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REVIEW ARTICLE

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Exploring m6A-RNA methylation as a potential therapeutic strategy for acute lung injury and acute respiratory distress syndrome

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Abstract

N^6-methyladenosine (m6A) is the most common methylation modification in mammalian messenger RNA (mRNA) and noncoding RNAs. m6A modification plays a role in the regulation of gene expression and deregulation of m6A methylation has been implicated in many human diseases. Recent publications suggest that exploitation of this methylation process may possess utility against acute lung injury (ALI). ALI and its more severe form, acute respiratory distress syndrome (ARDS) are acute, inflammatory clinical syndromes characterized by poor oxygenation and diffuse pulmonary infiltrates. This syndrome is associated with microvascular endothelial dysfunction, subsequent pulmonary hypertension and may ultimately lead to mortality without rigorous and acute clinical intervention. Over the years, many attempts have been made to detect novel therapeutic avenues for research without much success. The urgency for the discovery of novel therapeutic agents has become more pronounced recently given the current pandemic infection of coronavirus disease 2019 (COVID-2019), still ongoing at the time that this review is being written. We review the current landscape of literature regarding ALI and ARDS etiology, pathophysiology, and therapeutics and present a potential role of m6A methylation. Additionally, we will establish the axiomatic principles of m6A methylation to provide a framework. In conclusion, METTL3, or methyltransferase-like 3, the selective RNA methyltransferase for m6A, is a hub of proinflammatory gene expression regulation in ALI, and using a modern drug discovery strategy will identify new and effective ALI drug candidates targeting METTTL3.

KEYWORDS
acute lung injury, Acute Respiratory Distress Syndrome, m6A-RNA methylation

INTRODUCTION

Acute Respiratory Distress Syndrome (ARDS) is a severe lung condition that can result from a range of lung or systemic medical conditions, including pneumonia, sepsis, trauma, or aspiration.[1] ARDS is characterized by a sudden onset of severe breathing difficulty, rapid breathing, and low blood oxygen levels.[1] The syndrome is associated with microvascular endothelial dysfunction and neutrophil infiltration, which can lead to unacceptably high mortality rates without rigorous and acute clinical intervention.[2] Given the ongoing coronavirus disease 2019 (COVID-2019) pandemic, the urgency for the discovery of novel therapeutic agents has become more pronounced. Recent studies have suggested that methylation of mammalian messenger RNA (mRNA) on N^6-methyladenosine (m6A) can robustly regulate gene expression,[3] and deregulation of m6A methylation has been implicated in many human diseases,[3] including ARDS.[4,5] This review summarizes the current literature on m6A RNA modification and its clinical implications in multiple diseases. Additionally, we discuss the current landscape of literature regarding ARDS etiology, pathophysiology, and therapeutics, and present a potential role of m6A in ARDS pathogenesis. Targeting the m6A methylation process may be a promising strategy against acute lung injury (ALI).

PART I. INTRODUCTION TO M6A METHYLATION

m6A is the most common methylation modification in mammalian mRNA and noncoding RNAs that plays a role in the regulation of gene expression.[3] This modification consists of methylation of the amino group at the N^6 position of the adenine nucleobase in RNA. The modification sites contain a specific recognition sequence: DRACH motif (D representing G/A/U, R representing G/A, and H representing A/C/U).[6] These sequences are enriched in the coding sequence (CDS), 3′-untranslated regions (3′-UTRs) and 5′-untranslared regions (5′-UTRs) of mRNA.[7] These modifications are found with especially high frequency near the stop codon regions. This reversible modification has a primary role in the regulation, stabilization, and translation of mRNA.[6] The dynamic modification takes place in the nucleus of the cell using specific methyltransferase machinery. The methylation process is catalyzed by a heterodimer core complex consisting of two well-known m6A methyltransferases, methyltransferase 3 (METTL3) and methyltransferase 14 (METTL14).[8] METTL3 is a 70 kDa core methyltransferase which contains the catalytic core responsible for the removal and transfer of a methyl group from S-adenosyl methionine (SAM) to the N^6 amine position of an adenine nucleobase (Figure 1). METTL14 functions as an adaptor protein which complexes into a heterodimer with METTL3 and then binds to the RNA consensus sequence, anchoring the complex to that sequence.[8] Wilms' tumor 1-associating protein (WTAP) is a splicing factor which behaves as an additional structural component in this complex known as a "writer complex" and is also known to recruit METTL3 and METTL14.[8] This multiprotein complex is essential to the cell and alterations or removal of this complex leads to cell death (Figure 2). Global knockdown of METTL3, for example, leads to the death of early embryos,[9] however a knockout in mouse embryonic cells greatly reduces the frequency of m6A peaks and results in the loss of damaged embryonic stem

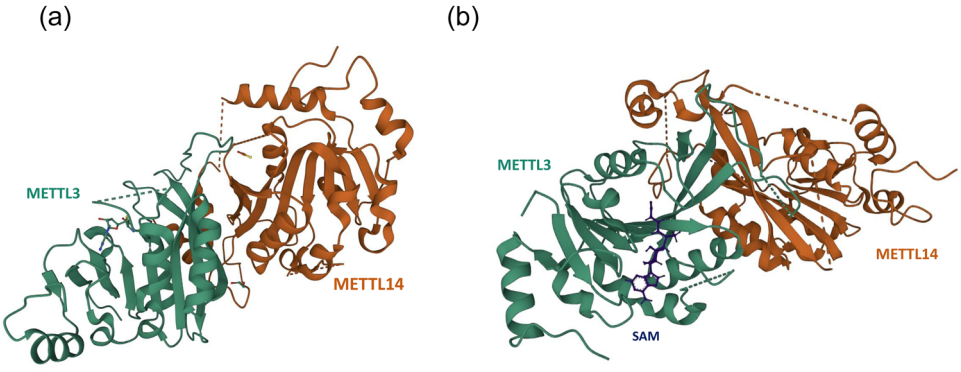

FIGURE 1 (a) METTL3-METTL14 complex. ~200 kDa Methyltransferase complex (70 kDa METTL3). METTL3 and METTL14 form a heterodimeric methyltransferase complex within the nuclear RNA that catalyzes RNA methylation. (b) METTL3-METTL14 complex bound to S-adenosyl methionine (SAM). METTL3 is the catalytically active subunit with a binding pocket for SAM and METTL14 functions as the structural component of the complex that facilitates substrate recognition.

