of transcription factors and subsequent gene expression (3). The proteins that lay these epigenetic marks are termed ‘writers’; those that interact with these marks, ‘readers’, and those that mitigate the marks, ‘erasers’ (4). Tightly condensed chromatin, termed heterochromatin, consists of predominantly ‘inactive’ genes; in contrast, open chromatin, termed euchromatin, consists of predominantly active genes (5, 6).

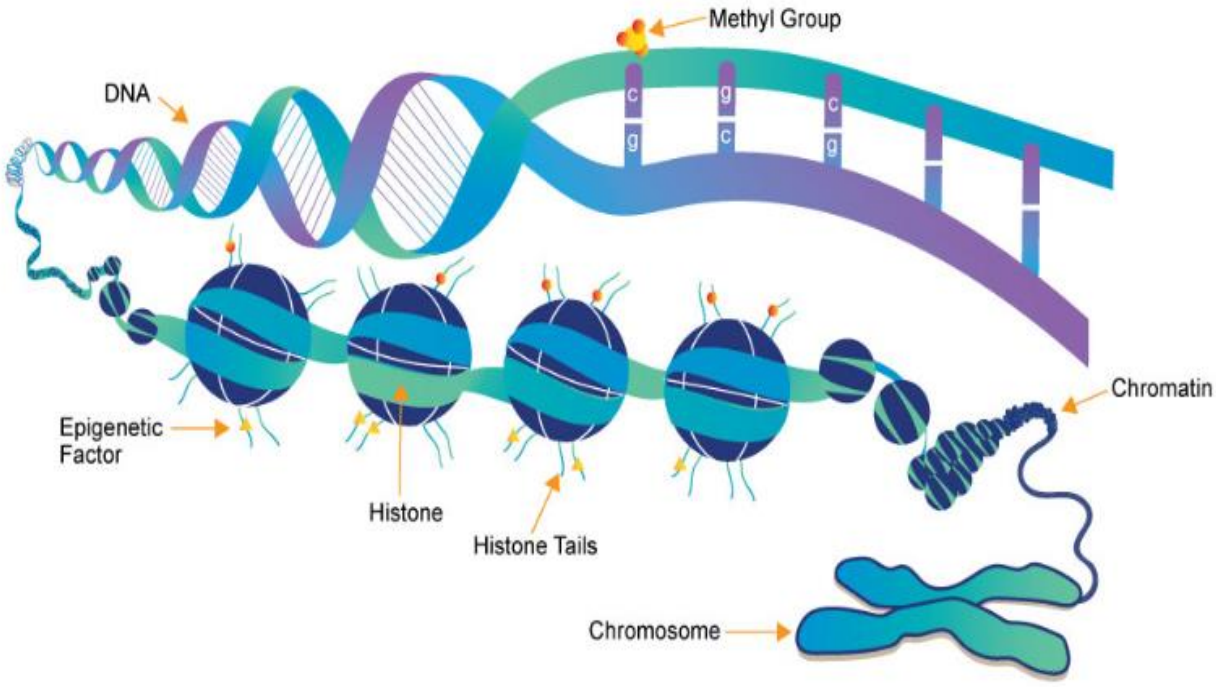


Figure 1. A high-level overview of chromatin composition and epigenetic marks (7).

Although RNA modifications resemble DNA modifications and can affect eventual protein expression, they are not widely considered a part of ‘epigenetics’; and neither are non-coding RNAs. In fact, an operational definition of epigenetics calls for a modification to be ‘stably heritable’ in order for it to be considered ‘epigenetics’ (8), although alternate definitions including non-heritable modifications continue to be used (9). Conceptually, however, rather than worry about the semantics of different definitions, it is helpful to consider the modifications that eventually influence biological processes without a change in the genetic code as a whole, and classify them as modifications at the histone/ DNA (pre-transcriptional), RNA (post-transcriptional) or protein (post-translational) levels (while recognizing that no formal definition of epigenetics encompasses all these features).

DNA methylation

DNA methylation is a heritable epigenetic mark that involves the covalent addition of a methyl group to the 5-carbon position of cytosine by DNA methyl transferases (DNMTs), and usually occurs on cytosines that precede a guanine ('CpG') (10). A majority of these CpG dinucleotides are located at 'CpG islands': CpG- rich regions that are present in around 70% of all gene promoters (11). Increased methylation of CpG islands usually leads to transcriptional silencing of the corresponding gene, either via preventing binding of transcription factors or via the recruitment of methyl-CpG binding proteins that interact with repressive histone modifying enzymes (10). While DNMT3A and DNMT3B are involved in *de novo* methylation, DNMT1 is involved in maintenance (12). Once a methyl mark is laid, the enzymes that are involved in active demethylation are the Ten-Eleven Translocation (TET) dioxygenase enzymes (TET 1,2,3), which belong to the family of Fe^{2+} and 2-oxoglutarate dependent enzymes (13). They accomplish demethylation via a series of oxidation reactions, from 5-methylcytosine (5-mC) to 5-hydroxymethylcytosine (5-hmC) to 5-formyl cytosine (5-fC) to 5-carboxyl cytosine (5-caC). 5-fC and 5-caC are then excised by the enzyme thymine DNA glycosylase, after which they are replaced with unmodified cytosine by the base exchange repair (BER) pathway (Figure 2) (13-15). Recent developments in methylation profiling techniques such as oxidative bisulphite sequencing (16), HELP-GT assay (17) and hMeDIP sequencing (18), has enabled genome-wide identification and differentiation of oxidative 5-hmC methylation marks, in addition to 5-mC. Studies have shown that the inhibitory effect of methyl mark on regulated genes is lost even upon oxidation to 5-hmC (17). However, the relatively distinct expression/function patterns of the three TET enzymes (and their isoforms) are currently being delineated (19).

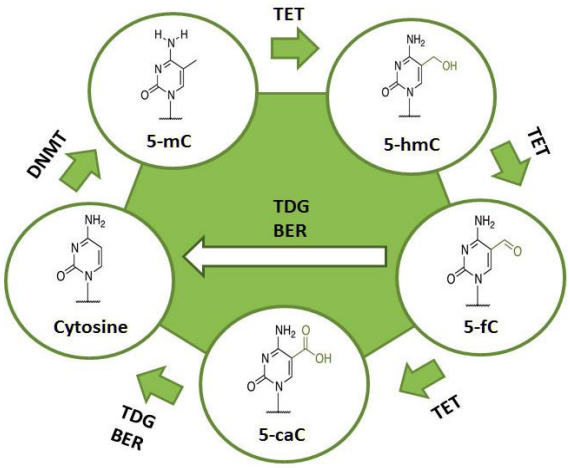


Figure 2. The DNA cytosine methylation cycle (DNMT: DNA methyl transferases; TET: Ten-Eleven Translocation enzymes; 5-mC: 5-methylcytosine; 5-hmC: 5-hydroxymethylcytosine; 5-fC: 5-formyl cytosine; 5-caC: 5-carboxyl cytosine) (20).

Dysregulation of DNA methylation in cancer

Global and gene specific changes in methylation patterns compared to normal tissue of origin are characteristic findings in cancers. Although many cancers have been shown to be, in general, characterized by global hypomethylation, hypermethylation of CpG islands leading to silencing of tumor suppressors has been frequently reported in cancers. Classic examples are the hypermethylation of Rb gene in retinoblastoma (21); p16 gene in breast, lung, colorectal carcinomas (22); and E-cadherin in RCC (23). On the other hand, hypomethylation of tumor promoters leading to enhanced expression has also been reported in malignancies (24-26). Furthermore, hydroxymethylcytosine, albeit a much less abundant mark than methylcytosine, has been shown to be relatively reduced in several cancers (27) compared to the tissue of origin, affecting gene expression in cancer cells.

Mutations or expression levels of DNMTs and TET enzymes do not fully explain the degree of methylation/hydroxymethylation aberrancies in cancer (28). That said, mutations have been reported in these enzymes in cancers. Recurrent *DNMT3A* loss of function mutations have been reported in AML, associated with losses of DNA methylation (29, 30). Also, *TET2* is frequently mutated in hematologic (both myeloid and lymphoid) malignancies (31). Because TET enzymes use 2-oxoglutarate as a co-substrate, they are susceptible to competitive inhibition by accumulation of metabolic intermediates such as hydroxyglutarate, succinate or fumarate, as a result of inactivating mutations or deletions of genes encoding their metabolizing enzymes like succinate dehydrogenase and fumarate hydratase (32, 33).

Azacytidine (Aza; Vidaza) and decitabine (5-aza-2′-deoxycytidine), the archetypal DNMT1 inhibitors, are cytosine analogues in which carbon at the C-5 position of the pyrimidine ring is substituted with nitrogen. When incorporated into DNA, they bind DNMT1 irreversibly, resulting in DNMT1 degradation and consequently demethylation of DNA. While decitabine mostly incorporates into DNA, about 80-90% of Aza incorporates into RNA. These agents are currently used in the clinical setting in higher-risk myelodysplastic syndromes. Interestingly, apart from causing demethylation and re-expression of silenced tumor suppressor genes, a global increase of H3 and H4 acetylation has also been observed with Aza treatment, indicating that mechanisms other than direct DNA demethylation may contribute to its anti-neoplastic effect, and also indicating a close co-operation between DNA and histone marks (27). Furthermore, demethylation induced enhanced expression of endogenous retroviruses (ERVs) likely also contributes to anti-tumor effects by increasing immune recognition.

Histone modifications

Histone modifications are also important mechanisms of regulation of gene transcription. Each one of the octamer core histones in a nucleosome (H2A, H2B, H3, and H4) has a tail extension, which are targets of histone modifications. A wide range of histone post-translational modifications are known to occur, including acetylation, methylation, ubiquitination,phosphorylation and others (34, 35) (Figure 3) . Such modifications can affect chromatin remodeling and gene regulation through two central mechanisms: one, neutralization of amino acid charge; and two, serving as docking sites for proteins that then regulate chromatin structure (34, 36).

Acetylation or *phosphorylation* of lysines on histones neutralizes the positive charge of lysine, thereby weakening interactions between the N terminus of the histone and the negatively charged phosphate group of DNA, to increase chromatin accessibility (37, 38). Histone acetyl transferases (HATs) lay the acetyl group, whereas histone deacetylases (HDACs) remove them (27). HATs are classified into Type A (acetylate chromatin bound histones and nuclear proteins) or Type B (acetylate newly translated histone H3 and H4). HDACs are divided into 4 classes based on homology and structure: classes I, II and IV are Zn^{2+}-dependent HDACs while class III is made up of NAD-dependent sirtuins (3, 27, 36).

Methylation of histones by histone methyltransferases can either activate or repress transcription, depending on the amino acid being methylated and the presence of other methyl or acetyl groups in the vicinity. For example, trimethylation of histone H3 at lysine 4 (H3K4me3) is a signal for transcriptional activation, whereas dimethylation of histone H3 at lysine 9 (H3K9me2) is a signal for transcriptional silencing (39). The two major classes of histone methyltransferases are lysine methyltransferases (KMTs) and protein arginine methyltransferases (PRMTs)(27). As an opposing mechanism, histone demethylases remove the histone methyl mark. The two classes of lysine demethylases (KDMs) that demethylate histones are the amine oxidases and the 2-oxoglutarate dependent Jumonji domain (JmjC) containing demethylases (3, 27, 36).

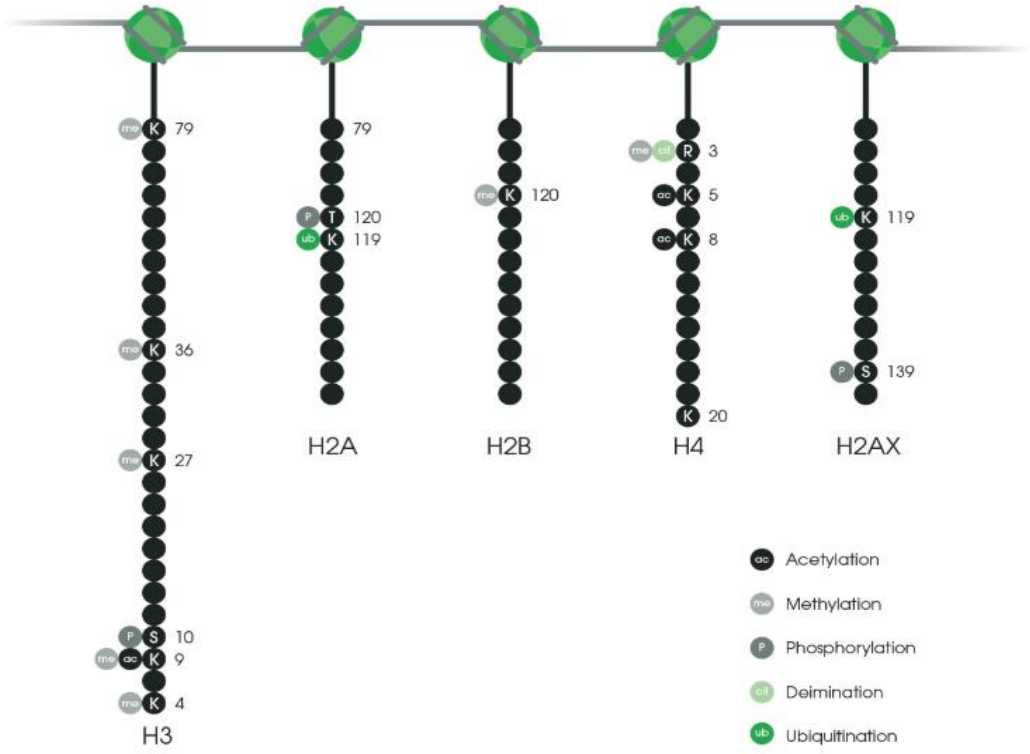


Figure 3. The most common histone modifications (40).

