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**Fuhrman Nuclear Grade-defined Multiomic Characterisation of Clear Cell Renal Cell
Carcinoma**

By

TATSUTO ISHMARU

DISSERTATION

Submitted in partial satisfaction of the requirements for the degree of

DOCTOR OF PHILOSOPHY

in

Cancer Biology and Bioinformatics

in the

OFFICE OF GRADUATE STUDIES

of the

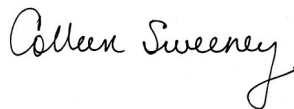
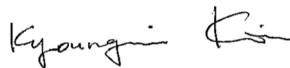
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Table of Contents

Title page	i
Acknowledgement.....	ii
Table of Contents	iii
 Abstract	 1
 Chapter 1 : Introduction	 2
Clear Cell Renal Cell Carcinoma	2
Fuhrman Nuclear Grades and the History of RCC classification	5
Cancer Chemotherapy and Alternative Therapeutics Against ccRCC	10
Cancer Biomarkers	15
Cancer Bioinformatics and Personalised Medicine	18
Proteomics	20
Metabolomics	23
Protein Moonlighting	30
Research Objectives	35
References	37
 Chapter 2 : VHL status dictates the response of Renal cell carcinoma (RCC) to Cyclin Dependent Kinase (CDK) inhibitor Roscovitine <i>in vitro</i>	 48
Abstract	48
Introduction	49
Materials and Methods	50
Results	52
Discussion	58
References	66
Figures	68
 Chapter 3 : Fuhrman nuclear Grade-defined ccRCC proteome	 84
Abstract	84
Introduction	84
Materials and Methods	89
Results	94
Discussion	121
Summary	144
References	146
Figures and tables	158
 Chapter 4 : Effects of Argininosuccinate Synthetase (ASS1) silencing on ccRCC metabolic reprogramming and morphology	 199
Abstract	199
Introduction	200

Materials and Methods	202
Results	205
Discussion	223
References	238
Figures and tables	243

Chapter 5 : Novel Immunohistochemical classification of clear cell renal cell carcinoma exploiting the grade-dependent metabolic reprogramming by retargeting of moonlighting proteins	288
Abstract	288
Introduction	288
Materials and Methods	291
Results	292
Discussion	298
References	303
Figures	307

Chapter 6 : Conclusion	314
References	322
Figure	323

ABSTRACT

Kidney cancer or renal cell carcinoma (RCC) is the 6th most common type of cancer in men and the 9th common cancer in women in the United States and claims nearly 15,000 lives annually. 75% of total RCC cases are clear-cell renal cell carcinoma (ccRCC) which is refractory to the conventional chemotherapy and there is currently no effective cure against ccRCC except for surgical removal. While Fuhrman nuclear grading has been widely used as a prognostic tool for ccRCC, its subjective nature hinders precise and reliable patient-specific prognosis. The overarching aim of this dissertation is to characterize the molecular components of Fuhrman nuclear grades to understand the molecular underpinning of ccRCC chemoresistance and to identify proteomic and metabolomic signatures of ccRCC that can be harnessed to establish novel diagnostic tools complimentary to the Fuhrman nuclear grading and to identify potential novel grade-defined pharmacological targets. This dissertation first aims to characterize the molecular mechanisms behind the ccRCC chemoresistance using Roscovitine (Seliciclib) as an example of conventional chemotherapy via cyclin dependent kinase (CDK) inhibition. Second, Fuhrman nuclear grade-defined proteomes are characterised to identify a set of proteins that can be used as diagnostic biomarkers and grade-specific pharmacological ccRCC targets. Third, the potential role of Argininosuccinate Synthetase (ASS1), whose expression was suppressed across all Fuhrman grades, in ccRCC pathogenesis is investigated. Additionally, in order to establish novel diagnostic criteria, this study aims to characterize the grade-dependent subcellular localization of novel ccRCC biomarker proteins that are found to behave as moonlighting proteins.

CHAPTER 1: INTRODUCTION

1.1 Clear cell Renal Cell Carcinoma

Renal cell carcinoma (RCC) is currently the 6th most common cancer in men and the 10th in women in the United States. RCC can be classified into several subtypes, and the underlying molecular mechanisms and mortality vary significantly among them. [1] Of all the RCC subtypes, clear cell Renal cell carcinoma (ccRCC) comprises a majority of the RCC cases in the US and worldwide, accounting for approximately 75% of the total cases. The remaining 25% includes chromophobe RCC, papillary RCC, tuberous sclerosis complex, and hereditary leiomyomatosis / RCC (HLRCC). These subtypes are discussed at the end of this section.

There is a preponderance of cases in males than females. [2] Additionally, in the US, there is a racial predilection for Hispanics and Native Americans amongst the ccRCC cases, [3] which suggests genetic, epigenetic, and environmental factors as well as socioeconomic determinants behind the disease. This implies that ccRCC presents as a synthetic manifestation of endogenous factors and the well-documented threat of health inequity. [4-6] In addition to the sporadic ccRCC, there are familial syndromes whose symptoms include RCC. The most widely documented example is von Hippel Lindau (VHL) disease. [7] VHL disease patients carry one inactivating germline mutation, therefore somatic one-hit on the other VHL allele is sufficient to render the patient VHL-null. As a result, ccRCC exhibits high HIF activity [8] and defective p53-dependent apoptosis. [9, 10]

Since its first description by Miril in 1810, several attempts have been made to classify RCC into subtypes first according to its appearance [11] and later by mutations, [1] Currently, RCC are classified into several subtypes including clear cell RCC (ccRCC), papillary RCC, and chromophobe RCC (chRCC) based upon histological characteristics as revealed by conventional histological staining. [1, 12] ccRCC exhibits its characteristic clear cytoplasm due to accumulation of cytoplasmic lipid and polysaccharide content. [13] On the other hand, papillary RCC consists of cuboidal mono-columnar cells with solid cytoplasm, and chromophobe RCC shows non-clear cytoplasm with pleomorphic nuclei. [14] As mentioned above, familial cases of ccRCC such as seen in VHL disease exhibit biallelic inactivation of *VHL* gene. Similarly, the majority of sporadic ccRCC cases lack functional VHL genes due to germline inactivation and somatic mutation or two somatic mutations. VHL functions as the E3 ubiquitin ligase that recognizes HIF α subunits and targets them to polyubiquitination and subsequent proteasomal degradation. [15] VHL is a HIF α -specific E3 ubiquitin ligase, and its biallelic inactivation leads to accumulation of HIF α protein and the consequent sustained activation of VEGF signalling, which leads to the extensive neovascularization commonly seen in ccRCC. In addition to its cardinal function as the E3 ubiquitin ligase, VHL functions as a chaperone for p53 to increase its stability and pro-apoptotic activity. Thus, conventional chemotherapy is of limited efficacy in the case of ccRCC whose VHL is inactivated and hence exhibits reduced apoptotic induction. [9, 10]

ccRCC is refractory to conventional chemotherapy due to inactivation of von Hippel Lindau (*VHL*) gene as exemplified by the familial von Hippel Lindau disease whose patient is born with monoallelic VHL inactivation due to mutations. A postnatal mutation in the second copy of VHL in the sister chromosome makes the cell VHL null or hypomorph. The resultant loss

of VHL, a cofactor and stabiliser of p53, renders the cell incapable of apoptotic induction in case of genotoxic insults. [9, 10] Consequently, accumulation of further mutations in the absence of apoptosis is thought to underlie the ccRCC induction and development.

Papillary RCC (pRCC) was first reported by Hirsch in 1918, [16, 17] and comprises 15 to 20% of all RCC incidence. [1] They originate from the renal pelvis and are characterised by their papillary morphology and avascularity, which contrasts themselves with ccRCC. [1, 18] They can be classified into two types; basophilic (Type 1) and eosinophilic (Type 2) based on their histological characteristics. The latter has cytoplasm that is stained with eosin while basophilic type 1 is not stained. Type 1 carries mutations in the *MET* gene while Type 2 chromophobe RCC harbours mutations in such genes as *CDKN2A* and *SETD1*. [1, 19] For pre-metastatic disease, pRCC has better prognostic outcomes compared with ccRCC, although there is no difference when metastasis is involved. [20]

Chromophobe RCC, originally described by Thoenes and others in 1985, [21] is thought to originate from the distal nephron, [22, 23] and makes up approximately 5% of all RCC cases. As the name suggests, these RCCs have cytoplasm that are only lightly stained by the conventional histological staining methods such as hematoxylin & eosin.

Tuberous sclerosis arises from an autosomal dominant mutation of *TSC1/2* genes, which are classical tumour suppressor genes in the mTOR pathway. [24] Under physiological conditions, the mTOR pathway integrates cell division and nutrient availability. Inactivating mutations of *TSC1/2* genes in tuberous sclerosis patients lead to uncontrolled cell division regardless of the nutrient status. [25] Although the majority of the renal manifestations of

tuberous sclerosis are renal cysts and angiomyolipoma, up to 4% of the patients develop RCC including ccRCC. [26]

Hereditary leiomyomatosis and renal cell carcinoma (HLRCC) is a rare familial autosomal dominant tumour-predisposition syndrome where heterozygous mutation in fumarate hydratase (FH) results in cancers originating from multiple organs including the kidney, skin, and uterus. [27] There are approximately 300 families reported who are affected by this syndrome. This type of RCC also often shows papillary morphology. [1] Biallelic inactivation of FH results in fumarate accumulation, which in turn activates HIF1 α signalling and NRF2 transcriptional activity. NRF2 target genes are involved in redox regulation, detoxification, and metabolism such as fatty acid synthesis and heme degradation. [28] Consequently, NRF2 activation results in reduced iron metabolism such as ferroptosis. [29] These changes in turn aid cancer cell survival and tumour growth.

HLRCC harbours an autosomal dominant mutation in fumarate hydratase, which results in sustained NRF2 activation. [30] In addition, deficiency in FH activity results in an increase in mitochondrial fumarate, its substrate, which causes modifications in mitochondrial DNA metabolism enzymes, faulty DNA replication, and eventual mitochondrial loss, resulting in cells relying on glycolysis for ATP production, which is a hallmark of aerobic glycolysis or the Warburg effect. [31]

1.2 Fuhrman Nuclear Grades and the history of RCC classification

RCC is a heterogeneous cancer [1], and as such, has attracted a number of attempts to classify tumours further into subtypes over the past century since it was first described as a lipoma by Grawitz in 1883. [32] Like many other cancers, two families of classification schemes were devised over the years. The staging system, such as TNM staging classifies RCC according to the spread of the cancer with respect to its origin. [33,34] In the case of the TNM system, Stage 1 RCC is confined to the kidney, while Stage 3 sees invasion of perinephric tissues and Stage 4 RCC would have metastasised beyond the Gerota's fascia. On the other hand, RCC grades are defined solely by cytological features of RCC.

These grading schemes aimed to facilitate the RCC diagnosis, prognosis, and management. A number of groups have proposed easy-to-use ccRCC grading systems. In this context, the word “grading” is used to refer to classification systems based on any morphological features of RCC. [35 – 45, 48] In addition, grading criteria for other types of RCC have recently been put forward. [14] These grading criteria employed the cutting-edge technology of the time and the available knowledge about RCC to classify it in order to develop better prognostic and therapeutic approaches. Hand and Broders (1932) used percentage degree of cellular differentiation and gross morphology to classify 193 cases into four grades where the lowest grade exhibited 70 to 100% differentiation while the highest grade showed 0 to 25% differentiation. [35] Melcow (1944) employed location such as pelvic, parenchymal and capsular renal carcinoma, and morphology such as papillary and non-papillary renal carcinoma to classify 199 cases. Each of the location categories were further sub-categorised. This is the first report to mention clear cell carcinoma and suggest the tubule system as its origin. [36] Foot and Humphreys used the origin of RCC to classify them into four groups, and further

subdivided each category by morphology. [37] Griffiths and Thackray (1949) classified 103 RCC cases into three grades based on histological criteria such as the degree of papillary or adenomatous formation, cellular irregularity, mitotic frequency, and the degree of hyperchromatic or darker nuclei. This is one of the first attempts to include nuclear features in an RCC grading system. [38] Murphy and Fishbein, on the other hand, classified 90 cases into RCC into clear type and granular type using a variety of staining including hematoxylin and eosin. [46] Claes examined 92 RCC cases and classified them into clear cell-type, plasmic cell-type, and intermediary cell-type, of which clear cell types were associated with lower mortality. In addition, high erythrocyte sedimentation rate (ESR), which is positively correlated with inflammation, was associated with poor prognosis. [47] Arner and colleagues studied 197 renal adenocarcinoma and proposed a four-tier malignancy grading system based on the degree of differentiation and macroscopic demarcation, which showed good correlation with postoperative survival. [40] Interestingly, the majority of the clear-cell type were in Grade 3.

Böttinger proposed three-class RCC grades defined by degree of differentiation, in which the second grade was further divided into two classes based on the degree of circumscription, which may correlate with the degree of intactness of the tumour tissue extracellular matrix. [41] In addition, the author notes that high ESR without metastasis correlates with a favourable prognosis including high rate of survival, although metastasis itself was indicative of poor prognosis. [41] Through study on 309 cases, Skinner and colleagues proposed a four-grade system in which the grades were defined solely by nuclear morphology with the grade 1 being indistinguishable from the normal cells while the grade 4 RCC contained many “bizarre giant nuclei”. [48] Thus, Skinner’s grading system is similar to that proposed by Fuhrman twelve years

later, although it lacks the semi-quantitative nature of the criteria adapted by Fuhrman and colleagues. This apparent lack of precision may in part arise from the inclusion of the granular types of RCC. Amtrup and colleagues attempted to first classify 76 RCC according to their prognosis, with 8 year-survival cut-offs with operation and unoperated groups, and investigated the tumours' cell type, presence or absence of necrosis, and structure. [42] Surprisingly, while they found the correlation between cell type and prognosis as well as between necrosis and prognosis, no correlation was found between structure and prognosis. This may be due to the small sample size and cytological heterogeneity within each group. In comparison of four grading systems, however, nuclear architecture was found to have better prognostic capacity than histologic type of tumour growth or Arner's malignancy grading. [49]

In the backdrop of the aforementioned studies, Fuhrman and colleagues examined 103 RCC cases and first classified them into three group based on cell types; clear cells (ccRCC), dark (granular cells, and mixture of the two types in which the minority comprised at least 25% of the total cells. Subsequently, they established a four-grade system of ccRCC classification based on nuclear features. That is, low grade RCC exhibited small, round and regular nucleus with no nucleoli detectable under a high magnification light microscope. Their diameters were approximately 10µm. On the other hand, the higher grades showed larger nucleus with prominent nuclei detectable under a low magnification. Grade 4 RCC, which is the highest grade, exhibited pleiomorphic and multi-lobular nuclei with heavy chromatin clumps. Additionally, Fuhrman and colleagues noted that the grade 4 tumours often contained spindle-shaped sarcomatoid cells. Intermediate two grades had a slightly larger (15mm) nuclei with irregular outline and nuclei detectable under high magnification (Grade 2) and an even larger

nuclei and nucleoli detectable under low magnification with more irregular outline (Grade 3), respectively, which suggests gradual changes in nuclear envelope and nuclear lamina architecture and molecular compositions. This grading system requires conventional hematoxylin eosin staining and a light microscope both of which are available in a standard medical laboratory. Additionally, inclusion of semi-quantitative criteria such as numerically defined nuclear size and nucleolar size allowed a degree of standardisation of classification across laboratories. These features made the Fuhrman nuclear grading the most prevalent ccRCC grading system over the past 40 years.

Since publication of the Fuhrman nuclear grading, several groups have attempted to revise the Fuhrman grading as well as to devise a novel grading scheme. Letourneux and colleagues argues that Fuhrman grading is unnecessarily and unverifiably detailed. [45] Rosenkrantz et al. (2010) used Letourneux grading to suggest that diffusion coefficient may be used to distinguish low and high grade ccRCC. [50] Computer-assisted imaging is becoming more utilised. [51] More recent classifications have used MRI combined with machine learning. [52] As seen above, morphological criteria have been widely used in RCC classification and prognosis with varied degree of success over the past century. Such criteria, however, contain a significant degree of subjectivity. Furthermore, the observations based on them are not amenable to direct clinical translation such as chemotherapy which first requires molecular target identification. Although grade-defined and stratified large-scale statistical studies can suggest effective approaches, such studies often lack their mechanistic underpinnings. Over the past three decades, chemometric approaches such as proteomics and metabolomics have made sufficient advances to allow unequivocal identification of polypeptides and other

metabolites. [53,54] This in turn allowed characterization of complex biochemical systems of hitherto uncharacterizable complexity such as cells and tissues, leading to biomarker discovery at an unprecedented speed. These chemometric technologies have successfully been applied to the ccRCC research, as discussed in Chapter 2 and 4.

1.3 Cancer Chemotherapy and Alternative Therapeutics Against ccRCC

Rate of cell division is one of the characteristics that defines a cell type and must be tightly regulated. For instance, colorectal epithelial cell progenitors maintain a high rate of cell division to replace damaged epithelial cells. On the other hand, terminally differentiated neurons do not undergo further cell division. Therefore, there are cell type specific mechanisms that define the rate of cell division. [55, 56] When these mechanisms fail, cells divide uncontrollably through sustained proliferative signalling, which is a hallmark of cancer. [57]

Exploitation of this hallmark of cancer has led to the development of a number of chemotherapeutic agents. [58] A major type of chemotherapy relies on the fact that the high rate of proliferation imposes upon the cancer with a need to increase its rate of anabolism such as DNA synthesis. DNA chelating or cross-linking agents such as platinum-based alkylating agents including cisplatin, carboplatin, and oxaliplatin fall under this category. [59] Anthracyclines such as doxorubicin inhibits proliferation by inhibiting topoisomerase 2. [60] Both classes of chemotherapeutic agents interfere with the DNA synthesis, thus preventing the transition through the G2 checkpoint. In contrast, there are the synthetic cyclin-dependent

kinase (CDK) inhibitors such as Dinaciclib and R-Roscovetine (seliciclib) [61] This class of chemotherapeutic agents inhibit CDKs and prevent the cells from going through the cell cycle. Ultimately, all classes of chemotherapeutic agents are designed to induce cell death through apoptosis selectively in the cancer cells.

In addition, chemotherapeutic approaches targeted at the hyperangiogenic nature of ccRCC has been attempted with limited success. [62, 63] These include VEGF inhibitors such as Sunitinib [64] and sorafenib. [65] They function as kinase inhibitors with moderate specificity that can target VEGFR and PDGFR as well as c-Raf. [66, 67] Examples of mTOR inhibitors include temsirolimus and everolimus. [68, 69] VHL directly interacts with RAPTOR, an mTOR pathway effector. [70] This leads to Raptor polyubiquitination and degradation and the subsequent mTORC1 signalling downregulation. Conversely, in ccRCC, VHL inactivation leads to mTOR hyperactivation. These mTOR inhibitors have been used against ccRCC although their overall response rate remained low in monotherapy. [71] While individual uses of these chemotherapeutic agents against ccRCC have seen only limited successes, [72] combinatorial approach using mTOR and VEGF inhibitors have seen improved therapeutic outcome. [73]

Although many chemotherapeutic agents have been successfully used against a limited range of cancers, ccRCC has proven resistant to such conventional chemotherapeutic reagents. ccRCC has thus far remained resistant because of its defective apoptotic pathway due to its VHL-negative status, which renders it p53-hypomorph. [9] Chemotherapy has shown significant cytotoxic and therefore therapeutic effects against a large range of cancers, yet many types of cancers remain out of reach of the existing chemotherapy. Furthermore, it is not without side effects. To circumvent them, recent attempts included cancer immunotherapy. [74] There are

several distinct approaches including those based on PD-1/PD-L1 pathway [75, 76], natural killer (NK) cells, [77] and Chimaeric Antigen Receptor (CAR)-T cells. [78]

The discovery of PD-1 in 1992 opened a new era of cancer research aiming to educate the activated CD4+ and CD+ T-cells and B cells to target the cancer cells [79] Cancers upregulates PD-L1 expression, thus activating the immune checkpoint via PD-1 activation. This suppresses the immune activation, effectively creating immune-privileged region around and within the cancer tissue. Attempts have been made to disengage system against many types of cancers, [80, 81] although an effective anti-PD-L1 therapy against ccRCC remains elusive. [82]

NK cells comprise another promising platform for immunotherapy. Originally discovered in 1975, [83] They are cytotoxic and cytokine-producing lymphocytes and orchestrate the late stage of immune response via a large set of activating and inhibitory receptors. [84] For example, LAIR1-Collagen interaction and KLRG-Cadherin interaction inhibit NK cell activation, while β 2-Integrin (CD11-CD18)-ICAM interaction and α 4 β 1 integrin-VCAM1 interaction activate NK cells. [84] Through combination of these interactions, NK cells recognise cancer cells and, upon activation, induce cytotoxicity specifically in the targeting tumour cells. [85] This characteristics is well suited for therapeutics against solid cancers such ccRCC.

CAR-T cells comprise the third category of cancer immunotherapy. Unlike NK cells, CAR-T cells are genetically engineered by introducing a chimaeric antigen receptor (CAR) which is constructed such that it recognises a target-cell specific antigen. The CAR has both the extracellular immunoglobulin-derived antigen recognition domain as well as T-cell receptor-derived intracellular activation domain. [86] Given the mutation-driven basis of cancer

pathogenesis, CAR-T cells have long been expected to be a novel and effective cancer therapy. [78] As of March 2022, there are six instances of FDA-approved CAR-T therapies, although none of them target a solid tumour such as RCC. Recently, efforts have been made to integrate the CAR-T cell approach with the NK cell-based cancer therapy by creating CAR-NK cells, which reduced ovarian cancer xenograft growth in mice. [87]

Cancer is characterised by a high rate of uncontrolled proliferation. Such pathological proliferation comes with increased needs for anabolism such as DNA, lipid, and protein syntheses. Thus, metabolic reprogramming is another hallmark of cancer. [88-90] Cancer metabolic reprogramming is multimodal and occurs at different points along its development.

Aerobic glycolysis, also known as the Warburg effect when considered in the context of cancer, is a classic example of such metabolic reprogramming. Cancer cells increase its dependency on glycolysis for ATP production despite growing under an oxygen-replete condition by apparently foregoing more efficient ATP synthesis through mitochondrial oxidative phosphorylation. This apparent incongruity has long remained a mystery, but recent research has suggested that such dependency on glycolysis can shuttle metabolic intermediates such as aspartate and pyruvate to anabolic processes such as DNA synthesis, amino acid synthesis, and lipid synthesis. Although the mechanisms underlying the initiation of the Warburg effect is not yet completely elucidated, in hereditary leiomyomatosis and renal cell carcinoma (HLRCC), inactivating mutations in fumarate hydratase, a TCA cycle that converts fumarate into malate, results in fumarate accumulation in mitochondria. Accumulated fumarate modifies mitochondrial DNA modification enzymes, which causes failed DNA replication and eventual mitochondrial dysfunction, leading to increased glycolytic dependence of HLRCC cells. [31]

More recently, a number of groups have taken advantage of the altered cancer metabolism to devise a therapy that would selectively target the cancer cells. Arginine depletion is such an example. In humans, arginine is a semi-essential amino acid; it is an essential amino acid for growing children and for adults in recovery from an injury or an illness. Although humans can synthesise the amino acid through the urea cycle, the body's demand for the amino acid exceeds its synthesis during the period of high growth and regeneration when it must be supplemented through dietary means. [91] A wide range of cancers are known to silence Argininosuccinate Synthetase (ASS1), the rate-determining enzyme in the urea cycle, through epigenetic mechanisms or harbour inactivating biallelic mutation in the gene, thus making them arginine auxotroph. Many groups have taken advantage of this to deplete arginine in the media through the use of pegylated arginine deiminase (ADI-PEG20) against a wide range of cancers and other proliferative diseases with varying degree of success, [92-96] although arginine deprivation may also stunt T-cell activation and curtail cancer immunosurveillance. [97] The tumorigenic function of ASS1 silencing in ccRCC has at least in part be ascribed to altered nitrogen metabolism by directing aspartate to pyrimidine synthesis and reducing nitric oxide (NO) synthesis. [98]

Lipids encompass a vast group of structurally diverse molecules. In addition to its classical role as the primary component of biological membranes, it serves as components of signal transduction pathways and source of ATP when sugars are scarce. These modalities of the lipid metabolism are also reprogrammed in cancer. [99] The omic approaches have identified significant differences in fatty acid compositions including their chain length and

saturation, which is reflected in sphingolipid structural differences. [99] In addition, phosphatidylglycerol with monounsaturated fatty acid is elevated in RCC. [100, 101]. While lipidome-targeted cancer therapy is still under development, a number of candidates have emerged, [99] and several drugs are specifically developed against RCC including A939572 which targets Stearoyl-CoA Desaturase-1 (SCD1) and Sphingomab against Sphingosine-1-Phosphate. [102, 103] Given cytosolic lipid droplets are a cardinal anatomical feature of ccRCC, lipid metabolic reprogramming underlying their development is likely to hold clues in developing novel therapeutics against ccRCC. In particular, mammalian Target of Rapamycin (mTOR) pathway and Peroxisome Proliferator-Activated Receptor Alpha (PPAR α) control expression of many lipogenic enzymes, [104] and offer attractive pharmaceutical targets. It should, however, be noted that structurally diverse lipid molecules are likely to contribute to diverse areas of ccRCC pathobiology, and precise assignment of pathobiological functions to each class of lipids is paramount.

As described above, metabolic reprogramming gives growth advantages to the tumours, it concomitantly creates tumour specific metabolic and cell biological signatures. That is, in adopting to its high proliferation characteristics, cancer cells have become reliant on metabolic and signalling pathways that are less used under physiological conditions, which can be exploited as chemotherapeutic targets.

1.4 Cancer Biomarkers

Cancer is a complex and constantly changing pathology. Therefore, simple and clear criteria for diagnosis and prognosis is vital. Traditional cancer diagnoses often relied on accidental discovery during routine examinations. In many cases, the cancer had already advanced to a stage that is often inoperable nor incurable. Once diagnosed, the physicians rely on further characterisation of the tumour to devise the most effective treatment options. Historically, cancer stages and grades have played important roles in prognosis. The staging takes into account the spread of cancer. On the other hand, grading often was done based on the cancer's cellular anatomical features. In the recent decades, molecular biomarkers have become increasingly available. For example, breast cancers can be classified by presence or absence of Estrogen Receptor (ER) or human Epidermal Growth Factor Receptor 2 (HER2) among other immunoreactivities. [105] On the other hand, thus far there is no viable biomarkers for many other types of cancers including glioblastoma [106] and ccRCC. [107]

Biomarkers can be defined as easy to obtain indicators of biological specimens that can be used to classify samples. [108] More specifically, biomarkers are often defined as molecules whose presence or absence characterised or indicated a physiological or pathological condition.[109] In the twentieth century, most of the cancer biomarkers relied on their morphological as well as positional characteristics. For example, ccRCC can be characterised in terms of stages, which are defined by the location and thus spread of the tumour. TNM staging system is a universal cancer staging system that is widely used. The lower stage cancers are confined to the primary site, while the high stage cancers had metastasised past the capsule and to distant sites. [33, 34] On the other hand, as mentioned above, nuclear grades can be assigned based on the nuclear morphology upon specific conventional staining. Fuhrman

nuclear grades, devised by Susan Fuhrman and her colleagues in 1982 is the most widely used biomarkers for ccRCC treatment and prognosis. [43] The Fuhrman nuclear grading system is based solely on the nuclear morphology upon standard haematoxylin and eosin (H&E) staining and comprises 4 grades. Grade 1 consists of small round nuclei with no visually detectable nucleolus. Grade 2 ccRCC exhibits nuclei of varying size, although larger than grade 1. The nuclear outlines are slightly irregular with nucleoli that are detectable only at high magnifications. Grade 3 ccRCC contains large nuclei with hyperchromasia and nucleoli that are visible to low magnification. Grade 4 ccRCC exhibits pleiomorphic multi-lobuled nuclei with chromatin clumping and highly conspicuous nucleoli. The Fuhrman nuclear grades, however, are often subjective as the grading depends on the condition of the specimens such as the extent of fixation. Thus, it left the field of the ccRCC researchers in want of a more objective set of biomarkers.

The technical development in omics over the past several decades equipped the cancer researchers with novel chemometric approaches that allowed unbiased and quantitative approaches to describe cells and tissues at molecular details. Although not without its own shortcomings, this has opened a new avenue of identifying and quantifying molecular cancer biomarkers. [See Section 1.6: Proteomics, Section 1.7: Metabolomics, and Chapters 3 and 4] These mass spectrometric approaches can easily be applied to a wide variety of sample forms from gases to liquids to solids. Our group and others have taken advantage of this characteristics of omics to analyse fresh frozen ccRCC samples, formalin-fixed paraffin-embedded (FFPE) ccRCC samples and patient serum and urines to identify novel biomarkers and dysregulated biochemical pathways. [98, 110 – 117]

1.5 Cancer Bioinformatics and personalised medicine

Organisms are complex and dynamic systems. While this complexity often presents to us in the form of the beauty of the Nature, it has also challenged scientists and clinicians in search of accurate diagnosis and effective treatments and cures with roadblocks and setbacks.

For most of the past two hundred years, scientific advances were fundamentally made through reductionism, which poses that complex systems such as organisms can be understood by first reducing its complexity and looking at their components, which are inherently simpler and thus easier to characterise and understand than the system as a whole. The system as a whole, in turn, can be understood by summation of the information obtained through studying each component. Therefore, scientists attempting to understand complex and hitherto unexplained phenomena used the scientific reductionistic approach coupled with descriptive and experimental methods has often been the method of choice for the biologists. These include molecular biology, gene expression analysis, and RNAi to name but a few. Each of these approaches were sparked with technical advancements in a discipline distinct from biology yet with implications for biology. Bioinformatics is no exception.

Bioinformatics, a term first coined in 1970 by Paulien Hogeweg and Ben Hesper [118] and defined as the study of informatics processes in biotic systems, [119] integrates biology with computer science and statistics in order to analyse big data that are produced through state-of-the-art technologies such as DNA, RNA, and protein sequencing and chemometrics. [120] Biological systems are inherently complex systems. The human genome is estimated to

contain approximately 30,000 genes, which can produce proteome of further complexity through such processes as alternative splicing and post-translational modification.

Furthermore, proteins can form larger biomolecular complexes whose biological functions depend on their subunit compositions. Each cell type in an organism contains a unique set of proteins through a defined gene expression pattern and protein profile. Advent of mass DNA and RNA sequencing has led to characterisation of the complex and diverse nature of the genome [121, 122] and the corresponding transcriptome of a number of species. [123, 124]

This, in combination with the development of mass spectrometry, [53] has led to the development of proteomics. [125] Furthermore, many proteins are enzymes which catalyse biochemical reactions. Consequently, the change in proteomic profile suggests that metabolites are in constant flux in a dynamic biological system such as cells, tissues, and organisms. This has led to the application of mass spectrometry-based chemometric approach to study metabolomes [126]

Over the past decade, the maturation in each modality of omic research has led to more integrated multi-omic approaches, which encompasses genomics, epigenomics, transcriptomics, proteomics, metabolomics, and fluxomics needs to be studied to have more comprehensive understanding of physiological and pathological states. [127]

Overall, these omics and bioinformatics studies synergised with more traditional reductionistic approaches to revealed cellular heterogeneity within cancers. Furthermore, bioinformatic approaches confirmed the prior findings that the previously known pathological changes to fundamentally contributed to disease progression such as cancer including kidney cancer [128, 195] as well as diabetes and cardiovascular diseases. [129]

Further improvement in detection sensitivity has allowed researchers to study multiomes at the single-cell and organellar resolutions, which

significantly contributed to our understanding of tissue architecture and dynamics such as cancer heterogeneity. [130]

As discussed above, omics approach opened the avenue to study individual organism rather than a population. Personalised medicine or individualised medicine is built on the findings that, although individuals within a species share significant similarities, the genome is significantly different between the individuals. Furthermore, an individual can undergo time-dependent dynamic changes in response to environmental factors such as diurnal changes and seasonal changes. These differences contribute to change in disease susceptibility and outcome. Thus, the personalised medicine contrasts in its approach to that by the traditional statistical population-based medicine.

1.6 Proteomics

Organisms are complex yet highly and dynamically organised mixture of biomolecules including proteins, and pathogenesis is often a result of change in the biomolecule composition. The human genome encodes at least 18,000 genes, many of which encode multiple protein isoforms through alternative splicing and alternative translation. Therefore, our understanding of any biological phenomenon relies on the chemical identification and cataloguing of complex protein mixtures, a chemometric approach known as proteomics.

Proteome is defined as the collection of proteins within a system such as cells, tissues, or organisms, and is a portmanteau between 'protein' and a Greek word 'ome', the latter of which refers to a collection of entities with common characteristics. The word itself was

derived from genome, or the collection of genes within an organism. [131] Proteomics is the study of proteome. Proteomics aims to catalogue the entire proteins present within a system. It also studies how the proteome functions within a biological system through the interplay of its constituent proteins in order to achieve the physiological functions. Similarly, a disease proteome characterises the differences in the proteome in comparison to the physiological state. It can shed light into the pathobiology of the disease and contribute to novel therapies and treatments. [132] Since its inception in the 1980s [53] The technology has contributed extensively to our understanding of biological systems. [54] Applications include cancer biology [133, 134] and COVID-19 pathology and therapeutics. [135]

Mass spectrometry had been successfully used to identify and quantify small organic compounds, but its application to study proteome had been limited due to the large size of the biomolecules which would decompose during the vaporisation and ionisation processes prior to the mass spectrometric analysis. The breakthrough came when matrix-assisted laser desorption/ionization (MALDI) method was established to protect the biomolecules during ionisation of high-molecular weight organic molecules such as proteins and polypeptides. The ionised fragments are subsequently separated in a magnetic field and are individually detected. The location of the detection is determined by the time-of-flight (TOF) method, where mass-to-charge ratio (m/z) is determined by the distance a fragment travels in a defined magnetic field. Since mass and charge can be calculated for any polypeptides or proteins of known amino acid sequence, the detected peptides can be identified. Once the genome is sequenced, any encoded protein sequence can be deduced for any organisms including human and mice.

Proteomics has been used extensively over the past three decades and contributed to major advances in our understanding of complex biological systems. In its nascent period, it relied on two-dimensional (2D) gel electrophoresis to separate protein mixtures. Proteins were visualised as a “spot” in the 2D-gel, which was subsequently extracted and identified by MALDI-TOF analysis [110, 136] While this was a significant step forward in studying the complex protein mixtures such as cells and tissues, only high abundance proteins, which could be visually detected on the 2D-gel and comprise a small fraction of proteins in the samples, were extracted, analysed, and identified. This resulted in a frustratingly shallow description of the proteomes.

Proteomic approaches can be roughly classified into two categories: shotgun proteomics and targeted proteomics. In the current study, shotgun proteomics was used to analyse the grade-defined ccRCC proteomes along with normal kidney cortex proteome. Shotgun proteomics was named after shotgun DNA sequencing, which was developed several years earlier, where a mixture of overlapping DNA fragments is sequenced in parallel, [137] which can be applied to such complex genomes as that of human [138] The shotgun proteomics, in contrast to the targeted proteomics, is not suitable to quantify protein of interests, but is suited to create a catalogue of the entire proteome being studied. In addition, by counting spectrum associated with the protein or the polypeptide, semi-quantitative analyses may be performed. Single liquid chromatography (LC) or multidimensional LC (LC/LC) followed by tandem mass spectrometry (MS/MS) followed by database search. [139] This multidimensional protein identification technology (MudPIT) can be applied to analyse complex protein mixtures such as tissue extracts. [140] Additionally, a protease of known enzymatic specificity can be used to

reduce the size of the polypeptides in the mixture. Since the digestion locations can be computationally predicted, the m/z for the digestion products can be calculated. Assuming no mutation, the sequence of each proteolytic fragment can be computationally deduced. In the current study (Chapter 3), trypsin was used. Trypsin cleaves polypeptide chain at the peptide bond after the carboxyl group of positively charged amino acids, namely arginine and lysine. Finally, presence and quantity of a protein is measured by counting the number of proteolytic fragments belonging to each protein. This method requires pre-established database of m/z . A peptide needs a unique m/z to be unambiguously identified. In other words, to be identified, a protein needs at least one proteolytic fragment with a unique m/z . [141] In addition, it relied on technological advances such as thin capillary columns with diameters in 10-100 μ m. [142]

While a very powerful technique, shotgun proteomics comes with its own shortcomings: first, the dynamic range of the current mass spectrometers is too narrow to cover all the proteins and peptide fragment species present. [143] Second, the peptide database may be incomplete. Furthermore, the peptide databases often do not cover potential mutant proteins. Lastly, the databases may not cover all naturally existing post-translational modifications. [144] These technical limitations hinder complete description of the proteomes.

1.7 Metabolomics

Biological systems such as organisms and cells are complex biochemical beings. Therefore, in order to comprehensively understand the pathophysiology of an organism such as development and cancer, a comprehensive understanding of the biochemical reactions that

occurs, or fails to occur, in the biological system is paramount. This in turn implies that characterisation of the comprehensive list of reactants and products is necessary to understand the biological system in question. Approximately 10% of the human genome are predicted to encode enzymes which catalyse biochemical reactions. [145] Each cell type has a unique metabolic signature which often results in their unique anatomical features. For example, brown adipose cells accumulate lipid droplets in their cytoplasm. On the other hand, under pathological conditions, the physiological metabolic network is rewired. For instance, the majority of cancers are known to rewire its metabolic network such that they rely on glycolysis for ATP synthesis even under normoxic conditions, a phenomenon known as Warburg effect after its discoverer. [146] The development of mass spectrometry and application of chemometrics to complex biological systems has allows the scientists to detect, identify, and quantify individual metabolites, or chemicals involved in the metabolism, present in a system [147, 148] Therefore, metabolomics, a comprehensive characterisation of metabolome or the complete list of metabolites present under physiological as well as pathological condition, was developed in the 90s and on, and has significantly contributed to our understanding of these phenomena.

The mass spectrometry-based chemometric methods, akin to the proteomic analysis, have been used for metabolomics. Unlike nucleic acids and proteins, which are respectively polymers of 5 (or sometimes 4) and 20 types of monomers, the metabolome, or the entire world of metabolites, consists of a diverse array of organic and bioinorganic compounds. On one hand, this contributes to the increased chemical complexity of a metabolome compared to its corresponding genome or proteome. Technologically, given proteins are a type of

metabolites, a similar setup to those used for proteomics can be adapted for metabolomic analysis, with the added advantage that the metabolites in question are often much smaller than the proteins and more amenable to ionisation without damaging themselves. On the other hand, metabolites overall encompass a much more diverse group of chemicals. This has led to development of different platforms for each group. Gas chromatography, for example, can be used to detect relatively small-molecular weight compounds of a wide range of chemical properties. These include, but are not limited to, amino acids, nucleic acids, fatty acids, and amines.

As was the case in proteomics, a molecule of metabolite is first ionised then is injected into a magnetic field. This results in the ionised metabolite to fly across the magnetic field before being detected at a position on the detector. The position that the metabolite is detected is a function of the time of flight (TOF), which in turn is a function of mass-to-charge ratio (m/z). Many metabolites have a unique m/z value and can be unequivocally identified, while others have the same m/z values and are not uniquely identified. Such m/z values are assigned to multiple metabolites, thus is not suitable for unambiguous identification of metabolites. For example, Both PC (18:2/18:2) and PC(18:0/18:4) can be denoted as PC(36:4) and have the same m/z . [149] While the library of identifiable metabolites is growing, it is far from complete and further efforts to expand the library coverage is desired. On the other hand, such diversity in chemical structures contribute to distinct chemical properties that the scientists can take advantage of by developing distinct platforms each of which has affinity for a specific subset of metabolites. It should be noted that proteins and nucleic acids such as genomic and mitochondrial DNA and mRNA are metabolites, these polymeric forms of nucleic

acids are customarily excluded from the metabolomics studies in part because their mass ranges are beyond the analytic capacity of mass spectrometry. Lastly, A metabolome is a direct reflection of the corresponding “enzymome”, defined here as the collective activity of enzymes present in the cells and tissues. While some enzymes show stringent specificity towards their substrates, many enzymes can catalyse the same biochemical reactions on a family of related molecules. For example, acetyl coenzyme A-acyl-carrier-protein transferase (EC 2.3.1.38) catalyses acetyl moiety transfer from acetyl-coenzyme A (acetyl-CoA) to acyl group of variable size attached to the carrier protein. This promiscuity further expands the complexity of the biological system under investigation.

The types of analytical platforms developed and used for metabolomics include gas chromatography, matrix-assisted laser desorption ionisation-time of flight (MALDI-TOF), electrospray ionisation, and hydrophilic interaction chromatography (HILIC). [150] For example, gas chromatography (GC) relies on the ability to ionise and vapourise each biomolecule or metabolites, which are subsequently analysed in magnetic-field driven mass spectrometry. Because GC is limited by the technical prowess to vapourise and ionise molecules, it is not suitable to larger metabolites that are prone to break ups, and thus is usually used to analyse small-molecular-weight metabolites. up to 650 Da. [151] These include, but are not limited to, amino acids, nucleic acids, fatty acids, and amines. Hydrophilic interaction chromatography (HILIC) is suited to analyse polar molecules, since it uses aqueous mobile phase for separation. [152] In the past decade, lipidomics, a sub-discipline of metabolomics that specifically studies the structurally complex and diverse family of lipids with mass spectrometry-based approaches, have become more and more commonly used in biology. [153]

Upon the collection of mass spectrometric spectral data, data analysis begins. The first step is data normalisation. Inadequate normalisation will result in skewed dataset and suboptimal downstream analyses, leading to misidentification of biomarkers and their quantification. [154] The purpose of the data normalisation is to standardise the variation across samples and eliminate technical errors that may mask biologically meaningful differences between groups. This can be done by sum or by mean and is employed by Metaboanalyst. [155, 156] Once the dataset is normalised, it can be put through algorithms to identify statistically significant differences in the form of metabolites present in different concentrations between distinct sample groups or different experimental conditions. These metabolites can be used as biomarkers to distinguish the two or more sample groups or experimental conditions. The choice for a given set of biomarkers is based on their power to distinguish different groups of samples. Discrimination between two or more groups of samples can be performed by principal component analysis (PCA) which is a non-supervised method, or partial least square discriminant analysis (PLS-DA), a supervised method. These models are often tested by conventional methods such as calorimetric methods to validate them whenever they are available.

For experimental biologists, a cardinal purpose of *in silico* analyses such as bioinformatics is hypothesis generation. Such hypotheses can take the form of testing whether the entire set of samples can be classified into two or more subsets, a question that can be answered using the aforementioned PCA or PLS-DA. A more prevalent use of bioinformatics, however, is subsequent pathway analysis and generation of hypotheses based on such an

analysis. These hypotheses are thereafter often tested by conventional experiment-based approaches. [157]

Regardless of the omics platforms and their subsequent directions, the *in silico* analyses is likely to produce a lengthy list of metabolites, some of which are false positive, and the rate at which such false identifications are made is called positive false discovery rate. This scenario is known as *multiple hypothesis testing* and must be handled properly to reduce false discoveries. In order to reduce the false positives, several statistical methods have been devised. Amongst them is q-value, [158] which is used in Chapter 4, to correct for the multiple hypothesis testing. Another popular method to control for multiple testing is Bonferroni correction, [159, 160] and was used in the proteomic study that formed a basis for the Chapter 3. [111]

Additional technological challenges in metabolomics lies in the inherently complex nature of family of similar compounds. For example, lipids encompass a large collection of organic molecules such as fatty acids, triglycerides, phospholipids, sphingolipids, diacylglycerols, and hormones. Each of these sub-families of lipids are composed of a large number of molecules, many of which have isomers. Therefore, unique identification of the species as well as their quantification still requires further development in the technology. Furthermore, suitable platforms for some organic molecules such as acyl-Coenzyme A (Acyl-CoA) are yet to be developed. Given the emerging complexity and importance of lipids in biological structures and signalling, development of such technologies is paramount and holds substantial hope to advance personalised medicine.

The metabolomic analysis flow described above serves as the foundation to analyse biology and pathobiology of metabolism across all organisms and conditions. It should, however, be noted that the impact of metabolomics is not limited to the conventional metabolites and its flux analysis. Metabolites can modify proteins to control their biochemical and biological activities through post-translational modifications (PTMs). Therefore, metabolomic analysis can shed light on protein and nucleic acid PTM. Protein PTMs with conventional metabolic intermediates and their physiological and pathological roles have been described, which further exemplifies the importance of metabolomic studies.

For example, α -ketoglutarate (α KG) is converted to succinyl-CoA by α -ketoglutarate dehydrogenase in the TCA cycle, an enzyme that catalyses a decarboxylation reaction which concomitantly produces NADH from NAD⁺. Succinyl-CoA plays a dual role. In addition to the traditional role as a TCA cycle intermediate, it transfers the succinyl moiety to Lysine residues as a form of post-translational protein modification, in which a positive charge is converted to a negative charge with concomitant increase in volume. [161, 162] This serves as a sensor for TCA cycle flux. Dysregulated protein lysine succinylation plays a causal role in such diseases as mitochondrial encephalomyopathy and esophageal squamous cell carcinoma. [163, 164] Desuccinylation is catalysed by SIRT5. [165]

Succinyl-CoA is in turn converted to fumarate via succinate. These sequential reactions are catalysed by succinyl-CoA synthetase which converts succinyl-CoA into succinate, and succinate dehydrogenase which converts succinate into fumarate. The former concomitantly produces GTP from GDP while the latter reduces FAD to FADH₂. Fumarate can modify a cysteine residue by converting it to S-(2-succinyl)cysteine, a process known as succination.

Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) succination causes irreversible inactivation of the glycolytic enzyme, [166] while Tubulin succination results in reduced microtubule polymerisation. [167]

These TCA cycle intermediates illustrate multifaceted biological roles of metabolites, which can coordinate otherwise unlinked metabolic pathways. These multifunctional metabolites consequently increase the complexity of the biological system.

As discussed above, in order to understand the biological systems at molecular and biochemical levels, comprehensive characterisation of the total collection proteins and metabolites is necessary. In other words, chemical identification and quantification of proteins and metabolites along with their covalent modifications are fundamental to our understanding of the biological systems being investigated. Technological advances in the past several decades coupled with the computational advancements have allowed researchers to use these chemometric approaches, collectively termed multi-omics, to understand the cells and organisms at an increasingly finer and more comprehensive scale.

1.8 Protein Moonlighting

The conventional view of one gene makes one protein serves one function has recently been challenged on multiple fronts. The traditional biology assigned a single function to a defined polypeptide. To increase the range of functions that can be performed, many genes encode multiple transcripts through alternative splicing or alternative promoters as well as alternative translation initiation site. Recent studies also showed that some proteins can

perform a secondary function unrelated to its originally ascribed function. This is known as protein moonlighting, and the proteins with such secondary functions are collectively known as moonlighting proteins. [168] It is a form of gene sharing in which multiple distinct biochemical or cell biological functions are attributed to the same protein. It is a distinct phenomenon from pleiotropism, in which a single function performed by a protein affects multiple targets and pathways. These may manifest from alternate translation (e.g., ENO1/MBP1), [169-171] novel post-translational modification (e.g., Cyt C), [172] novel binding partners (e.g., ACO1, GAPDH, TYMS, NDRG1), [173, 174] novel catalytic substrates (e.g., PGK1), [175] non-catalytic novel substrates (e.g., PGK1 in primer recognition and specific mRNA binding), [176, 177] and novel subcellular localizations leading to novel interacting partner and biochemical roles (e.g., PKM2). [178] It is interesting that some proteins such as PGK1 have multiple moonlighting functions, suggesting hidden functional flexibility for polypeptides, although most moonlighting functions so far seem species specific. (See below)

A diverse array of proteins falls under the category of moonlighting proteins. For example, hexose kinase was originally discovered as a polypeptide with enzymatic activity to phosphorylate glucose, which is the first step of glycolysis. [179-181] In mouse, hexokinase also functions as a bacterial N-acetylglucosamine receptor, which in turn activates NLRP3 inflammasome. [182] In another instance, translocation of pyruvate dehydrogenase complex controls nuclear acetyl-CoA concentration and consequently histone acetylation status. [183] Alternatively, a gene may be translated into protein from an internal ribosomal entry site (IRES) In the case of Tyrosyl-DNA phosphodiesterase 2 (TDP2), for example, IRES-driven translation results in deletion of nuclear localization signal without the loss of its enzymatic activity, thus

producing a cytosolic enzyme. [184] Third, a gene may produce an apparently identical polypeptide which possesses distinct functions. The Crystallin protein family presents illustrative examples. α -Crystallin functions as a structural protein as well as a heat shock protein in the lens. [185, 186] In cornea, ALDH1A1, [187] ENO1 [188] and lactate dehydrogenases [189] are among the enzymes which were found to perform structural function independently of its originally identified catalytic activities.

This functional plasticity in moonlighting proteins allows the cells to expand the extent of biochemical functions that they can perform without encoding additional proteins in their genome, thus confer energetic and metabolic advantages. On the other hand, unlike the primary protein functions which are often robustly catalysed by redundant proteins, such moonlighting functions are often fulfilled by a single gene within its gene family and is expected to be more prone to perturbation through mutations. Protein moonlighting is a more prevalent phenomenon than previously anticipated, and all glycolytic enzymes are now known to moonlight in various physiological contexts. [190]

The structural basis for a single gene or a single polypeptide to possess such an unexpected combination of functions is still under investigation. It most likely involves the dynamic flexibility of protein tertiary and quaternary structures. In this regard, it will be interesting to solve crystal, nuclear magnetic resonance (NMR), or electron microscopic (EM) structures and to identify the binding surface on the moonlighting protein for each of the binding partners. A unique binding surface may be created on a moonlighting protein through the use of PTM. Such PTM may induce conformational change in the moonlighting protein, creating a unique binding surface for different binding partners or distinct catalytic site for

different substrates. While not a PTM in a classical sense, this may be reminiscent of point mutations in enzymes which endows it with a novel product specificity. For example, a point mutation in isocitrate dehydrogenase 1/2 (IDH1/2) occurs in several cancers including glioblastoma. [191] IDH1/2 are TCA cycle isozymes that catalyses the NAD^+ -dependent decarboxylation of isocitrate to α -ketoglutarate (α KG). In addition, α KG can also be synthesised from glutamate via NAD^+ -dependent deamination catalysed by glutamate dehydrogenase, an anaplerotic reaction. α KG is a cofactor for DNA modifying enzymes such as ten-eleven-ten (TET) demethylases. This allows α KG to act as a nexus between metabolism and epigenetic control of transcription under physiological conditions. The mutant IDH1/2, however, harbours R132H mutation, and synthesises 2HG instead, which competitively inhibits the TET DNA demethylases, resulting in increase. [192] Because of these characteristics, 2HG is known as an oncometabolite which drives cancer progression. It should be noted that although ccRCC also exhibits an increased level of intracellular 2HG, this is due to reduction in L-2HG dehydrogenase (L2HGDH), and only the L-enantiomer of 2HG accumulates in ccRCC. [193]

To understand the structural basis of protein moonlighting, also informative is understanding the pH and ionic microenvironment of a particular moonlighting protein. These external conditions will likely modify the charge distribution on the surface, creating differential binding preference. Alternatively, a moonlighting protein may assume different conformation due to lack of proper sets of chaperone proteins or presence of incorrect sets of chaperones in ccRCC, and the cancer is able to capitalise on such misfolded proteins.

Interestingly, unlike the canonical functions, these moonlighting functions of proteins are often species specific. For example, PGK1 catalyses the conversion of 1,3-

bisphosphoglycerate to 3-phosphoglycerate with concomitant ATP synthesis in all species investigated, its mRNA binding function is reported so far only in *Homo Sapiens*, plasminogen binding function is reported only in *Candida Albicans* and Streptococci. [Moonprot database, [194] searched on 10 April 2022] This may simply reflect lack of more comprehensive investigation. On the other hand, it may reflect a fundamentally different biology or proteome in each species, some of which are more related to each other than others. Given the species-dependent variability in protein moonlighting, how the protein moonlighting evolved and whether such flexibility inadvertently endowed cells with the ability to become cancerous are questions that warrant further investigation.

It is becoming clear that cancers including ccRCC exploit protein moonlighting, which is a reflection of structural and functional flexibility that polypeptides possess. [195, 196] This expands the biochemical and cell biological functions that a given proteome can perform. Consequently, protein moonlighting allows integration of two cellular functions that are otherwise unrelated. The extensive use of moonlighting capacity of proteins in cancer demonstrates that the structure-function relationship of a protein is fundamentally dynamic and promiscuous. It, however, allows the organisms to achieve their biochemical goals by flexibly combining multiple functions of each component protein. On the other hand, such plasticity can be easily hijacked to create pathological conditions such as cancer. Therefore, such functional flexibility of proteins is both economically advantageous and existentially precarious for the cells and the organisms.

1.9 Research Objectives

Despite technological advances and resultant successful treatments of many types of cancers, ccRCC remains refractory to conventional chemotherapy and its mortality remains high.

Pharmacological innovations provide an ever-increasing number of candidate chemotherapeutics. With the advent of omics over the past three decades aided by technological and computational advances, it is now possible to describe the entire collection of proteins and metabolites in a tissue or even a single cell. This has allowed description of physiological and pathological state at far greater details than were ever possible.

This dissertation demonstrates the use of such technological advances in elucidating the molecular underpinnings of ccRCC. There are four research objectives investigated in this dissertation. First, chemotherapeutic potential of R-Roscovitine on ccRCC *in vitro* was investigated in order to test the efficacy of CDK inhibitors that had previously been proven useful against other types of cancers. Next, characterisation of Fuhrman nuclear grade-defined ccRCC proteomes was performed to apply the nascent discipline of proteomics to ccRCC to identify novel biomarkers and druggable targets. Third, metabolomics was employed to investigate the metabolic reprogramming following the silencing of Argininosuccinate Synthetase (ASS1), which was identified as one of the biomarkers in the grade-defined ccRCC proteomics discussed above and is also widely observed in other cancers. Finally, the potential

of using a subset of grade-defining ccRCC biomarkers as moonlighting proteins and subcellular location-based ccRCC grade-defined biomarkers was investigated.

Through these four research objectives, this dissertation aims to further define ccRCC proteo-metabolome and to clarify the ccRCC pathobiology to equip the researchers with novel knowledge that can be used to design novel therapeutics against ccRCC.

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CHAPTER 2

VHL Status Dictates the Response of Renal Cell Carcinoma (RCC) to Cyclin Dependent Kinase (CDK) Inhibitor Roscovitine *In Vitro*

Abstract

Renal cell carcinoma (RCC) accounts for 13000 annual deaths yet there is no effective chemotherapy in part due to incomplete understanding of the signal transduction pathways altered. VHL inactivating mutation is prevalent in RCC patients yet its role in apoptosis in RCC remains largely uninvestigated. To study the response of VHL-positive and VHL-negative RCC to Cell Cycle-dependent Kinase (CDK) inhibitors, we treated ACHN and 786-O RCC cell lines with Roscovitine, alone or in combination with Nutlin-3. Here we found that ACHN exhibits a profound caspase-3 mediated apoptotic response to a narrow range of Roscovitine while 786-O showed less pronounced caspase-3 independent cell death. These responses were p53-dependent and were accompanied by the abrogation of the survival signaling pathway. Furthermore, we identify concentration-dependent differential inhibition of CDK2 and CDK9 by Roscovitine as a potential basis for the narrow range of apoptotic response in ACHN, while VHL inactivating mutation is likely the contributing factor for the lack of Caspase-3 mediated apoptosis in 786-O. Lastly, Nutlin-3 only modestly augmented the cell death phenotype in 786-O. These observations indicate that Roscovitine is a potential chemotherapeutic agent against

a subset of RCC if the dosing is tightly controlled. These results provide further insight into our understanding into the effect of VHL mutation in RCC and into our needs to develop CDK2 specific CDK inhibitors.

Introduction

Kidney cancer, also known as renal cell carcinoma (RCC), is diagnosed in 36,000 patients and is the cause of death of 11-13,000 individuals yearly in the US. [1] While the treatment of metastatic RCC has long been considered dismal with only modest success using immunotherapeutic cytokines, the horizon is slowly changing with the advent of targeted therapeutic agents directed at such important signaling proteins as VEGF, mTOR, and HIF. In particular, the multi-kinase (including VEGF-R) inhibitors sunitinib and sorafenib have changed the outcome of patients who have been diagnosed with metastatic disease. However, as the molecular biology of RCC is being elucidated, more potential targets are being identified.

Cell cycle inhibitors have long been considered as a potential tool to interfere with the hyperproliferative phenotype that cancers exhibit. [2, 3] Classical DNA-damaging chemotherapeutics have been ineffective possibly due to an exuberant repair response seen in RCC as compared to other cancers; such a response can be defeated by manipulation of the CKI p21. [4] For this reason, we have examined pharmacologic competitive inhibitors of cyclin-dependent kinases (CDKs) to determine whether they may have salutary effects in RCC.

Several pharmacological cell cycle inhibitors have been developed and tested in other cancers with varying degrees of success, in most cases being limited by toxicities. These

pharmacological agents include AT-7519, P276-00 and Roscovitine, which are in either phase I or II clinical trials. For example, AT7519, which inhibits CDK1, 2, 4 and 5 as well as GSK3 β , is in a phase I clinical trial against advanced lymphoma. Roscovitine, also known as seliciclib or CYC202, is a purine derivative whose targets include CDK2, 5, 7, 8 and 9. [5] It is currently in a phase II clinical trial against non-small cell lung cancer. [6]

In order to address the potential use of Roscovitine as a therapeutic agent in RCC, we have undertaken an *in vitro* analysis of Roscovitine-treated RCC cell lines. We now show that Roscovitine causes apoptosis in the ACHN RCC cell line within a strikingly narrow concentration range, yet the VHL-mutant 786-O RCC cell line is more resistant to the apoptotic effect within the same concentration range. We further show that the mechanism of these effects is due to modulation of apoptotic and survival signaling pathways due to differential inhibition of the various CDK which are influenced by Roscovitine. Our data help explain the toxicities seen in clinical studies. Thus, the use of this pharmacological CDK inhibitor in RCC has considerable promise but dosing requirements will be of paramount importance in future studies. Furthermore, combinatorial therapy using Roscovitine as a component should hold great promise.

Materials and methods

Materials

RCC cell lines (ACHN, 786-O) as well as the normal renal epithelial cell line were purchased from ATCC (Manassas, VA). p53 Knockdown ACHN cell line was a gift from Dr. Jaime Modiano (U.

Minnesota). Roscovitine and Nutlin-3 and Ponceau S were obtained from Sigma-Aldrich (St Louis, MO). The anti-PARP, anti-phospho-CDK2, anti-Serine 46 p53, anti-phospho-Ser807/811 Rb, anti-total Caspase3 antibodies were purchased from Cell Signaling (Boston, MA). Anti-total p53 antibody was from Sigma-Aldrich. Anti-Phospho-Ser2 Pol II and anti-total-Pol II antibodies were purchased from Covance (Princeton, NJ). Supplies for SDS-PAGE and Western blotting were obtained from Bio-Rad and Pierce.

Cell culture

ACHN and 786-O were grown in DMEM (GIBCO) supplemented with 10% FBS, 10mM GlutaMax and antibiotics. The cells were plated in 6-well plates at the density of 2×10^5 /well 24 hours prior to Roscovitine treatment. The stock Roscovitine was dissolved in DMSO at 10mg/ml. The stock was diluted in the growth media to the designated concentration just prior to the application to the cells. The concentration of the DMSO never exceeded 20 μ g/ml. After grown in the presence or absence of Roscovitine for the specified amount of time, the cells were harvested in 200 μ l RIPA buffer supplemented with protease / phosphatase inhibitor cocktail (Pierce), and the protein concentration was determined by Lowry assay using DC protein assay (Bio-Rad).

Western blotting

10 μ g each of total protein were run on 7.5% or 12 % Tris-Glycine polyacrylamide gel. Following the electrophoresis, the protein was transferred on to a nitrocellulose membrane. An even transfer was visually inspected using Ponceau S staining. The nitrocellulose membrane was blocked with 5% non-fat milk / TBST for an hour at room temperature. The membrane was subsequently incubated with a primary antibody diluted in the 5% non-fat milk at 4 $^{\circ}$ C with

rocking overnight. After the incubation, the membranes were washed with TBST and were incubated with a corresponding secondary antibody diluted in the 5% non-fat milk / TBST for one hour, at RT. The membranes were washed, and the protein-antibody complex were treated with ECL-standard or ECL-Plus reagents and the chemiluminescence was detected using autoradiography films.

Quantification of apoptosis using Hoechst staining

Number of cells undergoing apoptosis was quantified by staining with Hoechst 33258 as described previously (4) with the following modification. Cells were plated on 8-well Labtek-II chamber slides at the density of 1×10^4 /well 24 hours before the start of Roscovitine treatment. The cells were harvested and stained with the Hoechst 33278 at the concentration of 10 $\mu\text{g/ml}$.

Results

Roscovitine causes RCC cell apoptosis in a VHL and p53 dependent manner

CDK inhibitors such as Roscovitine have been regarded as a promising set of chemotherapeutic agents, yet its application has been met with mixed results. [Guzi (2004)] In particular, lack of mechanistic understanding has resulted in haphazard application.

To determine the response of renal cell carcinoma (RCC) *in vitro* and its underlying mechanism, we applied Roscovitine to two RCC cell lines; VHL-wt ACHN and VHL-negative 786-O. Upon treatment with Roscovitine at concentration ranging from 0.1 $\mu\text{g/ml}$ to 40 $\mu\text{g/ml}$ for 24 and 36 hours, both cell lines exhibited apoptosis albeit with distinct patterns and degree. ACHN

showed profound apoptosis within a strikingly narrow range around 10 µg/ml while 786-O showed apoptosis across a broader range, but the maximum magnitude of the apparent apoptosis was reduced compared with that of ACHN (**Figure 1a**). In order to characterize the apoptotic response of these RCC cell lines, they were treated with the same concentration range of Roscovitine for a larger range of time course. The apoptosis in the Roscovitine-treated ACHN, measured as poly-ADP Ribose Polymerase (PARP) cleavage was first detected at 24 hours, and continued to 36 hours. Interestingly, the apoptosis was evident within a strikingly narrow range of Roscovitine concentration centering around 10 µg/ml yet was undetectable at 20 µg/ml and above (**Figure 1b**). In contrast, Roscovitine application caused apoptosis in 786-O cells across a wider concentration range starting as early as 12 hrs. The maximum of the PARP cleavage was still found at 10 µg/ml, but the cell line exhibited apoptosis across a wider concentration between 10 µg/ml and 40 µg/ml Roscovitine (**Figure 1b**).

In order to quantify the apoptosis, we used Hoechst staining of the apoptotic nuclei after 24-hour Roscovitine treatment. [4] In agreement with the PARP cleavage, as indicated as statistically significant increase in the apoptotic index defined as the percentage of apoptotic condensed nuclei, ACHN exhibited the highest degree of the condensed apoptotic nuclei when treated with 10 µg/ml Roscovitine. In contrast, 786-O cells exhibited little statistically significant change in the apoptotic index across the Roscovitine concentration range tested. (**Figure 2a**) Furthermore, activation of Caspase-3, observed as the catalytically active cleaved caspase fragments, was noted in ACHN cells treated with 10 µg/ml Roscovitine. (**Figure 2b**) The appearance of cleaved caspase-3 in 786-O cells under the identical condition was very modest. (**Figure 2b**)

To investigate the mechanism of the Roscovitine-induced apoptosis, we asked whether Roscovitine causes a change in p53 levels. While both cell lines are wild type with respect to p53 [7, 8] the 786-O cell line carries a missense mutation in VHL and hence is VHL negative. VHL has been shown to stabilize p53, [9] thus making 786-O an apparent p53-null. In agreement with these previous findings, Roscovitine treatment resulted in upregulation of p53 (**Figure 3a**). The upregulation precedes the onset of PARP cleavage and starts at 6 hours after Roscovitine treatment. Surprisingly, the upregulation is not limited to the apoptotic concentration of 10 $\mu\text{g/ml}$ but is broad spanning from 10 $\mu\text{g/ml}$ through 40 $\mu\text{g/ml}$ Roscovitine. 786-O shows a transient and minor upregulation of p53 at 12hr when treated with 10 $\mu\text{g/ml}$ and 20 $\mu\text{g/ml}$ Roscovitine, yet the level is downregulated by 24hr when treated with 10 to 40 $\mu\text{g/ml}$ Roscovitine, suggesting that VHL inactivation contributed to the destabilization of p53.

In order to ask whether the p53 is activated in the RCC cell lines, we asked if p53 is phosphorylated at Serine 46, which is known to be phosphorylated by DYRK2 and HIPK2 to activate the p53 transcriptional activity. [10, 11] Serine 46 is phosphorylated in ACHN when treated with 10 – 40 $\mu\text{g/ml}$ Roscovitine for 24 hours or longer. While The 20 and 40 $\mu\text{g/ml}$ Roscovitine treatment induces phosphorylation starting at 12 hours, the extent is minor (**Figure 3a**). In contrast, Serine 46 is not detectably phosphorylated in the 786-O cell line (**Figure 3a**).

In order to address whether the p53 is transcriptionally active, we measured the level of p21, a direct p53 target with short half-life. ACHN exhibited a basal level p21 which prevailed when treated with up to 1 $\mu\text{g/ml}$ Roscovitine for up to 36 hours. p21 was markedly upregulated in ACHN when treated with 10 $\mu\text{g/ml}$ Roscovitine for 12 hours and longer (**figure 3b**). Surprisingly,

the level of p21 was diminished to undetectable level when treated with 20 µg/ml Roscovitine and higher dose as early as 6 hours after the application, suggesting that p53 transcriptional activity was compromised. The p21 upregulation was absent in the VHL-negative 786-O (**Figure 3b**).

In order to ask whether p53 is necessary to cause apoptosis in RCC, we treated ACHN cells stably transfected with a p53 knockdown construct with the same Roscovitine concentrations. When treated for 30 hours, p53-knockdown ACHN exhibited a markedly reduced level of apoptosis (**Figure 4**). Surprisingly p53 level was further reduced at 10 µg/ml Roscovitine and above in a similar manner to that seen in Roscovitine-treated 786-O cells (**Figure 4**). This result indicates that the ACHN apoptosis upon Roscovitine treatment is p53-dependent. Interestingly, the apoptosis, while greatly reduced, was still present in p53 knockdown ACHN cells. This may indicate that the knockdown was incomplete and the residual p53 was able to induce apoptosis at 10 µg/ml Roscovitine treatment. Alternatively, this result suggests that there is an alternative p53-independent apoptotic pathway in ACHN.

RCC cell apoptosis induced by Roscovitine is accompanied by decreased survival signals

In order for cells to execute apoptosis, they must also decrease the survival signaling pathways such as AKT and Rb. AKT requires phosphorylation at Ser473 to be a fully active kinase to phosphorylate such targets as Rb, FOXO and Bad. [12, 13] Rb phosphorylation releases E2F from Rb, thereby allowing E2F to transcribe pro-survival genes.

Therefore, we asked whether p53-dependent apoptosis seen above is accompanied by decreased AKT and Rb signaling. Upon the Roscovitine treatment at 0.1- 40 $\mu\text{g/ml}$ for 2 – 36 hours, ACHN exhibited reduced AKT phosphorylation on Serine 473 (**Figure 5a**) and reduced Rb phosphorylation on Serine 807/811 (**Figure 5b**), which collectively reduce the pro-survival signaling pathway in ACHN. In agreement with this, XIAP level was reduced in the ACHN treated with 10 $\mu\text{g/ml}$ Roscovitine for 24 and 36 hours, but not at other Roscovitine concentrations tested (**Figure 5c**). Interestingly, the decrease in Rb phosphorylation, which started at 12 hours post Roscovitine treatment, preceded the decrease in AKT phosphorylation on Ser473 and total XIAP reduction which were not evident until 24 hours after the Roscovitine application.