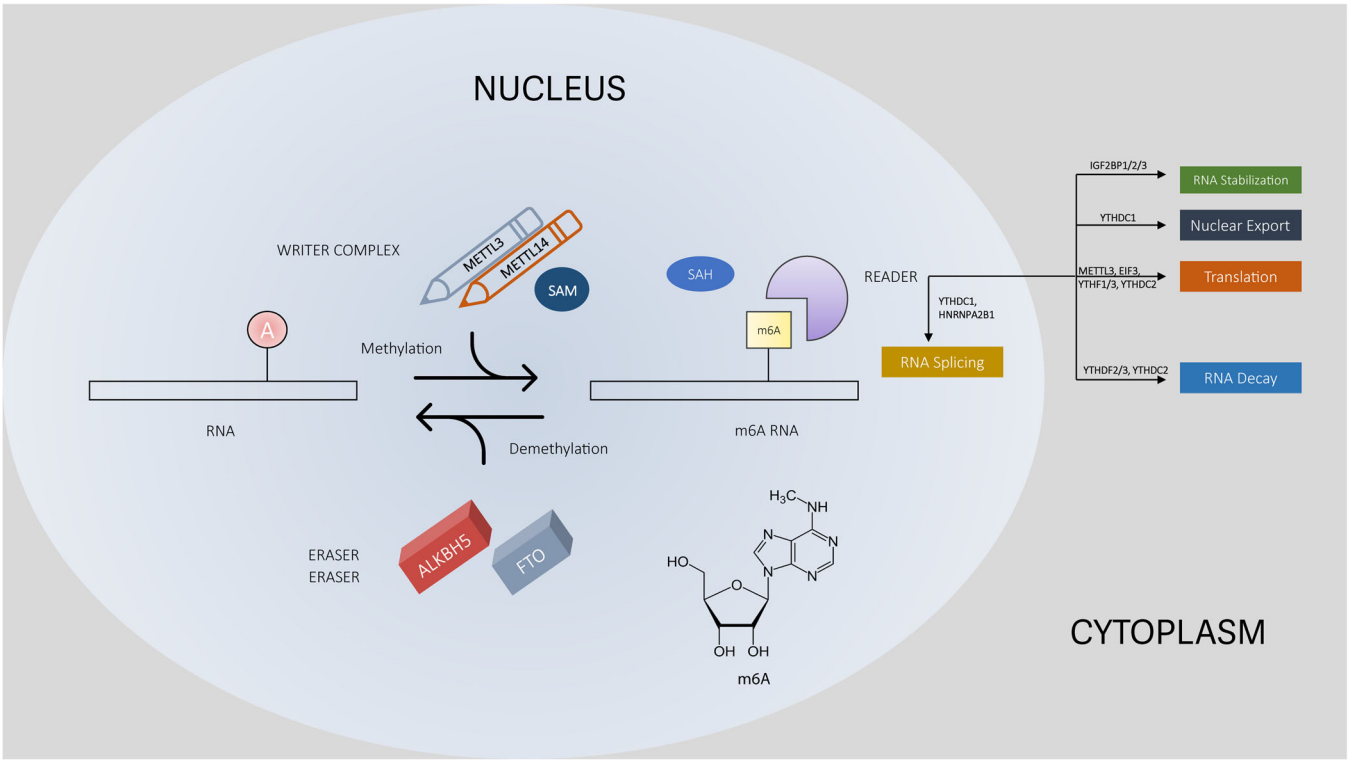

FIGURE 2 m6A modification mechanism (the life cycle of m6A RNA). m6A modification is facilitated by the "writer" complex consisting of methyltransferase 3 (METTL3), methyltransferase 14 (METTL14), and Wilms Tumor1-associated protein (WTAP) that make up the core along with regulatory cofactors (not pictured here). METTL3 acts as the core subunit that binds to S-adenosylmethionine (SAM) and catalyzes the transfer of a methyl group. METTL14 is an RNA-binding supportive unit that binds to METTL3 and WTAP allows for localization of the METTL3-METTL14 heterodimer to the nuclear speckle. m6A is a reversible modification as demethylase proteins alkB homolog 5 RNA demethylase (ALKBH5) and alpha-ketoglutarate-dependent dioxygenase (FTO), also known as erasers, remove methyl groups from the N6 position of adenine. m6A modifications are recognized by "reader" elements that ultimately decide the fate of the modified mRNA. A wide range of readers have been discovered and characterized. A few examples of readers and their functions are presented above. YTHDC1, HNRNPA2B1, and HNRNPC promote RNA splicing while YTHDF2/3 and YTHDC2 accelerate RNA decay, and so forth. m6A, N^6-methyladenosine; mRNA, messenger RNA.

cells (ESCs) to heal.[10] Other potential subunits of this complex have been identified in recent years as KIAA1429, RBM15, and METTL16. Their roles have not been fully elucidated, however, some data suggest that they may selectively identify binding sites for posttranscriptional regulation.[11,12] The discovery that m6A modifications are reversible was based on the identification of demethylase enzymes capable of removing the methyl group from the N^6 position of adenine nucleobases. Fat mass and obesity gene (FTO) and alkB homolog 5 (ALKBH5) are examples of well-characterized demethylases or "erasers" that operate to remove methyl groups from the N^6 adenine position on RNA sequences.[3] m6A modifications regulate gene expression through a wide array of operations. examples include alteration of pre-mRNA processing, enhancement of translation efficiency and mRNA stability, as well as promotion of mRNA nuclear transport. These effects are mediated by proteins known as "readers" capable of selectively identifying m6A and initiating a regulatory function on that mRNA.[3] Many m6A readers include proteins of the YTH (YT521-B homology) family (YTHDF1, YTHDF2, and YTHDF3) which function as cytosolic m6A readers that facilitate enhanced degradation and translation.[13] Although the YTH domain-containing proteins remain the most highly characterized family of readers, many others have since been identified. Two RNA-binding proteins (RBPs), heterogeneous nuclear ribonucleoprotein C (hnRNPC) and heterogeneous nuclear ribonucleoprotein G (hnRNPG), are capable of binding to sites in which m6A may alter the accessibility of proximal RNA sequences to facilitate the binding of RBPs.[14] The reversibility of m6A methylation has emerged as an important facet in epitranscriptomic regulation. DNA and protein have well-characterized reversible modifications and have received considerable attention in the

literature due to interest in the utilization of these modifications for drug discovery. RNA modifications, however, did not receive major attention in the field for many years for three reasons. First, before the advent of RNA modification characterization, it was thought that these modifications were static and nonreversible. Second, there was a lack of interest in pursuing RNA modification characterization as the short half-life of RNA in the cell made it an unlikely avenue for pursuit of translational medicinal studies. Third, many of the RNA modifications occur in structural tRNAs and ribosomal RNAs whereas investigators are more interested in protein-coding cellular mRNA. The discovery of demethylase proteins (FTO and ALKBH5) prompted interest in m6A methylation modifications. Once thought to be static and nonreversible, m6A methylation was now a dynamic and reversible modification with implications for drug discovery. Recall that m6A modifications on RNA are removed by FTO or ALKBH5, iron, and α-ketoglutarate-dependent dioxygenases that remove m6A via oxidative demethylation.[15–17]

IMPORTANCE/SIGNIFICANCE OF METHYLATION

Emerging clinical application of m6A RNA modification (Figure 3)

The exploitation of m6A modification possesses a potential new avenue for the treatment of human disease. In the last decade, there has been a particular interest in m6A methylation because of its frequency of occurrence (each mRNA sequence likely contains two to three sites of methylation[18]) as well as the discovery of dozens of proteins responsible for this modification. Given the multifaceted regulation of m6A on gene expression and its ability to modulate gene expression in every single human tissue studied thus far[19] it is no surprise that m6A methylation has emerged as a major regulator of biological processes in physiology and pathology. Deregulation of m6A methylation has been implicated in many human diseases. The role of METTL3 and METTL14-mediated m6A methylation in human

