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Oncometabolites in renal cancer: Warburg's hypothesis re-examined

Cissy Yong[1,3], **Grant D Stewart**[1,3], **Christian Frezza**[2]

[1]Department of Surgery, University of Cambridge, Cambridge Biomedical Campus, CB2 0QQ, UK

[2]Medical Research Council Cancer Unit, University of Cambridge, Cambridge Biomedical Campus, CB2 0XZ, UK

[3]Cambridge University Hospitals NHS Foundation Trust, Cambridge CB2 0QQ, UK

Abstract

The study of cancer metabolism has evolved vastly beyond the remit of tumour proliferation and survival, with an unveiling of the ostensible role of 'oncometabolites' in tumorigenesis. Simply defined, oncometabolites are conventional metabolites that when aberrantly accumulated have pro-oncogenic functions. Their discovery has led us to revisit the original, dispelled Warburg hypothesis, first postulated in the 1950s, of aberrant metabolism as an aetiological determinant of cancer. As such, the identification of oncometabolites alongside their attractive utilisation in diagnostics and prognostics, as novel therapeutic targets and as biomarkers of disease, has been intensely sought after in oncology. To date, fumarate, succinate and 2-hydroxyglutarate have been characterised as *bona fide* oncometabolites. Renal cell carcinoma (RCC) is an established example of a cancer type with extensive metabolic reprogramming during tumour initiation and progression. With oncometabolites postulated to be rooted in the oncological origins and drivers of tumorigenesis, in combination with all three of these oncometabolites remarkably implicated in RCC, this timely review synthesises the literature to date on oncometabolites in RCC, their oncogenic mechanisms and the clinical impact oncometabolites may have in the management of RCC.

Introduction

Cancers of the kidney account for an estimated 2.2% of the global burden of all cancers, which translates into more than 400,000 new diagnoses worldwide in 2018[1]. Renal cell carcinoma (RCC), a cancer of the kidney parenchyma is the most common solid tumour of the kidney and the most lethal of all urological malignancies[2]. Almost a third of all patients have metastatic dissemination at presentation and nearly half of all patients die from their disease[1,2]. RCC is increasingly recognised as a collection of renal cancer subtypes each with

Correspondence: Christian Frezza: cf366@mrc-cu.cam.ac.uk; Grant D Stewart: gds35@cam.ac.uk.

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C.Y. and C.F. conceptualised the review. C.Y. wrote the manuscript. G.D.S: contributed to the development of the clinical focus. G.D.S. and C.F. contribute to the final editing of the review. All authors discussed the content of the review.

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distinct histology, genetic and molecular alterations, different clinical courses, and therapeutic responses[3–5]. Recent single cell sequencing has shed light on the oncogenic events and cell of origin of this tumour type. Interestingly, the convoluted proximal tubular renal cell subtype was shown to be the likely common cell of origin in clear cell RCC (ccRCC) and type 1 papillary RCC (pRCC), suggesting that these tumours may arise from a common origin with divergent fates[6]. Recent studies have expanded upon the role of genomics in RCC tumour evolution[7–9]. Loss of chromosome 3p, a pathognomonic feature of ccRCC occurring in >90% of patients[10,11], was typically found to be the initiating driver event in sporadic ccRCC, arising as early as childhood in as little as a few hundred cells, preceding cancer diagnosis by up to 3-5 decades[7]. The *VHL* gene alongside chromatin-modifying genes *PBRM1, BAP1,* and *SETD2* are co-located in this lost chromosomal region and perhaps unsurprisingly, are the most prevalent somatic gene perturbations found in ccRCC, as patients are rendered vulnerable to complete (biallelic) inactivation of these genes during their lifetime[7,10,12]. Furthermore, this group have identified distinct evolutionary subtypes of ccRCC that correlate with clinical phenotypes and outcomes, which could be used to guide intervention and surveillance[8,9]. As genomic technology advances, the genetic perturbations implicated in ccRCC continue to expand and include somatic mutations in *TERT*[7,13], *PTEN*[10,12], *MYC*[14,15], and *mTOR*[12] signalling pathways as well as other numerous metabolic pathways[10], which will be discussed further in this review alongside subtype-specific genetic perturbations such as mutations in fumarate hydratase (FH) in type 2 pRCC[16].

RCC is increasingly recognised as a ‘disease of cell metabolism’. Before the advent of the ‘Omics’ era, at least twelve genes implicated in RCC pathogenesis were identified to have roles in fundamental metabolic processes [17,18]. One classic example in RCC is the ability of *VHL* inactivation to rewire the normal metabolic adaptation response to oxygen deprivation. Inactivation of *VHL* in ccRCC leads to the aberrant accumulation of the transcription factors hypoxia-inducible factor (HIF)1α and HIF2α despite normoxia[19,20], with resultant upregulation of pathways involved in glycolysis, fatty acid, and glycogen synthesis[21–23]. This example of the ‘metabolic reprogramming’ phenomenon observed in cancer cells, whereby metabolic adaptation facilitates the neoplasm’s capacity to meet its bioenergetic demands, such as uncontrolled proliferation and the acquisition of other hallmark traits of cancer, is now recognised as being fundamental in the malignant transformation of cells and also in the phenotypic evolution of tumours[24,25]. Interestingly, HIF has been found to be a common target for metabolic reprogramming in RCC by other genetic perturbations that affect FH[26–28], succinate dehydrogenase (SDH)[26,27,29], tuberous sclerosis complex[30], and more recently, fructose-bisphosphatase 1 (*FBP1*)[31]. *FBP1* gene encodes the key gluconeogenic enzyme fructose-1,6-bisphosphatase 1 (FBP1), which was found to directly interact with HIFs to restrain its transcriptional activity. The discovery that FPB1 is ubiquitously suppressed in ccRCC further supports the characterisation of RCC as a metabolic disease[31]. HIF being a common denominator associated with multiple RCC subtypes highlights it as a potential key candidate for RCC therapies. Further corroboration was provided by the landmark TCGA integrated platform analyses studying the genome, transcriptome and proteome of more than 400 ccRCC tumours[10]. This study highlighted the extensive metabolic reprogramming captured in ccRCC involving the upregulation of fatty

acid synthesis, pentose phosphate pathway, glutamine transporters, and downregulation of the tricarboxylic acid (TCA) cycle correlating to disease aggressiveness and worsened prognosis[10] (Fig. 1). Furthermore, subtype-specific metabolic gene alterations correlating to disease aggression and patient survival were identified in subsequent TCGA studies across the three major RCC subtypes (clear cell, papillary and chromophobe) supporting the principle of subtype-specific management and providing potential subtype-specific targets for novel therapies[5].

Given the metabolic nature of RCC and the emergence of metabolic reprogramming as a contemporary hallmark of cancer[32], a surge in the field of metabolomics has rapidly developed over the last decade. In general, metabolomics encompasses the ability to globally detect the metabolites present in a system (cell, tissue or organism) under a given set of conditions[33]. The field of metabolomics enables the study of the final downstream product of the genome and crucially captures the underlying environmental influences and external perturbations of a system[33,34]. Particularly in the setting of cancer, the tumour microenvironment notoriously has a profound effect on metabolism[35,36], therefore integrating the study of cancer metabolomics with other ‘omics’ studies enables a holistic approach to understanding cancer pathophysiology. The key metabolic pathways of interest in cancer, alongside a broad outline of these pathways are highlighted below with reference to RCC (Fig. 1).

A handful of metabolomic studies in RCC have been performed to date with the largest studies profiling a single cohort of 138 patients with ccRCC[37,38]. The metabolic profiles from these studies broadly corroborated several findings from the TCGA study of a network of metabolic shifts involving upregulation of glycolysis, pentose phosphate pathway, and glutamine uptake correlating with disease aggressiveness[37,38]. Integration of this metabolomic data with the TCGA dataset however highlighted a lack of linearity between enzyme expression and the corresponding catalysed metabolite levels[37]. This lack of linearity has been theorised to involve shunting of metabolites into alternate cancer-reprogrammed pathways[37,39]. As uncovered so far, physiological metabolism can be efficiently manipulated by RCC to provide the conditions needed for cancer cells to survive and proliferate. However, the identification of key genetic mutations in cancer cells encoding for enzymes in mitochondrial metabolism such as FH and SDH[16,40,41] paved the way for the next chapter of cancer metabolism, the discovery and evolution of the oncometabolite paradigm. By definition, oncometabolites are conventional metabolites that when aberrantly accumulated have pro-oncogenic capabilities that can contribute to tumorigenesis, especially via epigenetic dysregulation, as well as influence tumour phenotype and progression. Interestingly, of the short list of established *bona fide* oncometabolites, the majority have been implicated in hereditary and sporadic subtypes of RCC. This observation coupled with the strong metabolic paradigm in RCC, alongside the aggressive metabolic reprogramming underpinning RCC tumour progression, commands attention to this growing area of cross-over research in oncology and metabolism. This review is timely in synthesising the literature to date of *bona fide* oncometabolites in RCC, their proposed mechanisms of action, and the clinical impact oncometabolites may have in management of this important disease process.

Inception of the oncometabolite paradigm

The inception of the oncometabolite paradigm predates its contemporary nomenclature into the literature, and one could argue it began with the once dispelled but now revived Warburg hypothesis of aberrant metabolism as an aetiological determinant of cancer[42,43]. Based on the observation of excessive fermentation of glucose in mammalian cancer cells irrespective of the presence of oxygen (the observed phenomena later coined the 'Warburg effect'(Fig. 1).[44]), it was here that Warburg first postulated that this abnormal compensatory mechanism to counteract an irreversible injury of cellular respiration, induced cells into an undifferentiated state, giving rise to "cells that grow wildly- the cancer cells"[42,43]. In Warburg's view, the prime cause of cancer was mitochondrial dysfunction. Shortly thereafter however, in light of the discovery of mutated oncogenes and tumour suppressor genes, Warburg's hypothesis was soon dismissed and altered metabolism revised as a bystander effect secondary to these genetic perturbations identified in numerous cancers[45–48]. Warburg's hypothesis however came full circle at the pinnacle of this era when several genetic perturbations in genes encoding two key enzymes in the TCA cycle, *FH*[16] and *SDH* subunits[40,41,49] were implicated in the development of Hereditary Leiomyomatosis and Renal Cell Cancer (HLRCC) and hereditary Paraganglioma (PGL), respectively. Shortly after, seminal studies uncovered an unconventional and novel role, common to both fumarate and succinate, in deregulating the HIF pathway through direct inhibition of prolyl hydroxylases (PHD), enzymes involved in signalling HIF degradation[26,27,29]. Stabilisation of HIF1α/2α alongside upregulation of downstream HIF1 products such as VEGF and glucose transporter (GLUT1) were observed in these HLRCC tumours and *SDH*-deficient tumours, in the absence of *VHL* inactivation[26,27], providing support for the role of these aberrant metabolite accumulations in creating the hypoxic signatures and highly vascularised phenotype that are characteristic of these tumours[50–54]. These studies proposed an alternative mechanism to creating the notorious 'pseudo-hypoxic' milieu in the absence of *VHL* inactivation, which has been well established in *VHL*-mutant disease and a characteristic tumour phenotype associated with RCC and tumour aggression[55,56].