786-O also exhibited decreased survival signal, yet its response pattern, as investigated with Western blotting, was markedly different from that of ACHN (**Figure 5a-c**). 786-O exhibited a broader response to Roscovitine with regard to the Roscovitine concentration. Specifically, Rb phosphorylation on Ser807/811 and AKT phosphorylation on Ser473 were reduced at 10 $\mu\text{g/ml}$ Roscovitine and at higher concentrations of 20 $\mu\text{g/ml}$ and 40 $\mu\text{g/ml}$ (**Figure 5a**). A similar temporal and concentration-dependent pattern was seen in the total XIAP level reduction (**Figure 5c**). Interestingly, 786-O, like ACHN showed the Ser807/811 phosphorylation reduction and Akt Ser473 phosphorylation reduction starting the 12 hours, preceding the total XIAP level reduction which was not evident until 24 hours after Roscovitine application.

Roscovitine inhibits CDKs in ACHN and 786-O in a similar manner.

Roscovitrine is known to inhibit several CDKs including CDK2, 5, 7, 8 and 9. [14-16] CDK2 is a classical CDK involved in G1/ S transition, while CDK 7 and 9 phosphorylate the α subunit of RNA polymerase II (Pol II) at specific serine residues within the heptad repeats in the C-terminal domain. Specifically, CDK2 substrates include itself, which is phosphorylated at Threonine 166 as well as a number of proteins regulating the cell cycle. CDK7 and 9 phosphorylates Ser5 and Ser2 within the heptad repeats of RNA Pol II. [22] In order to understand the molecular mechanisms underlying the dose-dependent response of RCC to Roscovitrine *in vitro*, we analysed the CDK2 and CDK9 inhibition. In ACHN, CDK2 activity, as measured as the auto-phosphorylation on residue Threonine 160, diminishes starting at 12 hours after treatment with 10 μ g/ml Roscovitrine and higher (**Figure 6a**). At the highest concentration tested, the CDK2 inhibition was observed as early as 6 hours after the Roscovitrine application. (**Figure 6a**) Similarly, CDK2 autophosphorylation was inhibited in 786-O RCC cell line when it was treated with Roscovitrine at 10 μ g/ml and higher. As was the case in ACHN, the inhibition was detectable starting at 12 hours after the application, although there was a moderate inhibition at 6 hours after application when 40 μ g/ml Roscovitrine was applied.

The response of CDK9 activity to Roscovitrine was rapid and profound in both cell lines. (**Figure 6b**) CDK9 phosphorylates multiple serine residues within the C-terminal domain of the RNA Polymerase and the phosphorylation is necessary for the initiation of mRNA transcription. In ACHN, The Ser9 phosphorylation on the Pol II CTD was inhibited as early as 2 hours following the Roscovitrine application. There was a mild inhibition at 10 μ g/ml, while the inhibition was nearly complete at 20 μ g/ml Roscovitrine and above. On the other hand, in 786-O cells, the Ser9 phosphorylation was markedly reduced at as low as 10 μ g/ml Roscovitrine with further and

virtually complete inhibition at higher Roscovitine doses. As was the case in ACHN, Ser2 phosphorylation was reduced as early as 2 hours after the Roscovitine treatment in 786-O cell line. CDK7 inhibition was also blocked albeit at a lesser degree and with slower time course (data not shown).

Roscovitine-induced apoptosis in 786-O is moderately augmented by Nutlin-3 and is Caspase-3 independent

In contrast to the apoptosis in Roscovitine-treated ACHN, that in Roscovitine-treated 786-O is rather mild. ACHN is a VHL-wt while 786-O is a VHL mutant cell line with truncation due to a point mutation [7, 8] and VHL is reported to interact with and stabilize p53. [9] In the absence of VHL, p53 is destabilized and ubiquitinated for degradation, which is catalyzed by MDM2-p53 interaction. Thus, in order to disrupt the MDM2-p53 interaction, we treated 786-O simultaneously with varied concentration of Roscovitine and fixed concentrations of Nutlin-3 which were determined to cause p53 stabilization. The data showed slight augmentation in the apoptotic phenotype in the PARP cleavage across the Roscovitine concentration range determined to show weak apoptotic phenotype above (**Figure 7a and b**). The assessment of Caspase 3 cleavage, a direct measurement of apoptosis, revealed apoptosis at a negligible level after simultaneous Roscovitine and Nutlin-3 application (**Figure 7b**).

Discussion

RCC is the ninth prevalent cancer in the US with 16,000 annual deaths. Therefore, it is of great significance to study its molecular background and to characterize response of RCC cells *in vitro* to potential pharmacological anti-cancer agents. Due to its ability to target cell cycle, CDK inhibitors have been regarded as a potential chemotherapeutic agent against cancer whose hallmarks include aberrant cell cycle regulation. Roscovitine is a purine derivative with potent and specific CDK inhibitory effect against CDK2, 5, 7, 8, and 9. Thus we sought to characterize the response of RCC to Roscovitine *in vitro* in the hope to define a novel and effective therapeutic approach towards RCC.

In order to investigate the modulation of signal transduction pathways in the RCC cell lines by Roscovitine, we characterized p53, Rb and AKT pathways. The apoptosis in ACHN is accompanied by abolishment of AKT phosphorylation at residue Serine 473, increase in p53, increase in p53 phosphorylation at Serine 46 and as well as reduction in RB phosphorylation at Serine 807/811. The Roscovitine treatment of p53 knockdown ACHN showed reduced degree of apoptosis, suggesting that Roscovitine-induced apoptosis in ACHN is p53 dependent. Interestingly, a higher dose of Roscovitine treatment reduced CDK7 and 9 dependent phosphorylation of RNA polymerase II (Pol II) and abolished the apoptotic phenotype in ACHN cells. This observation further suggests that p53-dependent transcription, perhaps of proapoptotic genes, is necessary for the ACHN apoptosis. In contrast, Roscovitine is a poor inducer of apoptosis in VHL-negative 786-O cells. Lack of VHL activity is likely to result in the p53 destabilization hence the poor apoptotic phenotype of 786-O cells in comparison to ACHN cells. This led us to hypothesize that destabilization of MDM2-p53 would shuttle p53 away from ubiquitin-mediated degradation, thereby reverting the apoptotic pattern from that of

786-O to one which is more akin to that of ACHN characterized by narrow and profound PARP cleavage. The predicted effect was, however, not observed. Instead, upon destabilization of the MDM2-p53 interaction, the apoptosis was augmented across the Roscovitine concentration range where apoptosis was already weakly seen without the Nutlin-3 application. This may indicate that the cell death and its consequent PARP cleavage in 786-O is mediated primarily through the attenuated AKT activation, which is observed also across the broad Roscovitine concentration range of 10 µg/ml and above. Alternatively, it is possible that the activation of Caspase-3 and PARP cleavage occurred in a manner similar to that of ACHN yet was abolished by the time point that was tested (T = 24h).

Inhibition of CDK2 and CDK9 shapes the basis of the Roscovitine action on RCC *in vitro*

In agreement with the previous studies [5, 17] Roscovitine potently inhibited CDK2 and 9 in both ACHN and 786-O cells. ACHN and 786-O cells exhibited a similar time course and scale of inhibition. CDK2 in both cell lines showed reduced autophosphorylation at Threonine 160 starting 24 hours after Roscovitine treatment. The inhibition was detectable when the cell lines were treated with Roscovitine at 10 µg/ml (28.4µM) or higher. Higher Roscovitine concentrations did not result in an appreciably faster CDK2 inhibition.

On the other hand, Roscovitine inhibition of CDK9 activity, as measured by the Ser2 phosphorylation on RNA Pol II C-terminal domain, was observed in both cell lines when the cells were treated with Roscovitine at 20 µg/ml (56.8 µM) or higher. The inhibition, in contrast to that of CDK2, was swift, starting as early as 2 hours after the drug application.

The difference in the time course suggests that the CDK9 phosphorylation on RNA Pol II is rapidly reversed by a phosphatase. While CDK2 activating autophosphorylation persists for some time. This is in agreement with their biological roles; CDK2 is a cell cycle regulating kinase whose activation by cyclin E enables cells to progress through the G1-S checkpoint. To this end, CDK2 phosphorylates a number of target proteins including Nucleophosmin (B23) and itself, [18, 19] accounting for its need to stay in the phosphorylated active form for a long period of time. On the other hand, CDK9 is a component of RNA polymerase II holoenzyme, and its activity is required to initiate each transcription event upon the successful build-up of the holoenzyme. As such, it is necessary for the cells to quickly reverse Pol II phosphorylation by CDK9 in preparation for the next transcriptional event.

It should be noted that both CDK2 and CD9 are active in normal cells. Thus, further study should investigate the potential for Roscovitine to discriminate between cancerous cells such as ACHN and 786-O and normal cells such as HK-2 *in vitro*.

Serine 46 phosphorylation of p53 in the absence of Pol II inhibition correlates with the activation of caspase 3 and the induction of apoptosis.

HIPK2 is a stress-activated kinase whose targets include Serine 46 on p53. The residue has been shown to be phosphorylated by Roscovitine treatment of several cancer cell lines. Thus, it is interesting to see if this phosphorylation event mediates the pro-apoptotic function of Roscovitine,

A number of studies have indicated a central role in p53 to transduce the apoptotic stimuli initiated by Roscovitine in various cell lines. These cell lines include a RPMI-8226 myeloma cell line, MCF-7 breast cancer cell line, JAR choriocarcinoma cell line, and U2OS osteosarcoma cell line. [20] Our study in part corroborates with the aforementioned studies. Yet, at the same time, it suggests VHL negative RCC may undergo apoptosis in a p53 independent manner. It is also possible that Serine 46 phosphorylation or phosphorylation in general is not necessary for the p53-dependent apoptosis in RCC.

VHL-positive RCC activates apoptotic pathway upon Roscovitine treatment, but VHL-negative RCC undergoes cell death in a caspase-3 independent manner.

The current study revealed fundamental differences between the VHL-positive RCC and the VHL-negative RCC in response to Roscovitine. VHL was initially discovered as the ubiquitin ligase which targets HIF1 α to proteasomal degradation. [21] Later work revealed an additional role for VHL. It physically associates with p53 and keeps it in a stable conformation, protecting it from proteasomal degradation. [9] Our study lends support to this finding. In the VHL positive ACHN, total p53, its activating phosphorylation on Serine 46 as well as its target gene p21 is increased upon Roscovitine when applied within the range where CDK2 but not CDK9 is inhibited. On the other hand, in the VHL-negative 786-O cells, the same dose of Roscovitine failed to upregulate p53 and its activating phosphorylation. Consequently, there was no upregulation of p21, an indicator of p53 transcription. At the concentration where CDK2 and CDK9 are both inhibited, the magnitude of apoptosis was greatly reduced in ACHN, comparable

to that seen in 786-O. Since phosphorylation of the C-terminal domain of RNA Pol II by CDK9 is required for a successful initiation of transcription, [23] this is in agreement with the notion that transcription is required for the Roscovitine induced apoptosis.

It is of particular interest to note that 786-O shows a similar response to Roscovitine as was shown by the p53 knock-down ACHN. In particular, p53 protein is diminished upon treatment of Roscovitine at 10 µg/ml Roscovitine and above, and the magnitude of PARP cleavage is diminished. These observations further corroborate with the hypothesis that Roscovitine-induced apoptosis in ACHN is p53-dependent.

Roscovitine abrogation of survival signal by Roscovitine precedes the induction of apoptosis

In order for cells to execute a successful apoptotic event, it is necessary to turn off the survival signaling pathways such as AKT. [24] Thus, we investigated whether Roscovitine treatment would result in the abrogation of survival signaling via AKT phosphorylation, Rb phosphorylation and XIAP reduction. Our data shows that Serine 473 phosphorylation on AKT, Serines 807 and 811 phosphorylation on Rb as well as total XIAP were diminished specifically at the concentration where the cell death was observed in both ACHN and 786-O. That is, the AKT and Rb phosphorylation were abrogated at 10 µg/ml Roscovitine but not at higher concentration in ACHN. XIAP level was reduced at the same Roscovitine concentration. On the other hand, in 786-O AKT and Rb phosphorylation were reduced broadly when treated with Roscovitine at between 10 µg/ml and 40 µg/ml, which corresponds to the Roscovitine concentration range where 786-O cell death is observed. Since transcription of anti-apoptotic

proteins such as XIAP is required to keep cells from undergoing programmed cell death, it is possible that the higher concentrations of Roscovitine which are required to prevent RNA polymerase activation and subsequent transcription of proteins such as XIAP, could result in apoptosis by this mechanism.

Interestingly, in both cell lines, reduction in AKT phosphorylation and Rb phosphorylation precedes the reduction in XIAP level, suggesting a possible epistatic relationship.

Roscovitine, alone or in combination with P53-MDM2 interaction inhibitors may provide a promising treatment against RCC

Our data presented in this paper indicates that response of two RCC cell lines, ACHN and 786-O, to Roscovitine *in vitro* showed marked difference in their pattern, depending upon their genetic background, in particular, the status of VHL mutation. VHL was previously reported to directly interact with p53 and stabilize it thereby enhancing its activity as a transcriptional activator. This led us to hypothesize that destabilization of p53 in VHL-negative 786-O cells would result in the decreased p53 protein and its activity, thus leading to the lack of caspase-3 activation and the resultant diminished apoptosis.

In order to test this hypothesis, we treated 786-O cells simultaneously with Roscovitine and Nutlin-3, an antagonist of the p53-MDM2 interaction. Since MDM2 is a ubiquitin ligase specific for p53, thus directing p53 to proteasome-mediated degradation, disruption of the MDM2-p53 interaction is expected to increase p53 protein and its activity. [25 and **Figure 8**] Contrary to our expectation, the simultaneous Roscovitine-Nutlin-3 treatment resulted in only a modest

increase in Caspase cleavage and a minor Caspase-3 activation despite substantial increase in p53 (**Fig 7b**). This lack of apoptosis in Roscovitine-Nutlin-3 co-treatment suggests that further stimuli is required to activate the apoptotic pathway. One such candidate is post-translational modification of p53. In our study, Nutlin-3 treatment of 786-O with or without Roscovitine did not appreciably increase the activating phosphorylation of p53 on Serine 46. A recent report compared two distinct inhibitors of p53-MDM2 interaction and their impact on the apoptosis and related pathways. [26] The work indicates that RITA, but not Nutlin-3, induced the phosphorylation on p53, although both compounds are capable of upregulating the total p53. In light of this, Roscovitine-RITA co-treatment could provide a more effective therapy against RCC.

In summary, we have presented a thorough characterization of the response of renal cell carcinoma (kidney cancer) to Roscovitine or Roscovitine in combination with Nutlin-3 *in vitro*. Our data indicates that apoptosis in RCC is p53-dependent, and in the absence of its activity, as was seen in 786-O, the apoptotic phenotype is weak despite detectable PARP cleavage and cell death. Furthermore, our present study warrants further investigation of Roscovitine as a potential chemotherapeutic reagent against RCC.

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Figures

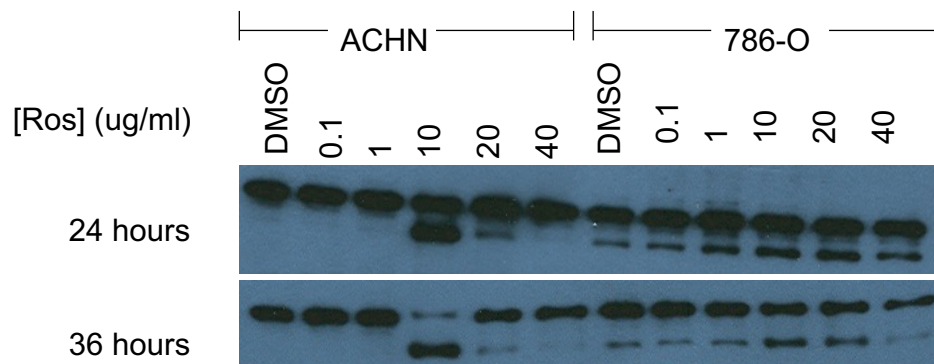


Fig. 1a PARP cleavage after of 24 and 36-hour Roscovitine treatment on ACHN & 786-O shows distinct patterns

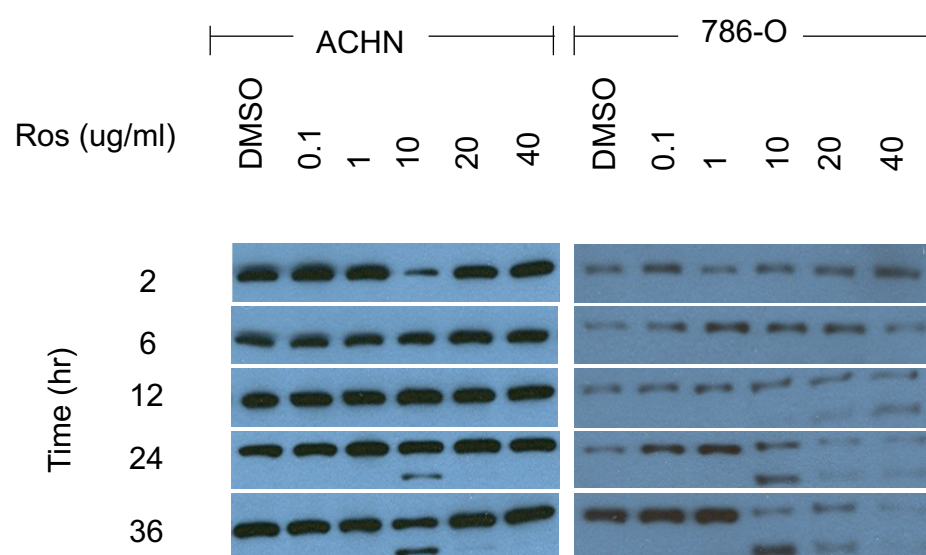


Fig. 1b PARP time course in ACHN and 786-O

Apoptotic Index of ACHN and 786-O RCC cell lines upon 24hr
Roscovitine treatment

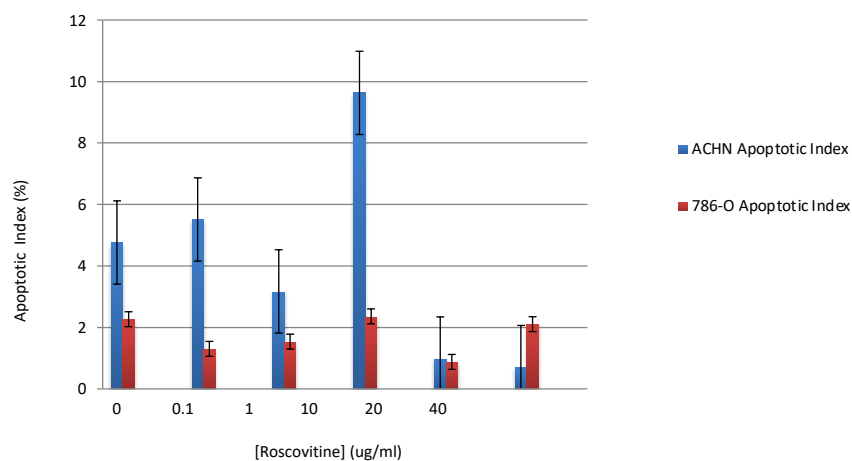


Fig. 2a : Hoechst staining detects apoptosis in ACHN
treated with 10ug/ml Roscovitine

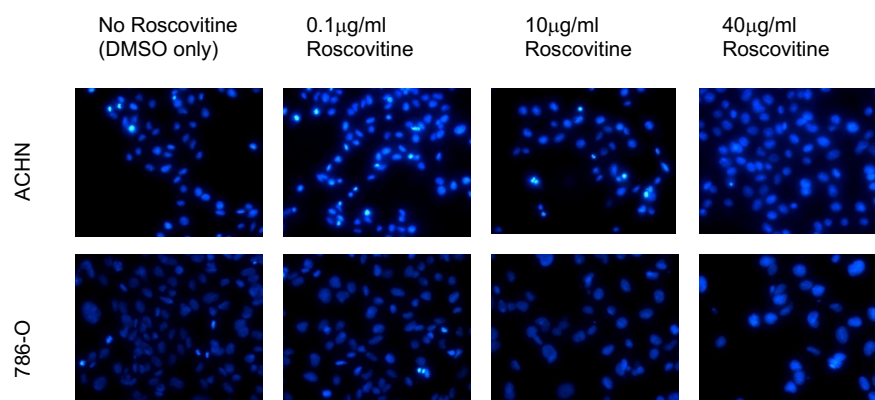


Fig. 2a : hoechst staining detects apoptosis in ACHN treated with 10 $\mu\text{g/ml}$ Roscovitine

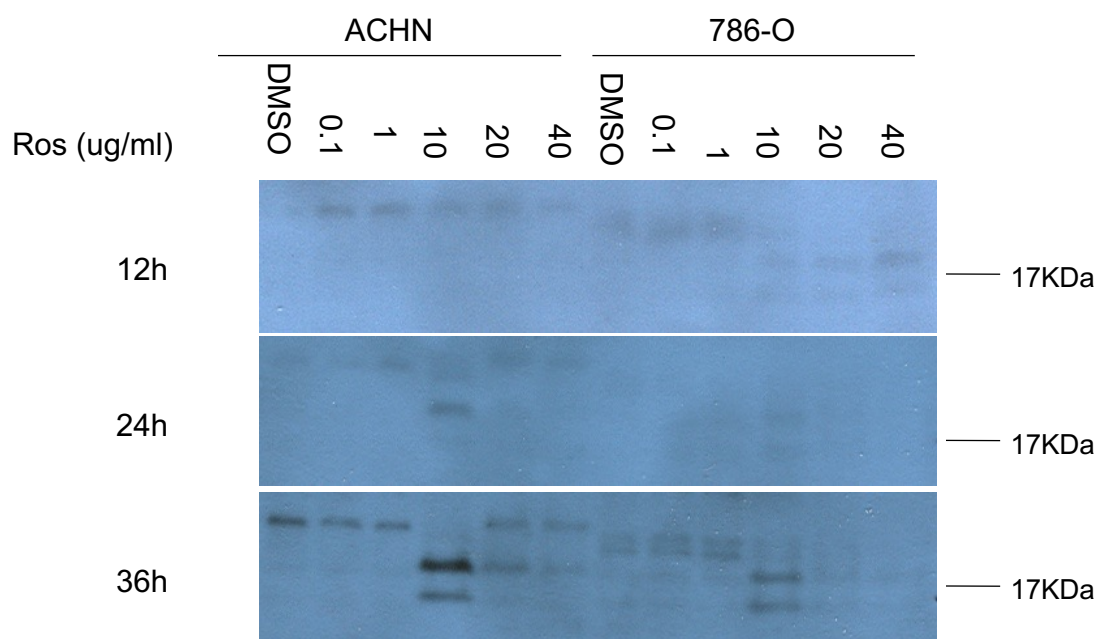


Fig. 2b Caspase-3 cleavage is pronounced in ACHN but is moderate in the 786-O

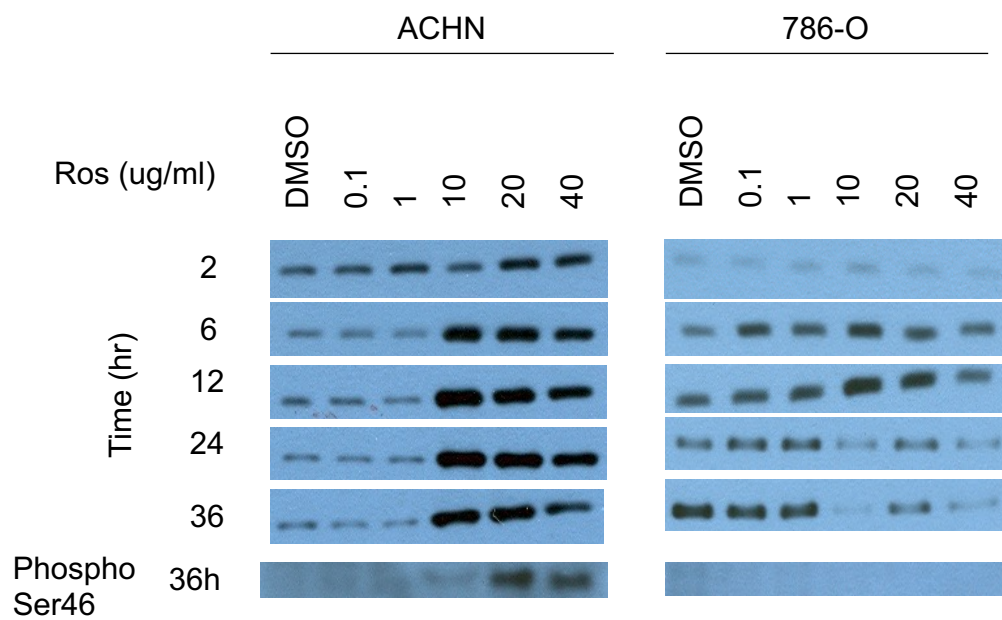


Fig. 3a Total p53 and phospho-Ser46-p53 time course in ACHN and 786-O upon Roscovitine treatment

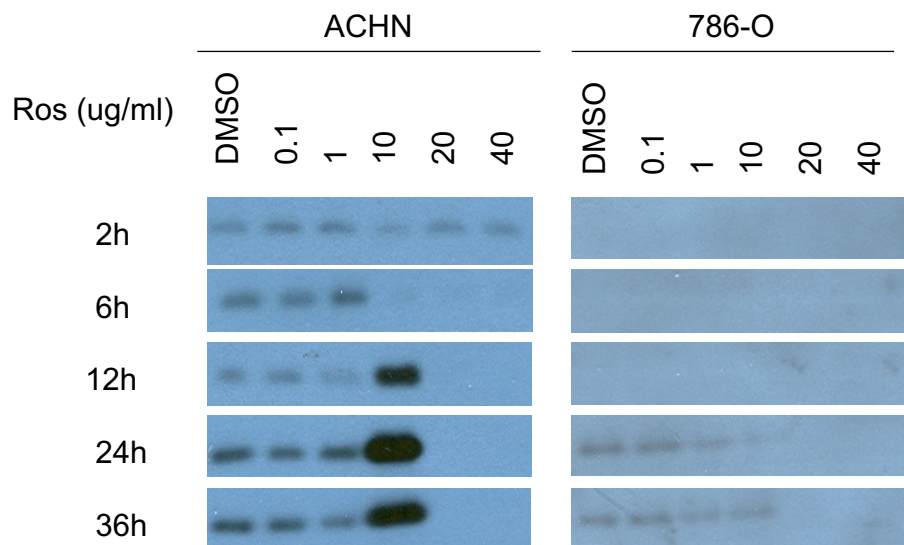


Fig. 3b Total p21 level reveals differential activation of p53 in ACHN and 786-O upon Roscovitine treatment

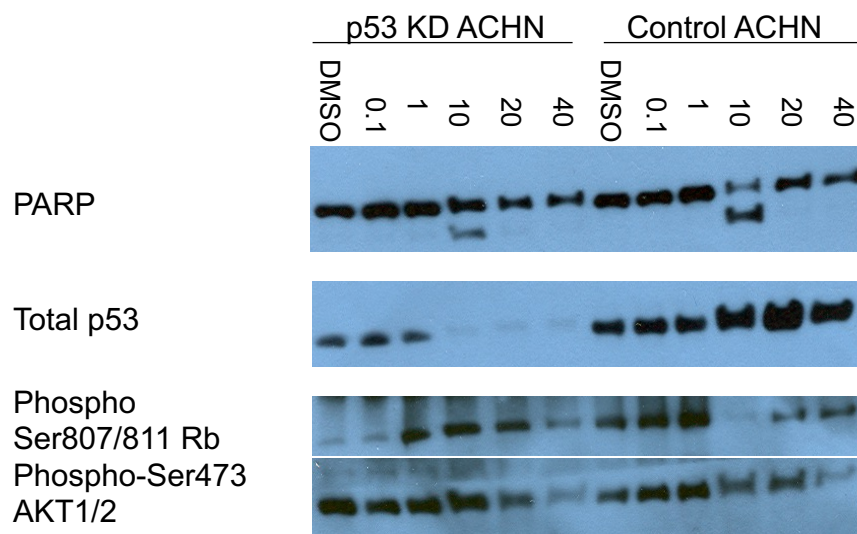


Figure 4 : Effect of p53 knockdown on 30h Roscovitine treatment on ACHN
 – p53 knockdown reduces apoptosis

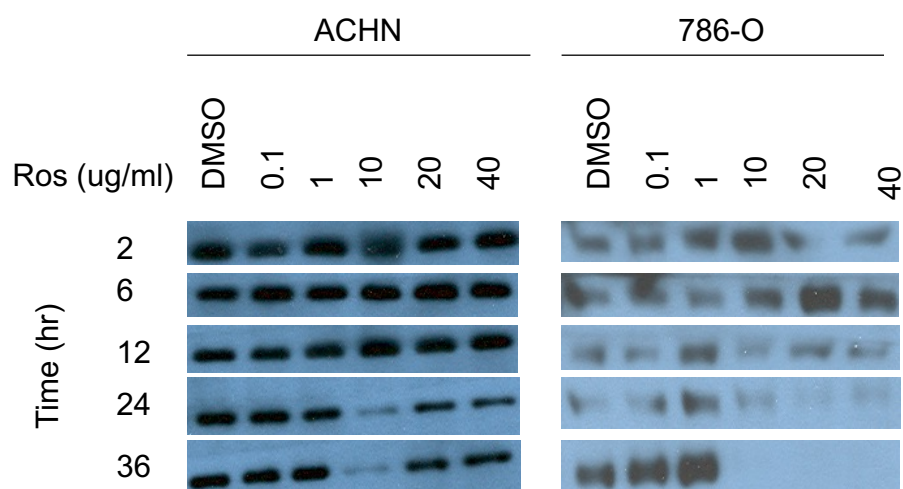


Fig. 5a Survival signals are decreased at times of maximal apoptosis in ACHN cells. AKT phosphorylation is reduced at times of maximal apoptosis in 786-O cells, although its pattern is distinct from that of ACHN

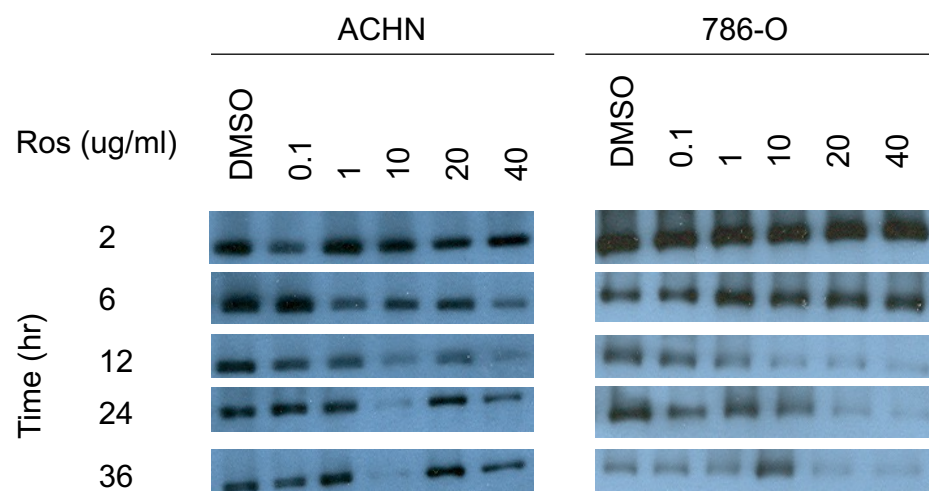


Fig. 5b Rb phosphorylation is decreased at times of maximal apoptosis in ACHN cells and 786-O

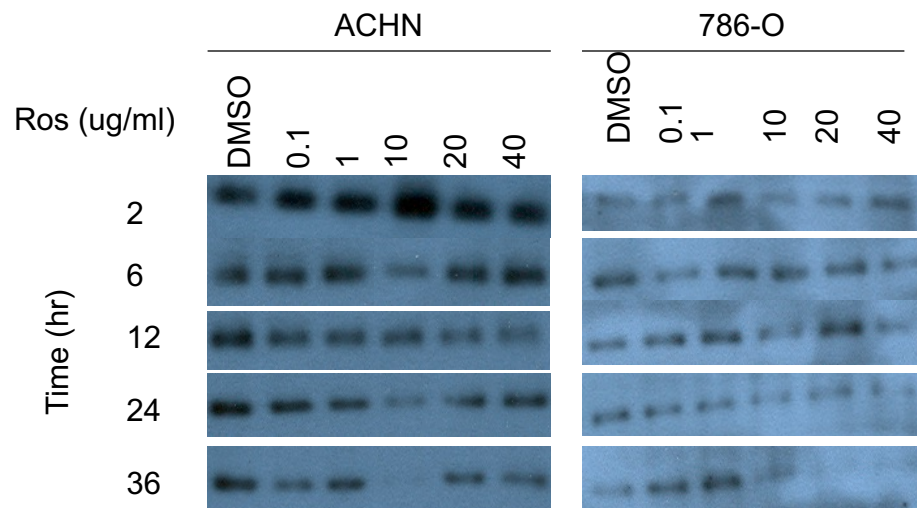


Fig. 5c XIAP is down at 10ug/ml Roscovitine in ACHN, while it is broadly reduced in 786-O by treatment with high Roscovitine concentration

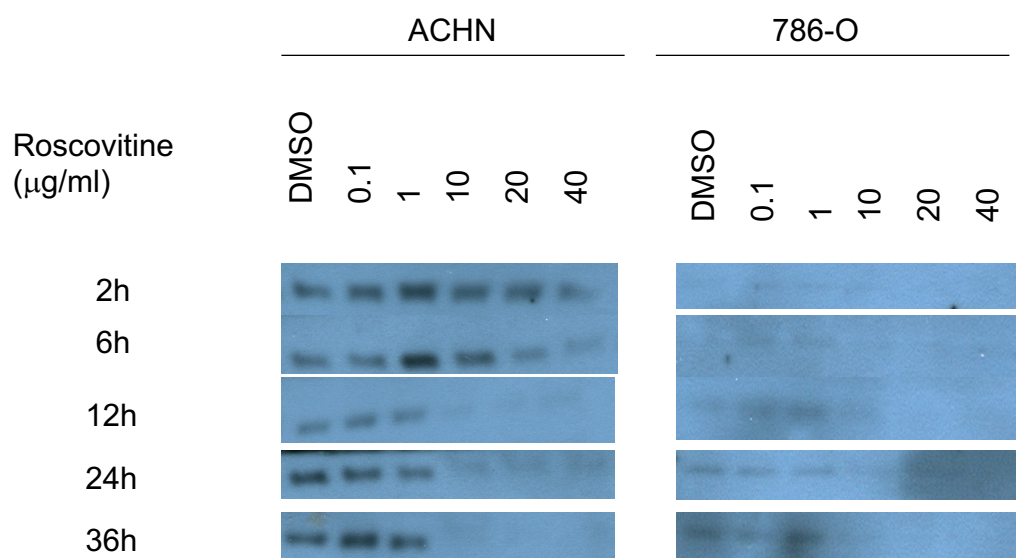


Fig. 6a Roscovitine inhibits CDK2 autophosphorylation and activation in ACHN and 786-O with similar time course and concentration dependency

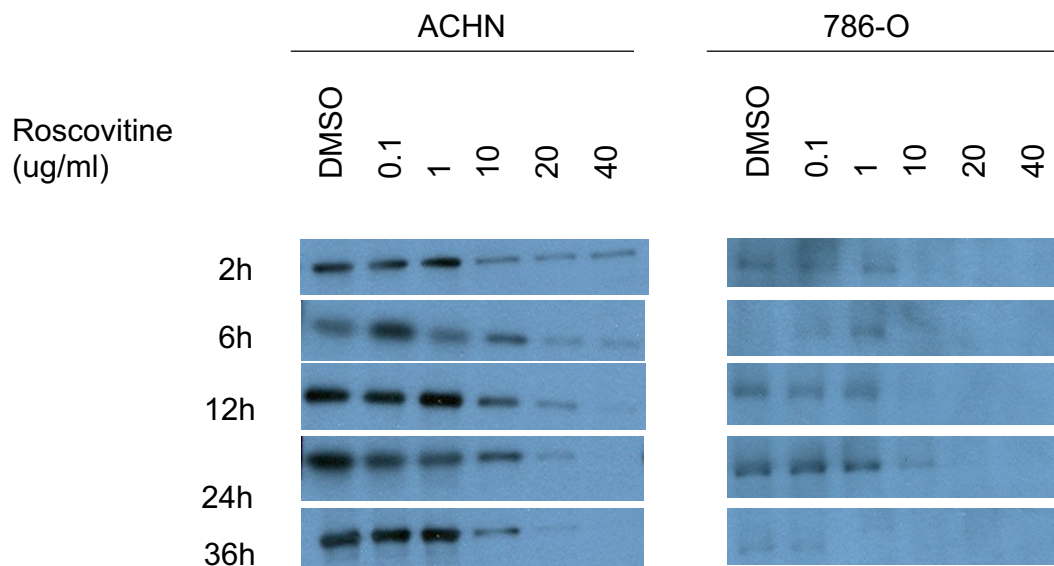


Fig. 6b Phosphorylation of Poll II by CDK9 is decreased at 10ug/ml and higher Ros as and results in the Pol II transcription inhibition. ACHN

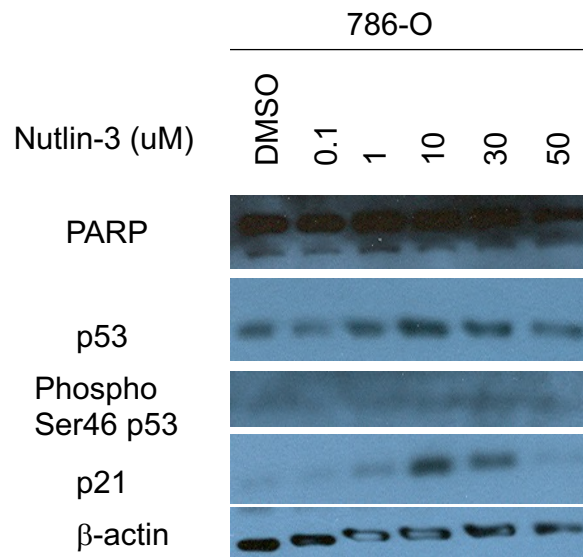


Fig.7a Nutlin-3 causes p53 stabilisation and p21 induction in 786-O *in vitro*

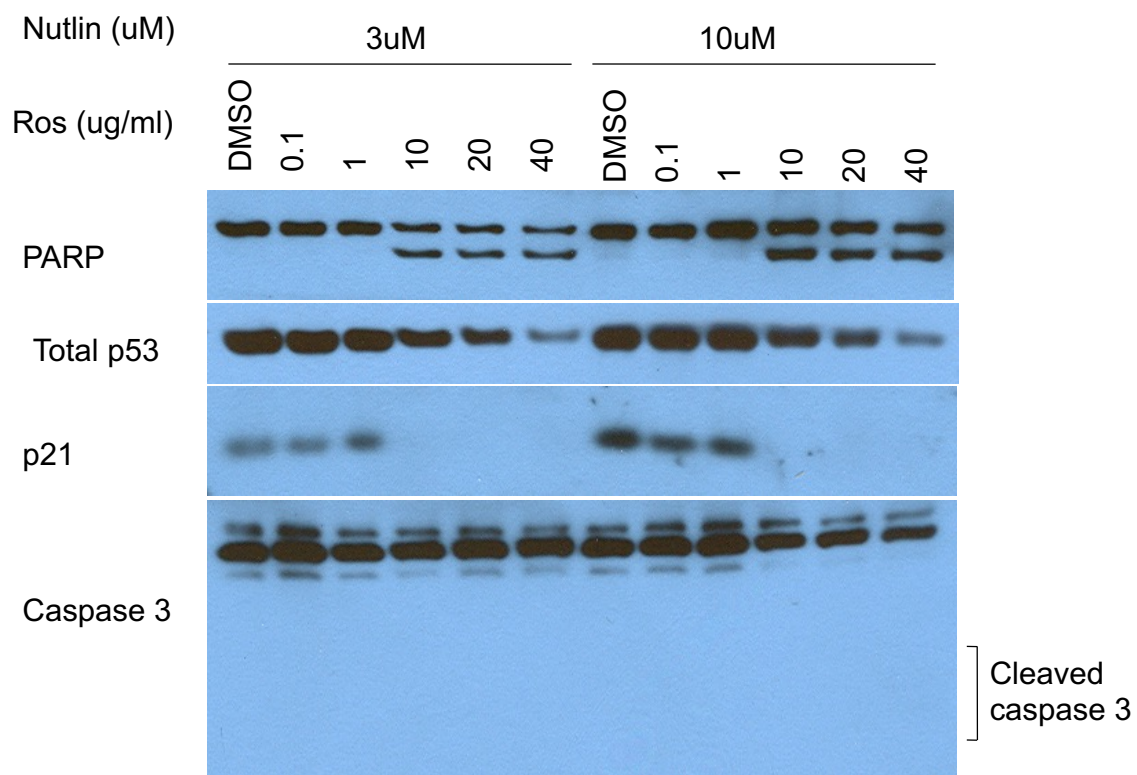


Fig.7b Nutlin slightly augment Roscovitine induced apoptosis in 786-O after 24h simultaneous treatment

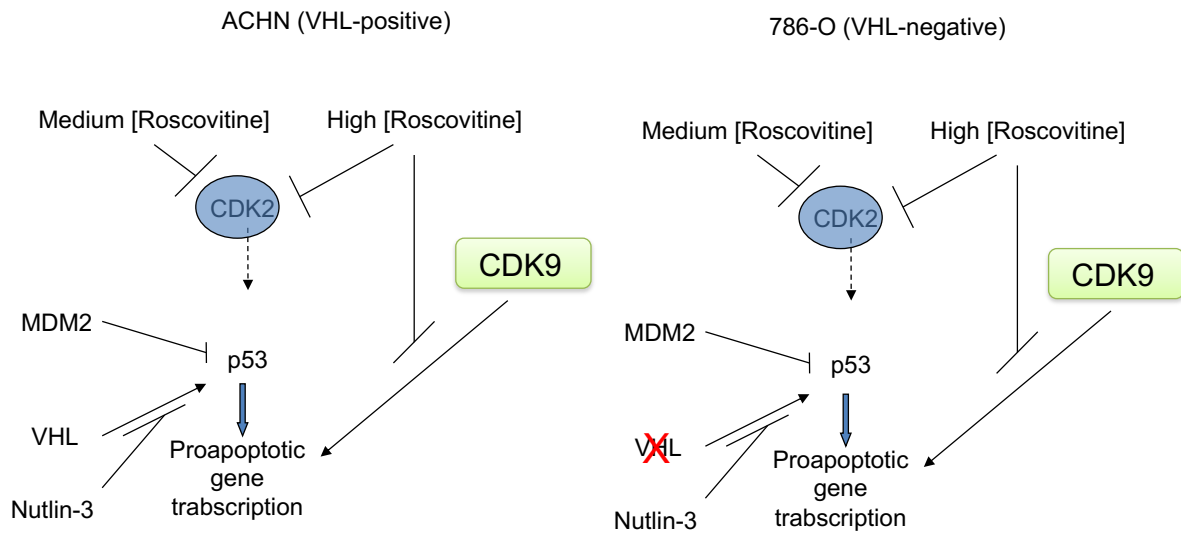


Fig. 8 VHL status of RCC influences response of RCC to Roscovitine

CHAPTER 3

Fuhrman Nuclear Grade-Defined ccRCC Proteome

Abstract

Kidney cancer is amongst the cancers most fatalities in the US and clear cell renal cell carcinoma (ccRCC) occupies 75% of the total cases. Biallelic inactivation of VHL renders ccRCC both hyper-angiogenic and resistant to conventional apoptosis-dependent chemotherapy. Fuhrman grades is a widely-used ccRCC prognostic classification based on nuclear morphology, but its subjective and coarse nature limits its precision and applicability. In order to identify novel ccRCC biomarkers and pharmacological targets. We undertook grade-defined proteomic analysis of ccRCC using formalin-fixed paraffin-embedded ccRCC samples. Our analysis identified 105 proteins that discriminated normal, low, and high Fuhrman grades with $p < 0.01$ in one-way ANOVA and 180 proteins with $p < 0.05$. A subset of the biomarkers were validated using Western blotting and immunohistochemistry. The enrichment analysis identified that biochemical pathways such as glycolysis, integrin signalling, and 5-hydroxytryptamine degradation were enriched in the grade-defined ccRCC biomarkers. Further improvements in detection and identification will likely aid discovery of additional graded-defining ccRCC biomarkers.

Introduction

Despite decades of medical advances, cancers still claim nearly 10,000,000 lives worldwide every year. Of these GLOBOCAN estimates that 180,000 deaths are caused by renal cell carcinoma (RCC) worldwide and 15,000 in US. [1] In the US, RCC is the 6th most common cancer among men and 8th most common cancer overall in US. While it ranks 12th in the number of cancer deaths, the total number of mortality due to RCC increased by 10% in US over the past decade. There are approximately twice as many cases in males than in females. Although many cancers have seen their mortality rates reduce drastically over the past decades, the RCC mortality rate reaches 7% in US and 15% overall worldwide. [1] RCC is composed of several subtypes including clear cell renal cell carcinoma (ccRCC), papillary RCC, and chromophobe RCC. Of these subtypes, ccRCC contributes to approximately 70% of the total cases. Genetically, ccRCC is characterised by the biallelic inactivation of VHL. VHL was originally identified as the E3 ubiquitin ligase [2] with tumour suppressor function, [3] but has since been shown to perform other functions such as the stabilisation of p53. [4] While the lack of HIF1 α E3 ubiquitin ligase activity was primarily thought to give rise to the ccRCC, the p53 chaperone activity of VHL is also likely to contribute to the oncogenesis. [4, 5] In the case of familial VHL syndrome, newborns inherit an autosomal dominant mutation in VHL gene. [6] Consequently, the affected individuals are prone to develop multiple neoplasms such as ccRCC and brain and spinal hemangioblastoma. [6, 7]

Our laboratory previously studied pan-ccRCC proteome in comparison to the normal kidney cortex. [8] The study elucidated 311 biomarker proteins that were differentially expressed between the ccRCC tissue and the normal tissue. [8] The proteins identified in the study

included six glycolytic enzymes (ALDOB, GAPDH, ENO2, PKM2, LDHA, and PCK2), six lipid metabolism enzymes (ECHS1, GATM, ANXA4, ANXA5, ACAT1, and FABP7), three protein folding chaperones (HSPB1, HSPCB, HSPD1), three enzymes involved in post-translational modifications (BHMT, ACY1, and ACAT1), two redox pathway enzymes (PRDX1 and AKR1A1), and two urea cycle enzymes (ASS1 and AGMAT). [8] Identification of several glycolytic enzymes as differentially expressed in ccRCC and normal tissue is in line with the Warburg effect. [9] Furthermore, representation of lipid metabolism, urea cycle, purine metabolism, and TCA cycle suggested global metabolic reprogramming, a hypothesis that was tested in a later study from our laboratory. [10]

In Perroud et al. (2006), the proteins were fractionated and isolated using two-dimensional (2D) gel electrophoresis before being identified with MALDI-TOF/TOF mass spectrometry. [8] This technique is relatively easy to implement in a laboratory, but lacks the resolution and sensitivity of such fractionation approaches as shotgun proteomics [11] In the few intervening years, the MALDI-TOF methods has become widely used in the proteomic analyses, allowing more sensitive and comprehensive proteomic analyses. This technological advancement also called for re-analysis of the ccRCC proteome.

As described in Chapter 1, the Fuhrman nuclear grading, first proposed in 1982 by Susan A Fuhrman and colleagues, has been the cardinal grading scheme for ccRCC prognosis over the past three decades since its publication. Based on the nuclear size, shape, and the presence of nucleoli as the grading criteria, the ccRCC samples are classified into four grades, in which the

higher grades are associated with poorer prognosis. [12] This grading, however, is crude and subjective, with inter-pathologist variations due to equipments and pathologists' experience. [13] Therefore, a more quantitative and objective criteria are desired.

In addition, conventional preservation and handling of patient specimens presented an additional challenge to the grade-defined proteomic characterisation of the ccRCC. Formalin-fixed paraffin-embedded (FFPE) samples have been the pathologists' choice of pathological sample preservation over the past century for its ease of handling and suitability to basic cytochemical staining for diagnostic purposes. This preservation method, however, cross-links proteins and renders it unsuitable for extraction and proteomic analyses. In order to utilise the vast archive of FFPE ccRCC tissues. Therefore, a novel extraction method was established and was compared to the fresh-frozen tissue-based proteomic analyses.

Aided by the recent technological advancement in untargeted proteomics, this chapter describes the characterisation of grade-defined ccRCC proteome, with the null hypotheses that 1) there is no difference in proteomic markers across grades, and 2) there is no difference in proteomic markers between normal and any grade of the ccRCC. Because Perroud et al. (2006) identified proteomic differences between the normal and the ccRCC tissues overall, it served as a reference and a positive control. First, a protocol to extract proteins from FFPE sections of human ccRCC specimens was established and validated against a patient matched frozen samples. Second, FFPE ccRCC sections for each Fuhrman nuclear grade and normal kidney cortex were procured and were analysed by mass spectrometry to identify grade-defining protein biomarkers. Overall, 105 grade-defining protein biomarkers were identified with $p < 0.01$ while 180 proteins were shown to distinguish between low and high grade ccRCC and the

normal tissue by their expression level as determined by the identified spectral counts. [14] The most dysregulated pathways based on the proteomic differences are also discussed.

A question that arises from this study is whether ccRCC progresses from a lower to higher grade, thus exacerbating its malignant phenotypes or each grade represents distinct and independent subtypes of ccRCC. While cancer grade transition is a contentious concept amongst the cancer pathologists and clinicians, several cases have been reported to support such a notion in other cancers. For example, in glioma, upregulation of VEGFR2 is necessary for progression of low-grade astrocytoma to high-grade glioma with concomitant increase in angiogenesis. [15] The transition from the low-grade to the high-grade transition is associated with change in gene expression profile such that the low grade is enriched in T-cell proliferation-related proteins, while the high-grade gene expression profile is related to mitosis. [16] In addition, extracellular matrix (ECM) modification-enzymes such as MMP2 and MMP9 were elevated in high-grade glioma, making them more prone to metastasis and aggressive tissue invasion. [17] Similar grade transition with concomitant gene expression changes has also been reported in the progression from intraductal papillary mucinous neoplasms to pancreatic ductal adenocarcinoma [18] and in prostate cancer. [19] In agreement with these studies, our data indicates incremental and grade-dependent changes in ccRCC proteome, which suggests such transition is likely in ccRCC, although gain-of-function and loss-of-function experiments must be conducted in ccRCC *in vitro* and *in vivo* to test such hypotheses.

The work presented in this chapter aims to establish methodologies for FFPE sample-based proteomics, characterise ccRCC proteomes in a Fuhrman nuclear grade-defined manner in order to identify novel grade-specific proteomic changes and drug targets as well as potential mechanisms underlying ccRCC pathogenesis and grade establishment.

Materials and Methods

Materials—After appropriate Institutional Review Board approval from University of California Davis, frozen and formalin-fixed paraffin-embedded (FFPE) clear cell renal cell carcinoma (ccRCC) tissues were obtained from the University of California Davis Medical Center in Sacramento, CA. The antibodies used in the study were mouse monoclonal anti-vimentin (VIM) (Dako), anti-SERPINH1 (Abcam), anti-phosphoglycerate kinase 1 (PGK1) (Santa Cruz Biotechnology), and anti-fructose-bisphosphate aldolase A (ALDOA) (Abnova) and rabbit polyclonal anti-ALDH1A1 (Abcam) and anti-AIFM1 (Abcam).

RCC Grading—All the FFPE RCC samples were sectioned and counterstained with haematoxylin and eosin (H&E), and all were re-graded by an experienced oncologic pathologist to standardize the grading of all specimens. The standard Fuhrman criteria of separation into four nuclear grades defined in order of increasing nuclear size, irregularity, and nucleolar prominence [12] were utilized.

Protein Extraction—For comparison between frozen samples and FFPE tissues, protein was extracted utilizing the Liquid Tissue Protein Prep kit (Expression Pathology, Gaithersburg, MD). For protein preparation from frozen tissue, RCC grading was done based on the Fuhrman nuclear grade scale on H&E-stained slides and compared with the frozen block. Pieces of tissue were cut off the block and boiled at 95 °C for 90 min in 20 µl of Liquid Tissue buffer (Expression Pathology). Subsequently 1 µg of trypsin was added, and the samples were digested overnight at 37 °C. The samples were spun at 12,000 relative centrifugal force (rcf) for 10 min, and the supernatant was transferred to a fresh tube. Protein preparation from FFPE tissues was performed with the Liquid Tissue Protein Prep kit (Expression Pathology) following the manufacturer's protocol.

For the grade-dependent proteomics experiments, the following protocol was used. Grading was done based on the FFPE sections counterstained with H&E. Unstained slides from adjacent sections were then deparaffinized and were used to collect regions of approximately 0.5 cm in diameter with an assigned nuclear grade. 10µm RCC FFPE sections were deparaffinized and hydrated through a xylene and ethanol series. The area corresponding to the adjacent H&E section was dissected using a 30-gauge needle and was transferred to 60 µl of RIPA buffer (150 mM NaCl, 10 mM Tris-HCl, pH 7.2, 2% SDS, 1% Triton X-100, 1% sodium deoxycholate, 5 mM EDTA). The sample was then heated at 100 °C for 20 min followed by a 3-h incubation at 80 °C with constant agitation to extract the protein. [20] The samples were chilled on ice for 1 min, and chloroform/methanol precipitation was then performed to remove salt and detergents. [21] After the addition of 40 µl of water, 400 µl of methanol was added, and after vortexing,

100 µl of chloroform was added. After vortexing, 300 µl of water was added. After a 1-min spin at 14,000 x g, the top aqueous layer was removed. Another 400 µl of methanol was added and mixed. After a 2-min spin at 14,000 x g, the supernatant was discarded, and the pellet was dried and suspended in 60 µl of 10 mM ammonium bicarbonate. Finally, samples were trypsin-digested overnight with 1 µg of sequencing grade trypsin (Promega). The protein concentration of the digested frozen and FFPE RCC samples was quantified using the microBCA Protein Assay Reagent kit (Pierce), and a total of 3µg from each sample was analysed by LC-MS/MS.

Immunoblotting—A section of tissue corresponding to what was used for LC-MS analysis was identified in an adjacent cut of the tissue block. These 10-µm FFPE sections were deparaffinized through a xylene and ethanol series, and 1 cm² of a tissue with known nuclear grade was harvested using a needle. The protein was decrosslinked and extracted using the QProteome FFPE protein extraction kit (Qiagen) according to the manufacturer's protocol. Immunoblotting was performed as reported previously. [22] Ponceau S staining was used to verify equal protein loading.

Immunohistochemistry—FFPE slides were deparaffinized through a xylene and ethanol series and were treated with 3% hydrogen peroxide, methanol prior to the antigen retrieval in sodium citrate buffer, pH 6. Following blocking, the slides were incubated with the primary antibodies at 4 °C overnight. After PBS washes, the slides were incubated with biotin-conjugated secondary antibodies followed by the avidin-biotin complex (ABC Elite kit, Vector Laboratories)

according to the manufacturer's instructions. The avidin-biotin complex was visualized using diaminobenzidine (Vector Laboratories). The sections were counterstained with hematoxylin and were coverslipped. Photographs were taken using 20x or 10x objective lenses on an Axiovert microscope (Carl Zeiss).

MS/MS Spectrometric analysis—The proteomic analyses were done by the UC Davis Proteomic Core. Briefly, MS/MS Spectrometric Analysis—Data were acquired using a Nano-LC-2D system (Eksigent) coupled with an LTQ (linear trap quadrupole) ion trap mass spectrometer (Thermo Finnigan) with separations accomplished on an in-laboratory fabricated fritless reversed phase microcapillary column (75 X 180 mm packed with Magic C18AQ, 3-mm beads with 100-Å pores; Michrom Bioresources) and vented column configuration. Each digested sample was transferred by the Eksigent autosampler to the on-line trap column (Zorbax 300SB C18, 5 X 0.3mm; Agilent) and desalted. Peptides were then eluted from the trap and separated by the aforementioned reversed phase microcapillary column at a flow rate of 300 nl/min and directly sprayed into the mass spectrometer. Buffer compositions used for reversed phase chromatography were as follows: buffer A, 0.1% formic acid in water; buffer B, 0.1% formic acid in 100% acetonitrile. Peptides were separated with a 48-min gradient (2–40% buffer B for 95 min, 40–80% buffer B for 12 min, and 80% buffer B for 13 min). MS/MS of the top 10 most intense ions was accumulated on the Thermo LTQ during each run. Peak lists were generated using the software Bioworks 3.3. The data were then analyzed with X!Tandem version 2008.01.01.1 and Ensembl human database version 49.36K (December 2006; 47,648 entries) based on human assembly NCBI 36 (October 2005). X!Tandem parameters were standard

(fragment ion mass tolerance of 0.40 Da and a parent ion tolerance of 1.8 Da; iodoacetamide derivative of cysteine was specified as a fixed modification). Deamidation of asparagine and glutamine, oxidation of methionine and tryptophan, sulfone modification of methionine, tryptophan oxidation to formylkynurenine of tryptophan, and acetylation of the N terminus were specified as variable modifications. A threshold of $-\log(\text{Expect scores}) = 2.0$ was used as a filtering criteria for X!Tandem; this is a standard value that is suitable for analysis with secondary statistical filtering such as clustering and enrichment calculation. Finally, results were imported into the software Scaffold (version 2_00_02; Proteome Software Inc., Portland, OR) for protein identification validation, normalization, and comparison of spectral count or occurrences (that is, the number of all redundant peptide hits for a given protein). Peptide identifications were accepted if they could be established at greater than 95.0% probability as specified by the Peptide Prophet algorithm [23] Proteins were assigned by the Protein Prophet algorithm, [24] and identifications were accepted if they could be established at greater than 95.0% probability and contained at least two identified peptides. Proteins that contained similar peptides and could not be differentiated based on MS/MS analysis alone were grouped to satisfy the principles of parsimony. The peptide false positive rate (FPR) was calculated using the Scaffold software. For each charge state, the incorrect assignments are tabulated to calculate the FPR_i using the following formula: $\text{FPR}_i = ((\text{Number assigned incorrect at 95\% probability}) / (\text{Total number incorrect assigned})) \times 100$ where i is the charge state. The assignment is called correct if it is associated with a protein that has a 95% probability according to the Protein Prophet algorithm [25] and a minimum of two peptides each with a

95% probability based on the Peptide Prophet algorithm. [26] The FPR is the sum of the values for each charge state.

Statistical and Pathway Analysis—To assess accuracy of protein quantitation, results of proteomics analysis of 10 samples per grade were averaged, and a coefficient of variance was calculated for each identified protein. Significant grade-dependent differentials were filtered with a one-way analysis of variance (ANOVA) [26, 27] using thresholds of F-distribution cumulative probability of 0.05 or 0.01 (two standard filtering thresholds). Statistically significant proteins were analyzed for molecular function, molecular process, and pathway enrichments using the Panther tools [28, 29] and Gene Ontology. [30, 31] Network and metabolic pathways were analysed using KEGG mapper [32] and Ingenuity Pathways Analysis (IPA) (versions 6 and 22; Ingenuity Systems Inc., Redwood City, CA).

Results

Comparison of frozen tissue samples and FFPE tissue samples for proteomic analysis

In order to preserve morphology which provides a wealth of information to clinicians, most of pathological samples are quickly fixed in formalin and embedded in paraffin, which allows preservation at room temperature for decades. As such, use of FFPE samples, unlike that of

frozen samples, would allow one to analyse samples from more patients collected over the past decades. Formalin, however, extensively modifies proteins through crosslinking ϵ -amino group of lysine with themselves or other amino groups present in DNA and RNA. [33] These covalent modifications alter the molecular weight of trypsin-generated polypeptide fragments whose fixed m/z values are fundamental to protein identification through mass spectrometry. At the time our grade-defined ccRCC proteomic analysis was performed, extraction of semi-comprehensive proteome from FFPE samples was still in its infancy, [34] which necessitated us to develop an in-hour protocol.

Despite being in its nascent stage, some methods had already been developed and commercialised including Liquid Tissue Protein Prep kit by Expression Pathology, Gaithersburg, MD. Comparison of proteomic analysis using the patient- and grade-matched frozen and FFPE ccRCC specimens from three patients produced a similar magnitude of proteome description from both the frozen and the FFPE samples. [14]. A total of 180 proteins were detected and identified. Of the 180, 126 proteins were present in both frozen and FFPE samples, while the frozen samples contained 42 proteins unique to the group and the FFPE samples produced 17 proteins unique to that group. No significant differences were observed in Panther molecular function analysis, although several proteins showed differential distribution between the fresh frozen and FFPE samples. Among them, 5 proteins, or less than 3% of the proteins detected, were detected at statistically significant levels between the frozen and FFPE samples. These proteins include HSPA8, ANXA4, TUBA1A, TUBB2C, and YWHAB, and were present at higher levels in the FFPE samples than in the frozen samples. Gene Ontology (GO) analysis of these proteins indicates that intracellular vesicles, in particular the extracellular exosome derived

from the vesicular transport is differentially represented, (**Table 1**) which suggests that FFPE samples may be a preferred source of exosome analyses. Overall, this analysis demonstrated that FFPE samples can satisfactorily be used for proteomic analysis in lieu of the conventional fresh samples. Due to cost considerations, we developed an in-house protocol for protein extraction as described in the Materials and Methods as described above, and further analysis was done according to the protocol.

Proteomic analysis of grade-defined FFPE ccRCC tissues

Using the protocol developed above followed by shotgun proteomics, we analysed 50 FFPE ccRCC samples for their proteomes. The 50 samples included 10 samples each from each of the four Fuhrman nuclear grades as well as the normal kidney cortex where the ccRCC is thought to originate from. [36] Although most samples came from different patients, FFPE sections from four patients contained a single Fuhrman grade and a normal kidney cortex tissue or tissues with two Fuhrman grades. In such instances, only the areas containing either the normal or one of the Fuhrman grades were scraped to obtain the samples for the proteomic analysis.

Overall, a total of 1,470,313 spectra were detected for analysis, resulting in the identification of 777 unique proteins which were represented with at least 2 peptide fragments. The average normalised spectral counts are shown in **Table 2**. [14] Of the 777 proteins identified, 105

showed differential distribution that is statistically significant with $p < 0.01$ using a one-way analysis of variance (ANOVA). (**Table 3**) This number increased to 180 proteins when we considered $p < 0.05$. (**Table 4**)

In order to quantify the protein levels, normalised spectral counts from 10 samples in a chosen grade were averaged. Each protein spectral count was normalised across the 50 samples using the total spectral counts for each sample. Furthermore, four proteins were used as endogenous internal controls. These proteins included Myosin Heavy Chain-9 (MYH9), Tubulin β Chain, Histone H4 (H4C1), and Peptidylprolyl Isomerase A (PPIA). The intra-grade averages of each of these proteins were used to normalise the raw spectral counts in order to compare samples across Fuhrman grades and against the normal samples. **Figure 9** illustrates the normalised spectral counts of four proteins for each of the 50 samples grouped into the normal and the four ccRCC grades. [14] As noted above, TUBB was one of the four proteins with least inter-grade variability. Yet it can clearly be seen that within a given grade, the number of identified spectral counts assigned to TUBB can vary widely. A similar magnitude of variability is seen in the other three proteins, namely Alanine Aminopeptidase (ANPEP), Lactate Dehydrogenase A (LDHA), and Carnosine Dipeptidase 2 (CNDP2) whose spectral counts were shown to vary across grades and can be used to distinguish between two or more grades and the normal tissue. One-way ANOVA ($p < 0.01$) indicated that these three proteins were differentially present between ccRCC with two or more Fuhrman grades, or between one or more of the ccRCC Fuhrman grades and the normal tissue.

In order to test the validity of the LC/MS-based shotgun proteomic analysis, We examined the inter-grade protein distribution of select proteins by Western blotting (**Figure 10** and [14]) and immunohistochemistry. (**Figure 11** and [14]) The FFPE ccRCC samples obtained from the UCDMC had been fixed and embedded by different personnel at different time points spanning two decades, which resulted in different degree of crosslinking and other chemical modifications that may interfere with the antibody-based detection such as the Western blotting and immunohistochemistry. To account for and minimise these technical variabilities, we uncrosslinked and extracted total proteins from four FFPE samples each of three grades including Normal, Grade 1 (low grade), and Grade 3 (high grade). Two criteria were applied to select the biomarkers to validate the proteomic analysis by immunoblotting. First criterion was based on the manufacturer's recommendation for use of detection of the target protein in paraformaldehyde (PFA)-fixed samples. The second was the statistically significant difference in the normalised spectral counts between their normal, the low Fuhrman grades (grade 1 and 2), and the high grades (grade 3 and 4). Based on these criteria, four potential ccRCC Fuhrman nuclear grade biomarkers were chosen as the target protein for the Western blotting-based validation. These included Aldolase A (ALDOA), Apoptosis Inducing Factor Mitochondria Associated 1 (AIFM1), Aldehyde dehydrogenase 1 Family Member 1A (ALDH1A1), Phosphoglycerate Kinase 1 (PGK1). Four samples each were randomly chosen from the normal, grade 1, and grade 3. Immunoblotting on the protein extract from each of these samples showed trends that approximately matched that shown in the proteomic analysis. ALDOA, which belongs to the UP class of the ccRCC grade-defined biomarkers in the proteomic analysis

(**Figure 2** and **Table 3**) showed increase in three out of four samples from Grade 1 and Grade 3 compared to the normal. Interestingly, amongst the 8 tumour samples. The Western blotting signal intensity roughly positively correlated with the normalised spectral counts, further validating the accuracy and precision of the proteomic analysis. AIFM1, which showed high fragment counts from the normal but essentially no counts from the tumour samples, thus belonging to the DOWN class, showed high counts in 3 out of 4 normal samples. (**Figure 2** and **Table 3**) On the other hand, all 8 tumour samples showed very low AIFM1 protein expression in the immunoblotting. ALDH1A1 belongs to the UPG1 class of the ccRCC biomarkers in which its expression is elevated in the low grade G1 and G2 samples while normal and high grade ccRCC samples showed lower expression in the proteomic analysis. (**Figure 2** and **Table 3**) In agreement with this, the immunoblotting showed extremely high expression levels in two out of four G1 samples. These two samples also showed high normalised spectral counts compared with the other two G1 samples used in the immunoblotting, which demonstrates the validity and sensitivity of the proteomic analysis. Finally, PGK1 showed elevated signals in the Western blotting in two out of 4 G1 samples and 1 out of 4 G3 samples while all normal samples had very low signal. This is in agreement with the proteomic analysis which showed that PGK1 belongs to the UP class of the ccRCC biomarkers. (**Figure 10** and **Table 3**)

Of note, there is a high degree of concordance in the normalised spectral count in the proteomic analysis and the relative intensity of the Western blotting signal from the corresponding sample. For example, the two G1 samples and one G3 samples with high anti-PGK1 immunoblotting signal also produced the highest normalised spectral count amongst the 12 samples in the proteomic analysis, further validating our proteomic analysis.

It should be noted that a large intra-grade variability exists which may be due to technical variabilities such as varied time and treatment between the sample collection and fixation, varied degree of crosslinking as well as variable time elapsed since the crosslinking and paraffin embedding.