Dysregulation of histone modifications in cancer

Histone deacetylases (HDACs) have been shown to be over-expressed in cancers resulting in loss of global histone acetylation and subsequent inhibition of tumor suppressor gene expression. Since HDACs (Class I, II, and IV) are Zn^{2+}-dependent, non-selective HDAC inhibitors target the zinc ion in the active site of HDACs to inhibit their enzymatic activity. Vorinostat (used in the treatment of cutaneous T cell lymphoma, CTCL), belinostat (for peripheral T cell lymphomas, PTCL) and panobinostat (for multiple myeloma) are all non-selective HDAC inhibitors that inhibit all Zn^{2+}-dependent HDACs. Selective HDAC inhibitors include romidepsin (used in treatment of CTCL and PTCL), which targets specifically class I HDACs, and ricolinostat (in clinical trials for multiple myeloma, lymphoma), which targets a specifically class II HDAC (27, 41) (Figure 4).

Histone acetyl transferases (HATs) have been reported to be inactivated via deletions or mutation in cancers. For example, around 40% of diffuse large B cell lymphoma (DLBCL) and follicular lymphoma (FL) have inactivating mutations or deletions of p300/CBP (cyclic AMP response element- binding protein) (42).

Both activation and inactivation of specific *Histone methyltransferases* has been demonstrated in cancer. Activating mutations of EZH2 (Enhancer of Zeste Homolog 2) which catalyzes di- and tri-methylation of H3K27 thereby repressing transcription of tumor suppressors has been reported in 22% of DLBCLs. EZH2 inhibitors have demonstrated efficacy in early phase trials in EZH2 mutated DLBCLs (43).

Put together, dysregulation of DNA methylation and histone modifications (particularly acetylation and methylation), constitute a bulk of the currently recognized, and targeted, 'epigenetic' aberrancies in cancer (Figure 4). This dysregulation in cancer allows the simultaneous reduction in expression of several 'tumor-suppressor' genes, causing their cumulative loss of function, even without any inactivating mutations in these genes.

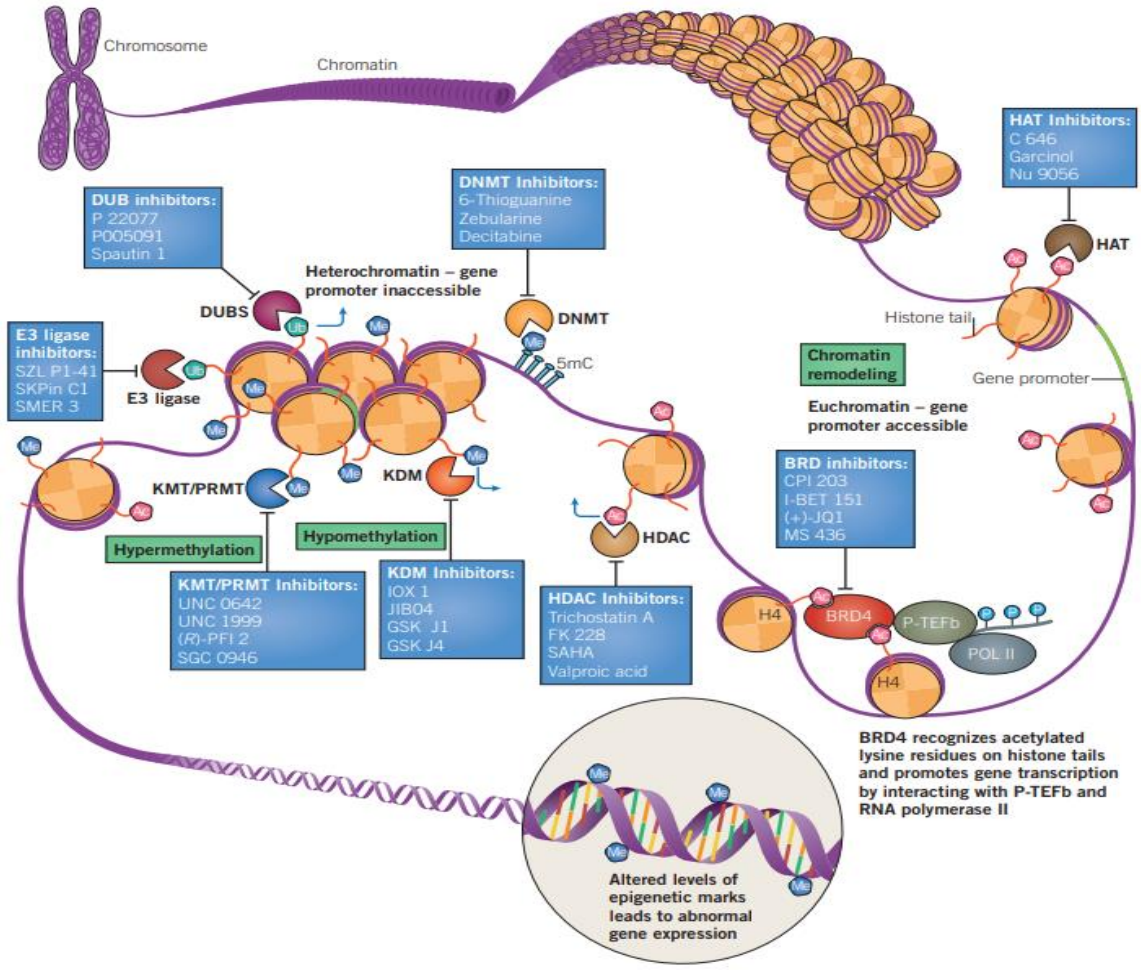


Figure 4. Epigenetic dysregulation in cancer and epigenetic therapeutic classes. Altered levels of histone and DNA epigenetic marks lead to abnormal gene expression, and 8 different classes of epigenetic therapeutic agents are either in clinical use or being investigated for potential benefit: DNMT inhibitors; HDAC inhibitors; HAT inhibitors; BRD inhibitors; KDM inhibitors; PRMT inhibitors; E3 ligase inhibitors; and DUB inhibitors (44). *(Abbreviations- DNMT: DNA Methyltransferase; HDAC: Histone Deacetylase; HAT: Histone Acetyltransferase; KMT: Histone lysine methyltransferase; KDM: Histone lysine demethylase; PRMT: Protein arginine methyltransferase; BRD: Bromodomain; DUB: deubiquitinating enzymes).*

VI.2 Immune evasion in cancer

Given the rapid clinical translation in the field of harnessing the immune system for therapeutic benefit, it would be beneficial to discuss this topic not only by throwing light on the different mechanisms adopted by cancer cells to evade immune killing, but also with a review/ perspective on the past, current, and future translation to the bedside. As related to this PhD study, one malignancy, renal cell carcinoma, is discussed here, recognizing that while several of the mechanisms overlap, there are significant differences in the degree to which a particular mechanism contributes to immune evasion between different cancers.

Clear cell renal cell carcinoma (ccRCC) accounts for ~75-80% of all RCC, and biallelic Von Hippel–Lindau (VHL) gene defects occur in ~75% of sporadic ccRCC (45). The molecular etiopathogenesis of VHL mutant metastatic RCC is a sequence of events which can be grouped under the following: (i) loss of VHL activity (germline/somatic mutation + inactivation of the wild-type copy); (ii) constitutive activation of the hypoxia-inducible factor (HIF) pathway due to loss of VHL activity and transcription of genes involved in angiogenesis, survival, anaerobic glycolysis, pentose phosphate pathway, epithelial–mesenchymal transition, invasion, and metastasis (iii) interactions of the HIF pathway with other oncogenic pathways; (iv) genome-wide epigenetic changes (potentially driven by an overactive HIF pathway) and the influence of epigenetics on various apoptotic, oncogenic, cell cycle regulatory and mismatch repair pathways (inhibition of multiple tumor suppressor genes); (v) immune evasion, partially caused by changes in the epigenome (45). These mechanisms interact throughout the pathogenesis and progression of disease, and confer resistance.

Renal cell carcinoma (RCC) is considered an immunogenic tumor, with immunotherapy being a part of its treatment landscape for decades. Although interleukin 2 (IL-2) and interferon alpha (IFN-α) were approved in the 1990s, they were effective in a small percentage of patients and had significant toxicity, often resulting in patients requiring intensive care for cytokine-driven hemodynamic instability (46, 47). The advent of checkpoint blockade has advanced the field of immunotherapy in RCC, both in terms of efficacy and safety (48, 49). However, several patients still fail to respond to single agent or combination checkpoint blockade. As such, a comprehensive understanding of immune evasion mechanisms in RCC is needed to help develop more effective combinations that provide mechanistic synergy and bring to light aspects that may not be currently countered in the clinical setting, representing potential opportunities for therapeutic exploration.

As mentioned above, ccRCC accounts for a large majority (~80%) of all RCC (45). Non-clear cell renal cell carcinomas (nccRCC) include papillary, chromophobe, collecting duct,

unclassified, and translocation carcinomas. In the 1990s, when IL-2 and IFN-α were studied in metastatic renal cancer, the selection criteria did not limit patients to clear cell histology (50, 51). Over the last two decades, however, after the Heidelberg classification of renal tumors (52) and starting with the randomized study investigating high dose vs low dose IL-2 in RCC (53), large randomized 'RCC' trials have limited the selection criteria to clear cell histology, unless otherwise specified. Therefore, where unspecified, 'RCC' mainly refers to clear cell histology.

Recently, the FDA approved two "checkpoint inhibitor plus tyrosine kinase inhibitor (TKI)" combinations in the first-line setting for advanced RCC: pembrolizumab (humanized anti-PD1 antibody) plus axitinib (small molecule TKI), and avelumab (fully human monoclonal anti-PD-L1) plus axitinib. Both combinations were tested against sunitinib (also a small molecule TKI) in large, randomized, multi-center trials, and demonstrated improved median progression-free survival with a 4-5 month margin and superior overall response rates (55-60%, in comparison with 26-35% with sunitinib) (54, 55). Although it remains unclear how the combinations fare in comparison with *sequential* treatment with single-agent TKIs (approved in the first-line setting) and single-agent anti-PD1 checkpoint inhibition (approved in the second-line setting), both combinations are attractive options particularly for patients with high disease burden and intermediate-poor risk, given the superior response rates. In these patients, the need for a quick disease burden reduction provides sufficient rationale to account for the increased risk of adverse effects, which are largely manageable except in a very small minority of patients. Notwithstanding these approvals, there is much room for improvement in combination strategies targeting immune evasion.

This section summarizes the currently known mechanisms of immune evasion in RCC, categorizing them as follows: loss of antigen-presenting ability; immune checkpoint signaling; tumor-associated gangliosides; tumor-associated metabolites; tumor-promoting immune cells in the microenvironment and their inhibitory cytokines; impaired immune cell trafficking; inhibitory cytokines released by tumor-promoting immune cells in the microenvironment, and metabolite- induced immunosuppression (depicted in Figure 5) (56).

Loss of antigen-presenting ability

Major histocompatibility complex (MHC) class I molecules are highly polymorphic proteins that bind to antigenic peptides and present these peptides to cytotoxic T cells. Co-ordinated antigen processing and presentation facilitates effective anti-tumor immune function (57, 58).

Abnormalities in MHC class I have been reported in different tumor types leading to dysfunction in various steps of antigen processing. These abnormalities include structural alterations or dysregulation of HLA class I antigens, and of different HLA class I- associated antigen processing machinery (APM) components. Loss of expression/ function of HLA and APM components resulting in poor antigen presentation is an important mechanism of escaping immune surveillance in cancers (57, 58).

ccRCC has been reported to have a partial loss of HLA class I molecules in 39% of cases, and complete loss in 6% (59). Down-regulation of genes needed for antigen processing, such as the transporters associated with antigen processing (TAP)1 and TAP2, and the proteasomal components low molecular weight proteins (LMP)2 and LMP7, has been reported in RCC (60). Furthermore, reduced expression of HLA heavy chain (HLAhc) and beta-2-microglobulin (*B2M*) in ccRCC is associated with metastatic spread and worse prognosis (59).

Immune checkpoint signaling

The B7 family of peripheral membrane proteins found on tumor cells and antigen presenting cells play a critical role in modulating T cell responses when paired with specific molecules on T cells (61). The growing B7 family consists of seven members: B7-1 (CD80), B7-2 (CD86), B7-DC (CD273, PD-L2), B7-H1 (CD274, PD-L1), B7-H2 (ICOS-L), B7-H3 (CD276), and B7-H4 (B7x, B7S1).

PD-L1 (Programmed death ligand 1) expressed on tumor cells and antigen presenting cells inhibits not only PD-1 (Programmed death 1)- expressing cytotoxic T cells (as previously thought), but also natural killer (NK) cells and dendritic cells, by inducing an exhaustion signature (62-65). The inhibitory PD-1/PD-L1 axis has been targeted in renal cell carcinoma with clinical benefit. Nivolumab, a human IgG4 monoclonal antibody (mAb) that blocks PD-1, has been approved in the second line setting after disease progression on a TKI (66). Nivolumab has also been approved in the first line setting in combination with Ipilimumab (a mAb that blocks CTLA-4) in intermediate and poor risk renal cell carcinoma (54). Pembrolizumab, a humanized anti-PD1 antibody, has been approved in the first line setting in combination with axitinib (small molecule TKI) (67). Avelumab, a fully human mAb that targets PD-L1, has also been approved in the first line setting in combination with axitinib (67). The expression of PD-L1 in RCC has been shown to have a weak correlation between primary tumor and metastatic sites (68), thereby making it a relatively poor biomarker for

patient selection for anti-PD-1/ PD-L1 immunotherapy, based on nephrectomy specimen expression. The PD-1/PD-L1 axis holds particularly significant therapeutic promise in sarcomatoid RCC, a highly aggressive form of RCC characterized by spindle cells, high cellularity and cellular atypia, which can be a component of either clear cell or non-clear cell histologies. In one study, intra-tumoral PD-1 and PD-L1 expression was found in 96% and 54% of sarcomatoid RCC, compared to 62% and 17% of clear cell RCC specimens (69). Furthermore, co-expression of PD-L1 on tumor cells and PD-1 on tumor-infiltrating lymphocytes (TILs) was found in 50% of all sarcomatoid RCC cases, compared to only 3% with clear cell RCC (69). In keeping with these findings, evidence from a single institution retrospective study indicates that checkpoint blockade may be very beneficial in sarcomatoid RCC, with an objective response rate of 62% and a complete response rate of 15% (70).