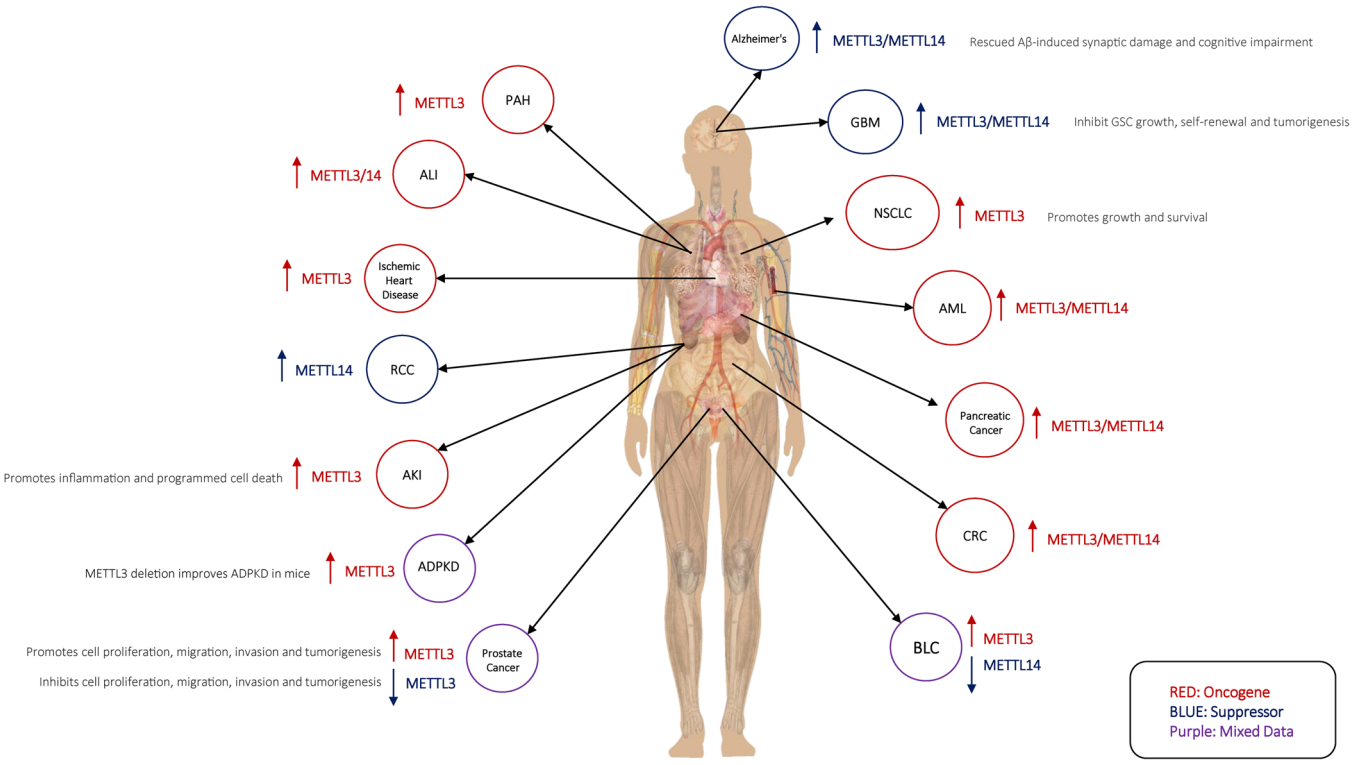

FIGURE 3 Role of METTL3 and METTL14-mediated m6A methylation in human pathology. METTL3 and METTL14 act as m6A regulators that can affect the progression of different human pathologies through oncogenic or suppressive methods. In several human cancers, for example, METTL3 plays an oncogenic role by upregulating oncogenes that support tumor progression. Conversely, METTL3 also behaves as a suppressor in the central nervous system (CNS) by upregulating tumor suppressor genes as well as genes that promote synaptic damage. METTL14 may enhance the growth and metastasis of pancreatic cancer through decreased expression of a vital oncogene through m6A modification. m6A, N^6-methyladenosine.

pathology is summarized in Figure 3. METTL3 and METTL14 act as m6A regulators that can affect the progression of different human pathologies through oncogenic or suppressive methods. In several human cancers, for example, METTL3 plays an oncogenic role by upregulating oncogenes that support tumor progression.[20] Conversely, METTL3 also behaves as a suppressor in the central nervous system (CNS), for example, by upregulating tumor suppressor genes as well as genes that promote synaptic damage.[21] These observations in the literature highlight the fact that METTL3-METTL14 are not globally uniform but rather, their roles in m6A modification produce specific downstream events based on the site of interest.

Role of m6A modifications in oncology

The role of m6A modification in the development and progression of cancer is described extensively in the literature. This includes but is not limited to, breast cancer (BC), bladder cancer, colorectal cancer, lung cancer, and liver cancer.[22] Additionally, new data supports m6A modification's role in treatment responses for oncologic conditions such as chemotherapy, radiation therapy, and immunotherapy. m6A modification regulates many components of malignancies including metastasis, proliferation, apoptosis, and resistance to therapeutics.[23,24] The literature contains reports of m6A modification regulators acting as both tumor promoters and tumor suppressors depending on the site and type of tumor. For example, METTL3 demonstrates potentially oncogenic activity in both bladder and uterine cancer, respectively[23] whereas METTL14, functions as a tumor suppressor in bladder cancer, demonstrating the variety of outcomes in the setting of m6A modulation regulator dysfunction.

m6A modification regulators as tumor promoters (oncogenes)

METTL14 enhances BC proliferation and progression through increased expression of CXC motif chemokine receptor 4 (CXCR4) and cytochrome P450 Family 1 Subfamily B Member 1 (CYP1B1).[25] METTL14 blocks acute myeloid leukemia (AML) differentiation through upregulation of MYB and MYC genes via m6A modification.[26] METTL14 also suppresses skin tumorigenesis through promotion of a global genome repair mechanism.[27] METTL3 promotes the growth and survival of non-small-cell lung carcinoma (NSCLC) through promotion of YAP translation and expression.[28] m6A modification regulators also function as tumor promoters through the downregulation of tumor suppressor genes. METTL3 increases hepatic cell carcinoma (HCC) tumorigenicity and metastasis through inhibition of suppressor of cytokine signaling 2 (SOC2) expression via m6A modification.[29] These findings were further corroborated following the observation that METTL3 was downregulated in sorafenib-resistant HCC.[30]

m6A modification regulators as tumor suppressors

m6A modification regulators are capable of tumor suppressor functions in specific human cancers. METTL3 and METTL14 downregulation increase osteosarcoma (OS) tumorigenesis and chemoresistance through increased expression of tripartite motif containing 7 (TRIM7), an oncogene.[31] In the setting of glioblastoma multiforme (GBM), METTL3-METTL14 complex inhibits cell growth, self-renewal, and tumorigenesis.[32] In endometrial cancer, loss of METTL14 increases the proliferation and tumorigenicity of endometrial cancer cells by altering their RNA stability and translation of AKT pathway regulators.[33]

