

(12) INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(19) World Intellectual Property
Organization
International Bureau

(10) International Publication Number

WO 2018/167780 A1

(43) International Publication Date
20 September 2018 (20.09.2018)(51) International Patent Classification:
C12Q 1/25 (2006.01) *G01N 33/574* (2006.01)Apt#01, Hyattsville, Maryland 20783 (US). **HANNEN-HALLI, Sridhar**; 409 Beech Avenue, Takoma Park, Maryland 20912 (US).

(21) International Application Number:

PCT/IL2018/050289

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(22) International Filing Date:

12 March 2018 (12.03.2018)

(81) Designated States (unless otherwise indicated, for every kind of national protection available): AE, AG, AL, AM, AO, AT, AU, AZ, BA, BB, BG, BH, BN, BR, BW, BY, BZ, CA, CH, CL, CN, CO, CR, CU, CZ, DE, DJ, DK, DM, DO, DZ, EC, EE, EG, ES, FI, GB, GD, GE, GH, GM, GT, HN, HR, HU, ID, IL, IN, IR, IS, JO, JP, KE, KG, KH, KN, KP, KR, KW, KZ, LA, LC, LK, LR, LS, LU, LY, MA, MD, ME, MG, MK, MN, MW, MX, MY, MZ, NA, NG, NI, NO, NZ, OM, PA, PE, PG, PH, PL, PT, QA, RO, RS, RU, RW, SA, SC, SD, SE, SG, SK, SL, SM, ST, SV, SY, TH, TJ, TM, TN, TR, TT, TZ, UA, UG, US, UZ, VC, VN, ZA, ZM, ZW.

(25) Filing Language:

English

(26) Publication Language:

English

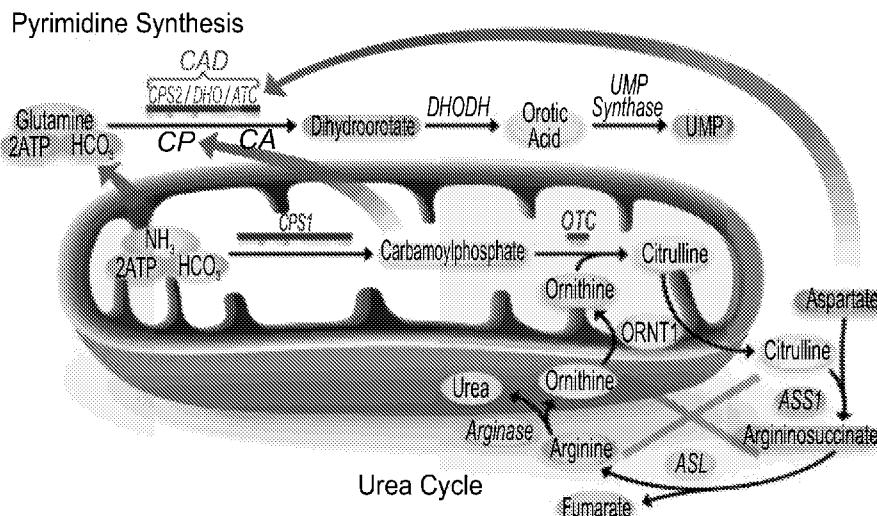
(30) Priority Data:

62/470,278 12 March 2017 (12.03.2017) US

(84) Designated States (unless otherwise indicated, for every kind of regional protection available): ARIPO (BW, GH, GM, KE, LR, LS, MW, MZ, NA, RW, SD, SL, ST, SZ, TZ, UG, ZM, ZW), Eurasian (AM, AZ, BY, KG, KZ, RU, TJ, TM), European (AL, AT, BE, BG, CH, CY, CZ, DE, DK, EE, ES, FI, FR, GB, GR, HR, HU, IE, IS, IT, LT, LU, LV, MC, MK, MT, NL, NO, PL, PT, RO, RS, SE, SI, SK, SM,

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(54) Title: METHODS OF PROGNOSING AND TREATING CANCER

Figure 1A

(57) Abstract: Methods of treating cancer are provided. Accordingly there is provided a method of treating cancer in a subject in need thereof, the method comprising determining a shift from the urea cycle to pyrimidine synthesis in a cancerous cell of the subject as compared to a control sample; and treating the subject with an immune modulating agent when the shift is indicated. Also provided are methods of prognosing cancer and treating cancer according to the prognosis.

TR), OAPI (BF, BJ, CF, CG, CI, CM, GA, GN, GQ, GW, KM, ML, MR, NE, SN, TD, TG).

Declarations under Rule 4.17:

- *of inventorship (Rule 4.17(iv))*

Published:

- *with international search report (Art. 21(3))*
- *before the expiration of the time limit for amending the claims and to be republished in the event of receipt of amendments (Rule 48.2(h))*
- *with sequence listing part of description (Rule 5.2(a))*

METHODS OF PROGNOSING AND TREATING CANCER

FIELD AND BACKGROUND OF THE INVENTION

The present invention, in some embodiments thereof, relates to methods of prognosing
5 and treating cancer.

Cancer diagnosis at early stage is essential when it comes to treatment outcome and survival, especially when it comes to highly malignant tumors. Clinically practiced methods for cancer diagnosis include general well-being of the patient, screening tests and medical imaging.

10 Cancer cells typically undergo metabolic transformations leading to synthesis of biological molecules that are essential for cell division and growth.

15 The urea cycle (UC) is a metabolic process which converts excess nitrogen derived from the breakdown of nitrogen-containing molecules to the excretable nitrogenous compound - urea. Urea, a colorless, odorless solid which is highly soluble in water and practically non-toxic is the main nitrogen-containing substance in the urine of mammals. Several studies have reported altered expression of specific UC components in several types of cancer and also indicated an association between the pattern of these UC components and poor survival or increased metastasis [see e.g. Chaerkady, R. et al. (2008) J Proteome Res 7, 4289-4298; Lee, Y. Y. et al. (2014) Tumour Biol 35: 11097-11105; Syed, N. et al. (2013) Cell Death Dis 4, e458; Miyo et al. (2016) Sci Rep. 6: 38415; Erez et al. (2011) Am J Hum Genet. Apr 8; 88(4): 402-421; Pavlova 20 et al. (2016) Cell Metab. 23(1): 27-47; Rabinovich, S. et al. (2015) Nature, 527(7578): 379-83; International Patent Application Publication No. WO 2016181393, US Patent Application Publication No. US 20150167094 and US Patent No. US 8440184].