CTLA-4 (cytotoxic T-lymphocyte-associated protein 4), an inducible receptor expressed by cytotoxic T cells binds with B7-1/ B7-2 on antigen presenting cells, leading to T cell inhibition (71, 72). As mentioned above, ipilimumab (anti-CTLA-4) has been approved in combination with anti-PD1 in the first line setting of advanced RCC (73).

B7-H4 is a negative regulator of cytotoxic T cell response (74), and masking of B7-H4 with a specific blocking antibody may increase the cytotoxicity of T cells in ccRCC (75). Soluble levels of B7-H4 are higher than normal in patients with non-metastatic RCC (76) and correlate with less differentiated tumors, higher invasive and metastatic potential, and a worse response to anti-VEGF therapy (76, 77). B7-H4-mediated inhibition of cytotoxic T cell response may be an important reason for the low efficacy of interleukin-2 and interferons in metastatic RCC (75). Strategies aimed at blocking B7-H4 are being developed to restore antitumor T-cell responses and to improve the efficacy of cancer immunotherapy (78, 79).

Tim-3 (T cell immunoglobulin and mucin-domain containing-3), an inhibitory receptor on T cells, plays a key role in inhibiting Th1 responses and the expression of cytokines such as IFN-γ. It has been found to be overexpressed in ccRCC, and is associated with worse prognosis (80).

LAG-3 (Lymphocyte activation gene-3), another inhibitory receptor on T cells, has been recently shown to be an important immune checkpoint in RCC (81). TILs and peripheral blood mononuclear cells isolated from RCC patients were studied with intracellular cytokine staining after *in vitro* stimulation in the presence or absence of PD-1 +/- LAG-3 or Tim-3–specific antibodies. PD1 blockade increased LAG-3 (but not Tim-3) expression. Dual blockade of PD-1 and LAG-3 (not PD-1 and Tim-3) increased IFN-γ release with *in vitro*

stimulation, suggesting that dual targeting of PD-1 and LAG-3 may be a promising therapeutic strategy in this malignancy (81).

HLA-G is a checkpoint molecule with emerging significance in cancers (82). Whether membrane bound or soluble, it strongly binds to its inhibitory receptors on effector immune cells (NK, T, B, monocyte/dendritic cells), and broadly inhibits the function of these cells (83). There are two main receptors for HLA-G on immune cells- ILT2 (expressed on monocytes/ dendritic cells, B cells, and some T and NK cells) and ILT4 (expressed on neutrophils and myeloid cells)(84). Aberrant induction of HLA-G expression has been demonstrated in several malignancies, including RCC, and is associated with worse prognosis (83-85).

HLA-E is a class Ib MHC molecule. It is overexpressed in several cancers, including RCC (86, 87). HLA-E binds to its receptors CD94/NKG2A, -B and -C on NK and T cells, and has a suppressive effect on these cells.

Put together, checkpoint interactions such as PD-1←→ PD-L1, CTLA4←→B7-1/2, TIM-3←→Galectin-9, LAG-3←→MCH II, play an important role in immune evasion of cancers.

Tumor-associated gangliosides

Gangliosides are structurally diverse acidic glycosphingolipids that are present in the plasma membranes and play an integral role in cell signaling, cell adhesion, and differentiation and growth (88). Increased expression of gangliosides and its shedding into the tumor microenvironment disrupts the normal functioning of T cells (89-91). Gangliosides derived from supernatants of RCC explants have been shown to inhibit IFN-γ production, downregulate Th1-type responses, and skew T-cell responses toward Th2-type (92). RCC-associated gangliosides have been shown to inhibit NFkB activation, and also reduce BCL-2 and BCL-X expression in effector T cells, thereby promoting apoptosis in these cells (93-95). Disialosyl globopentaosylceramide (DSGb5) is a ganglioside originally isolated from tissue extracts of RCC, and increased expression of DSGb5 is associated with lower recurrence-free survival in RCC (96). DSGb5 expressed on RCC cells binds to sialic acid-binding lg-like lectin 7 (Siglec-7) on NK cells, which leads to inhibition of NK cell cytotoxicity (97).

Tumor-associated metabolites

Tumor-associated metabolites play an important role in immunosuppression in the cancer microenvironment, particularly so in ccRCC. This malignancy expresses high levels of metabolites such as lactate, 2-hydroxylutarate and kynurenine metabolites (98), which have been shown to have an inhibitory effect on various effector cells and portend poor prognosis in ccRCC (70, 86, 99-102).

Tumor-promoting immune cells in the tumor microenvironment and their inhibitory cytokines

The tumor microenvironment (TME) is a heterogeneous network of cellular interactions that play a role in altering the course of tumorigenesis into either progression or suppression (103, 104). The TME in RCC typically has a prominent immune cell infiltrate, including CD8+ T cells, NK cells, macrophages and dendritic cells (105, 106). However, along with effector cells, the TME in RCC is also characterized by several suppressive immune cell types that aid immune escape:

-A unique subset of dendritic cells [CD209+CD14+CD163+] has been shown to be highly infiltrated in RCC tissues and associated with an unfavorable Th1 cell balance and advanced stage in RCC (107). These CD209+ dendritic cells in RCC are unusual because they co-express macrophage markers (CD14, CD163) (107). These cells secrete metalloproteinase 9 (MMP9) and crosstalk with T-cells to increase tumor-promoting TNF- α and reduce chemokines relevant for Th1-polarized lymphocyte recruitment.

-Regulatory T cells (Tregs) are CD4+CD25+FOXP3+ T cells that exert an immunosuppressive effect on anti-tumor immune cells (108). Tregs secrete suppressive cytokines such as transforming growth factor-β (TGF-β) and IL-10, express CTLA-4 and promote tumor progression (108). Presence of higher number of Tregs in the microenvironment is associated with a poor prognosis in RCC (107, 109), and a higher T effector/Treg ratio is associated with a lower recurrence rate (110). Tregs in RCC have higher CXCR4 expression and consequently, CXCR4 receptor antagonism reverses Treg immunosuppressive function (111). In mouse models of other tumor types, CXCR4 receptor blockade has been shown to induce tumor shrinkage and combinatorial efficacy with anti-PD1 (112, 113).

-Myeloid derived suppressor cells (MDSCs) have also been shown to play a role in immune evasion in RCC (101), and secrete suppressive cytokines such as IL-10 and TGF-β (114). Polymorphonuclear MDSCs and immature MDSCs are increased both within the RCC tumor and peripheral blood compared to normal control (101). Targeting PMN MDSCs with CXCR2

blockade enhanced the efficacy of anti-PD1 in a syngeneic renal cancer mouse model (Renca) (101). Similarly, targeting Interleukin 1β (a cytokine that attracts MDSCs), decreased intra-tumoral MDSCs, and delayed tumor growth (101).

Tumor-associated macrophages (TAMs) in RCC show a mixed M1/M2 phenotype. A higher M2 phenotype is associated with worse prognosis (75, 115), and M2 macrophages stimulate angiogenesis as well as aid invasiveness through the secretion of cytokines such as IL-10 and TGF-β (116, 117) as well as matrix metalloproteinases (115, 117).

The effector TILs in ccRCC have an anergic signature (from exposure to inhibitory cytokines, metabolites, gangliosides and checkpoints) characterized by high diacylglycerol kinase α (DGKα) expression, low activation of extracellular signal-regulated kinase (ERK), c-Jun N-terminal kinase (JNK) and AKT (118). DGKα is a physiologic inhibitor of T cell receptor (TCR) signaling and inhibition of DGKα leads to increased ERK activation and improved degranulation (118). This anergic signature may in part explain why, unlike in other tumors, a higher number of intratumoral CD8+ cells in ccRCC TME is actually associated with higher stage and worse prognosis (119). Of note, however, and importantly, this prognostic impact of intratumoral CD8+ T cells in ccRCC was reported prior to the era of immune checkpoint inhibition with anti-PD-1 and anti-CTLA-4.

Impaired immune cell trafficking

Cell adhesion molecules (CAMs) such as intercellular cell adhesion molecule-1 (ICAM-1), vascular endothelial cell adhesion molecule-1 (VCAM-1), E-selectin, and P-selectin are responsible for recruiting leukocytes and mediating leukocytes extravasation to inflammatory sites (120, 121). When RCC cells are enriched with TNF-alpha, they counter immune recognition by decreasing ICAM-1, VCAM-1 and E-selectin expression, thereby reducing endothelial attachment of peripheral blood lymphocytes and polymorphonuclear neutrophils (122, 123). Additionally, VHL tumor suppressor protein loss in RCC downregulates VCAM-1 transcription independent of hypoxia-inducible factor (HIF) and dependent on the NFκB signaling pathway (123). Several studies have demonstrated that higher levels of VCAM-1 are associated with better prognosis in RCC (123-126). Taken together, the ability of RCC to impair further leukocyte extravasation by reducing endothelial ICAM-1, VCAM-1 and E-selectin expression likely contributes to immune evasion to some extent. However, given the reasonably prominent immune cell infiltrate in RCC TME, this mechanism of immune evasion is likely of lower clinical importance.

Other mechanisms inhibiting effector CD8+ T cells and NK cells

RCC cells are capable of directly inducing apoptosis in activated T cells via the FAS/FASL interaction and subsequent activation of caspases (93, 127). Activated T cells displayed increased apoptosis when cultured in the presence of FasL+ RCC tumors, and the degree of apoptosis was significantly reduced by introduction of a neutralizing anti-FasL antibody (93).

NKG2D is an activating transmembrane glycoprotein receptor expressed on NK and T cells. The interaction of NKG2D with multiple ligands leads to the activation of NK cells and co-stimulation of CD8 T cells (128). RCC has been shown to have a marked under-expression of NKG2D ligands (85), thereby inhibiting this activation signal.

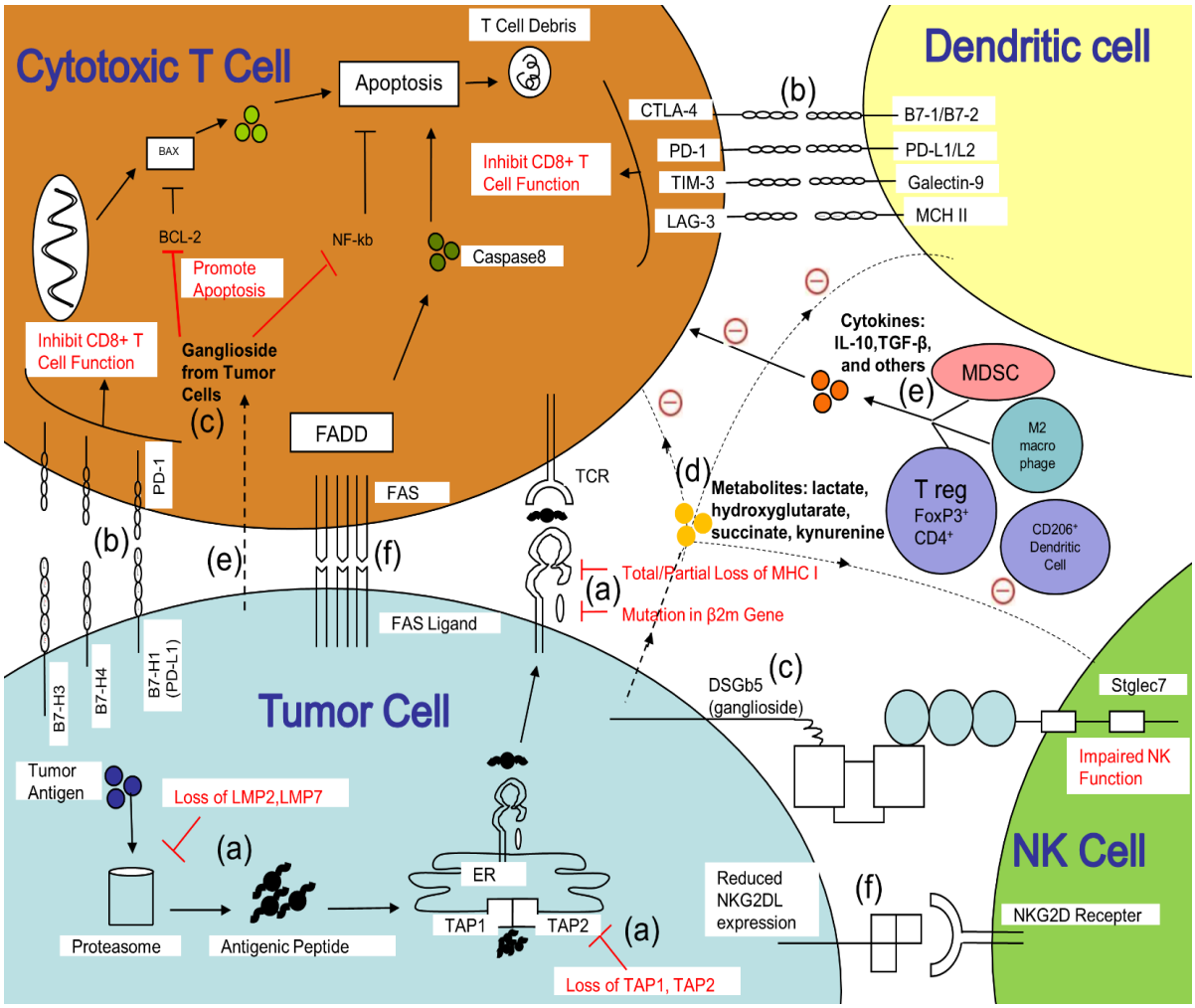


Figure 5. An illustration depicting the mechanisms of immune evasion in renal cell carcinoma. **(a) Poor antigen-presenting ability:** Changes in HLA expression/APM (antigen processing machinery) result in loss of antigen-presenting ability. **(b) Immune checkpoints:** cytotoxic cell inhibitory interactions such as PD-1 ←→ PD-L1, CTLA4 ←→ B7-1/2, TIM-3 ←→ Galectin-9, LAG-3 ←→ MCH II (as depicted). **(c) Tumor-associated gangliosides:** decrease lymphocyte expression of Bcl-2 and