Furthermore, the current grade-defined ccRCC proteomic analysis was largely in agreement with and confirmed the previous ccRCC proteomic analysis done in the Weiss lab, which identified 31 ccRCC biomarkers albeit in a grade-independent manner using 2D-gel electrophoresis and mass spectrometry. [8] Of the 31 previously identified ccRCC biomarkers, 18 proteins were also identified in the current analysis. These are summarised in **Table 5**. They include 3 biomarkers that are upregulated in all grades compared with the normal tissue, 12 that are downregulated in all Fuhrman grades in comparison to the normal tissue, and 3 proteins that are upregulated in low grades but show no statistical changes in the high Fuhrman grades relative to the normal tissue. It is noteworthy that all three proteins upregulated across all grades relative to the normal tissue are glycolytic enzymes. This is in agreement with the predictions from the Warburg effects. [9] It should be noted that several protein biomarkers identified in the initial gel electrophoresis-based study were not included in the current mass-spectrometry-based grade-defined ccRCC biomarker studies. These are indicated as Not identified in Table 5. This is surprising, given that the mass spectrometry is expected to have higher sensitivity than the gel electrophoresis. A potential reason for this is that although the peptide fragments from these proteins were detected in the current study, their m/z value was not unique to the fragment and thus was not able to be positively identified.

The proteomic analysis was further validated using immunohistochemistry. **(Figure 11)** The biomarkers were chosen based upon similar criteria as those used for the Western blotting validation discussed above. That is, 1) the biomarkers distinguished the normal, low grades, and/or high grades in the proteomic analysis, and 2) the antibodies had proven and published specificity to the protein in immunohistochemistry on PFA-fixed sections or was tested in house and proven to be specific to the protein antigen based on the Western blotting analysis on normal and RCC cell line lysates. While the immunohistochemical analysis had a general trend to agree with the proteomic analysis, variations in subcellular localisation made it hard to test, with certainty, the validity of the proteomic analysis. The grade-dependent differential subcellular localisation of some grade-defining ccRCC biomarkers, however, infers that protein moonlighting, a topic that will be discussed in detail in Chapter 5, plays a significant role in the ccRCC pathogenesis. [37, 54]

To summarise three instances of immunohistochemical analyses, Vimentin, which is an intermediate filament protein, showed increase in its expression across all ccRCC grades in comparison with the normal tissue **(Figure 11)**. In normal tissues, less than 10% of the cells are vimentin-positive; due to the lack of double immunohistochemistry with defined cell type markers, we were not able to elucidate the identity of the vimentin positive cells, although their location and abundance suggest that they are peritubular capillary cells or infiltrating macrophages, the latter of which is known to express and secrete Vimentin. [38] On the other

hand, the Vimentin immunohistochemistry on the ccRCC tissues showed a reticular pattern across all grades. (**Figure 11**) Based on their morphology, abundance, and location, these Vimentin positive cells are likely to be present in the cytoplasm of the ccRCC cells, although it is impossible, without double immunohistochemistry with defined cell markers, to exclude the possibility that these are hyperproliferative activated macrophages. Notably, grade 4 tissues show less reticular pattern of Vimentin positivity. Given that grade 4 ccRCC cells show the most aggressive metastatic potential, these Vimentin positive cells are expected to be the ccRCC cells, although further validation through double immunohistochemistry with a defined ccRCC marker is lacking.

AIFM1 is a mitochondrial-associated protein which is involved in apoptosis induction. AIFM1 showed well-defined grade-dependent spectral distribution in the proteomic analysis in which AIFM1 was highly detected in the normal tissues but was hardly present across all the ccRCC grades. In agreement with this, AIFM1 immunohistochemistry showed strong positive cytoplasmic staining in a majority of the proximal tubule epithelial cells in the normal tissues but was significantly reduced in all Fuhrman grades. (**Figure 11**)

Lastly, SerpinH1 expression was tested by immunohistochemistry. SerpinH1 is a serine protease inhibitor localised in the endoplasmic reticulum [39] and is involved in collagen biosynthesis. [40] In the proteomic analysis, it shows upregulation in all ccRCC grades compared to the normal tissues. (**Table 3**) Immunohistochemical analysis on the normal tissues show SerpinH1

positivity in the cytoplasm of a subset of tubular epithelial cells. It remains to be determined whether these tubular cells are of proximal or distal tubule origin. The immunohistochemical validation shows, however, only negligible to modest increase in grade 3 compared to the normal tissue while shows recognisable increase in grade 2 and more robust increase in grade 4. The renal tubule epithelium is moderately positive in the normal tissue. Grade 2 tissues show reticular distribution while the Grade 4 tissues show more robust cytoplasmic expression. Due to the lack of dual immunohistochemistry with cell-type specific markers, it is impossible to ascertain the identity of the Serpin H1 cells in the ccRCC tissues, but their morphology suggests that they are ccRCC cells.

In addition, six proteins identified in the current proteomic analysis were examined by immunohistochemistry for their validity as grade-defining ccRCC biomarker. These biomarker proteins include NDRG1, ENO1, PGK1, CNDP2, ALDOA, and ALDH1A1. They, however, showed grade-dependent differential subcellular distribution in addition to the grade-dependent expression level changes, suggesting that they may act as multifunctional moonlighting proteins. Therefore, their grade-defined immunohistochemistry will be discussed in Chapter 5.

One of the defining characteristics of a Fuhrman nuclear grade is the nucleoli. Their presence in higher grades and absence in lower grades is a cardinal criterion in the Fuhrman nuclear grading. [12] The size of the nucleoli increases in higher grades compared to a lower grade. Nucleoli are the subnuclear organelles that are responsible for such functions as ribosomal RNA

synthesis, ribosome assembly, RNA pol II transcription, stress response, and apoptosis. [41] The subnuclear organelle can be further divided into three compartments; the fibrillar centre, the dense fibrillar component, and the granular component, each with defined functions and biomarkers. [42, 43] Because the nucleolus is a defining feature of the Fuhrman nuclear grades, [12, 44] its component proteins are hypothesised to be among the grade-defined ccRCC biomarkers. Our proteomic studies identified at least two nucleolar proteins as grade-specific ccRCC biomarkers. One protein, Nucleophosmin, in particular had a statistically significant changes across all grades except for G1 when compared to the normal tissues. (**Table 2**, [14]) Due to the availability of specific and fixative-resistant antibodies, Western blotting was performed against Nucleophosmin to further confirm the proteomic analysis. (**Figure 12**) It showed no difference between the normal and the lowest grade, but its expression level was significantly increased in the higher grades as assessed by pair-wise t-test ($p < 0.0025$). [14] There is a moderately positive correlation between the increase in grade and that in the Nucleophosmin level despite intra-grade sample variability.

PANTHER analysis of ccRCC grade-defined proteome

A large subset of the biomarkers identified in the original analysis fell under the categories of dehydrogenases, which catalyses reduction-oxidation reaction such as biochemical conversion of hydrogen to hydroxyl to carboxyl group via carbonyl group. [14] This series of reactions are prevalent in the cellular metabolism with enrichment in the catabolic metabolism, which

suggest that proteomic reprogramming identified through the current work results in metabolic reprogramming, which correlates with and perhaps underlies the Fuhrman nuclear grading and Warburg effect. This hypothesis was later tested by our group and was reported in a separate publication. [10] The current proteomic analyses identified proteins that do not fall under the category of the dehydrogenases, thus, an updated enrichment analysis was expected to yield further insight into the ccRCC pathobiology. In order to perform enrichment analysis, the identified grade-defining proteins were analysed using PANTHER database. [28] Refinement in the Gene Ontology / Panther database since has allowed further in-depth analysis of the dataset. [29]

Our proteomic analysis identified 105 biomarkers that discriminated normal, low ccRCC grades, and high ccRCC grades. (**Table 3**) These 105 grade-defining biomarkers are enriched in Binding and Catalytic activity categories of GO Slim molecular functions. The former is suggestive of the correlation between the grades and the metastatic potential, while the latter suggests metabolic reprogramming as an underlying cause of the Fuhrman nuclear grade. In agreement with the molecular function analysis above, the protein class enrichment analysis showed enrichment in the class of metabolite interconversion enzyme, again suggesting the correlation between the Fuhrman nuclear grade and metabolic reprogramming. Glycolysis, integrin signalling, and 5-hydroxytryptamine degradation are the top 3 pathways enriched in the ccRCC grade-defining biomarkers. There are 8 ccRCC grade-defined biomarkers in Glycolysis pathway which includes GPI, PFKL, ALDOA, ALDOB, ALDOC, PGK1, ENO1, and ENO2, while integrin signalling pathway contributes 6 biomarkers including LAMA5, ACTN4, FN1, COL4A1, COL4A2,

and COL18A1. Further 5 ccRCC biomarkers belong to the 5-hydroxytryptamine degradation pathways; these include ALDH1A1, ALDH2, ALDH4A1, ALDH7A1, and ALDH9A1. (**Table 3, Figure 13a-c**)

Gene Ontology (GO)-based Panther analysis of the 180 biomarkers with $p < 0.05$ showed the same general trend as that done on the 105 biomarkers with $p < 0.01$. (**Table 4, Figure 13d-f**) This is in part because the latter is a subset of the former. Briefly, binding (GO: 0005488) and catalytic activities (GO: 0003824) are the most represented categories of Go-Slim molecular function. Greater than 50% of the biomarkers belong to the class of Metabolite interconversion enzymes (PC00262), although protein modifying enzyme and extracellular matrix proteins also are moderately represented. Amongst the pathways, glycolysis (P00024), integrin signalling pathway (P00034), and 5-hydroxydegradation pathway (P04372) are highly represented. 9 Glycolytic enzymes detected and classified as a ccRCC biomarkers with $p < 0.05$ include GAPDH, GPI, ENO1, ALDOA, ENO2, ALDOB, PFKL, ALDOC, and PGK1. 10 integrin pathway proteins determined as ccRCC biomarkers include LAMA5, VLC, ACTN4, MAPK1, FN1, COL1A1, COL4A1, COL4A2, COL14A1, and COL14A2. The 7 ccRCC biomarker proteins classified under the 5-hydroxytryptamine degradation pathway include ALDH1A1, ALDH1B1, ALDH2, ALDH4A1, ALDH7A1, ALDH9A1, and MAOB.

Our proteomic analysis showed that the biomarkers can be further classified into several classes using self-organising maps. [14] These classes are DOWN where the protein is

downregulated in all grades compared with the normal tissue, UP where the biomarker showed higher normalised spectral counts in all Fuhrman grades compared with the normal, but there was no statistically significant differences amongst the four Fuhrman grades, and G1 up where the spectral counts were significantly higher in low (G1 and G2) grades but no statistically significant differences were observed between the normal and the higher two (G3 and G4) grades. These are referred to as subclasses below.

In order to identify Panther molecular functions and pathways specifically up- or downregulated in each subclass, each of the three well-represented subclasses of ccRCC grade-defining biomarkers was analysed for enrichment. 66 out of 105 ccRCC biomarkers with $p < 0.01$ belong to the DOWN class where the protein was down regulated across all ccRCC grades compared with the normal tissue. (**Table 3, Figure 13g-i**) 42 proteins or over 60% of the biomarkers in this subclass belonged to Catalytic Activity category and further 13 biomarkers were classified into Binding category in the molecular function analysis. Protein class analysis showed enrichment with metabolite conversion enzymes (PC00262) and extracellular matrix protein (PC00102) categories being most represented, which approximately agrees with the analysis with all the ccRCC biomarkers. (**Figure 13b and 13e**) Pathway analysis, however, showed striking differences between the pan-ccRCC biomarker analysis and that of the DOWN subclass. For example, while Integrin signalling pathway (P00034) and 5-hydroxytryptamine degradation (P04372) were as highly represented in the DOWN subclass analysis as was in the pan-ccRCC biomarker analysis, glycolysis was hardly represented in the DOWN subclass. This agrees with the notion of Warburg effect or aerobic glycolysis in which the cancers rely more

heavily on the glycolysis for their energy production compared with their normal tissue counterparts.

Next, the UP subclass of the ccRCC biomarkers, which included 21 proteins, were analysed for their enrichment by the Panther database. (**Table 3, Figure 13j-l**) As was the case with the pan-ccRCC biomarkers and the DOWN subclass, UP subclass was enriched in binding (GO: 0005488) and catalytic activity (GO: 0003824). Interestingly, unlike the DOWN subclass, UP subclass was heavily enriched in binding category, while the representation from the catalytic activity was only moderate. Several protein classes were enriched in the UP subclass of ccRCC biomarkers, including metabolite interconversion enzyme (PC00262), intercellular signalling molecule (PC00207), and chaperones (PC00072) are the most represented. This is in agreement with the previous reports that cancers including ccRCC undergo metabolic reprogramming [10, 47] and exhibit unfolding protein response, [48] both of which require intercellular signalling. [49] Pathway enrichment analysis with the 21 UP biomarkers showed that the subclass is enriched in glycolysis. This is in agreement with the above analysis of the pan-ccRCC biomarkers and that of the DOWN biomarkers where the glycolytic proteins were largely lacking the representation. Unlike the DOWN subclass, there were only one component of the integrin pathway present in the UP ccRCC biomarkers, namely FN1.

UpG1 group comprises the third and the final subclass of ccRCC biomarkers with significant representation in the current study. 16 biomarkers identified in the present study belong to this

subclass. (**Table 3, Figure 13m-o**) The molecular function enrichment analysis showed, as was the case in the other two subclasses, the UpG1 subclass is enriched in catalytic activity (GO:0003824) and binding (GO: 0005488). Metabolic interconversion enzyme (PC00262) is the most represented protein class, followed by protein modifying enzyme (PC00260). Taken together with the analysis of UP and DOWN subclasses of ccRCC above, this suggests that metabolic reprogramming in ccRCC is a two-step process, with the first metabolic reprogramming associated with progression to low grade ccRCC, while the second results in the high grade ccRCC. Abnormal recruitment of protein modifying enzymes may underly the pathogenesis of low grade ccRCC. Glycolysis is the most represented pathways in the UpG1 subclass with GPI and PFKL included in this category, although other known cancer-related pathways such as pentose phosphate pathway was moderately represented.

Finally, IDH2 was the lone member of UpG3 subclass, which showed upregulation in high grades compared to the normal and low grade ccRCC. Due to the paucity of biomarkers, no further PANTHER analysis was performed on this subclass.

KEGG pathway analysis of ccRCC grade-defined proteome

In order to graphically summarise the proteomic differences and understand the pathobiology behind the grade-dependent differences in ccRCC proteome through pathway analysis, the grade-defining markers were mapped on KEGG map. [Figure 14a-e] Enzymes highlighted in red indicates those whose levels were increased across all ccRCC grades compared to the normal tissue, while blue indicates proteins downregulated in all ccRCC grades. Proteins upregulated in low ccRCC grades (Grade 1 and Grade 2) but were comparatively low in normal and high grade ccRCC are highlighted in yellow.

The overall proteomic reprogramming is summarized in **Figure 14a** and shows that a large portion of metabolism is affected, including glycolysis, TCA cycle, and the lipid metabolism. Selected examples are shown in **Figure 14b-e**. Glycolytic enzymes are increased while gluconeogenic enzymes are reduced across all grades, in agreement with the Warburg effect. Some glycolytic steps are catalysed by multiple enzymes, and they often show changes across grades that are distinct from their counterparts. This phenomenon is known as isozyme switch or isozyme shift, and its potential importance in ccRCC pathogenesis is discussed further in the Discussion section below. It should be noted that while glycolytic enzymes that contributes to the conversion of glucose to pyruvate are often upregulated in ccRCC across all grades, those in the pyruvate catabolism are downregulated across all grades. This suggests that the proteomic reprogramming is likely to shunt pyruvate to other catabolic pathways such as to lactate and acetyl-CoA. Enzymes involved in fatty acid degradation, also known as β -oxidation were also broadly downregulated across all grades. (**Figure 14c**) This is in agreement with the lipid accumulation and intracellular lipid droplet formation that is characteristic of ccRCC. Our proteomic analysis also found that a large number of extracellular matrix (ECM)

molecules changes protein levels in ccRCC compared with the normal tissue. **(Figure 14d)**

Fibronectin was upregulated across all ccRCC grades, while Laminins and Collagens were downregulated across all grades. These shift in ECM composition is likely to alter integrin signalling and cell adhesion properties in ccRCC, although no change in integrin protein levels were detected in our proteomic analyses. In agreement with the wealth of research indicating the importance of HIF signalling dysregulation in ccRCC, a large number of proteins encoded by HIF-regulated genes showed differential expression in ccRCC compared with the normal tissue. **(Figure 14e)**

To further understand the impact of proteomic reprogramming in ccRCC, Metascape was used to perform further enrichment analysis. **(Figure 15)** Metascape is a suite of online portal to analyse OMICS data. [50] Although the online metabolome analysis software encompasses a suite of online bioinformatic analysis tools, a majority of them are based on or similar to Gene Ontology analysis described above and are omitted here for clarity. Transcriptional regulatory relationships unraveled by sentence-based text-mining (TRRUST) is a database for transcription factor – target sequence interaction built based on Medline abstracts. [51, 52] Comparison of grade-specific proteins is expected to uncover transcriptional network that underlie the grade-defined ccRCC proteomes.

TRRUST analysis on the 180 proteins with ANOVA $p < 0.05$ highlighted HIF1 α , SP1, CEBP α , MYC, RELA, Androgen Receptor (AR), and NF κ B1 as the top transcription factors whose targets are enriched in the grade-defined ccRCC biomarkers. **(Figure 15a)** Analysis on the 105

biomarkers with ANOVA $p < 0.01$ identified an overlapping set of transcription factors including HIF1 α , MYC, SP1, AR, CEBP α . (Figure 7b) This is in agreement with the well-established notion that HIF pathway is dysregulated in ccRCC due to biallelic VHL inactivation. MYC targets include several nuclear proteins such as Nucleolin and Nucleophosmin as well as histone subunits. RELA is the p65 subunit of NF κ B. Studies from our group have previously identified NF κ B dysregulation and the resultant immune modulation, which agrees with this transcriptional target analysis. [53] Androgen Receptor is an X-linked nuclear receptor for male sex hormones, androgens, such as testosterone. Given the preponderance of ccRCC in males, such enrichment in androgen signalling in ccRCC is intriguing and warrants further investigation.

Furthermore, the TRRUST analysis on the UP subclass of 21 proteins showed that the UP subclass is enriched in HIF1 α targets with no other transcriptional factor with significantly enriched targets. This reinforces the findings that HIF1 pathway is aberrantly upregulated. **(Figure 15c)** In contrast, the 66 biomarkers of the DOWN subclass are enriched in SP1, MYC, and RELA targets. **(Figure 15d)** The TRRUST analysis of the UPG1 class of ccRCC biomarkers did not identify significant transcription factor interactions with them. (Data not shown) This may result from the small number of biomarkers in the group that are controlled by multiple transcription factors, thereby making any transcription factor statistically insignificant in the TRRUST criteria.

It is interesting that the TRRUST-identified transcription factors have significant overlap with the proteins that are direct first-order neighbors with the 180 ccRCC grade-defined biomarkers as identified in our initial publication. [14] These include cMYC and HIF1 α , which have well documented roles in ccRCC development and carcinogenesis in general. [54, 55] Many of these hidden nodes are transcription factors. This is likely due to two reasons. First,

transcription factors often have a number of target genes which are represented as high number of interactions with their neighbours. Second, since the transcription factors often act within a small subcellular compartment, their copy numbers are often low.

Transcription factor network forms hidden nodes, and may orchestrate and underly the pathobiology of ccRCC grades.

Pathway analysis of the grade-defined ccRCC biomarkers produced an intricate network of the biomarker proteins. Of the 180 protein biomarkers with $p < 0.05$, 27 proteins were used to interrogate the IPA Knowledge Base to incorporate the 64 first-order direct neighbours into the network. (Figure 8a, reproduced from [14])

Interestingly, the biomarker proteins often lie at the periphery of the interaction map and are in turn joined by other proteins not unidentified in the current proteomic study but are widely known to interact with the identified biomarkers. These proteins not included in the identified ccRCC biomarkers serve as “hidden nodes” in the protein-protein interaction map, and can shed light on the molecular basis of ccRCC pathology.

These transcriptional factors include cMYC,

1/2 α , HNF1 α , HNF4 α , p53, PPAR α , PPAR γ , and SREBF1. They are discussed below in the context of their known and potential contribution to ccRCC pathogenesis and Fuhrman nuclear grading criteria.

cMYC

Our grade-defined proteomics identified cMYC and nMYC, its neuronal paralogue, as a hidden node. [14] It was initially discovered in the late 1970s as a viral gene that caused a variety of tumors when inserted into the avian hosts, thus becoming an original member of oncogenes with transcriptional activity. [56 - 58] In part based on the presence of E-Box sequence (CACGTG), the canonical cMYC binding site in their promotor, up to 15% of the whole human genome was predicted to be cMYC target, [59] through its heterodimerization with MAX. [60]

The apparent broad target specificity that the simple hexameric cMYC binding sequence suggests additional modes of target selectivity. Chromatin regulation as well as transcriptional cofactors are among such potential mechanisms. Indeed, a network of chromatin remodeling machinery has been shown to influence cMYC transcriptional activity. In particular SWI/SNF chromatin remodeler has been shown to directly interact with cMYC. For example, SNF5, a tumor suppressor, directly interacts with cMYC and prevents its interaction with the E-box within the target gene promoter. [61] In myeloid leukemia, RUNX1 represses cMYC expression via SWI/SNF complex subunit switch. [62] Whether such a mechanism also operates in the context of ccRCC remains to be seen. Interestingly, mutations in a set of chromatin remodelling

genes contributes to ccRCC pathogenesis and influence patient survival. These genes include BAP1, PBRM1, and SETD1. [63]

While the target identification was aided by the deceptively simple hexameric sequence and the maturation of genomic sequencing, its molecular underpinnings evaded our understanding until 2015 when two groups reported that its principal function is not the selection of targets as is often described for other classic transcription factors such as p53, but rather to effect change in magnitude of expression for genes that had already been turned on. that is, cMYC acts like a volume amplifier rather than a ON/OFF toggle switch in direct and indirect manners. [64-66] This agrees with the findings that MYC activates a set of genes while silences another. [59]

Interestingly, nMYC, the neuronal paralogue of cMYC, was recently reported to control translation by regulating eIF4E expression, and its dysregulation caused medulloblastoma in mouse in a Hsp70-dependent manner [67] Indeed, in our grade-defined proteomic analysis, HSP70/HSPA8 was significantly increased in the high-grade ccRCC (G3 and G4) but not in the low-grade ccRCC (G1 and G2) when compared to the normal tissue. [14] Given the importance of metabolic reprogramming in ccRCC, [10] gliolastoma, [68] and medulloblastoma, [69] it is interesting to investigate whether the three distinct cancers employ similar translation-based mechanisms develop their malignancy.

PGC1 α

One of the cMYC transcriptional targets is Peroxisome Proliferator-activated receptor γ Coactivator-1 α (PGC1 α). [70] It is a transcription factor whose activity is modulated in several cancers including ccRCC, [71] and was identified as one of the hidden nodes in our network analysis. In ccRCC, PGC1 α activity is suppressed in a HIF-dependent manner, resulting in mitochondrial defects and avoidance of oxidative stress. [71] In prostate cancer, PGC1 α acts as a negative regulator of cMYC expression and consequently of polyamine synthesis, [72] suggesting the possibility that it may form a negative feedback loop in ccRCC, and its dysregulation may contribute to ccRCC induction and progression. Interestingly, suppression of the urea cycle in ccRCC leads to polyamine synthesis inhibition, [73] and a similar PGC1 α -cMYC axis may operate in ccRCC.

HIF1/2 α

HIF α forms a heterodimer with HIF β in response to hypoxia to orchestrate gene expression changes. There are two isoforms of HIF α , HIF1 α and HIF2 α , encoded in the human genome, and HIF1 α /HIF β heterodimer and HIF2 α /HIF β heterodimer target distinct but overlapping sets of genes. [74] In particular, HIF2 α has been shown to carry out pro-tumorigenic gene expression programmes including enhanced cMYC transcriptional activity, [75] and Oct-4 induction. [76]

In ccRCC, The HIF pathway is hyperactive due to biallelic inactivation of VHL, HIF α -specific E3 ubiquitin ligase. Since HIF pathway controls the expression of proangiogenic genes

such as VEGF, this leads to highly vascularised phenotype of ccRCC and aids its tumour development.

p53

In addition to its function as HIF α E3 ubiquitin ligase, VHL acts as p53 stabilising protein through direct interaction, [4] and its inactivation and resultant p53 hypomorph phenotype renders VHL-negative ccRCC resistant to conventional chemotherapy. (Chapter 2 and [5]) p53 is an archetypal tumour repressor whose loss-of-function mutations results in familial cancer syndromes such as Li-Fraumeni syndrome. [77] This was subsequently attributed to its role as a gene that codes for a protein enriched in transformed cell lines. [78] It encodes for a transcription factor whose inactivation due to mutations is a hallmark of many types of cancer. [79 – 81] Although *bona fide* p53 loss-of-function mutations are rare in ccRCC, most ccRCC harbors that of VHL. [82 – 84] Since VHL is a p53 binding protein which stabilises p53, [4] VHL-negative ccRCC effectively behaves as p53 hypomorph. [5] p53 expression is activated by several inputs including genomic insults, [85] and hypoxia. [86] In turn, p53 integrates these signals and controls a diverse array of including apoptotic pathways, CDK inhibitors, stemness, and metabolic enzymes. [87, 88] p21/WAF1/CIP1 is a p53 transcriptional target and principally acts as a CDK inhibitor through its direct interaction with the CDKs, [89, 90] resulting in G1 cell cycle arrest [91] This interaction is lost in cancer cells, thus endowing them with uncontrolled proliferation. [90] p21 belongs are family of intrinsically unstructured protein. [92] The structural flexibility has endowed p21 with a wide range of interacting partners, [93] and has

been speculated as a potential therapeutic targets against ccRCC. [94] p53 forms a hidden node in the network analysis based on our 105 grade-defined ccRCC biomarkers. (**Figure 16a and 16g**) This suggests that p53-controlled pathways mentioned above underlie the molecular mechanisms contributing to the features that define the Fuhrman nuclear grading criteria.

PPAR α / γ

Peroxisome proliferator-activated receptors (PPAR) are a family of nuclear hormone receptor with three human members: PPAR-alpha (PPAR α), PPAR-beta/delta (PPAR β / δ), and PPAR-gamma (PPAR γ). Of the three members, PPAR α and PPAR γ were identified as hidden nodes in our grade-defined ccRCC proteomic analysis. Subsequent study by our group identified important functions of PPAR α in ccRCC development, [95 – 97] and its pharmacological inhibition induced cell cycle arrest and apoptosis. [96] Mechanistically, these effects results from inhibition of several metabolic pathways including fatty acid β -oxidation, oxidative phosphorylation, and glycolysis, which may be mediated by miR-21. [97]

PPAR α was originally identified as a nuclear receptor activated by hepatocarcinogen, a peroxisome proliferator. [99] This is in agreement with our finding that several dehydrogenases identified as a grade-defined ccRCC biomarker are involved in xenobiotic metabolism which may be involved in chemoresistance. [14] In addition, PPAR α has been identified as a master regulator of peroxisome lipid metabolism. [100] Given the cytosolic lipid droplet accumulation is a hallmark of ccRCC, further investigation into PPAR α dysregulation in ccRCC is warranted.

PPAR γ physically interacts with PGC1 α , [101] and is known to control several pathways involved in the development of a range of cancers, [102 – 104] although mixed reports exist for its mechanistic and direct involvement in ccRCC. [105, 106]

In addition, our analysis identified PGC1 α , a PPAR γ co-activator, as an additional hidden node. PGC1 α plays a central role in mitochondrial biogenesis, an organelle known to be dysregulated in many forms of cancer, resulting in abrogated apoptosis, [107] ferroptosis, [108] and the dependency on glycolysis, notwithstanding the aerobic conditions (Warburg effects). [9, 109]

While no reports thus far links PGC1 α with ccRCC, its pivotal role in metabolic regulation is well-documented, [110, 111] and further investigation into its potential roles in ccRCC development is expected to identify additional dysregulated pathways in ccRCC and novel drug targets.

An intriguing question that arises from these three hidden nodes is whether peroxisome is involved in ccRCC pathogenesis. Although PPAR γ was recently reported to be dispensable for ccRCC, [106] potential redundancy among the PPAR family cannot be ruled out.

HNF1/4a

Hepatocyte nuclear factors are a family of nuclear factors originally identified for enrichment in hepatocyte. [112] In the current study, HNF1a and HNF4a were identified as hidden nodes in the network analysis. Although no known mechanistic role for HNF1a has been identified in

ccRCC pathogenesis, it is among the genes that harbour most genomic alterations in metastatic RCC. [113] HNF1 α binds to α - and β -fibrinogen promoters in hepatoma, [112] and a similar mechanism may operate to modulate ECM. Another member of the HNF family, HNF4a transcript is reduced in ccRCC. [114] HNF4a transcription is positively and directly regulated by VHL, and HNF4a in turn positively regulates ALDH2 transcription. ALDH2 was identified as one of the grade-defining ccRCC biomarkers in our present proteomic analysis, where the ALDH2 protein level was reduced across all the ccRCC grades by 6-fold or more.

SREBF1

Sterol Regulatory Element-1 Binding Transcription Factor (SREBF1) was originally identified as a nuclear transcription factor that binds to the SRE-1 element in the Low Density Lipoprotein (LDL) Receptor gene. [115] Subsequent works by a number of groups identified SREBF1 as a master regulator of lipid metabolism. [116] Interestingly, SREBF1 activation through PI3K-AKT-mTOR signalling, a pathway with well characterised involvement in cancer initiation and progression, suppresses ferroptosis. [117] In prostate cancer, SREBF1 activity is synergistically regulated by Androgen Receptor (AR) and mTOR pathways, and its inhibition results in curtailed expression of lipogenic genes such as FASN and SCD1. [118] In ccRCC, such metabolic dysregulation can result in accumulation of lipids and contribute to the clear-cell phenotype. While the work presented in Chapter 4 argues that ASS1 silencing in ccRCC contributes to its clear-cell phenotype, the possibility that ASS1 silencing synergises with SREBF1 to promote other features of ccRCC can not be ruled out.

In addition, the *in silico* analysis presented in this chapter identified the AR signalling as a potential pathway whose dysregulation manifests as the proteomic changes identified through our current analysis. **(Figure 15a and 15b)**. Therefore, further investigation is warranted to interrogate whether the AR-mTOR-SREBF1 axis is dysregulated in ccRCC.

Discussion

Renal cell carcinoma (RCC) is classified into several types including clear cell renal cell carcinoma (ccRCC), chromophobe RCC, and papillary RCC. ccRCC occupies roughly 75% of the total cases and is morphologically characterised by clear cytoplasm filled with lipid droplets [35] (Foster et al. (1994), Hsieh et al. (2017), genetically by biallelic inactivation of *VHL* gene, [3, 119 – 120] by DNA methylation, [121] and is pharmacologically refractory to conventional chemotherapeutic reagents (Ishimaru et al. (2010) and Chapter 1). Our laboratory previously characterised proteomic signature of ccRCC, which yielded 31 markers. [8] The current study expands the findings in Perroud *et al.* (2006). Fuhrman nuclear grading is a prevalent and semi-quantitative, if subjective, diagnostic and prognostic criteria for ccRCC, and more objective and quantitative biomarkers are needed. We hypothesised that each Fuhrman nuclear grade has their distinct proteome, which in turn, is different from the normal kidney proximal tubule epithelium from which the ccRCC is thought to originate from. Liquid chromatography / mass spectrometry-based shotgun proteomics was used to analyse protein extracts from patient-derived FFPE samples. This study found that there are significant differences in proteomes of

lower two grades (G1 and G2) and those of the higher two grades (G3 and G4), which are distinct from the normal kidney proteome. On the other hand, there was no statistically significant proteomic differences within the lower two grades and the higher two grades; this corroborates with other ccRCC nuclear grading schemes found that ccRCC should be classified into two, rather than four, grades. [122]

Briefly, many glycolytic enzymes were found to be upregulated across all grades, including Phosphohexose isomerase (GPI), Phosphofructokinase (PFKL, PFKP, and PFKM), Aldolases (ALDOA and ALDOC), Glyceraldehyde-3-phosphate dehydrogenase (GAPDH), phosphoglycerate kinase (PGK), phosphoglycerate mutase (PGM), Enoases (ENO1 and ENO2), Pyruvate kinase (PKM1/2) and Lactate dehydrogenase (LDHA) were all found to be increased in ccRCC compared to the normal kidney cortex tissue. This corroborates with the Warburg effect. [9] On the other hand gluconeogenic enzymes such as Phosphoenolpyruvate Carboxykinases (PCK1 and PCK2) and TCA cycle enzymes such as Pyruvate Carboxylase (PC), Aconitases (ACO1, ACO2), Succinate-CoA Ligase subunits (SUCLG1, SUCLG2), and Succinate Dehydrogenases (SDHA, SDHB) were downregulated in all ccRCC grades compared with the normal kidney tissue. Furthermore, Argininosuccinate synthetase (ASS1), the ATP-dependent and rate-limiting enzyme in the urea cycle [123] was downregulated across all grades compared with the normal tissue. The role of ASS1 silencing in ccRCC was further studied and is reported in Chapter 5. This proteomic study also suggested that γ -glutamyl transpeptidase (GGTLA1) was downregulated across all grades. This suggests increase in glutathione, which was later confirmed by the complimentary metabolomic study by our laboratory. [Wettersten et al. (2015)] Lastly, the proteomic analysis suggested a shift in lipid metabolism. β -oxidation enzymes in such as Acyl-

CoA dehydrogenases (ACADVL, ACADM, ACADSB, ACSM2A, ACSM2B, ACSL1, HADH), Enoyl-CoA Hydratase (ECHS1), Hydroxysteroid 17-Beta Dehydrogenase 10 (HSD17B10), and Acetyl-CoA Acyltransferase 2 are downregulated across all grades, although ACADVL shows modest upregulated in low grade ccRCC. On the other hand, ACLY was upregulated across all grades with more prominent upregulation observed in the low grade ccRCC. In addition, fatty acid synthesis pathway enzyme such as Fatty Acid Synthase (FASN) and Annexin A1 (ANXA1) showed potential increase in ccRCC compared to the normal tissue. ANXA1 inhibits Phospholipase A2 (PLA2), [124] which is expected to result in accumulation of phospholipoids. Overall, the reduction in β -oxidation and augmentation in fatty acid synthesis is expected to result in lipid accumulation. This correlates with the clear-cell phenotype of ccRCC, which results from intracellular lipid droplet accumulation and growth. Thus, Proteomic reprogramming in ccRCC is associated with the Fuhrman nuclear grades and may underlie the development of the clear-cell phenotype in ccRCC.

Validity and limitation of shotgun proteomics in biomarker search

A strength of shotgun proteomics is that the approach can analyse complex systems such as a cell proteome and a tissue proteome in detail and in a semi-quantitative manner. While the technique was originally established in the early 1990s, the technology saw true maturation in the past decade. The present study was originally performed when the shotgun-based proteomics was still in its adolescence. [125] First, this led to severely incomplete coverage of polypeptides in the reference database. In addition, ccRCC-specific mutations that are not

included in the database will not be correctly detected and assigned. Second, a large range in protein abundance spanning several orders of magnitude poses a challenge. Some proteins such as cytoskeletal proteins and canonical histones are abundant and highly represented in the detected set of the proteomic fragments while other classes of proteins such as transcription factors are present in the samples at much lower abundance and can go undetected. This is because the analysable dynamic range in polypeptide abundance was severely limited due to less advanced fractionation methods as well as less sensitive detection systems than those available today. Furthermore, FFPE samples show patient-to-patient and block-to-block variation due to varied fixation and embedding parameters as well as storage conditions. Furthermore, cancer tissues such as those preserved in FFPE blocks are composed of more than one cell types and includes non-cancerous cells such as endothelial cells and various immune cells. Inevitably, the resultant proteome is an average of those of the thousands of cells present in the sample. To circumvent this complication, laser-capture microdissection coupled with single-cell proteomics was developed to focus on proteome of a desired cell type. [126, 127] Native top-down proteomics allowed proteomic investigation of posttranslational modifications. [128] Furthermore, proteomic methods to quantitatively study the dynamic protein kinetics potential dynamic nature of the nuclear grades, such as isobaric tags for relative and absolute quantification (iTRAQ) have been developed, [129] and has been applied to study cancer biomarkers. [130] Proteins can also be labelled with deuterated water (D_2O) to study its dynamics using mass spectrometry. [131] These techniques opened an avenue to investigate the dynamic nature of proteomics and its influence on such phenomena as the Fuhrman nuclear grades.

Because of the technological limitations of the time and advancements thenceforth, a proteomic revisit to the molecular components of the Fuhrman nuclear grades may be warranted and is expected to yield a deeper description of their proteomic differences.

Identification of 5-HT metabolism as a novel metabolic pathway dysregulated in ccRCC

Tryptophan is an essential aromatic amino acid and forms one of the 20 building blocks of proteins. It can be degraded via two pathways: Kynurenine pathway and 5-HT pathway. [132] In the former, tryptophan is converted to kynurenine by Indoleamine 2,3, Dioxygenases (IDO1 and IDO2) and Tryptophan Dioxygenase. Kynurenine has immunosuppressive functions, and its function in ccRCC was extensively characterised by our group previously. [10, 95, 133 – 134] Briefly, Kynurenine and quinolinate, two downstream metabolites in Tryptophan degradation pathway were elevated in ccRCC patient urine as well as in ccRCC tissue. [10, 95] These metabolites are known to have immunosuppressive functions, [135] and may aid ccRCC development by suppressing immunity against ccRCC.

Alternatively, Tryptophan can be converted to 5-hydroxytryptamine (5-HT) through a two-step reaction catalysed by Tryptophan Hydroxylase and Aromatic L-Amino Acid Decarboxylase (DDC). [136] 5-HT is further degraded into 5-hydroxyindole acetaldehyde by Monoamine

oxidase. 5-hydroxyindole acetaldehyde is subsequently converted to 5-hydroxyindole acetic acid (5-HIAA) by an aldehyde dehydrogenase. [136] 5-HT, also known as serotonin, is well established as a neurotransmitter. [137] Recent research, however, elucidated more diverse biological function for the biomolecule, including cardiovascular functions, bladder control, and platelet aggregation. [138] Most importantly 5-HT is linked it to progression of several cancers. [139] For instance, 5-HT promotes colorectal cancer (CRC) cell proliferation, [140] and protects CRC cells from DNA damage. [141] In breast cancer, 5-HT stimulates proliferation through 5-HT 2A receptor *in vitro*. [142] In RCC, a 5-HT level is elevated in a subset of patients, but its pathobiological significance remains to be elucidated. [143, 144]

In our updated bioinformatic analysis with Gene Ontology, several enzymes in the serotonin-degradation pathway were found to be downregulated. (**Figure 13** and [14]) These include ALDH2, ALDH4A1, ALDH7A1, and ALDH9A1. In addition, DDC in 5-HT synthesis pathway was downregulated. On the other hand, ALDH1A1, a 5-HT degrading enzyme was upregulated in low grade ccRCC. Furthermore, MAOB, which converts 5-HT to 5-hydroxy-3-indolacetaldehyde (5-HIAL) was upregulated in G1 and G2 compared to the normal kidney cortex but was undetected in the higher grades. Taken together, these observations suggest that low grade ccRCC may have lower 5-HT metabolism than the normal kidney proximal epithelium. On the other hand, high grade ccRCC may accumulate 5-HT by reducing the 5-HT catabolic enzymes. Given the recent studies which have found that 5-HT regulates a number of cancer-related cell biology including proliferation and metastasis, further investigations on the role of 5-HT in ccRCC

pathology, in particular its contribution to the features relevant to the Fuhrman nuclear grading, is warranted. [139, 145, 146]

Metabolic Isozyme switch as a basis of proteomic shift and metabolic reprogramming across Fuhrman nuclear grades

The human genome encodes approximately 18,000 protein-coding genes, [147] many of which encode multiple isoforms through alternative splicing or alternative promoters, which contributes to diversified function and regulation of each gene product. In addition, multiple isozymes, encoded by distinct but usually evolutionarily related genes, catalyses the same biochemical reaction but often possess distinct biochemical properties from each other in terms of their substrate specificity and regulation. [148] In addition, these isozyme genes often contain distinct enhancers and promoters, allowing differential regulation of their expression, which in turn often results in differential tissue distribution.

Cancers exploits these endogenous flexibilities encoded in the human genome, which at least in part explains the molecular basis of the oncogenesis. That is, isozyme switch is a molecular basis of cancer development. [149] ccRCC is no exception. Our proteomic analysis identified several glycolytic and TCA cycle steps where isozyme switch was observed, [14, 54] with concomitant metabolomic reprogramming. [10] 1These isozyme switches across the Fuhrman

ccRCC grades and the implications to their potential mechanistic contributions to ccRCC metabolic reprogramming and ccRCC pathobiology are discussed below in the context of their potential mechanisms to contribute to ccRCC metabolic reprogramming and cancer development.

Glycolytic isozymes

1. Phosphofructokinases

Phosphofructokinases (PFK) catalyse phosphorylation of fructose-6-phosphate; PFK1 phosphorylates fructose-6-phosphate to form fructose-1,6-phosphate, a glycolytic intermediate, while PFK2 produces fructose-2,6-bisphosphate, an activator of PFK1. There are three PFK1 isozymes, all of which were detected in this proteomic study. These are named after the organ in which they are most prevalently expressed; the platelet type isoform (PFKP), the liver type isoform (PFKL), and the muscle type isoform (PFKM). Of these three isozymes, PFKP and PFKL were detected in at least 8 samples in at least one grade, while PFKP was detected in up to two samples in a grade. PFKL exhibited an archetypical G1UP distribution where its protein levels in G1 and G2 were up 6.8 to 9.2-fold compared with the normal tissue, while the PFKL in G3 was up moderately by 2.2-fold. PFKL in G4 showed reduction by 5-fold. (**Table 3**) PFKP on the other hand, showed 107-fold increase in G1 and 88-fold increase in G2 compared with the normal tissue. Unlike that of PFKL, the marked increase in PFKP spectral count

continued into higher grades, with G3 and G4 ccRCC exhibiting 51-fold and 27-fold increase compared with the normal tissue. This indicates that ccRCC in general upregulates PFK1 expression, with both PFKP and PFKL upregulated in low grade ccRCC while high grade ccRCC relies predominantly on PFKP for the conversion of glucose-6-phosphate to glucose-1,6-phosphate to drive the glycolysis to contribute to the Warburg effect.

Interestingly, FBP1, the gluconeogenic enzyme which catalyses the conversion of F-1,6-P to F-6-P, thus counteracting the PFK1-catalysed reactions, is reduced 4-fold in G1 and G2, and is reduced further in higher grades. FBP1 expression is known to be protective against ccRCC through glycolytic flux reduction in catalytic-activity-dependent manner and restricts pentose-phosphate pathway and proliferation in a catalysis-independent manner. [150] Thus, PFK1 isozyme switch is likely to synergise with FBP1 reduction during ccRCC development.

2. Aldolases

Aldolases catalyse the cleavage of fructose-1,6-bisphosphate, an aldol, into glyceraldehyde-3-phosphate and dihydroxyacetone phosphate (DHAP), which is an energetically neutral reaction largely at equilibrium. [151] There are three isozymes, Aldolase A (ALDOA), Aldolase B (ALDOB) and Aldolase C (ALDOC), which differ in their substrate specificity as well as in their tissue distribution in the physiological condition. [152] In adults, ALDOA is expressed predominantly in muscle and erythrocytes, ALDOB in the liver, and ALDOC in the brain. Albeit at lower levels, ALDOA and ALDOB are present in normal kidney. [152, 153] Specifically, ALDOA is expressed in more highly in distal tubules under physiological conditions, although moderate

immunoreactivity can be seen in the proximal tubule. [The Protein Atlas, 154] In contrast, ALDOB is expressed almost exclusively in the proximal tubules. [The Protein Atlas, 154] Functionally, ALDOA and ALDOC has high affinity for fructose-1,6-bisphosphate, thus catalysing glycolytic reaction, while ALDOB preferentially uses fructose-1-phosphate, which is derived from fructose via its phosphorylation. [155 – 157]

In our proteomic analysis, ALDOA peptide counts were increased in low grade ccRCC by 4-5 folds, while it was further increased to approximately 6- folds in the high grade ccRCC compared to the normal tissue. A similar trend was seen in ALDOC proteomic spectral counts which increased 4-fold in G1 and 5-folds in higher grades compared with the normal tissue. On the contrary, ALDOB spectral counts decreased by 8-folds in G1 and 12-folds in G2. Furthermore, there was no ALDOB fragments detected and identified in G3 and G4. (**Table 3**) Taken together, this suggests that There is an isozyme switch within the aldolase family from ALDOB in normal kidney epithelium to ALDOA and ALDOC This is likely to result in the preference glycolytic usage of glucose-derived fructose-1,6-bisphosphate over fructose-derived fructose-1-phosphate, thus diverting the latter to anabolic pathways such as nucleic acid synthesis for proliferation. Interestingly, fructose-1-phosphate activates glycogen synthetase and increases intracellular glycogen, a cardinal driver of the cytosolic droplet biogenesis. [158] Additionally. fructose itself has been reported to contribute to cancer growth. [159]

In addition to the glycolytic cleavage of fructose-1,6-bisphosphate, ALDOA has catalytic activity-dependent and -independent moonlighting functions. These include activity modulation of AMPK [160] and FBP2 [161], binding to and reorganization of actin filament, [162] activation of Wnt signalling, [163] and cell cycle progression. [164] Inhibition of ALDOA in pancreatic and

breast cancer cells *in vitro* leads to caspase activation and apoptosis through inhibition of its catalytic activity-independent moonlighting functions. [165] The moonlighting functions of aldolases and its use for ccRCC grading will be further discussed in chapter 5.

3. Enolases

Enolases catalyse reversible conversion between 2-phosphoglycerate and phosphoenolpyruvate, thus contributing to both glycolysis and gluconeogenesis. The human genome encodes three ENO isozymes, namely ENO1 (Enolase 1, also known as alpha enolase or on-neural enolase), ENO2, (gamma enolase or neuronal enolase), and ENO3 (beta enolase or muscle enolase). As the name suggests, ENO2 is predominantly expressed in neurons and ENO3 in muscles, respectively, under physiological conditions, while ENO1 is more broadly expressed.

In this grade-defined proteomic analysis, ENO1 showed increased expression in all four grades compared to the normal tissue. More precisely, it was more highly expressed in lower grades with G1 and G2 ccRCC showing ENO1 increase of 3.7 and 2.3-folds while the higher two grades showed 1.4-fold and 1.8-fold increase compared to the normal tissue. (**Table 3**) ENO2 showed no expression in the normal tissue, while it was highly expressed in the lower two grades, although it retained moderate to high expression in the higher grades. ENO3 was not detected in our proteomic analysis. Taken together, this shows that ccRCC increases the enolase activities compared to the normal tissue. While the normal tissues employ ENO1 exclusively for the conversion of glycerate-2-phosphate to phosphoenolpyruvate while the ccRCC uses both ENO1 and ENO2.

In addition to ENO1 transcript, *ENO1* gene encodes a shorter transcript from an alternatively and in-frame translated promoter. The truncated form is translated in a cap-independent manner and encodes Myc-promoter Binding Protein 1 (MBP1). [166] MBP1 is a transcriptional repressor that binds to the cMYC promoter and downregulates its expression. This dual functionality of ENO1 as both glycolytic enzyme and as cMYC translational regulator makes it a moonlighting protein, a peculiarity which is further studied and discussed in chapter 5. MBP1 isoform is detectable only in the lower two Fuhrman grades as well as the normal tissue but not in the ccRCC with higher Fuhrman grades, which may contribute to elevated cMYC level in high grade ccRCC, thus promoting a more aggressive phenotype. This indicates the ccRCC exploits posttranscriptional mechanisms to regulate cMYC expression, which may contribute to the ccRCC progression.

4. *Pyruvate kinases*

Pyruvate kinases (PK) catalyses the conversion of phosphoenolpyruvate to pyruvate with concomitant ATP synthesis, which is a rate-determining step in the glycolysis. There are two PK homologues in the human genome; PKLR and PKM, each of which encode two variants. PKLR encodes PK isoforms expressed in the liver, red blood cells and the kidney, while the PKM encodes two isoforms PKM1 and PKM2, which are splice variants. Under physiological conditions, PKM1 is expressed in muscles while PKM2 is chiefly an embryonic isoform with additional expression in kidney, lung, and adipocytes in adult.

In our proteomic studies, PKM1/2 isoforms are elevated 3- to 3.7-folds across all Fuhrman grades compared with the normal tissue, while PKLR showed moderate downregulation in low grade ccRCC and complete loss of expression in the high ccRCC grades. (**Table 3**) While our proteomic data on Fuhrman grade-defined PKLR expression lacks statistical significance to classify it as a grade-defining biomarker, there is a grade-dependent downregulation of PKLR and concomitant isozyme switch to PKM1/2 in the ccRCC. Due to technical limitations, the two isoforms encoded by each gene were indistinguishable.

PKM2 is another example moonlighting protein which can perform functions that were not originally attributed to it. These functions include gene transcription as a heterodimer with Oct4, [167] β -catenin, [168] and HIF1 α . [169] Therefore, simple increase in the spectral count is not satisfactory confirmation of increase in its contribution to the glycolytic flow, although this is in agreement with the Warburg effect.

5. Lactate dehydrogenases

Lactate dehydrogenases (LDH) catalyses the reversible conversion of pyruvate to lactate. The human genome encodes four isozymes, LDHA, LDHB, LDHC, and LDHD. [170] LDHA encodes LDH-M protein, a muscle-specific isoform, while LDHB encodes LDH-H which is predominantly expressed in the heart. LDHC forms homotetrametric enzyme while LDHA and LDHB can produce homo- or hetero- tetramers with any stoichiometry. LDHA/LDH-M has higher affinity for pyruvate while LDHB/LDH-H preferentially binds to lactate. Thus, cells can dictate the flow of pyruvate-to-lactate conversion by expressing differential ratio of LDHA/LDH-M and

LDHB/LDH-H. Higher LDHA expression prefers lactate production while higher LDHB is expected to result in lactate-to-pyruvate conversion.

In this proteomic analysis, LDHA was found to be elevated across all grades with the highest elevation seen in the lower Fuhrman grades, which saw 6- to 6.3-fold increase in LDHA. The higher two grades saw more moderate 4- to 5-fold increase. On the other hand, LDHB was decreased by up to 2-fold across all nuclear grades compared with the normal tissue. This predicts the ccRCC preferentially converts pyruvate to lactate; this was later confirmed by the metabolomic analysis in our laboratory, which showed increase in lactate in ccRCC compared to the normal tissue in a grade-dependent manner. [10]

Long regarded as a glycolytic byproduct with no biological significance, [171] lactate has seen renewed interests in cancer research. [172] Lactate dehydrogenase (LDH) levels are inversely related to response to checkpoint inhibitors such as PD-1 and PD-L1 in melanoma and non-small-cell lung cancer patients. [173] Once secreted, cancer-derived lactate can activate their own GPR81/HCAR1 as well as GPR81/HCAR1 on the immune cells and result in cancer angiogenesis and immune evasion as well as chemoresistance. [174] In addition, lactate signalling through GPR81 on adipocytes results in inhibition of lipolysis. That is, triglyceride hydrolysis into fatty acids and a glyceride is reduced. Additionally, fructose-derived lactate downregulates β -oxidation. [159] These findings are in agreement with the clear-cell phenotype of ccRCC in which cells accumulate lipids in intracellular lipid droplets. [174] In addition, lactate suppresses autophagy via FIP200. [175] Furthermore, lactate can modify gene expression through histone lactylation [176, 177] In this context, it is interesting that isozyme switch from in pyruvate kinases (PK) from PKM1 to PKM2 reroutes glucose metabolism to lactate

production. [178] Furthermore, lactate transporter expression is dysregulated in cancers including RCC. [179] Therefore, together with altered mitochondrial metabolism, [180] pathological lactate accumulation is likely to modify gene expression pattern and acidify its tumour microenvironment, [172] which enhances angiogenesis and suppress anti-cancer immunity in the ccRCC microenvironment. [181 – 183]

It should be noted that LDHA is yet another example of moonlighting proteins. [184, 185] In addition to its function in the lactate fermentation, it localises to the nucleus to regulate transcriptional programme including Histone 2B. [186] In our current proteomic analysis, Histone H2B type 2-F showed marginal yet progressive increase across all Fuhrman grades compared to the normal tissue. This correlates with the consistent elevation of LDHA spectral counts in all ccRCC grades and further proteomic interrogation is warranted.

In addition, upon reactive oxygen accumulation, LDHA translocates to the nucleus to convert α -ketobutyrate to α -hydroxybutyrate and contribute to cervical cancer growth. [185] Interestingly, in our *in vitro* model of Argininosuccinate synthetase (ASS1)-deficient ccRCC, 5-hydroxybutyrate was elevated at the whole cell level. (See Chapter 4)

Furthermore, LDHA converts α -ketoglutarate, a TCA cycle intermediate to L-2-hydroxyglutarate in hypoxia, [187] and at acidic pH, [188] two conditions associated with cancer and in particular with ccRCC.

6. Phosphoenolpyruvate Carboxykinases

Phosphoenolpyruvate Carboxykinases catalyse the bidirectional conversion of phosphoenolpyruvate and oxaloacetate, and functions as the entry point for the gluconeogenesis. The human genome encodes two isozymes; PCK1 and PCK2. [189] Both isozymes are significantly downregulated in the ccRCC across all grades in the current proteomic analysis, [14] which supports the Warburg effect hypothesis. [9]

7. Other glycolytic isozymes that were not detected in the current study, e.g., HK1 vs HK2

The shotgun proteomics is based on the identification of peptide fragment through its mass-to-charge ratio (m/z). This limits the potential of peptide identification. Some peptides may have the twice the charge of another that is half in its mass, thus producing the same m/z value. This precludes unambiguous identification of the origin for a detected proteolytic fragment.

Furthermore, presence of hitherto unknown post-translational modifications further complicates the peptide identification. Glucose transporters and hexokinases are expected to be present in the analysed samples but were not detected by the current proteomic studies.

These proteins, however, are known to contribute to and to regulate the glycolytic flux and further refinement and enrichment in the proteolytic fragment database is expected to shed additional lights on the presence and modulations of other glycolytic proteins.

TCA cycle isozymes

1. Glutamic-oxaloacetic transaminases

In addition to the aforementioned glycolytic enzymes, one TCA cycle enzyme was found to exhibit grade-dependent isozyme switch. Glutamic-oxaloacetic transaminases (GOT) catalyse the trans-amination of oxaloacetate, a TCA cycle metabolite, into aspartate at the expense of glutamate which is converted to α -ketoglutarate, another TCA cycle intermediate, in a pyridoxal phosphate dependent manner. This effectively forms a metabolic shunt which produces aspartate that can be exported out to the cytoplasm for anabolic pathways. Between aspartate and α -ketoglutarate to form oxaloacetate and glutamate in a pyridoxal phosphate-dependent manner. Two isozymes encode for the enzymatic activity: GOT1 and GOT2. GOT1 is cytoplasmic while GOT2 is mitochondrial. In our proteomic studies, GOT2 is downregulated across all grades, potentially indicating mitochondrial dysfunction. GOT2 is an integral component of the malate-aspartate shuttle which shuttles electrons across the NADH-impermeable mitochondrial inner membrane. Several inborn disorders of the malate-aspartate shuttle have been reported, including those originating from GOT2 deficiency. [190] The affected patients show central nervous system disorders such as microcephaly. [191] The pathobiological function of GOT2 reduction in ccRCC remains to be elucidated.

On the other hand, the cytosolic GOT1 level does not show significant reduction compared to the normal tissue except in the grade 4 ccRCC. Interestingly, majority of the cancer upregulates GOT1 expression. [192] and GOT1 inhibition induced ferroptosis-based cell death in pancreatic ductal adenocarcinoma. [193] On the other hand, a recent study identified GOT1 as a target of a ferroptosis-related long non-coding RNA. [194] In the same study, GOT1 expression

was reported to be reduced across all grades. The precise role of GOT1 reduction in ccRCC warrants further investigation.

ccRCC Proteomic data mining using QIAGEN Ingenuity pathway analysis (IPA)

In order to further interrogate the grade-defined ccRCC proteomics data, it was analysed using Ingenuity pathway analysis (IPA). The initial analysis was carried out in 2009 and was published in our earlier publication, (**Figure 16a** and [14]) while an updated analysis was carried out during the dissertation preparation.

The initial analysis identified several transcription factors which formed “hidden nodes” as described above.

In the recently updated analysis, the IPA analysis of the same set of 180 ccRCC grade-defined markers produced 12 sub network which are known to have mechanistic contribution to ccRCC pathogenesis or to that of other types of cancer. (**Figure 16b**) These include lipid metabolism, [195, 196] NF-kB / actin cytoskeleton, [53, 197] HIF-Vimentin-ERK network, [3, 6, 14] HNF4A network, [114, 198] Extracellular matrix, [199, 200] 14-3-3 / RAS network, [201 – 204] and S100/LDL network. [205, 206]

Next, the proteomics data was further analysed with IPA by comparing the high grade (G3/G4) proteome to the proteome of the normal kidney proximal tubule epithelium. (**Figure**

16c) As shown in **Figure 16d** and in agreement with the KEGG analysis and pre-existing literature, HIF1 network was predicted to be upregulated. Consequently, FN1 is upregulated and cytoskeletal proteins ACTN4, VCL, and SPTAN1 were down regulated. On the other hand, mitochondrial proteins were shown to be downregulated. (**Figure 16e**) In addition, HSD17B10-SOD interactome showed biphasic activation/inhibition profile in which SOD interactome such as ENO2 and MPO are upregulated in the high grade ccRCC in comparison to the normal tissue while HSD17B10 interactome, most of which comprises a part of β -oxidation pathway, were downregulated in high-grade ccRCC. (**Figure 16f**)

While further IPA analysis was not performed due to limited access to the programme, the analysis described above confirms that the result of our proteomic analysis is supported by works from other labs as well as ours.

It should be noted that the samples for the present proteomic analysis were obtained by scraping FFPE sections with razor blades instead of through single-cell micro-capture dissection. In addition, each tumour sometimes consisted of mixed grades, although regions with relatively high enrichment of one grade was chosen for the analysis. These technical limitations resulted in each sample being composed of different cell types including ccRCC cells of mixed Fuhrman grades, endothelial cells, and immune cells. Therefore, one must be aware that change in the detected protein levels across grades may reflect change in cell type compositions rather than bona fide upregulation or downregulation of the proteins in the ccRCC of an assigned nuclear grade. In order to more rigorously characterise ccRCC-specific grade-defined proteomic analysis, single-cell proteomics coupled with validation with defined cell type markers is highly desired.

Hypotheses on potential ccRCC grade-defining biomarkers whose spectral counts did not meet the cutoff

While we reported 105 grade-defining biomarkers with $p < 0.01$ and 180 biomarkers with $p < 0.05$, the total spectral counts identified covered as many as 777 proteins. Although many of these proteins were detected in multiple samples in each grade, they were excluded from the originally published statistical analysis due to our stringent cutoff criteria of presenting a fragment at least in 7 out of 10 samples with $p < 0.01$ or $p < 0.05$ with Bonferonni correction. They, however, can provide a useful source of hypotheses that can be experimentally tested, and select examples are discussed below. With further advancement in peptide detection and identification as well as sample collection and preparation, it is expected that some of these obscure proteins will become *bona fide* ccRCC biomarkers.

The following section discusses potential biomarker candidates whose fragments were identified in the current mass spectrometric analyses but that did not meet the stringent statistical criteria for outright inclusion in the published list of ccRCC biomarkers. [Perroud et al. (2009)] Since they did not meet the statistical criteria, they were excluded from the published list of the ccRCC grade-defined biomarkers. Based on studies on ccRCC, other cancers, or other diseases and physiological systems by others, however, they show potentials for further experimental studies and validation, which may lead to their being included in the grade-defined ccRCC biomarkers in the future.

Glutamate is well known as one of the twenty amino acids used in protein synthesis as well as an excitatory neurotransmitter. Recent studies have shown that it also has anaplerotic function which cancers manipulate to their advantage. Glutamate can be synthesised from such amino acids as glutamine via deamination reaction as well as from arginine, proline, and histidine. In addition, amination of α -ketoglutarate, a TCA cycle intermediate, forms glutamate. On the other hand, deamination-dehydrogenation of glutamate by Glutamate Dehydrogenase (GDH) forms α -ketoglutarate when TCA activity is low. In our proteomic study, GLUDP5, was identified to be downregulated in all ccRCC grades. Furthermore, the magnitude of downregulation was larger in the grades 3 and 4 compared with the grades 1 and 2. Because GLUDP5 is a pseudogene, it is likely that this is a database error and the true identity of these spectral fragments is GLUD1, which catalyses the conversion of glutamate to α -ketoglutarate. This indicates that by shutting down GLUD1, ccRCC shunts glutamate away from the energy-producing TCA cycle and electron transport chain towards anabolic amino acid and protein syntheses, and this shift is higher as the Fuhrman grade progresses to a higher grade. Glutaminase (GLS) is also downregulated in all grades except the grade 3. This may reflect the heterogeneity of ccRCC across Fuhrman grades or may be an artefact of sample collection in which the grade 3 samples had high-GLS contaminating non-ccRCC tissues.

A defining feature of the Fuhrman nuclear grade is the presence and the size of the nucleoli, which increases as the grade increases. It consists of three compartments: the fibrillar centre, the dense fibrillar component, and the granular component. Each of them has defined markers. [207] In this study, at least two nucleolar proteins were identified, namely NCL and NPM1. They were detected in multiple samples in each grade in the current study. Although

none of them showed statistical significance as measured by one-way ANOVA ($p < 0.05$), they showed moderate increase across all grades compared to the normal tissue.

NCL localises to the dense fibrillar components while NPM1 is present in granular centre.

Increase in NCL and NPM1 protein levels may contribute to increased number and size of nucleoli in ccRCC compared with the normal kidney proximal tubule epithelium. Furthermore, such upregulation in NCL and NPM1 may contribute to dysregulated nucleoli functions, which in turn play a part in the ccRCC pathobiology.

Nucleosomes are histone hetero-octamers around which DNA is wrapped around to form compact chromatin for storage in the limited space within the nucleus. While the canonical composition of the core histone octamers is two H2A-H2B which sandwiches (H3-H4)₂ tetramer. [208] H1 subunits is the linker histone which, although not an integral member of the histone octamer, interacts with the histone octamer and the DNA to determine the chromatin compaction state, thus regulating gene expression [209] The human genome encodes multiple variants of the core histone subunits as well as of the H1 histone, and the choice of a particular variant contributes to nucleosomal structural change [210] and the consequent outcome of gene expression. [211] The histone code hypothesis postulates that histone post-translational modifications mostly found in the N terminal and C-terminal tails of each histone defines the chromatin state as well as the gene expression activities. [212] Since some of the residues modified by these PTM are missing in certain variants, variant switch can have profound effects on gene expression even when the same chromatin modifying enzymes are expressed. In the present study, total of ten histone variants were identified including three H1 variants (HIST1H1C encoding H1.2, HIST1H1E encoding H1.4, HIST1H1B encoding H1.5), five H2 variants

(H2AFX encoding Histone H2A.x (H2a/x), Histone 2H2BF encoding H2B type 2-F, Histone H2AFV encoding H2AV (H2A.F/Z), H2AFY encoding core histone macro-H2A.1, HIST2H2AC encoding H2A type 2-C), and one H4 variant (H4_Human encoding Histone H4). None of these were included in the final list of 180 ccRCC grade-defining biomarkers, since their ANOVA values fell above the cut-off ($p < 0.05$), although Histone H1.4 was moderately downregulated in high grade ccRCC while H1.5 was upregulated in these samples, suggesting a potential variant switch. Additionally, Histone H2B type-F was moderately upregulated across all grades compared with the normal tissue. With the technological advancements in the intervening years, it is interesting to revisit the original spectral data to identify additional fragments belonging to these and other histone variants. If the differential expression in histone variants is statistically significant, further investigation into the loci at and conditions under which these variants exist may shed further mechanisms in ccRCC pathogenesis.

The potential ccRCC grade-defining biomarker proteins discussed above, albeit not statistically significant in the original analysis, are potential source of additional novel ccRCC biomarkers. Further expansion in identifiable peptide database is expected to contribute to identification of such biomarkers in the future.

Any scientific investigation is at the mercy of technological advancement and its resultant state of the art of the time. In the case of this study on grade-defined ccRCC proteomics, it was conducted at the time when untargeted proteomics was still in its infancy and the peptide databases to identify the analysed peptides based upon their mass spectrometric spectra were

vastly incomplete. In the intervening years, the databases have been expanded and improved. [213, 214] In addition, single-cell proteomics was established through the use of micro-capture dissection and more sensitive detectors, which in turn allowed subcellular proteomic analyses. [215] These advances have been applied to several cancers, but ccRCC is still in want of such detailed and well-defined proteomic investigation. It is exhilarating that Fuhrman nuclear grades correlates with change in proteome-wide changes, in particular the enzymes, which corroborates well with the subsequent metabolomic study from our lab. Yet given the morphological bases of the Fuhrman grade strictly lies in the nucleus, it is intriguing to investigate grade-defined ccRCC nuclear proteome. Since subcellular fractionation reduces the proteomic complexity, such subtle changes as nucleolar proteins and histone variants which could not be detected with statistical significance in the current study may become within our reach.

Summary

ccRCC is morphologically characterised by lipid droplet-filled cytoplasm which gives clear transparent appearance when observed with conventional cytological staining under a microscope. Genetically, it is genetically characterised by biallelic inactivating VHL mutation which not only contributes to highly vascularised morphology through hyperactive HIF pathway, [3, 84, 216, 217] but due to destabilised p53 and the resultant curtailed apoptotic response, [4, 5] which renders it refractory to conventional chemotherapy. [5, 35, 218] A

promising potential approach is to devise a pharmacological intervention to restore the p53 activity. [5] Alternatively, the work presented in this chapter suggests that proteomic reprogramming in ccRCC is associated with Fuhrman nuclear grades. Given such proteomic reprogramming, novel targets that circumvents the need for apoptosis may provide more druggable targets against ccRCC. This study suggests that there are hundreds of such targets, and combinatorial and personalised pharmacological intervention to manipulate ccRCC metabolome may hold great promise in the future.