25 International Application Publication No. WO 2016181393 discloses that loss of the UC enzyme argininosuccinate synthetase (ASS1) promotes cancer proliferation by diversion of its substrate aspartate towards CAD enzyme. CAD enzyme, a trifunctional protein comprising carbamoyl-phosphate synthase 2 (CPS2), aspartate transcarbamylase (ATC) and dihydroorotase, mediates the first three reactions in the *de-novo* synthesis pathway of pyrimidines. Several studies have reported altered expression of CAD in several types of cancer [see e.g. Poliakov et al. (2014) Genet Res Int. 2014: 646193; International Patent Application Publication No. WO 30 2013096455; and US Patent Application Publication No. US 20140087970].

SUMMARY OF THE INVENTION

According to an aspect of some embodiments of the present invention there is provided a method of treating cancer in a subject in need thereof, the method comprising:

(a) determining a shift from the urea cycle to pyrimidine synthesis in a cancerous cell of the subject as compared to a control sample; and

(b) treating the subject with an immune modulating agent when the shift is indicated.

According to an aspect of some embodiments of the present invention there is provided a method of potentiating cancer treatment with an immune modulating agent in a subject in need thereof, the method comprising:

(a) determining a shift from the urea cycle to pyrimidine synthesis in a cancerous cell of the subject as compared to a control sample; and

(b) treating the subject with an agent which induces a pyrimidines to purines nucleotide imbalance when the shift is indicated.

According to an aspect of some embodiments of the present invention there is provided a method of treating cancer in a subject in need thereof, the method comprising:

(a) determining a shift from the urea cycle to pyrimidine synthesis in a cancerous cell of the subject as compared to a control sample; and

(b) selecting an immune modulation agent for treating the subject when the shift is indicated.

According to some embodiments of the invention, the shift is determined by level of purine to pyrimidine transversion mutations.

According to some embodiments of the invention, the shift is determined by a level and/or activity of a urea cycle enzyme and/or a CAD enzyme.

According to some embodiments of the invention, the urea cycle enzyme is selected from the group consisting of ASL, ASS1, OTC, SLC25A15, CPS1 and SLC25A13.

According to an aspect of some embodiments of the present invention there is provided a method of prognosing cancer in a subject diagnosed with cancer, the method comprising determining a level of purine to pyrimidine transversion mutations in a cancerous cell of the subject as compared to a control sample, wherein the level of the purine to pyrimidine transversion mutations above a predetermined threshold is indicative of poor prognosis, thereby prognosing cancer in the subject.

According to an aspect of some embodiments of the present invention there is provided a method of monitoring efficacy of cancer therapy in a subject, the method comprising determining a level of purine to pyrimidine transversion mutations in a cancerous cell of the subject undergoing or following the cancer therapy, wherein a decrease in the level of the purine to pyrimidine transversion mutations from a predetermined threshold or in comparison to the level in the subject prior to the cancer therapy, indicates efficacious cancer therapy.

According to an aspect of some embodiments of the present invention there is provided a method of prognosing cancer in a subject diagnosed with cancer, the method comprising determining a level and/or activity of a urea cycle enzyme SLC25A15 in a cancerous cell of the subject as compared to a control sample, wherein the level and/or activity of the SLC25A15 below a predetermined threshold is indicative of poor prognosis, thereby prognosing cancer in the subject.

According to an aspect of some embodiments of the present invention there is provided a method of monitoring efficacy of cancer therapy in a subject, the method comprising determining a level and/or activity of a urea cycle enzyme SLC25A15 in a cancerous cell of the subject undergoing or following the cancer therapy, wherein an increase in the level and/or activity of the a urea cycle enzyme SLC25A15 from a predetermined threshold or in comparison to the level and/or activity in the subject prior to the cancer therapy, indicates efficacious cancer therapy.

According to some embodiments of the invention, the cancer is selected from the group consisting of thyroid cancer, hepatic cancer, bile duct cancer and kidney cancer.

According to an aspect of some embodiments of the present invention there is provided a method of prognosing cancer in a subject diagnosed with cancer, the method comprising determining a level and/or activity of at least two urea cycle enzymes selected from the group consisting of ASL, ASS1, OTC, SLC25A15, CPS1 and SLC25A13 in a cancerous cell of the subject as compared to a control sample, wherein the urea cycle enzymes comprise ASS1 and SLC25A13 the at least two is at least three; wherein the level and/or activity of the ASL, the ASS1, the OTC and/or the SLC25A15 below a predetermined threshold and/or the level and/or activity of the CPS1 and/or the SLC25A13 above a predetermined threshold is indicative of poor prognosis, thereby prognosing cancer in the subject.

According to an aspect of some embodiments of the present invention there is provided a method of monitoring efficacy of cancer therapy in a subject, the method comprising determining a level and/or activity of at least two urea cycle enzymes selected from the group consisting of ASL, ASS1, OTC, SLC25A15, CPS1 and SLC25A13 in a cancerous cell of the subject undergoing or following the cancer therapy, wherein the urea cycle enzymes comprise ASS1 and SLC25A13 the at least two is at least three; wherein an increase in the level and/or activity of the ASL, the ASS1, the OTC and/or the SLC25A15 and/or a decrease in the level and/or activity of the CPS1 and/or the SLC25A13 from a predetermined threshold or in comparison to the level and/or activity in the subject prior to the cancer therapy indicates efficacious cancer therapy.

According to some embodiments of the invention, the cancer is thyroid, stomach and/or bladder cancer and the at least two urea cycle enzymes are selected from the group consisting of OTC, SLC25A15 and SLC25A13.

According to some embodiments of the invention, the cancer is prostate cancer and the at least two urea cycle enzymes comprise ASS1 and CPS1.

According to some embodiments of the invention, the cancer is lung and/or head and neck cancer and the at least two urea cycle enzymes are selected from the group consisting of ASL, OTC, CPS1 and SLC25A13.

According to some embodiments of the invention, the cancer is hepatic, bile duct and/or kidney cancer and the at least two urea cycle enzymes are selected from the group consisting of ASL, ASS1, OTC and SLC25A15.

According to some embodiments of the invention, the cancer is breast cancer and the at least three urea cycle enzymes comprise ASS1, OTC and SLC25A13.