Bcl-xL, and also inhibit NF-kb to prompt T cell pro-apoptotic events. DSGb5 (a membrane ganglioside on RCC cells) binds to Siglec-7 on NK cells to dampen cytolytic activity. **(d) Tumor- associated metabolites:** metabolites such as lactate, hydroxyglutarate, kynurenine, succinate etc. released by RCC cells inhibit effector immune cells. **(e) Tumor-promoting immune cells in the microenvironment and their inhibitory cytokines:** such as IL-10 and TGF-beta, released by T regs, MDSCs, and M2 macrophages. **(f) Other mechanisms inhibiting effector CD8+ T cells and NK cells:** Reduced expression of NKG2D ligands and immune checkpoints also contribute to reduced NK cytolytic activity. Direct induction of CD8 T cell apoptosis via FAS←→FASL and activation of caspases. *(Abbreviations- LMP: latent membrane protein; TAP1: transporter 1; PD-1: Programmed cell death protein 1; PD-L1: Programmed death-ligand 1; TIM-3: T cell immunoglobulin and mucin domain-containing protein 3; LAG3: Lymphocyte-activation gene 3; DSGb5: disialosyl globopentaosylceramide; Siglec7: Sialic acid-binding immunoglobulin-like lectin-7; Bcl-2: B-cell lymphoma 2; BAX: Bcl-2-associated X).(56)*

VI.3 Metabolic dysregulation in cancer

Our understanding of the role of aberrant cancer metabolism in fostering the various aspects of cancer progression has evolved significantly over the last decade. The six 'hallmarks of cancer metabolism' (129), which provide a conceptual framework to understand the process, are as follows:

1. deregulated uptake of glucose and amino acids,
2. use of opportunistic modes of nutrient acquisition,
3. use of glycolysis/TCA cycle intermediates for biosynthesis and NADPH production,
4. increased demand for nitrogen,
5. metabolite-driven gene regulation, and
6. metabolic interactions with the microenvironment.

Deregulated uptake of glucose and amino acids

Otto Warburg, in 1927, postulated the shift in cancer cell metabolism toward glycolysis as the primary source of energy gain, in what is now known as the Warburg Effect (130). The upregulation of glucose transporters, primarily GLUTs 1, 3, and 4, has been observed in several cancers (131-135). Despite its lower yield of ATP when compared to that of oxidative phosphorylation, glycolysis is by far the most time-effective process to generate additional energy (135). Since glycolysis is a cytoplasmic process, energy is generated for unchecked proliferation, migration, and invasion, bypassing the need for mitochondrial transport (136). Several oncogenes upregulate glucose transporter expression (135).

Glutamine serves as an important source of reduced nitrogen and as a source of carbon. The reduced nitrogen is used for biosynthetic reactions, and the carbon is used to replenish the tricarboxylic acid (TCA) cycle intermediates, producing glutathione as a precursor to

nucleotides and lipid synthesis via reductive carboxylation (129, 137, 138). Glutamine has also been reported to play a role in the uptake of essential amino acids. The import of leucine, an essential amino acid, through the localized neutral amino acid antiporter (LAT1) was shown to be coupled with simultaneous efflux of glutamine (139). The upregulation of transcription factors c-myc and E2Fis a principal driver of glutamine transport and utilization by proliferating cells. These transcription factors enhance transcription of glutamine transporters ASCT2 and SN2, and also facilitate conversion of glutamine to glutamate (140-142).

Use of opportunistic modes of nutrient acquisition

Cancer cells also possess the ability to recover amino acids from the immediate extracellular microenvironment. Imbibing extracellular proteins via macropinocytosis (143, 144), entosis of entire living cells (145, 146), phagocytosis of apoptotic bodies (147), are some of the opportunistic modes of nutrient acquisition. In addition, cancer cells can also withstand long periods of nutrient deprivation via the self-catabolic process of macroautophagy (148).

Use of glycolysis/TCA cycle intermediates for biosynthesis and NADPH production

Metabolic intermediates from glycolysis (mainly) and TCA cycle (partly) constitute the major source for rapid biosynthesis of macromolecules, membranes, and organelles in cancer cells (Table 1).

Table 1. Use of glycolysis/TCA cycle intermediates for biosynthesis and NADPH production.

Glycolysis intermediate	Biosynthetic process
Glucose-6-phosphate	Pentose Phosphate Pathway Used for NADPH production and ribose-5-phosphate- an integral component of nucleosides (149)
Fructose-6-phosphate	Hexosamine biosynthesis Used in heparan sulfate and hyaluronic acid biosynthesis, potentiation of receptor-mediated signaling, and stabilization of certain proteins (150)

Dihydroxyacetone phosphate	<u>Glycerol-3-phosphate biosynthesis</u> Used in phospholipid synthesis
3-Phosphoglycerate	<u>Serine biosynthesis</u> Up to 50% of glucose-derived carbon is used in serine biosynthesis and subsequent use: -as a carbon donor for the one carbon cycle and biosynthesis of nitrogenous bases -biosynthesis of S-adenosyl methionine- the main substrate for all cellular methylation reactions -as a major source of cellular NADPH (151, 152)
TCA cycle intermediate	**Biosynthetic process**
Citrate	Transported to cytosol via the tricarboxylate carrier and converted to Acetyl CoA and oxaloacetate -Acetyl CoA is used for fatty acid/ cholesterol biosynthesis -Oxaloacetate is converted to malate which is imported to mitochondria to maintain anaplerosis (153)
Oxaloacetate	'Non-essential' amino acids - aspartate and asparagine (154)

Increased demand for nitrogen

Along with the increased carbon demands, a proliferating cell also has greatly enhanced nitrogen demands. Glutamine, a non-essential amino acid whose uptake in increased by the transcriptional activity of oncogenes such as *c-myc*, serves as the major nitrogen donor in a proliferating cancer cell. Its amide group is a key donor of nitrogen for the biosynthesis of purine and pyrimidine bases. In addition, aspartate, which is a distal catabolite of glutamine, is used in the formation of purine and pyrimidine rings.

Metabolite-driven gene regulation

Metabolites play a key role in epigenetic modifications (Table 2), thereby having a marked influence on gene regulation. In addition to these epigenetic modifications, certain metabolic intermediates also function as co-substrates or competitive inhibitors of enzymes involved in epigenetic regulation or transcription factor processing (Figure 6). This hallmark of cancer metabolism is of particular significance with regards to this PhD study.

Table 2. Metabolite-driven gene regulation.

Metabolite	Gene regulation process
Acetyl CoA (derived from citrate and acetate)	Histone acetylation→ increased accessibility of genomic DNA for the assembly of transcriptional complex (155)
Crotonyl CoA (derived from lysine and tryptophan)	Histone crotonylation→ activates gene expression even more potently than acetyl marks (156)
S-Adenosyl methionine (derived from serine catabolism)	Histone methylation, DNA (cytosine) methylation, mRNA (adenosine) methylation (157, 158)
Succinyl-CoA, Malonyl CoA, Propionyl CoA, Butyryl CoA, Formyl CoA	Histone succinylation, malonylation, propionylation, butyrylation, formylation (159-161)→ ?effect

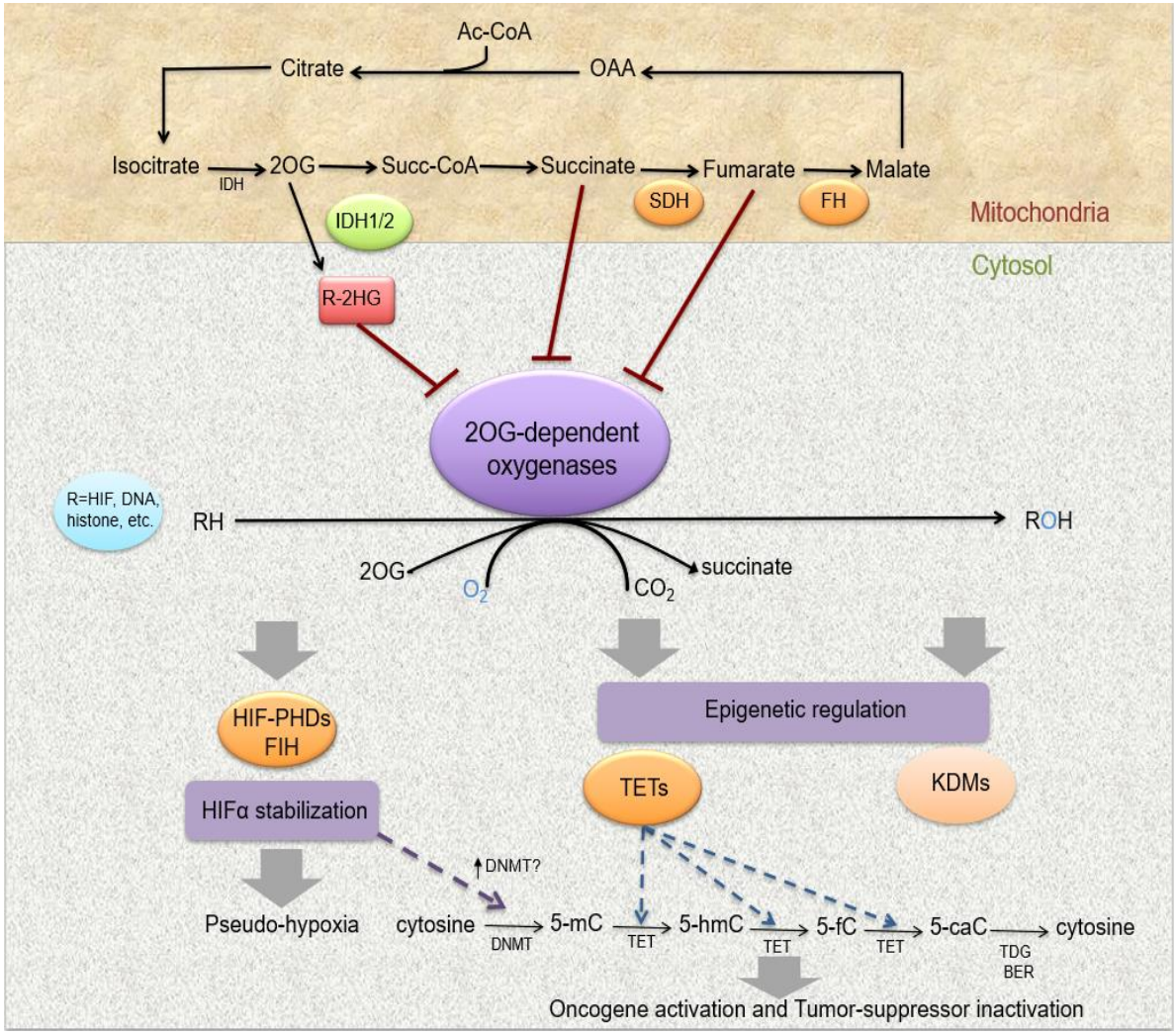


Figure 6. TCA cycle intermediates competitively inhibiting 2-oxoglutarate dependent enzymes, including Ten-Eleven-Translocation enzymes involved in DNA demethylation and Hypoxia Inducible Factor (HIF) prolyl hydroxylases involved in post-translational regulation of HIF. HIF may be involved in the increased expression of DNMT in some cancers, particularly the enhanced expression of DNMT1 in ccRCC (162)*(Abbreviations: 2OG: 2-Oxoglutarate; 2HG: 2-Hydroxyglutarate; HIF: Hypoxia Inducible Factor; DNMT: DNA methyl transferases; TET: Ten-Eleven Translocation enzymes; 5-mC: 5-methylcytosine; 5-hmC: 5-hydroxymethylcytosine; 5-fC: 5-formyl cytosine; 5-caC: 5-carboxyl cytosine; KDM; Lysine Demethylases; SDH: Succinate dehydrogenase; FH: Fumarate hydratase; MDH: Malate dehydrogenase).*

Metabolic interactions with the tumor microenvironment

The metabolites secreted by cancer cells have a profound effect on the microenvironment, promoting an immune-permissive environment, angiogenesis, degradation of extracellular matrix, thereby playing a critical role in progression, invasion and metastasis of cancer cells (Table 3).

Table 3. Metabolites that interact with the tumor microenvironment.

Metabolite	Microenvironment
Lactate	-Inhibits dendritic cells and cytotoxic T cells (163-165) -Macrophage polarization from M1 to M2 (immune-suppressive subtype) (166) -Promotes angiogenesis (induces VEGF secretion from stromal cells, and HIF-1 activation in endothelial cells) (167, 168)
Kynurenine	-promotes regulatory T cell phenotype (169) -promotes degradation of the extracellular matrix and invasion (170)
Acidification of the extracellular space (via lactate, H^+ and CO_2)	-stimulates proteolytic activity of matrix metalloproteinases (MMP) and cathepsins, promoting tumor invasion (171)

VI.4 ccRCC as a model for resistant cancers

While most malignancies demonstrate initial sensitivity to conventional chemotherapeutic agents and subsequent resistance, ccRCC is relatively unique in its upfront, innate, chemoresistance as well as resistance to conventional radiotherapy (172-174). As a result, it serves as a compelling model to study mechanisms of resistance and strategies to overcome or evade resistance mechanisms.