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Figures

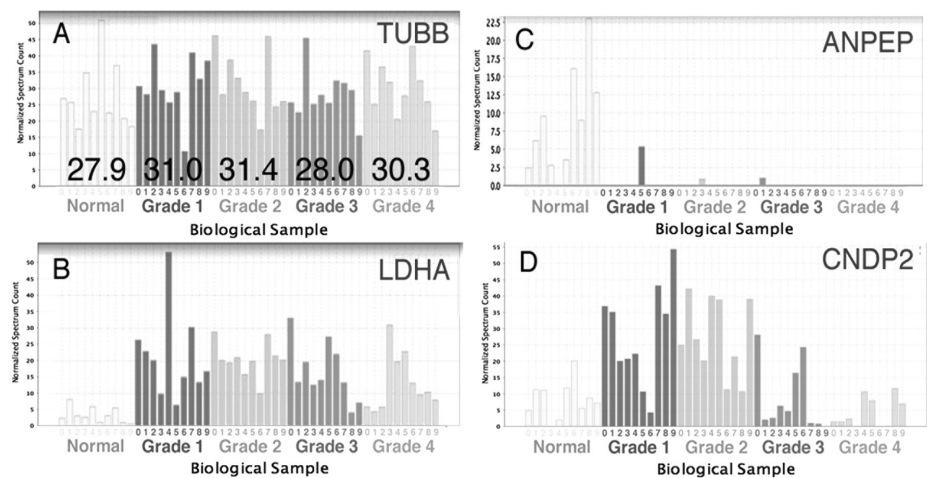


Fig. 9 : comparison of normalized spectral counts of select proteins across Fuhrman grades

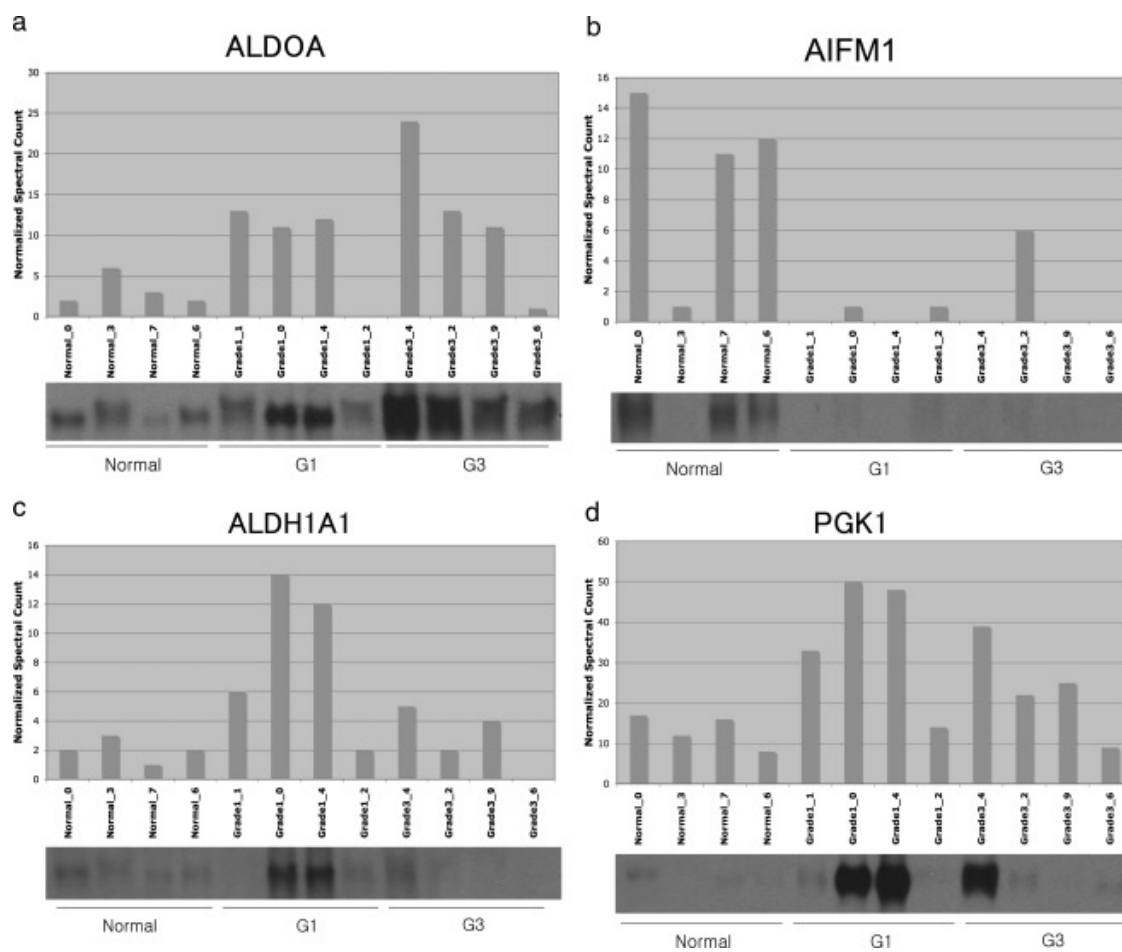


Fig. 10 Inter-grade comparison of select protein biomarker distribution measured by Western blotting

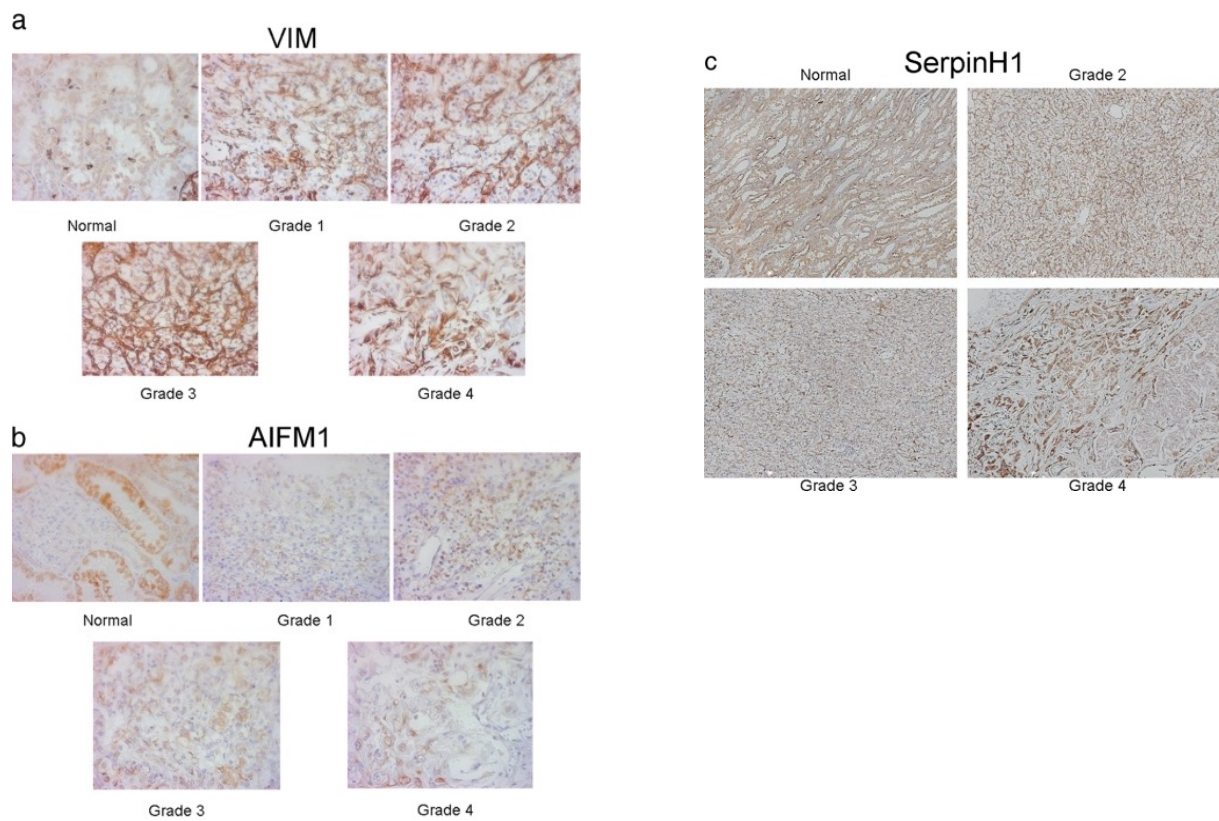


Fig.11 Inter-grade comparison of select biomarker distribution as investigated by immunohistochemistry

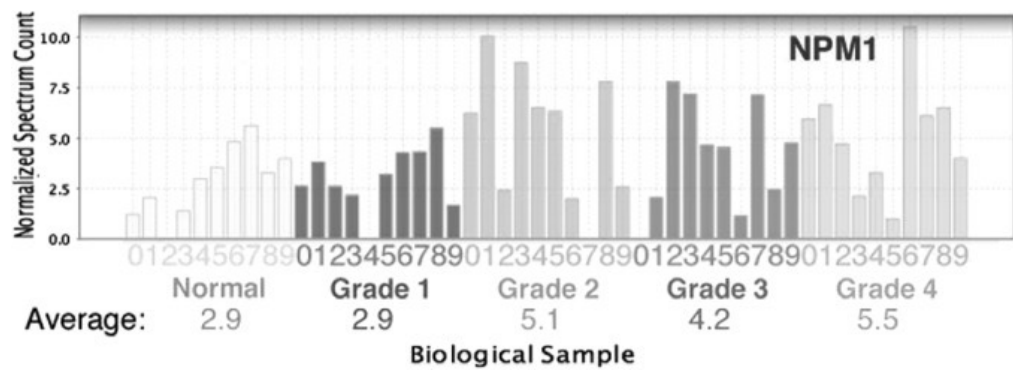


Fig.12 : grade-defined Nucleophosmin protein expression pattern and its inter-sample variation

Fig.13 PANTHER analysis of grade-defined ccRCC proteomes

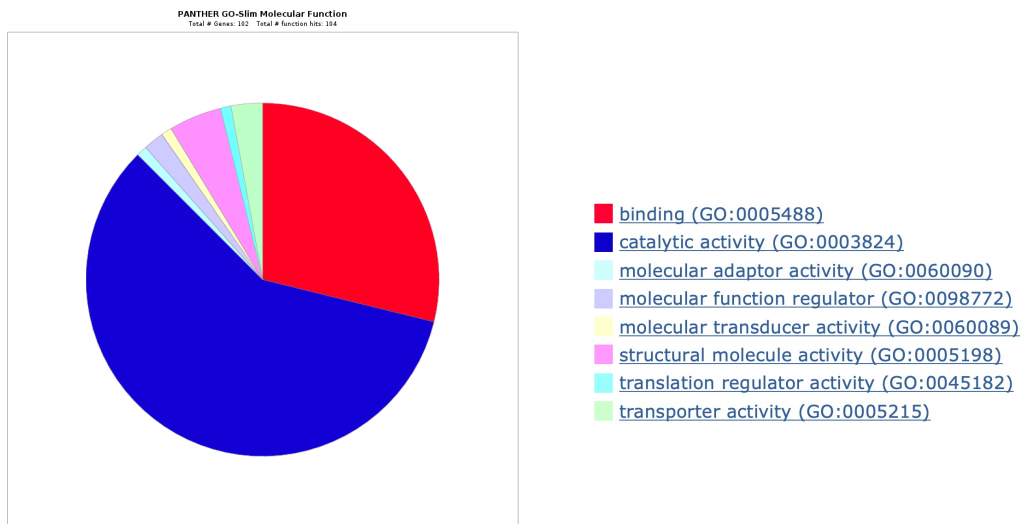


Fig 13a: Panther Go-Slim Molecular functions on the 105 biomarkers with $p < 0.01$

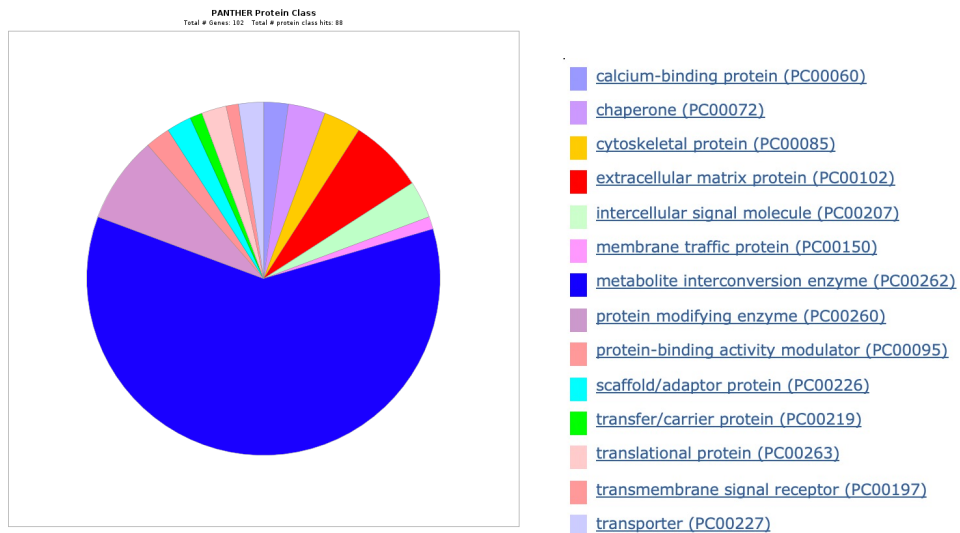


Fig 13b: Panther Protein Class on the 105 Biomarkers with $p < 0.01$

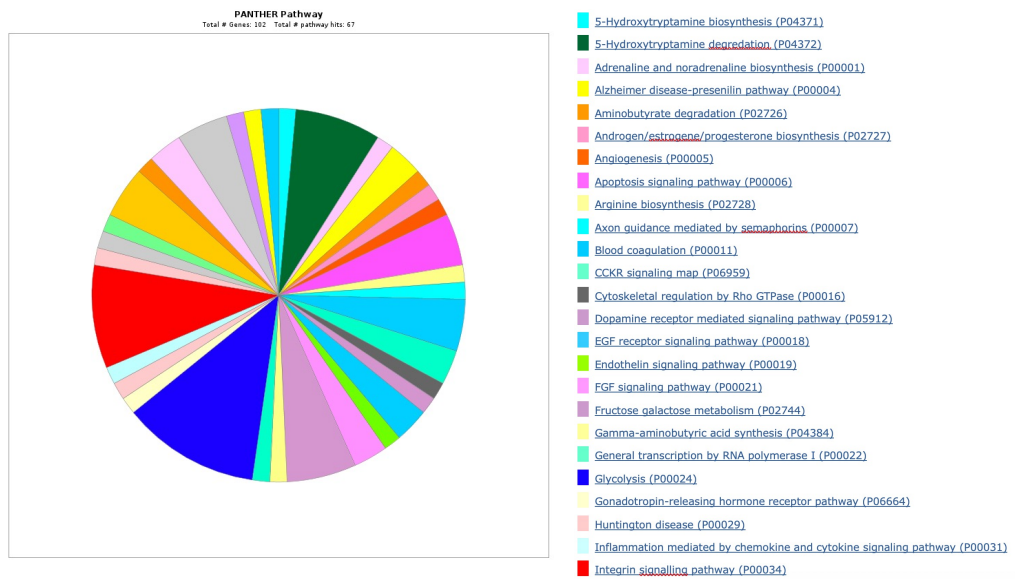


Fig 13c: Panther pathway analysis on the 105 biomarkers with $p < 0.01$

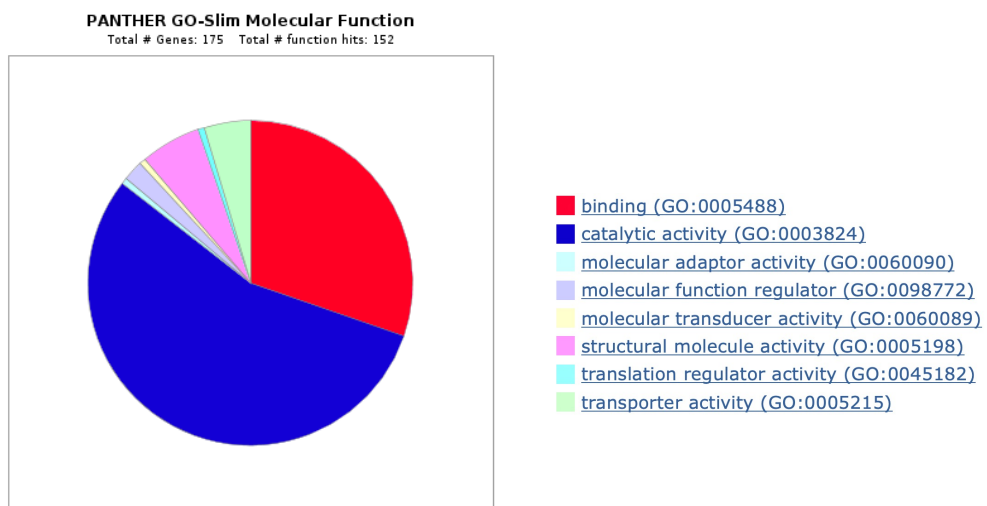


Fig 13d: Panther Go-Slim Molecular functions on the 180 biomarkers with $p < 0.05$

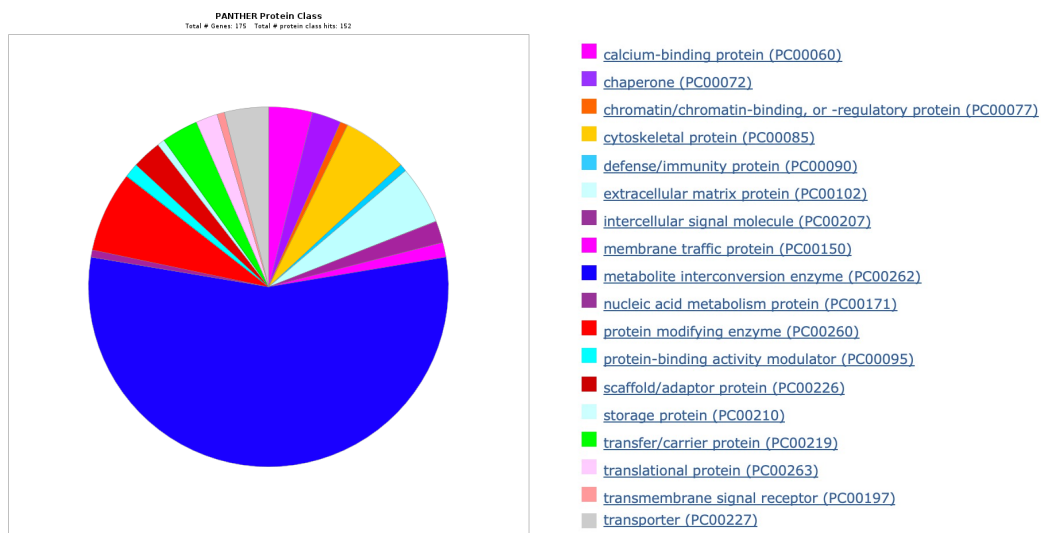


Fig 13e: Panther Protein Class on the 180 biomarkers with $p < 0.05$

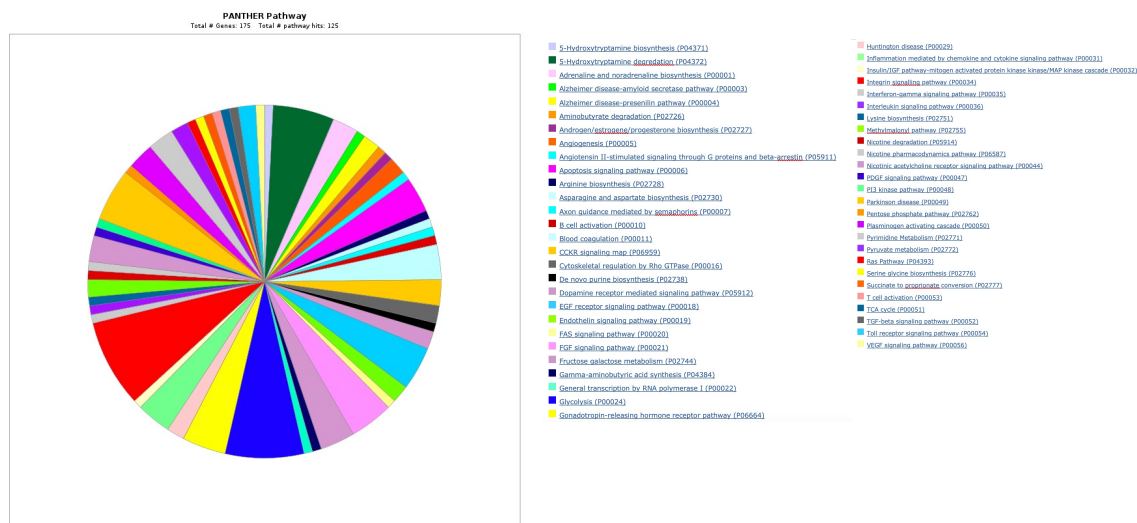


Fig 13f: Panther pathway analysis on the 180 biomarkers with $p < 0.05$

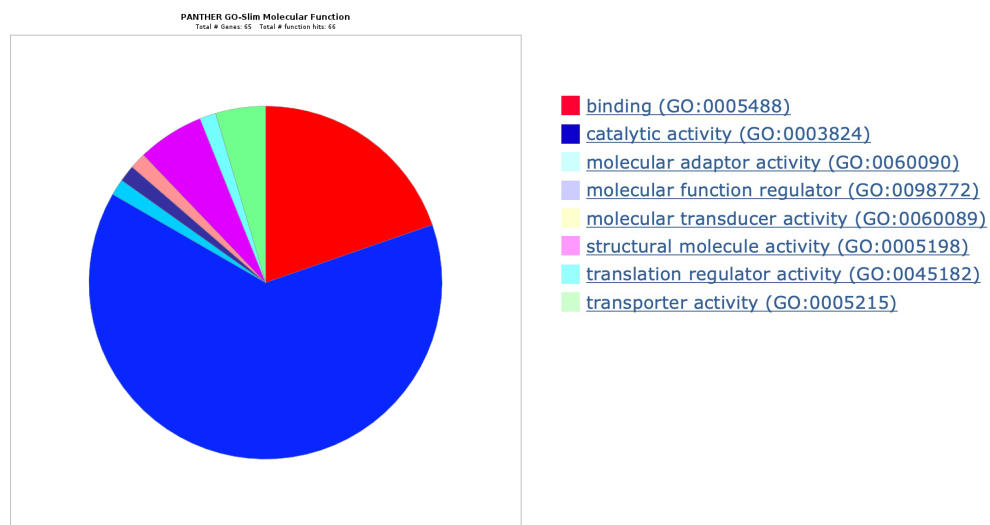


Fig 13g: Panther Go-Slim Molecular functions on the 66 DOWN biomarkers $p < 0.01$

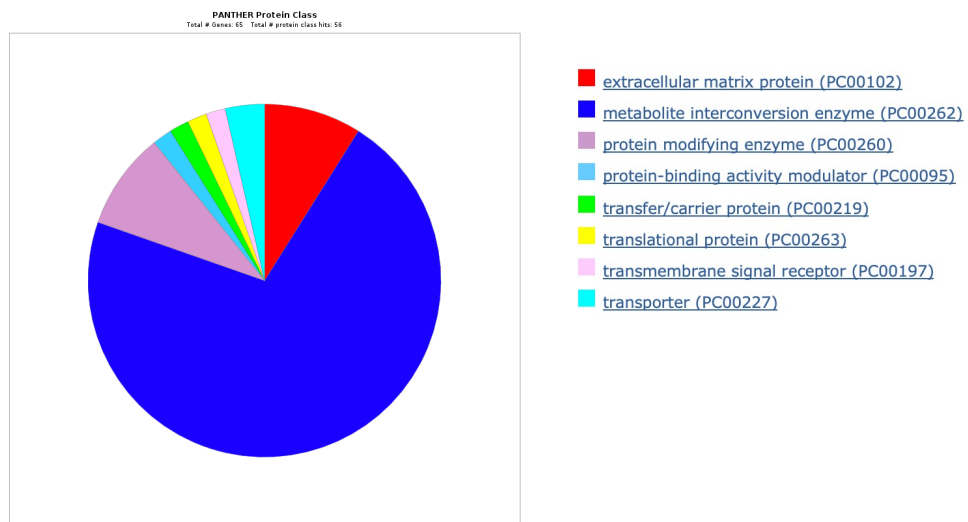


Fig 13h: Panther Protein Class on the 66 DOWN biomarkers $p < 0.01$

Fig.13 PANTHER analysis of grade-defined ccRCC proteomes

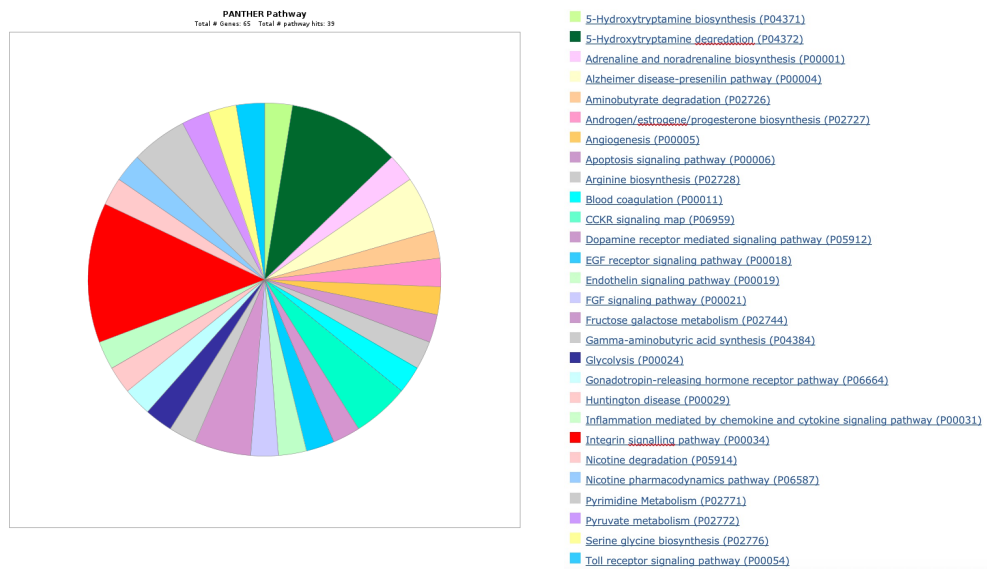


Fig 13i: Panther pathway analysis on the 66 DOWN biomarkers $p < 0.01$

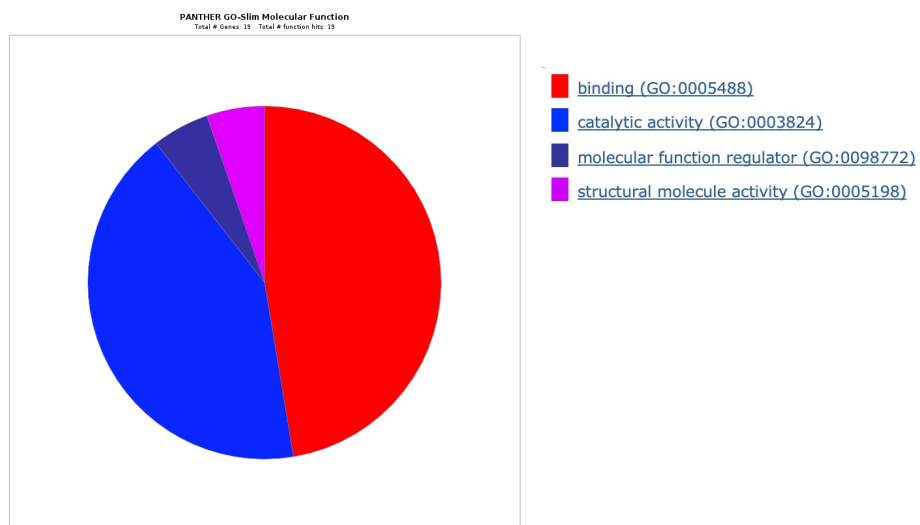


Fig 13j: Panther Go-Slim Molecular functions on the 21 UP biomarkers with $p < 0.01$

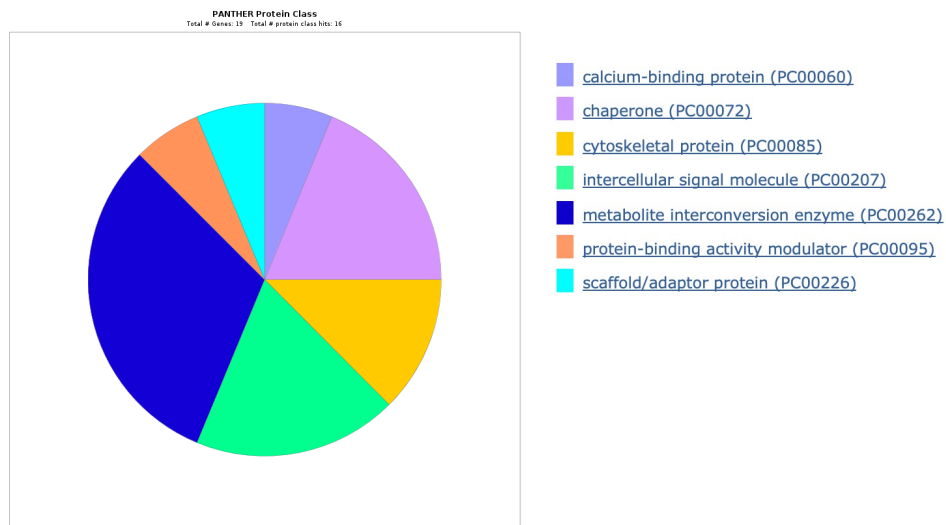


Fig 13k: Panther Protein Class on the 21 UP biomarkers with $p < 0.01$

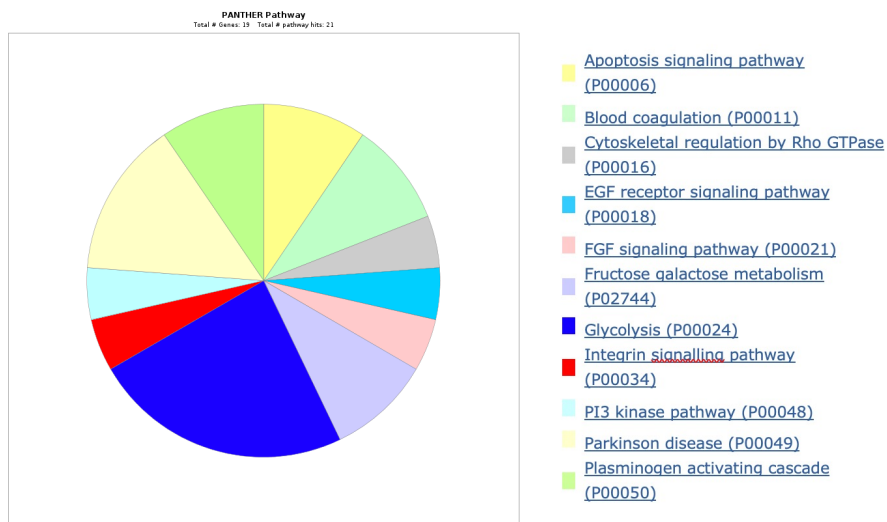


Fig 13I: Panther pathway analysis on the 21 UP biomarkers with $p < 0.01$

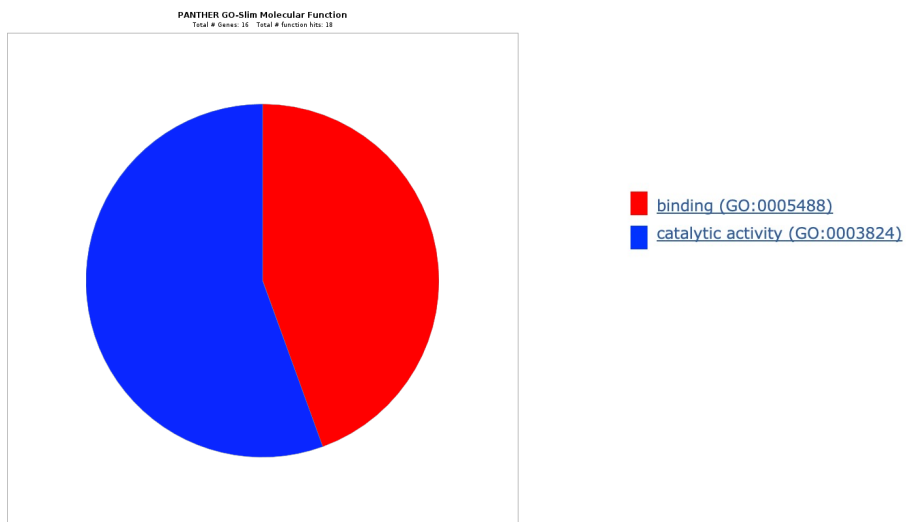


Fig 13m: Panther Go-Slim Molecular functions on the 21 UP biomarkers with $p < 0.01$

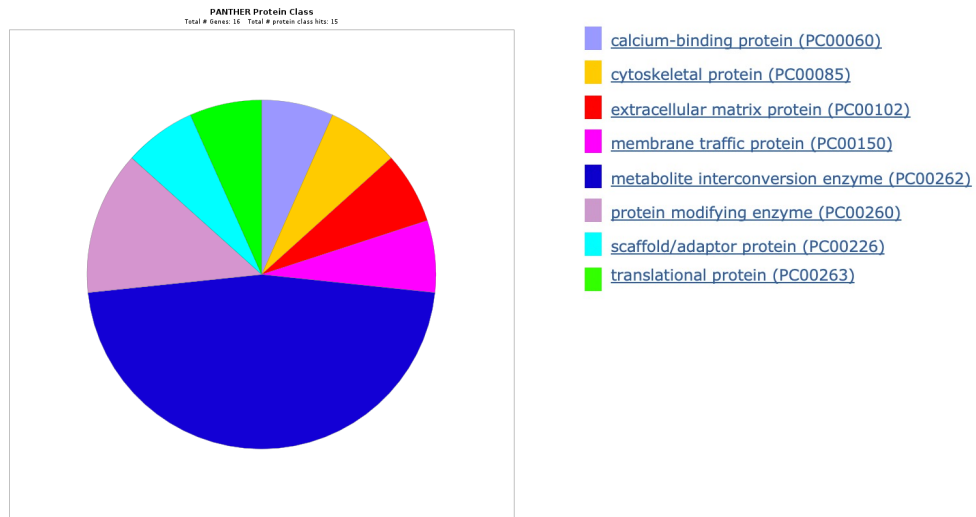


Fig. 13n: Panther Protein Class on the 21 UP biomarkers with $p < 0.01$

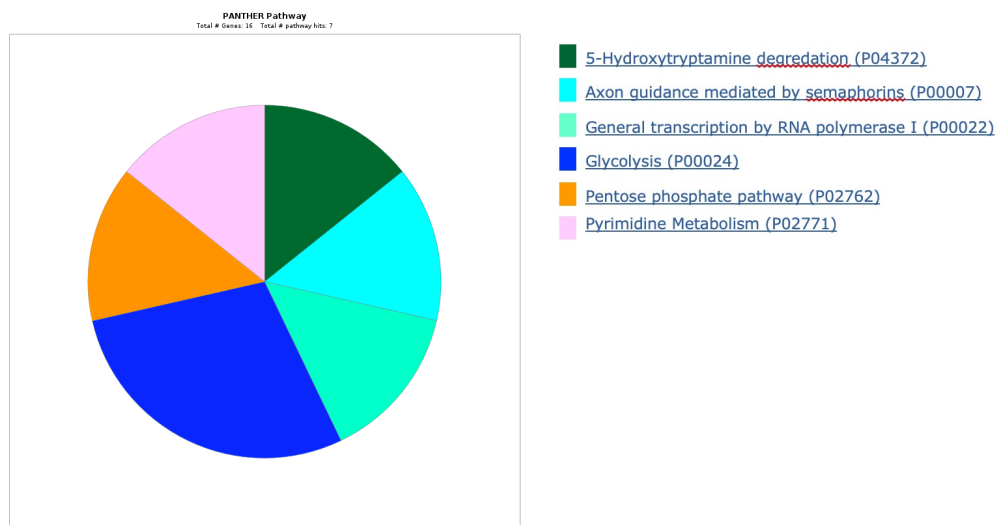


Fig 13o: Panther pathway analysis on the 21 UP biomarkers with $p < 0.01$

Fig. 14: KEGG pathway analyses

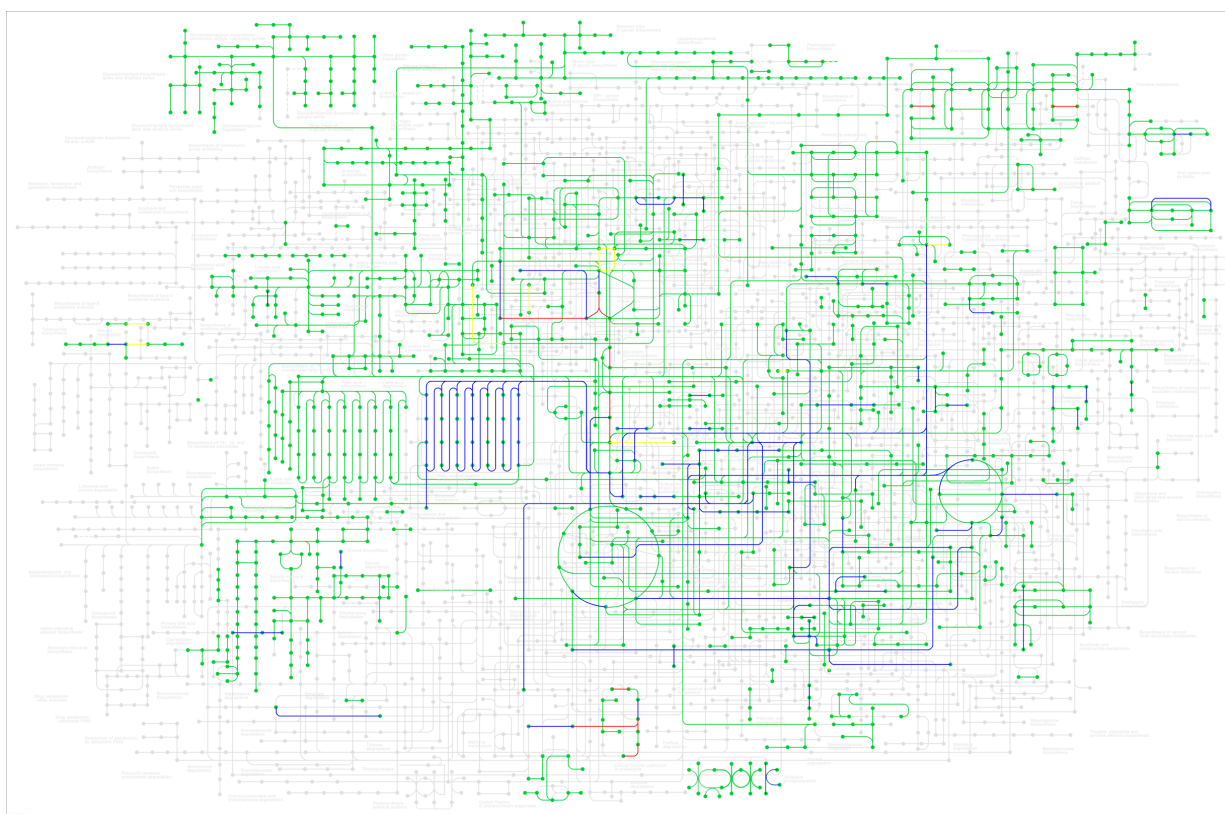


Fig 14a: Overall metabolism

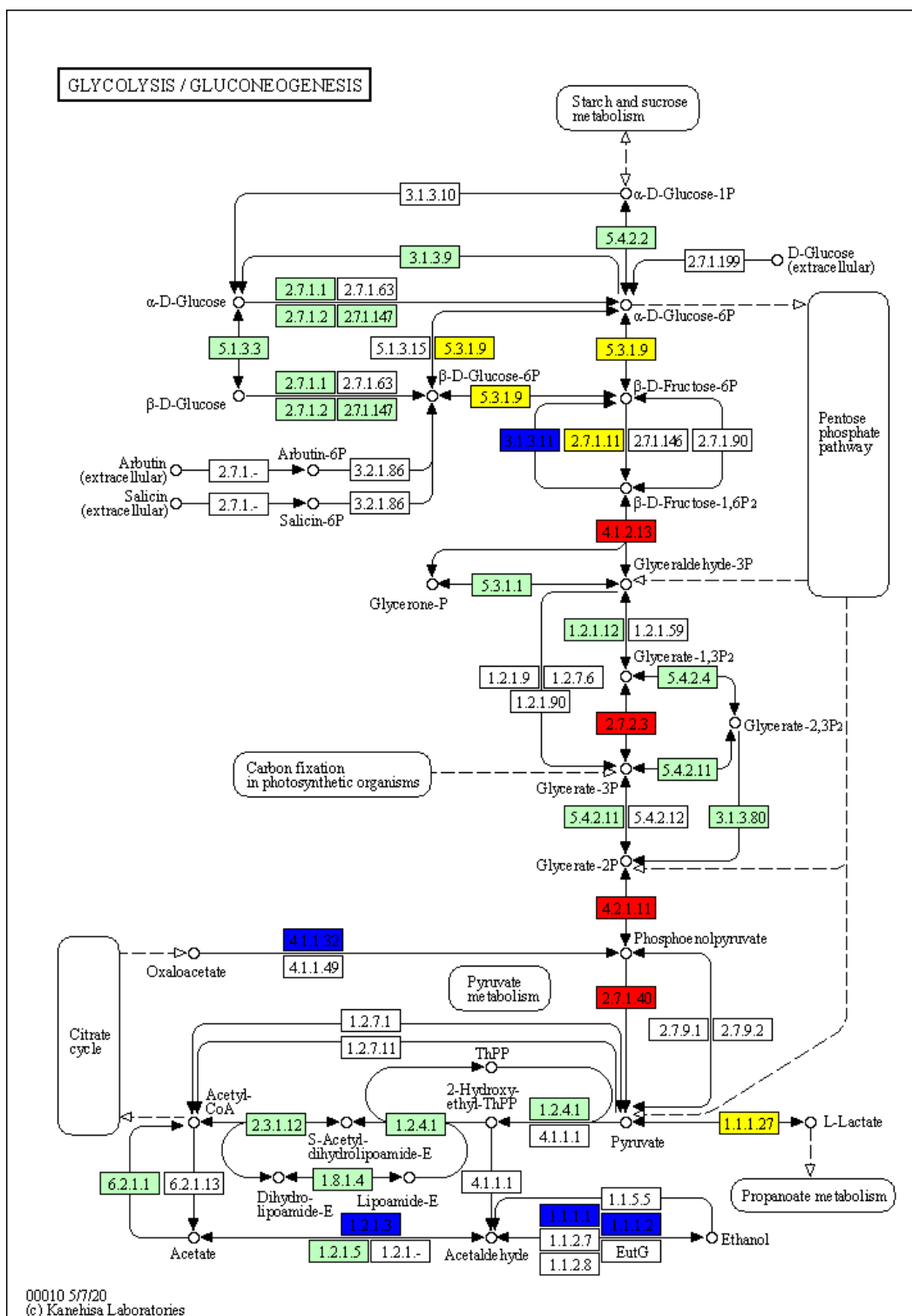


Fig 14b: KEGG pathway analysis on glycolysis and gluconeogenesis

FATTY ACID DEGRADATION

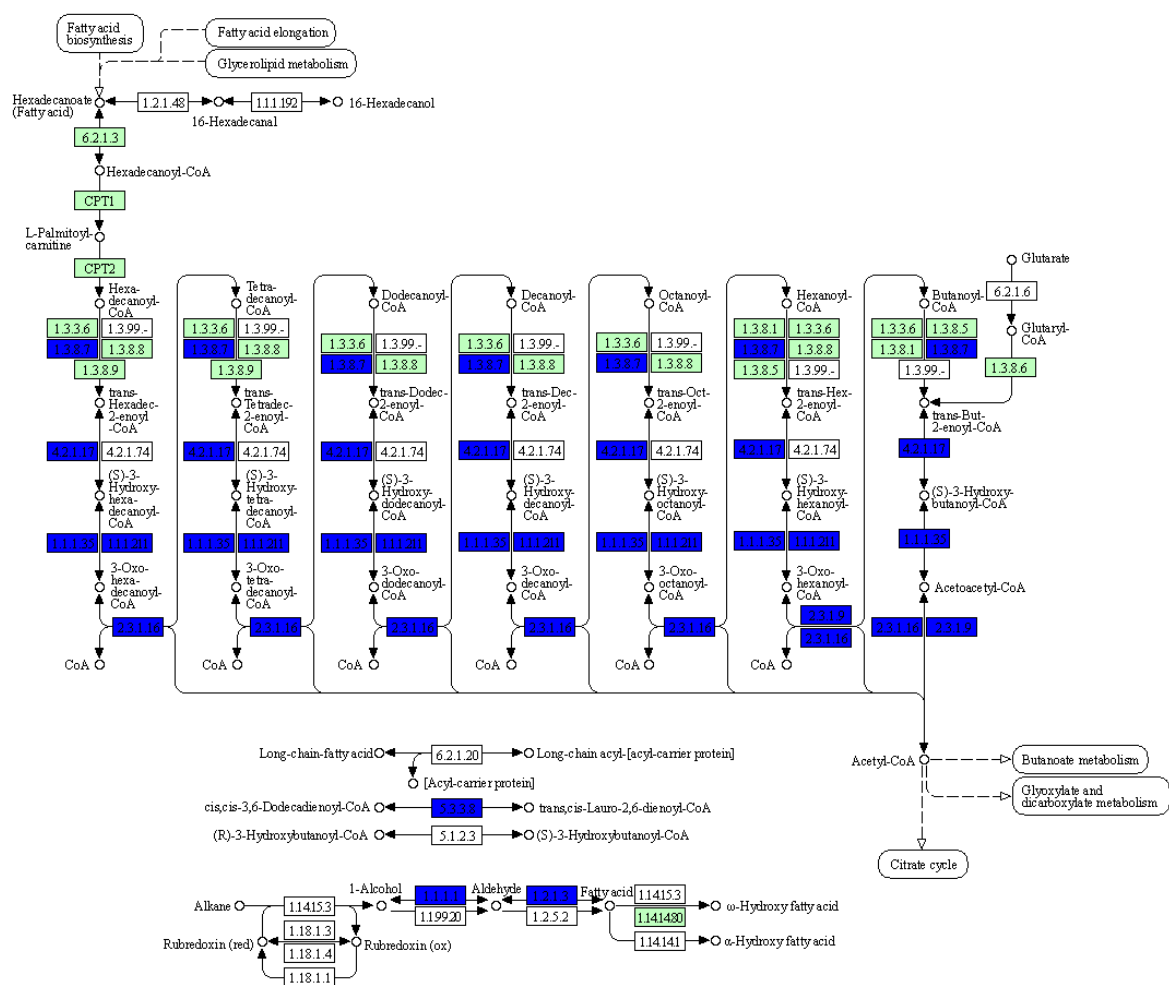


Fig 14c: KEGG analysis of β -oxidation

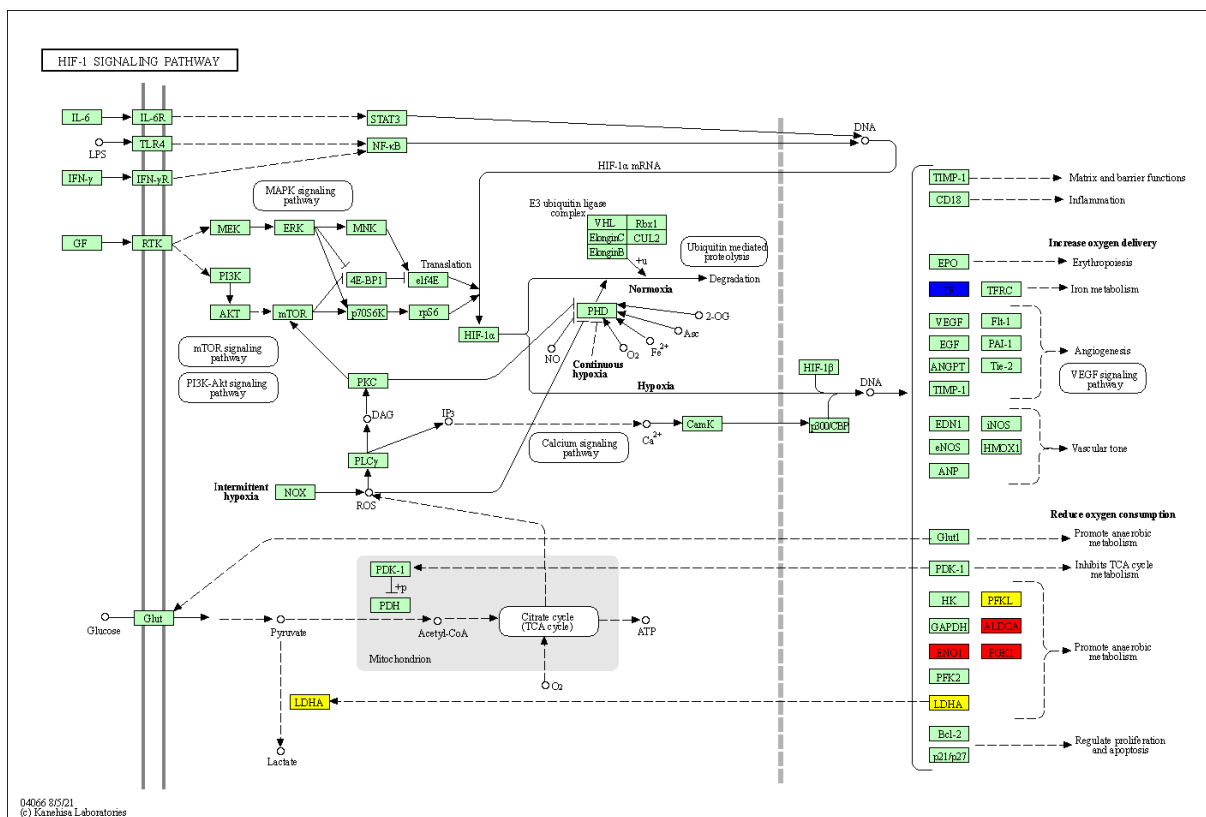
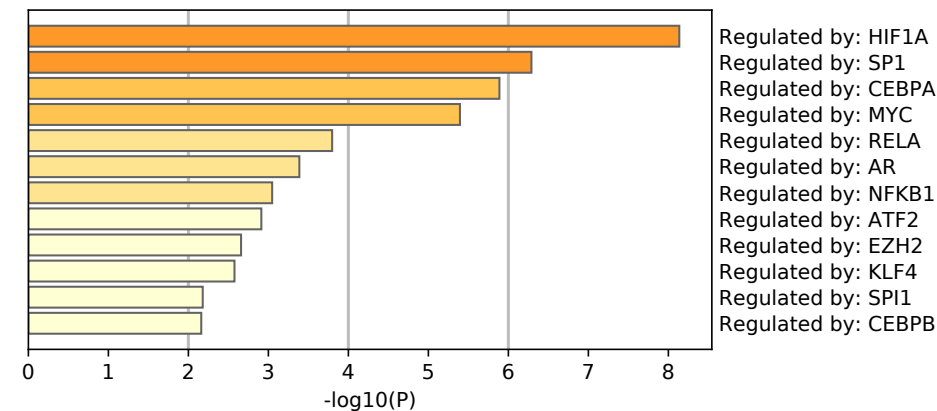


Fig. 14e KEGG pathway analysis : HIF pathway

Fig. 15 Metascape analysis (TRRUST Analysis)



GO	Regulated by	Count	%	Log10(P)	Log10(q)
TRR00484	HIF1A	9	5.1	-8.1	-5.8
TRR01256	SP1	15	8.5	-6.3	-4.2
TRR00109	CEBPA	6	3.4	-5.9	-3.9
TRR00780	MYC	7	4	-5.4	-3.5
TRR01158	RELA	9	5.1	-3.8	-2.1
TRR00011	AR	5	2.8	-3.4	-1.8
TRR00875	NFKB1	8	4.5	-3.0	-1.5
TRR00023	ATF2	3	1.7	-2.9	-1.4
TRR00290	EZH2	3	1.7	-2.7	-1.2
TRR00674	KLF4	3	1.7	-2.6	-1.2
TRR01264	SPI1	3	1.7	-2.2	-0.85
TRR00110	CEBPB	3	1.7	-2.2	-0.83

Fig 15a: TRRUST analysis on the 180 biomarkers with $p < 0.05$

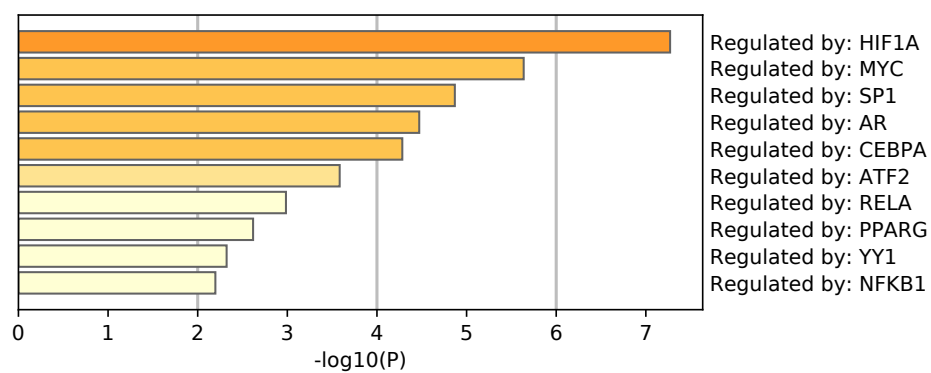
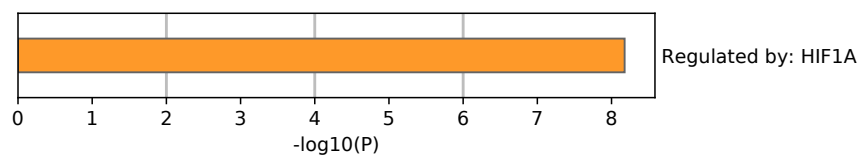


Fig 15b: TRRUST analysis of the 105 ccRCC biomarkers with $p < 0.01$



GO	Regulated by	Count	%	Log10(P)	Log10(q)
TRR00484	HIF1A	5	24	-8.2	-5

Fig 15c: TRRUST analysis of the the 21 UP biomarkers with $p < 0.01$

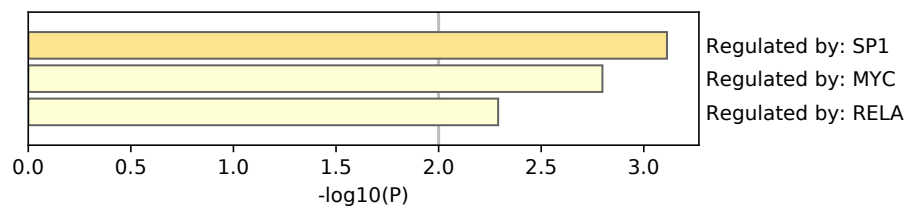


Figure 15d: TRRUST analysis of the 66 DOWN biomarkers $p < 0.01$

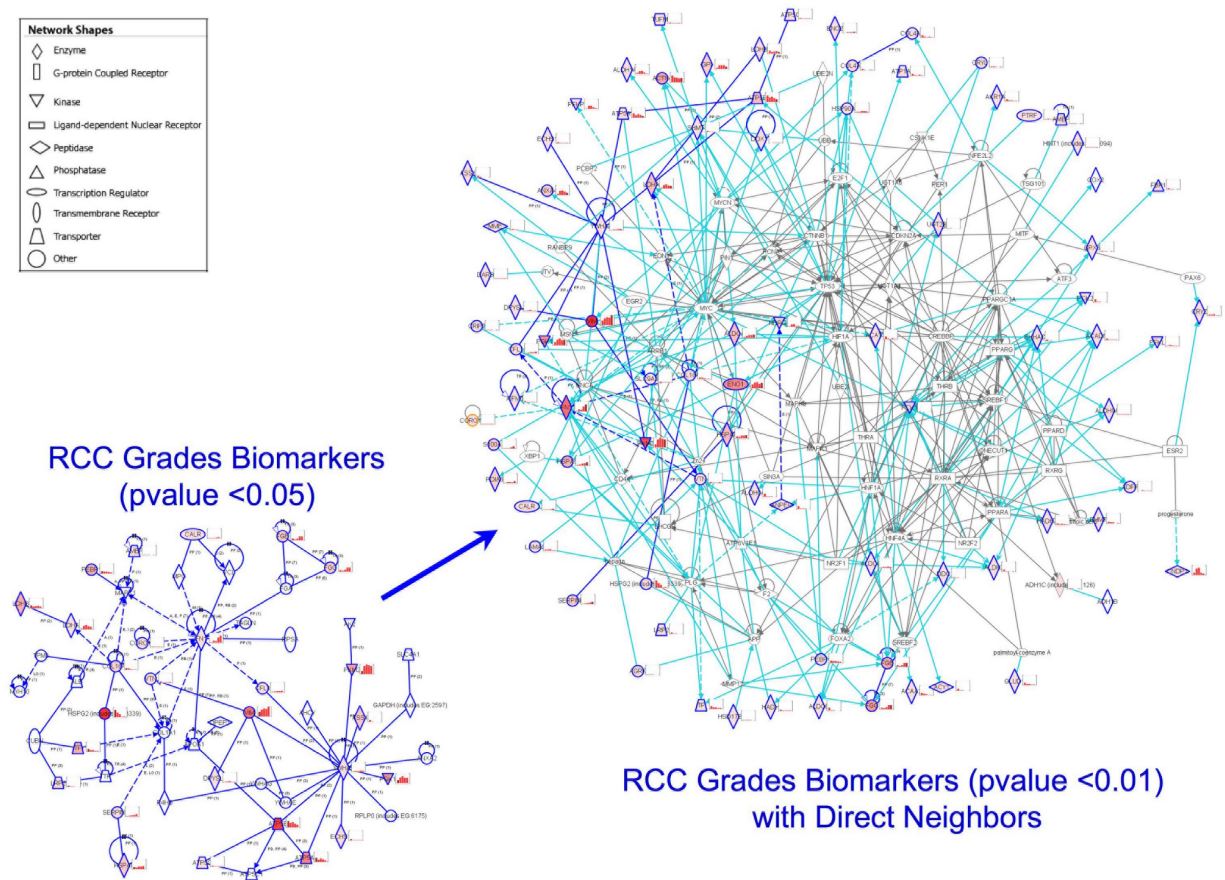


Fig.16a ccRCC proteomic interaction map (from Figure 6, Perroud et al. (2009))

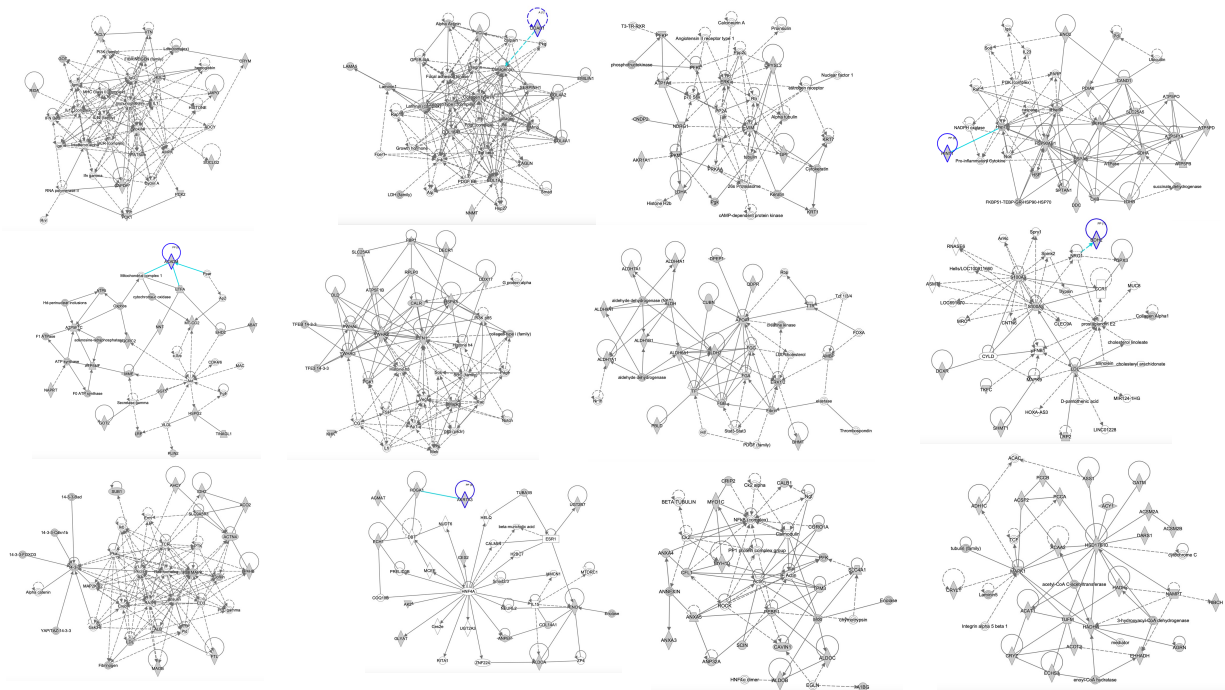


Figure 16b: 12 IPA subnetworks based on the 180 proteins with $p < 0.05$

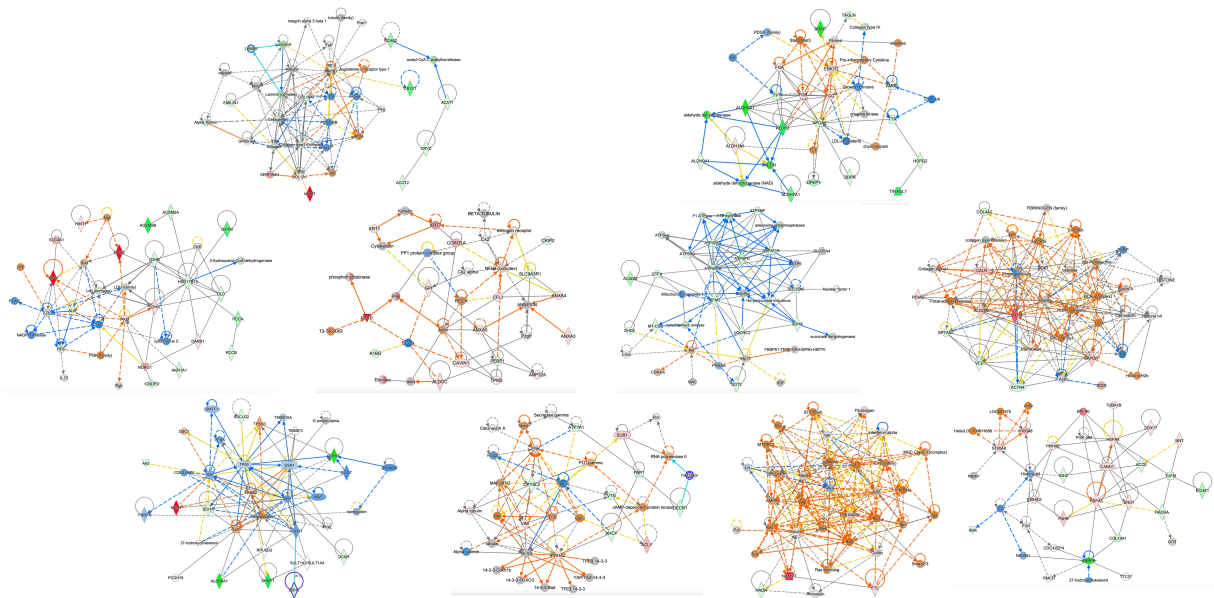


Fig.16c: IPA network analysis on 180 biomarkers high grade vs Normal fold change and ANOVA p-value