According to an aspect of some embodiments of the present invention there is provided a method of prognosing breast cancer in a subject diagnosed with breast cancer, the method comprising determining a level and/or activity of at least two urea cycle enzymes selected from the group consisting of ASS1, OTC and SLC25A13 in a cancerous cell of the subject as compared to a control sample,

wherein the level and/or activity of the ASS1 and/or the OTC below a predetermined threshold and/or the level and/or activity of the SLC25A13 above a predetermined threshold is indicative of poor prognosis, thereby prognosing the breast cancer in the subject.

According to an aspect of some embodiments of the present invention there is provided a method of monitoring efficacy of cancer therapy in a subject diagnosed with breast cancer, the method comprising determining a level and/or activity of at least two urea cycle enzymes selected from the group consisting of ASS1, OTC and SLC25A13 in a cancerous cell of the subject undergoing or following the cancer therapy, wherein an increase in the level and/or activity of the ASS1 and/or the OTC and/or a decrease in the level and/or activity of the SLC25A13 from a predetermined threshold or in comparison to the level and/or activity in the subject prior to the cancer therapy, indicates efficacious cancer therapy.

According to some embodiments of the invention, the method further comprising determining a level and/or activity of a CAD enzyme, wherein the level and/or activity of the CAD enzyme above a predetermined threshold is indicative of poor prognosis.

According to some embodiments of the invention, the method further comprising determining a level and/or activity of a CAD enzyme, wherein a decrease in the level and/or activity of the CAD enzyme from a predetermined threshold or in comparison to the level in the subject prior to the cancer therapy, indicates efficacious cancer therapy.

According to some embodiments of the invention, the CAD is activated CAD.

According to some embodiments of the invention, the method comprising corroborating the prognosis using a state of the art technique.

According to some embodiments of the invention, there is provided a method of treating cancer in a subject in need thereof, the method comprising:

- 5 (a) prognosing the subject according to the method of the present invention; and
 (b) treating the subject with a cancer therapy according to the prognosis.

According to some embodiments of the invention, there is provided a method of treating cancer in a subject in need thereof, the method comprising:

- 10 (a) prognosing the subject according to the method of the present invention; and
 wherein when a poor prognosis is indicated
 (b) treating the subject with a cancer therapy.

According to some embodiments of the invention, there is provided a method of treating cancer in a subject in need thereof, the method comprising:

- 15 (a) prognosing the subject according to the method of the present invention; and
 (b) selecting a cancer therapy for treating the subject based on the prognosis.

According to some embodiments of the invention, there is provided a method of treating cancer in a subject in need thereof, the method comprising:

- 20 (a) prognosing the subject according to the method of the present invention; and
 wherein when a poor prognosis is indicated
 (b) selecting a cancer therapy for treating the subject based on the level of the purine to pyrimidine transversion mutations, the urea cycle enzyme and/or the CAD enzyme.

According to some embodiments of the invention, the predetermined threshold is at least 1.1 fold.

According to some embodiments of the invention, the control sample is a non-cancerous cell of the subject.

According to some embodiments of the invention, the control sample is a cancerous cell with a level and/or activity of the urea cycle enzyme, the purine to pyrimidine transversion mutations and/or the CAD enzyme similar to levels and/or activity of same in a healthy cell of the same type.

30 According to some embodiments of the invention, the cancer is selected from the group consisting of hepatic cancer, osteosarcoma, breast cancer, colon cancer, thyroid cancer, stomach cancer, lung cancer, kidney cancer, prostate cancer, head and neck cancer, bile duct cancer and bladder cancer.

35 According to some embodiments of the invention, the cancer is selected from the group consisting of hepatic cancer, osteosarcoma, breast cancer and colon cancer.

According to some embodiments of the invention, the cancer therapy comprises a therapy selected from the group consisting of L-arginine depletion, glutamine depletion, pyrimidine analogs, thymidylate synthase inhibitor and mammalian target of Rapamycin (mTOR) inhibitor.

According to some embodiments of the invention, the cancer therapy comprises an immune modulation agent.

According to some embodiments of the invention, the cancer therapy further comprises an agent which induces a pyrimidines to purines nucleotide imbalance.

According to some embodiments of the invention, the method further comprising treating the subject with an agent which induces a pyrimidines to purines nucleotide imbalance when the shift is indicated.

According to some embodiments of the invention, the method further comprising treating the subject with the immune modulation agent.

According to some embodiments of the invention, the immune modulation agent comprises anti-PD1.

According to some embodiments of the invention, the immune modulation agent comprises anti-CTLA4.

According to some embodiments of the invention, the agent which induces a pyrimidines to purines nucleotide imbalance comprises an anti-folate agent.

According to some embodiments of the invention, the anti-folate agent comprises methotrexate.

According to some embodiments of the invention, the purine to pyrimidine transversion mutations are non-synonymous purine to pyrimidine transversion mutations.

According to some embodiments of the invention, the determining the level of purine to pyrimidine transversion mutations is effected at the genomic level.

According to some embodiments of the invention, the determining the level of the enzyme is effected at the transcript level.

According to some embodiments of the invention, the determining the level of the enzyme is effected at the protein level.

Unless otherwise defined, all technical and/or scientific terms used herein have the same meaning as commonly understood by one of ordinary skill in the art to which the invention pertains. Although methods and materials similar or equivalent to those described herein can be used in the practice or testing of embodiments of the invention, exemplary methods and/or materials are described below. In case of conflict, the patent specification, including definitions, will control. In addition, the materials, methods, and examples are illustrative only and are not intended to be necessarily limiting.

BRIEF DESCRIPTION OF THE SEVERAL VIEWS OF THE DRAWINGS

Some embodiments of the invention are herein described, by way of example only, with reference to the accompanying drawings. With specific reference now to the drawings in detail, it is stressed that the particulars shown are by way of example and for purposes of illustrative discussion of embodiments of the invention. In this regard, the description taken with the drawings makes apparent to those skilled in the art how embodiments of the invention may be practiced.

In the drawings:

Figures 1A-E demonstrate the association between the urea cycle (UC) enzymes and CAD. Figure 1A is a schematic representation demonstrating that the UC enzymes alternate substrates with CAD. Figure 1B shows a representative photograph and a bar plot summarizing the crystal violet staining which indicates increased proliferation of cultured fibroblasts extracted from ORNT1 deficient (ORNT1D) or OTC deficient (OTCD) patients as compared to fibroblasts extracted from healthy controls. The Y-axis represents fold change of the staining at 48 hours in comparison to time 0 ($P \leq 0.05$, student t-test), $n = 4$ biological repetitions. Figure 1C is a western blot photograph demonstrating increased levels of CAD and phosphorylated CAD in fibroblasts extracted from ORNT1D and OTCD patients as compared to fibroblasts extracted from healthy patient (NF). Figure 1D is a plot showing decreased expression of ASS1 and increase expression of SLC25A13 and CAD in fibroblasts extracted from healthy patients following human Cytomegalovirus (CMV) infection as measured by ribosome profiling. Y-axis represents expression normalized to non-infected control. Figure 1E demonstrates high homology and identity between the UC enzymes and CAD. Protein domain structures were annotated using the NCBI BLAST and conserved domain search server (www.ncbi.nlm.nih.gov/Structure/cdd/wrpsb.cgi). Results show high homology between the proximal UC enzymes proteins CPSI and OTC, and two CAD domains CPS2 and ATC, respectively.