Dang *et al* however was the first group to coin the term 'oncometabolite' to describe the potential tumorigenic role of pathological accumulations of the metabolite D-2-Hydroxglutarate (D2HG)[57]. Under physiological settings, 2-hydroxyglutarate (2HG) exists in two natural isoforms L- and D- (L2HG and D2HG respectively), both are minor metabolic by-products produced via distinct biological mechanisms and are normally kept at unappreciable levels by conversion back to α-ketoglutarate (αKG) via the respective L-/D2HG dehydrogenase (L2HGDH/ D2HGDH) enzymes[58–61]. In Dang et al's study, abnormally elevated D2HG levels (up to tens of *μmol per gram of tissue*)were expressed by>100-fold greater in patients with malignant gliomas harbouring a single mutant copy of the isocitrate dehydrogenase (*IDH*) 1 gene compared to malignant gliomas with wildtype *IDH* genes[57]. Wildtype *IDH* encodes the TCA cycle enzyme responsible for the reversible oxidative carboxylation of isocitrate to αKG. Mutation of the *IDH1* gene confers a gain-of-function neomorphic activity of the IDH enzyme that catalyses the reduction of αKG to D2HG, leading to its accumulation[57]. Interestingly, in patients with inborn errors of 2HG metabolism, elevated levels of L2HG have been associated with brain tumours[62,63] as well

as one case of Wilm's tumour (nephroblastoma)[63,64], whereas D2HG accumulations in this cohort has not been associated with cancer[65]. Overall, these initial studies galvanized a myriad of 'oncometabolite'-focussed studies that have expanded upon these findings, establishing a core group of *bona fide* oncometabolites. Currently, this consists of fumarate, succinate, L2HG and D2HG[48,59,66,67]. These oncometabolites are increasingly associated with numerous malignancies, including neuroendocrine tumours[49,68,69], brain tumours[70,71], haematological malignancies[72,73], head and neck squamous cell carcinoma[74], and our topic of interest, hereditary and sporadic forms of renal cell carcinoma (RCC)[59,66,67].

Pathogenesis of oncometabolite accumulation

Endogenous origins: tumour suppressors and oncogenes

Identification of loss-of-function mutations in genes encoding the key TCA cycle enzymes SDH and FH, which lead to the accumulation of succinate and fumarate respectively, as well as a gain-of-function mutation in IDH, which leads to the accumulation of D2HG, has led to appreciation of how these genetic perturbations act as tumour suppressor genes and oncogenes[27,48,66,75]. Both *FH* and *SDH* mutations in tumours follow the Knudson's 'two hit' hypothesis of tumorigenesis[76]. In patients with heterozygous germline mutations for *SDH* or *FH* i.e. inheritance of one mutated allele, the loss of heterozygosity (LOH) i.e. loss of the remaining wildtype allele, seems to be the clinching factor in tumorigenesis[48,66,75] and in both cases, converge on the predisposition to the development of PGL/phaeochromocytomas (PCC)[68,77–80]. The SDH enzyme is composed of four subunits (SDHA, SDHB, SDHC and SDHD) as well as two assembly factors (SDHAF1 and SDHAF2), each encoded by distinct genes across multiple chromosomes[81]. Loss of heterozygosity in multiple subunits of *SDH* predisposes to a variety of cancers including SDH-deficient RCC, a very rare and aggressive disease[5,78,79,82–84], whereas LOH in patients with heterozygous *FH* germline mutations predisposes to HLRCC[16,85,86], an autosomal dominant hereditary cancer syndrome characterised by cutaneous and uterine leiomyomas and a highly aggressive form (type 2) of pRCC[87]. Interestingly, distinct clinical phenotypes are also observed in *FH*- and *SDH*-deficient diseases. Homozygous germline mutations in *FH* give rise to fumaric aciduria, a rare metabolic disease associated with infantile encephalopathy, brain malformations and neonatal polycythaemia without an associated cancer predisposition[88,89], whereas homozygous germline mutations of *SDHA* cause severe neurological dysfunction and cardiomyopathy[78]. This divergence in the clinical phenotypes observed suggest that the 'two-hit' mutational timing and tissue-specific nature of mutations may be crucial to cancer predisposition. As such, it has been suggested that oncometabolites may be insufficient in themselves for oncogenic transformation[59]. Potentially confounding this notion is the finding that patients with inborn errors of metabolism such as fumarate aciduria often do not survive long enough[90,91] for potential malignancies to manifest. An interesting point to discuss that currently eludes our knowledge is what drives or does not drive cancer in certain tissues upon LOH in an exquisitely-specific nature as evidenced by diseases such as HLRCC and SDH-deficient diseases. Current hypotheses have been recently discussed[92], and, building upon this insight, we postulate a concept of 'LOH tolerance' in 'permissive' tissues that propagate tumourigenesis due to their capability to be more flexible (such as the ability to metabolically adapt and/or compensate as a result of

these genetic perturbations e.g. reversal of the activity of the urea cycle enzyme arginosuccinate lyase (ASL) in FH-deficient cells which funnels accumulated fumarate into aberrant urea metabolism [93]), whereas 'LOH intolerance' in a small proportion of cells and or tissues, results in lethality without further propagation or replication of these genetic perturbations and thus attenuating tumorigenesis in these tissues. Needless to say, further understanding into this current conundrum of what gives rise to distinctive patterns of tissue-specificity in cancer may reveal tissue-specific vulnerabilities that may impact greatly on future management of these clinically challenging diseases.

In contrast to *SDH* and *FH*, *IDH1* and *IDH2* genes, which encode the compartment-specific isoform of the IDH enzyme in the cytosol and mitochondrial respectively[94], express a dominant pattern of oncogenic behaviour. Somatic mutations in only one copy of the *IDH* gene i.e. retention of one wildtype copy of *IDH*, were observed in patients in multiple cancers including gliomas[71,95] and acute myeloid leukemia[96], leading to the neomorphic gain-of-function activity in converting αKG into D2HG[57]. More recently, D2HG accumulation as a result of loss-of-function mutations in *D2HGDH* has been observed in a small subset of large B-cell lymphoma[91], implicating both the synthesis and conversion of D2HG in its accumulation in cancer. Using the cBioPortal Database[97], <1% of *IDH1/2* mutations are found in large-scale cancer genomic studies of RCC such as the TCGA dataset[97,98]. Although 2HG was identified to be significantly accumulated in human ccRCC tissues, >90% of this was in the L2HG isoform[99], suggesting D2HG is unlikely to have a significant role in RCC pathogenesis. Reduced expression of L2HGDH, an enzyme that catalyses the conversion of L2HG into αKG, was found to contribute to the accumulation of L2HG in patients with ccRCC[99]. LOH of the *L2HGDH* gene (which resides on chromosome 14q and noted to be a commonly deleted region in ccRCC[100,101]) correlated with reduced protein expression of L2HGDH and accumulation of 2HG, providing support that *L2HGDH* may also function as a tumour suppressor in RCC[101]. Furthermore, loss of *L2HGDH* conferred a worse prognosis in patients with ccRCC compared to those with *L2HGDH,* with preliminary metabolomic profiling suggesting that increasing levels of L2HG are associated with RCC tumour progression, further corroborating its role as an oncometabolite[101].

More recently, mutations in several genes, αKG dehydrogenase (*αKGDH*)[102], lipoic acid synthase (*LIAS*) [102] and lipoyltransferase-1 *(LIPT1)*[103] have also been implicated in 2HG accumulation. These genes encode enzymes required for the proper functioning of the αKGDH-complex (αKGDHC), which catalyses the conversion of αKG to succinyl-coenzyme A in the TCA cycle. The truncated TCA cycle due to these mutations promoted the production of L2HG from accumulated αKG[102,103], with evidence of downstream oncometabolite activity inhibiting PHDs leading to HIF stabilisation and HIF1-targeted gene activation including VEGF and GLUT1[102]. Interestingly, in patients with homozygous germline mutations of enzymes of lipoic acid synthesis, L2HG accumulations lead to suppression of PHD activity and subsequent HIF1 activation[102]. However as this is also a rare inborn errors of metabolism diseases and is generally lethal at a young age[102,104] it may also preclude any potential oncometabolite-associated tumour development. Characterisation of heterozygous germline mutations in this setting may provide additional insight for the tumorigenic role of L2HG accumulation in αKGDH/LIAS mutations. Table 1. highlights the

genetic mutations in oncometabolite-associated RCC subtypes, the clinical features and potential therapeutic strategies which will be discussed further in this review.

Exogenous origins: environmental factors

Remarkably, in the absence of oncogenic mutations, oncometabolites have been demonstrated to accumulate in cells and to induce oncogenic transformation[105]. Identifying the environmental factors linked to oncometabolite accumulation may shed important light on how they impact or predispose individuals to cancer risk. Hypoxia-induced production of oncometabolites via “off-target”, substrate-promiscuous activity of the enzymes lactate dehydrogenase A (LDHA)[106] and malate dehydrogenase (MDH)[106,107] on glutamine-derived αKG[23,106] results in L2HG accumulation, whereas promiscuous activity of D-3-phosphoglycerate dehydrogenase (PHGDH) catalyses the conversion of αKG to D2HG in *IDH*-wild type breast cancer cells[108], demonstrating alternative pathways for 2HG accumulation in response to exogenous stimuli. In addition, acute ischaemic preconditioning *in vivo* resulted in 2HG accumulations in mouse myocardium[109]. Although modest accumulations of L2HG in hypoxic cells were observed compared to that in cancer cells[23], hypoxia-induced L2HG accumulation was sufficient and necessary for exerting recognised ‘oncometabolite functions’ such as the repressive trimethylation of the histone protein, histone 3 lysine 9 (H3K9me3)[106], in which upregulated levels were similarly observed in patients with *IDH*-mutant gliomas[110]. Histone methylation affects chromatin organisation and regulation of gene transcription[110,111] and, *in vitro,* L2HG-induced hypermethylation of H3K9 has been demonstrated to block cellular differentiation in non-transformed astrocytes[110], supporting its role in epigenetic regulation. Independent of hypoxia, acidic conditions have also been observed to drive L2HG accumulation, augmenting the promiscuous activity of LDH1/MDH2 activity as well as inhibiting the activity of L2HGDH *in vitro*[112].