Apart from the dysregulated hypoxia signaling (secondary to VHL function loss), ccRCC has been found to be an epigenetically unique solid tumor, characterized by genome-wide hypermethylation that confers worse prognosis (175). Motif analysis of aberrantly hypermethylated regions revealed enrichment for binding sites to transcription factors involved in hypoxia signaling (175), suggesting a link between aberrant methylation and the pseudo-hypoxic signature in ccRCC. A comprehensive understanding of these two central features, therefore, is essential to discerning the pathogenesis and progression of this disease, and exploring therapeutic strategies.

VII. Sequential exploring of the unknowns (Aims of the PhD investigation):

What is the role of DNA methylation in RCC pathogenesis?

What is the role of dysregulated hypoxia signaling in RCC pathogenesis?

What is the comprehensive understanding, to date, of key molecular pathogenetic features in ccRCC?

How can these aberrancies be targeted?

1. **Shenoy N*,** Pagliaro L: Sequential pathogenesis of metastatic VHL mutant clear cell renal cell carcinoma: putting it together with a translational perspective." ***Ann Oncol*,** 2016 Sep;27(9):1685-95. PMID:27329246. *corresponding

What is the status of DNA hydroxymethylation (5hmC) in RCC?

Is there a prognostic role for 5hmC in RCC?

What is the mechanistic basis for loss of 5hmC and gain of 5mC in RCC?

Rather than suppressing DNMT enzymes, can we enhance demethylation (via increased hydroxymethylation) by activating the TET enzymes using ascorbic acid (AA)?

Can AA reactivate tumor-suppressor genes silenced by methylation?

What is the protein-level interaction between the TET-2 protein and AA in the presence and absence of the TET inhibitory metabolite L-2-hydroxyglutarate.

Does high dose AA have an RCC proliferation inhibitory effect in vitro and in vivo?

Are the epigenetic changes induced by AA in RCC tumors in vivo?

2. **Shenoy N***, *et al*. Ascorbic acid-induced TET activation mitigates adverse hydroxymethylcytosine loss in renal cell carcinoma. ***J Clin Invest*** 2019 Mar 4, 130:1612-1625. *co-corresponding

Does the AA-induced DNA demethylation have a role in other malignancies?

Can it be used as a chemotherapy-sensitizer in lymphoma?

3. **Shenoy N***, *et al*. Upregulation of TET activity with ascorbic acid induces epigenetic modulation of lymphoma cells. ***Blood Cancer J***. 2017 Jul 21;7(7): e587. *co-corresponding

What are the different mechanisms of anti-cancer activity of AA in relation to pharmacokinetics?

What is the history and evolution of ascorbic acid as an anti-cancer agent?

What are the future directions for exploring AA as an anti-cancer agent, in light of new emerging data?

4. **Shenoy N*,** *et al*. Ascorbic acid in cancer treatment: Let the phoenix fly. ***Cancer Cell*.** 2018 Nov 12;34(5):700-706.(Review) PMID: 30174242. *****co-corresponding

Can we use the demethylating property of AA and increase the expression of endogenous retroviral elements in cancer cells, thereby making them more immunogenic (i.e. using AA to enhance immune recognition of cancer)?

Is there an additive or synergistic effect of AA with anti-PD1 immunotherapy?

5. Luchtel R, Bhagat T, Pradhan K, Jacobs WR, Levine M, Verma A, **Shenoy N*.** High-dose Ascorbic acid synergizes with anti-PD1 in a Lymphoma mouse model**.** ***Proc Natl Acad Sci USA*.** 2020 Jan 21;117(3):1666-1677 *senior corresponding

Technical observations and offshoot projects during the PhD investigation

6. **Shenoy N*** *et al*. Drugs with anti-oxidant properties can interfere with cell viability measurements by assays that rely on the reducing property of viable cells. ***Lab Invest***. 2017 Feb 27. *Pathobiology in focus.* PMID: 28240748. *co-corresponding

7. **Shenoy N*** *et al*. Association between renal cell carcinoma and myelodysplastic syndromes: epigenetic underpinning? ***Clin Genitourin Cancer***. 2018 Dec.*co-corresponding

Appendix:

8. **Shenoy N***. HIF1α is not a target of 14q deletion in clear cell renal cancer. *Under review* at ***PLOS Genetics*** *single author

(Yellow highlighted articles represent original scientific investigations as first/ corresponding author. Turquoise highlighted articles represent comprehensive reviews as first/ corresponding author)

VIII.1 Sequential pathogenesis of metastatic VHL mutant clear cell renal cell carcinoma: putting it together with a translational perspective

(Shenoy N*, Pagliaro L. *Ann Oncol*, 2016*;* *corresponding)(45)

[Personal contribution: conceptualization; literature search; drafting manuscript; editing manuscript; visualization (figure); correspondence. This article was submitted as a single author-paper to a Nature reviews journal (and rejected), prior to submission to Annals of Oncology after Dr. Pagliaro's edits]

This comprehensive review cum perspective article details the step-wise molecular pathogenesis and progression of ccRCC, with a perspective on translational strategies.

The review groups the sequence of events in the molecular etiopathogenesis of VHL mutant metastatic RCC as follows: (i) loss of VHL activity (germline/somatic mutation + inactivation of the wild-type copy); (ii) constitutive activation of the hypoxia-inducible factor (HIF) pathway due to loss of VHL activity and transcription of genes involved in angiogenesis, survival, anaerobic glycolysis, pentose phosphate pathway, epithelial–mesenchymal transition, invasion, and metastasis (iii) interactions of the HIF pathway with other oncogenic pathways; (iv) genome-wide epigenetic changes (potentially driven by an overactive HIF pathway) and the influence of epigenetics on various apoptotic, oncogenic, cell cycle regulatory and mismatch repair pathways (e.g. through inhibition of multiple tumor suppressor genes); (v) immune evasion, partially caused by changes in the epigenome.

The review also reflects on the massive gap between our understanding of the molecular biology and (then) accepted standard of care in metastatic ccRCC, and presents ideas for better translational research involving therapeutic strategies with combinatorial drug approach, targeting different aspects of the pathogenesis.

This review was an extension of prior experimental work demonstrating genome-wide aberrant adverse hypermethylation in ccRCC (175) as well as comprehensive reviews on the role of DNA methylation in ccRCC (176) and dysregulated hypoxia signaling (177).

VIII.2 Ascorbic acid-induced TET activation mitigates adverse hydroxymethylcytosine loss in renal cell carcinoma

(Shenoy* *et al. J Clin Invest*, 2019; *co-corresponding)(178)

[Personal contribution: conceptualization, leadership and direction; design; data acquisition; data analysis and interpretation; drafting manuscript; editing manuscript; funding; correspondence]

We had previously demonstrated the adverse prognostic impact of DNA hypermethylation in RCC. This study unraveled the mechanisms and prognostic impact of loss of DNA hydroxymethylation in ccRCC, and comprehensively explored the potential of ascorbic acid in reversing the epigenetic aberrancy. (179)

Loss of 5hmC is an independent adverse prognostic biomarker in ccRCC

We found that loss of 5hmC is associated with aggressive clinicopathologic features and is an independent adverse prognostic factor in ccRCC through analysis of 576 primary ccRCC cases. It also predicts a shortened time to metastatic disease after surgical resection for localized disease. We showed that a grading of 5hmC immunohistochemistry (IHC) based on intensity (absent, mild, moderate and marked) or based on percent positive tumor cells, can be used as a strong tool to predict outcomes and could potentially be integrated in prognostic models, therapeutic decisions as well as clinical trial designs in the future. Given that this is a simple IHC test, it could potentially be adopted universally as a prognostic biomarker in this malignancy. Efforts are ongoing to determine if 5hmC can be a useful prognostic biomarker in other malignancies.

Loss of 5hmc in ccRCC is due to metabolic inhibition of TET enzymes

As discussed previously, Ten-Eleven Translocation (TET) enzymes are dioxygenase enzymes involved in active demethylation through a series of oxidation steps, the first of which is 5mC to 5hmC. We showed that gain of 5mC and loss of 5hmC in ccRCC is not due to mutational or transcriptional inactivation of TET enzymes, but by their functional inactivation by l-2-hydroxyglutarate (L2HG), an oncometabolite that accumulates largely due to the deletion and under-expression of l-2-hydroxyglutarate dehydrogenase (L2HGDH) (Figure 7). L2HG competes with 2-oxoglutarate (2OG), a necessary co-substrate of the TET enzymes (180). We found that the *L2HGDH* gene, located on chromosome 14q, is deleted in 41% of all ccRCC. Furthermore, we found a strong correlation between loss of L2HGDH

and loss of 5hmC in ccRCC (IHC). Also, loss of L2HGDH conferred worse prognosis (TCGA).

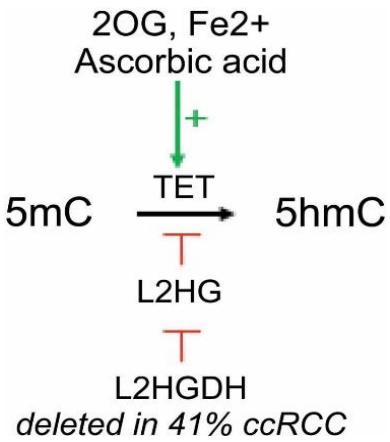


Figure 7. Schematic representation depicting the mechanism of 5mC gain and 5hmC loss in ccRCC. *L2HGDH* (l-2-hydroxyglutarate dehydrogenase) deletion and under-expression is common in ccRCC, resulting in the accumulation of L2HG (l-2-hydroxyglutarate), a competitive inhiitor of TET enzymes, which use 2-oxoglutarate as a substrate for oxidation of 5mC to 5hmC.

Ascorbic acid restores 5hmC in ccRCC and inhibits ccRCC proliferation *in vitro* and *in vivo*

Ascorbic acid (AA) is a cofactor for the TET enzymes, reducing the enzyme-bound iron from Fe^{3+} to Fe^{2+}. AA had been previously shown to cause TET-mediated demethylation of embryonal stem cells (181). We therefore hypothesized that AA could be used as an epigenetic targeting agent in ccRCC given the genome-wide aberrant methylation that is present in that cancer type. Indeed, we found that AA treatment increases TET activity, reduces 5mC and increases 5hmC in ccRCC. AA treatment was found to result in ccRCC proliferation inhibition *in vitro* and in a xenograft model *in vivo*. Histologic examination of the xenografts treated with intravenous AA revealed increased intra-tumoral 5hmC and enhanced differentiation.

The following were some important considerations and findings while studying the effects of ascorbic acid as an anti-cancer agent *in vitro* and *in vivo*:

A. Is the *in vitro* effect from AA or H_2O_2?

Although AA is an anti-oxidant, in the presence of free catalytic ions in culture media, it produces hydrogen peroxide (H_2O_2), a pro-oxidant, through the following reactions:

1) Ascorbate reduces catalytic metal ions such as ferric ions to ferrous ions.

$AscH^-$ (ascorbate) + $Fe^{3+} \rightarrow Asc^{*-}$ (ascorbate radical) + Fe^{2+}

2) The ferrous ions react with oxygen to form superoxide radical.

$Fe^{2+} + O_2 \rightarrow Fe^{3+} + O_2{}^{*-}$

3) Superoxide radicals then dismutes to H_2O_2 and O_2.

$O_2{}^{*-} + O_2{}^{*-} + 2H^+ \rightarrow H_2O_2 + O_2$

H_2O_2 is toxic to cancer cells. It is therefore important to neutralize H_2O_2 with catalase prior to studying epigenetic (or other cofactor related) effects of AA. This is particularly true with high doses (millimolar concentrations) of AA. Through catalase control and TET 1/2/3 knockdown experiments, we found that the demethylation effects of AA are independent of H_2O_2 and dependent on TET enzymes in ccRCC.

B. Dosing considerations

While designing both *in vitro* and *in vivo* experiments using AA, it is also important to consider the history and evolution of ascorbic acid as an anti-cancer agent and the available pre-clinical and clinical data. Oral AA has previously been tested as an anti-cancer agent and has failed (182, 183). There was no indication (either in the series by Cameron/Pauling in which oral AA was used after the first 7–10 days of intravenous AA treatment or in the Mayo studies in which only oral AA was used) to suggest that hypermethylated malignancies such as kidney cancer fared any better with oral AA treatment. On the other hand, pharmacologic AA is emerging as a promising agent in the treatment of established cancers, both in preclinical animal models (AA administered intraperitoneally) and more recently in early phase clinical trial data (AA administered intravenously at doses around 1g/kg 2–3 times/week) (184). Intraperitoneal and intravenous administration of ascorbate in mice has been shown to achieve ‘pharmacologic’ plasma concentrations in the millimolar range – over 100 times that with oral dosing – by bypassing the tight gastrointestinal regulation (185, 186). Intraperitoneally administered AA and not oral AA resulted in tumor shrinkage in a murine hepatoma model (185).

It is therefore important to design *in vitro* and *in vivo* experiments taking into account the pharmacokinetics of parenteral AA in humans and mice (a cycle of ‘bench to bedside and bedside to bench’). Furthermore, at these high concentrations of AA *in vitro*, it is essential to

neutralize H_2O_2 with catalase in order to study non-free radical effects. Our xenograft study was the first to use tail vein injections (5d/wk) to administer AA in order to further mimic intravenous AA treatment in humans (178). The prior animal model studies with parenteral AA were done with intraperitoneal administration. Although the intraperitoneal route attains plasma concentrations much higher than that with oral administration, it is not as high as that with the IV route with the same dose. The bioavailability fraction of intraperitoneal administration is around 0.62 (185).