Figures 2A-E demonstrate that downregulation of UC enzymes increases cancer proliferation and pyrimidine synthesis. Figure 2A is a western blow photograph demonstrating the extent of OTC downregulation using several shRNAs in HepG2 hepatic cancer cell line. Figure 2B shows a representative photograph and a bar plot summarizing the crystal violet staining which indicates increased proliferation of HepG2 hepatic cancer cells transduced with OTC shRNA, as compared to HepG2 hepatic cancer cell transduced with an empty vector (EV). The Y-axis represents fold change of the staining at 48 hours in comparison to time 0 (* $P \leq 0.05$, ** $P \leq 0.01$, student t-test), $n = 3$ biological repetitions. Figure 2C is a bar plot demonstrating

increased uracil to urea ratio in HepG2 hepatic cancer cells transduced with OTC shRNA, as compared to HepG2 hepatic cancer cell transduced with an empty vector (EV), (****P ≤ 0.0001, student t-test), n = 3 technical repetitions using GCMS. Figures 2D-E are bar plots demonstrating increased uracil to urea ratio (Figure 2D) and increased pyrimidine to purine ration in 5 osteosarcoma cells transduced with ASS1 shRNA, as compared to osteosarcoma cells transduced with an empty vector (EV), (*P ≤ 0.05, ****P ≤ 0.0001, student t-test), n = 3 biological repetitions.

Figures 2F-H demonstrate that specific dysregulation of UC enzymes facilitates cancer proliferation. Figure 2F shows western blot photographs demonstrating the specific UC 10 perturbations induced in different cancer cells [i.e. downregulation of OTC (shOTC) or ORNT1 (shORNT1) or overexpression of citrin (OE-Citrin)] and the resultant effect on CAD activation compared to control cells transfected with empty vector (EV). Figure 2G upper left bar plot is a quantification of crystal violet staining showing increased proliferation of different cancer cells following the indicated UC perturbations. Figure 2G lower left bar plot shows that rescue 15 experiments for the specific UC perturbation reverses the proliferative phenotype. Figure 2G right bar plots show RT-PCR quantification for the changes in UC genes RNA expression levels following transfection with the specific rescue plasmid versus control plasmids. Figure 2H left bar plots show enhanced synthesis of labelled M+1 uracil from 15N-a-glutamine in HepG2 cancer cells transduced with OTC shRNA and SKOV cancer cells transduced with ORNT1 20 shRNA as compared to controls transduced with empty vector. Figure 2H right bar plots show in vivo growth of HepG2 transduced with OTC shRNA and SKOV transduced with ORNT1 shRNA xenografts compared to xenografts transduced with an empty vector.

Figures 3A-E demonstrate that dysregulation of the UC genes (denoted herein as UCD) 25 in cancer activates CAD and correlates with worse prognosis. Figure 3A shows relative expression of 6 UC genes in tumors from the cancer genome atlas (TCGA) with respect to their expression in healthy control tissues. Most tumors have aberrant expression of at least 2 UC components in the direction that metabolically supplies the required substrates for CAD activity [that is, decreased expression of ASL, ASS1, OTC and/or ONRT1D (SLC23A15) and/or increased expression of CPS1 and/or SLC25A13, P<2.67E-3]. Tumor type's abbreviations are as 30 follows: THCA - Thyroid cancer, STAD - Stomach adenocarcinoma, PRAD - Prostate cancer, LUSC - Lung squamous carcinoma, LIHC - Liver hepatocellular carcinoma, KIRP - Kidney renal papillary cell carcinoma, KIRC - Kidney renal Clear Cell Ca, KICH - Kidney chromophobe, HNSC- Head Neck Squamous Cell Carcinoma, CHOL - cholangiocarcinoma, BRCA - breast cancer, BLCA - Bladder cancer. Figure 3B shows immunohistochemistry images 35 of cancer tissues with their respective healthy tissue controls stained with the indicated UC

components or PCNA as a marker for proliferation, showing inverse correlation between the expression of UC genes and the proliferation marker. Magnification x10. Figure 3C shows bar plots summarizing staining intensity of the PCNA positive cell count and UC proteins. Each staining was calibrated and repeated in two technical repetitions per patient sample in each slide (intensity OD level was compared in a matched T-student test). Figure 3D is a graph demonstrating that UCD-scores (X-axis, equally divided into 5 bins) are positively correlated with CAD expression. Each paired consecutive bins were compared using the Wilcoxon rank sum test. Figure 3E is a Kaplan-Meier survival curve showing that UCD is associated with worse survival of patients computed across all TCGA samples (i.e. pan cancer analysis).

Figures 4A-E demonstrate that UCD in cancer correlates with tumor grade. Figure 4A is a schematic representation demonstrating the direction of UC enzymes expression that supports CAD activation (represented in blue arrows). The resulting changes in metabolites' levels following these expression alterations are represented by red arrows. Figure 4B shows immunohistochemistry images of cancer tissues with their respective healthy tissue controls stained with OTC Magnification X10; and a bar plot summarizing OTC staining intensity. Each staining was calibrated and repeated in 2 technical repetitions per patient sample in each slide (intensity OD level was compared in a matched T-student test, ****P ≤ 0.0001). Figure 4C shows immunohistochemistry images of thyroid cancer tissues stained with ORNT1 Magnification X10; and a bar plot summarizing ORNT1 staining intensity; demonstrating that low levels of ORNT1 are associated with more advanced thyroid tumor grades. Each staining was calibrated and repeated in 2 technical repetitions per patient sample in each slide (intensity OD level was compared in a matched T-student test, ***P ≤ 0.001). Figure 4D is a Kaplan-Meier survival curve showing that CAD is associated with worse survival of patients computed across all TCGA samples (i.e. pan cancer analysis). Figure 4E shows a Cox regression analysis of the UCD-score and CAD expression, demonstrating that both variables are independently significant.