Succinate accumulation has also been observed in cancer cells grown under hypoxic conditions within a 3D tumour model[113] and in animal models subjected to ischaemia-reperfusion injury *in vivo* [114–116]. Succinate oxidation has been shown to contribute to cardiac injury at reperfusion[114,116,117] via the generation of reactive oxidative species (ROS)[114]. However, rapid resolution of the accumulated succinate in these tissues back to baseline in this setting[114,116,117] means chronic effects of succinate accumulation in tissues, analogous to SDH-deficient tumours, is challenging to study. Nevertheless, succinate accumulation observed in hypoxic retinas of rodents leads to an upregulation of angiogenic proteins such as VEGF in a HIF-independent manner via activation of the succinate receptor GPR91, suggesting alternative mechanisms for stimulating angiogenesis by succinate in this setting[115]. Of note, the hypoxic induction of oncometabolite accumulation may be propagated and amplified by the oncometabolites themselves as they can stabilise HIF expression through direct inhibition of the PHD enzymes involved in signalling HIF degradation[19,26,27,29,29,118–120]. In addition, succinate may also participate in an alternative positive-feedback system of reinforcing the *HIF1α* signalling loop[121]. *HIF*-dependent expression of micro-RNA210 (miR-210) in lung adenocarcinoma cells *in vitro* was demonstrated to target the *SDHD* subunit and impair SDH function, and the ensuing

succinate accumulation in turn leads to HIF stabilisation through inhibition of PHDs perpetuating this hypoxic phenotype[121].

Aside from hypoxia, mitochondrial dysfunction arising from glucose toxicity[122–124] has been shown to result in oncometabolite production. Fumarate accumulation causing fumarate-dependent protein succination was observed in adipose tissues of hyperglycaemic mice[124,125], analogous to the succination features exhibited by fumarate accumulation in cancer cells. Interestingly, a similar succination phenotype was also observed in the adipose tissue of obese, insulin-resistant, non-hyperglycaemic mice[128], proposing other potential exogenous sources of oncometabolite production. Furthermore, evidence of succinate accumulation has been found in bone marrow stromal cells of diabetic mice, stimulating osteoclastogenesis through activation of the succinate receptor GPR91[129]. Of note, mitochondrial dysfunction has been linked to the metabolic syndrome, a distinct cluster of conditions including obesity, diabetes, hypertension, and hyperlipidaemia[130], which in turn, has also been causally linked to RCC and are factors correlated with an increased risk of RCC[131,132]. This link provides a nidus for detailed investigations into the cross-talk between environment-induced oncometabolite accumulation and their tumorigenic role in RCC. In addition, infection has also been implicated in the exogenous production of oncometabolites, demonstrating stimulation of succinate accumulation in macrophages[133,134], causing downstream HIF stabilisation and upregulation of HIF-targeted gene transcription such as interleukin 1β, a key pro-inflammatory signalling molecule[133]. Overall, further elucidation of the magnitude and mechanisms by which exogenous factors have on oncometabolite production and the subsequent sequalae, independent of oncogenic mutations, will be a critical step forwards in understanding and ameliorating the role of these factors in tumorigenesis and tumour evolution.

Mechanistic actions of oncometabolites in RCC

Oncometabolites exhibit a multitude of downstream pro-oncogenic functions. The functions that converge on a group of downstream pro-oncogenic pathways will be discussed first, followed by unique functions specific to each oncometabolite (Fig. 2).

Common downstream pro-oncogenic pathways

A characteristic trait shared between succinate, fumarate, and 2HG is their ability to competitively inhibit αKG-dependent dioxygenases (αKGDDs)[66,135] through their structural similarity to αKG, an essential co-substrate for enzyme activity[70,118]. αKGDDs are a superfamily of enzymes involved in a diverse plethora of biological processes. The most studied αKGDDs related to oncometabolite signalling consist of PHDs, for which inhibition is involved in the induction of the ‘pseudohypoxic’ milieu[27,48,66,67]; the Jumonji C domain-containing histone lysine demethylases (KDM)[70,110,118,137], and the ten-eleven translocation (TET) enzyme family of 5-methylcytosine (5mc) hydroxylases, involved in histone and DNA demethylation respectively[70,110,118,137,138], in which characteristic hypermethylated phenotypes are associated with altered gene expressions including the regulators of epithelial-to-mesenchymal transition (EMT), a hallmark of tumour aggressiveness and metastatic progression[137,139](Fig. 2).

As highlighted above, the initial evidence supporting the concept of oncometabolites was their role in inducing a pseudohypoxic milieu observed in SDH-deficient PGL/PCC and *FH*-deficient HLRCC[26,27,29,120]. Through direct inhibition of PHD[19], hydroxylation of HIF1α/2α subunits by PHD is inactivated, ultimately culminating in aberrant HIF stabilisation with downstream upregulation of HIF-targeted genes such as VEGF and GLUT1[26,27]. Notably, these tumours tend to exhibit intense vascularisation and hypoxic gene signatures in keeping with a pseudohypoxic tumour phenotype[50–54]. Interestingly, HIF1α/2α inactivation in *SDHB*-deficient osteosarcoma cells significantly impaired tumour growth in a mouse xenograft model[140] whereas HIF1α/2α inactivation in a FH-deficient mouse model of renal cyst disease exacerbated or failed to ameliorate this phenotype respectively[141]. Overall, these studies highlight the complex role of HIF/pseudohypoxia in tumorigenesis suggesting it may be context dependent e.g. cell specific[66]. Whilst L2HG has also been shown to inactivate PHDs and aberrantly stabilise HIF1α[102,142,143], the activity of D2HG on PHD remains contentious. Unexpected agonistic activity of D2HG on PHDs has been observed *in vitro*[102,143], however these findings are challenged due to the non-enzymatic oxidation of D2HG to αKG activity observed *in vitro*[144], which would provide the necessary co-substrate for PHD activation in this situation.

Oncometabolite also have a role in epigenetic alterations through direct inhibition of KDMs[110,137] and TETs[138], which are groups of enzymes responsible for histone and DNA demethylation respectively. This oncometabolite function leads to changes in chromatin structure and function that lead to hypermethylation phenotypes that alters the expression of a wide range of genes involved in cellular differentiation and acquisition of malignant features. Of note, the epigenetic effects of DNA and histone methylation on transcriptional activity are challenging to distinguish as they are often interdependent and inter-regulated[145]. Generally speaking, histone hypermethylation e.g. due to KDM inhibition, results in either transcriptional gene repression or activation (also known as repressive or active marks) depending on the type of histone residues and the number of methyl groups added[66,145]. DNA methylation at ‘CpG islands’ (clusters of dinucleotide sequence of a cytosine followed by a guanosine nucleotide in the 5’-3’ direction, often found in promoter regions upstream of transcription sites) usually represses downstream gene transcription[66,145]. Cytosine methylation in position 5, also known as 5-methylcytosine (5mC) undergoes oxidation by TET enzymes that convert 5mC to hydroxylated 5mc (5hmC)[66,145]. This reaction primes the cytosine to demethylation, generating unmethylated cytosine (5C). In general, global DNA hypomethylation, leading to inappropriate transcriptional activity and chromosomal instability, coupled with specific patterns of hypermethylated CpG promoter islands, especially upstream of tumour suppressor genes resulting in repressed expression, is characteristic of many tumour types[145–147].

Several studies to date have investigated the oncometabolic effects on histone/DNA hypermethylation through inhibition of KDM and TETs in *FH*, *SDH* and *IDH*-mutant tumours[70,110,118,137,142,148–150]. *SDH*-deficient tissues from patients with PGL and PCC demonstrated high levels of repressive histone marks (H3K27me3)[137]. In addition, succinate accumulation in *SDHB*-knockout chromaffin cells[137] and *SDHB*-knockdown murine ovarian cancer cells[151], as well as 2HG accumulation in *IDH1* mutant cells[70,110], demonstrated KDM and TET inhibition with characteristic hypermethylation phenotypes associated with

suppression of cellular differentiation[110,137] and activation of EMT, through up- or downregulation of positive and negative EMT regulator genes respectively[137,151]. HLRCC-derived *FH*-deficient cells also elicited an EMT signature in keeping with *SDH*-deficient cells[137], via fumarate-induced TET-mediated epigenetic suppression of miR-200, a short RNA molecule with tumour suppressive effects on EMT gene expression by modulating mRNA translation[139]. This EMT phenotypic switch induced by oncometabolite accumulation in *FH*- and *SDH*-mutant RCC tumours is no doubt a contributing factor to their clinically aggressive behaviour. *In vivo*, *SDHA*- and *FH*-silencing in mouse hepatocytes led to succinate and fumarate accumulation respectively, with evidence of KDM and TET inhibition and regulation of target gene expression[118]. DNA hypermethylation linking to repression of specific-lineage differentiation has also been observed in patients with *IDH1/2* mutant chondrosarcoma[149] and acute myeloid leukaemia[148]. Furthermore, accumulations of D2HG resulted in increased DNA methylation (5mc) with concurrent decreased DNA hydroxymethylation, indicating TET inhibition, was observed in human *IDH1* glioma tissue[70] and in ectopic expression of *IDH1/2* mutations in various cell types, which blocked cellular differentiation[148–150].

Identification of specific DNA hypermethylation patterns within a subset of colorectal cancers[152] gave rise to the CpG island methylator phenotype (CIMP)-associated cancer subtypes, characterised by their extensive epigenomic aberrations and distinct biology[150,152,153], which has been increasingly recognised in other malignancies including gliomas (G-CIMP) [150,153,154] and more recently, in a subset of type 2 papillary RCC (CIMP-RCC)[5,155]. Interestingly, G-CIMP tumours are tightly associated with IDH1 mutations[150,154] and introduction of mutant-IDH1 into human primary astrocytes leads to an accumulation of D2HG, inhibition of TET and reproduced a DNA hypermethylation profile that mirrored the changes observed in G-CIMP[150]. The recent characterisation of CIMP-RCC has been associated with early-onset disease and perhaps unsurprisingly, germline or somatic mutations of the *FH* gene[5,155]. Given the role of oncometabolites in αKGDD inhibition, including on TET enzymes, it is plausible that fumarate accumulation may be causally linked to the hypermethylated state in CIMP-RCC. Given that CIMP-RCC conferred the worst prognosis of all the RCC subtypes[5] and the highly aggressive nature of *FH*-deficient RCCs[156–159], in combination with the ineffectiveness of current RCC therapies in advanced *FH*-deficient RCC[157,160], understanding the molecular underpinnings of this disease process is warranted to find more effective strategies to treat these patients, such as the potential use of histone and DNA methylation inhibitors, which will be discussed in the next section.