C. Interaction of AA with TET enzymes

It is very difficult to determine the concentration of AA needed for its maximal effect on the TET enzymes with conventional experiments. The reasons are several – Michaelis constant (Km) shifts of ascorbate transporters, decreased/mutated enzyme/transporter copy numbers, the rate of intracellular/extracellular oxidation, Km shifts and copy numbers of TET enzymes. Therefore, we used the fluorescence quenching technique with recombinant TET-2 and AA, which reflects conformational changes to the protein induced by its binding with AA. We found that >90% quenching of recombinant TET-2 fluorescence is obtained with 132μM AA. We also studied the dynamics of fluorescence quenching of the recombinant TET-2 with oncometabolite L2HG and co-substrate 2OG in the presence and absence of AA. We found that the quenching efficiency of 2OG is higher than that of L2HG, indicating that the substrate specificity of the TET-2 protein is 2OG over L2HG. Furthermore, the fluorescence quenching of the TET enzyme with AA was largely unaffected by the presence of L2HG, suggesting that the effect of AA is unperturbed by L2HG. Indeed, the ccRCC cell line 786-O had markedly higher intracellular L2HG levels compared to the immortalized kidney cell line HKC-8, and AA treatment of 786-O cells still caused TET enzyme-dependent demethylation and increase in 5hmC.

D. Epigenetic reprogramming or oxidative stress?

Two mechanisms of AA-induced anti-cancer activity have gained prominence: TET-mediated demethylation and H_2O_2-induced oxidative stress. *In vitro* cancer cell cytotoxicity with short-term exposure to high dose AA is almost solely due to H_2O_2, given the complete reversal with catalase. However, it is important to recognize that for anti-oxidant AA to form pro-oxidant H_2O_2, free catalytic metal ions are required (as shown in reactions 1–3 above). While these are plentiful *in vitro*, they may be restricted in the tumor microenvironment. H_2O_2 has been shown to be generated in the extracellular fluid compartment but not within tumors in animal

models. In our xenograft study, we showed that high dose intravenous AA increases 5hmC within ccRCC tumors as well as enhances differentiation. The increased intra-tumoral 5hmC, enhanced differentiation and delayed tumor growth with high-dose AA, when taken together, suggest that epigenetic reprogramming is an important mechanism of intravenous AA-induced anti-cancer activity.

Mice have a functional L-gulonolactone oxidase (GULO) enzyme which enables them to produce L-AA from L-gulono-1,4-lactone. In humans, the GULO enzyme turned non-functional over the course of evolution, making AA an essential dietary vitamin. The finding of enhanced intra-tumoral 5hmC with intravenous AA despite the presence of a functional GULO enzyme suggests that supra-physiologic plasma concentrations of AA are needed even for optimal cofactor functions of AA within tumors, not just for potential oxidative stress.

VIII.3 Upregulation of TET activity with ascorbic acid induces epigenetic modulation of lymphoma cells

(Shenoy* *et al*. *Blood Cancer J*, 2017; *co-corresponding)(187)

[Personal contribution: conceptualization and direction; design; data acquisition; data analysis and interpretation; drafting manuscript; editing manuscript; correspondence]

Loss of function of TET enzymes can be secondary to inactivating mutation or hypoactivity of normal TET enzymes via competitive inhibition by metabolic intermediates. In lymphoma, a cancer of the lymphatic system, TET-2 mutations are found most commonly in the 'T-cell lymphomas'. In angioimmunoblastic T-cell lymphoma, TET-2 has been reported to be mutated in up to 76% of patients, with 50% harboring 2 or 3 mutations within the *TET-2* gene (188). Angioimmunoblastic T-cell lymphoma patients also have a high frequency of *IDH2* mutations that can inhibit TET activity through the formation of 2-hydroxyglutarate (188). TET-2 mutation rate is also as high as 38% in peripheral T-cell lymphoma-not otherwise specified (189), and 13% in diffuse large B-cell lymphoma (DLBCL), the most common type of non-Hodgkin lymphoma (190).

We therefore wanted to investigate whether AA could bring about similar epigenetic changes in lymphoma cells as observed in renal cell carcinoma cells (Paper 2 in this thesis), and if these epigenetic changes could re-activate important tumor-suppressor(s) in lymphoma cells.

AA enhances TET activity in lymphoma cells leading to genome-wide demethylation and increase in 5hmC fraction

We showed that AA increases activity of the TET enzymes in LY-1 (DLBCL) and Karpas 299 (T-cell) lymphoma cell lines using the ELISA-based *Epigentek* TET activity kit. Consistent with the increase in TET activity, DNA extracted from treated vs control cells revealed that AA treatment decreased global 5mC and increased 5hmC levels in lymphoma cells. The experiments were conducted such that the dose and exposure time mimicked the bioavailability curves of IV AA (191), and also considering potential differences between plasma concentrations and that of the tumor microenvironment.

AA-induced epigenetic effects are independent of hydrogen peroxide

H_2O_2 is formed when AA is added to the media (reactions detailed above). We wanted to ensure that the oxidative reaction of 5mC to 5hmC was not contributed to by the diffusion of H_2O_2 into the lymphoma cells, but a direct effect of AA. We found that addition of a high dose

of catalase sufficient to fully neutralize H_2O_2 did not alter the amount of 5hmC fraction produced by the same dose and exposure of AA, further indicating that the 5hmC production was independent of H_2O_2.

AA reactivates SMAD1, a critical contributor of chemosensitization in DLBCL cells

SMAD1, a component the TGF/BMP pathway, is an important tumor suppressor in DLBCL cells and known to be suppressed by methylation (192). Reactivation of epigenetically silenced SMAD1 with the DNMT inhibitor azacytidine, as well as SMAD1 transfection, had been shown to induce chemosensitization to conventional anti-tumor agents (192).

We sought to determine whether AA-induced demethylation could also reactivate SMAD1 expression. Indeed, treatment of LY-1 cells with pharmacologic doses of AA led to the reactivation of SMAD1. Furthermore, addition of catalase had no effect on SMAD1 transcript abundance, again indicating that AA-induced SMAD1 upregulation was independent of H_2O_2. Pre-treatment of lymphoma cells with AA resulted in increased sensitivity to cisplatin and doxorubicin, two commonly used chemotherapeutic agents in DLBCL.

Low plasma AA levels are frequent in lymphoma patients with high bulk disease

We then studied plasma AA level in 34 lymphoma patients (31 Non-Hodgkin, 3 Hodgkin). We found that 64% (9/14) of patients with clinical high-burden lymphoma had low AA levels (mean=0.39 mg/dl; range 0–1.2 mg/dl) compared to 5% (1/20) of those with low burden disease (mean 1.1; range 0.2–2; $P<0.001$).

VIII.4. Ascorbic acid in cancer treatment: Let the phoenix fly

(Shenoy N* *et al. Cancer Cell*, 2018. *co-corresponding)(184)

[Personal contribution: conceptualization; literature search and review; drafting manuscript; editing manuscript; correspondence]

This review-cum-perspective article (i) highlights salient aspects of the evolution of AA in cancer treatment (Table 4), (ii) provides insights into the pharmacokinetics of AA, (iii) describes mechanisms of its anti-cancer activity in relation to the pharmacokinetics, (iv) outlines promising preclinical and clinical evidence, and (v) recommends future directions for the investigation of AA in cancer.

Table 4. Evolution of ascorbic acid studies in cancer.

Cameron/Pauling Studies	Mayo Clinic Studies	AA pharmacokinetics and early-phase clinical trials	Studies on H_2O_2 mechanism	Studies on epigenetic mechanism
Ewan Cameron and Linus Pauling described retrospectively and in case reports that patients with advanced cancer had survival benefit and symptomatic relief using high-dose ascorbate (10 g/day i.v. followed by oral) (193-195)	Two double-blind placebo-controlled prospective trials performed at the Mayo Clinic using the same dose of ascorbate, but orally only, failed to confirm these results, and oral ascorbate was dismissed as an anti-cancer agent (182, 183)	Oral ascorbate, even at high doses, was found to produce plasma concentrations that were tightly regulated by gastrointestinal absorption, but i.v. administration bypassed this control until the kidney restored homeostasis. Maximum tolerated doses of oral (~18 g daily) ascorbate produced plasma concentrations of ~100–200 μM. Intravenous ascorbate was found to produce plasma levels hundreds of times higher than those produced by the maximum tolerated dose of oral ascorbate (191, 196) Early-phase clinical trials indicate that i.v. ascorbate at 1 g/kg over 1.5–2 hours two to three times weekly is well tolerated and may enhance chemosensitivity as well as decrease chemotherapy-related side effects (197-201)	Plasma concentrations achieved by i.v. dosing found to act as a prodrug for hydrogen peroxide (H_2O_2) in the extracellular space. High, but not physiologic, doses of ascorbic acid were selectively toxic to cancer cells *in vitro* and *in vivo* (186, 198, 202, 203)	Ascorbate functions as a cofactor and increases the activity of the TET enzymes causing DNA demethylation. This function results in the re-expression of tumor-suppressor genes in cancer cells, promotion of stem cell differentiation and inhibition of leukemogenesis, and increase in expression of endogenous retrovirus transcripts (178, 187, 204-206)

VIII.5 High-dose ascorbic acid synergizes with anti-PD1 in a lymphoma mouse model

(Luchtel *et al*.... Shenoy*, *PNAS* 2020; *senior corresponding)(63)

[Personal contribution: conceptualization, leadership and direction; design; data acquisition; data analysis and interpretation; drafting manuscript; editing manuscript; funding; correspondence]

DNA methyltransferase inhibitors (DNMTIs) are being investigated in combination with anti-PD1 therapy in hematologic malignancies (207). Enhanced endogenous retroviral expression and cancer testis antigen expression induced by demethylation results in increased tumor recognition by immune cells (208-212). Having shown that AA causes demethylation and corresponding increase in the hydroxymethylation fraction of lymphoma cells (187) (Paper 3 in this thesis), we hypothesized that AA may be an optimal demethylating agent for combination with anti-PD1 therapy as it has also been shown to enhance the function of immune cells such as natural killer (NK) cells, macrophages, and dendritic cells (213, 214). In contrast, effects of DNMTIs on immune cells have been inconsistent, with some studies indicating an inhibitory effect (215-217).

In this study, we characterized genome wide high-resolution methylation changes, endogenous retroviral expression, and PD-L1 expression changes in lymphoma cells with high-dose AA treatment and the subsequent effect on sensitivity to cytotoxic T cell-mediated killing. Given that T lymphocytes exhibit an enrichment of 5-hydroxymethylcytosine (5hmC) at gene bodies during differentiation and development (218), we determined the direct effects of AA on CD8+ T cells with regards to global 5hmC changes and cytotoxic function. Finally, we investigated antitumor effects of AA alone and in combination with anti-PD1 therapy in a syngeneic lymphoma mouse model and determined the changes in the tumor immune microenvironment.

AA treatment leads to genome-wide demethylation and increased endogenous retroviral expression in lymphoma cells

We further characterized the hypomethylation effect of AA in lymphoma cells with high-resolution methylation analysis using the HELP (Hpa II tiny fragment enrichment by ligation-mediated PCR) assay that relies on differential restriction digestion of methylated CpGs followed by high-throughput sequencing analysis. Unsupervised clustering showed that AA treatment led to significant changes in cytosine methylation patterns between control and

AA-treated lymphoma cells. Specifically, AA treatment led to global loss of cytosine methylation.

Next, we next assessed the expression of human endogenous retroviruses (HERVs) by RNA sequencing. HERVs have been shown to be up-regulated by DNMT inhibition (204). HERVs increase immune recognition of tumor cells, trigger an interferon response by induction of the viral defense pathway and enhance checkpoint blockade antitumor activity (219, 220). Consistent with the global loss of methylation, the majority (70%) of differentially expressed HERVs were increased in lymphoma cells following AA treatment. Global methylation analysis of HERVs up-regulated with AA treatment revealed that ~60% of the loci were demethylated upon AA treatment. However, there was no change in methylation at the *CD274* locus, encoding PD-L1. Also, there was no increase in PD-L1 expression with AA treatment in any of the 4 DLBCL cell lines tested.

AA pretreatment of lymphoma cells leads to increased sensitivity to CD8+ T cell cytotoxicity; AA treatment of CD8+ T cells leads to increase in its cytotoxic activity against lymphoma cells

Pretreatment of lymphoma cells with high-dose AA significantly increased their immunogenicity, evidenced by increased percent killing of lymphoma cells by 15% and 21% of control by CD8+ T cells when combined at 5:1 and 10:1 effector: target cell ratios. Similarly, AA pretreatment of healthy donor-derived CD8+ T cells led to a 3.8-fold increase in their cytotoxic activity against lymphoma cells, as measured with the lactate dehydrogenase (LDH) cytotoxicity assay (further validated by a flow cytometry-based cytotoxicity assay). This was associated with a significant increase in 5hmC levels in AA-treated CD8+ T cells.

High-dose AA treatment synergizes with anti-PD1 immunotherapy in a syngeneic lymphoma mouse model, resulting in significant tumor proliferation inhibition

Using the A20 lymphoma syngeneic mouse model, we treated tumor-bearing mice with vehicle, anti-PD1, high-dose AA, or the combination of high-dose AA and anti-PD1 until the tumor volume endpoint was met. Daily treatment was administered from day 10 (after appearance of tumors) until the tumor size endpoint was met. Tumor volume (cubic millimeters) was monitored every 2 days over the duration of the study by caliper. Given the highly aggressive nature of the A20 lymphoma mouse model, humane endpoint was reached in one mouse in the vehicle group on day 19 (after only 9 d of treatment). To facilitate

comparison between the treatment groups, all mice in the 4 groups were killed on day 19 and tumors excised and weighed.

Compared to AA and anti-PD1 single agents, we found that AA+anti-PD1 therapy resulted in greater inhibition of tumor growth over time as well as markedly lower final tumor weight. Because the observed effect of combined AA and anti-PD1 therapies was greater than the expected additive effect, we applied the coefficient of drug interaction (CDI) formula (221-224) to calculate synergy using mean tumor weight measurements whereby CDI<1 indicates synergism, with CDI<0.7 indicating a significantly synergistic effect. The CDI between high dose AA and anti-PD1, using the mean tumor weight measurements was 0.63, indicating a significantly synergistic effect (Fig 3E). This effect is particularly significant given the aggressive nature of this model and short treatment duration (10 days) after appearance of tumors. Furthermore, we also demonstrated that intra-tumoral 5-hmC can be increased *in vivo* through high-dose AA, consistent with the *in vitro* findings.