Figures 5A-G demonstrate that UCD in cancer increases nitrogen utilization. Figure 5A shows metabolic modelling which predicts decreased urea excretion (left panel) and increased nitrogen utilization (right panel) with increased CAD activity, at high biomass production (that is, higher cell proliferation) conditions. Figure 5B shows bar plots demonstrating increased pyrimidine pathway metabolites' in urine of breast or colon tumors bearing mice (n=37) as compared to control mice (W/Tumor); n=11), (*P<0.05, **P<0.01, Mann-Whitney test). Figure 5C shows plots demonstrating the distribution of the ratio of pyrimidine to purine metabolites for samples with low and high UCD-scores (top and bottom 15 %). The plot on the left shows the results for hepatocellular carcinoma (HCC) tumors and the plot on the right for Breast cancer

(BC) tumors. Figure 5D is a plot showing urea plasma levels in children with different cancers. The dashed red line demonstrates the normal age matched mean urea value. Figure 5E is a plot showing urea plasma levels in patients with prostate cancer (PCa, n=519) as compared to age matched patients with benign prostate hyperplasia (BPH, n=257), ****P<0.0001, Mann-Whitney test. Figure 5F shows metabolic modelling which predicts a significant increase in metabolic flux reactions involving pyrimidine metabolites following UCD. Figure 5G shows western blot photographs and their quantification bar plots demonstrating that the increased pyrimidine pathway metabolites' in urine of colon tumors bearing mice shown in Figure 5B correlates with UCD in the tumors compared to control healthy colon.

Figures 6A-D demonstrate that tumors with UCD have increased transverse coding mutations. Figure 6A is a bar plot demonstrating that downregulation of ASS1 in osteosarcoma cancer cells using shRNA increases pyrimidine to purines ratio as compared to osteosarcoma cancer cells transduced with an empty vector (EV), (****P-value<0.0001, two way ANOVA with Dunnett's correction). Figure 6B is a plot demonstrating that UCD (UC-dys) increases DNA purine to pyrimidine transversion mutations at a pan-cancer scale and across different tumor types compared to tumors with intact UC (UC-WT). Figure 6C is a plot demonstrating that UCD samples show a higher fraction of nonsynonymous purine to pyrimidine transversion mutations as compared to UC-WT across all TCGA data (P<4.93E-3). Such a significant bias is not present for any of the other transversion mutation types (Y->Y, R->R, and Y->R). Figure 6D shows a Cox regression analysis demonstrating that only R->Y mutation levels are significantly associated with survival (while overall mutation levels and Y->R mutation levels are not).

Figures 7A-F demonstrate that UCD increases transversion mutations in tumors. Figure 7A is a bar plot demonstrating that downregulation of OTC in hepatic cancer cells using shRNA increases pyrimidine to purines ratio as compared to hepatic cancer cells transduced with an empty vector (EV), as measured by LCMS Bars represent the mean of >2 biological repeats, *P<0.05, one way anova with dunnet correction. Figure 7B is a plot demonstrating that tumors with UCD (UC-dys) have significantly higher number of transversion mutations from purines to pyrimidines on the coding (sense) DNA strand versus tumors with intact UC (UC-WT), Wilcoxon rank sum P<2.35E-3, while such a significance is not observed for transition mutations. Figure 7C is a plot demonstrating that UCD is associated with higher number of purine to pyrimidine transversion mutations across different cancer types [each circle denotes the UCD and transversion mutation bias levels in a given cancer type, (overall Spearman correlation = 0.58, P<0.01)]. Figure 7D is a plot demonstrating that tumors with UCD have significantly greater fractions of transversion mutations from purines to pyrimidines at the mRNA level, based on 18 breast cancer samples (Wilcoxon rank sum, **P<0.001). Only those variants that were

detected as a somatic mutation in the exome sequence and were mapped in the corresponding RNA sequence were considered. Figure 7E is a plot representing genome wide proteomic analysis of 42 breast cancers demonstrating a significantly increased R->Y mutation rates in UCD tumors as compared to tumors with intact UC (Wilcoxon rank sum P<0.02). Figure 7F is a 5 plot demonstrating that *CAD*, *SLC25A13* and *SLC25A15* genes' expression are among the top 10 % of genes that correlate most strongly with DNA purines to pyrimidines transversion mutations.

Figure 8 is a bar plot demonstrating that specific UC perturbations induced in different cancer cells [i.e. downregulation of OTC (shOTC), ORNT1 (shSLC25A15) or ASS1 (shASS1) or overexpression of citrin (Citrin OE)] increases pyrimidine to purines ratio as compared to 10 control cancer cells transduced with an empty vector (EV), as measured by LCMS. Shown is a representative of the mean of more than two biological repeats. (*P≤0.05, ** P≤0.01, one way ANOVA with Dunnet's correction).

Figure 9 is a bar graph demonstrating that specific UC perturbations induced in different cancer cells [i.e. downregulation of OTC (shOTC), ORNT1 (shSLC25A15) or ASS1 (shASS1) 15 or overexpression of citrin (Citrin OE)] increases purines to pyrimidines (R->Y) mutations using a Fisher's exact test.

Figures 10A-F demonstrate that UCD score correlates with response to immune modulation therapy (ICT). Figure 10A demonstrates that UCD-scores are significantly higher in human patients responding to anti-PD1 (left panel) and anti-CTLA4 (right panel) therapies 20 (orange) compared to non-responders (grey) (Wilcoxon ranksum P<0.05). Figure 10B shows ROC curves demonstrating higher predictive power of pyrimidine-rich transversion mutational bias (PTMB, AUC=0.77, blue) compared to mutational load (AUC=0.34, red) in predicting the response to anti-PD1 therapy (Roh et al., 2017). Figures 10C-E demonstrates that anti-PD1 25 therapy is more efficient in UCD tumors, as determined in an in-vivo syngeneic mouse model of colon cancer. Specifically, control MC-38 mouse colon cancer cells (EV) or MC-38 mouse colon cancer cells transduced with ASS1 shRNA (shASS1) were inoculated into C57BL6 mice injected intraperitoneally with anti-PD1 immunotherapy (N=20 mice, 5 mice in each group). Figure 10C demonstrates tumor volume 22 days following inoculation (Wilcoxon ranksum P<0.007). Figure 10D shows CD8 T cells infiltration in the tumors excised on day 21 following inoculation, as 30 evaluated by flow cytometry analysis (Wilcoxon ranksum P=0.01 and 0.3, respectively for shASS1 and EV). Figure 10E demonstrates tumor growth over time in the shASS1 group with or without anti-PD1 (P<0.01, ANOVA with Dunnett's correction). Figure 10F is a schematic representation summary the "*UCD effect*": while in normal tissues excess nitrogen is disposed as urea, in cancer cells most nitrogen is utilized for synthesis of macromolecules, with pyrimidine

synthesis playing a major role in carcinogenesis and effecting patients' prognosis and response to ICT.