Of note, several studies have demonstrated that oncometabolites have varying IC_{50} values (half maximal inhibitory concentration, a measure of the potency of a substance to inhibit a specific biology process/function by 50%) for different αKGDDs[70,118,138,142] suggesting that oncometabolite type and accumulation levels may determine the precise nature of downstream oncogenic processes in a given cell. Beyond the common inhibition of αKGDDs, we have begun to appreciate the distinct biological functions of individual metabolites, those that are especially relevant to RCC tumorigenic capabilities will be discussed next.

Distinct downstream effects of oncometabolites

Fumarate—Fumarate has demonstrated the most versatility of the *bona fide* oncometabolites to date, impacting on oncogenic signalling, antioxidant response, and phenotype switching (Fig. 2). In addition to the direct inhibition of PHD enzymes that facilitate pseudohypoxia induction, fumarate has also been shown to drive a hypoxic phenotype on a transcriptional level through the non-canonical activation of NFkB, a family of transcription factors that can promote HIF1α transcription. This signalling pathway is dependent on fumarate activation of Tank-binding kinase 1 (TBK1), an enzyme that phosphorylates p65 (a subunit of NFkB) with subsequent NFkB activation. Furthermore, inhibition of TBK1/p65 axis in *FH*-deficient RCC cells blocked HIF1α expression and reduced cellular invasion *in vitro*, suggesting a novel target for treatment in *FH*-deficient RCC[158]. This finding supports the critical tumorigenic role of HIF1α and pseudohypoxia in aggressive RCC subtypes such as *FH*-deficient RCC (HLRCC)[16,55,162]. In a similar fashion, silencing HIF1α in HLRCC-derived (FH-deficient) RCC cell lines diminished the invasive properties of these cells[163]. Potentially contradicting this theory, the genetic inactivation of HIF1α/2α in *Fh1* (murine FH)-deficient mice was shown to exacerbate, or failed to ameliorate, the renal cyst phenotype respectively, suggesting alternate mechanisms for oncogenesis in *FH*-deficient cells[141].

An alternative candidate oncogenic pathway in *FH*-deficient disease via the stabilisation of the Nuclear factor (erythroid-derived 2)-like 2 (Nrf2) antioxidant pathway has been proposed[141,157]. A distinct feature of *FH*-deficient tumours is the ability of accumulated fumarate to modify a wide range of proteins[126,164]. The post-translational protein modification triggered by fumarate, known as succination, can impair protein function, and is caused by fumarate reacting with specific cysteine residues on proteins, producing S-2-succino-cysteine (2SC) residues[126,164]. A key target of succination is the protein Kelch-like ECH-associated protein-1 (KEAP1). Removing KEAP1's repressive effects on the transcription factor Nrf2 results in upregulation of Nrf2-dependent genes involved in antioxidant pathways that regulates the cells ability to adapt to oxidative stress[141,157]. In keeping with this, Nrf2 and downstream Nrf2-targeted genes were found to be upregulated in HLRCC-derived type 2 pRCC tumour cells[141], highlighting potential alternative targets for treatment strategies in this aggressive disease[141,157]. On the contrary, succination of the antioxidant glutathione in *FH*-deficient RCC cells, depletes the antioxidant capacity of these cells, rendering them susceptible to endogenous accumulation of ROS[165] with subsequent stabilisation of HIF1α[165] and induction of cellular senescence[166]. Senescence is a state of irreversible growth arrest linked to tumour suppressive activation and thought to be a protective phenotype against cancer[167]. Ablation of a key mediator of senescence, p21, in *Fh1*-deficient mice induced the transformation of benign renal cysts into hyperplastic lesions suggesting that this fumarate-induced senescent event needs to be overcome for renal tumorigenesis to proceed[166]. Although ROS in itself can activate the Nrf2 signalling pathway through KEAP1 inhibition[168], fumarate-dependent succination of KEAP1 seems to be the predominant mechanism for Nrf2 activation in these *FH*-deficient cells[165,166].

Furthermore, although not within the context of *FH*-deficiency, fumarate has also been found to bind directly to glutathione peroxidase 1 (Gpx1), activating this ROS scavenging

enzyme in cancer cells with upregulated glutamate dehydrogenase 1 (GDH1) expression[169]. GDH1 maintains the levels of TCA cycle intermediates by catalysing the conversion of glutamate to αKG and subsequently to fumarate, where it can bind to Gpx1 and confer a proliferative advantage to cancer cells by regulating redox homeostasis[169]. Furthermore, GDH1 inhibition attenuated cancer cell proliferation and tumour growth *in vivo*[169]. It is plausible, given the nature of fumarate accumulation in *FH*-deficient tumours and that glutamine entry into the TCA cycle (via GDH1) is a dominant pathway in this setting[170,171], GDH1 inhibition in the setting of *FH*-deficiency may also ameliorate these pro-tumoural effects. Nevertheless, these studies in *FH*-deficient cells demonstrate that they have highly adapted and intrinsic mechanisms to combat redox stress in a multi-layered approach that confers tumour survival. Tying the HIF and Nrf2 pathways together was the identification of the Abelson Murine Leukaemia viral oncogene homolog 1 (*ABL1*) upregulated in *FH*-deficient tumours[160]. Fumarate-mediated activation of ABL1 occurs via the suppression of the protein phosphatase PTPN12 via oxidative stress[172], which leads to activation of the Nrf2 antioxidant pathway and the mTOR/HIF1α hypoxic signalling pathway in *FH*-deficient RCC cells[160]. Furthermore, ABL1 inhibition suppressed the invasion capacity and growth of these cells *in vitro* and *in vivo*[160,172]. As ABL1 is upstream of two major pathways implicated in *FH*-deficient tumours, this may suggest that multimodal treatment strategies i.e. targeting multiple pathways, may be beneficial in these diseases.

Another unique oncometabolic feature of fumarate is its ability to directly modulate cellular metabolism. Conventionally, fumarate participates in several major interlinked pathways such as the TCA cycle and the urea cycle[93,173]. Accumulation of fumarate in *FH*-deficient cells has been shown to reverse the activity of the urea cycle enzyme arginosuccinate lyase (ASL)[93]. Normally, argininosuccinate is produced from citrulline and aspartate via argininosuccinate synthetase (ASS) in the urea cycle, which is then converted into fumarate and arginine via argininosuccinate lyase (ASL). Reversal of ASL activity results in an accumulation of argininosuccinate and renders *FH*-deficient cells auxotrophic for arginine[93]. Expectedly, arginine depletion led to reduced cellular survival and proliferation *in vitro*[93]. In addition, the loss of FH leads to a complex metabolic rewiring pattern involving the diversion of increased glutamine uptake into the haem synthesis/degradation pathway, which critically sustains mitochondrial NADH levels and mitochondrial membrane potential[174]. Targeting this unique *FH*-deficient haem pathway, in particular the enzyme haem oxygenase 1, which catalyses the degradation of haem, thus rendered a selective synthetic lethality to *FH*-deficient cells, cleverly sparing normal (wildtype FH) tissues[174]. These two studies[93,174] highlight how *FH*-specific liabilities can be meaningfully manipulated to provide novel strategies to treat *FH*-deficient tumours such as in patients with HLRCC. Perhaps unsurprisingly in RCC, given the multitude of ways in which fumarate lives up to its deserving oncometabolite status, an increased gene expression of *FH* is correlated with better survival outcomes[10], and correspondingly, *FH* gene suppression correlates with very poor prognosis[5,139,155]. Furthermore, FH is found to be suppressed in a large subset of patients with ccRCC, which correlates with EMT and poor prognosis[139]. Therefore, identifying the pervasive sequalae of fumarate accumulation in these tumours can be utilised as a nidus for the development of more effective and targeted therapies that are required for the management of *FH*-deficient RCC.

Succinate—Besides the inhibitory role of succinate against αKGDDs shared with fumarate and 2HG, succinate also exhibits distinct oncometabolite features that may impact on the phenotype of *SDH*-deficient tumours. Activation of the succinate receptor GPR91 by high levels of succinate has been shown to upregulate angiogenic proteins including VEGF in a HIF-independent manner in hypoxic retinal ganglion cells of rodents[115], and induce an angiogenic phenotype in human endothelial cells *in vitro* and in transgenic zebrafish *in vivo*[115,175]. Activation of this succinate/GPR91 signalling axis may also be an important pathway in tumour angiogenesis[115,175] and highlights the ability of succinate to exhibit hormone-like traits. Through activation of the GPR91 pathway, elevated circulating levels of succinate have been implicated in renovascular hypertension via activation of the renin-angiotensin-system (RAS) in kidneys[176,177]. Interestingly, hyperglycaemia was also found to trigger this pathway, potentially implicating it in the pathophysiology of diabetic nephropathy[178]. The succinate/GPR91 signalling axis has also been implicated in the pathological hypertrophy of ischaemic cardiomyocytes[179] and activation of fibrosis in ischaemic-induced liver damage[180].

Succinate, similar to fumarate, has been linked to a role in the post-translational protein modification known as protein succinylation (different from fumarate-induced *succination*)[181–183]. However, as it has been observed that succinylation results from succinyl-CoA reacting with the lysine residues in proteins[182,183], it is likely that succinate accumulation in *SDH*-deficiency, which can equilibrate with succinyl-CoA, is the underlying mechanism behind this link[182]. Interestingly, the accumulation of D2HG competitively inhibits SDH activity in *IDH1*-mutant fibrosarcoma cells, causing a hypersuccinylated phenotype and apoptosis resistance[184], two established hallmarks of cancer[24]. Re-expression of the desuccinylase SIRT5, as well as glycine supplementation led to reversal of this hypersuccinylated phenotype and slowed oncogenic growth *in vitro*[184]. Mechanistically, glycine depletes the availability of succinyl-CoA to succinylate proteins, by condensing directly with succinyl-CoA to form 5-aminoevuilinc acid which enters the haem biosynthesis pathway[184]. Remarkably, type 2 pRCC tumours with *FH*-mutations were also found to be hypersuccinylated compared to *FH*-wildtype RCC[184], demonstrating the likely convergence of oncometabolites onto this process. In addition, several key metabolic enzymes such as MDH and IDH2, as well as histones[185] are found to be targets of protein succinylation[182,186], possibly suggesting an autoregulatory role in metabolism and perturbation of the cellular epigenome, however these functional effects are yet to be fully elucidated[133,186,187]. Lastly, SDH-deficient cells have also been identified to exhibit dependency on pyruvate carboxylase (PC) to funnel pyruvate into the truncated TCA cycle for aspartate biosynthesis, an important precursor in sustaining cellular growth[188,189]. Furthermore, silencing PC expression attenuated SDH-deficient tumour growth *in vivo* in a mouse model[188]. This coupled with an increased mRNA expression of *PC* in a range of human SDH-deficient tumours including PC protein expression in SDH-deficient RCC highlights a potential target for synthetic lethality in SDH-deficient RCC[188].