The role of m6A modification regulator dysfunction in oncologic drug sensitivity and resistance

Acquired chemoresistance purports a major hindrance to effective and successful cancer therapy. The role of m6A methylation in the growth and proliferation of neoplasms has received considerable attention in the literature over the past few years. The last 5 years have also seen an unprecedented focus on the role of m6A modifications in acquired chemoresistance following the discovery of technology allowing researchers to identify m6A enrichment sites on RNA.[34]

Chemotherapy

5-Fluorouracil (5FU) is a commonly used antineoplastic drug used in a variety of different cancers.[35] 5FU is often given concomitantly with other chemotherapeutic agents as part of a therapeutic regimen (an example is FOLFOX for CRC-Folinic acid, Fluorouracil, and Oxaliplatin). METTL3 knockdown increases 5FU sensitivity in pancreatic ductal adenocarcinomas.[36] Another common antineoplastic agent is Gemcitabine, a pyrimidine analogue. Knockdown of METTL3 enhances sensitivity toward gemcitabine in pancreatic cancer cells.[36] Taken together, there is some evidence to suggest a potential benefit to the addition of a METTL3 inhibitor to a specific chemotherapeutic regimen susceptible to higher

rates of resistance. This hypothesis requires additional work to determine the implication of the observations.

Immunotherapy

One study identified that METTL3 depletion in dendritic cells impaired their maturation and resulted in weakened costimulatory signals which ultimately reduced T-cell stimulation.[37]

Radiotherapy

Radiation therapy is a primary treatment regimen for a variety of different cancers. It is a noninvasive method of targeting cancer via direct DNA damage. Many cancer therapies include concomitant use of chemotherapeutic drugs with radiation therapy followed by surgery in solid tumors.[38] Increased METTL3 expression appears to induce radiation therapy resistance in GBM. One study found that GBM stem-like cells enhanced METTL3 expression and induced resistance against radiation through increased SOX2 expression.[39] Similarly, METTL3 selective knockdown in pancreatic cancer cell lines resulted in sensitization to radiotherapy.[36]

Role of m6A RNA methylation modification in other pathologies

Alzheimer's

Perturbations in m6A signaling are suspected to play a role in Alzheimer's disease (AD) due to dysregulation of RNA and protein expression profiles in the brain. m6A controls RNA stability, splicing, translation, and trafficking. One study found METTL3 among a subset of m6A regulatory genes which were significantly dysregulated in the human AD brain (postmortem AD samples).[40]

Inflammatory bowel disease (IBD)

IBD is characterized by inflammation of the intestinal mucosal barrier and a prolonged immune response.[41] There is abundant literature to support m6A's role in the regulation of intestinal mucosal immunity,[42] yielding speculation that m6A modification may play a role in the pathogenesis of IBD. Studies are underway to investigate the utility of m6A modification in the diagnosis and treatment of IBD. METLL14 deficiency in T cells has been linked to the development of spontaneous colitis in mice likely because of T-regulatory cell (Treg) dysfunction. Decreased expression of Rorγ T in METTL14 deficient Treg cells impairs the induction of naïve T cells into Tregs-a conclusion based on a study that demonstrated weakened colitis phenotype following an adoptive transfer of wild-type Tregs.[43] Another investigation into the regulatory effect of m6a methylation on T cells concluded that METTL3-deficient T cells failed to expand homeostasis in a mouse model of lymphatic adoptive transplantation and resulted in T cells being unable to mature beyond a naïve state. This ultimately prevented colitis in mice and further supports the premise that m6A methylation may contain a clinical application aimed at distinguishing subtypes of colitis and appropriate management.[44] Additionally, one human study involving samples collected from 236 pediatric IBD patients demonstrated colonic epithelium DNA methylation patterns that were evident in patients with Crohn's Disease or Ulcerative Colitis.[45]

Cardiovascular disease (CVD)

CVD is currently the leading cause of morbidity and mortality worldwide. Despite major advancements in the understanding and treatment of the conditions, it remains a severe public health issue.[46,47] The advent of new technologies has uncovered a major component of CVD that was previously less well understood. New molecular biology techniques have allowed for the exploration of genetic and epigenetic influences in the etiology and pathophysiology of CVD. This novel frontier of CVD research has confirmed the role of epigenetics in CVD. There is specific interest as of recent regarding the posttranscriptional regulation of CVD-related RNA.[48] There is emerging evidence to indicate that m6A modification is closely related to the occurrence and progression of CVDs, including cardiac hypertrophy, heart failure, ischemic heart disease, aortic aneurysm, vascular calcification, and pulmonary hypertension.

m6A and vascular disease

The umbrella term of vascular pathology encompasses diseases such as atherosclerosis, pulmonary hypertension, aortic dissection, and (to some capacity) ALI/acute respiratory distress syndrome. m6A methylation plays a role in the pathogenesis of vascular conditions through endothelial cell regulation, inflammation, and the proliferation of vascular smooth muscle cells.[48] Endothelial cell inflammation plays an inciting role in the development of both coronary atherosclerosis as well ALI. Jian et al. discovered increased RNA m6A methylation levels in a tumor necrosis factor-α (TNF-α) induced model of endothelial cell inflammation through METTL14 upregulation.[49] Similarly, Chien et al. found that METTL3 promoted

TNF-α-mediated inflammation in endothelial cells through upregulation of nucleotide-binding domain leucine-rick repeat pyrin domain containing 1 (NLRP1) and down-regulation of Kruppel-like factor 4 (KLF4) following m6A methylation.[50] METTL3 also stimulates monocyte response and facilitates monocyte-endothelial cell adhesion through induction of peroxisome proliferators-activated receptor (gamma) coactivator 1α (PGC-1α) mRNA degradation.[48] Studies also support a potential role for METTL3-METTL14 complex in the promotion and progression of pulmonary arterial hypertension (PAH). m6A levels are reportedly significantly elevated in a rat model of PH. m6A methylated genes that are upregulated in PH have been identified as those involved in the inflammatory sequenced, endothelial cell receptor activation, and lung development.[51,52] Knock-down of METTL3 and METTL14 delayed the progression of pulmonary hypertension through the inhibition of pulmonary arterial smooth muscle cell proliferation and migration.[48] The data on m6A modification in vascular disease is growing at an unprecedented rate and is rapidly demonstrating the link between m6A modification and endothelial vascular pathologies. Although further study is required for the characterization of specific regulatory mechanisms of methylases in vascular disease, methylase inhibition may provide a potential avenue for drug discovery.

m6A and cardiac pathologies

Cardiac pathologies encompass a wide range of conditions labeled as atherosclerosis, arrhythmia, heart failure, and ischemic heart disease, to name just a few. Literature on m6A methylation's role in the pathogenesis of cardiac disease is robust. The landscape of data on hypertensive heart disease and heart failure is especially comprehensive. Dorn et al. demonstrated that METTL3-mediated m6A modification is significant for maintaining cardiac homeostasis and normal cardiac function and revealed increased m6A methylation in cardiomyocytes under hypertrophic stimulation.[48,53] METTL3-overexpressing mice exhibited marked cardiac hypertrophy but not accelerated dysfunction during pressure overload stress. The inhibition of m6A blocked cardiomyocyte hypertrophy by deleting METTL3.[48,53]