Figures 11A-D demonstrate the impact of CAD and PTMB on ICT response and HLA-peptide presentation. Figure 11A demonstrates the expression of CAD is less associated with 5 ICT response than UCD both in anti-PD1 (Hugo et al., 2016) (left panel) and anti-CTLA4 (Van Allen et al., 2015) (right panel) cohort (Wilcoxon ranksum P=0.71 and 0.45, respectively). Figure 11B shows peptidomics analysis which demonstrates that UCD cell lines have higher MS/MS intensity than control cell lines (Wilcoxon ranksum P<0.001). Figure 11C demonstrates that UCD cell lines have more hydrophobic peptides than control cell lines (Wilcoxon ranksum 10 P<0.0002). Figure 11D demonstrates that hydrophobic peptides (hydrophobicity score > 80-percentile) are more abundant (MS/MS intensity) than non-hydrophobic peptides (hydrophobicity-score < 20-pervcentile) in UCD cell lines (Wilcoxon ranksum P<1E-6) but not in control cell lines (Wilcoxon ranksum P=0.14).

Figures 12A-E demonstrates that UCD perturbed mouse colon cancers respond better to 15 ICT. Figure 12A shows western blot photograph and a quantification bar graph demonstrating that MC-38 mouse colon cancer cells infected with different shASS1 clones demonstrate downregulation of ASS1 at the protein level as compared to control cells infected with an empty vector (EV). Figure 12B is a RT PCR quantification bar graph demonstrating decreased ASS1 levels in MC38 infected with different shASS1 clones as compared to MC38 infected with EV. Figure 12C is a bar graph demonstrating that *in vivo* tumor growth was enhanced in MC38 20 transduced with shASS1 as compared to the growth of MC38-EV tumors 22 days following inoculation. Figure 12D shows CD4 T cells infiltration in the tumors excised on day 22 following inoculation, as evaluated by flow cytometry analysis (N=20 mice, 5 mice in each group, Wilcoxon ranksum P>0.4 both for shASS1 and EV). Figure 12E demonstrates tumor 25 growth over time in the control group (EV) with (red) or without (blue) anti-PD1 (ANOVA P>0.12).

DESCRIPTION OF SPECIFIC EMBODIMENTS OF THE INVENTION

The present invention, in some embodiments thereof, relates to methods of prognosing 30 and treating cancer.

Before explaining at least one embodiment of the invention in detail, it is to be understood that the invention is not necessarily limited in its application to the details set forth in the following description or exemplified by the Examples. The invention is capable of other embodiments or of being practiced or carried out in various ways.

Cancer cells typically undergo metabolic transformations leading to synthesis of biological molecules that are essential for cell division and growth.

Whilst reducing the present invention to practice, the present inventors have uncovered a direct link between alterations in the expression levels of urea cycle (UC) enzymes and increased expression levels of activated CAD enzyme in cancer accompanied by a genome-wide shift in mutational bias toward pyrimidines. Moreover, these changes were found to be correlated with higher tumor grade and decreased survival and therefore are indicative of poor prognosis.

As is illustrated hereinunder and in the examples section, which follows, the present inventors show that fibroblasts from patients with deficiency in the UC enzymes OTC or SLC25A15 are more proliferative and exhibit elevated levels of activated CAD (Example 1, Figures 1A-E). Following, the present inventors have uncovered that downregulation of the UC enzymes ASS1 or OTC in cancer cells using shRNA resulted in increased proliferation and pyrimidine synthesis (Example 2, Figures 2A-D). The inventors then developed a computational method to quantify the extent of the metabolic redirection from the UC towards CAD and pyrimidine synthesis (denoted herein as UCD) by calculating a UCD-score which sums up expression of 6 UC genes - ASL, ASS1, CPS1, OTC, SLC25A13 and SLC25A15. In this way, the inventors show that a majority of tumors harbour expression alterations in at least two UC components in the direction that enhances CAD activity (Example 2, Figures 3A-D and 4A-4B). More particularly, up-regulation of CPS1 and/or SLC25A13; and/or down-regulation of ASL, ASS1, OTC and/or SLC25A15 were detected in different kinds of cancer and were associated with increased CAD expression and activity and pyrimidine synthesis. Most importantly, UCD (and the UCD-score) and activated CAD expression, independently, were associated with higher tumor grade and decreased cancer survival (Example Figures 3E, 4B-E). The inventors further demonstrate that UCD in cancer is also associated with increased pyrimidine to purine ratio (Example 3, Figure 5C) and with increased purine to pyrimidine transversion mutations at the DNA, RNA and protein levels (Example 4, Figures 6A-B, 7A-F). In addition, relative to samples with normal UC activity, in UCD samples the purines to pyrimidine mutations have a greater tendency to be non-synonymous, i.e. they change the encoded amino acid (Example 4, Figure 6C). Importantly, the increased purine to pyrimidine mutation rate was associated with patient survival, independent of the rate of overall mutations (Example 4, Figure 6D).

Moreover, the inventors present several computational modeling and experimental studies of urine and plasma samples, which show increased levels of pyrimidine synthesis metabolites (Uracil, Thymidine, Orotic acid and Orotidine) and decreased levels of urea in urine and plasma samples of tumor bearing mice and cancer patients, respectively, compared to cancer-free mice and patients (Example 3, Figures 5A-B, 5D-E).

Consequently, according to some embodiments, the presence of a shift in the metabolic urea cycle (UC) to CAD enzyme associated with a mutation bias toward pyrimidines, can be used as a marker for prognosing and treating cancer.

In addition, as purine to pyrimidine transversion mutations and particularly non-synonymous purine to pyrimidine transversion mutations result in presentation of new tumor-associated antigens that can activate the host immune response toward the cancer, the present teachings suggest that cancers prognosed and/or monitored according to some embodiments of the present invention are more susceptible to treatment by immune modulation which can deprive the tumor of its protective immune suppression that enables the cancer to evade the host immune response toward the new antigens.