As highlighted earlier, although SDH-deficient renal tumours represent a rare (0.2% of all RCCs) and recently recognised distinct RCC subtype (WHO 2016 Classification), overall it is a highly aggressive tumour with a younger onset of disease (mean age 37- 46years) with

the majority of tumours likely to harbour *SDHB* mutations (82%), although mutations in all four subunits and the assembly factor SDHAF2 have been implicated as tumour suppressor genes in the pathogenesis of RCC[79,83,84,190–192]. Several common features in keeping with HLRCC renal tumours (highly aggressive, younger onset) makes SDH-deficient RCC as challenging to manage[82,191]. Although within the remit of ccRCC, increased gene expression of SDHB, SDHC, SDHD have been correlated with better patient survival outcomes[10]. In a similar manner, elucidating the common and individual sequalae of succinate accumulation in the setting of RCC will form the basis for future strategies in targeting this cohort.

2-hydroxyglutarate—The recent discovery and elucidation of L2HG accumulation in ccRCC[99,101] highlights the relevance of distinguishing the tumorigenic role of this oncometabolite. As discussed, 2HG exists in two isoforms (L2HG/D2HG), produced by distinct biological mechanisms that are differentially upregulated in cancers and are found to exhibit distinct features beyond αKGDD enzyme inhibition[59,67]. Interestingly, studies of 2HG in leukaemic cells has yielded conflicting results. In multiple *IDH*-wildtype leukaemic cell lines, dose-dependent inhibition of cell proliferation and viability were demonstrated upon addition of D2HG[193]. However, exogenous D2HG added at a comparable concentration in similar *IDH*-wildtype cells demonstrated a contrasting phenotype of cell proliferation and leukaemic transformation in another study[105]. This disparity has been partly attributed to the discrepancy in *in vitro* conditions used in these experiments[193]. However, in support of an anti-tumoural effect, D2HG accumulation (either exogenous addition or endogenously through *IDH* mutation) demonstrated attenuated progression of the disease and increased survival in an *in vivo* xenograft model of leukaemia[193]. Mechanistically, D2HG competitively inhibits the fat mass and obesity-associated protein (FTO)[193], the first identified mRNA demethylase and member of the αKGDD family[193,194], which in turn downmodulates the expression of targeted genes, such as *MYC, RARA and ASB2*, normally involved in promoting cell growth and transformation[193,195]. Interestingly, these findings were also recapitulated in glioma cells as well as with exogenous L2HG[193], suggesting a convergence in function of these 2HG isoforms as well as in phenotypic effects across multiple cancers. Furthermore, direct inhibition of ATP synthase and subsequent downregulation of mTOR signalling by D2HG accumulation in *IDH1*-mutant glioma cells *in vitro* and *in vivo* suggest growth suppressive functions of D2HG and further corroborates the anti-tumoural effects of D2HG[196]. This phenomenon may partially marry up the correlation between *IDH*-mutations in gliomas and improved patient prognosis[196,197].

One rationale for the convincing simultaneous pro- and anti-tumoural roles of 2HG, is that its effects are contingent on the specific cancer and/or specific stage in tumour evolution (i.e. tumour initiation versus tumour progression)[193,196]. Supporting this notion, accumulations of D2HG and L2HG were observed in colorectal cell lines in the absence of *IDH or D-/L2HGDH* mutations[198]. Dissecting their individual roles in this setting revealed that D2HG, but not L2HG, was found to have pro-tumoural roles in EMT gene upregulation through KDM inhibition and subsequent histone hypermethylation, as well as in the acquisition of invasive and migratory phenotypes in these cells[198]. Furthermore, this phenotype was ameliorated by the addition of a glutaminase inhibitor[198], signifying that D-/L2HG

accumulation in this context is dependent on glutamine-derived anaplerotic flux. In keeping with these findings, colorectal cancer specimens demonstrated elevated D2HG levels, with increased levels of D2HG correlated with higher frequency of distant metastases[198]. Ascertaining the IC_{50} levels for these isoforms within this context may add insight into this disparity between D-/L2HG, which as discussed, shows variation between oncometabolites, target enzymes and experimental conditions[66,70,118,138,142]. Overall, these studies highlight that both 2HG isoforms can converge on a range of non-metabolic functions such as DNA/histone hypermethylation[70,101,110,142,148], however also potentially diverges in respects to the pro- and anti-tumoural effects as well as the 2HG isoform implicated[101,102,105,110,112,193,195,196,198]. Thus, it would be imperative for future studies elucidating the role of 2HG isoforms in tumours and the potential clinical applications associated, to be cancer and tumour stage specific.

Clinical applications of oncometabolites in RCC

Unravelling the oncogenic identity of a small group of seemingly innocuous metabolites has given rise to the oncometabolite paradigm, whereby aberrant accumulations of member metabolites have demonstrated potent pro-oncogenic capabilities that impact on tumorigenesis, tumour phenotypes and progression. Naturally, a number of potential clinical applications utilising oncometabolites has surfaced as a result. This section highlights the areas in which oncometabolites may have a role in clinical practice (Fig. 3), particularly in regard to the management of RCC.

Novel therapeutic targets

Multiple targets within oncometabolite-associated pathways for therapeutic intervention have been highlighted. These can broadly be divided into targeting oncometabolite accumulation i.e. production and/or degradation pathways, or targeting the downstream sequalae either in the broad sense e.g. DNA hypomethylation agents[149], or specific pathways e.g. arginine deprivation in *FH*-deficient RCC[93,199].

Targeting oncometabolite accumulation—Targeting the pathways that contribute to oncometabolite accumulation has shown promising results to date with evidence of translation into clinical practice[200]. The development of specific mutant-IDH1/2 inhibitors demonstrating reduction in D2HG levels with reversal of the DNA/histone hypermethylation profile and phenotypic reversal of the cellular differentiation block in leukaemia in pre-clinical studies is one such example[105,201–203]. Further supported by clinical studies[200,204], this effort has resulted in the recent approval for their use in the management of *IDH*-mutant acute myeloid leukaemia[200].

Another rapidly emerging area of interest is the use of glutaminase inhibitors in oncology. Cancer cells are long recognised to rely on glutamine as an essential fuel source and biosynthetic precursor to support the demands of rapid growth, survival and stress in cancer cells[171]. Glutamine has several fates, however the conversion of glutamine to αKG as an anaplerotic source is of particular relevance, in which the first step from glutamine to glutamate is catalysed by the enzyme glutaminase[171]. In addition, cancers with defective mitochondria such as *FH*- and *SDH*-deficient RCC, predominantly utilise glutamine-derived

αKG in a reductive carboxylation manner (reversal of the canonical TCA cycle flow, Fig. 1), allowing these cells to bypass the truncated TCA cycle and replenish essential TCA cycle intermediates such as citrate, which is cleaved to form acetyl-coenzyme A for lipid biosynthesis[170,171]. In addition, glutamine-derived αKG appears to be the dominant source for 2HG production in several cancer subtypes including breast [205], chondrosarcomas [206], colorectal cancer , and RCC [101], as well as being the main source of fumarate in *FH*-deficient RCC cells[170,171]. As highlighted, L2HG is significantly accumulated in human ccRCC tissues[99], partly attributable to the LOH of the *L2HGDH* gene[99] as well as the promiscuous activity of MDH2 on predominantly glutamine-derived αKG[101]. Targeting the 'production' pathway of 2HG accumulation i.e. glutamine/MDH2 axis via pharmacological or genetic inhibition resulted in significantly reduced L2HG levels and suppression of the migratory phenotype in multiple RCC cell lines with restoration of epigenetic TET activity, as evidenced by elevated DNA 5hmc levels *in vitro*[101]. Moreover, glutaminase inhibition *in vivo* demonstrated suppression of tumour growth, adding to the evidence base that targeting glutamine in this setting profoundly affects L2HG accumulation with suppression of tumour phenotype. Of note, several other independent studies have investigated glutaminase inhibition in the wider context of RCC, including in *VHL*-mutant and *VHL*-wildtype RCC, also demonstrating tumour growth suppression *in vivo*[15,207,208]. These studies have facilitated the translation of glutaminase inhibitors into several phase 1/2 clinical studies either as monotherapy or in combination with approved therapeutic agents and have included *FH*- and *SDH-deficient* RCC subtypes, as well as in several metastatic RCC cohorts with early promising results[209–211]. Although it is highly unlikely that the effects of glutaminase inhibition in RCC are purely mediated through L2HG (as not all RCC tumours accumulate L2HG), given that the loss of *L2HGDH* and accumulation of L2HG confers a worse prognosis in patients with ccRCC[101] and concurrently, upregulation of the glutamine transporter correlates with aggressive ccRCC and worse prognosis[10], it would be of immense value to ascertain whether there is crossover talk, given the ability of L2HG to significantly modulate the epigenetic cell state, as well as determine whether glutaminase inhibition has a more profound effect in L2HG-associated RCC tumours given the oncometabolite gamut of capabilities. In addition to glutamine/MDH2 axis inhibition, genetic restoration of *L2HGDH* also demonstrated suppression of tumour growth *in vivo*[101]. These findings highlight several vulnerabilities on both sides of L2HG accumulation that can be exploited for the development of targeted therapies in L2HG-associated RCC. Establishing whether use of multiple approaches to reduce L2HG levels have a synergistic effect or not may impact on strategic management of this subset of RCC tumours. Of note, given that L2HG has no known physiological role[58–61], specific targeting of L2HGDH may be preferable over MDH2 given that MDH has a physiological role and targeting this enzyme may lead to undesirable systemic effects.