PART II. NOVEL TARGET FOR ALI WARRANTED

Definition

ALI and its more severe form ARDS are acute, inflammatory clinical syndromes characterized by poor oxygenation and diffuse pulmonary infiltrates.[54] This syndrome is associated with microvascular endothelial dysfunction and subsequent pulmonary hypertension and may ultimately lead to mortality without rigorous and acute clinical intervention.[1] Before the 1990s, ALI and ARDS existed in both the literature as a clinical syndrome consisting of specific definitions with wide variances across different institutions worldwide. This created inconsistencies in the definition of ALI/ARDS and affected clinicians' ability to properly diagnose, classify, and then treat the condition effectively. In 1994, after decades of inconclusive definitions, the American-European Consensus Conference Committee recommended the adoption of a consensus definition for ALI/ARDS as meeting the following conditions: PaO_2/FiO_2 ratio of less than 300 mmHg and a pulmonary wedge pressure <18 or no clinical evidence of left atrial hypertension.[54,55] In 2012, the updated "Berlin" definition removed the requirement for wedge pressure <18 and included positive end-expiratory pressure (PEEP) or continuous positive airways pressure (CPAP) of greater than or equal to 5.[54,55] Additionally, the Berlin definition excludes the term ALI. Today, however, both ALI and ARDS are used interchangeably, with ARDS denoting a more severe prognosis and stricter definitions ($PaO_2/FiO_2 \leq 200$ mmHg).[55]

***$\mathbf{PaO_2/FiO_2}$:** PaO_2 is traditionally used as the most precise measurement of patient oxygenation in patients with ALI/ARDS, but its accuracy is corrupted with communicated as PaO_2/FiO_2 ratio

- No organ in the body detects P_{aO2}/F_{IO2}, whereas several respond to miniscule changes in P_{aO2} (carotid bodies).
- P_{aO2}/F_{IO2} plays no role in any biological process, whereas arterial oxygen saturation directly determines oxygen delivery to the brain and myocardium
- More easily calculated from information routinely available in patients' charts.

Epidemiology

Data extrapolated from large, prospective population-based cohorts of ALI studies and estimated 200,000 cases per year in the United States with a mortality rate of close to 40%.[56] More recent studies now suggest a mortality risk of 29%–42%. These rates must be interpreted with caution, as an ALI/ARDS consensus definition was not adopted until 1994. Additionally, the incidence has been difficult to assess due to nonuniform definitions, variation in etiology, geographical variation, and inadequate documentation. The nature of the underlying clinical disorder is an important determinant of outcome. For

example, sepsis has higher mortality than major trauma (43% vs. 11%), whereas pneumonia and aspiration are intermediate risk factors (36% and 37%, respectively).[56] This data, however, is variable and will be discussed further in the section on pathophysiology. Racial inequalities in disease burden occur in African Americans and Hispanics who have a higher 60-day mortality rate (33%) compared to Caucasians (27%).[57] This increased risk of death is independent of age, gender, ventilation strategy, lung injury etiology, comorbidities, or degree of hypoxemia. For African Americans, the severity of illness at presentation appeared to moderate this higher mortality risk. The cause of race and ethnicity-related differences in disease outcome is likely derived from "social determinants of disease."[58] Supporting this claim is the resolution of higher mortality rates in African Americans after controlling for illness severity. It is, therefore, probable that the differences in mortality observed are due to delayed care and delayed diagnosis[57] as opposed to genetic factors. Age is a risk factor for the development of ARDS but does not increase the risk of mortality. The LUNG-SAFE (The Large observation study to Understand the Global impact of Severe Acute Respiratory Failure) study failed to show an independent relationship between age and mortality in ARDS after controlling for variables such as risk, severity, and comorbidity.[59] Chronic alcohol use and smoking are both independent risk factors for ARDS.[60–62] Chronic alcohol use results in weakened pulmonary immunity, epithelial dysfunction, and high permeability pulmonary edema. Cigarette smoke promotes initiation of the inflammatory cascade which further aggravates the pulmonary system in ARDS.

Etiology

ARDS/ALI represents a heterogenous clinical syndrome resulting from various etiologies. First reported in 1967, clinicians (Ashbaugh and colleagues) described a cohort of patients that developed similar patterns of acute-onset respiratory failure coupled with similar histopathologic findings despite differences in the primary insults or etiologies.[63] The heterogeneity of ARDS presents a major challenge in diagnosis and treatment currently. ARDS can occur secondary to both pulmonary (direct) and extrapulmonary (indirect) triggers. Pneumonia is the most common pulmonary cause of ARDS and often results in the use of mechanical ventilation. ARDS that occur secondary to a direct insult to the pulmonary system such as pneumonia are characterized by alveolar collapse, fibrinous exudate, and edema of the alveolar walls. Causes of nonpulmonary or indirect ARDS include sepsis, trauma, hemorrhagic shock, and drug toxicity. Although ARDS can be subdivided based on etiology, it is difficult to differentiate between the two in clinical practice due to physiological overlap.[64] This substantial overlap makes it difficult to identify mortality differences based on etiology. One observation that appears to be well supported in the literature, however, is that ARDS secondary to pneumonia may be associated with higher rates of mortality likely related to difficulties in proper management of acute pathologies such as alveolar collapse and fibrinous exudate which tend to occur to a lesser degree in extrapulmonary ARDS. Other causes of lung injury include ventilator-induced lung injury (VILI) which can occur because of suboptimal mechanical ventilatory settings.[65] More recently, new categories of ARDS have emerged such as E-cigarette or vaping associated lung injury (EVALI) and the pandemic of severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) related ARDS.[66–68]

Pathogenesis, pathophysiology, and histopathology

The hallmark of ALI and ARDS is acute inflammation that causes disruption of lung endothelial and epithelial barriers. Although the pathogenesis is still only partly understood, it is well known that damage to the pulmonary endothelium is responsible for increased permeability and endothelial dysfunction culminating in alveolar collapse.[1] ARDS progresses through three stages each represented by stereotypic responses. The primary acute phase of ARDS is characterized by alveolar-capillary damage, pulmonary microvascular endothelial dysregulation, and subsequent alveolar collapse.[54] This initial phase occurs immediately following injury and is termed the exudative phase due to the high permeability pulmonary edema that occurs. This edema prohibits efficient gas exchange and ultimately leads to hypoxemia.[54] In this initial phase, hyaline membrane formation also takes place. Cellular characteristics of ALI include loss of alveolar-capillary membrane integrity, excessive transepithelial neutrophil migration, and release of proinflammatory and cytotoxic mediators. Upregulation of proinflammatory cytokines occurs as a direct response to ongoing cellular injury. Microvascular endothelial injury leads to increased capillary permeability which then permits the efflux of protein-rich fluid into the peribronchovascular interstitial space and ultimately reaches to distal airspaces of the lung. Transepithelial neutrophil migration is an important feature of ALI because neutrophils are the primary perpetrators of inflammation. Key histologic changes