High-dose AA and anti-PD1 treatment combination leads to increase in tumor CD8+ T cell and macrophage infiltration, enhanced granzyme B production by cytotoxic cells, and enhanced Interleukin 12 production by antigen-presenting cells

Analysis of our syngeneic B-cell lymphoma model treated with AA and anti-PD1 revealed several important changes within the tumor immune microenvironment:

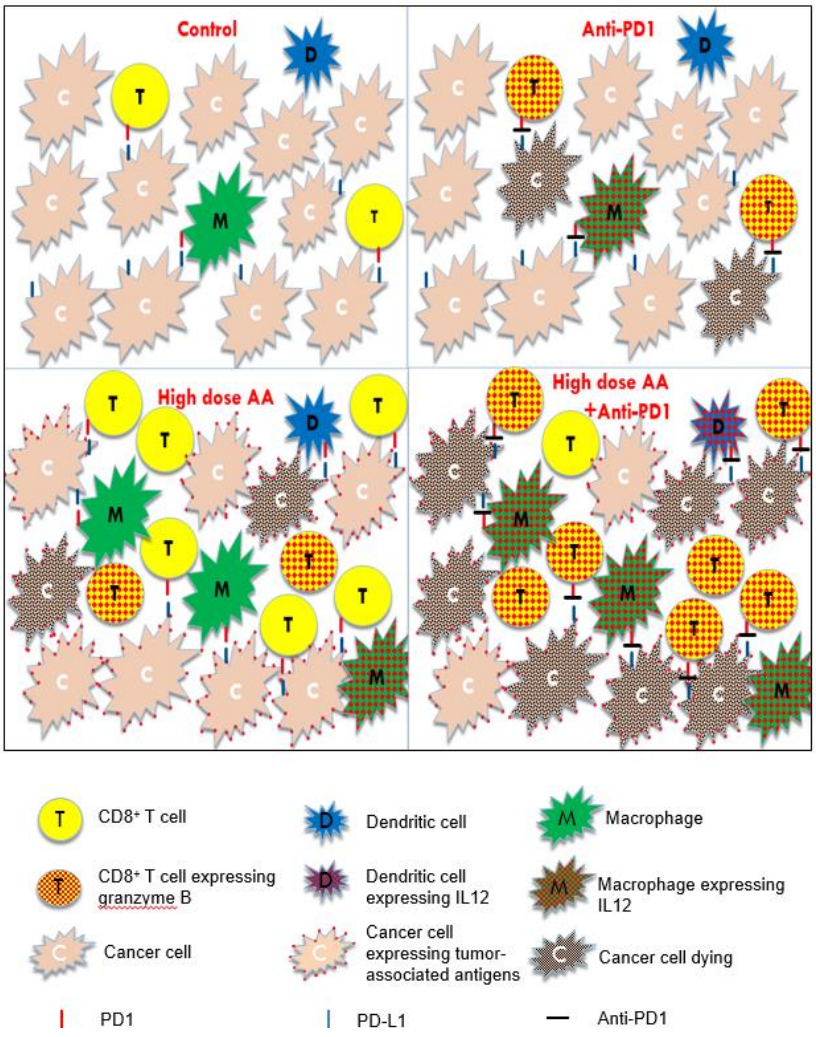
First, AA treatment, both alone and in combination, significantly increased CD8+ T cell infiltration compared with vehicle and single agent anti-PD1.

Second, granzyme B expression was markedly higher with combined AA + anti-PD1 than AA or anti-PD1 alone. An inverse exponential relationship was observed between granzyme B and final tumor weight, indicating an exponential growth of tumor with decreasing granzyme B expression. This is consistent with reported utility of granzyme B as a biomarker for response to immunotherapy in humans (225). Although the cytotoxic T cell infiltration with single agent AA was much higher than anti-PD1, the granzyme B expression was similar between both groups. Together, these data suggest that AA functions to recruit cytotoxic T cells to the tumor but anti-PD1 is more potent than AA at inducing a cytotoxic response.

Third, we found that within each treatment group, the contribution of NK cells towards granzyme B expression was at least as much as CD8+ T cells. The combination treatment markedly upregulated granzyme B expression in both CD8+ T cells and NK cells.

Fourth, we observed significantly increased intratumoral macrophage infiltration in mice that received AA, alone and in combination. In the combination group only, expression of IL12, a cytokine produced by macrophages and dendritic cells, that stimulates cytotoxic cells (CD8+ T cells and NK cells) was markedly upregulated.

Put together, this study shows that AA treatment 1) increases immunogenicity of lymphoma cells; 2) enhances intratumoral infiltration of CD8+ T cells and macrophages; and 3) synergizes with anti-PD1 checkpoint inhibition in a syngeneic lymphoma mouse model via marked activation of both cytotoxic cells (cytotoxic T cells and NK cells) and antigen presenting cells (Figure 8). The data provide a compelling rationale for testing combinations of high-dose AA and anti-PD1 agents in patients with aggressive B cell lymphoma and in preclinical models of other malignancies.



C: cancer cell; **T**: CD8+ T lymphocyte; **M**: Macrophage; **D**: Dendritic cell.

→**Single agent anti-PD1:** blocks the inhibitory PD-1/ PD-L1 axis thereby activating the sparse infiltrated cytotoxic T lymphocytes, NK cells (not depicted) and macrophages.

→**Single agent high dose AA:** enhances tumor immune recognition, and increases macrophage and cytotoxic T cell infiltration into the tumor. However, despite the increased immune cell numbers, the inhibitory effect of PD-1/PDL-1 axis on cytotoxic cells and macrophages cannot be abrogated by AA.

→**Anti-PD1+High dose AA:** The combination treatment results in both increased immune infiltration and enhanced activation of antigen presenting cells and cytotoxic cells, resulting in marked tumor shrinkage.

Figure 8. Graphical summary of the synergistic effects of high dose AA and anti-PD1.

VIII.6 Drugs with anti-oxidant properties can interfere with cell viability measurements by assays that rely on the reducing property of viable cells.

(Shenoy* *et al. Lab Invest.* 2017 *co-corresponding) (226) [Pathobiology in Focus section]

[Personal contribution: conceptualization; design; data acquisition; data analysis and interpretation; drafting manuscript; editing manuscript; correspondence]

Cell viability assays such as Cell Titer Blue and Alamar Blue rely on the reducing property of viable cells to reduce the reagent dye to a product which gives a fluorescent signal. The manufacture-recommended protocols do not take into account the possibility of the reagent substrate being reduced directly to the fluorescent product by drugs with an anti-oxidant property. After suspecting spurious results while determining the cytotoxic potential of ascorbic acid against a renal cell cancer (RCC) cell line, we aimed to establish that drugs with anti-oxidant property can indeed cause false-negative results with the current protocols of these assays by direct reduction of the reagent substrate. We also aimed to counter the same with a simple modification added to the protocol.

Through our experiments, we conclusively demonstrated that drugs with anti-oxidant properties can indeed interfere with cell viability measurements by assays that rely on the reducing property of viable cells (Figure 9). We also reported that a simple modification in the protocol (removal of the media containing the drug and washing the cells prior to incubation with fresh media and Cell Titer Blue reagent) can prevent spurious results with these otherwise convenient assays.(step-by-step protocol in Protocol Exchange (227))

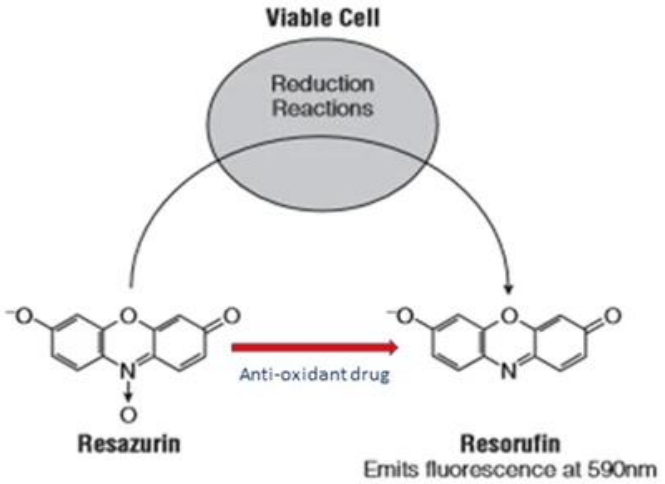


Figure 9. Depiction of interference of anti-oxidant drugs on Cell Titer Blue and Alamar Blue assays.

How this seemingly obvious phenomenon escaped detection during the development of resazurin-based cell viability assays, and its theoretical impact on cancer research:

The resazurin reduction test has been used for more than 60 years to detect contamination of milk, and to assess the quality of semen. In the 1990s, it began gaining popularity as a convenient method of assessing cytotoxicity. However, before the year 2000, it was unclear whether the resazurin dye was reduced within the cells or in the 'reduced medium' from increased cell growth. In the year 2000, O'Brien *et al*. reported that when the dye was added to the reduced medium separated from cells after 24–48 h incubation, it did not get converted to the fluorescent resorufin. They detected fluorescence in the cytoplasm of cells on addition of the dye, albeit at a quantity not large enough to be detected by the fluorescent microplate reader. They therefore concluded that the dye likely enters the cytoplasm where it is reduced to the fluorescent product, which is then excreted into the medium giving the fluorescent signal (228). Furthermore, an analysis of hepatic subcellular fractions suggested that resazurin can be reduced by mitochondrial, cytosolic and microsomal enzymes (229). When this was extrapolated into cancer research to test cell viability, the question of how the redox property of drugs tested would affect the dynamics seems to have escaped widespread attention. It was assumed that the dye could only get reduced intracellularly. The Cell Titer Blue protocol even mentions 'cell washing, removal of medium and multiple pipetting steps are not required' and states that a 'Test compound control' is optional.

A majority of anti-cancer drugs developed, initially go through cell viability assays or proliferation assays. The decision to pursue further research with these drugs is often based on their effects on cell viability or proliferation. One therefore wonders how many indigenous agents with high redox potential tested with assays that rely on the viable cells' reducing property had spurious results. Therefore, the addition of a simple step, as elaborated in the protocol, can eliminate such spurious results from these otherwise convenient assays. Based on our report, we recommended that this step be incorporated in the protocols of these assays, especially when testing a drug with high redox potential, and to always have a 'test compound' control without cells.

VIII.7 Association between renal cell carcinoma and myelodysplastic syndromes: epigenetic underpinning?

(Shenoy* *et al. Clin Genitourin Cancer.* 2018 *co-corresponding)

[Personal contribution: conceptualization, leadership and direction; design; data acquisition; data analysis and interpretation; drafting manuscript; editing manuscript; correspondence]

Having determined that renal cell cancer is characterized by marked aberrant hypermethylation and is therefore epigenetically similar to myelodysplastic syndromes (MDS), and after clinical observations of patients with a personal history of both malignancies, we sought to explore a potential association between the two, using Mayo Clinic's 'Advanced Cohort Explorer' database.

We found that the prevalence of MDS in patients > 65 years with a personal history of nephrectomy for RCC was ≈8.4 times that of the age-concordant general population based on the Dusseldorf registry (28/6490 or 395/100,000 vs. ≈ 47/100,000; P < .001), and 3.07 times that of the age-concordant patient population at Mayo Clinic (28/6490 or 395/100,000 vs. 128.4/100,000; P < .001).

We therefore demonstrated a strong association between RCC and MDS. Patients with a history of RCC have a substantially increased risk of developing MDS compared with the general population, a factor that must be considered in the survivorship care plan in these patients.

IX. Future research directions

A. CLINICAL

As an outcome of the above work, there are four phase 2 clinical trials (2 ongoing, 2 pending regulatory approvals) to investigate the potential of high dose IV ascorbic acid in cancer:

1. Randomized phase II trial of intravenous ascorbic acid (AA) as an adjunct to pazopanib for metastatic and unresectable clear cell renal cell carcinoma (ccRCC): A study of Academic and Community Cancer Research United (ACCRU) GU1703. *(Clinicaltrials.gov Identifier: NCT03334409)* [Shenoy: Co-PI] (230)

2. IV Ascorbic acid and combination chemotherapy in treating patients with relapsed or refractory lymphoma *(ClinicalTrials.gov Identifier: NCT03418038)* [Shenoy: Co-PI]

3. High dose intravenous ascorbic acid as an adjunct to azacytidine in high-risk myelodysplastic syndromes (pending regulatory approvals) [Shenoy: PI]

4. High dose intravenous ascorbic acid plus nivolumab for relapsed/refractory diffuse large B cell lymphoma in patients having failed or ineligible for autologous transplantation (being evaluated for funding) [Shenoy: PI]

B. LABORATORY

Continuation of the above work would involve exploring several important, and exciting areas:

1. Investigating the combination of AA+anti-PD1 in pre-clinical models of other cancer types (using both syngeneic and humanized mouse models). This project is of immediate, and important, translational relevance for cancer patients. If the same response is seen with the combination in pre-clinical models of most/ all malignancies, it could be of immense benefit to patients with different types of cancer. If the response to the combination is different between cancer types, we plan to investigate mechanisms of resistance/ difference by using techniques such as CyTOF and single cell RNA sequencing to dissect the immune microenvironment changes induced by the combination. We will also attempt to determine biomarkers by correlating these findings with response, and subsequent validation of potential candidates.

2. Further investigating the mechanisms of AA-induced anti-cancer activity.

3. Further exploring the mechanistic basis of interactions between the metabolome and the epigenome in ccRCC and other malignancies. Specifically, our immediate next project investigates the contribution of a second oncogenic metabolite (apart from L2HG) in driving hypermethylation of ccRCC and its role in the pathogenesis and progression of this malignancy.

4. Exploring the biologic basis for the increased risk of myelodysplastic syndromes in patients with a history of renal cell carcinoma, with a long-term goal of risk stratification and early intervention to mitigate the increased risk.