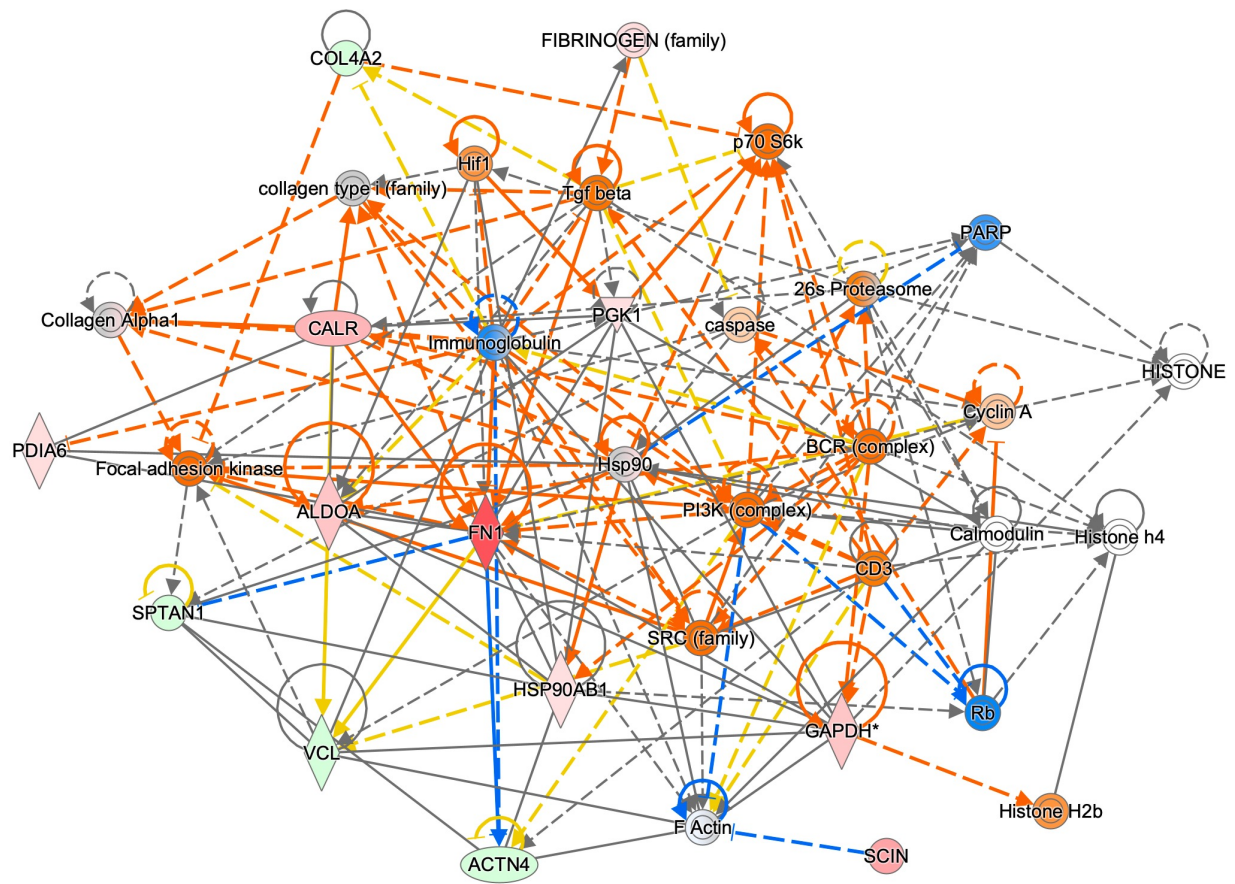


Fig.16d: HIF1-TGFβ-FN1-VCL-ACTN4 network

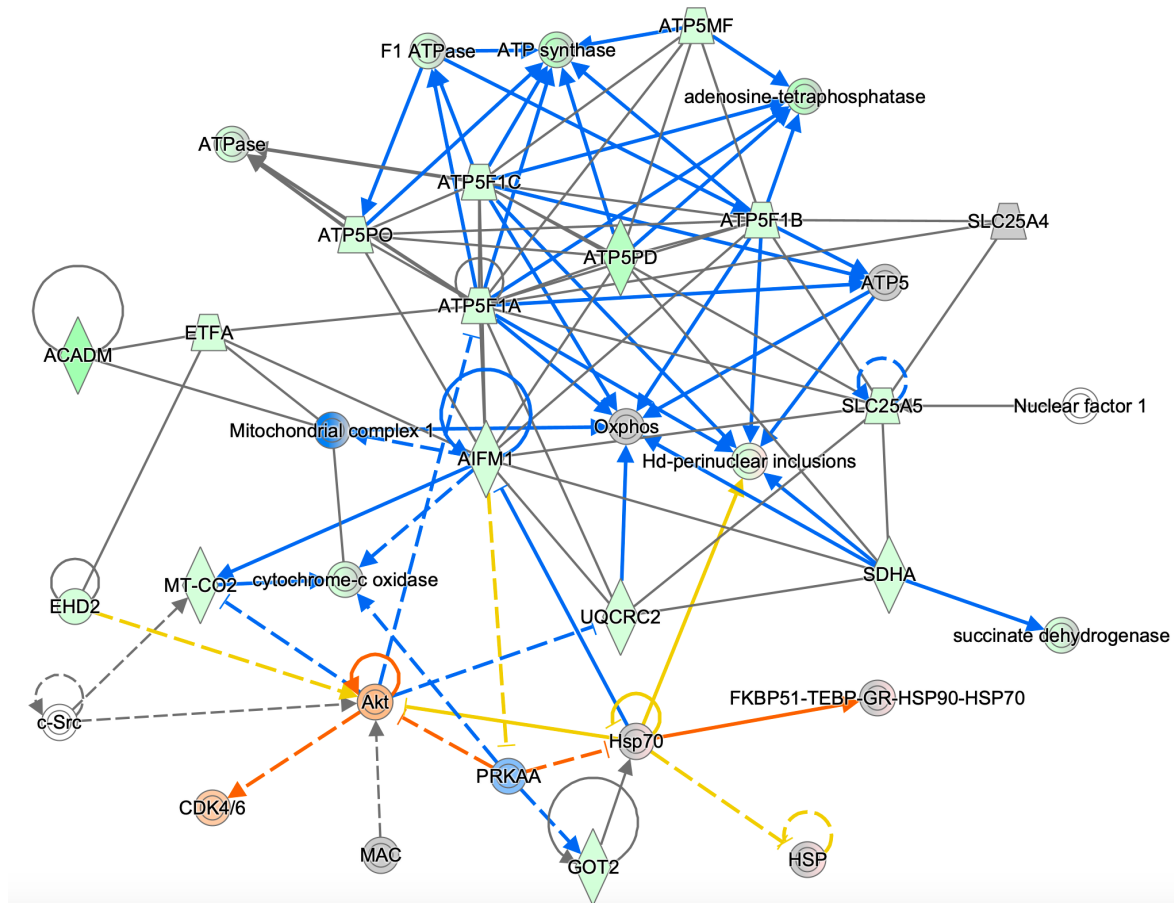


Fig.16e: Mitochondrial metabolism

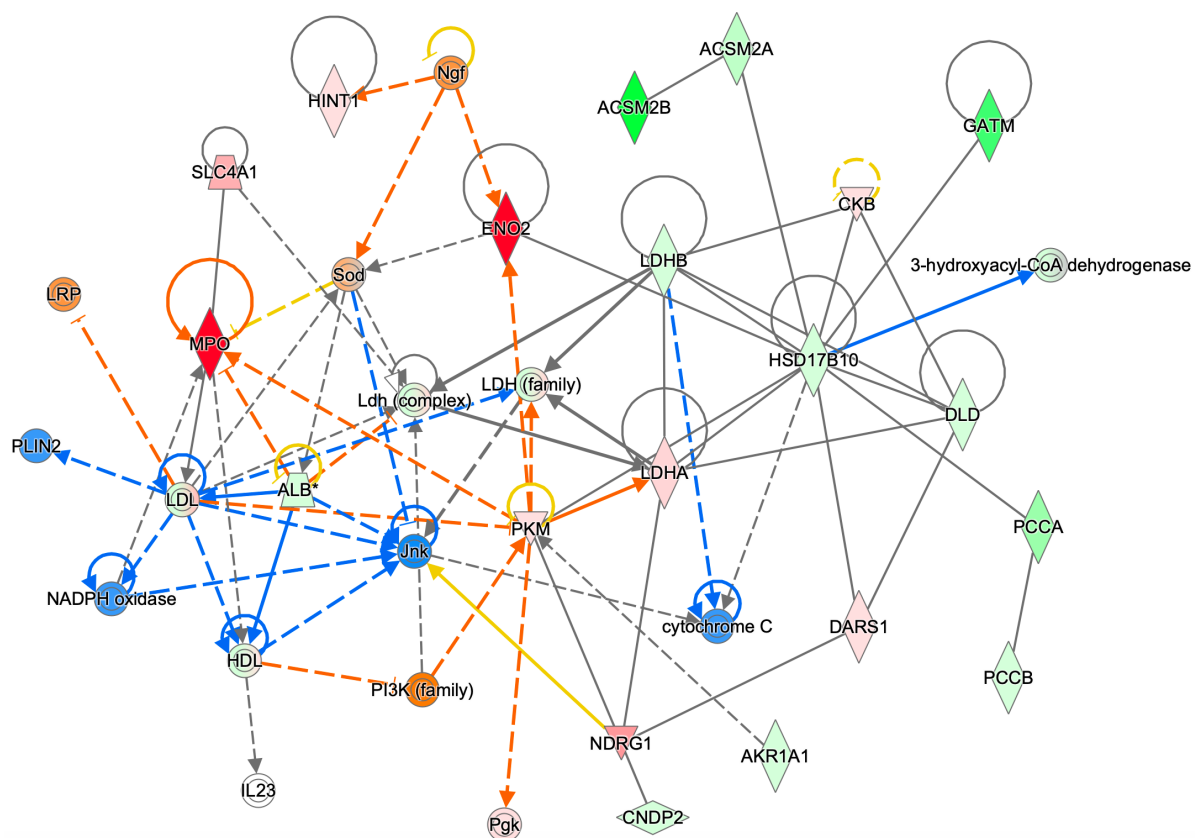


Figure 16f: β -oxidation – NDRG1 network

GO cellular component complete	Total	Detected	Expected	Fold Enrichment	+/-	Raw p value	FDR
cytoplasmic vesicle	2455	5	0.6	8.39	+	2.42E-05	9.62E-03
intracellular vesicle	2458	5	0.6	8.38	+	2.43E-05	8.06E-03
extracellular exosome	2098	5	0.51	9.82	+	1.10E-05	2.19E-02
extracellular vesicle	2118	5	0.51	9.72	+	1.16E-05	1.15E-02
extracellular membrane-bounded organelle	2120	5	0.51	9.71	+	1.16E-05	5.78E-03
extracellular organelle	2120	5	0.51	9.71	+	1.16E-05	7.70E-03
extracellular space	3392	5	0.82	6.07	+	1.22E-04	3.45E-02

Table 1: Gene Ontology (GO) analysis of proteins differentially detected from fresh-frozen and formalin-fixed paraffin-embedded (FFPE) human kidney sections

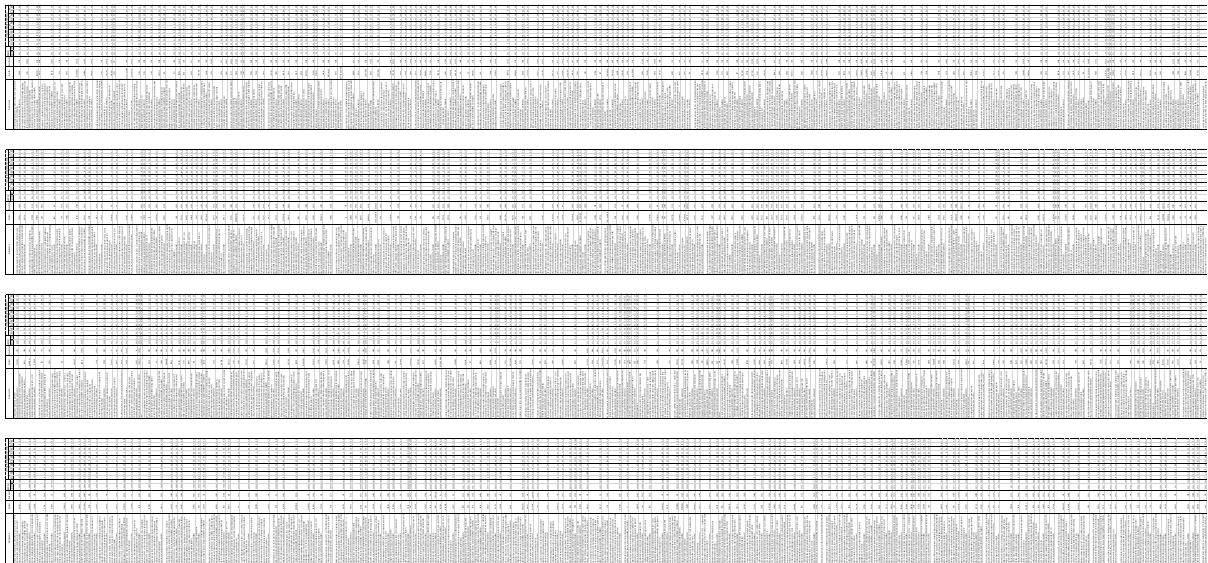


Table 2: Average normalised spectral count of grade-defined ccRCC FFPE samples

Protein Name	Symbol	Genes ID	CCM	ANOVA	Grade 3 dancer	Grade 4 dancer	Grade 5 dancer
Glycine amidotransferase	GATM	26782 Down	1.1E-09	1.7	2.6	1.2	0.0
Methylcrotonyl-CoA carboxylase	ADPM41	43292 Down	2.8E-09	14.9	1.8	0.4	0.3
Glutathione peroxidase 1 precursor	GPX3	28778 Down	3.6E-08	6	0.7	0.2	0.1
Adenylate dehydrogenase	ADH2	62127 Down	1.1E-07	7.3	0.6	1.1	0
Glutathione S-transferase A1	GSTA1	64761 Down	1.5E-07	1.5	1.5	1.1	0
3-Hydroxyphenyllactic acid synthase	SHMT1	64792 Down	7.7E-07	3.7	1.3	0.1	0
Collagen, type XVII, α 1	COL18A1	80781 Down	1.2E-06	7.2	4.3	2.5	0.7
ASG1	ASG1	8460 Down	1.7E-06	8.2	10.9	7	0
Argininosuccinate synthase	ASG1	8460 Down	1.7E-06	8.2	10.9	7	0
Asparaginase N	ANP3	3360 Down	2.1E-06	8.6	0.5	0.1	0
Al-Phosphate 5-carboxylate dehydrogenase	ADPM41	86592 Down	4.8E-06	9.6	0.4	0.3	0
Glutathione S-transferase A1	GSTA1	64761 Down	1.5E-07	1.5	1.5	1.1	0
Fructose-1,6-bisphosphatase 1	FBP1	21020 Down	6.6E-06	5.7	1.3	0.5	0.6
Succinyl-CoA ligase β chain	SUCLG2	88021 Down	1.4E-05	2.8	0.2	0.1	0.6
Phosphoenolpyruvate carboxykinase	PCK2	51066 Down	7.1E-05	1.3	1.2	0.4	0
Protein tyrosine phosphatase, binding protein 1	PTBP1	50373 Down	3.5E-05	11.9	9.4	6.8	3.7
Adenylate dehydrogenase	ADH2	37795 Down	5.0E-05	4.2	0.8	1.2	0.3
Glutathione peroxidase 3B	GPX3	28778 Down	3.6E-08	6	0.7	0.2	0.1
Protein tyrosine phosphatase, catalytic	BLNK1	65035 Down	1.0E-04	6.4	4.3	0	0.1
Beta-actin	ACT1	96 Down	2.0E-04	9.5	2.6	0.8	0
3-Hydroxyacyl-CoA hydratase	HACD1	26072 Down	2.0E-04	2	0.2	0	0.1
3-Hydroxyacyl-CoA dehydrogenase, type 2	ADPM41	43292 Down	2.8E-04	1.7	0.3	0.6	0.3
Collagen 2 (H1) chain precursor [arabid]	COL2A1	12746 Down	3.0E-04	10.6	4.3	2.3	2.5
ATP synthase D subunit	ATP5D	5390 Down	3.0E-04	3.6	0.3	0.5	0.4
ATP synthase D subunit	ATP5D	5390 Down	3.0E-04	3.6	0.3	0.5	0.4
Asparaginase-inducing factor 1	AFM1	9131 Down	5.0E-04	5.5	0.6	0.7	0.1
Myosin	MYO1	4311 Down	6.0E-04	2.4	0.1	0.1	0
Protein tyrosine phosphatase, catalytic	BLNK1	65035 Down	1.0E-04	6.4	4.3	0	0.1
Enoyl-CoA hydratase	ECHE1	18520 Down	6.0E-04	4.6	1	1.2	0.1
Acetate dehydrogenase	ADH1A1	10327 Down	6.0E-04	9.1	4.3	3.8	1.8
Collagen 2 (H1) chain precursor [arabid]	COL2A1	12746 Down	3.0E-04	10.6	4.3	2.3	2.5
Glutathione peroxidase 1	GPX1	27462 Down	8.0E-04	8.3	4.2	3.9	1.3
Acetyl-CoA synthetase	ACAT1	360 Down	9.0E-04	11.4	2.5	2.1	5.6
Glutathione peroxidase 1	GPX1	27462 Down	8.0E-04	8.3	4.2	3.9	1.3
Quinine endonuclease	QNE1	14229 Down	1.0E-03	3.8	3.9	5.3	1.2
Prenylate biosynthesis-like domain-containing protein	PHL1	64831 Down	1.1E-03	1.3	0.2	0.6	0
Hydroxyacyl-CoA dehydrogenase	HACD1	26072 Down	2.0E-04	2	0.2	0	0.1
Enoyl-CoA hydratase	ECHE1	18520 Down	6.0E-04	4.6	1	1.2	0.1
Collagen 2 (H1) chain precursor [arabid]	COL2A1	12746 Down	3.0E-04	10.6	4.3	2.3	2.5
Glutathione peroxidase 1	GPX1	27462 Down	8.0E-04	8.3	4.2	3.9	1.3
Acetyl-CoA synthetase	ACAT1	360 Down	9.0E-04	11.4	2.5	2.1	5.6
Glutathione peroxidase 1	GPX1	27462 Down	8.0E-04	8.3	4.2	3.9	1.3
Quinine endonuclease	QNE1	14229 Down	1.0E-03	3.8	3.9	5.3	1.2
Prenylate biosynthesis-like domain-containing protein	PHL1	64831 Down	1.1E-03	1.3	0.2	0.6	0
Hydroxyacyl-CoA dehydrogenase	HACD1	26072 Down	2.0E-04	2	0.2	0	0.1
Enoyl-CoA hydratase	ECHE1	18520 Down	6.0E-04	4.6	1	1.2	0.1
Collagen 2 (H1) chain precursor [arabid]	COL2A1	12746 Down	3.0E-04	10.6	4.3	2.3	2.5
Glutathione peroxidase 1	GPX1	27462 Down	8.0E-04	8.3	4.2	3.9	1.3
Acetyl-CoA synthetase	ACAT1	360 Down	9.0E-04	11.4	2.5	2.1	5.6
Glutathione peroxidase 1	GPX1	27462 Down	8.0E-04	8.3	4.2	3.9	1.3
Quinine endonuclease	QNE1	14229 Down	1.0E-03	3.8	3.9	5.3	1.2
Prenylate biosynthesis-like domain-containing protein	PHL1	64831 Down	1.1E-03	1.3	0.2	0.6	0
Hydroxyacyl-CoA dehydrogenase	HACD1	26072 Down	2.0E-04	2	0.2	0	0.1
Enoyl-CoA hydratase	ECHE1	18520 Down	6.0E-04	4.6	1	1.2	0.1
Collagen 2 (H1) chain precursor [arabid]	COL2A1	12746 Down	3.0E-04	10.6	4.3	2.3	2.5
Glutathione peroxidase 1	GPX1	27462 Down	8.0E-04	8.3	4.2	3.9	1.3
Acetyl-CoA synthetase	ACAT1	360 Down	9.0E-04	11.4	2.5	2.1	5.6
Glutathione peroxidase 1	GPX1	27462 Down	8.0E-04	8.3	4.2	3.9	1.3
Quinine endonuclease	QNE1	14229 Down	1.0E-03	3.8	3.9	5.3	1.2
Prenylate biosynthesis-like domain-containing protein	PHL1	64831 Down	1.1E-03	1.3	0.2	0.6	0
Hydroxyacyl-CoA dehydrogenase	HACD1	26072 Down	2.0E-04	2	0.2	0	0.1
Enoyl-CoA hydratase	ECHE1	18520 Down	6.0E-04	4.6	1	1.2	0.1
Collagen 2 (H1) chain precursor [arabid]	COL2A1	12746 Down	3.0E-04	10.6	4.3	2.3	2.5
Glutathione peroxidase 1	GPX1	27462 Down	8.0E-04	8.3	4.2	3.9	1.3
Acetyl-CoA synthetase	ACAT1	360 Down	9.0E-04	11.4	2.5	2.1	5.6
Glutathione peroxidase 1	GPX1	27462 Down	8.0E-04	8.3	4.2	3.9	1.3
Quinine endonuclease	QNE1	14229 Down	1.0E-03	3.8	3.9	5.3	1.2
Prenylate biosynthesis-like domain-containing protein	PHL1	64831 Down	1.1E-03	1.3	0.2	0.6	0
Hydroxyacyl-CoA dehydrogenase	HACD1	26072 Down	2.0E-04	2	0.2	0	0.1
Enoyl-CoA hydratase	ECHE1	18520 Down	6.0E-04	4.6	1	1.2	0.1
Collagen 2 (H1) chain precursor [arabid]	COL2A1	12746 Down	3.0E-04	10.6	4.3	2.3	2.5
Glutathione peroxidase 1	GPX1	27462 Down	8.0E-04	8.3	4.2	3.9	1.3
Acetyl-CoA synthetase	ACAT1	360 Down	9.0E-04	11.4	2.5	2.1	5.6
Glutathione peroxidase 1	GPX1	27462 Down	8.0E-04	8.3	4.2	3.9	1.3
Quinine endonuclease	QNE1	14229 Down	1.0E-03	3.8	3.9	5.3	1.2
Prenylate biosynthesis-like domain-containing protein	PHL1	64831 Down	1.1E-03	1.3	0.2	0.6	0
Hydroxyacyl-CoA dehydrogenase	HACD1	26072 Down	2.0E-04	2	0.2	0	0.1
Enoyl-CoA hydratase	ECHE1	18520 Down	6.0E-04	4.6	1	1.2	0.1
Collagen 2 (H1) chain precursor [arabid]	COL2A1	12746 Down	3.0E-04	10.6	4.3	2.3	2.5
Glutathione peroxidase 1	GPX1	27462 Down	8.0E-04	8.3	4.2	3.9	1.3
Acetyl-CoA synthetase	ACAT1	360 Down	9.0E-04	11.4	2.5	2.1	5.6
Glutathione peroxidase 1	GPX1	27462 Down	8.0E-04	8.3	4.2	3.9	1.3
Quinine endonuclease	QNE1	14229 Down	1.0E-03	3.8	3.9	5.3	1.2
Prenylate biosynthesis-like domain-containing protein	PHL1	64831 Down	1.1E-03	1.3	0.2	0.6	0
Hydroxyacyl-CoA dehydrogenase	HACD1	26072 Down	2.0E-04	2	0.2	0	0.1
Enoyl-CoA hydratase	ECHE1	18520 Down	6.0E-04	4.6	1	1.2	0.1
Collagen 2 (H1) chain precursor [arabid]	COL2A1	12746 Down	3.0E-04	10.6	4.3	2.3	2.5
Glutathione peroxidase 1	GPX1	27462 Down	8.0E-04	8.3	4.2	3.9	1.3
Acetyl-CoA synthetase	ACAT1	360 Down	9.0E-04	11.4	2.5	2.1	5.6
Glutathione peroxidase 1	GPX1	27462 Down	8.0E-04	8.3	4.2	3.9	1.3
Quinine endonuclease	QNE1	14229 Down	1.0E-03	3.8	3.9	5.3	1.2
Prenylate biosynthesis-like domain-containing protein	PHL1	64831 Down	1.1E-03	1.3	0.2	0.6	0
Hydroxyacyl-CoA dehydrogenase	HACD1	26072 Down	2.0E-04	2	0.2	0	0.1
Enoyl-CoA hydratase	ECHE1	18520 Down	6.0E-04	4.6	1	1.2	0.1
Collagen 2 (H1) chain precursor [arabid]	COL2A1	12746 Down	3.0E-04	10.6	4.3	2.3	2.5
Glutathione peroxidase 1	GPX1	27462 Down	8.0E-04	8.3	4.2	3.9	1.3
Acetyl-CoA synthetase	ACAT1	360 Down	9.0E-04	11.4	2.5	2.1	5.6
Glutathione peroxidase 1	GPX1	27462 Down	8.0E-04	8.3	4.2	3.9	1.3
Quinine endonuclease	QNE1	14229 Down	1.0E-03	3.8	3.9	5.3	1.2
Prenylate biosynthesis-like domain-containing protein	PHL1	64831 Down	1.1E-03	1.3	0.2	0.6	0
Hydroxyacyl-CoA dehydrogenase	HACD1	26072 Down	2.0E-04	2	0.2	0	0.1
Enoyl-CoA hydratase	ECHE1	18520 Down	6.0E-04	4.6	1	1.2	0.1
Collagen 2 (H1) chain precursor [arabid]	COL2A1	12746 Down	3.0E-04	10.6	4.3	2.3	2.5
Glutathione peroxidase 1	GPX1	27462 Down	8.0E-04	8.3	4.2	3.9	1.3
Acetyl-CoA synthetase	ACAT1	360 Down	9.0E-04	11.4	2.5	2.1	5.6
Glutathione peroxidase 1	GPX1	27462 Down	8.0E-04	8.3	4.2	3.9	1.3
Quinine endonuclease	QNE1	14229 Down	1.0E-03	3.8	3.9	5.3	1.2
Prenylate biosynthesis-like domain-containing protein	PHL1	64831 Down	1.1E-03	1.3	0.2	0.6	0
Hydroxyacyl-CoA dehydrogenase	HACD1	26072 Down	2.0E-04	2	0.2	0	0.1
Enoyl-CoA hydratase	ECHE1	18520 Down	6.0E-04	4.6	1	1.2	0.1
Collagen 2 (H1) chain precursor [arabid]	COL2A1	12746 Down	3.0E-04	10.6	4.3	2.3	2.5
Glutathione peroxidase 1	GPX1	27462 Down	8.0E-04	8.3	4.2	3.9	1.3
Acetyl-CoA synthetase	ACAT1	360 Down	9.0E-04	11.4	2.5	2.1	5.6
Glutathione peroxidase 1	GPX1	27462 Down	8.0E-04	8.3	4.2	3.9	1.3
Quinine endonuclease	QNE1	14229 Down	1.0E-03	3.8	3.9	5.3	1.2
Prenylate biosynthesis-like domain-containing protein	PHL1	64831 Down	1.1E-03	1.3	0.2	0.6	0
Hydroxyacyl-CoA dehydrogenase	HACD1	26072 Down	2.0E-04	2	0.2	0	0.1
Enoyl-CoA hydratase	ECHE1	18520 Down	6.0E-04	4.6	1	1.2	0.1
Collagen 2 (H1) chain precursor [arabid]	COL2A1	12746 Down	3.0E-04	10.6	4.3	2.3	2.5
Glutathione peroxidase 1	GPX1	27462 Down	8.0E-04	8.3	4.2	3.9	1.3
Acetyl-CoA synthetase	ACAT1	360 Down	9.0E-04	11.4	2.5	2.1	5.6
Glutathione peroxidase 1	GPX1	27462 Down	8.0E-04	8.3	4.2	3.9	1.3
Quinine endonuclease	QNE1	14229 Down	1.0E-03	3.8	3.9	5.3	1.2
Prenylate biosynthesis-like domain-containing protein	PHL1	64831 Down	1.1E-03	1.3	0.2	0.6	0
Hydroxyacyl-CoA dehydrogenase	HACD1	26072 Down	2.0E-04	2	0.2	0	0.1
Enoyl-CoA hydratase	ECHE1	18520 Down	6.0E-04	4.6	1	1.2	0.1
Collagen 2 (H1) chain precursor [arabid]	COL2A1	12746 Down	3.0E-04	10.6	4.3	2.3	2.5
Glutathione peroxidase 1	GPX1	27462 Down	8.0E-04	8.3	4.2	3.9	1.3
Acetyl-CoA synthetase	ACAT1	360 Down	9.0E-04	11.4	2.5	2.1	5.6
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Glutathione peroxidase 1	GPX1	27462 Down	8.0E-04	8.3	4.2	3.9	1.3
Acetyl-CoA synthetase	ACAT1	360 Down	9.0E-04	11.4	2.5	2.1	5.6
Glutathione peroxidase 1	GPX1	27462 Down	8.0E-04	8.3	4.2	3.9	1.3
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Prenylate biosynthesis-like domain-containing protein	PHL1	64831 Down	1.1E-03	1.3	0.2	0.6	0
Hydroxyacyl-CoA dehydrogenase	HACD1	26072 Down	2.0E-04	2	0.2	0	0.1
Enoyl-CoA hydratase	ECHE1	18520 Down</					

[illegible]

Table 4: grade-defining ccRCC biomarkers, their expression profile classification, and their grade-wise average normalised spectral counts ($p < 0.05$)

Protein	p-value	ANOVA Class
ACY1	0.01	DOWN
ASS1	0.01	DOWN
AGMAT	0.01	DOWN
BHMT	0.01	DOWN
GATM	0.01	DOWN
ECHS1	0.01	DOWN
PCK2	0.01	DOWN
AKR1A1	0.01	DOWN
ALDOB	0.01	DOWN
ACAT1	0.01	DOWN
CALB1	0.05	DOWN
ATP5H	0.05	DOWN
ENO2	0.01	UP
PKM2	0.01	UP
GAPDH	0.05	UP
LDHA	0.01	UPG1
ANXA4	0.01	UPG1
HINT1	0.01	UPG1
CLIC1	NA	Not identified
PRDX1	NA	Not identified
GSTO2	NA	Not identified
SUCLG1	NA	Not identified
FABP7	NA	Not identified
ANX5	NA	Not identified
HSPD1	NA	Not identified
HSPB1	NA	Not identified
HSPCB	NA	Not identified
CSTB	NA	Not identified
PSME2	NA	Not identified
TBB2	NA	Not identified
ATP5D	NA	Not identified

Table 5: grade-defining ccRCC biomarkers detected in Perroud et al. (2006) and their expression pattern in the current grade-defined mass spectrometry-based proteomic analysis

CHAPTER 4

Effects of Argininosuccinate Synthetase (ASS1) silencing on ccRCC metabolic reprogramming and morphology

ABSTRACT

Argininosuccinate synthetase (ASS1) catalyses the condensation reaction of aspartate and citrulline into argininosuccinate, the rate-determining step in the urea cycle. In our grade-defined ccRCC proteomic analysis, it was identified as one of the Fuhrman grade-defining biomarkers whose expression levels were found to be downregulated across all four Fuhrman nuclear grades compared to the normal tissue. The gene is also silenced in a variety of cancers. In order to investigate the significance of the ASS1 silencing in ccRCC pathobiology, ASS1 expression levels were modulated in normal and cancer cells *in vitro* and the resultant metabolomes were analysed by mass spectrometry. A wide range of metabolic pathways were shown to be modulated upon ASS1 overexpression or knockdown. ASS1 overexpression in 786-O cells resulted in 6-fold increase in N-acetylaspartate while ASS1 knockdown in HK2 cells showed 2.2-fold decrease in N-acetylaspartate. This suggests potential modulation in acetyl-CoA metabolism. Notably, ASS1 knockdown resulted in profound and global lipidomic changes. Choline plasmalogens such as PC(p-36:2)/PC(o-36:3) and PC(p-36:4)/PC(o-36:5) were reduced by up to three magnitudes of order while neutral lipids including sphingolipids such as GlcCer(d34:1) and triacylglycerols such as TG(58:1) were upregulated by up to 6.7-fold. Given

intracellular lipid droplets are composed of the core of neutral lipids surrounded by monolayer of polar lipids such as plasmalogens, the observed increase in neutral lipids and reduction in choline plasmalogens are hypothesised to synergise to decrease the surface-to-volume ratio of the lipid droplets. This in turn is hypothesised to result in larger intracellular lipid droplets characteristic of ccRCC. Together with the changes in N-acetylaspartate levels, our lipidomic analysis suggests ASS1 silencing in ccRCC is a key contributor to the clear-cell phenotype in ccRCC.

INTRODUCTION

While renal cell carcinoma (RCC) accounts for only 2% of the total cancer cases, it is the sixth leading cause of cancer death in US. This disparity in part comes from the lack of effective treatments against clear cell RCC (ccRCC), which comprises 75% of the total RCC cases.

A vast majority of ccRCC is characterised by constitutively active HIF signalling due to truncated and thus inactivated VHL protein, the E3 ubiquitin ligase for HIF1 α and HIF2 α . Morphologically, it exhibits clear cytoplasm due to accumulation of cytosolic lipid droplets. [1] In addition, VHL-hypomorph phenotype contributes to its inability to undergo p53-dependent apoptosis, thus demonstrating need to exploit other tumour vulnerabilities. [2, 3]

Fuhrman nuclear Grade was designed to classify ccRCC into four grades according to their nuclear morphology. [4] Low-grade ccRCC retains small round nuclei despite disrupted basolateral membrane structure typical of proximal kidney tubule. Higher grades show increasingly larger non-spherical shape, with the highest grade associated with multi-lobule nuclei. In addition, higher grade nuclei exhibit more prominent nucleoli. Fuhrman grades remained a widely used ccRCC diagnostic criteria due to good correlation with the prognosis and its ease of use that comes from simple and standard staining procedure. The grading scheme, however, is fundamentally a subjectively defined criteria, which lacks molecular definition. [5]

Our earlier proteomic studies identified Argininosuccinate Synthetase 1 (ASS1) as a ccRCC biomarker. [6] ASS1 is a urea cycle enzyme downregulated in cRCC across all Fuhrman nuclear grades. [7, 8] as well as in a number of other types of cancers. ASS1 silencing in ccRCC results in an increase in amino acid levels and reduction of toxic metabolites, [8] which in turn contributes to adaptation to hypo-nutrient and hypoxic cancer microenvironment and augments its proliferation.

In this chapter, using a combination of conventional molecular biology and metabolomics approaches, we investigated additional metabolites whose levels were altered upon exogenous ASS1 expression or its CRISPR deletion. This work shows that alterations in ASS1 status reprogrammes ccRCC lipidome, and ASS1 re-expression is sufficient to reduce lipid droplets in 786O ccRCC cells *in vitro*. We also confirm the prior finding that ASS1 deletion increases proliferation in low-nutrient medium. In summary, the study presented in this chapter provides evidence that ASS1 silencing increases proliferation with concomitant lipid

metabolic reprogramming and puts forward a hypothesis that modulation in acetyl-CoA level underlies the lipidomic reprogramming, contributing to the clear-cell phenotype of ccRCC.

MATERIALS AND METHODS

Cell lines

ASS1-786O cell line was created by transfecting pCMV6-Entry-hASS1 vector (Origene, cat# RC223189) using Lipofectamine 2000 (Thermo Fisher Scientific, Cat # 11668019). Stably transfected cells were selected against using G418 (Thermo Fisher Scientific, Cat# 11811031). ASS1-786O and the control 786O cell lines were cultured in RPMI-1440 supplemented with 1% or 10% FBS, Pen/Strep, and G418. ASS1-Knockdown (ASS1-KD) HK2 cell lines were gift from Dr. M Celeste Simon (U Pennsylvania) and was previously described. [8]

Immunoblotting

Immunoblotting was done according to a standard protocol. Briefly, cells were washed in ice cold PBS and collected in RIPA buffer supplemented with protease/phosphatase inhibitor cocktail (Sigma, cat#: P8340). The cells were lysed with a sonicator, and cell lysates were immunoblotted. The membranes were blocked using 5% non-fat dry milk / TBST for one hour at ambient temperature. Subsequently, they were incubated in the primary antibody with appropriate blocking agent in TBST at 4°C overnight. After brief washes, the membranes were

incubated in horseradish peroxidase-conjugated anti-mouse or anti-rabbit secondary antibodies. The signal was detected using ECL2 (Pierce, Cat#: 32134) and imaged with LAS-4000 imaging system (Fujifilm Life Sciences, USA).

Lipid droplet (LD) assay

LD-positive cells were counted under a microscope at the specified time. Minimum of three visual fields were counted and the fraction of LD-positive cells to the total cells were manually counted.

Metabolomic analysis

Metabolomics analysis was done by the UC Davis West Coast Metabolomics Center according to the standard protocols using GC-TOF (Primary metabolites), HILIC (Biogenic amines), and QTOF (Complex lipids).

GC-TOF was done on Gerstel CIS4 –with dual MPS Injector/ Agilent 6890 GC-Pegasus III TOF MS. 0.5µl samples were injected at the rate of 10µl/s. GC was performed using a 30m long 0.25mm i.d. Rtx-5Sil MS column set at constant flow of 1ml/min. The oven temperature was held at 50oC for 1 minute, then was increased to 330oC at the rate of 20oC/min. The temperature was held constant at 330oC for 5 mins. For Mass spectroscopic detection, a Leco Pegasus IV TOF mass spectrometer controlled by the Leco Chroma TOF software vs.2.32 (St. Joseph, MI) was used at the temperature of 280oC and the electron impact ionisation at 70V.

Following the sample preparation with a biphasic solvent system of cold methanol, methyl tert-butyl ether (MTBE), the biogenic amine HILIC analysis was done by analysis of polar phase of lipid extraction by ultra-high pressure liquid chromatography (UHPLC) on a Waters BEH Amide Column, interfaced to a SCIEX Triple TOF 6600 mass spectrometer (high resolution, accurate mass), with a 16.8-minute total run time. Data were collected in both positive and negative ion modes and were analyzed using MS DIAL open software for metabolome analysis.

For the lipidomic analyses, samples were extracted in MTBE with addition of internal standards, followed by ultra-high pressure liquid chromatography (UHPLC) on a Waters charged surface hybrid (CSH) column, [9] interfaced with a QTOF mass spectrometer (high resolution, accurate mass), with a 15-minute total run time. Data were collected in both positive and negative ion modes and were analyzed using Mass Hunter (Agilent). Approximately 400 lipids can be identified from plasma, with additional unknowns. The method is highly stable and has been validated on large datasets (>8,000 samples) collected over long time periods (> 1 year).

Statistical analysis

Statistical analysis was performed on Metaboanalyst. [10] Briefly, the datasets were normalised by sum or by median to account for the dynamic ranges of metabolites as well as between-sample variations. Volcano plot was created with fold-change cutoff of 1.2 or 2-fold and $p_{\text{adjusted}} < 0.10$. The normalised data were used to perform partial least squares discriminant analysis (PLS-DA) to calculate the latent variable based on covariance. Components 1 and 2

were used to create the scores plot and percentage represented was noted. Variable importance in projection values were calculated and cross-validation was performed.

Pathway enrichment analysis

The pathway enrichment analysis was performed on ChemRICH according to the developers' instructions. [11]

Pathway analysis

The pathway analysis visualisation was performed using MetaMAPP. [12]

RESULTS

Construction of ASS1-786O and ASS1-Knockdown (ASS1-KD) HK2

The parental 786O ccRCC cell line was stably transfected with full length human Argininosuccinate synthetase (ASS1) tagged with a DDK (FLAG) tag. The expression of the transfected ASS1 was validated using immunoblotting. (**Figure 17a**) Over expressed ASS1 shows significant increase in overall ASS1 level compared with the endogenous level. Additionally, ASS1 overexpression results in suppressed cMYC protein expression as reported previously. [13]

ASS1-KD HK2 (a gift from Dr. M. Celeste Simon) shows complete absence of the endogenous ASS1 although a minor non-specific band remains, which is in agreement with the original report. (**Figure 17b** and [8])

ASS1-786O Metabolomics

Primary Metabolites (GC-TOF) analysis

To investigate the roles of ASS1 in clear cell renal cell carcinoma (ccRCC), ASS1 was exogenously expressed in 786O ccRCC cell line, and metabolomics analysis was performed using three platforms, namely, GC-TOF (primary metabolites), LC-QTOF/GHILIC (biogenic amines), and LC-QTOF / CSH (complex lipids). Statistical analysis was performed using Metaboanalyst [10] In GC-TOF, We observed that 12 metabolites showed greater than 2-fold change in their levels with statistical significance ($p_{\text{adjusted}} < 0.10$) between the ASS1-786O and the control 786O cell lines. Of the 12 metabolites that displayed differential distribution between ASS1-786O and the control 786O, 6 showed increased levels in ASS1-786O compared with those in the control, while another 6 were decreased in ASS1-786O compared with the control. (**Figure 18a** and **Table 6a**). PLS-DA analysis showed that component 1 and 2 described 36% and 17.3 % of the data obtained for the total of 53.3%, which together were able to discriminate between the ASS1-786O samples and the Control-786O on the PLS-DA scores plot. (**Figure 18b** and **18c**) 15

metabolites with the highest VIP scores (**Figure 18d**) were used to successfully cluster the samples into two groups in the heatmap. (**Figure 18e**)

As discussed above, 12 metabolites were significantly increased in ASS1-786O while two metabolites showed statistically significant decrease in the ASS1-786O. (**Table 6a**) ChemRICH analysis indicated reduction in overall amino acids as well as their subclasses such as branched-chain amino acids and aromatic amino acids. On the other hand, purine nucleotides and sugar acids were increased in ASS1-786O. (**Figure 18f**). These metabolites clustered into three large clusters and additional three small clusters in our MetaMAPP analysis. Namely, sugar acids such as octodecylglycerol and hexadecylglycerol were increased in ASS1-786O and clustered together. Another cluster contained increased metabolites such as N-acetyl-aspartic acid and pyruvic acid as well as a number of amino acids that were reduced in ASS1-786O compared to the control 786O. (**Figure 18g**)

Of the six DOWN metabolites whose concentration was reduced in ASS1-786O compared with the control 786O, four are amino acids, and two are glycolysis derivatives including ribitol and glycerol-alpha-phosphate. Of the six UP metabolites, two are monoacylglycerols, which are glycerol derivatives and potential precursor to diacylglycerols (DAG) and triacylglycerols (TAG). Ribose, the remaining metabolite upregulated in the ASS1-786O compared with the control 786O is a monosaccharide and nucleic acid precursor derived ultimately from glycolysis. Ribose also serves as NAD and FAD precursor as well as mitochondrial energy source. [14]

The remaining three are acetyl derivatives including N-acetylaspartate. Notably, N-acetylaspartate showed a 7.3-fold increase in ASS1-786O compared with the control-786O. Further enrichment analysis with ChemRICH programme [11] showed that amino acids are decreased in ASS1-786O compared with the control 786O. In detail, of the four sub-categories of amino acids, aromatic amino acids, amino acids containing sulphur, and branched-chain amino acids were reduced in ASS1-786O compared with the control 786O, while acidic amino acids were relatively unchanged. In addition, several dicarboxylic acids were reduced in ASS1-786O. Finally, an enrichment analysis was performed on Metaboanalyst against the KEGG database. [15] The results indicated alanine, aspartate, and glutamate metabolism as well as pyrimidine metabolism to have a moderate enrichment in the identified metabolites, which collaborated with the analysis above. (Data not shown)

Biogenic amines

Our analysis of biogenic amines in ASS1-786O and control 786O identified 20 metabolites that were differentially present between the two groups analysed with at least 2-fold enrichment with $p_{\text{adjusted}} > 0.10$. (**Figure 19a**, and **Table 6b**) PLS-DA analysis discriminated ASS1-786O and control 786O with component 1 covering 55.0% of the data and component 2 representing 13.7% for the total of 68.7% (**Figure 19b** and **c**)

Of the 20 metabolites that showed greater than 2-fold changes with statistical significance (adjusted p -value < 0.10), 2 of them showed 2.4 and 2.6-fold decrease, while the

rest showed increase of greater than 2-fold. (**Table 6b**) Three acetyl compounds including N-acetylaspartylglutamic acid (NAAG), N-acetyl-D-lactosamine, and N-acetylglucosamine were increased 2.5 to 5.7-fold. In particular, NAAG, which is formed by condensation reaction between N-acetylaspartic acid and glutamic acid, showed 5.7-fold increase in ASS1-786O cells. compared to the control 786O cells. Since N-acetylaspartic acid and NAAG are metabolically interchangeable and are often quantified together as total NAA (t-NAA), [16] this NAAG enrichment is indicative of higher NAA level in the ASS1-786O compared with the control 786O, in agreement with the primary metabolites analysis described above. On the other hand, propionylcarnitine and N8-acetylspermidine showed 2.4 and 2.6-fold decrease in ASS1-786O in comparison to the control. VIP scores of the metabolites with the highest VIP scores ranged 1.2 to 1.5. (**Figure 19d**), and these metabolites were able to correctly classify the 11 samples to either the experimental (ASS1-786O, n=6) or the control (control-786O, n=5) as shown in the heatmap. (**Figure 19e**) ChemRICH-based enrichment analysis showed reductions in carnitine and its derivatives, ethanolamines, and amino acids in ASS1-786O compared with the control 786O. (**Figure 19f**) MetaMAPP analysis clustered the detected metabolites into three large clusters and five small clusters. One of the large clusters contained carnitine and its derivatives, which were all downregulated in ASS1-786O compared to the control 786O. Another cluster contained amino acids most of which were downregulated with two exceptions. The third large cluster contained nucleic acids. In addition, there was a small cluster of metabolites that included creatinine, a urea-cycle derivative, and another network with N8-acetylspermidine. Both creatinine and N8-acetylspermidine were reduced in ASS1-786O. Lastly, Galacto-N-biose

and N-acetyl-D-lactosamine, two metabolites increased in ASS1-786O compared with the control 786O, were clustered into another small cluster. (**Figure 19g**)

ASS1-KD HK2 metabolomics

In order to assess the effects of ASS1 null phenotype on the normal kidney epithelial cells, ASS1-KD HK2 cells (a gift from Dr. M. Celeste Simon, U of Pennsylvania) were analysed on three metabolomic platforms compared with the control HK2 cells. These platforms included GC-TOF (primary metabolites), HILIC (biogenic amines), and QTOF (lipidomics).

Primary metabolite analysis of ASS1-KD HK2

First, our analysis of ASS1-KD HK2 primary metabolome on GC-TOF identified 41 metabolites that were expressed with greater than 2-fold difference and the $p_{\text{adjusted}} < 0.10$ between the ASS1-KD HK2 and the control HK2. (**Figure 20a** and **Table 6c**) PLS-DA analysis showed that components 1 and 2 can discriminate the 6 ASS1-KD HK2 samples and the 5 control samples, with each representing 38.4% and 17.3% of the data for the total of 55.7% contributing to the scores plot. (**Figure 20b** and **20c**) Heatmap constructed with 28 metabolites with the highest VIP scores was able to correctly classify the 11 samples to two groups. (**Figure 20d** and **20e**)

Of the 41 metabolites identified in the ASS1-KD HK2 GC-TOF analysis, 7 metabolites were increased in ASS1-KD HK2 by the factor of 2 to 12-fold compared to its control while 34 were decreased in ASS1-KD by the factor of 2 to 9-fold. (**Table 6c**) Increased compounds include several nucleic acids and their derivatives including uridine (12-fold increase), guanosine (4.5-fold increase), adenosine (3.8-fold increase), inosine (2.6-fold increase), and uracil (2.1-fold increase). This indicates that ASS1 silencing is sufficient to reprogramme the metabolism to augment nucleic acid synthesis which in turn supports augmented proliferation of ccRCC. Amino acids were uniformly downregulated in ASS1-KD HK2 compared to the control HK2. The amino acids whose levels were significantly reduced in ASS1- KD HK2 compared with the control include cystine, histidine, lysine, serine, tryptophan, β -alanine, cysteine-glycine, aspartic acid, cysteine, methionine, phenylalanine, glycine, threonine, proline, tyrosine, and isoleucine. While this may seem counterintuitive based upon a similar reduction in the ASS1-786O compared with control-786O, this may be attributable to the fact that these two pairs inherently have distinct genetic background. That is, in addition to ASS1 silencing, 786O harbours several mutations, most notably VHL truncation due to a premature stop codon. These differences in genetic background may contribute to the discrepancy. In addition, tumour microenvironment-specific factors such as hypoxia may synergise with the ASS1 status to manifest in the metabolomic reprogramming *in vivo* that was not captured in the current *in vitro* metabolic analysis of ccRCC. In addition, putrescine, a polyamine, was found to be reduced 7.1-fold in ASS1-KD HK2, in agreement with a prior report. [8] Furthermore, creatinine, a downstream degradation product of arginine was reduced by 7.1-fold. This is in line with the reduction in arginine level due to ASS1 knockout. Of note, glucose-6-phosphate and fructose-6-

phosphate, two upstream glycolytic intermediates, were reduced in ASS1-KD HK2 by 7.7 and 4.8-fold, respectively, suggesting that ASS1 silencing may contribute to the Warburg effect.

Notably, N-acetylaspartate is reduced in ASS1-KD HK2 by 2.2-fold. (**Figure 20a**) Albeit a relatively small change, this contrasts with its increase in ASS1-786O, (**Figure 18a**) and suggests that a primary manifestation of ASS1 silencing in ccRCC is reduction of N-acetylaspartate. This results further suggests that acetylation of aspartate to N-acetylaspartate by N-acetyltransferase 8-Like (NAT8L) is inhibited or conversion of N-acetylaspartate to aspartate by Aspartoacylase (ASPA) is increased in ccRCC. Given that the conversion of aspartate to acetylaspartate involves equimolar consumption of acetyl-CoA, this working model predicts that acetyl-CoA level is altered in ccRCC compared. Specifically, given the decreased level of N-acetylaspartate in 786O, an *in vitro* model of ccRCC, compared with ASS1-786O where the ASS1 is exogenously over-expressed to mimic a normal kidney proximal tubule epithelium, acetyl-CoA level is predicted to be higher in the ccRCC compared with the normal proximal tubule epithelium. This hypothesis agrees with the observation that accumulation of cytoplasmic lipid droplets is a common and defining characteristics of ccRCC.

It should also be noted that adenosine-5-monophosphate (AMP) is reduced by 9.1-fold in ASS1-HK2 compared to the control HK2. It is a part of the ATP degradation pathway via ADP, and reduction in its level upon ASS1 deletion corroborates with the increase in ATP synthesis for DNA and RNA synthesis necessary for proliferation. In addition. AMP is an activator of AMP-activated kinase. [17, 18]

In addition, the enrichment analysis was performed using Chem RICH. **(Figure 4f)** In the ASS1-KD HK2 cells grown in the complete media, amino acids and sugar acids were reduced while purine nucleosides were increased compared with the control HK2. This increase in the nucleic acids seen in ASS1-KD HK2 agrees with a previous study which reported shuttling of aspartate to pyrimidine synthesis in melanoma and osteosarcoma. [19] Finally, pathway analysis with MetaMAPP clustered the metabolites into 5 large clusters and two smaller clusters. One of the large clusters contained amino acids which were uniformly downregulated in ASS1-KD HK2. Another cluster contained 19 metabolites, of which 4 nucleic acids showed statistically significant changes of more than 2-fold. Adenosine, guanosine, and inosine were increased in ASS1-KD HK2, while AMP was reduced. Another cluster contained 28 metabolites, of which two showed statistically significant differences between the ASS1-KD HK2 and the control HK2; behenic acid was moderately increased in ASS1-KD HK2, and 1-monopalmitin was considerably reduced with statistical significance. On the other hand, putrescine and spermidine were moderately to highly reduced in ASS1-KD HK2 and formed a smaller cluster. Another small cluster contained creatinine, which was significantly reduced in ASS1-KD HK2.

Biogenic amine analysis of ASS1-KD HK2

Metabolic reprogramming in ASS1-KD HK2 was also studied by HILIC analysis, which targets biogenic amines. **(Figure 21a-f)** The HILIC analysis identified 27 metabolites with the fold difference greater than 2 and p.adjusted p < 0.10. **(Figure 21a)** PLS-DA analysis was performed

in which components 1 and 2 represented 41.7% and 12.9% of the data, thus rendering the graph represent 54.6% of the total data. **(Figure 21b and 21c)** VIP scores were calculated for each metabolite, and 50 metabolites with the highest VIP scores were used to draw a heatmap, **(Figure 21d)** which was able to cluster the 11 samples into the correct group using Euclidean-Ward clustering. **(Figure 21e)** Enrichment analysis was performed on ChemRICH platform, which indicated increase in nucleic acid derivatives such as purine nucleosides and pyrimidines in ASS1-KD HK2 and reduction in trimethyl ammonium compounds and carnitines. **(Figure 21f)**

Among the 27 metabolites that were detected with greater than 2-fold differences and $p_{\text{adjusted}} < 0.10$. 13 metabolites were increased in ASS1-KD HK2 while 14 were reduced in the cell line compared to the control. **(Table 6d)** The 13 metabolites increased in ASS1-KD HK2 include 8 nucleic acids. 3'-deoxyadenosine was increased 22-fold in ASS1-KD HK2 while Uridine and cytidine were enriched by 16.4 and 12.1-fold, respectively. In addition, nicotinamide riboside, a nicotinamide adenine dinucleotide (NAD⁺) precursor, was increased by 9.8-fold. These observations suggest that ASS1 silencing in ccRCC contributes to adaptation to high rate of proliferation and metabolism typical of cancer which requires augmented nucleotide synthesis and protection against oxidative stress. Of the 14 metabolites downregulated in ASS1-KD HK2, beta-alanine and N-acetylmethionine are non-proteinogenic amino acids. Instead, they act as intermediate in the synthesis of proteinogenic amino acids, which corroborates with the altered amino acid levels in GC-TOF analysis described above. Creatine and creatinine, two intermediate metabolites of arginine degradation and urea synthesis was reduced by 2.6- and 2.3-folds respectively, indicating that ASS1 knockdown indeed reduces the urea cycle activity. Interestingly, three nucleotide monophosphates including AMP, uridine-5'-monophosphate

(UMP) and inosine-5'-monophosphate (IMP) are reduced in ASS1-KD HK2 by 2.6 to 3.3-fold, which suggests altered nucleic acid metabolism and folic acid cycle. [20] In addition, reduction in AMP level can potentially reduce the 5'-AMP-dependent kinase (AMPK) activity in ASS1-KD ccRCC, a model of ccRCC, thus integrating the ccRCC biogenic amine metabolome and its intracellular signalling and will be discussed in the Discussion below.

Of the 14 metabolites reduced in ASS1-KD HK2, four are carnitine and its derivatives. **(Table 6d)** This suggests that ASS1 knockdown resulted in reduced rate of β -oxidation. In addition, lyso-sphingomyelin, a sphingolipid and a potent inhibitor of protein kinase C (PKC), [21] was reduced 5.3-fold in ASS1-KD HK2, suggesting ASS1 silencing may diminish lipid-mediated PKC inhibition. Furthermore, phenylacetylglycine, a biomarker of phospholipidosis, [22] was increased by 2.2-fold in ASS1-KD HK2.

Finally, the ASS1-KD HK2 biogenic amine metabolomics was summarised using ChemRICH analysis **(Figure 21f)** and MetaMAPP analysis **(Figure 21g)**. ChemRICH analysis showed purine nucleotides and pyrimidines were increased while trimethylammonium compounds were reduced in ASS1-KD HK2 compared to the control HK2. The MetaMAPP network analysis showed a cluster of carnitines and its five derivatives which were uniformly reduced. Betaine, creatine, and creatinine were clustered into another network, whose members were also uniformly reduced in ASS1-KD HK2. The largest cluster contained nucleic acids which showed varied degrees and directions of changes. Nucleotides which are conventionally known to form DNA and RNA were increased in ASS1-KD HK2. These include 3'-deoxyadenosine, 6-methyladenosine, adenosine, cytidine, uridine, guanine, and guanosine. On

the other hand, AMP was reduced. These results are in accordance with the gas chromatography-based primary metabolite analysis described above (**Figure 20g**)

Lipidomic analysis of ASS1-KD HK2

ASS1-KD HK2 and its control were analysed for their lipidomes using QTOF mass spectrometer on the positive and negative ion modes. The former identified 120 metabolites with significant differential expression between the ASS1-KD HK2 and the control, while the negative mode identified no metabolites differentially expressed with statistical significance. (**Figure 22a** and **Table 6e**) Based on their statistical significance, only the metabolites identified in the positive mode were considered for the subsequent analysis. PLS-DA analysis clearly discriminated the two sample groups with the components 1 and 2 integrating 60.2% and 13.4% of the available data, respectively, with the total of 73.6% contributing to the scores plot. (**Figure 22b** and **22c**) Heatmap of top 30 metabolites by PLS-DA VIP score satisfactorily classified the 11 samples into their corresponding groups. (**Figure 22d** and **22e**)

In detail, the analysis identified 120 lipids distributed across 14 classes, (**Table 6e**) including 47 phosphatidylcholines (PC), 13 phosphatidylethanolamines (PE), 5 phosphatidylserines, 10 sphingomyelins (SM), 7 triacylglycerides (TAG), 19 triglycerides (TG), 1 phosphatidylinositol, 2 cholesterol esters (CE), 1 cholesterol, 1 N-acylsphingosine, 1 diacylglycerol, 3 glucosylceramides (GlcCer), 1 lactosylceramide, and 6 lysophosphatidylcholine (LPC). (See **table 6e**) Notably, CE, GlcCer, and LPC uniformly increase or decrease in fold

changes in ASS1-KD HK2 compared with the control HK2, while PC, PE, PS, SM, TAG, and TG showed varied changes. CE were reduced 3.7 to 6.7-fold, while GlcCer and LPC were increased 2.1 to 6.7- and 3.0 to 5.5-fold, respectively. It should be noted that while CEs were reduced in ASS1-KD HK2 compared with the control, cholesterol was increased 2.6-fold. In addition, diacylglycerol (DAG), a potent agonist of protein kinase C (PKC) was upregulated 3-fold in the ASS1-KD HK2 in comparison to the control HK2.

As many as 47 PC were detected to show statistically significant differences between the ASS1-KD HK2 and the control, although their fold changes varied in direction as well as in their magnitudes. For example, PC (18:1/18:3) was increased in ASS1-KD HK2 by the factor of 3.1 while PC (38:7) was reduced 129 times. Plasmalogen Phosphatidylcholines (PCp) such as highly unsaturated PC with short chains were markedly reduced in the ASS1-KD HK2, ranging from 938-fold (PC (p-34:2)/PC (o-34:3)) to 1912-fold (PC (p-36:4)/PC (o-36:5)). Unsaturated PC with longer acyl chains showed less marked, albeit uniform, reduction. 12 out of 13 PE with statistically significant changes showed 2 – 3-fold increase in ASS1-KD HK2 although at least one polyunsaturated PE (PE (38:5)) showed greater than 2-fold reduction in ASS1-KD HK2. Of the five PS detected, 4 showed increases of 2.4 – 3.0-fold in ASS1-KD HK2, while PS (40:5) showed a 4.3-fold reduction compared to that in the control. There were 10 SM detected with statistically significant differences between ASS1-KD HK2 compared with the control. Of these three species with the smallest acyl moieties were reduced in ASS1-KD HK2, namely SM (d30:1), SM (d32:1), and SM (d34:1). SM (d33:1) with an odd-numbered acyl chain, on the other hand, showed 2.3-fold reduction, suggesting preference for even-numbered acyl chains in the ASS1-KD HK2. SM

with larger acyl chains showed uniform decrease in the ASS1-KD HK2 compared with the control, ranging from 2.1-fold for SM (d43:2) to 11-fold for SM (d41:1).

There were 7 TAGs detected with statistically significant differences between ASS1-KD HK2. Saturated and monounsaturated TAGs showed increase of 2-3-fold in ASS1-KD HK2, while polyunsaturated TAGs showed reduction. Changes in TGs were more varied. Of the 19 TGs with significant changes, 6 showed increase of 2.3-4.4-fold in ASS1-KD HK2, while 13 showed decrease of 2-20-fold. Interestingly, Both B and C isomers of TG (56:5) were detected with statistically significant changes with B isomer exhibiting 4.3-fold increase and C isomer showing 21-fold decrease, which suggests that this is an enzyme-catalysed change.

ChemRICH enrichment analysis was used to analyse the lipid class enrichment. **(Figure 22f)** The results show that a large number of unsaturated PC and PE are highly enriched in ASS1-KD HK2, while LPC, saturated TG, and saturated PC showed higher levels in control HK2. SM, unsaturated TG, glycerophospholipids and PS showed mixed distribution, although the former two classes are generally reduced in ASS1-KD HK2 while the latter two classes showed increase. MetaMAPP network analysis showed reduction in a number of triacetyl glycerides, glycerophospholipids, sphingomyeline, and ceramides **(Figure 22g)**

Effects of ASS1 status and media conditions on ccRCC morphology

Given the extensive lipidome-wide reprogramming in ASS1-786O and that the intracellular lipid droplets are a cardinal feature of ccRCC, lipid droplets in ASS1-786O were assessed under

microscope. (**Figure 23a**) After 72 hours of culture in complete media (CM, 10% FBS) or serum-deficient quiescent media (QM, 1% FBS), Lipid-droplet (LD) positive cells and total cells were counted, and the percentages of the lipid-droplet positive cells were calculated from triplicates (**Figure 23b**). As expected, both cell lines show higher percentages of LD-positive cells when grown in QM compared with CM. Surprisingly, control cells show higher percentage of LD-positive cells compared with the ASS1-786O. 3.8% of ASS1-786O cells are lipid droplet-positive, while 13.9 % of control 786O cells contained cytoplasmic lipid droplets visible under the microscope. Under QM, 25.2% of ASS1-786O showed lipid droplets while 34.1% of the control 786O was lipid droplet positive. Overall, ASS1 deficiency resulted in 3.7-fold and 1.4-fold increase in lipid droplet-positive cells in CM and QM, respectively. In contrast, ASS1-786O grown in QM had 6.6-times more lipid droplet-positive cells than that grown in CM.

Similarly, control 786O grown in QM had 2.5-fold higher percentage of lipid droplet-positive cells compared to those grown in CM. When the ASS1 status and the nutrient condition was simultaneously taken into account, 34.1% of the control 786O cells grown in QM exhibited cytoplasmic lipid droplets while only 3.5% of the ASS1-76O grown in CM was lipid droplet-positive. Taken together, these results suggest that both ASS1 silencing and nutrient-deficient environment synergise to produce cytoplasmic lipid droplet.

Effects of ASS1 silencing under low nutrient conditions on ccRCC metabolome

In order to investigate the role of ASS1 silencing in a low nutrient condition that mimics the tumour microenvironment, primary metabolite GC-TOF analysis and lipidomic analysis were

repeated using ASS1-786O and control-786O cell lines grown under low nutrient conditions (1% FBS, RPMI-1640). While 10% FBS is usually used as a standard culture condition for 786O cell line, tumour microenvironment is known to exhibit low-nutrient conditions. [23]

Primary metabolites (GC-TOF)

GC-TOF analysis of ASS1-786O grown in QM condition identified 35 metabolites that had greater than 1.2-fold change in ASS1-786O with $p_{\text{adjusted}} < 0.10$. (**Figure 24a**) PLS-DA showed that components 1 and 2 describe 25.3% and 18.9% of the data, respectively for the total of 44.2%, and can discriminate between ASS1-786O and control 786O. (**Figure 24b and 24c**). A heat map was constructed using 25 metabolites with the highest VIP scores, which was able to classify the 11 samples to the correct class. (**Figure 24d and 24e**).

Of the 21 metabolites differentially expressed between the ASS1-786O and Control 786O grown in QM, 6 were increased in ASS1-786O by 1.23 to 1.66-fold, while 15 were decreased by the factor of 1.29 to 2.08 in the experimental cell line compared to the control. ($p_{\text{adjusted}} < 0.10$) The changes are smaller compared to the analysis done on cells grown in CM, (**Table 6a**) which may reflect the effect of nutrient deprivation. Alternatively, it is possible that the magnitude in changes was a result of smaller quantity of each QM sample analysed. The metabolites reduced in ASS1-786O include amino acids (e.g., alanine, cyanoalanine, trans-4-hydroxy-L-proline, asparagine, cystine, and threomine), TCA cycle intermediates (isocitric acid, malic acid) and sugar alcohols (e.g., lyxitol, ribitol, sorbitol, and arabitol) and a polyamine

(e.g., putrescine). The 6 metabolites increased in ASS1-786O compared with the control include cholecalciferol (Vitamin D) and 5,6-dihydropyrimidin-2(1H)-one, which is a uracil degradation intermediate.

Lipidomic analysis of ASS1-786O grown in nutrient-deficient media

Because the low nutrient condition (1% FBS) resulted in more pronounced changes in ccRCC morphology upon ASS1 re-expression in 786O cells compared to the re-expression in complete media (10% FBS), (**Figure 23**) we investigated the ASS1-induced metabolic reprogramming in ccRCC under the low nutrient condition using the positive CSH and the negative CSH modes (**Figure 25**). Surprisingly, Triglycerides (TG) are increased in ASS1-786O compared with the control-786O. In particular, TG(59:2) was increased 250-fold in ASS1-786O compared with the control-786O (**Table 6g**). This is unexpected given that triglycerides are main constituents of the lipid droplets which is elevated in Control-786O compared with ASS1-786O in QM. A potential explanation is that the control-786O further converts these TGs into other TG derivatives for lipid droplet formation, and the TG derivatives are not detectable in the current setup. 22 other mono- or polyunsaturated TGs were modestly elevated in ASS1-786O by 1.2 to 2.1-fold. In contrast, 7 saturated TGs, including TG(50:0) and TG (51:0), were moderately reduced by the factor of 1.2 to 1.4 in ASS1-786O compared with the control 786O. This suggests that ASS1 silencing increases saturated TGs at the expense of unsaturated TGs.

In addition, DG(38:6), a polyunsaturated diglyceride, was elevated in ASS1-786O by 45-fold while DG34:2 was moderately elevated by 1.3-fold. SM(d38:1.2) was elevated 82-fold in ASS1 along with two other SMs which were elevated 1.3-fold.

In contrast, lipids reduced in ASS1-786O compared to the control-786O under the low serum condition include ceramides and their derivatives such as glucosyl ceramides (GlcCer), dihexosylceramides (Hex2Cer), and Lactosylceramides. Lysophosphatidylcholines (LPC), Phosphatidylcholines (PC), Phosphatidylethanolamine (PE), sphingomyelins (SM) as well as unsaturated TGs described above were also reduced. (**Figure 25**)

It should be noted that smaller quantities of ASS1-786O / Control-786O samples grown in QM analysed compared with the ASS1-KD HK2 lipidomics grown in high nutrient (10% FBS) media potentially rendered the current analysis more prone to technical variations. Indeed, both GC-TOF-based primary metabolite analysis and the QTOF-based lipidomic analysis identified fewer number of metabolites that were statistically different between the two groups compared. For example, ASS1-786O / QM lipidomics identified 5 metabolites with FC > 2 and p.adjusted < 0.10 compared with 120 metabolites identified in ASS1-KD HK2/CM lipidomics. In order to account for this, a less stringent cut-off of 1.2-fold changes was used to include a larger number of metabolites for downstream analysis, which resulted in the inclusion of 206 lipid species.

Despite the technical discrepancies noted above, several noteworthy trends can be identified through comparison of lipidomic analysis on ASS1-KD HK2 in CM and ASS1-786O in QM. For example, diglycerides were increased 3.0-fold in ASS1-KD HK2 in CM compared to the

control, while they were on average reduced 23-fold in control-786O compared with the ASS1-786O in QM. Unsaturated TGs were unchanged to moderately increased in ASS1-KD in CM while reduced by 9-fold in the control 786O compared to the ASS1-786O.

In particular, under the low nutrient condition, the lipids reduced in ASS1-786O compared with the control include PCs, PEs, Lactosylceramides, glucosylceramides, Hex2cer, and LPE. While the increase in triglyceride upon ASS1 overexpression in 786O is unexpected, the detected yet unidentified lipids may account for this anomaly. Taken together, this lipidomic analysis supports the hypothesis that ASS1 silencing in ccRCC reprograms its lipid metabolism. Thus, it warrants further lipidomic analysis to quantitatively characterise the effects of ASS1 silencing on ccRCC lipidome and its interaction with tumour microenvironment such as hypoxia and hypo-nutrient conditions.

DISCUSSION

Kidney cancer results in over 76,000 cases and 13,000 deaths annually in the US. [24] 75% of the cases belong to clear cell renal cell carcinoma (ccRCC), majority of which harbours non-sense mutation in VHL, which functions as HIF α E3 ubiquitin ligase as well as a p53-associated protein to increase p53 stability and activity. Therefore, VHL inactivation prevalent in ccRCC renders the cancer resistant to the conventional p53-dependent apoptosis, [3] and its surgical

removal remains the only effective therapeutic option. Therefore, novel therapeutic approaches and targets are ardently sought. Argininosuccinate synthetase (ASS1) is the rate-determining urea cycle enzyme that catalyses the condensation reaction of aspartic acid and citrulline into argininosuccinate. It is universally silenced in ccRCC across all Fuhrman nuclear grades, [7] thus making it an ideal target against ccRCC. Previous studies by other groups have shown that ASS1 silencing is a universal phenomenon in various types of cancers. [25, 26] ASS1-deficient osteosarcoma had elevated pyrimidine levels as well as increased proliferation rate compared with its ASS1-proficient counterpart, which was further validated in melanoma, [19] while ASS1 silencing was found to give growth advantage to normal kidney epithelial cells *in vitro*. [8] In addition, ASS1 and ASL silencing leads to modulation in nitrogen oxide metabolism and signalling. [27] Given the central role that the urea cycle plays in orchestrating several central metabolic pathways, silencing of ASS1 broadly observed in ccRCC is hypothesised to result in dysregulation of a number of them, many of which remain to be identified and characterised.

In this study, we employed metabolomic analyses to further characterise the metabolomic reprogramming which results from the silencing of ASS1 in ccRCC, to identify pathways and proteins that may serve as druggable targets against ccRCC. In agreement with a previous study, [8] we found that amino acids were reduced in ASS1-786O compared with the control 786O as was shown in the enrichment of metabolites in amino acid metabolism including alanine, aspartate, and glutamate metabolism, and beta-alanine metabolism (**Figure 18** and **Table 6a**). Metabolites in pantothenate and CoA biosynthesis were also found to be enriched in

the metabolites differentially present. N-acetylaspartate was identified in both ASS1-786O and ASS1-KD HK2 to be differentially present compared with their respective control cells. N-acetylaspartate was increased in ASS1-786O compared with the control 786O while reduced in ASS1-KD HK2 compared to the control HK2, which suggests ASS1-silencing results in altered levels of acetyl-CoA, a substrate for N-acetylaspartate synthesis. (**Figure 18, Figure 20, Tables 1a, Table 6c**) The biogenic amine metabolomic analysis showed that carnitine and its derivatives are differentially present in ASS1-786O and ASS1-KD HK2 compared with their respective controls, suggesting that ASS1 silencing results in reprogramming in β -oxidation and fatty acid synthesis in ccRCC. (**Figure 19 and 21**) In agreement with the biogenic amine analysis, ASS1 deletion in HK2 and ASS1 over-expression in 786O profoundly reprogrammed their lipidome. (**Figure 22**) Plasmalogen phosphatidylcholines (PCp) are decreased in ASS1-deficient HK2 compared to its control by the factor of up to 2000. Finally, our morphological analysis of intracellular lipid droplets showed that their numbers is higher in ASS1-deficient control 786O compared to the ASS1-786O. The proportion of lipid droplet-positive cells were higher in each cell line when grown in QM compared with the same cell line grown in CM. (**Figure 23**) This may result from increase in the number of lipid droplets. On the other hand, it is plausible that this apparent increase is a consequence of lipid droplet fusion, thereby increasing the number of lipid droplets visible droplets while reducing their total number.

Taken together with the morphological analysis summarised above, our metabolomic analysis of ASS1 status in ccRCC can be summarised in the following model of metabolic reprogramming that contributes to the induction of clear cell phenotype and cancer-associated cell signalling

upon *ASS1* silencing. (**Figure 26**) Loss of *ASS1* expression leads to accumulation of intracellular neutral lipids. Concurrently, sphingolipid content is drastically reduced. Since intracellular lipid droplets are composed of the core of neutral lipids surrounded by a monolayer of phospholipids, the lipidomic reprogramming upon *ASS1* silencing presents ccRCC with shift in core-to-surface ratio in terms of lipid availability to the lipid droplet formation. That is, Manipulation of the production of different lipid classes may result in the increase in the average lipid droplet size. Pathobiological importance of such lipidomic reprogramming in ccRCC development remains a major area of investigation and a source of possible druggable targets against ccRCC.