observed in ARDS include alveolar edema in areas of the diseased lung due to injury of Type 1 pneumocytes. Under normal physiologic conditions, these pneumocytes provide additional protection to the vascular endothelium which functions to create a barrier for the regulation of fluid in and out of the alveolar airspace. Damage to these cells and increased permeability of the endothelium render the alveolar airspace to an influx of proteinaceous fluid and blood. The second phase, often referred to as the proliferative phase, occurs 7–10 days following initial injury and is characterized by interstitial inflammation and attempted lung healing.[54] Excessive or prolonged activation of neutrophils contributes to basement membrane destruction and subsequent increases in permeability of the alveolar-capillary barrier. Neutrophils also release damaging proinflammatory and proapoptotic mediators such as elastase, which appears to break down epithelial junctional proteins, further contributing to the inflammatory onslaught. Normally, types 1 and 2 alveolar epithelial cells form tight junctions with each other to selectively regulate the epithelial barrier and control the content and number of ions that travel into and out of the alveolar spaces. Damage to types 1 and 2 alveolar epithelial cells in the acute phase of lung injury leads to disruption of the normal fluid transport through downregulation of epithelial sodium channels and ATPase pumps and further contributes to the exudative damage that occurs in the acute phase of lung injury. The fibrotic phase occurs 21 days following initial injury and signals the end of the acute disease phase. The fibrotic phase is characterized by fibroproliferation and disordered healing.[54]

Diagnosis (evaluation) and management (Table 1)

In a clinical setting, ALI is observed by dyspnea and hypoxemia which continue to worsen 6–72 h after the initial injury and frequently results in an intensive care unit admission and subsequent placement on mechanical ventilation.[69] Dyspnea, or difficulty breathing, is often the first complaint from patients and is usually mild but progresses after 12–48 h to major respiratory distress requiring mechanical ventilation to prevent hypoxia. Other presentations on physical examination include tachypnea and increased efforts to breathe. Systemic evidence may also be present upon admission based on severity and include cyanosis, tachycardia, and cognitive impairment.

Although our understanding of ARDS/ALI mechanisms and pathophysiology has advanced markedly since the syndrome's first description in 1967, there have been but few candidates for pharmacological intervention and of those few, none have proven clinically significant for abrogating the pulmonary sequelae that occurs following injury. Currently, the general principle for management of ALI/VILI is treatment of the underlying medical or surgical condition and supportive treatment. However, solemnly do these methods work to attenuate the acute response which can lead to severe long-term outcomes and progressive lung fibrosis. The only A-rated recommendation by the American Thoracic Society (ATS) is to begin these patients on MV which then puts them at risk for additional lung injuries such as VILI[69] (Table 1).

Novel therapies warranted

Our concern is really with identifying a druggable target in the acute, early phase of ALI onset and specifically targeting these acute phase events such as increased endothelial barrier permeability and dysregulation. Effective treatments are limited to minimization of harm and prolonging survival time rather than the prevention of lung damage. Treatment in the acute phase of ALI relies upon supportive care including mechanical ventilation, an intervention which then leaves patients susceptible to the development of lung fibrosis.[70,71] Over the years, many attempts have been made to detect novel

TABLE 1 ARDS management recommendations (adapted from ATS 2017 Guidelines[61]). (A) ATS A-rated recommendation: a recommendation that is based on the highest level of clinical evidence and is considered highly reliable. (B) ATS B-rated recommendation: a recommendation that is based on a lower level of evidence, while still considered valuable and effective.

Mechanical ventilation	Fluid management	Neuromuscular blockade	Glucocorticoids
Prevent alveolar collapse (A)	Maintain low left atrial filling pressure (B)	Early administration of neuromuscular blockade (B)	Reduced pulmonary inflammation
• Optimize PEEP through titration • Increase MAP with inverse-ratio ventilation	• Fluid restriction • Diuretics	• Increased rate of survival • Increased ventilator-free days without increasing ICU-acquired paresis	• Current literature does not support the use of high-dose glucocorticoids

Abbreviation: PEEP, positive end-expiratory pressure

therapeutic avenues for research without much success. The clinical trials aimed at repurposing older Food and Drug Administration (FDA)-approved compounds have all been inconclusive. Failed clinical trials include surfactants, statins, inhaled nitrous oxide, and ACE-Inhibitors.[72–76] More recently, however, a latent class analysis published by Calfee et al.[77] suggests potential utility for the use of statins in patients falling under a specific clinical subtype of ARDS. Of note-our lab has also done work in the realm of novel signal transduction pathways in ALI using Simvastatin and other β-Hydroxy β-methylglutaryl coenzyme A (HMG CoA)-reductase inhibitors. Despite over 50 years of description in the literature, there remains a tremendous challenge in the identification of novel avenues for targeted therapy of ALI/ARDS for several reasons. Firstly, the diagnosis of the condition is complicated due to multi-protein involvement. Additionally, lung biopsy is perhaps the most common method of obtaining a definitive histologic diagnosis of ALI/ARDS which is invasive and impractical in an acute setting. Second, there is a major gap in knowledge of the mechanisms underlying ALI at the posttranscriptional level which hinders the development of novel therapeutics for treatment. Gene expression in response to ALI-related stimuli has been a concern for decades but only a handful of investigations have been performed to elucidate these mechanisms. Recently, new methods of elucidating the mechanisms of ALI have been reported. These include weighted gene coexpression network analyses and transcriptome profiling.[78] We remain optimistic that these novel techniques will shed light on the pathological mechanisms of ALI/ARDS and subsequently open doors for novel avenues of drug discovery.