Thus, according to a first aspect of the present invention, there is provided a method of treating cancer in a subject in need thereof, the method comprising:

- (a) determining a shift from the urea cycle to pyrimidine synthesis in a cancerous cell of the subject as compared to a control sample; and
- (b) treating said subject with an immune modulating agent when said shift is indicated.

According to another aspect of the present invention, there is provided a method of treating cancer in a subject in need thereof, the method comprising:

- (a) determining a shift from the urea cycle to pyrimidine synthesis in a cancerous cell of the subject as compared to a control sample; and
- (b) selecting an immune modulation agent for treating said subject when said shift is indicated.

The term “treating” refers to inhibiting, preventing or arresting the development of a pathology (e.g. cancer) and/or causing the reduction, remission, or regression of a pathology. Those of skill in the art will understand that various methodologies and assays can be used to assess the development of a pathology, and similarly, various methodologies and assays may be used to assess the reduction, remission or regression of a pathology.

As used herein the term “subject” refers to a mammal (e.g., human being) at any age or of any gender.

According to specific embodiments, the subject is a human subject.

According to specific embodiments, the subject is diagnosed with a disease (i.e., cancer) or is at risk of to develop a disease (i.e. cancer).

According to specific embodiments, the subject is not afflicted with an ongoing inflammatory disease (other than cancer). According to specific embodiments, the subject is not a pregnant female.

Cancers which may be prognosed, monitored and/or treated by some embodiments of the invention can be any solid or non-solid cancer and/or cancer metastasis. Examples of cancer include but are not limited to, carcinoma, lymphoma, blastoma, sarcoma, and leukemia. More particular examples of such cancers include, but not limited to, tumors of the gastrointestinal tract (colon carcinoma, rectal carcinoma, colorectal carcinoma, colorectal cancer, colorectal adenoma, hereditary nonpolyposis type 1, hereditary nonpolyposis type 2, hereditary nonpolyposis type 3, hereditary nonpolyposis type 6; colorectal cancer, hereditary nonpolyposis type 7, small and/or large bowel carcinoma, esophageal carcinoma, tylosis with esophageal cancer, stomach carcinoma, pancreatic carcinoma, pancreatic endocrine tumors), endometrial carcinoma, dermatofibrosarcoma protuberans, gallbladder carcinoma, Biliary tract tumors, prostate cancer, prostate adenocarcinoma, renal cancer (e.g., Wilms' tumor type 2 or type 1), liver cancer (e.g., hepatoblastoma, hepatocellular carcinoma, hepatocellular cancer), bladder cancer, embryonal rhabdomyosarcoma, germ cell tumor, trophoblastic tumor, testicular germ cells tumor, immature teratoma of ovary, uterine, epithelial ovarian, sacrococcygeal tumor, choriocarcinoma, placental site trophoblastic tumor, epithelial adult tumor, ovarian carcinoma, serous ovarian cancer, ovarian sex cord tumors, cervical carcinoma, uterine cervix carcinoma, small-cell and non-small cell lung carcinoma, nasopharyngeal, breast carcinoma (e.g., ductal breast cancer, invasive intraductal breast cancer, sporadic ; breast cancer, susceptibility to breast cancer, type 4 breast cancer, breast cancer-1, breast cancer-3; breast-ovarian cancer), squamous cell carcinoma (e.g., in head and neck), neurogenic tumor, astrocytoma, ganglioblastoma, neuroblastoma, lymphomas (e.g., Hodgkin's disease, non-Hodgkin's lymphoma, B cell, Burkitt, cutaneous T cell, histiocytic, lymphoblastic, T cell, thymic), gliomas, adenocarcinoma, adrenal tumor, hereditary adrenocortical carcinoma, brain malignancy (tumor), various other carcinomas (e.g., bronchogenic large cell, ductal, Ehrlich-Lettre ascites, epidermoid, large cell, Lewis lung, 20 medullary, mucoepidermoid, oat cell, small cell, spindle cell, spinocellular, transitional cell, undifferentiated, carcinosarcoma, choriocarcinoma, cystadenocarcinoma), ependymoblastoma, epithelioma, erythroleukemia (e.g., Friend, lymphoblast), fibrosarcoma, giant cell tumor, glial tumor, glioblastoma (e.g., multiforme, astrocytoma), glioma hepatoma, heterohybridoma, heteromyeloma, histiocytoma, hybridoma (e.g., B cell), hypernephroma, insulinoma, islet tumor, 25 keratoma, leiomyoblastoma, leiomyosarcoma, leukemia (e.g., acute lymphatic, acute lymphoblastic, acute lymphoblastic pre-B cell, acute lymphoblastic T cell leukemia, acute -megakaryoblastic, monocytic, acute myelogenous, acute myeloid, acute myeloid with eosinophilia, B cell, basophilic, chronic myeloid, chronic, B cell, eosinophilic, Friend, granulocytic or myelocytic, hairy cell, lymphocytic, megakaryoblastic, monocytic, monocytic-macrophage, myeloblastic, myeloid, myelomonocytic, plasma cell, pre-B cell, promyelocytic,

subacute, T cell, lymphoid neoplasm, predisposition to myeloid malignancy, acute nonlymphocytic leukemia), lymphosarcoma, melanoma, mammary tumor, mastocytoma, medulloblastoma, mesothelioma, metastatic tumor, monocyte tumor, multiple myeloma, myelodysplastic syndrome, myeloma, nephroblastoma, nervous tissue glial tumor, nervous tissue 5 neuronal tumor, neurinoma, neuroblastoma, oligodendrogloma, osteochondroma, osteomyeloma, osteosarcoma (e.g., Ewing's), papilloma, transitional cell, pheochromocytoma, pituitary tumor (invasive), plasmacytoma, retinoblastoma, rhabdomyosarcoma, sarcoma (e.g., Ewing's, histiocytic cell, Jensen, osteogenic, reticulum cell), schwannoma, subcutaneous tumor, teratocarcinoma (e.g., pluripotent), teratoma, testicular tumor, thymoma and trichoepithelioma, 10 gastric cancer, fibrosarcoma, glioblastoma multiforme; multiple glomus tumors, Li-Fraumeni syndrome, liposarcoma, lynch cancer family syndrome II, male germ cell tumor, mast cell leukemia, medullary thyroid, multiple meningioma, endocrine neoplasia myxosarcoma, paraganglioma, familial nonchromaffin, pilomatricoma, papillary, familial and sporadic, rhabdoid predisposition syndrome, familial, rhabdoid tumors, soft tissue sarcoma, and Turcot 15 syndrome with glioblastoma.