Targeting downstream oncometabolite-sequelae—Potential therapeutic strategies have been developed to tackle both broad oncometabolite-induced pathways as well as cancer-specific liabilities, that is, oncometabolite-associated phenomena observed in specific cancer types (Fig. 3). The general convergence of oncometabolites on the inhibition of αKGDD enzymes led to an early and straightforward strategy of overcoming competitive inhibition by administering αKG in excess. Studies in *SDH*-deficient cancer cells and in

RCC cells treated with exogenous fumarate, administration of αKG lead to a reversal of the HIF pseudohypoxic drive through restoration of PHD activity[212,213] as well as reversal of DNA 5mc accumulation, indicative of TET activity restoration[137]. Dose-dependent suppression of HIF1α and VEGF protein levels by αKG were also observed in lung cancer cells and in hepatocellular carcinoma cells *in vitro*[214,215]. Furthermore, αKG administered to human colorectal cancer cells under hypoxic conditions led to PHD-induced destabilisation of HIF expression and furthermore, exhibited PHD-dependent hypoxic cell death[216]. In corroboration, αKG exhibited anti-tumoural effects *in vivo*, suppressing tumour growth and angiogenesis in a lung cancer xenograft model[214]. Overall, these preclinical studies suggest that utilising αKG, in a wide variety of cancer subtypes, can meaningfully reverse oncometabolite-induced αKGDD inhibition at a molecular and phenotypic level. As epigenetic dysregulation and pseudohypoxia drive are strongly implicated in the pathogenesis and progression of RCC, broad targeting of αKGDD combating both these elements, warrants further investigation in this setting. In a similar vein, targeting the HIF transcription factors, drivers of the mutual pseudohypoxic phenotype observed in oncometabolite-associated RCC subtypes, may be another promising therapeutic approach in this setting. Attenuation of tumour growth upon HIF2α inhibition was demonstrated *in vitro* and in multiple RCC tumourgraft models[217,218]. These studies helped lay the foundation for the first human studies and clinical trials using HIF2α inhibition in patients with locally advanced and metastatic ccRCC with promising early results demonstrating two-thirds of patients having complete/partial response or stable disease with HIF2α inhibition[217,219]. Given the robust capability of oncometabolites to induce the HIF-signalling pathway independent of VHL-deficient RCC, these studies will prove insightful in the management strategies for targeting rare but aggressive oncometabolite-associated RCCs.

The development of DNA hypomethylation agents, also known as DNA methyltransferase (DNMT) inhibitors for clinical practice, such as 5-azacitidine, have demonstrated to improve outcomes and delay transformation in patients with high-risk myelodysplastic syndrome[220,221]. As highlighted, the hypermethylation phenotype is a characteristic trait amongst oncometabolite-associated tumours and DNMT inhibitors have demonstrated potential in ameliorating these associated phenotypes[137,149,222]. Studies using low doses of DNMT inhibitors have demonstrated impairment in cell growth, reversal of the migratory phenotype and restoration of cell differentiation in a range of *SDH*-knockout and *IDH1/2*-mutant cells *in vitro*[137,149,222], which is further evidenced by reversal of DNA methylation marks[222]. Furthermore, decitabine (a derivative of 5-azacitidine) demonstrated tumour growth suppression in *IDH1*-mutant glioma cells *in vivo*[222]. Whilst these studies provide a potential strategy for targeting DNA-related oncometabolite-induced epigenetic modifications, both DNA and histone methylation have a role in modulating transcriptional activity and therefore simultaneous targeting of multiple epigenetic modifiers may prove to be more strategic[66]. Of note, high doses of decitabine induced cytotoxicity in all cells[137], therefore careful characterisation of the desired therapeutic window will also be important for future studies.

The recent elucidation of the role of L2HG in RCC epigenetic dysregulation has added more insight into this disease process and potential therapeutic strategies. Interrogation of the

epigenetic effects demonstrated elevated levels of the trimethylated histone H3K27Me3 which corresponded with reduced levels of DNA 5hmc suggesting L2HG-induced KDM inhibition in RCC[101]. In conjunction, lowering L2HG levels in these cells leads to the re-expression of *H3K27Me3* target genes as well as polycomb repressor complex 2 (*PRC2*) target genes, which encodes a histone methyltransferase responsible for the repressive trimethylation of H327Me3[101,223]. Inhibition of PRC2 via knockdown of the PRC2 catalytic subunit, enhancer of zeste homologue 2 gene, in RCC cells with high 2HG-levels, resulted in reduced H3K27Me3 levels as well as reduced migratory abilities[101]. Furthermore, knockdown of *KDM6A*, a known H3K27 demethylase, in *L2HGDH*-wildtype RCC phenocopied the enhanced migratory properties of elevated L2HG-RCC cells, implicating *KDM6A* as a specific target for L2HG in RCC[101]. Interestingly, mutations, predominantly somatic, in *KDM6A* (also referred to as *UTX*) have been identified in renal cancer [224,225], suggesting that chromatin remodelling via oncometabolites may recapitulate the effects of other epigenetic modifiers mutated in RCC. In other words, oncometabolites and chromatic modifiers may converge towards the same gene signature. Due to the identification of mutations in epigenetic regulators, such as KDM6A/UTX in renal cancer[224,225], several studies have investigated the effects of DNMT inhibitors in renal cancer with encouraging results demonstrating growth inhibition[226] in *VHL*-mutant and -wildtype RCC cell lines. In addition, re-expression of silenced genes was observed in a dose-dependent manner with DNMT inhibition in several RCC cell lines[226,227], and moreover, re-expression of interferon (IFN) response genes in RCC cells via reversal of the gene silencing methylation by DNMT inhibitors augmented interferon-induced apoptosis *in vitro*[228]. In an early clinical study, combined DNMT inhibition and interferon therapy has demonstrated potential efficacy in the setting of metastatic RCC[229]. Overall, these studies demonstrate that targeting epigenetic modifiers in RCC has evidence of anti-tumoural effects that may also potentiate and synergise with other adjunctive therapies such as interferon therapy. Given that oncometabolites and other mutated epigenetic modifiers in RCC may converge towards the same gene signature, these studies are especially relevant to therapeutic tactics for targeting aggressive oncometabolite-associated RCC diseases.

The discovery of individual oncometabolite properties permits the development of novel therapeutic methods to manipulate these pathways for amelioration of pro-tumoural effects. As highlighted throughout this review, there are numerous targets and strategies that are oncometabolite- and cancer-specific, that provides the basis for further translational studies. **Table 1** provides a summary of the potential therapeutic targets for oncometabolite-associated RCC subtypes as discussed in this review. Whilst not novel, targeting the modifiable exogenous factors implicated in oncometabolite accumulation may ameliorate pro-tumoural effects of oncometabolites, particularly given the examples of dose-dependent effects on oncometabolite-induced downstream pathways highlighted this review. Although not fully elucidated, hyperglycaemic-induced oncometabolite production has the most compelling evidence in eliciting phenotypes analogous to those observed in oncometabolite-associated cancer cells[124–126,164] and thus studying the role of antidiabetic therapies such as metformin, may be of interest in oncometabolite-associated tumours. Of note, metformin is currently in oncological clinical trials in patients with breast and prostate cancer, although

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the data is challenging, it has demonstrated a degree of anti-tumoural activity with recognised roles in modulating numerous metabolic pathways [39,230].

Biomarkers of disease

Oncometabolite-associated metabolic imaging—Oncometabolites accumulate to millimolar levels in the tissue. The specific accumulation of these metabolites could be exploited to detect tumour masses using multiple metabolic imaging modalities. One recent advance has been in the development of hyperpolarised magnetic resonance imaging (hpMRI)[231,232]. Administration of isotopically labelled ^{13}C-glutamine in an *IDH1/2*-mutant chondrosarcoma xenograft mouse model with hpMRI enables visualisation of glutamine conversion to 2HG in real-time[206]. More strikingly, hpMRI was able to capture the suppression of 2HG accumulation in response to IDH inhibition[206]. A similar study performed recently in a ccRCC xenograft model utilised labelled ^{13}C-pyruvate to visualise the metabolic response of the glycolytic flux to lactate in response to mTOR inhibitors[233]. Capitalising on the unique oncometabolite properties in RCC, hpMRI has multiple potential applications. It can facilitate the diagnoses of oncometabolite-associated RCC subtypes such as L2HG-associated RCC, whilst concomitantly conferring prognostic information as well i.e. L2HG is associated with poorer patient prognosis and tumour progression[101]. Furthermore, dynamic assessment of oncometabolite levels in this setting could be used as biomarkers of therapeutic efficacy, and by elucidating the IC_{50} for the oncometabolites and αKGDDs[70,118,138,142] implicated in RCC, hpMRI could provide a means of monitoring progression or recurrence of the disease. In the wider context, given the basis of RCC as a metabolic disease process with extensive metabolic reprogramming associated with tumour progression, utilising hpMRI alongside selective tracers to identify malignant metabolic signatures, would facilitate more robust diagnoses of small and/or indeterminate renal lesions. The largely non-invasive and safe, non-ionising radioactive nature of hpMRI makes this a very attractive tool for development and translation into clinical practice. Similarly, other imaging modalities may also prove valuable in the diagnosis and management of oncometabolite-associated RCC, such as proton magnetic resonance spectroscopy (1H-MRS) and Positron Emission Tomography (PET) imaging. Successful detection using 1H-MRS of the oncometabolites succinate in patients with a variety of SDH-deficient tumours[234,235], and 2HG in IDH-mutant gliomas[236–238] has seen transition of 1H-MRS into clinical practice including in disease monitoring of IDH-mutant gliomas[237]. Although 1H-MRS has been explored in the setting of RCC patients to assess the general metabolic profile[239], capitalising on the knowledge that the majority of oncometabolites have been implicated in RCC provides strong evidence for further investigation and may hold promise for patients with rare and aggressive RCC subtypes such as in SDH-deficiency. In addition, a recent pre-clinical study capitalising on glutamine reliance in several RCC subtypes, have demonstrated the ability to dynamically assess ccRCC metabolism *in vivo* using PET imaging with the radiotracer ^{18}F-(2S,4R)4-fluoroglutamine (18F-FGln)[207]. In particular, this may also be a potential method of diagnosing and staging RCCs as well as stratifying patients that are likely to respond to glutaminase inhibition[207].

Optimising surgical oncology—Oncometabolite-associated biomarkers may also prove indispensable for optimising surgical oncology. Intraoperative mass spectrometry of the

oncometabolite 2HG has been used to guide brain tumour resections with promising results[240]. Identifying the presence of oncometabolites at tumour resection margins or "molecular margins" identifies the presence of tumour cells, thus providing a straightforward guide for the need for further resections[240]. More so, it provides the metabolite information within minutes and concurrently yields relevant information about genotype, tumour classification and potentially prognosis[240]. With multiple oncometabolites implicated in numerous RCC subtypes including in ccRCC[99,101] and partial nephrectomies the gold-standard treatment for localised RCC[241], utilising these methods may help optimise surgical margins in patients with oncometabolite-associated RCC undergoing partial nephrectomies. This may prove to be of great benefit as positive surgical margins have been demonstrated to correlate with tumour recurrence[242]. Furthermore, given the highly aggressive phenotypes of *FH*- and *SDH*-deficient RCCs in which a significant proportion present with bilateral disease, utilising intraoperative mass spectrometry concurrently may assist in meticulous tumour resections to help preserve renal function in these patients.