SARS-CoV-2 and COVID-19-related ALI

The urgency for the discovery of novel therapeutic agents has become more pronounced recently given the current pandemic infection of COVID-2019, still ongoing while this review is being written. COVID-2019 is caused by a novel RNA β-coronavirus known as severe acute respiratory syndrome coronavirus-2 (SARS-CoV-2). SARS-CoV-2 transmission is presumed to occur through either inhalation or contact with infected droplets (a less supported hypothesis is that transmission occurs via aerosolization/fecal-oral route).[79] Symptoms are vague and overlap with symptoms of the flu and common upper respiratory infections. The most common symptoms of patients with COVID-19 include fever, cough, and fatigue. Less commonly, some patients may experience gastrointestinal distress, headache, and altered smell or loss of smell.[80] COVID-19 presentation occurs along a spectrum with some patients experiencing little to no symptomology and others presenting with pneumonia and, in severely ill individuals, ARDS. ALI and ARDS represent the most severe form of the viral infection sustained by COVID-19.[80] SARS-CoV-2 surmounts an immune inflammatory response and in severe cases, may induce cytokine storm. Over the last few years, several therapeutic strategies have been investigated.[81] Remdesivir (GS-5734), a broad-spectrum antiviral agent, received emergency use authorization (EUA) from the FDA as it was the first antiviral agent to be tested against COVID-19 and showed initial success at inhibition of SARS-CoV-2 in primary human lung cell lines.[82] Clinical trials have also shown a reduction in recovery times and reduced all-cause mortality.[83] Tocilizumab, brand name Actemra, is a recombinant, fully humanized monoclonal antibody that targets soluble and membrane-bound forms of interleukin 6R (IL-6R). It is currently FDA-approved for the treatments of rheumatoid arthritis and Sjogren's but possesses systemic activity against autoimmune and inflammatory conditions. There were initial reports that correlated IL-6R levels with increased COVID-19 mortality.[84] However, the efficacy of COVID-19 infection remains unproven and in August of 2021, the NIH advised against its widespread use following conflicting reports and lack of properly designed clinical data.[85] Other strategies to rapidly identify therapeutics included repurposing of already FDA-approved drugs with suspected activity against other coronaviruses. A major example is the use of Quinine derivatives. Chloroquine and Hydroxychloroquine (CQ/HCQ) have been used as therapeutic agents for centuries and gained tremendous popularity during World War 2 as antimalarial drugs. Today, they are commonly used to treat autoimmune diseases, particularly Systemic Lupus Erythramatosus. There are many suspected potential mechanisms of action on coronavirus infection although these remain to be elucidated. One theory is the alteration in endosomal pH which prevents acidification and results in reduced viral entry to cellular cytoplasm.[86] CQ/HCQ were proven useful against SARS-CoV in the early 2000s and demonstrated activity against bat coronaviruses (HCoV-229E and HCoV-O43). Unfortunately, observational, and multicenter randomized, controlled trials reported that HCQ did not significantly reduce the chances of requiring intubation or mortality rate. As a result of these findings, treatment of COVID-19 with quinine derivatives is not recommended by the NIH.[86] The use of corticosteroids was also investigated in COVID-19-related ALI as steroids are a common therapy administered in the setting of ARDS despite the lack of literature to support its use. The most

influential clinical investigation of steroid use in COVID-19 ALI is the RECOVERY trial published in July 2020 which reported a reduction in death rates associated with the administration of dexamethasone in hospitalized patients requiring supplemental oxygenation (an observation that was only seen in patients on respiratory support).[87] The NIH still recommends the use of dexamethasone until discharge in patients hospitalized with COVID-19 requiring supplemental oxygen.[88]

As of January 2023, the list of therapeutic strategies aimed at combatting COVID-19 ARDS has become expansive. FDA has fully approved Actemra (Tocilizumab) and Olumiant (baricitinib) for hospitalized COVID-19 adult patients, as well as Veklury (Remdesivir) for COVID-19 in both adults and kids. Some medications received FDA EUA including (https://www.fda.gov/drugs/emergency-preparedness-drugs/coronavirus-covid-19-drugs) small molecule antivirals Paxlovid (nirmatrelvir and ritonavir) and Lagevrio (molnupiravir); SARS-COV-2 antibodies REGEN-COV (casirivimab and imdevimab) and Sotrovimab; immune modulators: Kineret (anakinra) and Olumiant (baricitinib). More new therapies are under clinical investigation including the use of Mesenchymal Stromal Cells,[89] Janus Kinase Inhibitors including ruxolitinib and baricitinib,[89] convalescent plasma therapy[90] (NCT04380935), angiotensin II receptor blockers[91] (NCT04337190), polyvalent immunoglobulin[92] (NCT04350580), tissue plasminogen activator[93] (NCT04453371), and pirfenidone[89] (NCT04653831).

It is currently unknown whether m6A methylation plays a major role in the etiology of COVID-19-related ALI. However, given the urgency for an effective treatment and the similarities in pathogenetic mechanisms involved in the inflammatory sequence of ALI, it would be reasonable to investigate the role of methylases in this severe, acute respiratory syndrome.

PART III. NEW STRATEGY FOR M6A TARGETING THERAPY OF ALI

Emerging evidence of a potential link between ALI and m6A

Pharmacological modulation of RNA methylation, in particular—by small-molecule modulators (inhibitors and/or activators) holds immense therapeutic potential and promise for advancing traditional and pulmonary medicine. Substantial literature exists to support the link between m6A modification and the development and progression of oncologic disorders, however, the relationship between m6A and pulmonary vascular pathologies remains largely unknown. m6A methylation has been associated with the pathogenesis of lung ischemia-reperfusion injury.[94] Activation of inflammatory pathways and endothelial barrier dysfunction has been associated with m6A modification.[95] A hallmark consequence of ALI is endothelial barrier dysfunction which precludes the inflammatory sequelae from leading to ineffective alveolar gas exchange. We sought to characterize ALI-related stimuli effects on m6A methylation to determine whether there is a potential link between m6A modification and the progression of ALI. ARDS is a common disease entity in critical care medicine and is still associated with a high mortality. A common rodent model of acute lung inflammation and ARDS is administration of lipopolysaccharide (LPS), either into the airways (direct, pulmonary insult) or systemically (indirect, extra-pulmonary insult). We confirmed that LPS induces increased m6A methylation in human pulmonary artery endothelial cells in a time dependent manner and identified METTL3 as the major player in upregulated RNA methylation.[96] We also confirmed that only m6A methylation on promoter sequences had a negative correlation with corresponding genes expression- further supporting our hypothesis that modulation of m6A methylation regulates endothelial cell gene expression and endothelial inflammatory response.[96] Additionally, we detected that METTL3 inhibition abrogates increased m6A methylation following LPS treatment and m6A demethylase inhibition increases endothelial permeability comparable to LPS. A few very recent studies supported this hypothesis that METTL3 contributed to PM2.5-induced lung inflammation via IL-24 upregulation,[97] and monocrotaline-induced pulmonary hypertension via GLUT4.[98] This knowledge in addition to the literature that supports m6A RNA methylation's role in the development of several human diseases allowed us to hypothesize that METTL3 (as well as the METTL-14-WTAP complex) is a potential avenue for investigation of novel ALI therapies.

m6A interventions in clinical trials (Table 2)

RNA epitranscriptomic modulation is a novel field of drug development that was only described in the last 3 years. Although still in its infancy, this field of therapeutics holds tremendous promise and paves the way for other epigenetic modulations in RNA in the treatment of human disease. As of 2022, no known METTL3 inhibitor has been studied in human. However, three independent drug companies have announced plans for phase 1 clinical trials of METTL3 inhibitors

TABLE 2 Clinical trials targeting m6A RNA modifications.

Intervention	Target	Indication	Phase	Trial Start Date	Company	Status*	Identifier
STM-2457	METTL3	AML	1	Unknown	STORM Therapeutics	No data	N/A
Unidentified	METTL3	AML, NSCLC	1	Unknown	Accent Therapeutics	No data	N/A
Unidentified	METTL3	AML	1	Unknown	Gotham Therapeutics	No data	N/A
STC-15 (oral)	METTL3	Advanced solid Tumor	1	2002	STORM therapeutics	Recruiting	NCT05584111

Note: STORM Therapeutics, Accent Therapeutics and Gotham Therapeutics announced at the RNA epigenetics conference in Cambridge, UK that they have developed small molecule inhibitors of METTL3-METTL14 complex which they anticipate investigating in Phase 1 clinical trials for oncologic indications following the discovery that RNA methylation plays a role in AML oncogenesis. STORM therapeutics is currently leading the field of RNA modulation therapeutic investigations following their proof-of-concept activity of the first, and as of November 2022, the only RNA methyltransferase inhibitor in an animal model. STC-15 is a highly potent and selective METTL3 inhibitor with activity against leukemic cells that have failed chemotherapy. STC-15 is the first molecule specifically targeting RNA methyltransferase to enter clinical development.