Phospholipids such as PC are primary constituents of the lipid monolayers that surround lipid droplets, while neutral lipids such as cholesterol esters, triacylglycerols (TAGs), and phosphatidylethanolamine (PEs) are found in the lipid droplet core. [28] During intracellular lipid droplet growth, droplet fusion is induced under low phospholipid conditions as neutral lipid -to- PC ratio is increased, [29] which is equivalent to increase in volume-to-surface ratio in total lipid droplets. This process is mediated at least in part by Perilipins, a family of membrane-associated proteins present on lipid droplets. [30] Perilipin-2/Adipophilin was upregulated in low-grade ccRCC in our grade-defined proteomic study (Chapter 3 and [7]) Conversely, neutral lipids such as ceramides, PCs, lyso-phosphatidylcholines (LPCs), and TAGs are increased in *ASS1*-KD HK2 compared with the control HK2. Therefore, the lipidomic data presented in this chapter suggests that down-regulation of *ASS1*, a hallmark of ccRCC across all Fuhrman nuclear grades, [7] results in downregulation of polyunsaturated phosphatidylcholines and concomitant upregulation in neutral lipids, which leads to increase in neutral lipid-to-PC ratio in ccRCC,

thereby facilitating the lipid droplet fusion and growth. The mechanistic underpinning and its significance in ccRCC pathogenesis of this lipidomic reprogramming beyond its contribution to clear-cell phenotype warrants further investigation. Given ASS1 status synergised with nutrient status to modulate the number and size of cytosolic lipid droplets, a cardinal feature of the ccRCC, it is hypothesised that ASS1 silencing is necessary for ccRCC to develop its clear-cell phenotype. Mechanistically, plasmalogen and neutral lipid metabolism are altered such that neutral lipids are increased while plasmalogens are reduced. Even less is known why ccRCC accumulates such intracellular lipid droplets. Given that colossal lipid droplets are a cardinal characteristic of ccRCC, their significance and mechanisms in conferring survival and growth advantage for ccRCC is of particular interest in ccRCC research. Further investigation should test whether such morphological change manifests in a more aggressive and invasive ccRCC phenotypes.

Intracellular lipid droplets and their central role in energy storage has been well established for several decades. [31 – 35] In addition, importance of lipid accumulation and lipid droplet growth in various cancers are beginning to be recognised, [36] although nowhere is the importance of lipid accumulation better recognised than in ccRCC where its cytosolic accumulation gives the characteristic clear cell appearance. On the other hand, the reasons why the ccRCC accumulates the cytoplasmic lipid droplets is a matter of decades-long debate, [34] and its molecular mechanisms have only recently been characterised albeit partially.

Recent studies suggest several mechanisms linked to the lipid droplet growth in ccRCC. Lipid droplet accumulation in ccRCC may be a consequence of inactivated autophagy through reduced MAP1S level. [37] Alternatively, lipid droplet accumulation is a consequence of

repressed β -oxidation via HIF signalling, which is activated through VHL inactivation in ccRCC. [38] Furthermore, upregulation in cholesterol uptake can also contribute to increased lipid content, which in turn results in increase in lipid droplet growth. [39] Interestingly, melatonin-dependent activation of PGC1A is repressed in ccRCC and results in reduced lipid autophagy and poor patient survival. [40] PGC1A was identified as a hidden node in our grade-defined ccRCC proteomics, (Chapter 3 and [7]) which suggests that Fuhrman nuclear grade-dependent lipidomic reprogramming is orchestrated by PGC1A. The overall process is regulated by Ancient Ubiquitous Protein 1 (AUP1) which targets sterol o-acyltransferase 1 and increases intracellular triglyceride in A498, an *in vitro* model of ccRCC. Jumonji Domain-Containing 6 (JMJD6) is upregulated in ccRCC, which in turn binds to diacylglycerol O-acyltransferase 1 (DGAT1) promoter and increase its gene expression. [70] In addition, a recent lipidomic analysis of ccRCC patient tissue in comparison to an adjacent normal tissue showed increase in unsaturation of acylcarnitines, cholesteryl esters, and diacylglycerols was observed. [41]

Interestingly, the lipidomic reprogramming seen upon genetic manipulation of ASS1 status agrees with our prior grade-defined ccRCC proteomics. (Chapter 3 and [7]) The proteomics analysis identified several proteins that were differentially expressed between normal kidney cortex tissue and ccRCC as well as across Fuhrman nuclear grades. For example, Perilipin2/ADRP, a lipid-droplet coating protein, was undetected in normal kidney cortex while its expression was upregulated in low-grade ccRCC and moderately detected in high-grade ccRCC. While Perilipin2/ADRP was downregulated in high-grade ccRCC, another member of the Perilipin family whose proteomic fragments were not identifiable in the tryptic fragment database in 2009 is predicted to be upregulated to promote the lipid droplet growth further.

Several members of the phospholipid-binding Annexin family were also differentially expressed in the grade-defined ccRCC proteomic study. These annexins include Annexin A2 (ANXA2), Annexin A3 (ANXA3), Annexin A4 (ANXA4), and Annexin A5 (ANXA5). ANXA2 and ANXA5 were upregulated across all Fuhrman grades compared with the normal tissue. ANXA4 was upregulated highly in low nuclear grades while the high grades showed only moderate upregulation compared with the normal tissue. On the other hand, ANXA3 was detected only in G4. [Perroud et al. (2009) and Chapter 3] The complementary nature of the protein levels between these Annexin paralogues suggests that ccRCC modulates the Annexin levels during its progression, which may underlie the lipid droplet growth and development of the clear-cell phenotype. Pathological importance of this switch warrants further investigation.

Additional lipid-interacting proteins showed grade-dependent differential expression in our proteomic analysis. These include Megalin and Apolipoprotein A1 which were down-regulated across all grades compared to the normal kidney cortex. Importance of these changes in the context of lipid droplet growth in ccRCC also merits further investigation.

Furthermore, it is of interest that the lipid droplet-filled cytoplasm and the resultant clear-cell phenotype is unique to ccRCC, while lack of ASS1 expression is a phenomenon seen universally across many cancers. [25, 42] This indicates that a second ccRCC-specific alteration synergises with the ASS1 silencing to manifest in the clear-cell phenotype. An obvious candidate is VHL which is inactivated in ccRCC but not in other types of kidney cancer nor in the broader spectrum of cancers. The identity of this second gene and its associated signalling pathways is of great significance for future investigation to identify ccRCC-specific druggable targets and to develop novel, effective, and specific ccRCC therapies.

In addition to the histological phenotype described above, the ASS1 silencing and the concomitant lipidomic programming is expected to reprogramme the ccRCC intracellular signalling. Signalling molecules such as diacylglycerol (DG(32:0), DAG), AMP, and ceramides showed statistically significant changes in their levels upon ASS1 over-expression or deletion. In addition, Acetyl-derivatives such as N-acetylaspartate showed statistically significant changes upon ASS1 re-introduction or deletion.

DG(32:0), a DAG, was increased 3.0-fold in ASS1-KD HK2 compared to that in the control HK2. DAG is an intracellular signalling molecule and activates protein kinase C (PKC) and protein kinase D (PKD). [43] PKC in turn activates NF- κ B pathway, [44 – 47] which is predicted to protect ccRCC from stress-induced apoptosis. On the other hand, DG(38:6) was found to be increased by 45-fold in ASS1-786O relative to control 786O when grown in nutrient deficient condition. Together, they seem to indicate that DG may be increased in ccRCC when grown in nutrient-replete condition, while decreased in the cancer cells when grown in nutrient-deficient conditions. Given this is based on a single DG species in each case, further analysis is required to determine whether DAG levels are indeed modulated upon ASS1 silencing. Given the importance of DAG in PKC signalling, [48, 49] and their importance in cancer development, [50] further investigation in its potential roles in ccRCC development is warranted.

Potential functions of ceramides in ccRCC development

Ceramides were increased in ASS1-KD HK2 compared to the control HK2 grown in nutrient-replete media, and conversely were reduced in ASS1-786O compared to its control when grown in low nutrient media which mimics the tumour microenvironment. (**Figure 22, Figure 25**)

Ceramides belong to sphingolipids and can function as tumour suppressor biomolecule. They are a component of biological membrane and can affect its physical properties. High ceramide contents favours decreased curvature. [51] This is expected to result in increased lipid droplet size. In addition, ceramides are a family of neutral lipids that are expected to be present in the core of lipid droplets.

There were six ceramide derivatives detected in our lipidomic analysis of ASS1-KD HK2 and the control HK2 grown in nutrient-replete media. All of them were increased in ASS1- KD HK2, with GlcCer(d34:1) showing the highest fold increase amongst all the lipids identified. This suggest that ceramides, a group of neutral lipids, are likely to form a major structural part of the lipid droplet core. On the other hand, it is also possible that these ceramides are major components of the phospholipid monolayer where increase in ceramide composition leads to decreased curvature and the resultant increase in the droplet size.

In addition to the subcellular structural implications, increased ceramide content in ccRCC is expected to influence intracellular signalling. The ceramide signalling can originate from extracellular or intracellular stimuli, and is known to positively orchestrate such responses as apoptosis. For example, ceramides cluster membrane proteins such as CD95 through their inclusion into ceramide-rich lipid rafts, and this is necessary for CD95-dependent apoptosis. [52] This contradicts with a hallmark of cancer, which states that cancers resist cell death. [71] This suggests that additional mechanisms exist that suppresses such ceramide-dependent pro-

apoptotic signalling. Alternatively, it is possible that the majority of ceramides are indeed downregulated in ASS1-KD HK2 in comparison to the control HK2, but were not detected or identified due to an incomplete lipidome database used in the current lipidomic analysis.

Notably, our grade-defined ccRCC proteomic studies were not able to detect any ceramide metabolism enzymes with statistical significance. [Perroud et al. (2009) and Chapter 3] Further investigation into this pathway in the context of ccRCC is expected to identify novel druggable targets against ccRCC.

AMP/AMPK signalling

ASS1-KD HK2 also showed decrease in nucleotide monophosphates, including AMP, in the primary metabolite analysis (GC-TOF) and biological amine analysis (LC-QTOF HILIC), AMP was reduced 9-fold and 3.3-fold, respectively. [Table 6c and 1d] In addition to being a degradation product of ATP, AMP acts as an activating co-factor for AMPK, which in turn serves as an intracellular energy level sensor. In hepatocytes which possess the complete urea cycle, antisense knockdown of ASS1 results in reduced AMP and concomitant reduction in AMPK autophosphorylation and activation in nutrient replete condition. [53] In addition, ACC phosphorylation was reduced in ASS1-knockdown hepatocytes, which together with the reduction in AMPK activation switched the cells from β -oxidation to lipogenesis. [53, 54] Such a switch favours lipid accumulation. Whether a similar biochemical mechanism contributes to the

lipid droplet formation and clear-cell phenotype establishment in ccRCC warrants further investigation.

Dysregulated acetyl-CoA metabolism in ccRCC

Acetyl-CoA is an important metabolic intermediate that orchestrates multiple metabolic pathways such as glycolysis, gluconeogenesis, TCA cycle, fatty acid synthesis, β -oxidation, amino acid synthesis and degradation, and glutathione metabolism. [55, 56] In addition, it acts as the acetyl group donor in protein acetylation, thereby regulating a variety of enzymatic pathways including HIF-1 pathway, AMPK pathway, and epigenetic control pathway. [55, 57, 58]

Interestingly, N-acetylaspartic acid (NAA), which was increased in ASS1-786O and reduced in ASS1-KD HK2 compared with their controls when grown in CM, was originally discovered as one of the most abundant metabolites in the brain where it is involved in myelin lipid synthesis. [59] Furthermore, studies on brain pathophysiology in such conditions as multiple sclerosis and glioma have successfully used total NAA (tNAA), which is the sum of NAA and N-acetylaspartylglutamic acid (NAAG), as a biomarker. [60, 61] The current metabolomic study on ccRCC found that NAA is increased in ASS1-786O when analysed with the primary metabolite mode while NAAG showed a comparable increase against the control 786O when analysed in the primary amine mode. In addition, it is integral in regulating the brown adipose tissue metabolism. [62] In the brain, NAA is synthesised by N-Acetyltransferase 8 (NAT8) and N-

Acetyltransferase-Like (NAT8L) from acetyl-CoA and aspartic acid and is degraded by Aspartoacylase (ASPA) into aspartic acid and acetic acid. [63] In normal kidney, ASPA is expressed predominantly in tubules while absent from the glomeruli. (The Human Protein Atlas, [64]) and NAA catabolic activity was previously found to be enriched in kidney compared to other organs, [65] which seems to maintain the NAA level low in kidney. Given that ASS1 deletion in normal kidney tubule epithelial cells results in further decrease in the NAA level with concomitant lipidomic reprogramming while exogenous ASS1 expression in ccRCC *in vitro* shows reverse effect on the NAA level, it is likely that ccRCC alters the NAA metabolism and its lipidome to reprogramme the cell signalling to gain growth advantage. Furthermore, the Acetyl-CoA metabolism is highly compartmentalised, [55] due in part to the fact that acetyl-CoA is impermeable to the inner mitochondrial membrane. Consequently, the mitochondrial acetyl-CoA pool is distinct from the nucleo-cytoplasmic acetyl-CoA pool where the nuclear pores allow free diffusion of acetyl-CoA between the nucleus and the cytoplasm. Thus, in order to detect the accurate change in the acetyl-CoA levels, subcellular fractionation may be necessary. Further study on this aspect of ccRCC metabolism is expected not only to result in novel therapeutics targeted towards ccRCC but also those potentially effective against obesity.

As described above, our metabolomic analysis and the complimentary cellular and biochemical assays revealed that ASS1 silencing in ccRCC results in changes the ccRCC metabolome with the most drastic changes observed in its lipidome. Furthermore, the Metaboanalyst enrichment analysis identified obesity as a category enriched in the metabolites differentially present upon ASS1 manipulation. (Data not shown) This raises the possibility that ASS1 activity may be dysregulated in obesity and may act as a causal agent. This is intriguing

because the defining characteristics of ccRCC is cytoplasmic build-up of lipid and lipid droplets, which may be regarded as analogous to the excessive fat tissue present in obese patients.

Indeed, several recent studies have suggested that ASS1 dysregulation may be related to obesity and to its various associated symptoms. Peripheral monocytes from obese patients exhibited reduced ASS1 expression. [66] Dietary supplementation of *Luffa cylindrica* increased the Ass1 expression in mice and concomitantly reduced hepatic lipid synthesis. [67] Furthermore, TLR4 knockout mice exhibit pancreatic proteome reprogramming including increased ASS1 expression with concomitant lipotoxicity-induced insulin secretion disorder. [68] In addition, unclassified hepatocellular adenoma in an obese patient was reported to show ASS1 over-expression. [69] While far from conclusive, these studies as well as our present study suggest that ASS1 dysregulation may underlie aspects of obesity development.

Overall, the metabolomic study discussed in this chapter suggests that ASS1 silencing results in a lipidomic-wide reprogramming, in particular, increase in neutral lipids and decrease in plasmalogens, and modulation in associated signalling pathways. This, in turn, favours lipid droplet fusions and resultant increase in their average size. **(Figure 26)** ASS1 is silenced in a wide range of cancers, although ccRCC uniquely exhibits the accumulation of intracellular lipid droplets. Previous studies by others indicated that ASS1 silencing can results in increased pyrimidine synthesis [19] and intracellular pyridoxal-5'phosphate as well as in reduction in toxic polyamines. [8] The present work suggests that ASS1 silencing can have more global impact on the metabolome including lipidomic reprogramming. **(Figure 26)** In addition, ASS1 silencing

resulted in reduced N-acetylaspartate (NAA) with concomitant changes in plasmalogen phosphatidylcholine (PCp), DAG, and ceramide levels. Interestingly, a recent study showed that double silencing of two urea cycle enzymes, ASS1 and argininosuccinate lyase (ASL) results in suppression of xenograft growth and metabolic reprogramming. [27] Thus, it will be interesting to investigate the mechanisms underpinning the lipidomic reprogramming downstream effects of ASS1 silencing which results from such metabolic reprogramming as well as the factors and mechanisms epistatic to the ASS1 silencing in ccRCC.

Conclusion

In this study, in order to identify altered and druggable metabolic pathways in ccRCC, we employed metabolomic approaches complimented with morphological analysis to investigate metabolic reprogramming upon ASS1 silencing that is observed across all Fuhrman nuclear grades in ccRCC. A metabolome-wide reprogramming was observed upon ASS1 deletion and reconstitution. In particular, ccRCC lipidome showed a drastic reprogramming, and lead to the model that *ccRCC silences ASS1 in order to modulate cellular lipid composition and acetyl-CoA availability to other pathways including lipid synthesis, resulting in cytoplasmic lipid droplet fusion and enlargement.* (Figure 26) Altered intracellular signalling such as ceramide, PKC and AMPK signalling was also implicated. Further study on the altered metabolic pathways will ultimately lead to identification of druggable targets against ccRCC. Given the universal nature

of ASS1 silencing in ccRCC, such targeted molecular therapeutic approaches are expected to show efficacy for a wide spectrum of ccRCC patients.