Cancer-specific oncometabolite-associated biomarkers—Finally, unique oncometabolite properties such as post-translational modifications of proteins and metabolic rewiring can be exploited for use as diagnostic or prognostic biomarkers. Capitalising on the ability of fumarate to induce protein succination (2SC), detection of the distinct 2SC protein modification signature on immunohistochemistry or cyst staining signifies fumarate accumulation and has been corroborated to be a robust diagnostic biomarker of FH deficiency, such as in HLRCC patients with ramifications for genetic testing [126,243]. In addition, metabolomic analyses of urine from *Fh1*-deficient mice and growth media of *FH*-deficient cells revealed consistently elevated levels of argininosuccinate as a result of fumarate-induced reversal of ASL activity, raising its potential as a urinary biomarker for the early detection of *FH*-deficient renal cancer[93]. Although this biomarker awaits validation, the non-invasive nature of sampling, the specificity to *FH*-deficiency metabolism and the straightforward detection methods make this an exciting and attractive diagnostic biomarker.

Conclusion

Although in its infancy, the oncometabolite paradigm has been gathering momentum over the last decade with a firm movement away from the traditional view of metabolism as a simple by-product of genetic perturbations that occur in cancer. A growing body of evidence has substantiated the roles of a small group of seemingly innocuous metabolites that when aberrantly accumulated are transformed into oncometabolites that possess a plethora of capabilities that can contribute to tumorigenesis and tumour progression. Given that RCC is an established metabolic disease process, it is of no surprise that multiple oncometabolites are implicated in renal cancer. In general, oncometabolites in RCC exert significant effects on chromatin remodelling and epigenetic dysregulation leading to characteristic hypermethylated phenotypes, inducing an EMT phenotypic switch and the propagation of a pseudohypoxic signature contributing to the aggressive features of these RCC subtypes. Furthermore, by elucidating the roles of oncometabolites, it permits the exploitation of these molecules and their associated signalling pathways for multiple clinical applications such as the development of novel targets or as biomarkers of disease.

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Key points

- Oncometabolites are aberrantly accumulated metabolites that possess pro-oncogenic capabilities that contribute to tumorigenesis via epigenetic dysregulation and can influence tumour progression through phenotypic switches such as EMT.
- L-2HG, fumarate and succinate are *bona fide* RCC oncometabolites. Exploitation of these oncometabolites and their downstream signalling effects are attractive targets for novel therapies and as biomarkers of disease.
- Chromatin remodelling via oncometabolites may recapitulate the effects of other epigenetic modifiers mutated in RCC thus converging on the same gene signature. Identification of these pathways involved will influence treatment strategy.
- Elucidation of the exogenous factors that give rise to oncometabolite production such as hyperglycaemia may prove to be a synergistic strategy in reducing oncometabolite levels and their subsequent sequelae.

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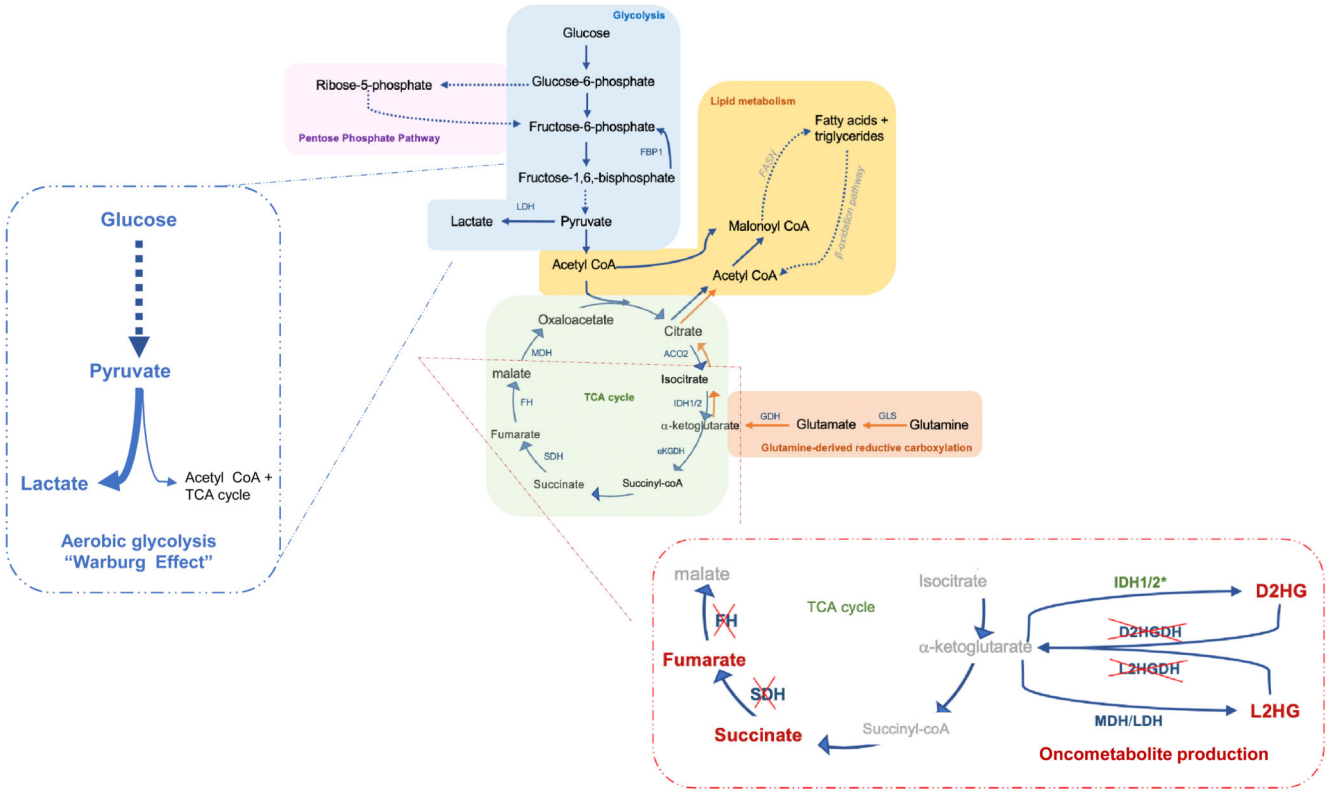

Figure 1. A simplified overview of key metabolic pathways studied in cancer metabolism, including the oncometabolite production pathways.

The key pathways are as summarised. **Glycolysis,** a series of pathways involved in glucose catabolism to pyruvate yielding intermediates for entry into PPP, lactate fermentation ('anaerobic glycolysis'), TCA cycle and lipid synthesis. In oncology, the **'Warburg effect'** (aerobic glycolysis) describes the upregulation of glycolysis observed in many cancers even in the presence of oxygen. *In ccRCC, upregulation of glycolysis correlates with poor prognostic outcomes in patient*[10]. **Pentose Phosphate Pathway (PPP),** is a branched pathway from glycolysis, provides reducing equivalents (NADPH) and precursors for nucleotide synthesis (building block of DNA/RNA). *Upregulation of PPP correlates with aggressive ccRCC and poor prognostic outcomes in patient*[10]. The **TCA cycle is a** series of reactions that fully oxidise carbohydrates, lipids and proteins and generates reducing equivalents (NADH) for the electron transport chain to generate ATP. TCA cycle intermediates provides a source of precursors for lipids and amino acid biosynthesis. Anaplerosis is the process of replenishing the TCA cycle intermediates. *Downregulation of TCA cycle genes correlates with aggressive ccRCC and poor prognostic outcomes in patients*[10]**. Lipid Metabolism** pathways, lipid synthesis is required for energy stores and synthesis of cell membrane components whereas lipid degradation (β-oxidation) is required for release of energy stores. *Upregulation of fatty acid synthesis correlates with aggressive ccRCC and poor prognostic outcomes in patients*[10]. **Glutamine-derived reductive carboxylation**, glutamine is metabolised to α-ketoglutarate for entry into the TCA cycle in a reversed flow of the canonical TCA cycle (orange arrows), citrate can be extracted for lipid synthesis. This is an essential metabolic pathway that supports the growth of cancer cells

with mitochondrial defects such as *FH*-deficient RCC[178]. **Oncometabolite production pathways**, loss-of-function mutations in *FH* and *SDH* genes encoding the respective TCA cycle enzymes lead to an accumulation of fumarate and succinate. 2HG exists in two isoforms (D2HG/L2HG). D2HG is accumulated by gain-of-function neomorphic activity of *IDH* enzymes. L2HG is accumulated by promiscuous activity of MDH/LDH activity. Loss-of-function of the enzymes D-/L2HGDH which catalyse the oxidation of D-/L2HG to α-ketoglutarate also result in the accumulation of D2HG and L2HG respectively.