Abbreviations: AML, acute myeloid leukemia; METTL3, m6A methyltransferases, methyltransferase 3; NSCLC, non-small-cell lung carcinoma.

*status is as of 11/2022.

for the treatment of AML (Table 2).[22] Barbieri et al. identified METTL3 as an essential gene for the growth of AML cell lines and that downregulation of METTL3 results in failure to establish leukemia in immunodeficient mice.[99] This novel data confirmed METTL3 as a potential therapeutic target for AML. STORM Therapeutics, an independent company, has focused on METTL3 inhibitors for the treatment of AML. Using high throughput screening methods, biophysical screens, and mass spectrometry, they identified small molecules with access to METTL3 binding. Following a successful screen, they tested lead compounds in a mouse model of AML with oral administration of their lead compounds reducing splenomegaly and quantity of circulating monocytes. These promising outcomes were also seen in patient-derived xenografts which grew more slowly following treatment with their METTL3 inhibitor. Currently, STORM is the only company that has established proof of concept animal studies and has validated the approach of METTL3 inhibition as an avenue for drug discovery. STM2457 being investigated by STORM, which is aiming to put it in phase trials by 2022.[22] Currently, they are recruiting for an ongoing phase 1 trial of oral STC-15, a METTL3 inhibitor, to evaluate the safety, pharmacokinetics, pharmacodynamics, and clinical activity in patients with advanced solid tumor malignancies (Table 2). In early 2022, STORM therapeutics also announced that they were awarded a grant by Innovate UK for the research and development of novel drugs against a unique SARS-CoV2 protein using their RNA epigenetic platform. These investigations may shed light on the potential utility of METTL3 inhibition in SARS-CoV2. Accent Therapeutics and Gotham Therapeutics have also announced plans for phase 1 clinical trials utilizing their METTL3 inhibitors in 2022 (Table 2).

Plan of action

We propose a new strategy to first identify ligands by virtually screening molecular libraries for modulators of the RNA methyltransferase METTL3-14-WTAP complex and then to characterize their binding properties as well as effects on enzymatic activity with the intention of ultimately choosing a lead compound for animal studies of ALI. In conventional drug discovery, libraries containing a few million compounds are screened in high-throughput assays to find compounds that can be used as leads for further development. These compounds typically have low potency and specificity for the biological target. Analogues of the lead compounds are then made and assayed in a medicinal chemistry program so that they could develop advanced compounds with high potency and specificity. The screening process could take months or years before the identification of a lead compound. Medicinal chemistry focusing on altering structure–activity relationships could then take several years. Additionally, physical drug screening libraries are limited only to compounds that are available from commercial catalogues. However, there is an alternative strategy as of recently in which a virtual library can be used to identify virtual compound leads using a structure-based drug design screen. In short, a virtual screen takes a 3D structure of our target protein (METTL3) and identifies whether a ligand can "dock" or bind efficiently. Unlike the traditional physical screens, this virtual process is significantly less expensive and allows investigators to complete a screen of millions of compounds within just a few months. A virtual screening campaign can be proposed to combat the issues involved in the usage of traditional screens in our investigation for a small molecule inhibitor of METTL3. Small molecules will be identified with efficient and

excellent ability to bind to the SAM pocket of METTL3. We anticipate the discovery of several compounds with the ability to bind to METTL3 and plan to characterize their binding properties through our established binding affinity assay. Compounds with the most efficient binding capabilities will undergo a functional enzymatic activity assay to identify whether they selectively inhibit methyltransferase activity. Lead candidates with efficient binding and confirmed methyltransferase activity inhibition will be further investigated in an LPS-induced animal model of ALI or VILI for proof-of-concept studies. Beyond the scope of this thesis is the hope that perhaps a lead compound with demonstrated METTL3 inhibitory activity and abrogation of ALI in an animal model may be considered for future phase I clinical trials in humans.

Limitation of m6A as a therapeutic target

Similar to other therapeutic strategies targeting transcription factors or RNA binding proteins, m6A modulators, such as METTL3 inhibitors, have the potential to alter the expression of a collection of genes, without functional specificity, leading to unexpected off-target effects or potential side effects of the therapy. RNA m6A methylation is a dynamic and complex process involving multiple enzymes and pathways, and targeting any one component of this system may have unintended consequences on other cellular processes. Moreover, since METTL3 is expressed ubiquitously in multiple organs and tissues, RNA m6A methylation is not limited to cancer cells or lung endothelial cells, and targeting it may affect normal or other cell types as well. Therefore, further research is necessary to develop specific and targeted approaches for RNA methylation as a therapeutic target. The focus of drug development should be prioritized for severe diseases with no or limited available therapy, including cancer and ARDS.

PART IV. SUMMARY

ALI and its more severe form, ARDS, are acute inflammatory conditions with unacceptably high mortality rates that ultimately lead to progressive lung damage and may result in impaired lung function. Treatment modalities are limited and there are no FDA-approved pharmacological interventions capable of abrogating microvascular endothelial damage in the acute phase of the syndrome that ultimately leads to impaired pulmonary dysfunction and in many cases, death. Identification of novel therapeutic avenues for drug discovery is challenging due to multi-protein involvement and posttranscriptional mechanisms which are not fully understood. We aimed to characterize the post-transcriptional gene expression patterns following ALI-related stimuli to elucidate the mechanisms of this heterogenous clinical syndrome and identify a novel therapeutic target for the treatment of ALI/ARDS. Recently, m6A RNA methylation has been identified as a posttranscriptional modification with a potentially strong link to the contribution of many human diseases including cancer and pulmonary hypertension. These findings led us to conclude that the inhibition of METTL3, the active methyltransferase protein in the m6A modification process, holds immense therapeutic potential and promise for the treatment of ALI. A new strategy is proposed to first identify ligands by virtually screening molecular libraries for modulators of the RNA methyltransferase METTL3-14-WTAP complex and then to characterize their binding properties (kinetic screen) as well as effects on enzymatic activity (functional assay) with the intention of ultimately choosing a lead compound for testing in well established and validated animal model of ALI.

NOTE FOR LITERATURE SEARCH AND INCLUSION

A systematic search strategy was employed to ensure a comprehensive and thorough analysis of the existing literature body on this new and exciting research topic. First, an initial database search was performed using the keywords and phrases of each section. The databases searched include NIH PubMed and Clarivate Web of Science. Next, the reference lists of relevant articles were imported to an Endnote library. Finally, all articles were screened for relevance based on their title and abstract.

AUTHOR CONTRIBUTIONS

Reem Faraj and Ting Wang drafted the manuscript. All authors edited, revised, and approved the final version of the manuscript.

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CONFLICT OF INTEREST STATEMENT

The authors declare no conflict of interest.

ETHICS STATEMENT

N/A.

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