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Figures and Tables

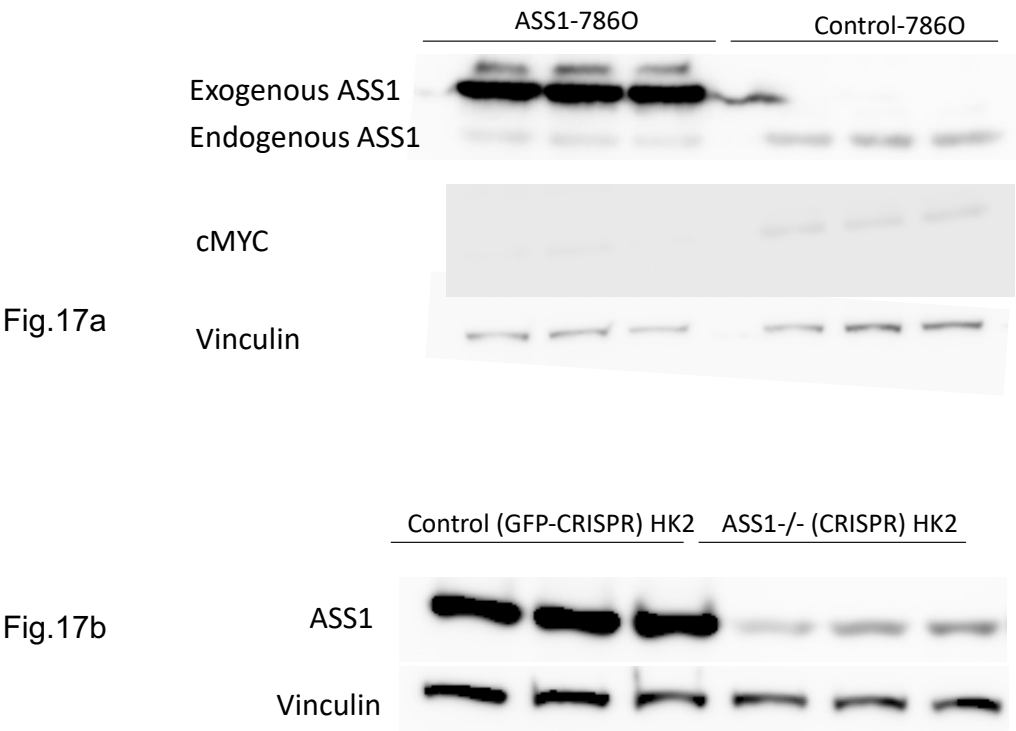


Fig.17 ASS1 and cMYC expression levels in (a) ASS1-786O and (b) ASS1-KD HK2

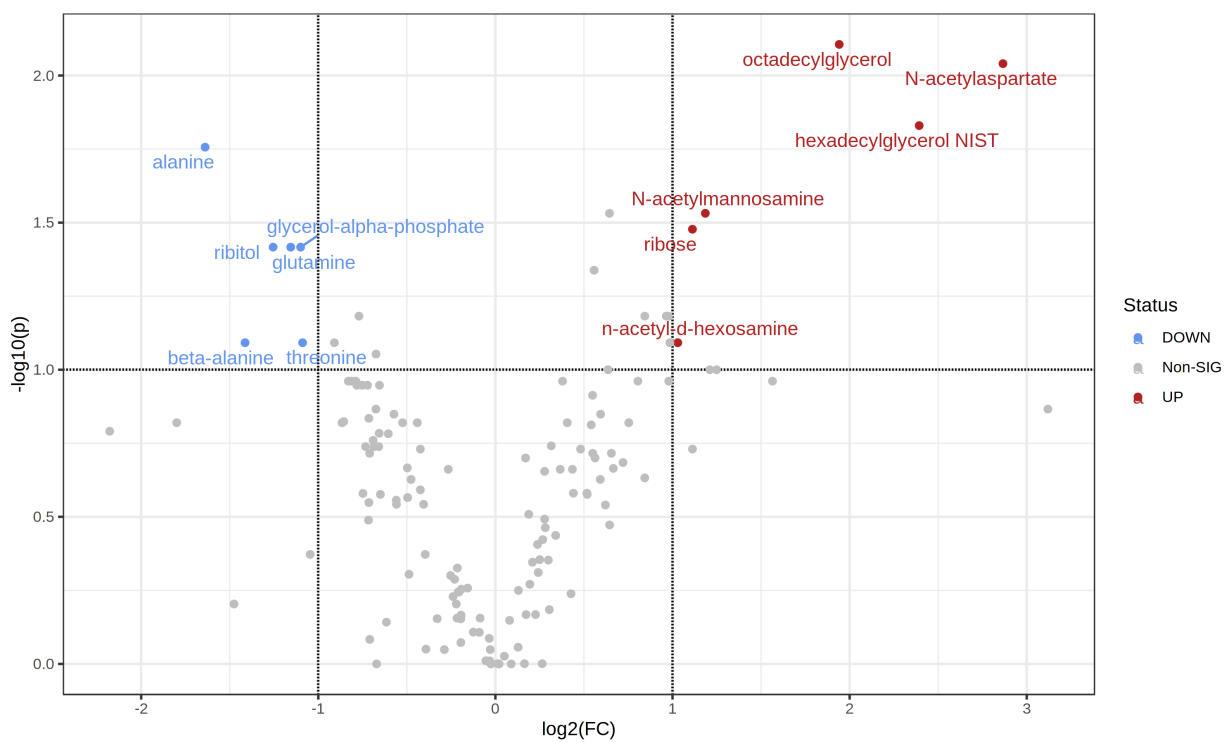


Fig.18a: Primary metabolites volcano plot on ASS1-786O vs control 786O

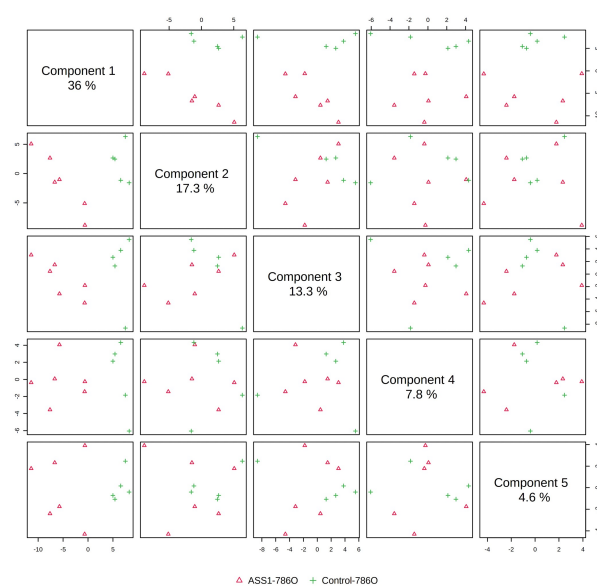


Fig.18b: PLS-DA overview

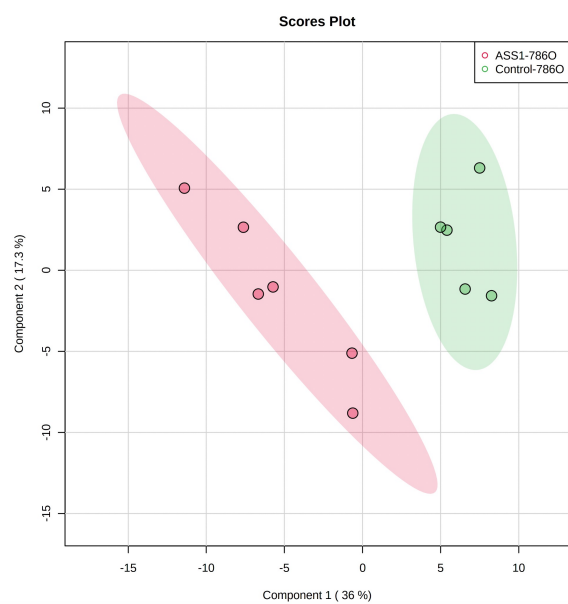


Fig.18c: PLS-DA 2D score plot

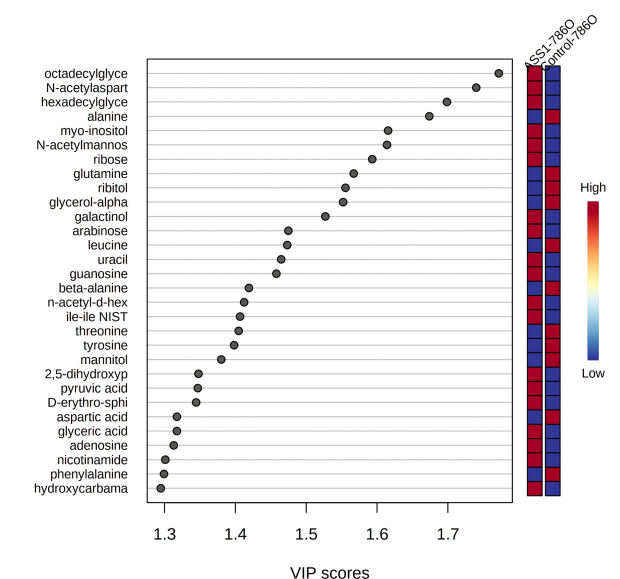


Fig.18d: Variable important projection (VIP) score

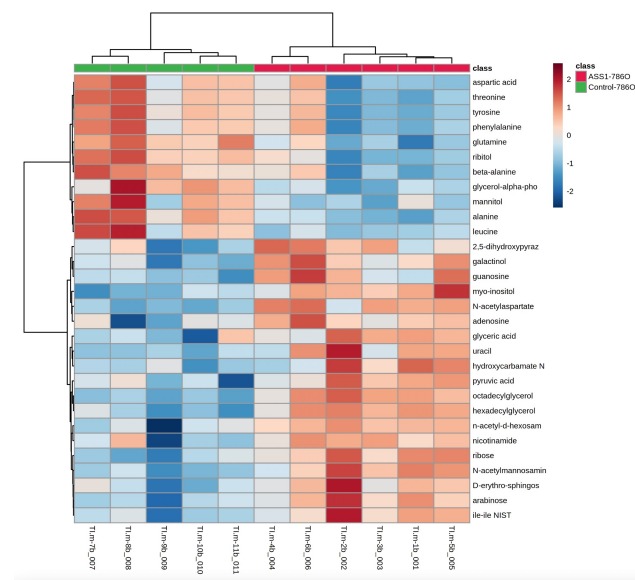


Fig. 18e: Heatmap

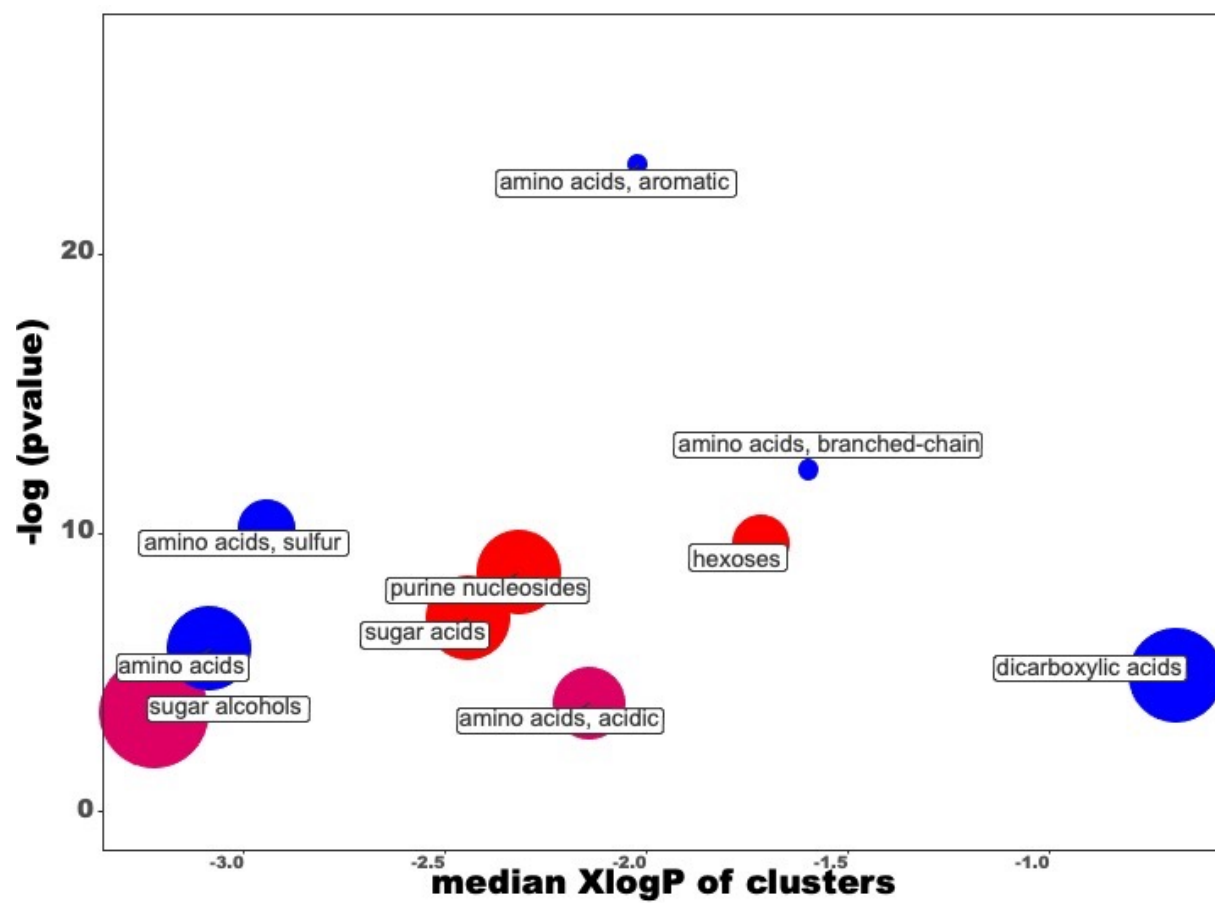


Fig. 18f: ChemRICH enrichment analysis

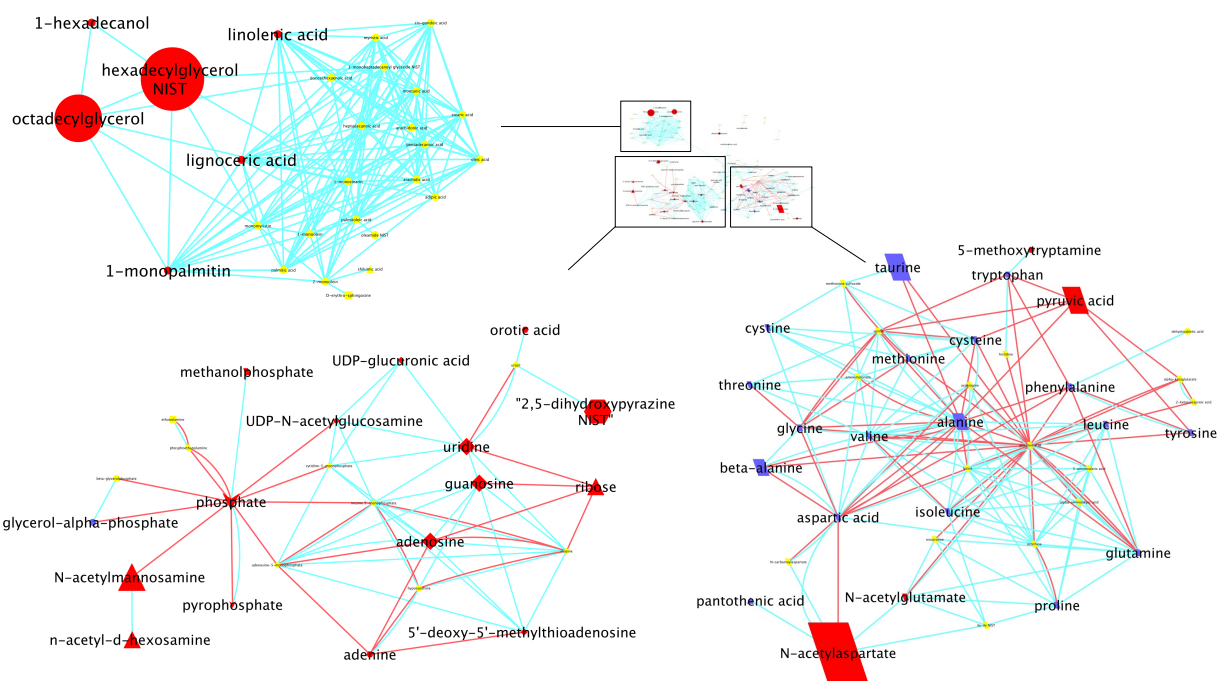


Fig.18g ASS1-786O primary metabolites network analysis

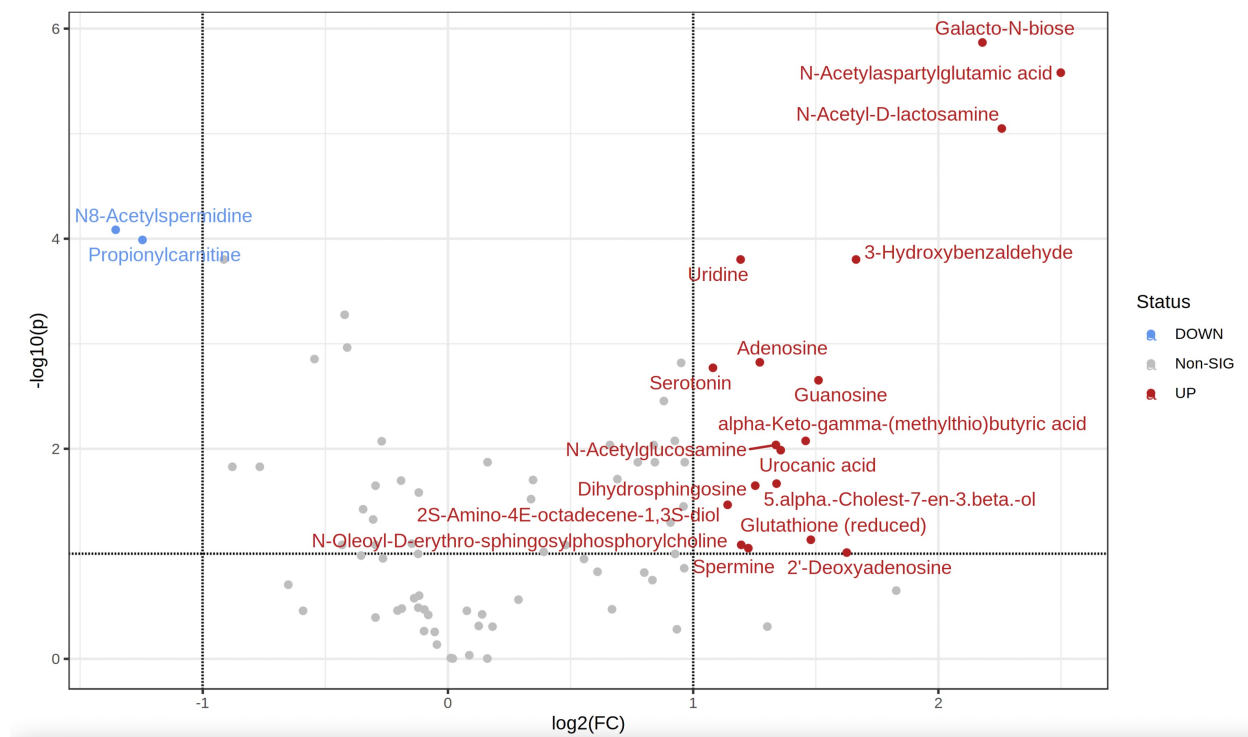


Fig.19a: Biogenic amines volcano plot on ASS1-786O vs control 786O

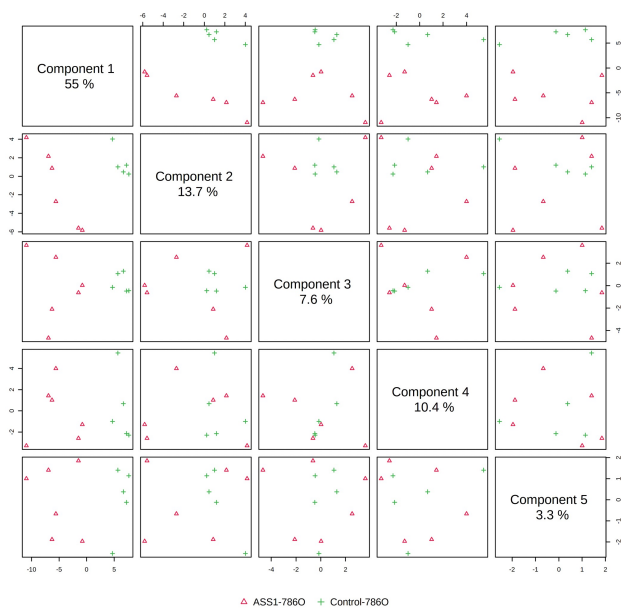


Fig.19b: PLS-DA overview

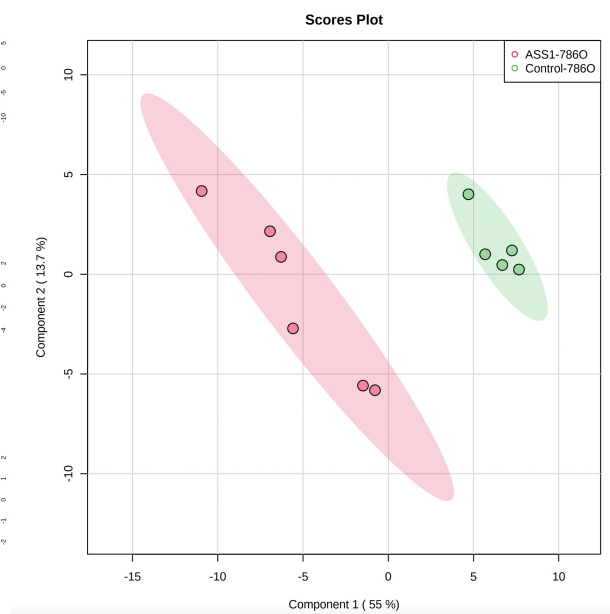


Fig.19c: PLS-DA 2D score plot

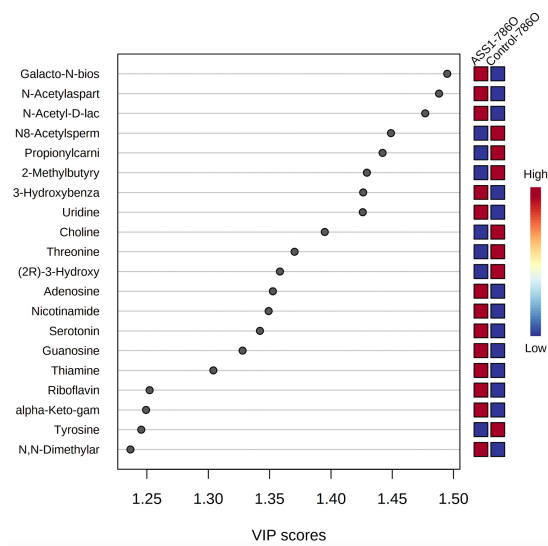


Fig.19d: Variable important projection (VIP) score

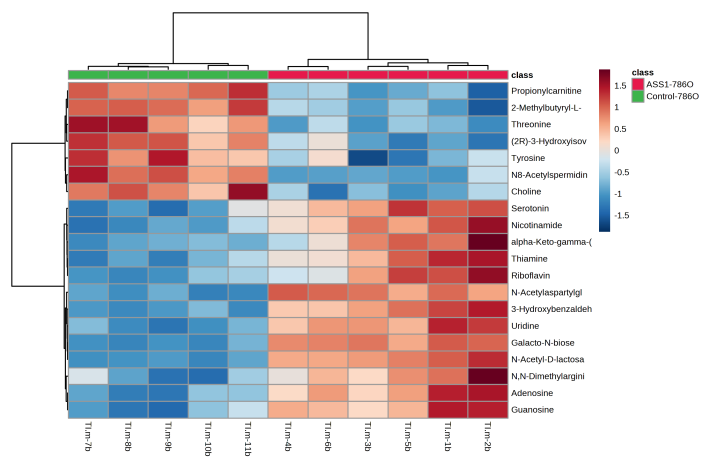


Fig.19e: Heatmap

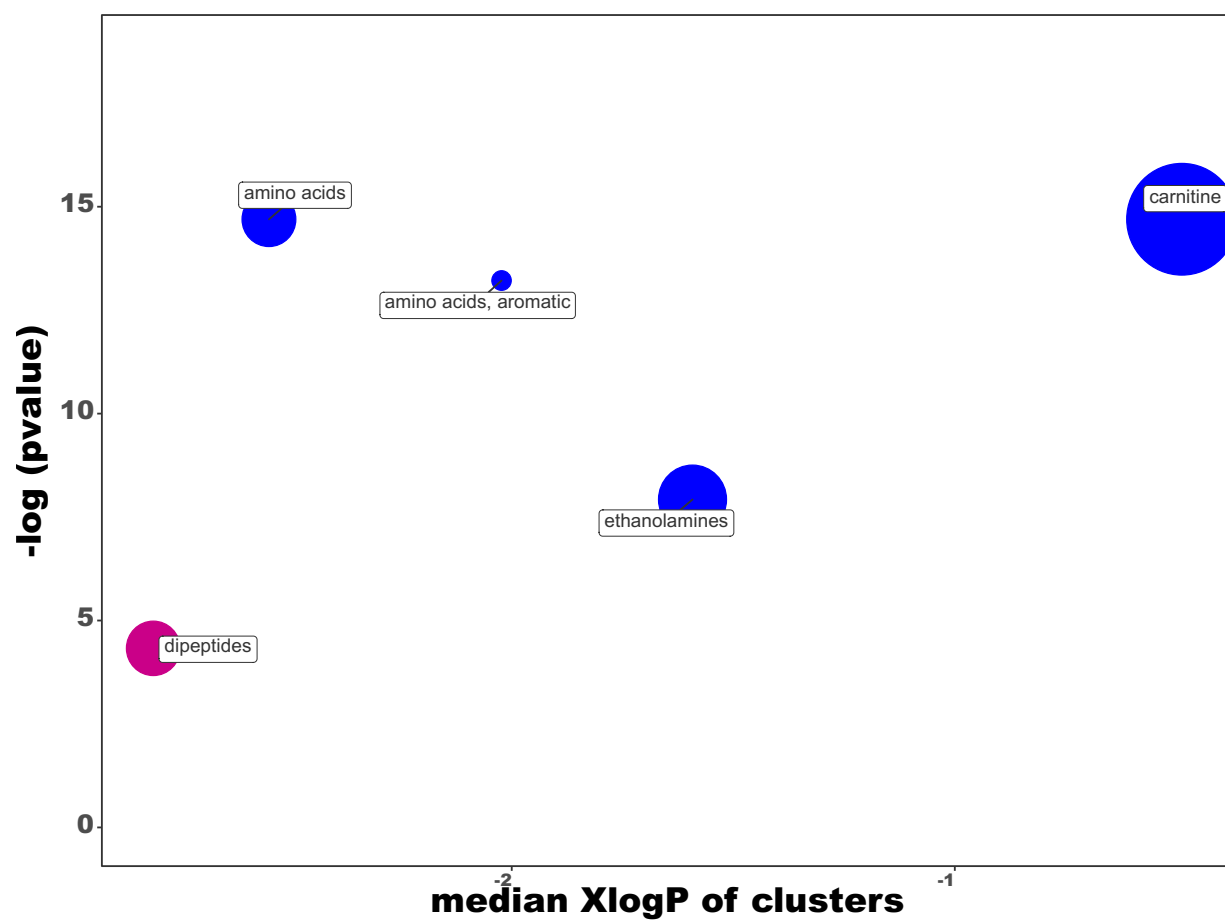


Fig.19f: ChemRICH enrichment analysis

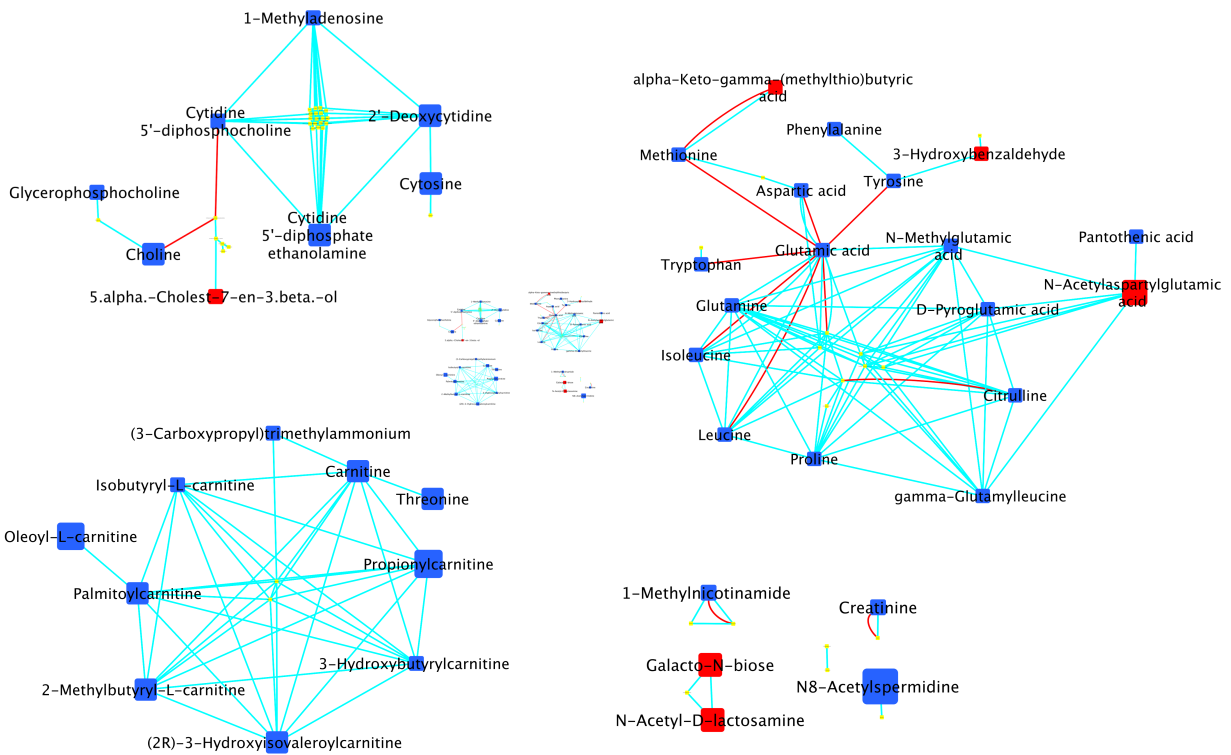


Fig.19g: ASS1-786O biogenic amines network analysis

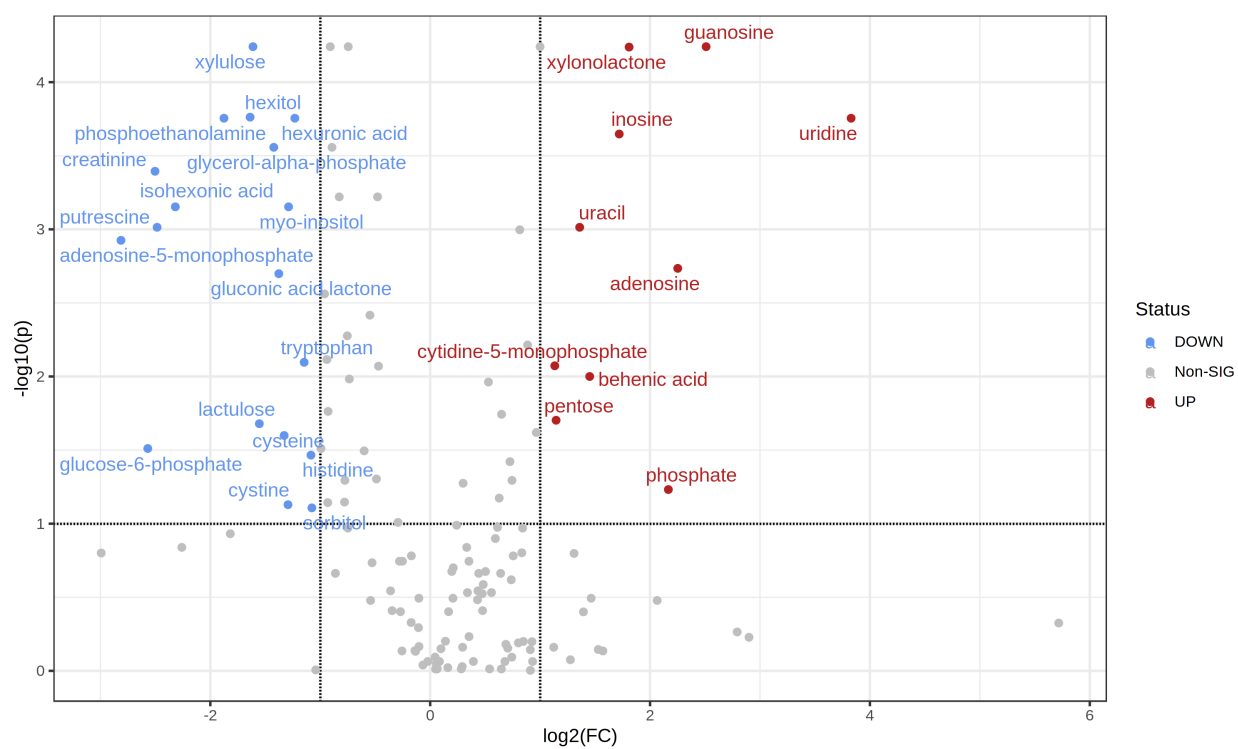


Fig. 20a: Primary metabolites volcano plot on ASS1-KD HK2 vs control HK2

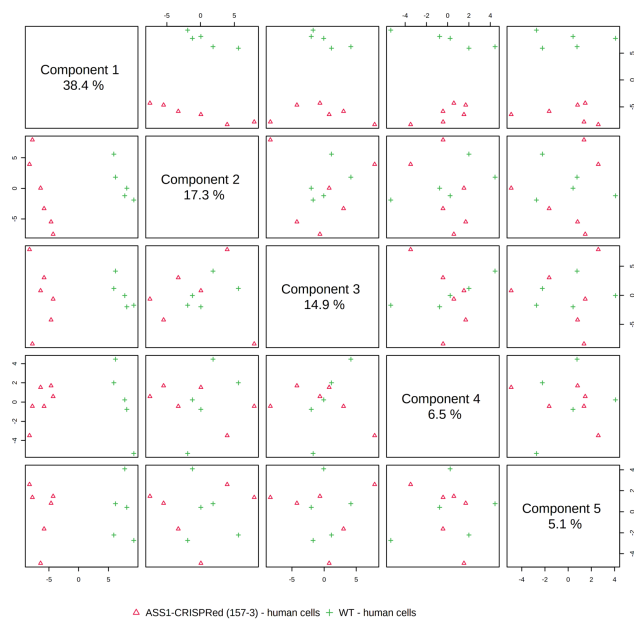


Fig.20b: PLS-DA overview

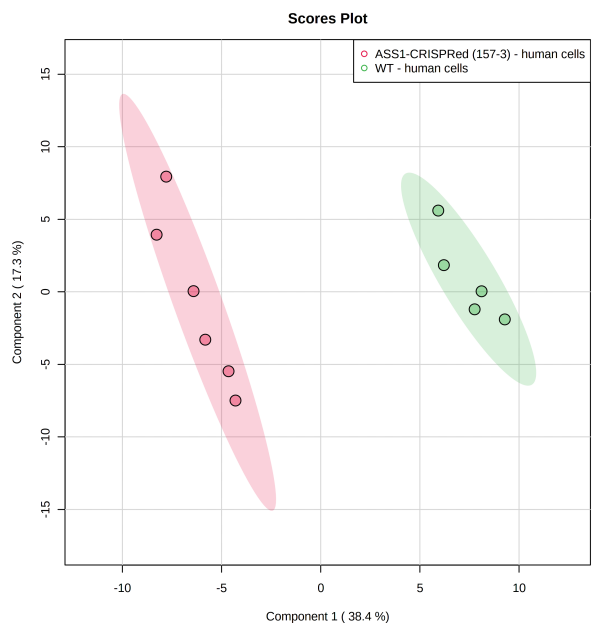


Fig. 20c: PLS-DA 2D score plot

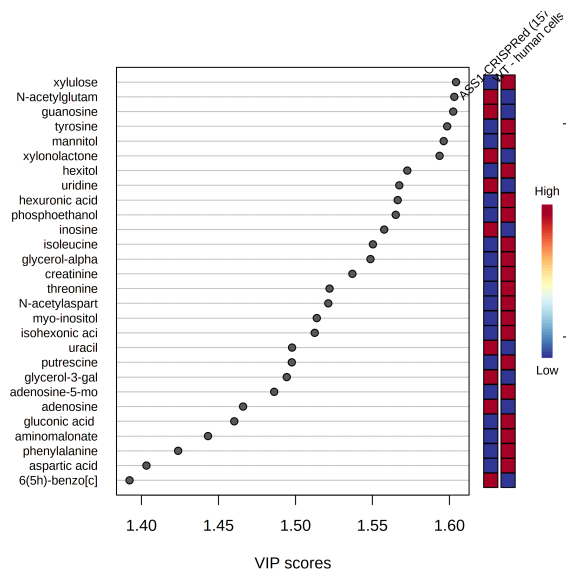


Fig. 20d: Variable important projection (VIP) score

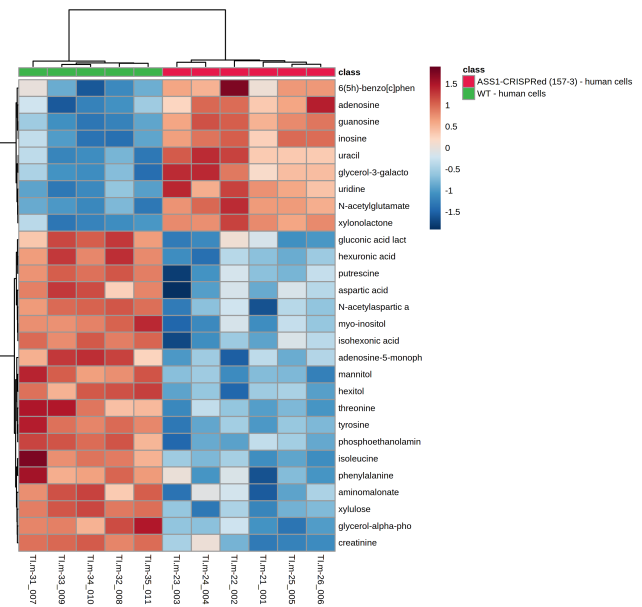


Fig. 20e: Heatmap

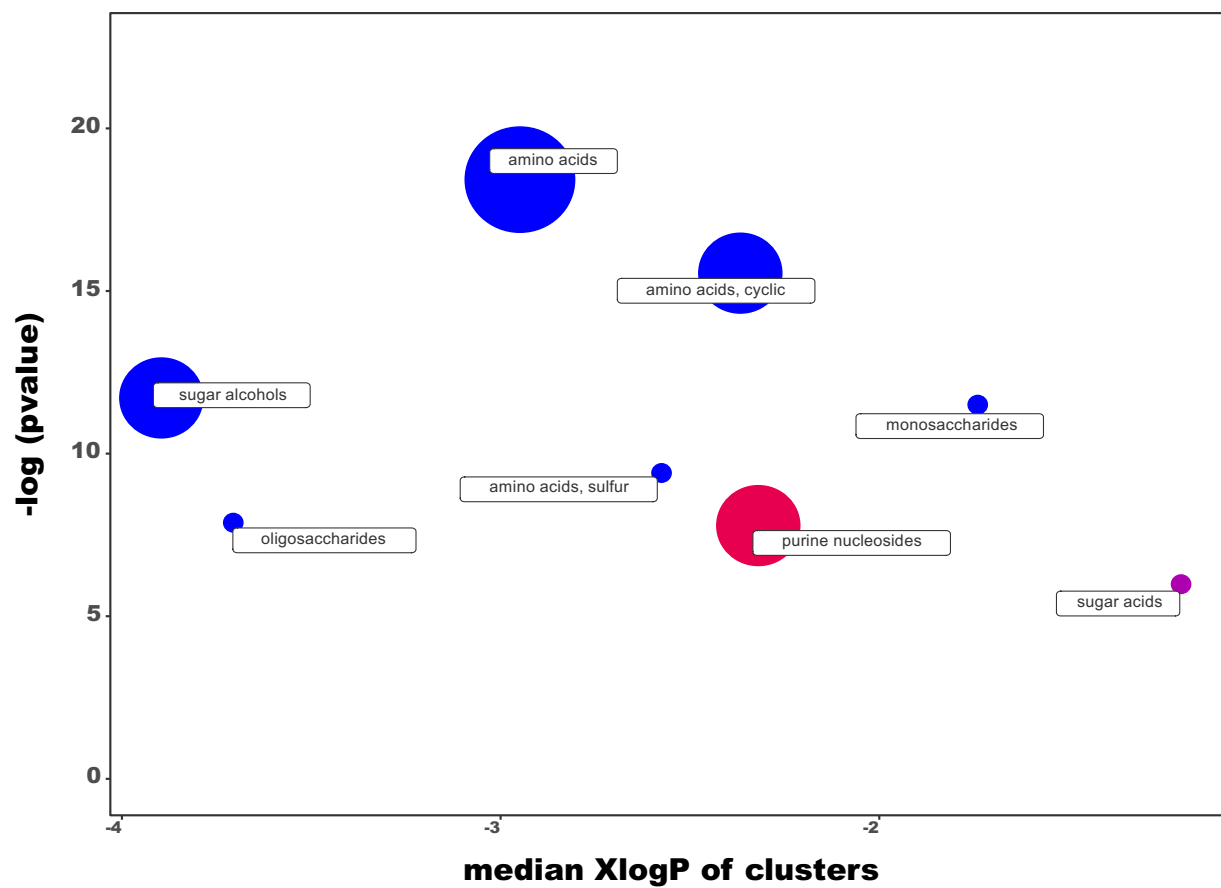


Fig. 20f: ChemRICH enrichment analysis

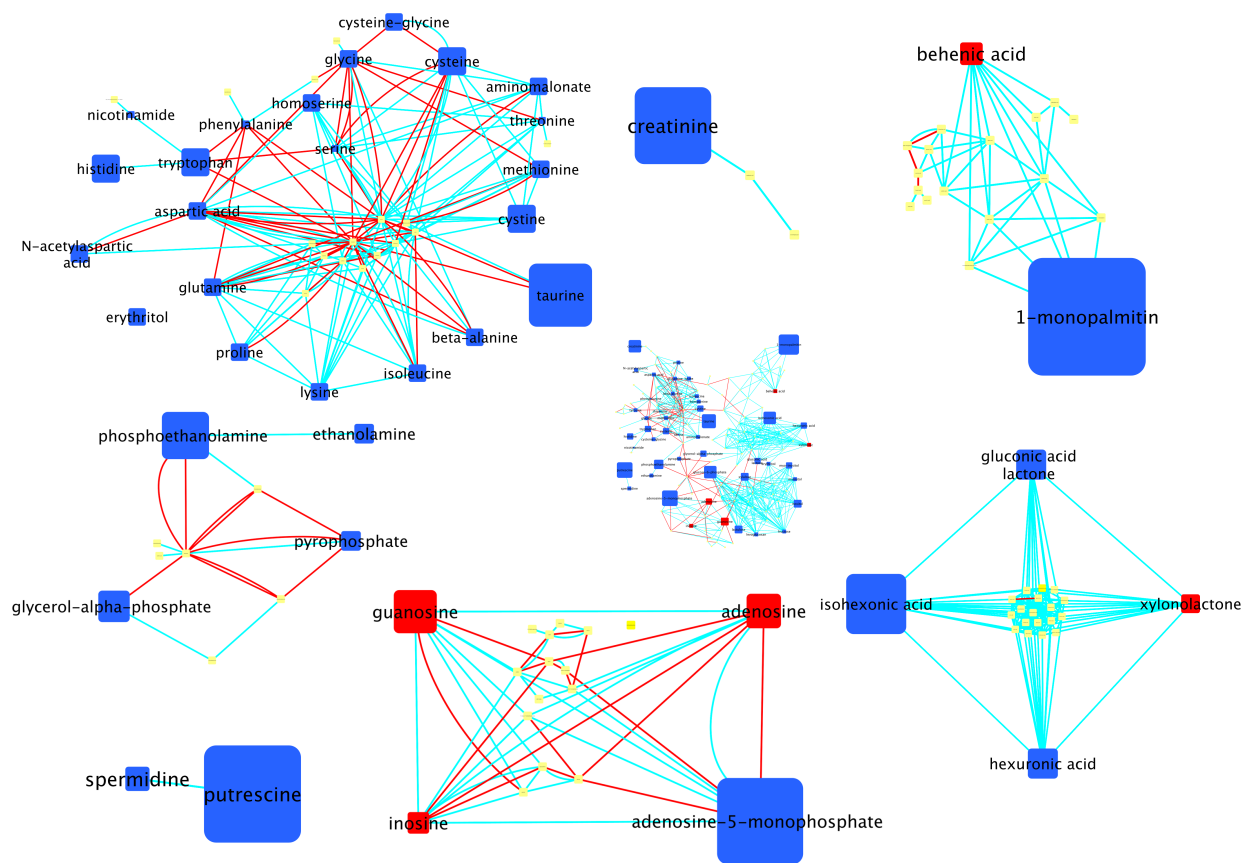


Fig. 20g: ASS1-KD HK2 primary metabolites MetaMAPP network

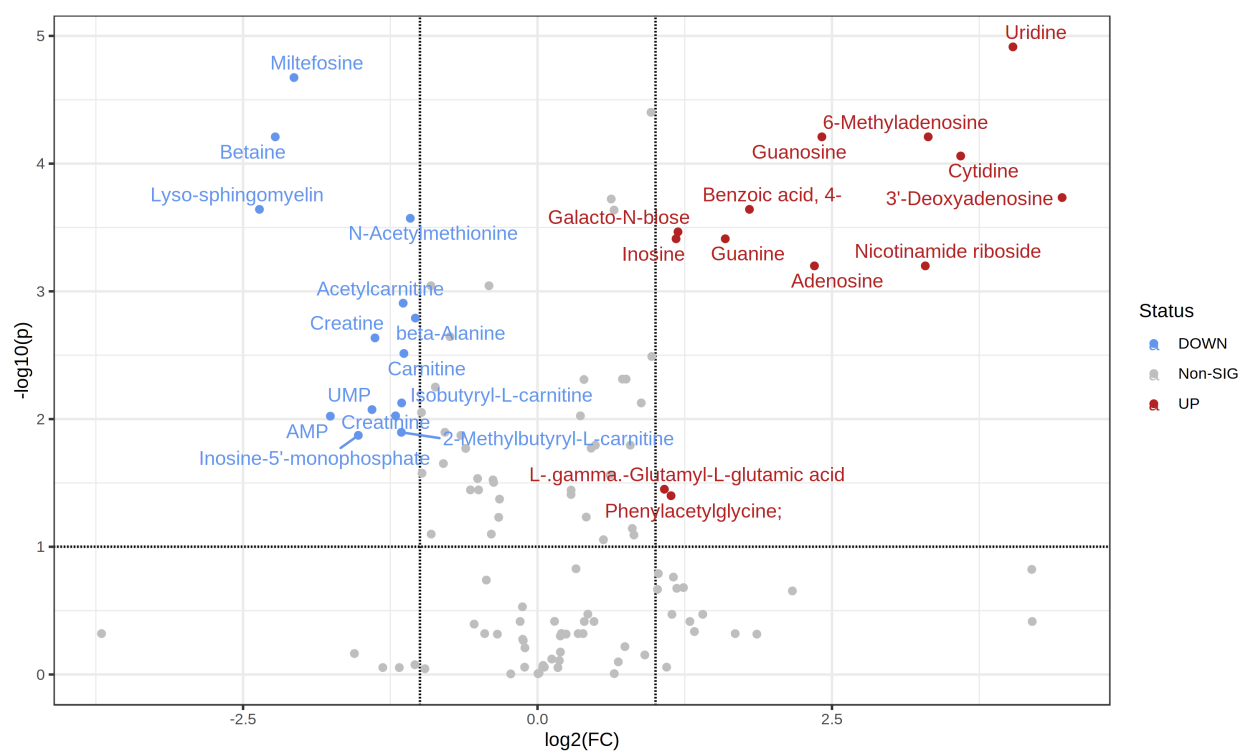


Fig.21a: Biogenic amines volcano plot on ASS1-KD HK2 vs control HK2

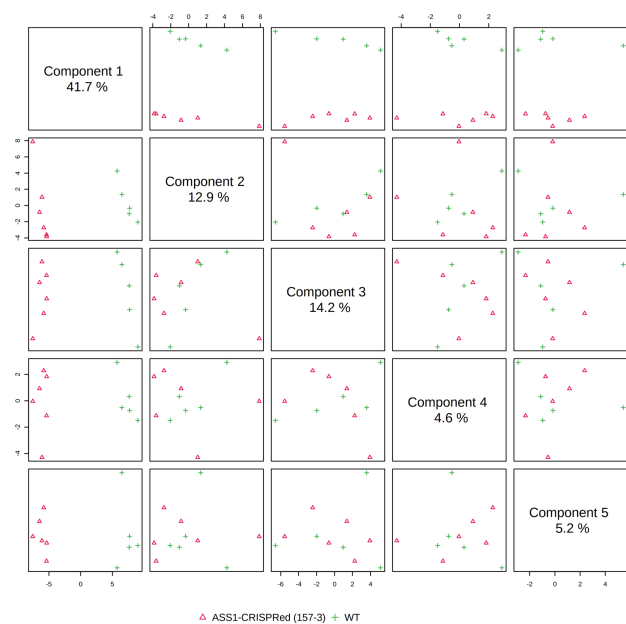


Fig.21b: PLS-DA overview

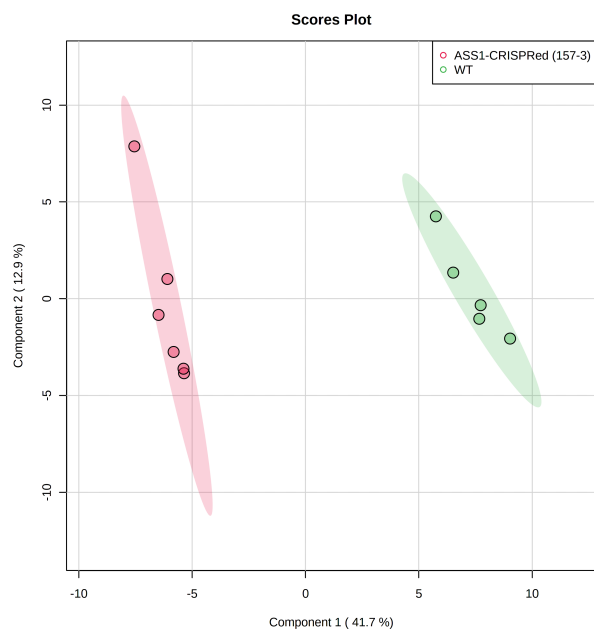


Fig.21c: PLS-DA 2D score plot

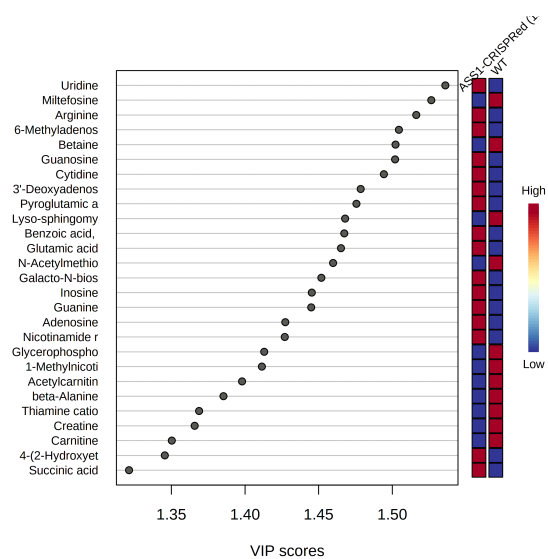


Fig. 21d: Variable important projection (VIP) score

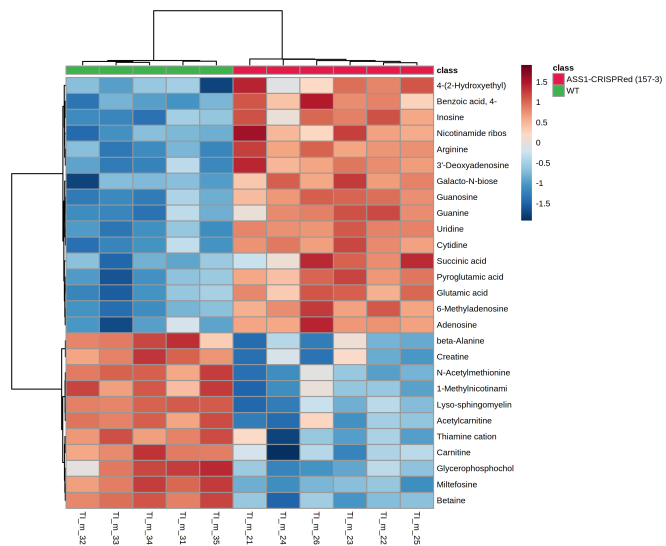


Fig. 21e: Heatmap

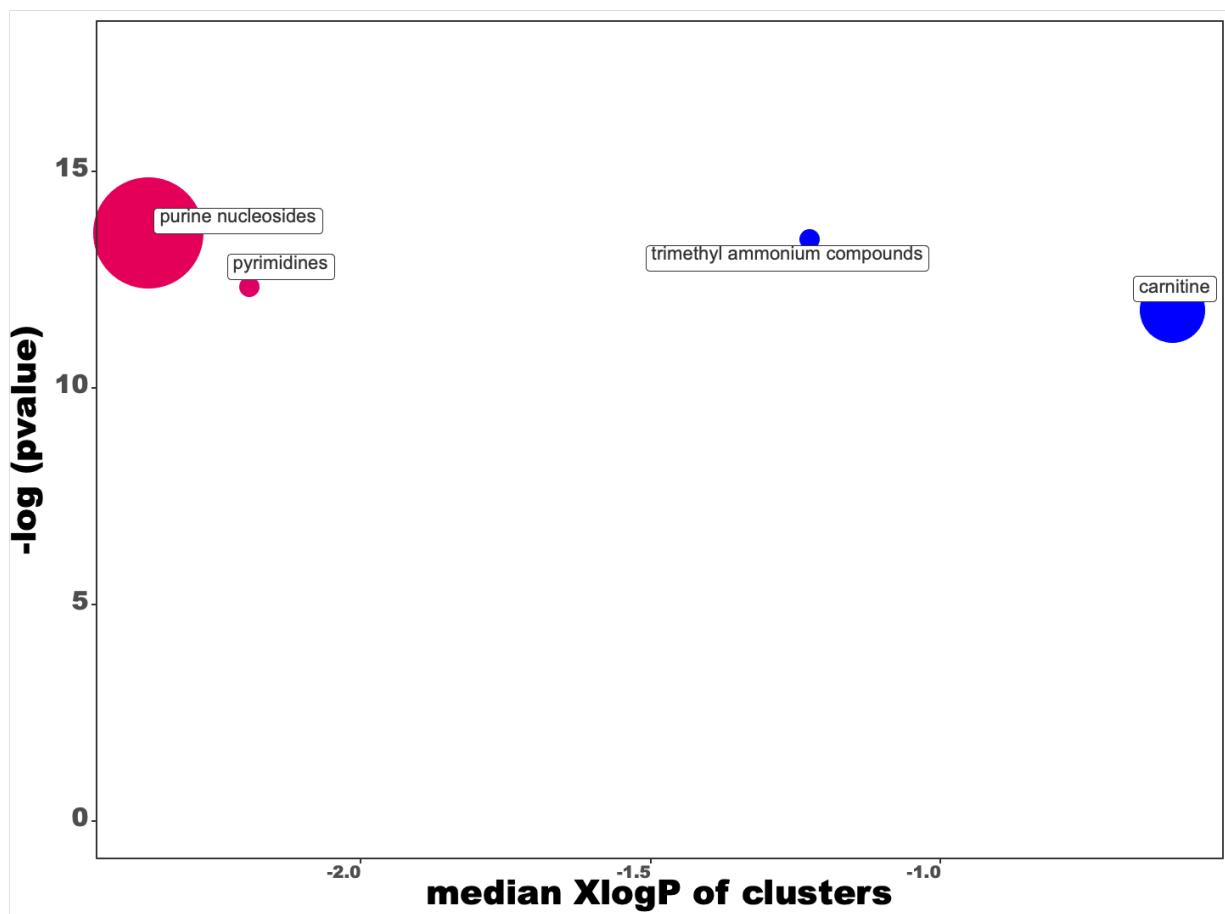


Fig. 21f: ChemRICH enrichment analysis

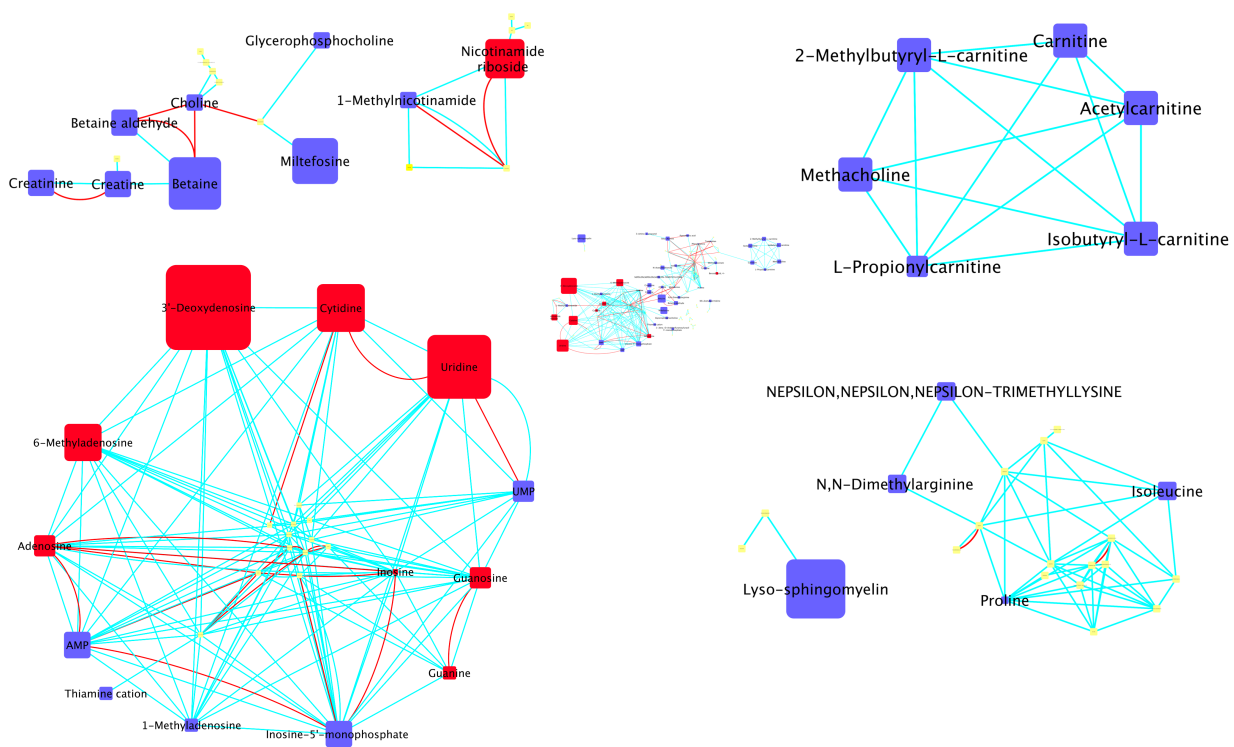


Fig. 21g: ASS1-KD HK2 biogenic amines MetaMAPP network analysis

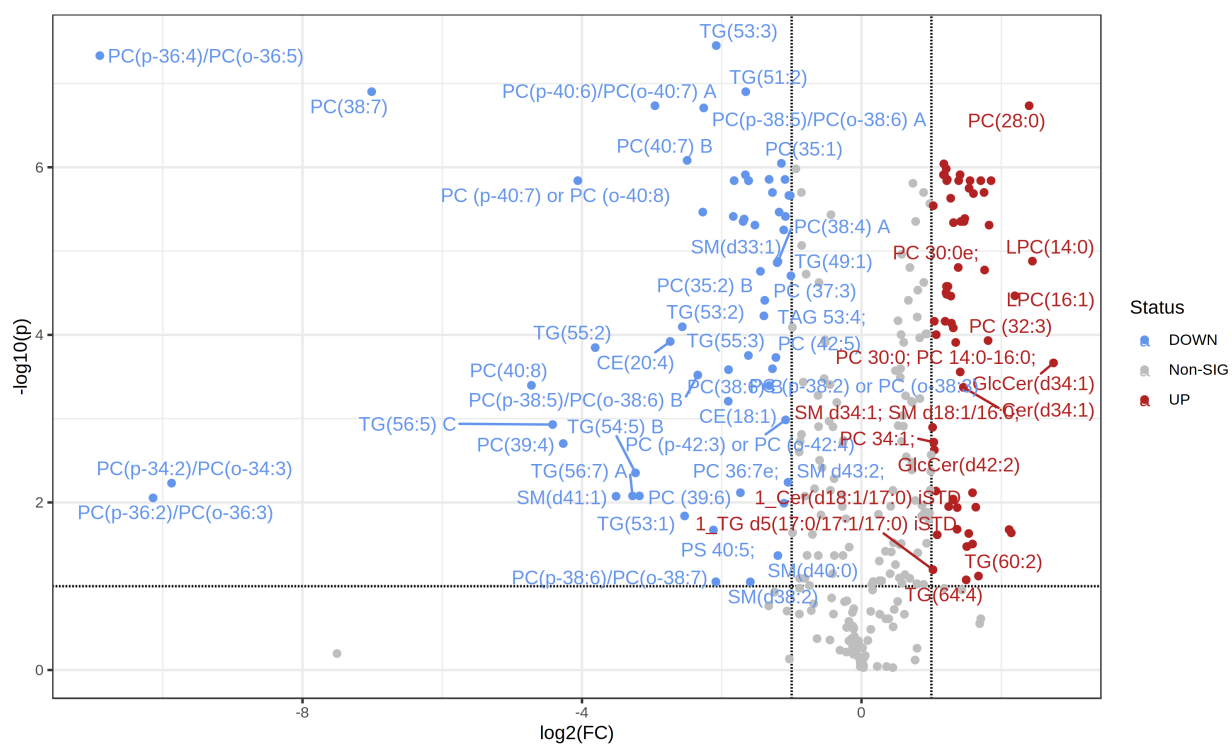


Fig.22a: Lipidomics volcano plot on ASS1-KD HK2 vs control HK2

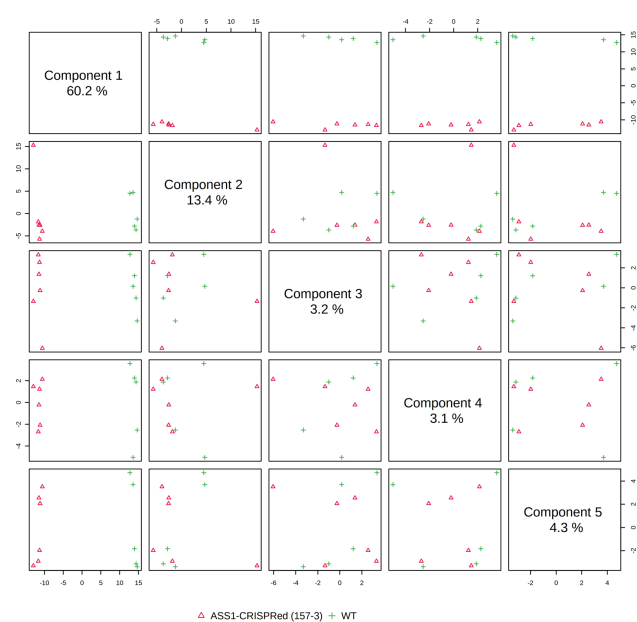


Fig.22b: PLS-DA overview

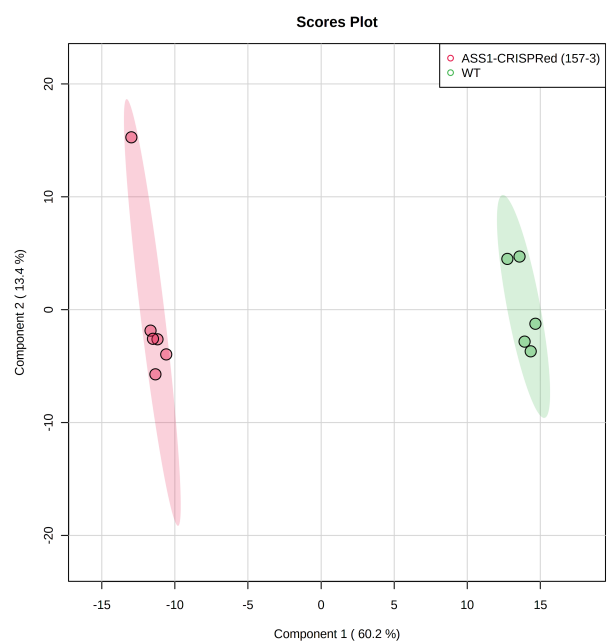


Fig.22c: PLS-DA 2D score plot

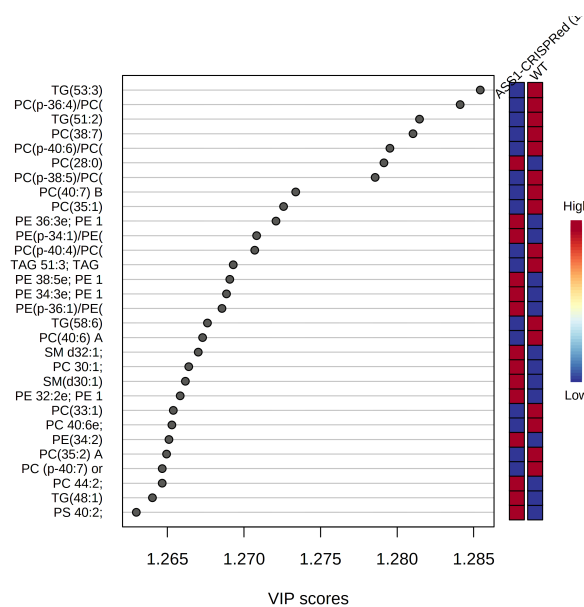


Fig.22d: Variable important projection (VIP) score

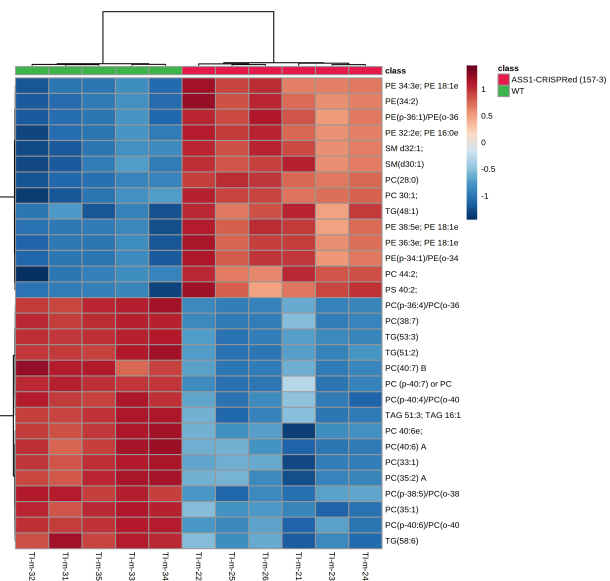


Fig.22e: Heatmap

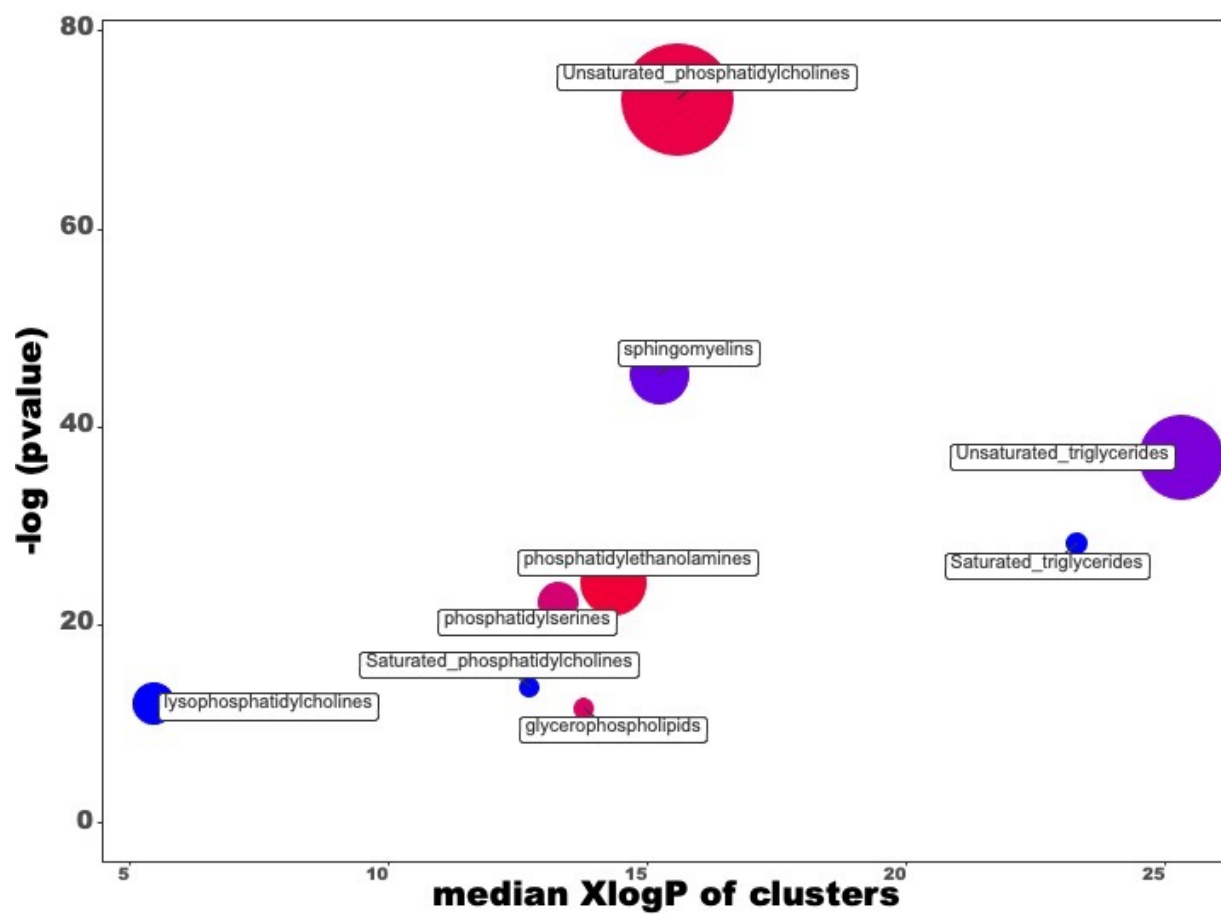
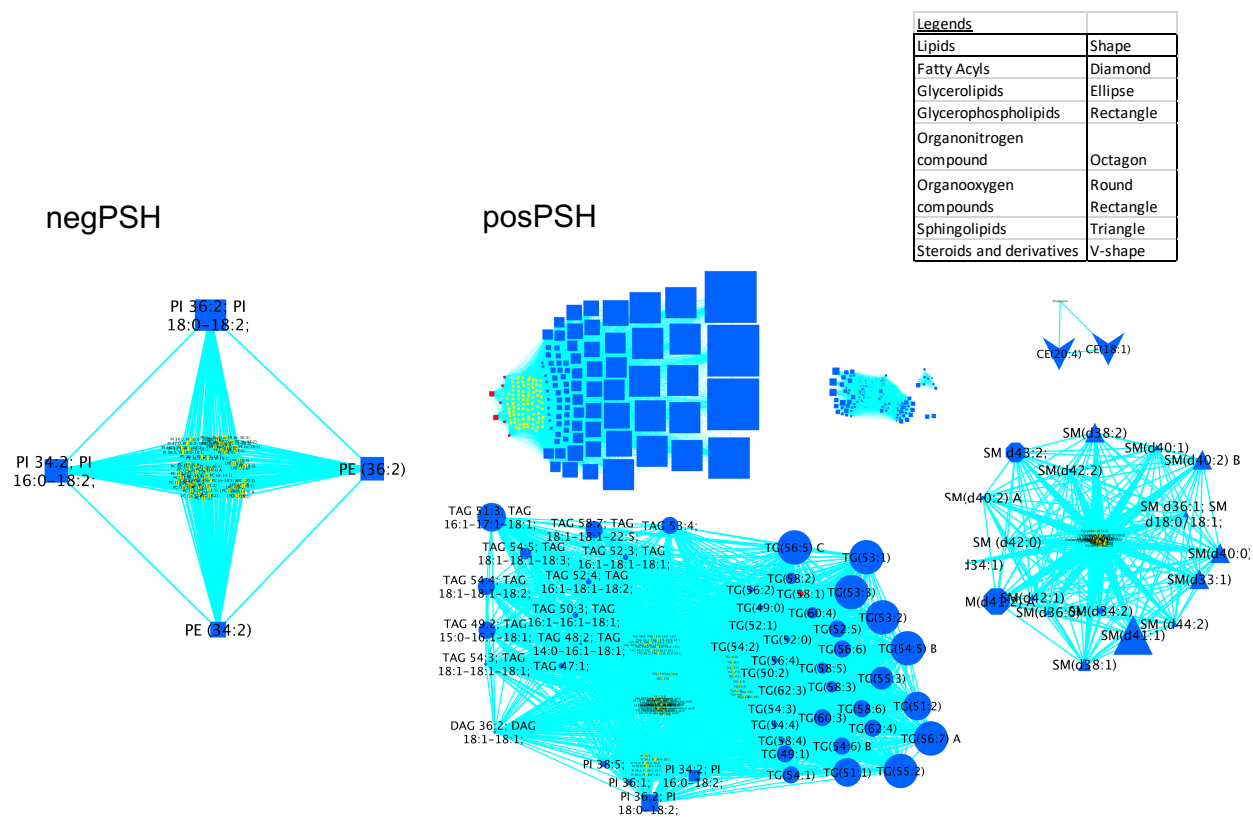


Fig.22f: ChemRICH enrichment analysis



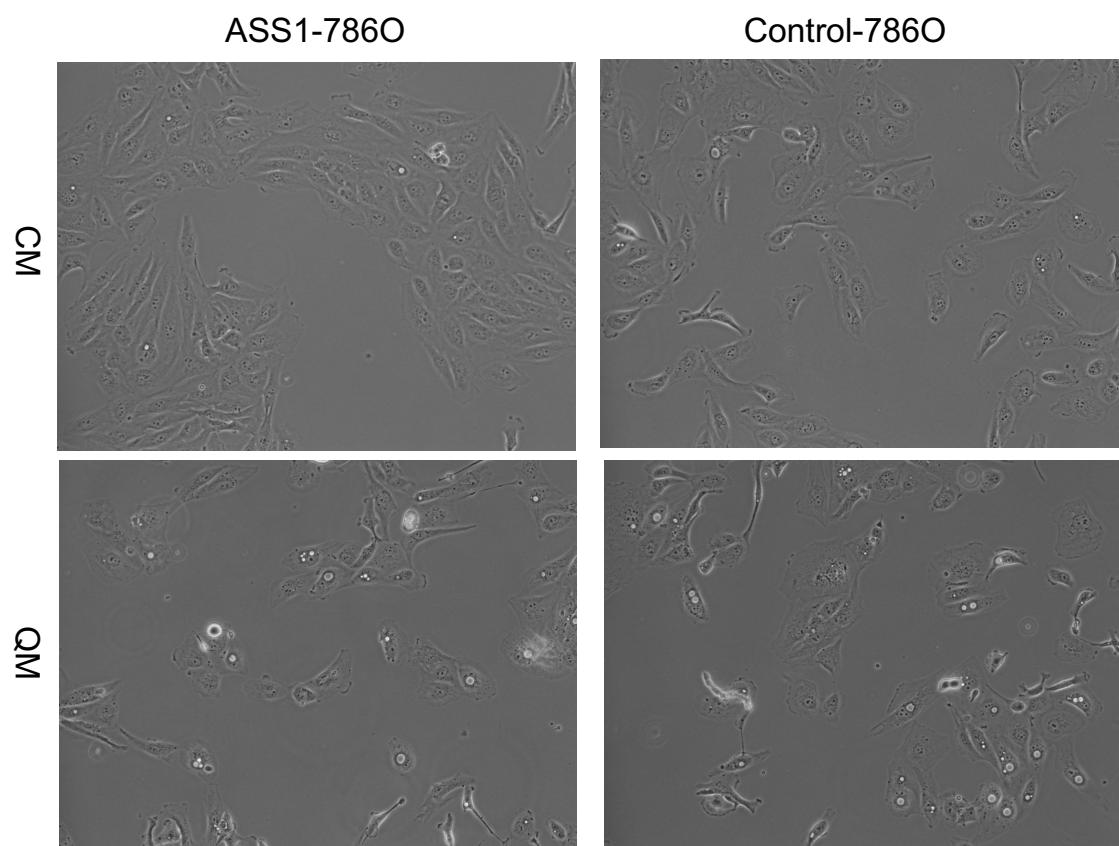
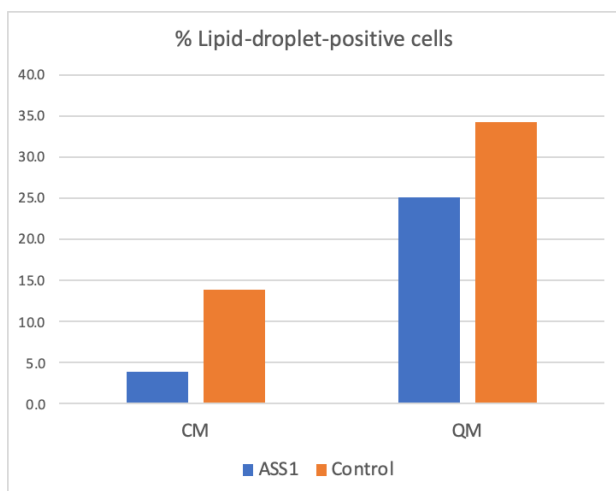


Fig.23a: Morphology of ASS1-786O and Control 786O in CM and QM



% of lipid-droplet positive cells (n=3)			
	ASS1	Control	
CM	3.8	13.9	
QM	25.2	34.1	
		Ratio	p value
	Cont/QM vs ASS1/CM	8.9	4.3E-05
	ASS1/QM vs ASS1/CM	6.6	1.23E-03
	Cont/CM vs ASS1/CM	3.6	0.075
	Cont/QM vs Cont/CM	2.5	8.25E-03
	Cont/QMvs ASS1/QM	1.4	0.023

CM : complete media, 10% FBS/DMEM
QM : Quiescent media, 1% FBS/DMEM
ASS1: ASS1-786O
Cont: Control-786O (empty-vector transfected)

Fig.23b: percentage of Lipid droplet (LD) positive cells

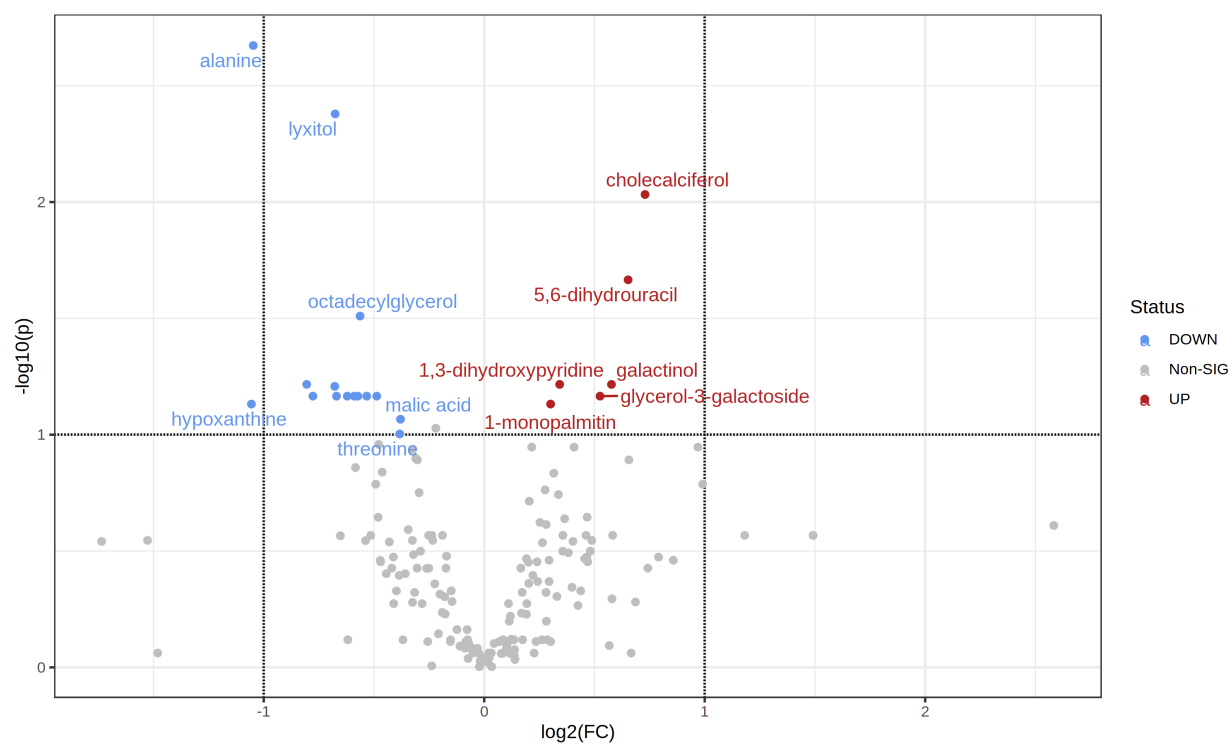


Fig.24a: Volcano plot of lipidomic analysis on ASS1-786O grown in low-serum media

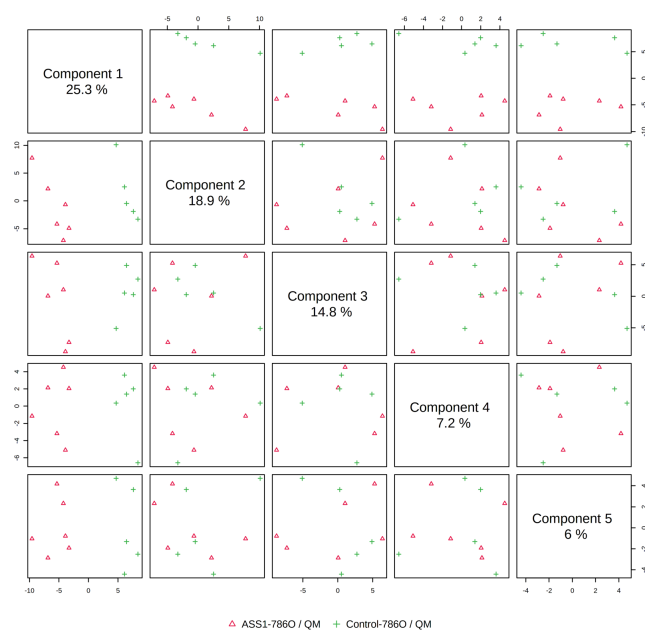


Fig.24b: PLS-DA overview

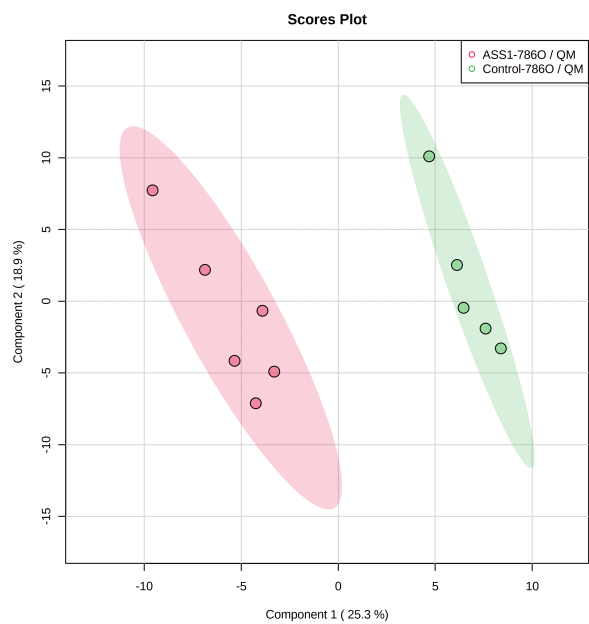


Fig.24c: PLS-DA score plot

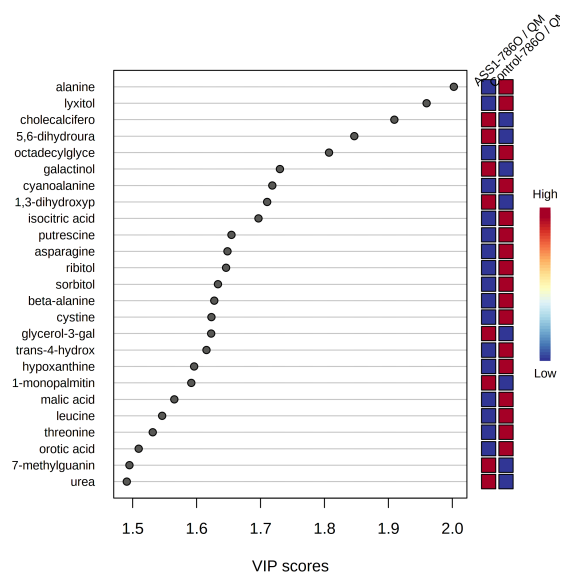


Fig.24d: Variable important projection (VIP) score

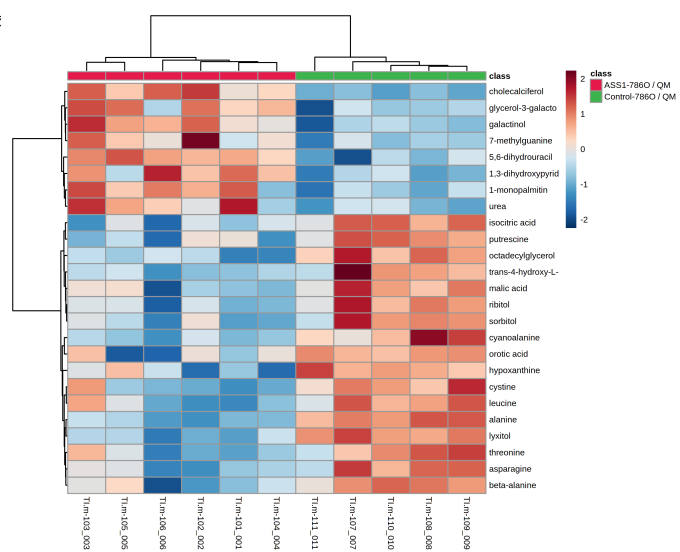


Fig.24e: Heat map

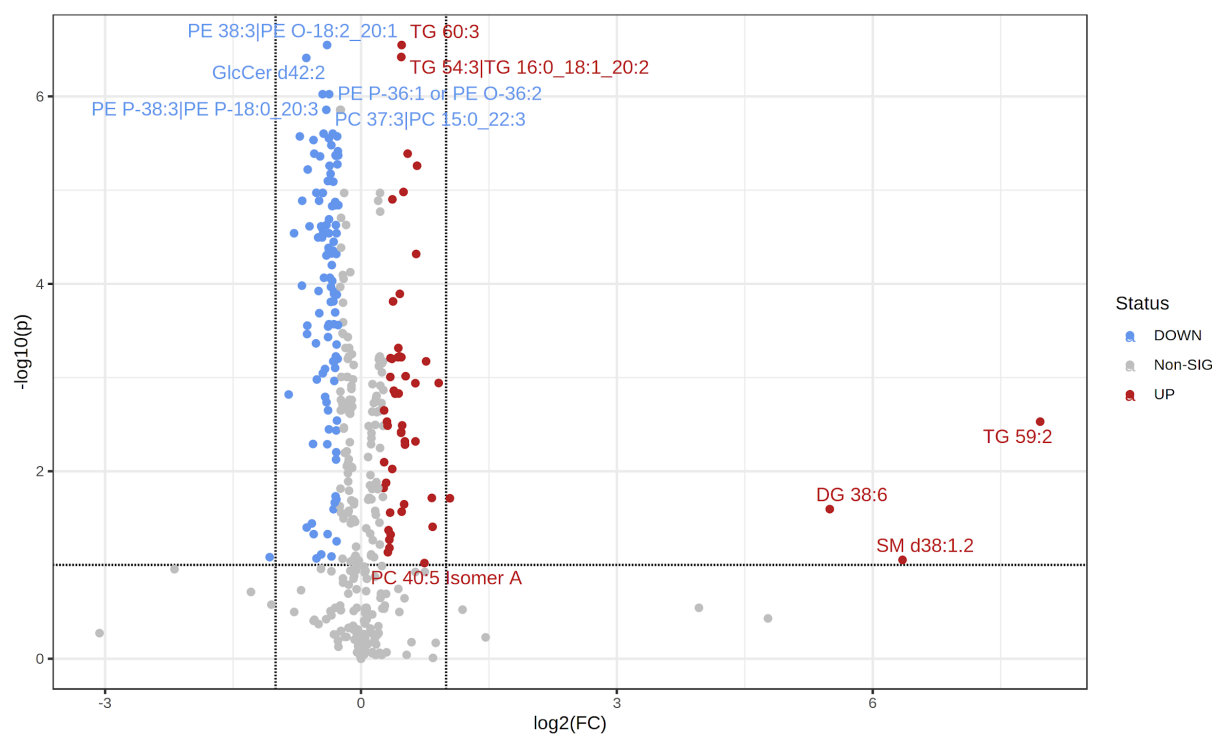


Fig.25a: Volcano plot on the lipidomic analysis of ASS1-786O grown in low-serum media, posCSH. Adjusted p-value <0.10, FC > 1.2

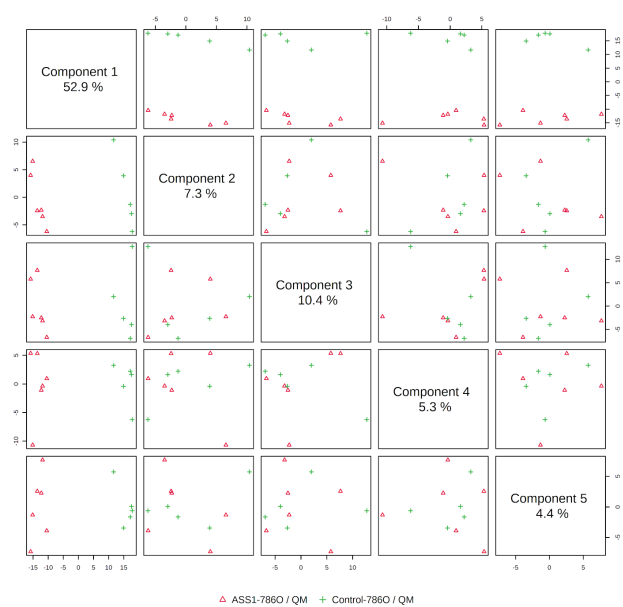


Fig.25b: PLS-DA overview

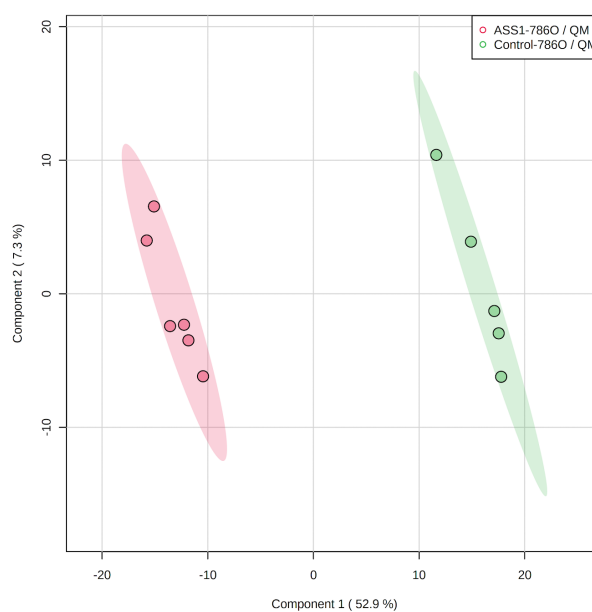


Fig.25c: PLS-DA score plot

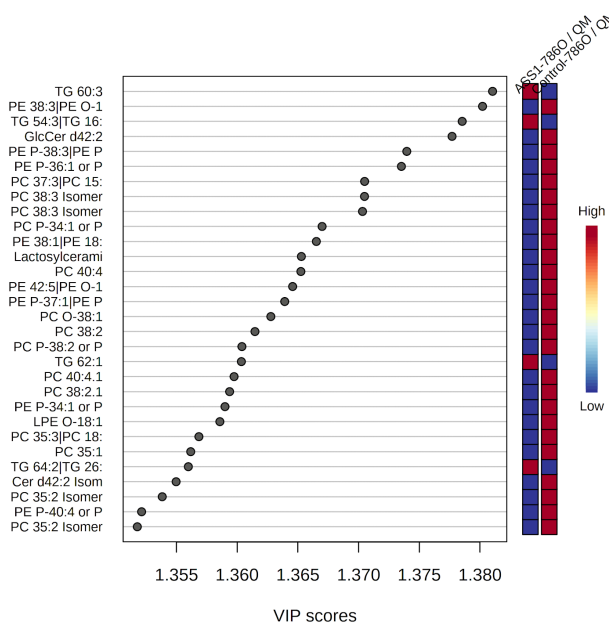


Fig.25d: Variable important projection (VIP) score

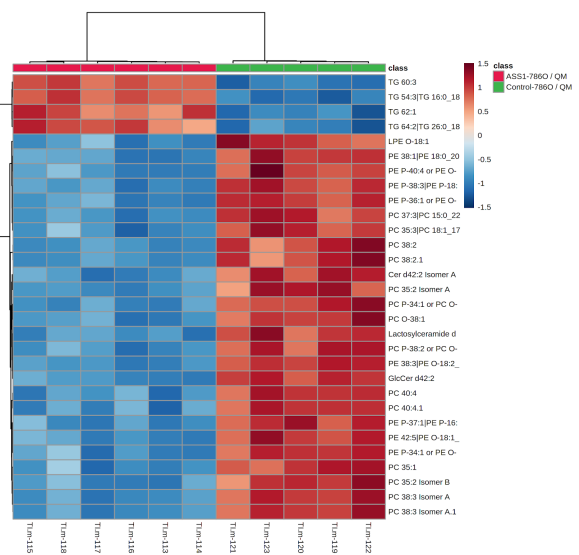


Fig.25e: Heat map

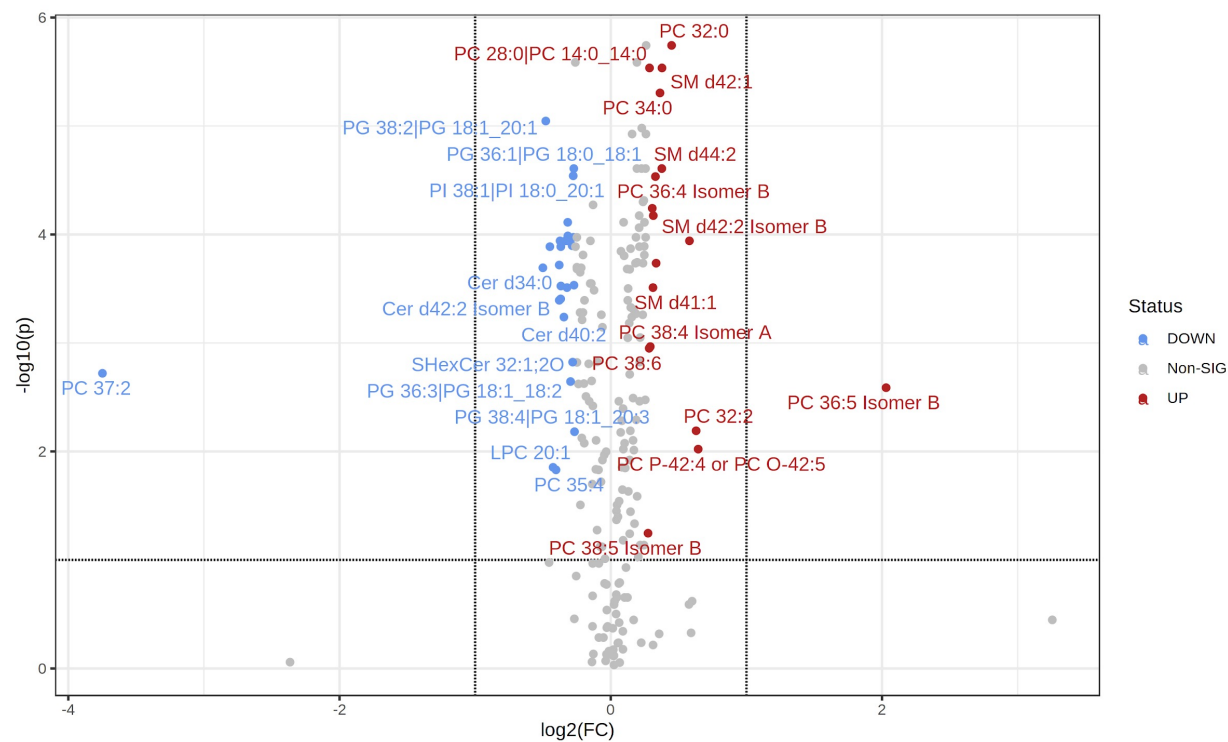


Fig.25f: Volcano plot on the lipidomic analysis of ASS1-786O grown in low-serum media, negCSH. Adjusted p-value < 0.10, FC > 1.2

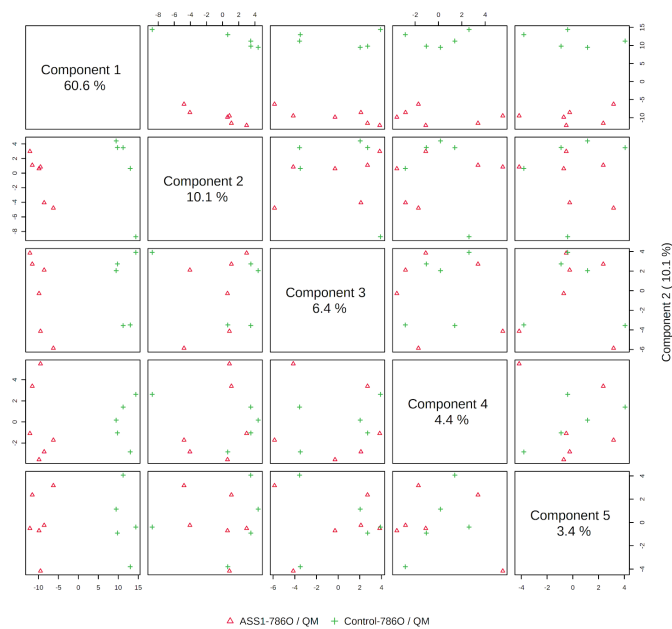


Fig.25g: PLS-DA overview

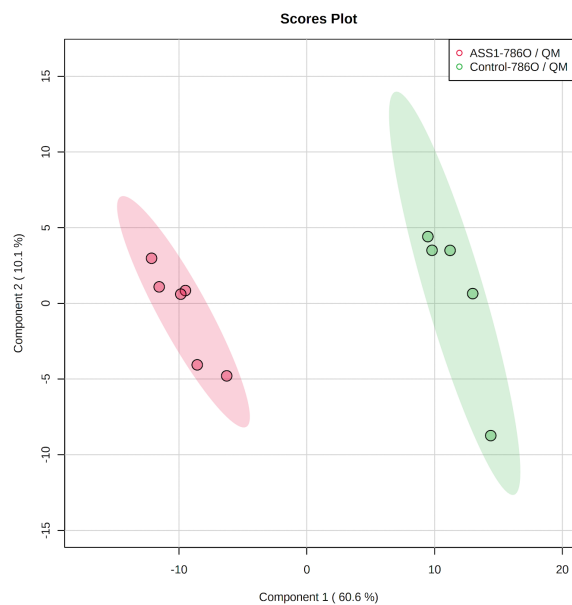


Fig.25h: PLS-DA score plot

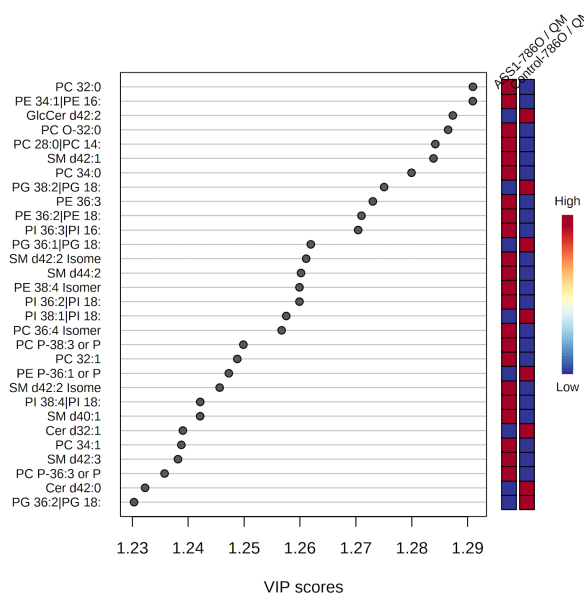


Fig.25i: Variable important projection (VIP) score

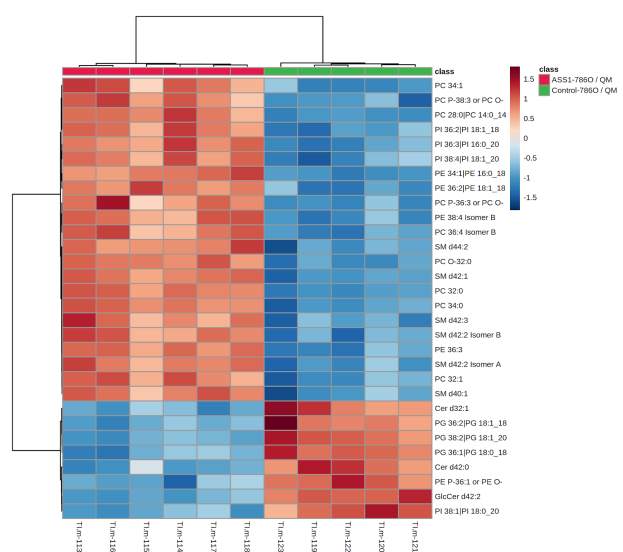


Fig.25j: Heat map

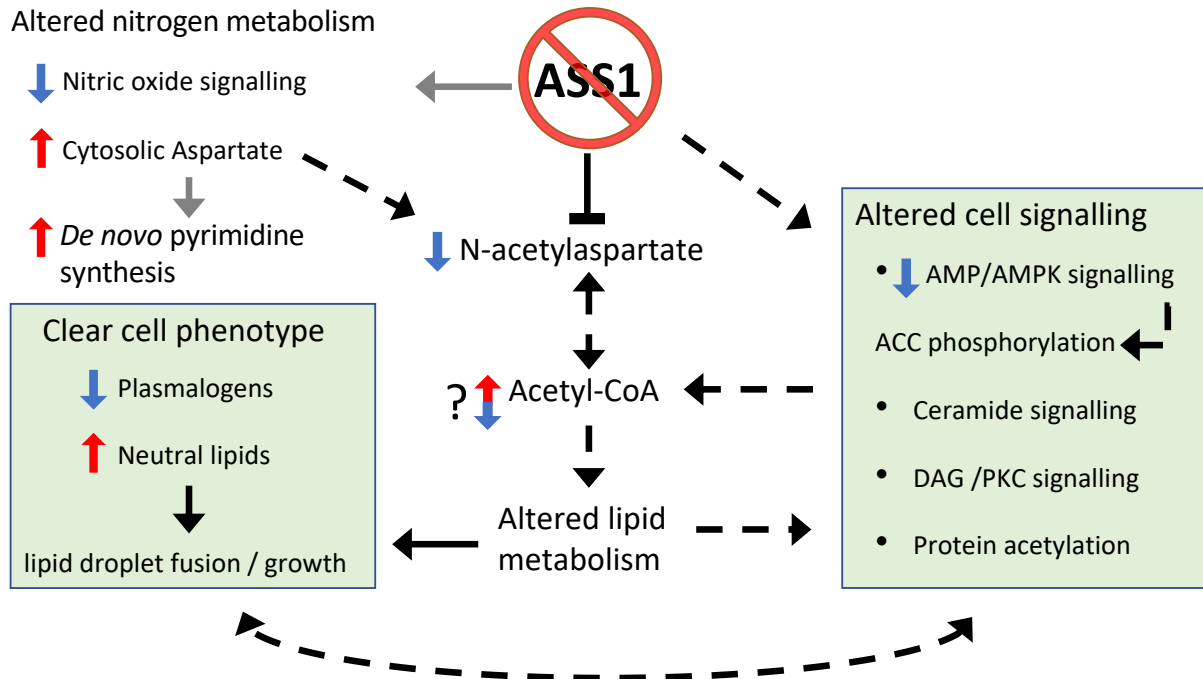


Fig.26: Summary – ASS1-silencing in ccRCC results in multi-faceted metabolic reprogramming encompassing acetyl-CoA metabolism and lipid metabolome

Metabolite name	FC	log2(FC)	p.adjusted	-LOG10(p)
N-acetylaspartate	6.04	2.60	2.35E-04	3.63
hexadecylglycerol NIST	5.48	2.45	1.93E-03	2.71
octadecylglycerol	3.86	1.95	2.74E-03	2.56
pyruvic acid	2.49	1.31	8.04E-02	1.09
N-acetylmannosamine	2.35	1.23	4.47E-02	1.35
2,5-dihydroxypyrazine NIST	2.33	1.22	6.93E-03	2.16
ribose	2.14	1.10	5.84E-02	1.23
n-acetyl-d-hexosamine	2.10	1.07	2.46E-02	1.61
arabinose	2.00	1.00	8.04E-02	1.09
tyrosine	0.48	-1.06	3.13E-05	4.51
cysteine	0.46	-1.11	6.96E-03	2.16
glycerol-alpha-phosphate	0.44	-1.18	1.98E-03	2.70
threonine	0.43	-1.23	5.31E-05	4.27
glutamine	0.40	-1.32	1.20E-04	3.92
ribitol	0.38	-1.39	5.31E-05	4.27
beta-alanine	0.31	-1.67	2.33E-03	2.63
alanine	0.31	-1.70	3.13E-05	4.51
taurine	0.21	-2.23	4.47E-02	1.35

Table 6a: Metabolites differentially present in ASS1-786O and control 786O ccRCC cell line as detected in the primary metabolite analysis

Metabolite name	FC	log2(FC)	p.adjusted	-LOG10(p)
N-Acetylaspartylglutamic acid	5.65	2.50	2.63E-06	5.58
N-Acetyl-D-lactosamine	4.78	2.26	8.93E-06	5.05
Galacto-N-biose	4.53	2.18	1.36E-06	5.87
3-Hydroxybenzaldehyde	3.17	1.66	1.58E-04	3.80
2'-Deoxyadenosine	3.09	1.63	9.73E-02	1.01
Guanosine	2.85	1.51	2.23E-03	2.65
Glutathione (reduced)	2.79	1.48	7.35E-02	1.13
alpha-Keto-gamma-(methylthio)butyric acid	2.75	1.46	8.41E-03	2.08
Urocanic acid	2.56	1.36	1.03E-02	1.99
5.alpha.-Cholest-7-en-3.beta.-ol	2.53	1.34	2.15E-02	1.67
N-Acetylglucosamine	2.53	1.34	9.19E-03	2.04
Adenosine	2.41	1.27	1.50E-03	2.82
Dihydrosphingosine	2.38	1.25	2.24E-02	1.65
Spermine	2.34	1.22	8.83E-02	1.05
N-Oleoyl-D-erythro-sphingosylphosphorylcholine	2.29	1.20	8.22E-02	1.09
Uridine	2.29	1.19	1.58E-04	3.80
2S-Amino-4E-octadecene-1,3S-diol	2.20	1.14	3.42E-02	1.47
Serotonin	2.12	1.08	1.69E-03	2.77
Propionylcarnitine	0.42	-1.25	1.03E-04	3.99
N8-Acetylspermidine	0.39	-1.36	8.23E-05	4.08

Table 6b: Biogenic amines differentially present in ASS1-786O and control 786O

Metabolite name	FC	log2(FC)	p.adjusted	-LOG10(p)
uridine	11.6	3.54	5.64E-04	3.25
guanosine	4.51	2.17	1.13E-03	2.95
adenosine	3.83	1.94	1.34E-02	1.87
xylonolactone	2.75	1.46	1.21E-03	2.92
inosine	2.61	1.38	1.34E-02	1.87
benenic acid	2.19	1.13	2.16E-02	1.66
uracil	2.07	1.05	2.43E-02	1.61
pyrophosphate	0.49	-1.02	9.11E-02	1.04
maltotriose	0.48	-1.05	7.24E-02	1.14
glycine	0.48	-1.06	2.51E-02	1.60
levoglucosan	0.48	-1.07	2.16E-02	1.66
mannitol	0.47	-1.10	1.71E-03	2.77
aspartic acid	0.46	-1.12	7.89E-03	2.10
N-acetylaspatic acid	0.45	-1.15	5.62E-03	2.25
lysine	0.45	-1.16	2.05E-02	1.69
isoleucine	0.43	-1.23	1.67E-03	2.78
erythritol	0.42	-1.24	1.34E-02	1.87
tyrosine	0.42	-1.25	9.08E-04	3.04
fructose	0.41	-1.27	1.17E-02	1.93
proline	0.41	-1.29	2.05E-02	1.69
aminomalinate	0.41	-1.30	1.17E-02	1.93
sorbitol	0.41	-1.30	5.35E-02	1.27
cysteine-glycine	0.38	-1.40	1.84E-02	1.74
histidine	0.36	-1.47	2.00E-02	1.70
tryptophan	0.36	-1.47	1.30E-02	1.89
hexuronic acid	0.33	-1.59	2.87E-04	3.54
cystine	0.32	-1.64	4.35E-02	1.36
myo-inositol	0.32	-1.64	1.23E-03	2.91
cysteine	0.31	-1.69	2.05E-02	1.69
glycerol-alpha-phosphate	0.29	-1.77	9.08E-04	3.04
gluconic acid lactone	0.29	-1.77	2.87E-04	3.54
xylulose	0.26	-1.95	5.64E-04	3.25
hexitol	0.25	-1.98	2.87E-04	3.54
lactulose	0.22	-2.21	3.61E-03	2.44
phosphoethanolamine	0.21	-2.26	7.24E-05	4.14
fructose-6-phosphate	0.21	-2.28	7.93E-02	1.10
isohexonic acid	0.16	-2.66	9.08E-04	3.04
creatinine	0.14	-2.79	1.13E-03	2.95
putrescine	0.14	-2.85	8.02E-04	3.10
glucose-6-phosphate	0.13	-2.91	2.73E-02	1.56
adenosine-5-monophosphate	0.11	-3.17	9.08E-04	3.04

Table 6c: Metabolites differentially present in ASS1-Knockdown (KD) HK2 and control HK2 kidney epithelial cell lines as detected in the primary metabolite analysis

Metabolite name	FC	log2(FC)	p.adjusted	-LOG10(p)
3'-Deoxyadenosine	21.9	4.45	1.84E-04	3.73
Uridine	16.4	4.04	1.22E-05	4.91
Cytidine	12.1	3.59	8.72E-05	4.06
6-Methyladenosine	9.97	3.32	6.17E-05	4.21
Nicotinamide riboside	9.80	3.29	6.32E-04	3.20
Guanosine	5.33	2.41	6.17E-05	4.21
Adenosine	5.10	2.35	6.32E-04	3.20
Benzoic acid, 4-	3.48	1.80	2.28E-04	3.64
Guanine	3.02	1.59	3.88E-04	3.41
Galacto-N-biose	2.29	1.19	3.42E-04	3.47
Inosine	2.26	1.18	3.88E-04	3.41
Phenylacetylglycine;	2.19	1.13	3.98E-02	1.40
L-.gamma.-Glutamyl-L-glutamic acid	2.11	1.08	3.54E-02	1.45
beta-Alanine	0.49	-1.04	1.62E-03	2.79
N-Acetylmethionine	0.47	-1.08	2.68E-04	3.57
Carnitine	0.46	-1.13	3.07E-03	2.51
Acetylcarnitine	0.45	-1.14	1.24E-03	2.91
Isobutyryl-L-carnitine	0.45	-1.15	7.48E-03	2.13
2-Methylbutyryl-L-carnitine	0.45	-1.16	1.27E-02	1.90
Creatinine	0.43	-1.21	9.44E-03	2.03
Creatine	0.38	-1.38	2.31E-03	2.64
UMP	0.38	-1.41	8.43E-03	2.07
Inosine-5'-monophosphate	0.35	-1.52	1.34E-02	1.87
AMP	0.30	-1.76	9.49E-03	2.02
Miltefosine	0.24	-2.07	2.12E-05	4.67
Betaine	0.21	-2.23	6.17E-05	4.21
Lyso-sphingomyelin	0.19	-2.36	2.28E-04	3.64

Table 6d: Biogenic amines differentially present in ASS1-KD HK2 and control HK2

Metabolite name	FC	log2(FC)	p.adjusted	-LOG10(p)	Metabolite name	FC	log2(FC)	p.adjusted	-LOG10(p)
GlCer(d34:1)	6.72	2.75	2.16E-04	3.67	PC(p-36:4)/PC(o-36:5)	5.23E-04	-10.90	4.65E-08	7.33
LPC(14:0)	5.45	2.45	1.32E-05	4.88	PC(p-36:2)/PC(o-36:3)	8.97E-04	-10.14	8.82E-03	2.05
PC(28:0)	5.28	2.40	1.84E-07	6.73	PC(p-34:2)/PC(o-34:3)	1.07E-03	-9.87	5.88E-03	2.23
LPC(16:1)	4.58	2.20	3.42E-05	4.47	PC(38:7)	7.76E-03	-7.01	1.25E-07	6.90
TG(58:1)	4.42	2.14	2.31E-02	1.64	PC(40:8)	3.78E-02	-4.72	4.00E-04	3.40
TG(56:5) B	4.33	2.11	2.11E-02	1.68	TG(56:5) C	4.67E-02	-4.42	1.18E-03	2.93
PC 30:1	3.62	1.85	1.44E-06	5.84	PC(39:4)	5.19E-02	-4.27	1.98E-03	2.70
LPC(16:0)	3.55	1.83	4.90E-06	5.31	PC (p-40:7) or PC (o-40:8)	5.99E-02	-4.06	1.44E-06	5.84
PC (32:3)	3.51	1.81	1.17E-04	3.93	TG(55:2)	7.12E-02	-3.81	1.42E-04	3.85
LPC(18:1)	3.39	1.76	1.68E-05	4.77	SM(d41:1)	8.76E-02	-3.51	8.43E-03	2.07
PE 32:1e; PE 16:0e/16:1;	3.37	1.75	2.00E-06	5.70	TG(56:7) A	0.10	-3.28	8.36E-03	2.08
SM(d30:1)	3.27	1.71	1.44E-06	5.84	TG(54:5) B	0.11	-3.23	4.44E-03	2.35
TG(60:2)	3.19	1.67	7.56E-02	1.12	PC (39:6)	0.11	-3.18	8.36E-03	2.08
PC 36:4; PC 18:1-18:3;	3.11	1.64	1.14E-02	1.94	PC(p-40:6)/PC(o-40:7) A	0.13	-2.96	1.84E-07	6.73
LPC 20:1-SN2;	3.04	1.60	2.06E-06	5.69	CE(20:4)	0.15	-2.74	1.20E-04	3.92
TAG 48:0; TAG 14:0-16:0-18:0;	3.01	1.59	7.68E-03	2.12	TG(53:2)	0.17	-2.56	8.01E-05	4.10
DG(32:0)	3.01	1.59	3.14E-02	1.50	TG(53:1)	0.17	-2.53	1.45E-02	1.84
PE 32:2e; PE 16:0e/16:2;	2.94	1.55	1.44E-06	5.84	PC(40:7) B	0.18	-2.50	8.28E-07	6.08
PS 40:2;	2.91	1.54	1.78E-06	5.75	PC(p-38:5)/PC(o-38:6) B	0.20	-2.34	3.02E-04	3.52
1_ Cholesterol d7 STD	2.90	1.54	2.35E-02	1.63	PC(40:6) B	0.21	-2.27	3.43E-06	5.47
TAG 50:0; TAG 16:0-16:0-18:0;	2.84	1.51	3.36E-02	1.47	PC(p-38:5)/PC(o-38:6) A	0.21	-2.26	1.96E-07	6.71
TG(64:4)	2.83	1.50	8.39E-02	1.08	PS 40:5;	0.23	-2.12	2.13E-02	1.67
PE 34:1e; PE 18:0e/16:1;	2.79	1.48	4.09E-06	5.39	PC(p-38:6)/PC(o-38:7)	0.24	-2.08	8.85E-02	1.05
Lactosylceramide (d18:1/24:1(15:2))	2.76	1.47	4.43E-06	5.35	TG(53:3)	0.24	-2.08	3.53E-08	7.45
Cer(d34:1)	2.75	1.46	4.23E-04	3.37	CE(18:1)	0.27	-1.91	6.20E-04	3.21
PS 34:2;	2.68	1.42	4.43E-06	5.35	PC(38:6) B	0.27	-1.90	2.60E-04	3.58
PC 30:0; PC 14:0-16:0;	2.66	1.41	2.77E-04	3.56	PC(p-38:4)/PC(o-38:5) A	0.28	-1.84	3.86E-06	5.41
PE 34:3e; PE 18:1e/16:2;	2.66	1.41	1.23E-06	5.91	PC(35:2) A	0.28	-1.82	1.44E-06	5.84
PE(34:2)	2.62	1.39	1.44E-06	5.84	PC 36:7e;	0.30	-1.73	7.65E-03	2.12
PC 30:0e;	2.61	1.38	1.57E-05	4.80	TG(51:1)	0.31	-1.69	4.43E-06	5.35
PC 36:2;	2.59	1.37	1.15E-02	1.94	SM(d41:2) A	0.31	-1.68	4.15E-06	5.38
Cholesterol	2.58	1.37	2.09E-02	1.68	TAG 51:3; TAG 16:1-17:1-18:1;	0.32	-1.66	1.23E-06	5.91
GlCer(d42:1)	2.55	1.35	1.23E-04	3.91	TG(51:2)	0.32	-1.66	1.25E-07	6.90
PE 34:1; PE 16:0-18:1;	2.49	1.32	4.58E-06	5.34	PC(33:1)	0.33	-1.62	1.44E-06	5.84
PC(30:0)	2.49	1.31	8.25E-05	4.08	TG(55:3)	0.33	-1.62	1.76E-04	3.75
PC 34:4; PC 16:1-18:3;	2.48	1.31	9.11E-03	2.04	PC 40:6e;	0.33	-1.61	1.44E-06	5.84
PE 36:5e; PE 18:3e/18:2;	2.44	1.29	7.25E-05	4.14	SM(d38:2)	0.33	-1.59	8.86E-02	1.05
PS 36:2; PS 18:1-18:1;	2.43	1.28	3.45E-05	4.46	PC(p-40:5)/PC(o-40:6)	0.35	-1.52	4.90E-06	5.31
PS 40:1;	2.42	1.28	2.34E-06	5.63	PC(35:2) B	0.37	-1.45	1.74E-05	4.76
PC 36:5; PC 18:2-18:3;	2.37	1.25	1.12E-02	1.95	TAG 53:4;	0.38	-1.40	5.94E-05	4.23
PE 36:2; PE 18:1-18:1;	2.36	1.24	2.64E-05	4.58	PC(37:3)	0.38	-1.38	3.87E-05	4.41
SM d32:1;	2.34	1.23	1.40E-06	5.85	TG(58:6)	0.40	-1.32	1.39E-06	5.86
PC 44:2;	2.33	1.22	1.44E-06	5.84	PC (p-38:2) or PC (o-38:3)	0.40	-1.32	4.03E-04	3.39
PI 38:3;	2.33	1.22	3.28E-05	4.48	PC 37:2;	0.41	-1.27	2.00E-06	5.70
TG(46:0)	2.32	1.22	2.64E-05	4.58	TAG 58:7; TAG 18:1-18:1-22:5;	0.41	-1.27	2.53E-04	3.60
PE(p-34:1)/PE(o-34:2)	2.32	1.21	1.04E-06	5.98	PC(42:5)	0.43	-1.23	1.86E-04	3.73
TG(64:2)	2.31	1.21	3.12E-05	4.51	SM(d33:1)	0.43	-1.21	1.38E-05	4.86
PC 32:1; PC 16:0-16:1;	2.29	1.20	6.88E-05	4.16	SM(d40:0)	0.44	-1.20	4.30E-02	1.37
PE 38:5e; PE 18:1e/20:4;	2.28	1.19	1.23E-06	5.91	PC(38:4) A	0.44	-1.20	1.33E-05	4.88
PE 36:3e; PE 18:1e/18:2;	2.26	1.18	9.10E-07	6.04	SM(d40:2) B	0.44	-1.18	3.43E-06	5.47
PE(p-36:1)/PE(o-36:2)	2.26	1.17	1.23E-06	5.91	PC(35:1)	0.45	-1.15	8.97E-07	6.05
1_ LPC(17:0) STD	2.12	1.08	2.43E-02	1.61	PE 38:5;	0.46	-1.11	5.59E-06	5.25
PC 34:2; PC 16:1-18:1;	2.10	1.07	9.94E-05	4.00	PC(p-40:6)/PC(o-40:7) B	0.46	-1.11	1.02E-02	1.99
1_ Cer(d18:1/17:0) STD	2.09	1.06	7.30E-03	2.14	PC(40:6) A	0.47	-1.09	1.40E-06	5.85
GlCer(d42:2)	2.06	1.04	2.36E-03	2.63	TAG 49:2; TAG 15:0-16:1-18:1;	0.47	-1.09	3.86E-06	5.41
PC 42:1;	2.06	1.04	6.88E-05	4.16	PC (p-42:3) or PC (o-42:4)	0.47	-1.09	1.03E-03	2.99
PC 34:1;	2.05	1.03	1.91E-03	2.72	SM d43:2;	0.48	-1.05	5.75E-03	2.24
TAG 44:1; TAG 12:0-14:0-18:1;	2.04	1.03	2.88E-06	5.54	PC(38:5) A	0.49	-1.04	2.17E-06	5.66
1_ TG d5(17:0/17:1/17:0) STD;	2.03	1.02	6.35E-02	1.20	TG(56:6)	0.49	-1.02	2.17E-06	5.66
SM d34:1; SM d18:1/16:0;	2.02	1.02	1.27E-03	2.90	TG(49:1)	0.50	-1.01	1.97E-05	4.71

Table 6e: Lipidomic analysis of ASS1-KD HK2 and control HK2

Metabolites	FC	log2(FC)	p.adjusted	-log10(p)
cholecalciferol	1.66	0.73	9.28E-03	2.03
5,6-dihydrouracil	1.57	0.65	2.16E-02	1.67
galactinol	1.49	0.58	6.08E-02	1.22
glycerol-3-galactoside	1.44	0.53	6.83E-02	1.17
1,3-dihydroxypyridine	1.27	0.34	6.08E-02	1.22
1-monopalmitin	1.23	0.30	7.39E-02	1.13
malic acid	0.77	-0.38	8.58E-02	1.07
threonine	0.77	-0.38	9.93E-02	1.00
putrescine	0.71	-0.49	6.83E-02	1.17
sorbitol	0.69	-0.53	6.83E-02	1.17
octadecylglycerol	0.68	-0.56	3.09E-02	1.51
beta-alanine	0.67	-0.57	6.83E-02	1.17
ribitol	0.66	-0.59	6.83E-02	1.17
cystine	0.65	-0.62	6.83E-02	1.17
asparagine	0.63	-0.67	6.83E-02	1.17
lyxitol	0.63	-0.68	4.18E-03	2.38
isocitric acid	0.63	-0.68	6.20E-02	1.21
trans-4-hydroxy-L-proline	0.58	-0.78	6.83E-02	1.17
cyanoalanine	0.57	-0.81	6.08E-02	1.22
alanine	0.48	-1.05	2.13E-03	2.67
hypoxanthine	0.48	-1.06	7.39E-02	1.13

Table 6f: Primary metabolite analysis of ASS1-786O and control 786O grown in low-nutrient media

CHAPTER 5

Novel Immunohistochemical classification of clear cell renal cell carcinoma exploiting the grade-dependent metabolic reprogramming by retargeting of moonlighting proteins

Abstract

Cancer cells utilize moonlighting or neomorphic capacity of metabolic enzymes and their interacting proteins to reprogram metabolism. Subcellular retargeting of those enzymes often accompanies such reprogramming. Since clear cell renal cell carcinoma (ccRCC) changes their proteomic landscape according to their Fuhrman nuclear grades, immunochemistry was used in this study to interrogate whether such retargeting occurs in a grade-defined manner by evaluating PGK1, ENO1/MBP1, ALDOA, ALDH1A1, CNDP2 and NDRG1 immunoreactivity, discovered using non-targeted proteomic analysis. Our data show that these proteins undergo grade-dependent subcellular retargeting, which can be used to more precisely and objectively define ccRCC nuclear grades. Based on the dynamic redistribution of these proteins, we propose a mechanistic model of ccRCC subtypes as defined by the Fuhrman nuclear grades. Furthermore, we show that immunohistochemical approach can elucidate tumor heterogeneity. Together, this study indicates that immunohistochemistry of grade-delineating proteins is a powerful tool to more precisely and objectively define ccRCC nuclear grades.