Abbreviations: αKGDH- α-ketoglutarate dehydrogenase, ACO2 – aconitase, CoA – coenzyme A, ATP- adenosine triphosphate, ccRCC – clear cell RCC, D2HG – D-2-hydroxyglutarate, D2HGDH – D-2HG dehydrogenase, FBP1 – fructose-1,6-bisphosphatase 1, FASN - fatty acid synthesis, FH- fumarate hydratase, GDH – glutamate dehydrogenase, GLS – glutaminase, IDH- isocitrate dehydrogenase, L2HG – L-2-hydroxyglutarate, L2HGDH – L2HG dehydrogenase, LDH – lactate dehydrogenase, MDH- malate dehydrogenase, RCC- renal cell carcinoma, SDH- succinate dehydrogenase, TCA – tricarboxylic acid

Key: Dashed arrows = multiple enzyme-catalysed reactions between these two metabolites, * = mutant form of enzyme, red crosses = loss-of-function

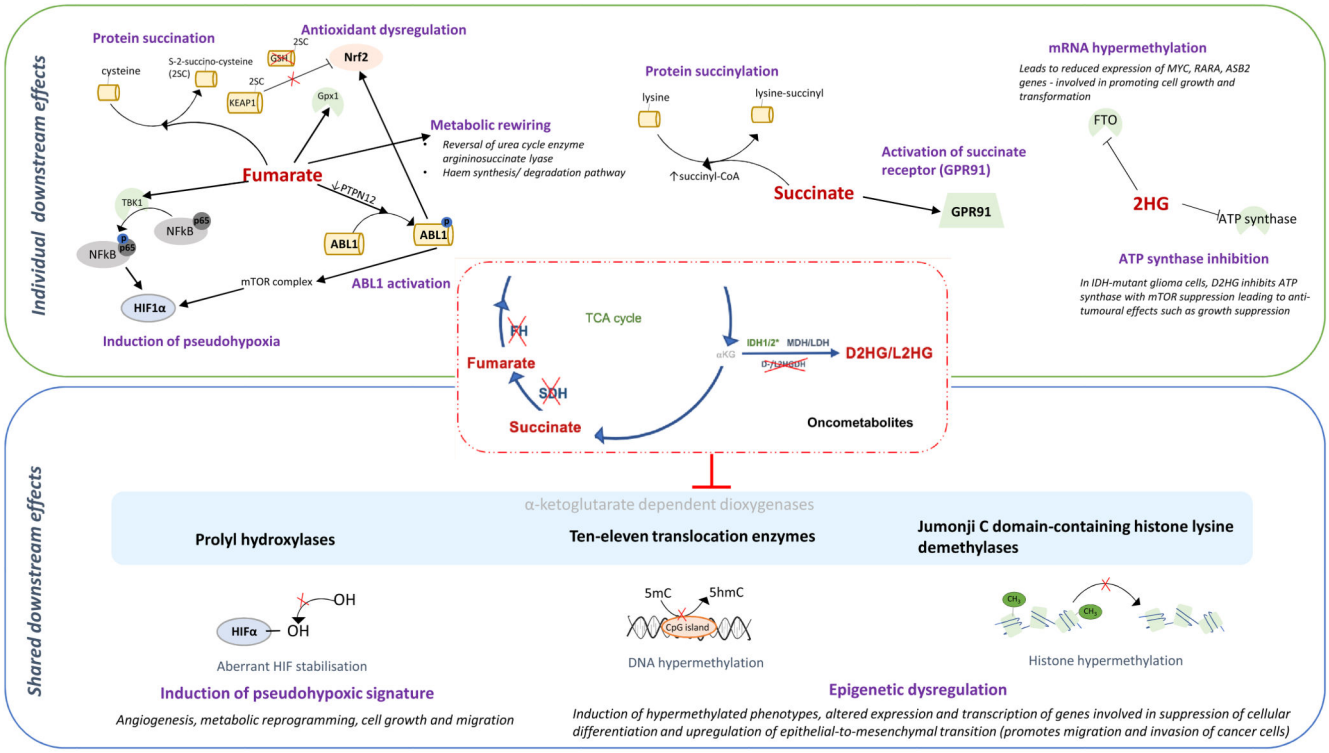

Figure 2. Summary of the shared and individual oncometabolite downstream signalling pathways.

The oncometabolites fumarate, succinate, and 2HG and their production pathways are highlighted (red box). These oncometabolites converge on shared downstream effects through the inhibition of α-ketoglutarate dependent dioxygenases (bottom blue box). These oncometabolites also exhibit distinct, divergent downstream effects, which are summarised for each oncometabolite (top green box).

Abbreviations: αKG – alpha-ketoglutarate, 2SC – S-2-succino-cysteine, 5hmc -5-hydoxymethylcytosine, 5mc-methylcytosine, ABL1 - Abelson Murine Leukaemia viral oncogene homolog 1, CpG – cytosine-guanosine dinucleotide, D2HG – D-2-hydroxyglutarate, D2HGDH – D-2HG dehydrogenase, FH- fumarate hydratase, FTO – fat mass and obesity-associated protein, GLS – glutaminase, GPR91 – G-protein coupled receptor 91, Gpx1 – glutathione peroxidase 1, HIF- hypoxia-inducible factors, IDH- isocitrate dehydrogenase, KEAP1 = Kelch-like ECH-associated protein-1, KDM- histone demethylases, L2HG – L-2-hydroxyglutarate, L2HGDH – L2HG dehydrogenase, LDH – lactate dehydrogenase, MDH- malate dehydrogenase, mRNA -messenger RNA, mTOR – mammalian target of rapamycin, MYC - NFkB – nuclear factor kappa-light-chain-enhancer of activated B cells, Nrf2 -Nuclear factor (erythroid-derived 2)-like 2, OH – hydroxyl group, P – phosphorylated, PHD- prolyl hydroxylases, PTPN12 - protein tyrosine phosphatase non-receptor type 12, RCC- renal cell carcinoma, SDH- succinate dehydrogenase, TCA – tricarboxylic acid, TKB1- tank-binding kinase 1, TET – ten-eleven translocation enzymes, VEGF – vascular endothelial growth factor

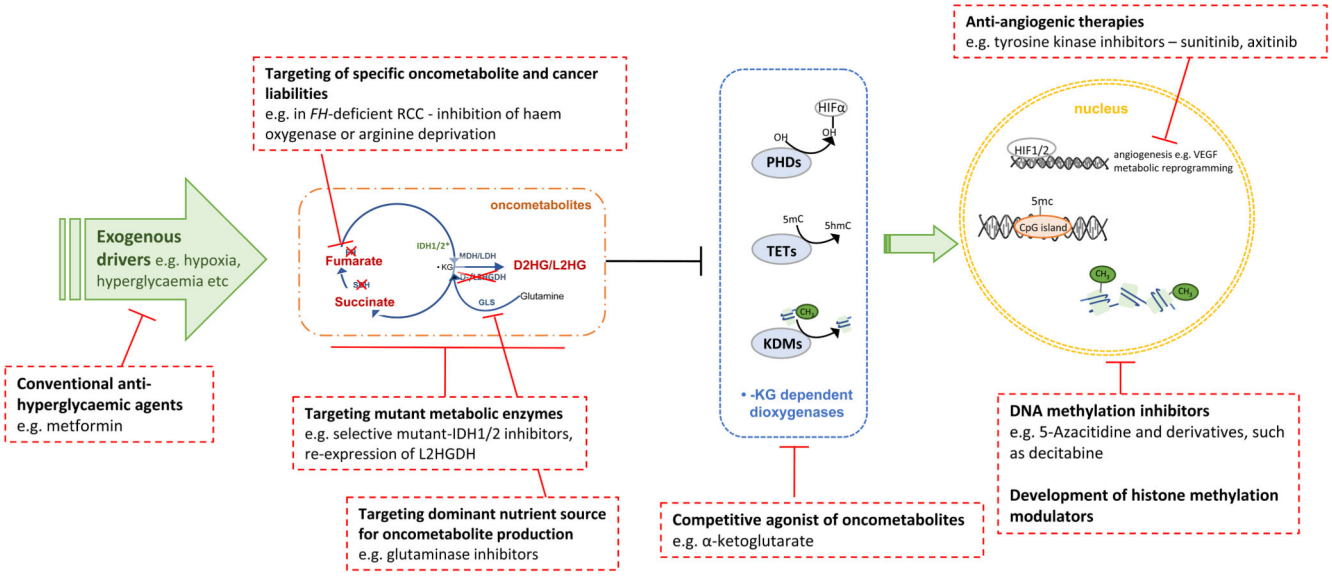

Figure 3. Overview of oncometabolite-associated pathways highlighting potential therapeutic targets.

This schematic provides an overview of the different stages in which oncometabolites can be exploited for therapeutic intervention, also highlighting the opportunity for multimodal or multi-layered synergistic approaches. This ranges from targeting the exogenous drivers of oncometabolite production (green arrow), to the nutrient sources and enzymatic perturbations involved in oncometabolite accumulation (red box), to the downstream enzymatic, epigenetic and phenotypic phenomena (blue and yellow boxes).

Abbreviations: αKG – alpha-ketoglutarate, 5hmc -5-hydoxymethylcytosine, 5mc-methylcytosine, CpG – cytosine-guanosine dinucleotide, D2HG – D-2-hydroxyglutarate, D2HGDH – D-2HG dehydrogenase, FH- fumarate hydratase, GLS – glutaminase, HIF-hypoxia-inducible factors, IDH- isocitrate dehydrogenase, KDM- histone demethylases, L2HG – L-2-hydroxyglutarate, L2HGDH – L2HG dehydrogenase, LDH – lactate dehydrogenase, MDH- malate dehydrogenase, OH – hydroxyl group, PHD- prolyl hydroxylases, RCC- renal cell carcinoma, SDH- succinate dehydrogenase, TET – ten-eleven translocation enzymes, VEGF – vascular endothelial growth factor

Key: Red dashed boxes = therapeutic strategies, * = mutant form of enzyme, red crosses = loss-of-function

Table 1

Oncometabolite-associated RCC subtypes, clinical features and potential therapeutic strategies

Oncometabolite	Gene mutation	Clinical features	Potential therapeutic strategies	References
Fumarate	FH (tumour suppressor)	HLRCC-associated RCC (14-18% develop pRCC) Highly aggressive and early metastasis Early onset Bilateral Mainly papillary but also described as solid, tubulocystic, cribiform or cystic	Arginine deprivation Haem oxygenase inhibition ABL1 inactivation Targeting TBK1/p65 axis GDH1 inhibition Glutaminase inhibition	4,16,26,87,93,156,169,174,199,245
Succinate	SDHA SDHB (82%) SDHC SDHD SDHAF2 (tumour suppressors)	SDH-deficient RCC (0.2% of all RCC) Early onset (mean age 37-46yo) Associated aggressive phenotype Bilateral RCC (26%) Associated with paraganglioma (25%)	SIRT expression Exogenous glycine PC inhibition	4,77–79,83,84,184,188,190–192,246–249
L2HG	L2HGDH (tumour suppressor)	ccRCC Associated with Wilms' tumour	L2HGDH re-expression Glutaminase inhibition MDH2 inhibition	64,99,101

Abbreviations: ABL1 - Abelson Murine Leukaemia viral oncogene homolog 1, ccRCC – clear cell RCC, FH- fumarate hydratase, GDH – glutamate dehydrogenase, HLRCC – Hereditary Leiomyomatosis and Renal Cell Cancer, L2HG – L-2-hydroxyglutarate, L2HGDH – L2HG dehydrogenase, MDH2 – malate dehydrogenase 2, PC – pyruvate carboxylase, pRCC – papillary RCC, RCC- renal cell carcinoma, SDHAF- succinate dehydrogenase assembly factor, SDH- succinate dehydrogenase, SIRT - silent mating type information regulation 2 homolog, TKB1- tank-binding kinase 1