C. A few critical remarks in conclusion

1. The data revealing synergy between AA and anti-PD1 (63) highlight the limitations of using immunocompromised mouse models to comprehensively study AA- induced anti-cancer activity. Prior *in vivo* studies with parenteral AA in cancer (as a single agent and in combination with chemotherapy) were performed using immunocompromised models, which could not capture the effect of AA on anti-cancer immunity (immune recognition and function) and also could not determine how that aspect is affected in combination with chemotherapy. As such, even if it turns out that the randomized trials exploring IV AA in combination with chemotherapy are negative, it should not impede exploration of IV AA in combination with immunotherapy (specifically anti-PD1). With the mechanistic synergy between high dose AA and anti-PD1 being clearly delineated (63), the translational promise of the combination is perhaps higher than that with high dose AA and chemotherapy, and should be subjected to rigorous clinical exploration.

2. The increased intra-tumoral 5hmC, enhanced differentiation and delayed tumor growth with high-dose intravenous AA in an RCC xenograft model (178), when taken together, suggest that epigenetic reprogramming is an important mechanism of IV AA-induced anti-cancer activity. If H_2O_2 (a pro-oxidant) was the main mechanism of IV AA-induced anti-cancer activity, enhanced differentiation and increased tumoral 5hmC (reflective of AA's anti-oxidant cofactor function in TET enzymes- reducing Fe^{3+} to Fe^{2+}) would not be the expected histopathologic features.

3. It is important to recognize that for anti-oxidant AA to undergo auto-oxidation and subsequently generate pro-oxidant H_2O_2, free catalytic metal ions are required (as shown in reactions 1–3 above, page 33) (231). While these free catalytic metal ions are plentiful *in vitro*, they may be restricted in the tumor microenvironment. H_2O_2 has been shown to be generated with parenteral AA in the extracellular fluid compartment *in vivo* in one study (186), but not within tumors in animal models.

   A historical perspective and critical analysis of AA dosing regimens: Ewan Cameron, Allan Campbell, and Linus Pauling used IV AA (at 10g/ day) only for 7-10 days followed by oral AA (at 10 g/ day), and reported survival benefit and symptomatic relief in patients with advanced cancer in retrospective studies (193-195). The IV AA dose used was 10g daily given over 24 hours. In a pharmacokinetic study of vitamin C (196), 10 g IV ascorbate given over 40 minutes (i.e. 250mg/min) produced a peak plasma concentration of 5.6 millimolar AA. However, considering the expected simultaneous renal excretion, the 10g IV AA given over 24 hours in the Cameron/ Campbell/ Pauling series would be expected to produce peak AA concentrations only in the high micromolar or perhaps low millimolar range, which would then be expected to produce minimal amounts of ascorbate radical and H_2O_2 in the tumor microenvironment (as per measurements reported in (203)). After the first 10 days, all patients in the Cameron/ Campbell/ Pauling series received only oral ascorbate, which would be expected not to generate any H_2O_2. Furthermore, Cameron and Campbell stated the following: "*With increasing experience, we now tend to believe that the intravenous regime is probably unnecessary as a routine measure, and need only be employed in clinical situations, where vomiting, anorexia, or other complications of malignancy preclude oral administration*" (193). They also stated that the few patients in their series who received IV AA above 10g daily (up to 45g daily) had no clear therapeutic advantage (193). In keeping with these statements, many patients in their series only received oral AA. And when Mayo Clinic shared the planned randomized clinic trial protocol with Pauling, there was no specific objection to using oral AA (232). For all the above reasons, attributing the difference in outcomes of the Cameron/ Campbell/ Pauling studies and the Mayo clinic studies primarily to a difference in the route of administration and H_2O_2 generation, as has been repeatedly suggested in literature, would not only be an oversimplification but also grossly inaccurate. That said, neither the Mayo Clinic investigators in the 1970s and 80s nor Cameron and Pauling were aware of the striking pharmacokinetic differences between IV and oral AA (196). Early phase clinical trials and pilot studies have since demonstrated that IV AA at 1-1.5g/kg given 2-3 times weekly (a regimen primarily borne out of practice in the integrative medicine circles rather than a phased bench to bedside translation (233,

234)) is well tolerated and may enhance chemosensitivity as well as decrease chemotherapy related side effects (reviewed in (184)).

4. In the *Cancer Cell* review (184), we had indicated that the documented reports of high dose AA-induced oxidative hemolysis in G6PD patients was an indication of oxidative effects of high dose AA (in potential support of the H_2O_2 mechanism against cancer). However, over the last year and during the preparation of this thesis, I realized that there is an alternative, non-H_2O_2- related explanation for the high dose AA-induced oxidative hemolysis in G6PD deficient individuals: dehydroascorbic acid (DHA), oxidized vitamin C. While ascorbate ($AscH^-$), the dominant form of vitamin C in plasma, is transported into cells by Na+-dependent vitamin C transporters (SVCTs- SVCT1 and SVCT2), DHA is transported via Na+-independent facilitative glucose transporters (GLUTs) followed by intracellular reduction (235-239). Plasma DHA concentrations are very low (under 2uM) under physiologic conditions, and plasma glucose (which also uses GLUTs to be transported into cells), is significantly higher at 2-5 mM (237, 239). Therefore, intracellular ascorbate concentrations in most cells is determined by ascorbate uptake by SVCTs. However, red blood cells lack SVCTs (240) and rely on DHA uptake via GLUTs followed by intracellular reduction, to maintain membrane structural integrity (241, 242). G6PD is a key regulatory enzyme of the pentose phosphate pathway that produces NADPH and reduced glutathione (the major antioxidant machinery). With low G6PD, the amount of DHA entering the red cells with high doses of AA administration likely overwhelms the capacity for intracellular reduction resulting in oxidative membrane damage and hemolysis. Therefore, high dose AA-induced oxidative hemolysis in G6PD patients may have very little (or nothing) to do with H_2O_2 generation.

5. Intra-tumoral factors that could significantly affect ascorbate concentrations within cancer cells and immune cells as well as affect optimal cumulative AA cofactor function within these cells such as: intra-tumoral perfusion, transporter (SVCT1/ 2) expression and function, microenvironment composition and pH, Km shifts of enzymes etc. are yet to be comprehensively explored. These factors may account for the requirement of high doses of AA given parenterally (intravenously in humans) for its anti-cancer activity.

6. Given the unknown factors above, and suggestion of potential benefit with high dose IV AA in early phase trials as reviewed in (184), it would be prudent to continue clinical exploration with high dose IV AA at 1g/kg given 2-3 times/ week as recommended in (184), simultaneously with mechanistic exploration of the unknowns in the pre-clinical setting and

with correlative studies in trials. However, following this IV AA administration schedule is particularly challenging in the middle of a pandemic. A possible solution to reduce the number of healthcare center visits while keeping up with the schedule is tele-monitored home infusion (after adequate in-person instructions during administration of the first few doses in a monitored setting). The regimen has been shown to be safe in early phase trials in patients with no G6PD deficiency and adequate renal function.

In conclusion, the implication of having a new therapeutic agent that is safe, universally available and accessible, with the potential of anti-cancer activity across multiple tumor types in combination with conventional therapy (chemo/ immuno- therapy), would be substantial. However, it must pass traditional response metrics in well-designed, rigorous, clinical trials before widespread use, and these trials must be conducted without delay.

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XI. Appendix

XI.1 HIF1α is not a target of 14q deletion in clear cell renal cancer (Shenoy N*. *under review at PLOS Genetics.* *single author)

Clear cell renal cell carcinoma (ccRCC) is the only malignancy in which Hypoxia Inducible Factor 1-alpha (HIF1α) has been stated to have a tumor-suppressive role. The claim was primarily based on proliferation studies in ccRCC cell lines and their xenograft models in nude mice with HIF1α manipulation, published in 2011 (243). While the data were compelling, they stood in contrast with the well-established tumor-promoting properties of HIF1α in other malignancies (via angiogenesis, metabolic adaptation, resistance and survival)(177, 244, 245). The paper further stated that loss of *HIF1A* gene (which is located at 14q23.2, and encodes HIF1α) was a 'target' of 14q loss in kidney cancer (14q deletion is seen in up to 40% of ccRCC). Following this landmark paper, the poor prognosis of 14q deleted RCC patients was commonly attributed to loss of *HIF1A* (246). From a translational standpoint, inhibiting HIF1 as an anti-cancer strategy has since been viewed as murky at best, and indeed likely harmful in kidney cancer.

Recently, however, HIF-2 inhibition-resistant ccRCC patient-derived xenograft tumors were found to have high HIF1α levels, raising the question whether HIF1α indeed played a tumor promoting/resistance conferring role in these ccRCC tumors (https://doi.org/10.1016/j.eururo.2017.10.007). Then, within the last year and half, the tumor suppressive role of *L2HGDH* (L-2-hydroxyglutarate dehydrogenase) was characterized and reported in ccRCC- based not only on mechanistic *in vitro* and *in vivo* studies (247) but also on patient histology, tumor methylation and survival data (178). L2HGDH is a flavin adenine dinucleotide (FAD)-dependent enzyme that oxidizes L-2-hydroxyglutarate (L2HG) to alpha-ketoglutarate. Loss of L2HGDH in ccRCC (via deletions and under-expression) leads to the accumulation of L2HG, which is an oncometabolite that inhibits the TET enzymes, resulting in an adverse loss of 5hmC and gain of 5mC. Furthermore, this characterization of L2HGDH as a tumor suppressor was much more comprehensive than HIF1α.

This manuscript reports that *L2HGDH* is located reasonably close to *HIF1A* on 14q (at 14q21.3), and is deleted in nearly 95% of 14q deletions in ccRCC involving the *HIF1A* locus (an observation that triggered this investigation). Furthermore, the copy number of *HIF1A* correlates much stronger with *L2HGDH* expression (Rho=0.55) than its own gene expression (Rho=0.27) revealing that there is a high degree of preserved-allele compensation of *HIF1A* in 14q deleted RCC (compared to *L2HGDH* as well as other potential 14q tumor suppressors identified in this manuscript). Therefore, genetic loss of *HIF1A* in ccRCC is associated with a

markedly greater reduction in *L2HGDH* expression than its own gene expression. In addition, when genetic loss of *HIF1A* occurs without genetic loss of *L2HGDH* (which occurs in <5% of 14q deletions), the survival is significantly greater (nearly two times) than when there is simultaneous genetic loss of both (p=0.007). Finally, HIF1α data from this large cohort of ccRCC patients (TCGA) at the mRNA (n=530) and protein (n=444) levels showed that it has no impact on survival. Therefore, put together, the data strongly indicate that HIF1α is not a target of 14q deletion, and hence a tumor suppressor, in ccRCC.

This finding represents a significant conceptual advance for the field with high translational relevance. Moreover, controversially, it happens to refute a claim made by Dr. William Kaelin (recipient of the 2019 Nobel Prize in Medicine) that *HIF1A* is a target of 14q deletion in ccRCC. Therefore, importantly, the manuscript calls for a need to take into consideration differences between the biology of human cancers and that of cell lines/xenografts in nude mouse models, while assigning an overall tumor suppressive/promoter role for a particular protein in human cancers. By analogy, when a drug is considered to be a promising anti-cancer agent based on *in vitro* and *in vivo* models but then fails to demonstrate any anti-tumor effect in humans, it is no longer considered an effective anti-cancer agent.

XI.2 Research ethics review checklist

FORM UPR16

Research Ethics Review Checklist

Please include this completed form as an appendix to your thesis (see the Research Degrees Operational Handbook for more information

UNIVERSITY OF PORTSMOUTH

Postgraduate Research Student (PGRS) Information			**Student ID:**	952200
PGRS Name:	Dr. Niraj K. Shenoy			
Department:	Pharmacy and Biomedical Sciences	**First Supervisor:**	Dr. Sassan Hafizi	
Start Date: (or progression date for Prof Doc students)	**1 October 2019**			
Study Mode and Route:	Part-time ☒ Full-time ☐	MPhil ☐ PhD ☒	MD ☐ Professional Doctorate ☐	

Title of Thesis:	Understanding and targeting epigenetic dysregulation, aberrant metabolism, and immune evasion in cancer with ascorbic acid
Thesis Word Count: (excluding ancillary data)	11,784

If you are unsure about any of the following, please contact the local representative on your Faculty Ethics Committee for advice. Please note that it is your responsibility to follow the University's Ethics Policy and any relevant University, academic or professional guidelines in the conduct of your study

Although the Ethics Committee may have given your study a favourable opinion, the final responsibility for the ethical conduct of this work lies with the researcher(s).

UKRIO Finished Research Checklist:
(If you would like to know more about the checklist, please see your Faculty or Departmental Ethics Committee rep or see the online version of the full checklist at: http://www.ukrio.org/what-we-do/code-of-practice-for-research/)

a) Have all of your research and findings been reported accurately, honestly and within a reasonable time frame?	YES ☒ NO ☐
b) Have all contributions to knowledge been acknowledged?	YES ☒ NO ☐
c) Have you complied with all agreements relating to intellectual property, publication and authorship?	YES ☒ NO ☐
d) Has your research data been retained in a secure and accessible form and will it remain so for the required duration?	YES ☒ NO ☐
e) Does your research comply with all legal, ethical, and contractual requirements?	YES ☒ NO ☐

Candidate Statement:

I have considered the ethical dimensions of the above named research project, and have successfully obtained the necessary ethical approval(s)

Ethical review number(s) from Faculty Ethics Committee (or from NRES/SCREC):	N/A

If you have *not* submitted your work for ethical review, and/or you have answered 'No' to one or more of questions a) to e), please explain below why this is so:

This work is completed in regards to a PhD by publication project. All relevant studies are published. All work was done in accordance with the relevant ethics approvals from respective institutions

Signed *(PGRS)*:	**Date:** 13 May 2020

UPR16 – April 2018