Introduction

Renal cell carcinoma (RCC) is the 6th most common cancer amongst men and 10th amongst

women with over 65,000 estimated cases overall, and it result in 15,000 estimated deaths in the US annually, [1 – 3] and more than 140,000 deaths worldwide. [1] RCC can be classified into several subtypes including clear cell RCC (ccRCC), papillary RCC, and chromophobe RCC (chRCC) based upon histological characteristics as revealed by conventional staining. [4, 5] ccRCC consists of 75% of the entire RCC incidence, and exhibits its characteristic clear cytoplasm due to accumulation of cytoplasmic lipid and polysaccharide content. [6]

While localized ccRCC responds well to surgical intervention, metastasized ccRCC presents clinical challenges due to its refractory nature to conventional chemotherapy. Thus, early detection and intervention is paramount. Once detected, the tumor conventionally is graded using Fuhrman nuclear grade [7] in order to assist further prognostic assessments. The Fuhrman grading classifies ccRCC into four grades based upon size and shape of nucleus as well as presence and size of the nucleoli after hematoxylin and eosin counterstain. It is a diagnostic criterion that employs Hematoxylin and Eosin (H&E), a simple and inexpensive staining procedure, and thus has been widely adopted and significantly contributed to the advancement and standardization of ccRCC diagnosis. More recent efforts developed modified criteria for RCC grading including 2004 WHO classification [8] and 2012 WHO/ISUP grading. [9] They are, however, solely based upon microscopic observation of simple morphological criteria including the presence and size as well as shape of nucleus and nucleolus, thus leaving the room for diagnostic error and variation amongst pathologists.

Omics is a relatively novel yet rapidly growing field of research that aims to comprehensively characterize a biological system or “ome”. [10] Biological systems are composed of complex mixtures of biomolecules with a vast array of molecules present in a wide dynamic range, and their comprehensive detection and identification rely on technological advances on sensitivity and databases. Over the past two decades, our group and others have applied the technology to characterize the cancer omes. In particular, proteo-metabolomic approaches were used to identify protein and metabolites that can serve as grade- or stage-specific RCC biomarkers [11 –

17] These approaches, however, regarded tissues as a homogeneous entity and sought to identify biomarkers that discriminate between normal and cancer tissues or between two or more Fuhrman grades and corresponding normal tissues. For decades, it was known that some proteins, and in particular metabolic enzymes, can relocate to a non-canonical subcellular location to perform neomorphic functions unrelated to its originally ascribed biological function. [18, 19] Recent studies indicated that this phenomenon is widespread and many metabolic enzymes are localized at two or more distinct subcellular compartments. [20, 21] This suggests that such retargeting reflects metabolic reprogramming and may form a basis for cancer progression.

ccRCC is often clinically classified using nuclear grades as well as the stages. Fuhrman nuclear grading is the most widely used nuclear grading scheme for ccRCC, yet its subjective nature as well as ambiguous boundary criteria has found many clinicians and researchers wanting. Furthermore, conventional chemotherapy has been ineffective against ccRCC, leaving the need to identify additional druggable targets and to develop novel chemotherapeutic agents. In order to address these issues, we have previously performed grade-defined proteomics. [11] The study identified up to 180 proteins whose expression level can be used as biomarkers to distinguish between normal tissues, low grade (Grade 1 (G1) and Grade 2 (G2)) ccRCC, and high grade (Grade 3 (G3) and Grade 4 (G4)) ccRCC. The 180 proteins encompass metabolic enzymes, cytoskeletal elements, extracellular matrix components, signal transduction machinery, proteins chaperones, adaptor proteins, and nuclear proteins.

Here we present a further analysis of a subset of these grade-defining proteins which show subcellular re-targeting depending on the grades. Many of these proteins have previously been identified as moonlighting proteins, and their moonlighting functions suggests that exploitation of such neomorphic functions by the ccRCC may contribute to ccRCC pathogenesis. Furthermore, in combination with the availability of specific monoclonal antibodies against these biomarker proteins, this study forms a basis for more precise ccRCC grading.

We report that metabolic enzymes are targeted to distinct compartments depending on ccRCC Fuhrman nuclear grades, and propose a novel ccRCC grading scheme based upon

conventional immunohistochemistry. This combinatorial scheme successfully distinguishes the four Fuhrman grades based upon presence or absence of a particular antigen in conjunction with their subcellular localization. Their subcellular localization is often distinct between grades and can be unequivocally determined, thus reducing the personal errors and bias in ccRCC grading and diagnosis. Notably, majority of the antibodies used in this study are monoclonal and commercially available, thus allowing standardized implementation worldwide. While the ENO1/MBP1 and anti-CNDP2 antibodies used in this study are polyclonal, their monoclonal successors have since been developed. Furthermore, the new immunochemistry (IHC)-based grading identifies re-targeting of metabolic enzymes, and proposes new mechanisms in ccRCC progression.

Materials and Methods

After appropriate Institutional Review Board approval from University of California Davis, formalin-fixed paraffin-embedded (FFPE) clear cell renal cell carcinoma (ccRCC) tissues were obtained from the University of California Davis Medical Center in Sacramento.

Following antibodies were used in this study; anti-PGK1/2 antibody (Santa Cruz Biotechnology), anti-ALDH1A1 (Abcam), and anti-CNDP2 (AbNova), anti-NDRG1 (Proteintech), anti-ENO1/MBP1(Abcam), and anti-ALDOA (Abnova).

Haematoxylin and eosin staining were done according to a standard protocol [22] as previously described. [9]

Immunohistochemistry was done as previously described [9] Briefly, FFPE slides were deparaffinized through a xylene and ethanol series and were treated with 3% hydrogen peroxide/methanol prior to the antigen retrieval in sodium citrate buffer, pH 6 in order to inactivate endogenous peroxidases. Following blocking, the slides were incubated with the primary antibodies at 4°C overnight. After PBS washes, the slides were incubated with biotin-conjugated secondary antibodies followed by the avidin-biotin complex (ABC Elite kit, Vector Laboratories) according to the manufacturer's instructions. The avidin-biotin complex was visualized using diaminobenzidine (Vector Laboratories). The sections were counterstained with hematoxylin and were coverslipped. Photographs were taken using 20× objective lenses on an Axiovert microscope (Carl Zeiss).

Results

Immunohistochemical analysis was performed against several protein biomarkers identified in our grade-defined proteomic analysis. [9] These biomarker proteins include Phosphoglycerate Kinase 1(PGK1), Alcohol Dehydrogenase 1 A1 (ALDH1A1), Cytosolic nonspecific dipeptidase (CNDP2), N-Myc Downregulated Gene 1 (NDRG1), Enolase 1 (ENO1) /Myc-Binding Protein 1 (MBP1), and Aldolase A (ALDOA).

Phosphoglycerate Kinase 1 (PGK1)

PGK1 is a ubiquitously expressed glycolytic enzyme that converts 1,3-bisphosphoglycerate into 3-phosphoglycerate and concomitantly generates ATP from ADP. Although this reaction is catalyzed exclusively in the cytoplasm, the protein has previously been detected in the nucleus where, in conjunction with Annexin II, [23] it acts as a subunit of primer recognition protein, [24] and modulates DNA polymerase activities. [25, 26] In addition, colon cancer secretes PGK1 as a proangiogenic factor *in vitro*. [27] Finally, in glioblastoma under hypoxia and ERK pathway activation, PGK1 translocates to mitochondria and acts as a protein kinase that phosphorylates pyruvate dehydrogenase kinase 1 (PDHK1) which in turn phosphorylates and inhibits pyruvate

dehydrogenase (PDH), thus inhibiting mitochondrial conversion of pyruvate to acetyl-CoA. [28]

In the present study, PGK1 immunoreactivity was seen in tubule cytosol with occasional perinuclear staining in the normal kidneys. **(Figure 27)** In addition, glomeruli and Bowman's capsules were moderately PGK1-positive. Majority of nuclei in G1 ccRCC tissues were intensely PGK1-positive. **(Figure 27)** In contrast, majority of grade 1 cells had the cytosol that was largely devoid of PGK1 immunoreactivity, although some cytoplasm stained weakly positive for PGK1. **(Figure 27)** G2 tissues exhibited intensely PGK1 positive nuclei with sparse cytosolic staining. **(Figure 27)** In contrast, G4 tissues were largely devoid of PGK1 staining with less than 10% of the cells being PGK1-positive. **(Figure 27)** The PGK1 immunoreactivity was confined to cytosol within the PGK1-positive G4 cells. The overall percentage of PGK1-positive cells were higher in low grade (G1-G2) cells and lower in the G4 with the normal kidney tissue being moderately PGK1-positive, which correlates with our earlier proteomic analysis [9]

Alcohol Dehydrogenase 1 A1 (ALDH1A1)

ALDH1A1 is a cytoplasmic component of retinoid acid metabolic pathway and oxidises retinal into retinoic acid. [29] In several cancers, it serves as a cancer stem cell marker. [30, 31] While no distinct function for nuclear ALDH1A1 has been reported, its nuclear localization is associated with oxidative stress in astrocytes. [32]

ALDH1A1 is broadly expressed in renal tubule epithelial cell cytoplasm while their nuclei are virtually devoid of the signal. **(Fig. 28)** The cytoplasmic staining is distributed in a punctate manner, suggesting its presence in an organelle such as mitochondria. Bowman's capsule as well as a subset of glomerular endothelial cells are also ALDH1A1-positive. In G1 tissue, majority of the cytoplasm is ALDH1A1-negative while the immunoreactivity is associated with cell membrane. A minority subset of nuclei is also ALDH1A1-positive. Although a small subset of cells possesses ALDH1A1-positive cytoplasm, the staining is more diffuse rather than

punctate. G2 tissues show a similar but more pronounced staining pattern to that of G1 tissues with immunoreactivity associated with nuclei and cell membrane. In contrast, G3 tissues show minimal staining in the nuclei and most of the immunoreactivity is associated with cell membrane, although a subset of cells is seen with diffuse cytoplasmic staining. Finally, G4 tissues exhibit the lowest immunoreactivity of all the grades.

Cytosolic nonspecific dipeptidase (CNDP2)

CNDP2 is a dipeptidase that was originally identified to hydrolyze L-carnosine into β -alanine and L-histidine. [33] It has since been discovered to possess broader substrate specificity with preference for Cys-Gly dipeptide, thus catalyzing the last step of glutathione degradation. [34] In addition, it reverse proteolyzes N-lactoyl-amino acids from lactate and amino acids. [35] Down-regulation of CNDP2 in gastric cancer contributes to increased cell proliferation and inhibition of apoptosis and cell cycle arrest. [36] Concomitantly, CNDP2 up regulates p38 expression and JNK activation. [36] In contrast, CNDP2 inactivation resulted in ERK activation. [36]

Moderate CNDP2 immunoreactivity was detected in the cytosol of normal kidney proximal tubule epithelium. (**Fig. 29**) In contrast, normal tubule nuclei were virtually CNDP2-negative. Distal tubules exhibited more reduced immunoreactivity. Additionally, there are some CNDP2-positive cells in the glomeruli, which may correspond to podocytes or a subset of cells belonging to the Bowman's capsule. In the G1 tissues, CNDP2 is detected in the majority of nuclei as well as in a small fraction of cytoplasm. It should be noted that majority of CNDP2-positive nuclei are surrounded by CNDP2-negative nuclei. G2 ccRCC cells exhibit CNDP2-positive nuclei and cell membrane while the majority of cells contain CNDP2-negative cytoplasm. In contrast, in G3 cells, nuclei are nearly completely negative. The overall immunoreactivity is reduced in G3 compared to G1 and G2. G4 tissues exhibit further diminished CNDP2 staining in their sarcomatous cytoplasm, which supports our earlier proteomic study, [11] along with the sarcomatous morphology, which is in agreement with the

report by Fuhrman and others. [7]

N-Myc Downregulated Gene 1 (NDRG1)

NDRG1/Cap43 is a transcriptional target of N-MYC and cMYC as well as p53 [37, 38] and retinoic acid signalling. [39] It acts as a tumor metastasis suppressor [40, 41] and an autophagy suppressor. [42] An interactome analysis suggested an extensive network of interacting partners and supported its pleiotropic functions, [43, 44] including E-Cadherin recycling [45] and RA-induced differentiation of leukemic cells via C/EBP β . [46] Additionally, NDRG1 downregulates NF- κ B expression, thus resulting in suppressed angiogenesis and tumor growth in pancreatic cancer. [47]

Our immunohistochemical study shows that NDRG1 is detected in two subcellular compartments in normal proximal tubule epithelial cells. (**Fig. 30**) First, it is concentrated along the basolateral membrane, although it is absent from the apical membrane. The second pool of NDRG1 is distributed broadly throughout the cytoplasm. There is a large variation in cytoplasmic NDRG1 staining. G1 tissue loses the basolateral membrane-associated pool, and instead is uniformly membranous in a subset of cells, although the signal is weaker than the basolateral membrane of the normal epithelial cells. Additionally, a minority subset of G1 nuclei is NDRG1-positive. A large fraction of G2 cells contain NDRG1-positive nuclei along with NDRG1-positive membrane. In addition, NDRG1 immunoreactivity is present in cytoplasm in a reticular manner. G3 ccRCC cells show less NDRG1 immunoreactivity than their G2 counterpart. In particular, the G3 nuclei are essentially devoid of NDRG1, and the protein is present in the cytosol of a small subset of cells in a perinuclear manner. G4 tissues also show diminished NDRG1 expression. The immunoreactivity is present at a reduced level in the cytoplasm of a small subset of cells.

Enolase 1 (ENO1) /Myc-Binding Protein 1 (MBP1)

ENO1/MBP1 are two distinct in-frame polypeptides translated from two distinct ATG start codons on a single mRNA. That is, MBP1 is an N-terminus-truncated form of ENO1. ENO1 is a glycolytic enzyme present in the cytoplasm, and catalyzes the conversion of 2-phosphoglycerate into phosphoenolpyruvate. [48] In addition, it acts as a plasminogen receptor on the cell surface. [49] Furthermore, it is a hypoxia protein upregulated in response to hypoxic stress in endothelial cells. [50] At least in yeast, it also acts as a heat shock protein. [51] In contrast, MBP1 is a nuclear protein without catalytic activity. It acts as a transcriptional repressor, which binds to the cMYC promoter and represses its transcription. [52] While the translational control of MBP1 protein synthesis is not yet fully understood, it is down regulated by microRNA-363 in breast cancer and gastric cancer. [53, 54]

In normal kidney epithelial cells, ENO1/MBP1 is present throughout the cytoplasm and a large fraction of nuclei of proximal tubule epithelial cells as well as in the bowman's capsule of the glomeruli. (**Fig. 31**) A small subset of glomerular endothelial cells also stained positive for ENO1/MBP1. G1 tissues are characterized with strong nuclear staining and moderate cell membrane staining while the majority of cells exhibit ENO1/MBP1-negative or weakly-positive cytoplasm. (**Fig. 31**) A similar pattern to G1 tissues is seen in the G2 tissue stained against ENO1/MBP1 with majority of the signal being present in the nucleus and cell membrane, although the signal is more diffuse than that seen in G1 tissue. (**Fig. 31**) The G3 tissues show far fewer signals in the nucleus while the cell membrane remains moderately positive for ENO1/MBP1. (**Fig. 31**) There is more heterogeneity in the G3 tissues compared with lower grades with approximately half of the cells exhibiting negative or only weakly positive staining for ENO1/MBP1. Notably, majority of the nuclei are ENO1/MBP1 negative. G4 ccRCC tissues show even more pronounced heterogeneity. Some cells contain large ENO1/MBP1 negative nucleus with ENO1/MBP1 positive cytoplasm, while others contain weakly positive nuclei and sarcomatous cytoplasm with varying degree of ENO1/MBP1 immunoreactivity. (**Fig. 31**) These observations correlate well with the normalized proteomic spectral count for ENO1/MBP1 obtained from the previous grade-defined ccRCC proteomics. (**Fig. 31** and [11])

Aldolase A (ALDOA)

ALDOA is a glycolytic enzyme belonging to aldolases family, which include three isozymes, A, B, and C. ALDOA catalyze the cleavage of fructose-1,6-bisphosphate into dihydroxyacetone-phosphate and glyceralate-3-phosphate. As a moonlighting protein, ALDOA has several non-catalytic functions. In cytoplasm, ALDOA also functions as a WASP-binding protein. [55] Second, through eventual lactate production under anaerobic conditions, ALDOA inhibits PHD hydroxylase, thus stabilizing HIF α protein and increasing its transcriptional activity. [56] In non-small cell lung cancer cells *in vitro*, ALDOA knockdown concomitantly inhibited proliferation, MAPKK1/2 pathway activation, and reduced nuclear localization of pyruvate kinase M2 (PKM2). [57] It correlated with cell proliferation in squamous lung cancer cell *in vitro*, [58] and abrogation of the moonlighting function leads to apoptosis. [59]

ALDOA immunoreactivity in normal kidney tubule epithelium exhibits mainly cytosolic distribution in a granular manner. The signal is more concentrated around nucleus. (**Fig. 32**) Within glomeruli, some endothelial cells are moderately positive while the Bowman's capsule is devoid of immunoreactivity. In contrast, in G1 tissues, ALDOA immunoreactivity is largely seen in a subset of nuclei. The ALDOA-positive nuclei are smaller and rounder than the ALDOA-negative nuclei, which are larger and exhibit more irregular outline. In G2 tissues, ALDOA reactivity is present mostly in the cell membrane with additional perinuclear staining. G3 tissues show cytosolic and perinuclear staining. The cytosolic staining often exhibits fibrous rather than diffuse appearance. In G4, ALDOA staining is largely cytosolic and delineates the sarcomatous morphology. There is some nuclear staining, which seems to localize to sub-nuclear organ such as nucleoli.

Based upon our grade-defined immunohistochemical study combined as described above with functional studies by others using various systems, we propose the following model of ccRCC

pathobiology as summarised in **Fig. 33**.

Discussion

Renal cell carcinoma (RCC) is the 7th common malignancy worldwide of which over 90% is of clear cell type. [1] Due to *VHL* inactivation and the resultant hypervascularization and ineffective apoptotic induction, ccRCC are often refractory to conventional chemotherapy. Thus, early diagnosis with surgical intervention is currently the most effective treatment.

Conventionally, Fuhrman nuclear grading is used to assess, diagnose, and prognose ccRCC patients. While it has seen successes, its subjective nature and crude criteria have been major issues against unequivocal assignment of the nuclear grades. This, however, presented opportunities to the omic era that enabled simultaneous identification of multiple druggable targets.

In this study we interrogated subcellular localization of a subset of previously identified grade-defining biomarkers [11] in order to test the hypothesis that change in subcellular localization accompanies change in Fuhrman grades. It demonstrates that subcellular localization of the grade-defining biomarkers indeed differs between grades, suggests that protein moonlighting and thus offers a more accurate surrogate for the conventional Fuhrman grading.

Protein moonlighting is defined as non-canonical functions of proteins, [19, 60] a well-documented and widespread phenomenon that is employed by a number of enzymes including most of those that are classically considered glycolytic or tricarboxylic acid (TCA) cycle enzymes

[18, 61 – 65] While a subset of these enzymes perform the identical or a similar biochemical function at the novel location upon their subcellular re-targeting, many of them exhibit unexpected and unrelated biochemical or cell biological activities that are unrelated to their canonical enzymatic function. For example, PGK1 was originally discovered as a cytosolic glycolytic enzyme, [66] while it functions as a subunit of the primer recognition particle in the nucleus [24] and as a protein kinase in the mitochondria. [28] Thus, the observed translocation of metabolic enzymes between grades as described above has the potential to not only activate dormant pathway or inactivate pre-existing pathway, but also create a neomorphic cancer specific pathways. That is, in addition to providing a new grading scheme, distinct subcellular localization across grades reflects grade-specific fundamental pathobiology.

The expression and subcellular localization of the following enzymes were studied; Phosphoglycerate Kinase 1 (PGK1), Aldehyde Dehydrogenase 1 Family Member A1 (ALDH1A1), Carnosinase-like Non-specific Dipeptidase 2 (CNDP2), Enolase 1 / c-Myc Binding Protein 1 (ENO1/MBP1), and Aldolase A (ALDOA). (**Figure 33**)

In normal kidneys, PGK1, and CNDP2 and ALDOA was distributed solely in the cytoplasm of renal tubule epithelial cells. PGK1 and CNDP2 staining was diffuse while ALDOA showed more granular distribution. NDRG1 was detected in the basolateral membrane as well as throughout the cytoplasm. ENO1/MBP1 was positive in all the cytosol and in a subset of nuclei. ALDH1A1 distribution was also cytosolic, although it exhibited more punctate staining, indicating potential association with an organelle. The cytoplasmic distribution of PGK1, ALDOA, and ENO1 suggests that, in the normal kidney epithelial cells, these enzymes primarily perform their canonical functions as glycolytic enzymes. The nuclear staining from ENO1/MBP1 IHC likely derives from MBP1, which is predicted to downregulated cMYC expression. The punctate cytoplasmic staining of ALDH1A1, an enzyme which oxidizes retinaldehyde into RA suggests importance of localized RA signaling in normal kidney epithelium. Membrane-associated NDRG1 immunoreactivity suggests its potential association with E-cadherin to maintain

epithelial integrity.

In grade 1 ccRCC, in addition to MBP1, four proteins including ALDOA, ALDH1A1, PGK1 and CNDP2, are detected in the nucleus. These changes suggest increased proliferation, localized nuclear RA production, DNA synthesis upregulation via increased primer recognition, nuclear glutathione degradation, respectively. Specifically, PGK1 immunoreactivity was present largely in the nuclei with a minor cytoplasmic staining. ALDH1A1 is mostly associated with cell membrane in G1 and a subset of nuclei and cytoplasm is ALDH1A1-positive. CNDP2 immunoreactivity is detected in the majority of the nuclei and a small subset of cytoplasm, although the CNDP2-positive nuclei are usually associated with CNDP2-negative cytoplasm. NDRG1 was present in a subset of nuclei as well as in the cytoplasm and the cell membrane, although the latter had lost apico-basal patterning and NDRG1 immunoreactivity is distributed uniformly along the membrane. ENO1/MBP1 exhibits more pronounced nuclei staining than in the normal epithelial cells, while the cytoplasmic staining is either absent or markedly reduced in G1. Finally, ALDOA is present mostly in a subset of nuclei. It should be noted that G1 tissue presents mixture of ALDOA-positive and –negative nuclei, with ALDOA-positive nuclei exhibiting smaller and rounder morphology.

G2 tissues exhibit a similar distribution to G1 with the exception of the presence of cytosolic ALDOA expression. G2 ccRCC is composed of a large fraction of intensely PGK1-positive nuclei with minimal cytosolic PGK1. The nuclear PGK1 signal intensity seems higher in the G2 tissue than in the G1 tissue. ALDH1A1 expression was associated with cell membrane and the nucleus. Analogous to PGK1, the pattern was similar to but more pronounced than in G1. Similar to the G1 tissues, CNDP2 immunoreactivity was present in the nucleus and cell membrane, while the cytoplasm was mostly negative for CNDP2. G2 tissues contained higher percentage of NDRG1-positive nuclei while sharing the cytosolic and uniform membranous distribution with the G1 tissues. ENO1/MBP1 was detected in the cell membrane and the nucleus. ALDOA signal was mostly distributed in the cell membrane and cytosol with minor perinuclear distribution. While

the overall pattern of grade 2 ccRCC immunoreactivity is qualitatively similar to that of Grade 1 ccRCC, the semi-quantitative parameters (*i.e.*, signal intensities) are more pronounced. This is in agreement with the notion that cMYC, which was identified as one of the hidden nodes in the earlier grade-defined ccRCC proteomic analysis, [Perroud et al. (2009)] functions as an amplifier of already active genes rather than as a classical on-off switch. [67 – 69]

G3 ccRCC differs from the lower ccRCC grades and the normal tissue in that most of the protein tested loses their nuclear localization. Specifically, ALDOA, ALDH1A1, PGK1, and CNDP2 are solely cytosolic. In contrast, MBP1 is present in the nuclei in a punctate staining, suggesting its potential nucleolar localization. The nucleus is largely devoid of ALDH1A1 staining, and the immunoreactivity is instead associated with the cell membrane. In addition, a small subset of cells shows ALDH1A1 staining in the cytoplasm. Similarly, the nuclei are largely negative for CNDP2 staining, which is associated with cell membrane and a subset of cytoplasm. In contrast to the lower grades, grade 3 nuclei are negative for NDRG1 staining. The overall NDRG1 staining is weaker than grade 1 and 2, and is seen in the cytoplasm of a small subset of cells as well as in their cell membrane. Reduction in NDRG1 level may reprogramme metabolism via its interacting partners, although the mechanistic details remain to be elucidated. ENO1/MBP1 staining is associated with a subset of cytoplasm, while the nucleus is ENO1/MBP1-negative. Unlike in G2 tissues, ALDOA staining in G3 tissue is mostly cytosolic with fibrous appearance, suggesting its interaction with cytoskeleton via WASP, which inactivates its catalytic activity. [55] In addition, there is a perinuclear ALDOA staining.

Finally, G4 tissues share similar immunohistochemical patterns to those of the G3 tissues with the following differences. The cytoplasmic ALDOA is distributed in a diffuse pattern, suggesting de-inhibition of its catalytic activity. In addition, NDRG1 is present in the nucleus in addition to the cytoplasm and nucleoli show MBP1-immunoreactivity. PGK1 staining was generally low with occasional cytosolic staining. ALDH1A1 was similarly lowest in its expression in the G4 tissues compared to other grades. CNDP2 expression is confined to cytoplasm and shows a

similar reduction in expression to that of ALDH1A1 compared to those in the lower grades. NDRG1 expression is further reduced and is highly heterogeneous with a subset of cells exhibiting weakly NDRG1-positive cytoplasm and nucleus. ENO1/MBP1 immunoreactivity is present in the cytoplasm of sarcomatous cells whose nuclei are ENO1/MBP1-negative, while a subset of cells contains smaller ENO1/MBP1-positive nuclei despite sharing the sarcomatous cytoplasm.

By employing conventional immunohistochemistry, we have reported that a number of ccRCC grade-defining proteins show distinct subcellular localization as summarized below, which may in part underlie previously reported metabolic reprogramming. [12] A number of metabolic enzymes have been shown to have one or more moonlighting functions. [61] Many of these moonlighting enzymes exhibited grade-dependent changes in protein expression levels, [11] although they were not included in the current immunohistochemical study. It is expected that several of these biomarker proteins also will exhibit grade-dependent changes in sub-cellular localization, which in turn is instructive in ccRCC progression.

In summary, this immunohistochemical study demonstrates that retargeting of metabolic enzyme occurs in ccRCC across the four Fuhrman grades in comparison to the normal kidney proximal tubule epithelial cells, thus suggesting that protein moonlighting is an underlying mechanism of ccRCC grade progression, wherein the cancer cells repurpose and utilize metabolic enzymes and non-enzymatic proteins in a neomorphic manner. This can be used to reinterpret and refine the Fuhrman nuclear grading for more precise diagnosis and to devise novel pharmaceutical interventions.

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Figures

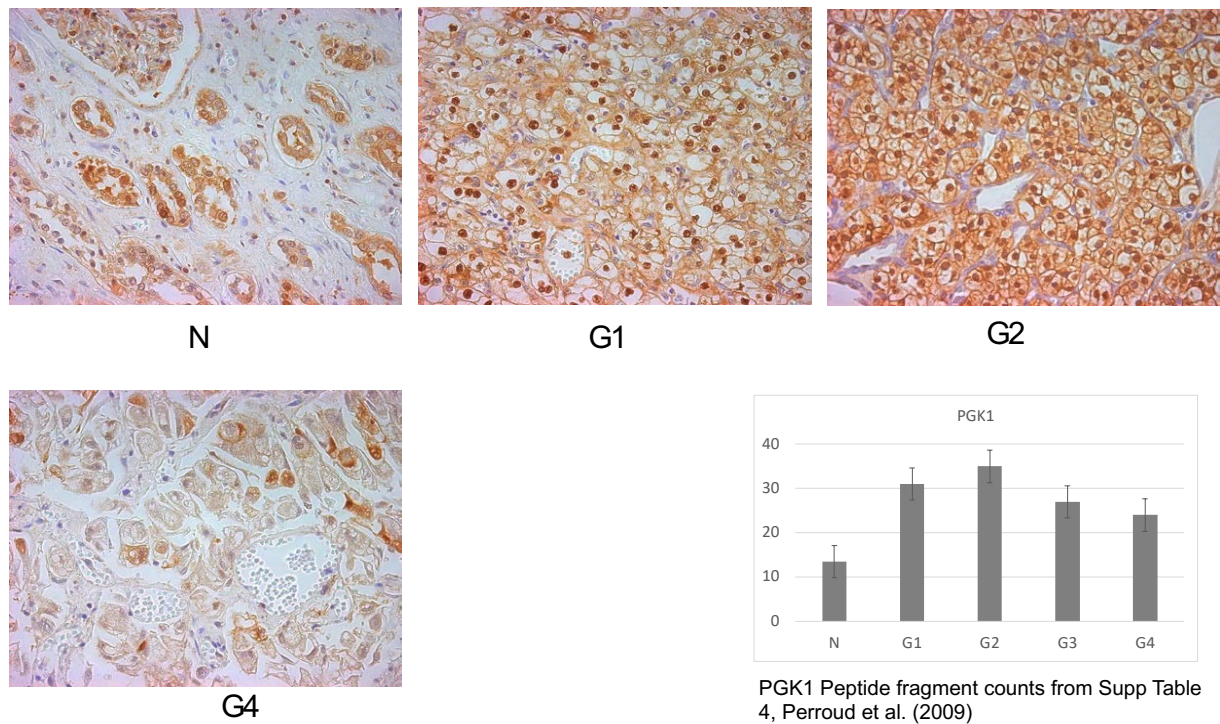


Fig.27: Phosphoglycerate Kinase 1 (PGK1) immunohistochemistry on ccRCC FFPE sections.

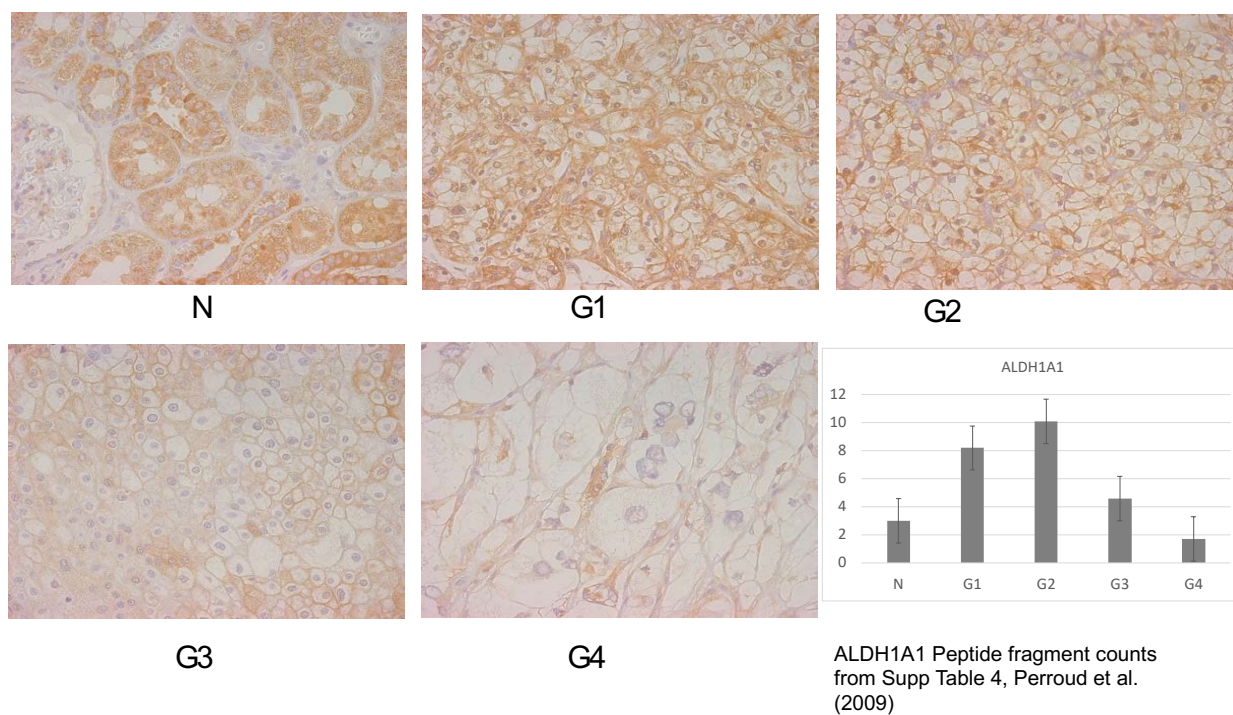


Fig.28: ALDH1A1 immunoistochemistry on ccRCC FFPE sections

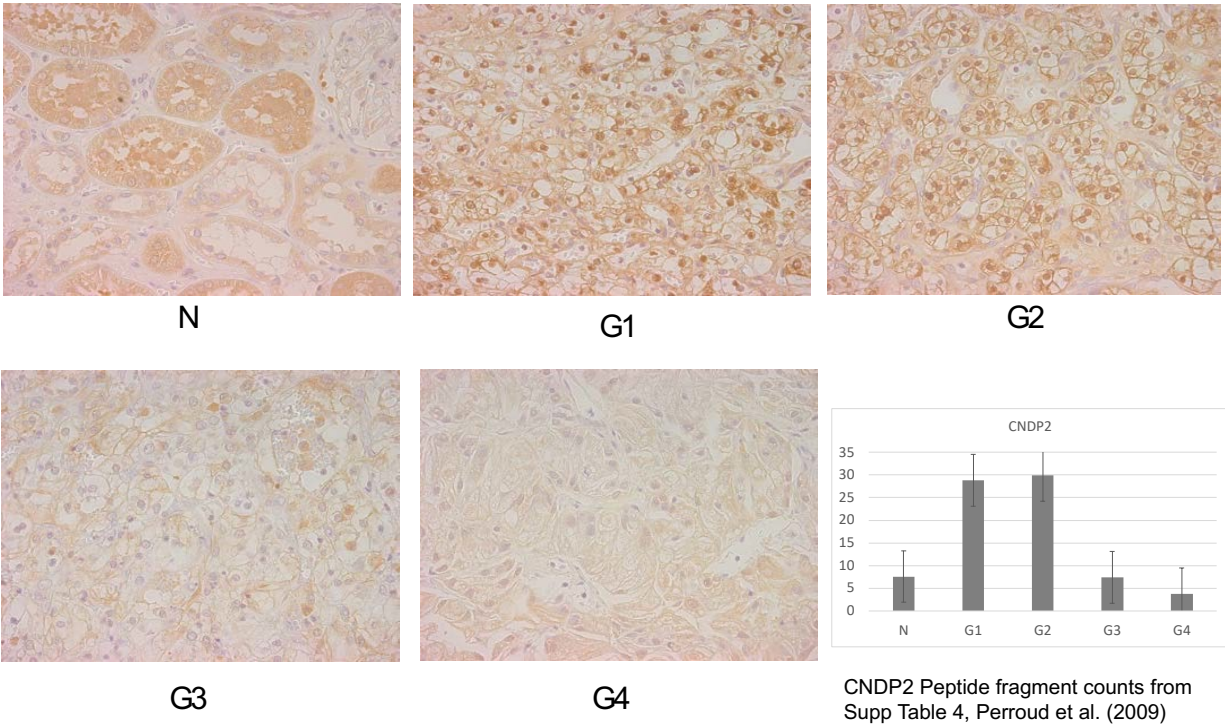
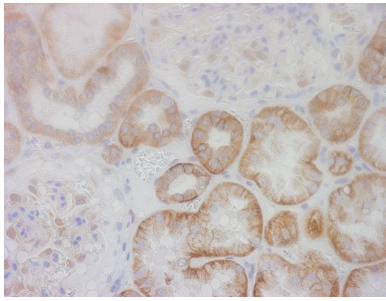
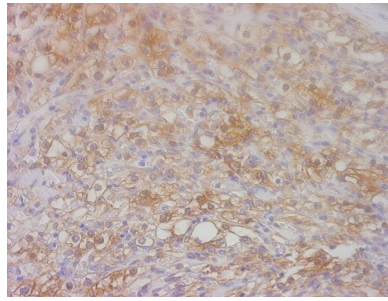


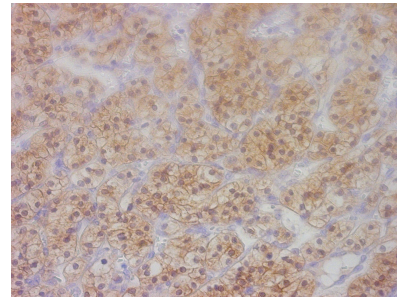
Fig.29: Cytosolic Nonspecific Dipeptidase 2 (CNDP2) immunohistochemistry on ccRCC FFPE sections



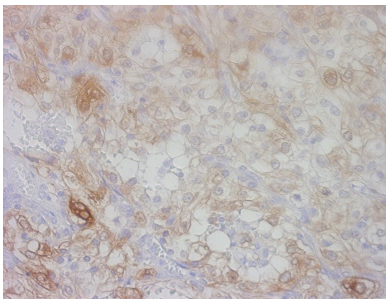
N



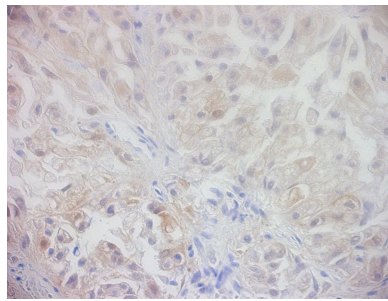
G1



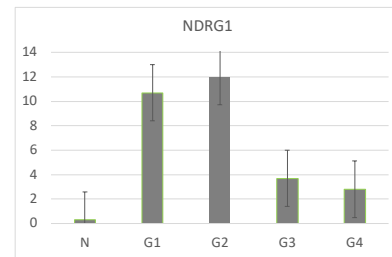
G2



G3

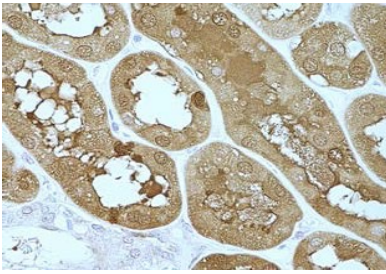


G4

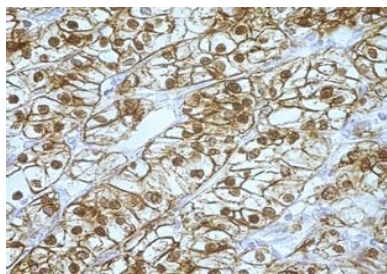


NDRG1 Peptide fragment counts from
Supp Table 4, Perroud et al. (2009)

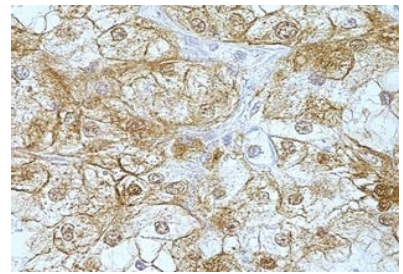
Fig.30: N-Myc Downstream Regulated Gene 1 (NDRG1)
immunohistochemistry on ccRCC FFPE sections



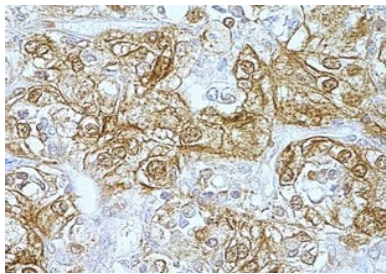
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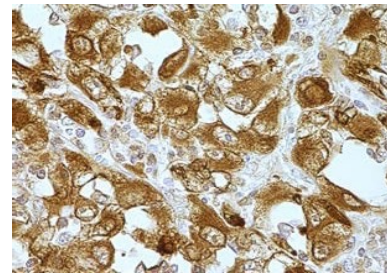
G1



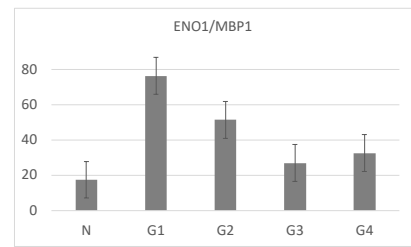
G2



G3



G4



ENO1 / MBP1 Peptide fragment counts
from Supp Table 4, Perroud et al. (2009)

Fig.31: Enoase 1 (ENO1) / Myc Binding Protein 1 (MBP1)
immunohistochemistry on ccRCC FFPE sections

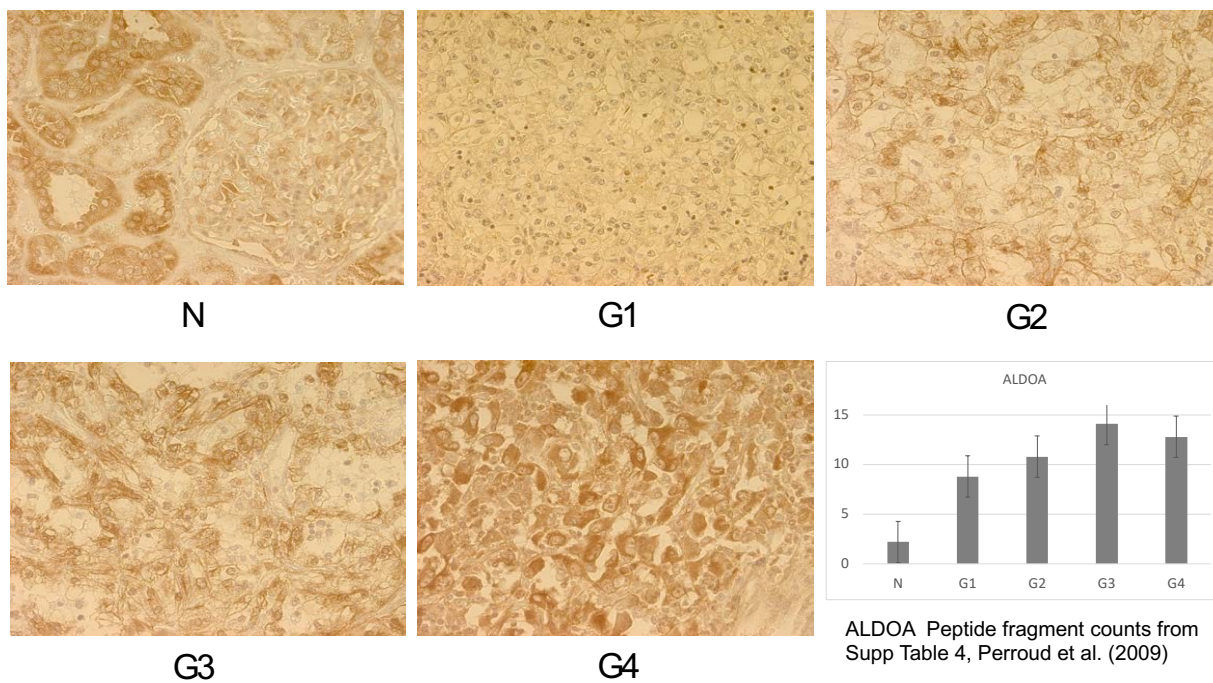


Fig.32: ALDOA immunohistochemistry on ccRCC FFPE sections

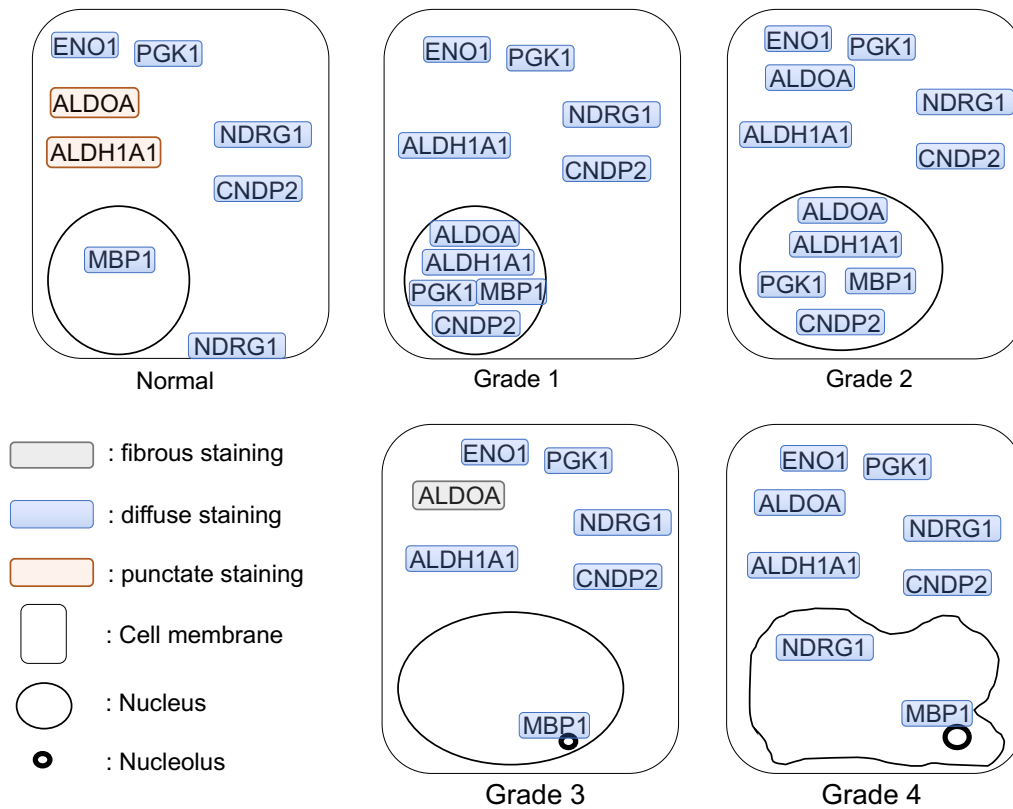


Fig. 33: Subcellular localization of ccRCC grade-segregating biomarker proteins

Chapter 6

Conclusion

Renal cell carcinoma (RCC) is the 9th leading cause of cancer death in the US, and ccRCC comprises roughly three quarters of all cases. Globally, RCC accounts for 180,000 deaths annually which ranks it the 9th in incidence amongst men, and 14th amongst women. Global Cancer Statistics (GLOBOCAN) 2020 ranks RCC 13th in fatality amongst both men and women. (<https://gco.iarc.fr/>, [1]) RCC can be further classified into subtypes including clear cell renal cell carcinoma (ccRCC), chromophobe RCC, and papillary RCC based on their morphology and staining characteristics. 75% of the cases are composed of the ccRCC subtype. Anatomically, ccRCC is characterised with clear cytoplasm filled with lipid droplets, which gave it the name “clear cell”, abbreviated as “cc”. Although a number of attempts were made to establish ccRCC chemotherapy, its overarching genetic defects, namely VHL inactivation, has rendered it refractory to conventional chemotherapy and hyper-angiogenic. Consequently, Surgical intervention at an early stage remains the only effective cure and the mortality rate remains high, thus making identification of novel druggable targets paramount. Recent advances in "omics" technologies fueled by biotechnological and computational advances have made omics such as proteomics and metabolomics have added advantages in identifying such drug targets, and such novel approaches are expected to give renewed momentum in development and clinical application of ccRCC-specific pharmacological intervention which thus far remain stagnant. The overarching aim of this dissertation is to characterise ccRCC using up-and-coming multi-omic approaches such as proteomics and metabolomics, and identify potentially

druggable proteins and pathways to abate ccRCC. We tested the efficacy of Roscovitine, a semi-specific CDK inhibitor (Chapter 2), characterised grade-defined ccRCC proteomes (Chapter 3), Identified the contribution of ASS1 silencing to the development of its clear-cell characteristics, and developed a novel ccRCC grading scheme based on the grade-dependent differential subcellular localisation of several ccRCC grade biomarkers identified in the proteomic study (Chapter 5).

In our work on the effect of Roscovitine (Seliciclib, CYC202) on ccRCC, chemotherapeutic potential of Roscovitine on ccRCC was studied *in vitro*. [2] While various chemotherapeutic approaches have been established for a number of cancers, ccRCC evades such approaches. I tested the efficacy of Roscovitine to serve as a ccRCC chemotherapy. 786O cell line, a model system for ccRCC, however, proved refractory to Roscovitine. Instead, Nutlin-3 proved effective in inducing apoptosis, demonstrating its VHL-negative status and the resultant inability to stabilise p53 as the underlying mechanism for its chemotherapeutic resistance. This indicates that future development in p53-stabilising reagent in combination with more target-specific CDK inhibitor may hold hope as a successful ccRCC chemotherapy. Next-generation pharmacological approaches against ccRCC may target one or more select targets downstream of CDKs. Alternatively, as described in Chapter 3, each Fuhrman nuclear grade has distinct proteomes in addition to the distinct morphological characteristics, suggesting that future ccRCC chemotherapy tailored to each grade may have more success.

In order to identify novel druggable targets against ccRCC, Fuhrman nuclear grade-defined proteomics was studied using the shotgun proteomics on FFPE sections. We established a new protocol and showed that FFPE samples are viable and widely available starting materials to characterise disease proteome.

Our Fuhrman nuclear grade-defined ccRCC proteomics identified 180 protein biomarkers that distinguished grades with $p < 0.05$, and 105 proteins with $p < 0.01$. [3] Amongst the 105 biomarkers, 66 proteins were downregulated across all four grades compared with the normal kidney cortex, 16 were upregulated only in the lower two grades, and 21 protein biomarkers were upregulated across all grades. The UP category was enriched in glycolytic enzymes, while Integrin signalling pathway and 5-hydroxytryptamine degradation pathway were enriched in the DOWN class. The UPG1 class was enriched in glycolytic enzymes, although the smaller size of this class makes the analysis less reliable. With the recent availability in monoclonal antibodies raised against them, these biomarkers are expected to provide the basis for easy and standard assays such as immunohistochemistry and Western blotting to more clearly define the ccRCC in each patient.

Shotgun proteomics assumes the samples are homogeneous biomolecular cocktail without any internal structures and substructures. This neglects the contribution of cellular architecture and molecular complexes to its physiological functions, thus is an inaccurate and insufficient description of a biological system. This is an inherent flaw in proteomics. To circumvent this limitation, subcellular and organellar proteomics are becoming more and more widely used. Application of these more cutting-edge proteomic approaches is expected to yield

more grade-defining ccRCC biomarkers including those that can distinguish each grade and the normal proximal tubule epithelial cells.

In Chapter 4, Argininosuccinate Synthetase (ASS1), the rate-limiting enzyme of the urea cycle and one of the ccRCC biomarkers identified in Chapter 3, was studied in the context of ccRCC development *in vitro*. ASS1 catalyses the condensation reaction of aspartate and citrulline to form argininosuccinate, but its expression is negligible in many cancers including ccRCC.

Our metabolomics analysis using gas chromatography, hydrophilic interaction chromatography, and reverse-phase lipid chromatography-quadrupole time-of-flight mass spectrometry revealed metabolome-wide reprogramming upon ASS1 reconstitution in 786O and ASS1 deletion in HK2. For example, N-acetylaspartate was significantly increased in ASS1-786O compared to the control 786O, while it was reduced in ASS1-KD HK2 compared to the parental HK2. Aspartate, on the other hand, was reduced in ASS1-786O compared to the control 786O as well as in ASS1-KD HK2 compared to the parental HK2. Other amino acids such as tyrosine, threonine, and beta-alanine also showed similar trends. This is surprising, but can be attributed to the nutrient and oxygen-replete conditions that these cells were grown. In addition, these two pairs of cell lines differ in their genetic background. For example, both ASS1-786O and the control 786O are VHL-deficient, while both ASS1-KD HK2 and the parental HK2 cell lines are VHL-wild type.

Most significant changes, however, were detected in the lipidomic analysis, which showed drastic reprogramming in ASS1-KD HK2 against the parental HK2 under nutrient-replete conditions as well as ASS1-786O compared to the control 786O under a nutrient-deficient condition. Taken together, this work complements reports by the Simon group, [4, 5] and further expands the scope of the metabolic reprogramming upon ASS1 silencing in ccRCC. Most notably, this work demonstrates that the kidney proximal tubule epithelial cells undergo lipidomic reprogramming concurrently with the decrease in N-acetylaspartate. While the cytoplasmic lipid droplet accumulation in ccRCC may instinctively suggest increase in lipid content, the most drastic change happened in plasmalogen class of phospholipids which decreased by three orders of magnitude, although neutral lipids such as glycerols increased by factor of 8 upon ASS1 deletion in HK2. Given lipid droplets consist of the core of neutral lipid encapsulated by a phospholipid monolayer, [6] and assuming that the detected lipidomic changes primarily manifests as change in lipid droplet morphology, our lipidomic analysis suggests that ccRCC metabolic reprogramming shifts the intracellular lipid composition to favour decrease in total lipid droplet surface area and increase in total lipid droplet volume. Geometrically, this can be achieved by fusing multitude of miniature lipid droplets to form a small number of gargantuan lipid droplets. (**Figure 1**) That is, ASS1 silencing may contribute to the clear cell phenotype due to the cytoplasmic lipid droplet fusion and resultant enlargement. This, in turn, is likely to have an effect to reduced lipid-droplet-surface-dependent signalling. [7] Lipid droplet surface pathobiology can serve as a further interrogation target into ASS1 silencing, acetyl-CoA, and acyl-CoA metabolism and lipid metabolism will shed light into the metabolic vulnerability in ccRCC. In agreement with this hypothesis, our proteomic studies

described in Chapter 3 showed grade-dependent decrease in LRP2 and APOA1, two vesicle surface-associated proteins, in ccRCC compared with the normal kidney cortex. On the other hand, ADFP/PLIN2, a lipid-droplet-associated protein showed low-grade-specific increase, suggesting grade-dependent differences in lipid droplet functions. Recent study showed APOA1 induction upon SARS-CoV-2 infection. [8, 9] Further investigation is warranted to elucidate the nature of cell signalling modulation upon lipid droplet fusions during ccRCC development and its possible shared molecular underpinning with COVID-19 and its neurological symptoms such as brain fog.

A surprising finding from our study on grade-defined ccRCC proteomics and its subsequent immunohistochemical validation was that many ccRCC grade biomarkers changed subcellular location as well as expression level as the grade progressed. This suggested these ccRCC grade-defining biomarkers act as moonlighting proteins. Functionally, such protein moonlighting can confer them with novel functions beyond the increase or decrease in total amount across grades, since changes in subcellular localisation often suggests distinct sets of binding partners and enzyme substrates, and in turn, distinct biological outcome. Majority of these proteins were indeed previously identified as moonlighting proteins, albeit in systems unrelated to ccRCC and kidney epithelia.

In our study of subcellular localisation of several ccRCC grade biomarkers, we identified several proteins with such potential moonlighting functions as indicated by shift in subcellular distribution. This was described in Chapter 5 as novel diagnostic criteria to complement the

conventional nuclear morphology-based Fuhrman grading. In addition, distinct subcellular locations from that in the normal kidney proximal epithelia imply distinct binding partners and cellular functions for these proteins in ccRCC compared with the physiological proximal tubule epithelia. Since binding to different proteins will inherently utilise different binding surfaces, novel drugs may be developed to directly target such binding surfaces to interfere with the pathogenic protein-protein interactions.

Mechanistically, their contribution to ccRCC pathogenesis is not a simple function of their increased levels resulting in upregulation of the original function or absence resulting in its downregulation, but rather a result of their neomorphic functions. Since each grade shows distinct combinations of their subcellular distribution, it is expected that their moonlighting functions will confer cancer cells with their distinct characteristics. If the protein is also present in its physiological subcellular location, its contribution to the overall ccRCC pathogenesis needs to be considered in a combinatorial manner. Further investigation of such functional integration between cardinal and moonlighting functions will likely lead to identification of novel druggable targets.

In summary, this work simultaneously illustrates a small step forward in our search for ccRCC treatment and demonstrates the need for novel ccRCC biomarkers that can aid us to novel therapeutics, and identifies a number of such protein biomarkers. Furthermore, many of the proteomic biomarkers show differential subcellular distribution, suggesting that protein moonlighting is at least in part responsible for ccRCC development and grade establishment.

This work integrated conventional chemotherapeutic approaches and immunohistochemistry with contemporary multi-omic analyses and strongly suggests that integration of these two schools of approaches is indispensable to develop personalised medicine.

Despite the decades of continual scientific progress, of which I hope this thesis is a part of, ccRCC remains a formidable foe with dismal prognosis unless discovered at an early stage and treated by surgical means. The work presented in this dissertation aimed to utilise omic approaches to describe ccRCC at the molecular level with unprecedented details to identify novel biomarkers that can be used for prognosis and treatment. While scores of proteins and metabolites were identified as such ccRCC biomarkers, the underlying molecular mechanisms that contribute to ccRCC pathogenesis remain far from complete or clear. Further advancement in omics technology as well as other pertinent innovation is urgently needed.

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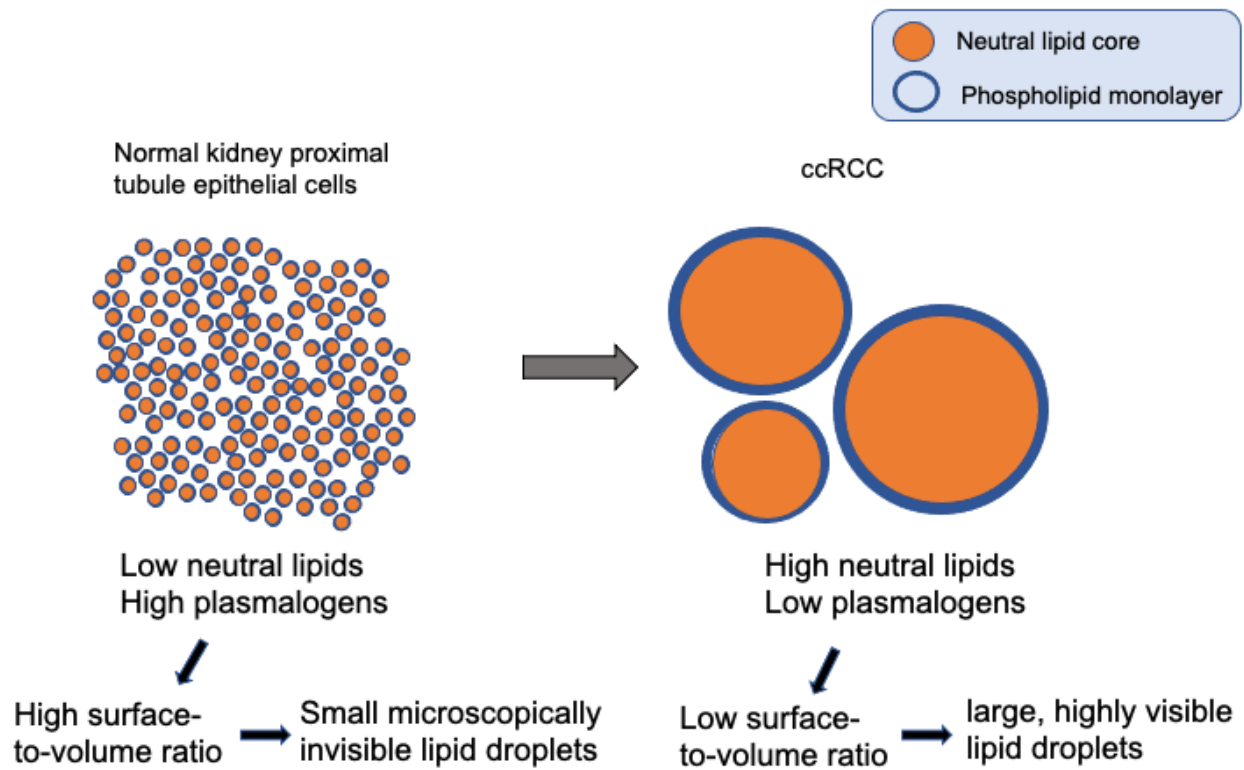


Fig. 34: ASS1 deletion drives lipid droplet aggregation and growth through lipidomic reprogramming