According to specific embodiments, the cancer is carcinoma.

According to specific embodiments, the cancer is selected from the list of cancers presented in Figure 3A, each possibility represents a separate embodiment of the present invention.

20 According to specific embodiments, the cancer is selected from the group consisting of hepatic cancer, osteosarcoma, breast cancer, colon cancer, thyroid cancer, stomach cancer, lung cancer, kidney cancer, prostate cancer, head and neck cancer, bile duct cancer and bladder cancer, each possibility represents a separate embodiment of the present invention.

According to specific embodiments, the cancer is selected from the group consisting of 25 hepatic cancer, osteosarcoma, breast cancer and colon cancer, each possibility represents a separate embodiment of the present invention.

According to specific embodiments, the cancer is selected from the group consisting of thyroid cancer, hepatic cancer, bile duct cancer and kidney cancer, each possibility represents a separate embodiment of the present invention.

30 According to specific embodiments, the cancer is lung cancer.

According to specific embodiments, the lung cancer is lung squamous carcinoma.

According to specific embodiments, the cancer is a hepatic cancer.

According to specific embodiments, the hepatic cancer is hepatocellular carcinoma.

According to specific embodiments, the cancer is not hepatocellular carcinoma.

35 According to specific embodiments, the cancer is kidney cancer.

According to specific embodiments, the kidney cancer is kidney renal papillary cell carcinoma.

According to specific embodiments, the kidney cancer is kidney renal clear cell carcinoma.

5 According to specific embodiments, the kidney cancer is Kidney chromophobe.

According to specific embodiments, the cancer is a head and neck cancer.

According to specific embodiments, the head and neck cancer is Head Neck Squamous Cell Carcinoma.

According to specific embodiments, the cancer is bile duct cancer.

10 According to specific embodiments, the bile duct cancer is cholangiocarcinoma.

According to specific embodiments, the cancer is thyroid cancer.

According to specific embodiments, the cancer is not thyroid cancer.

According to specific embodiments, the cancer is breast cancer.

As mentioned, the methods of some embodiments of the present invention comprise
15 determining a shift from the urea cycle to pyrimidine synthesis in a cancerous cell of the subject.

As used herein the phrase “a cell of the subject” refers to at least one cell (e.g., an isolated cell), cell culture, cell content and/or cell secreted content which contains RNA and/or proteins of the subject. According to specific embodiments, the cell is comprised in a tissue or an organ.

According to specific embodiments, the cell is a cancerous cell, which can be primary or
20 metastatic.

It should be noted that the cell may be isolated from the subject (e.g., for *in-vitro* detection) or may optionally comprise a cell that has not been physically removed from the subject (e.g., *in-vivo* detection).

According to specific embodiments, the determining is effected *in-vivo* using detection
25 methods suitable for the subject (e.g. human) body (e.g. using antibodies, such as the antibodies e.g. conjugated to a label or to a detectable moiety).

According to other specific embodiments, the determining is effected *in-vitro* or *ex-vivo*.

Thus, according to specific embodiments, the method comprising obtaining the cell prior to the determining.

30 According to specific embodiments the cell is comprised in a biological sample.

The phrase “biological sample” as used herein refers to any cellular biological sample which may express a UC enzyme and CAD enzyme. Examples include but are not limited to, a cell obtained from any tissue biopsy, a tissue, an organ, a blood cell, a bone marrow cell, body fluids such as blood, saliva, spinal fluid, lymph fluid, rinse fluid that may have been in contact
35 with the tumor, the external sections of the skin, respiratory, intestinal, and genitourinary tracts,

urine and feces. According to specific embodiments, the biological sample is an in-situ sample (i.e. of the cancer).

According to specific embodiments, the method of the present invention comprises obtaining the biological sample prior to the determining.

5 The biological sample can be obtained using methods known in the art such as using a syringe with a needle, a scalpel, fine needle biopsy, needle biopsy, core needle biopsy, fine needle aspiration (FNA), surgical biopsy, buccal smear, lavage and the like. According to specific embodiments, the biological sample is obtained by biopsy.

10 It will be appreciated that a specific cell type may be further isolated from the biological sample obtained from the subject. Methods of isolating specific cell types are well known in the art including, but not limited to, density gradient centrifugation, flow cytometry and magnetic beads separation. Thus, for example, a cancerous cell can be isolated from the biological sample by e.g. tumor specific markers.

15 As used herein, the phrase "a shift from the urea cycle to pyrimidine synthesis" refers to a change in the metabolic balance characterized by a decrease in the urea cycle (UC) activity and an increase in pyrimidine synthesis in a cell as compared to a control sample, which may be manifested in e.g. alterations in level and/or activity of a UC enzyme, increased level and/or activity of CAD enzyme, decreased levels of UC metabolites, increased levels of pyrimidines and/or an increased level of purine to pyrimidine transversion mutations.

20 According to specific embodiments, a shift from the urea cycle to pyrimidine synthesis is indicated when the change (e.g. alterations in level and/or activity of a UC enzyme gene, increased level and/or activity of CAD enzyme, increased level of purine to pyrimidine transversion mutations) is above or below a predetermined threshold (depending on the component analyzed).

25 As used herein the phrase "predetermined threshold" refers to a level and/or activity (typically a range) of a component (e.g. a UC enzyme gene, CAD enzyme, purine to pyrimidine transversion mutations) that characterizes a healthy sample of cell. Such a level can be experimentally determined by comparing samples with normal levels of the component (e.g., samples obtained from healthy subjects e.g., not having cancer) to samples derived from subjects diagnosed with cancer. Alternatively, such a level can be obtained from the scientific literature and from databases.

According to specific embodiments, the increase/decrease above or below a predetermined threshold is statistically significant.

30 According to specific embodiments, the predetermined threshold is derived from a control sample.

Several control samples can be used with specific embodiments of the present invention. Typically, the control sample has a balance of UC to pyrimidine synthesis representative of a healthy biological sample.

According to specific embodiments, the control sample contains a level and/or activity of 5 a UC enzyme comparable to a healthy biological sample.

According to specific embodiments, the control sample contains a level and/or activity of a CAD enzyme comparable to a healthy biological sample.

According to specific embodiments, the control sample contains a level of purine to pyrimidine transversion mutations comparable to a healthy biological sample.

10 Since biological characteristics depend on, amongst other things, species and age, it is preferable that the control sample is obtained from a subject of the same species, age, gender and from the same sub-population (e.g. smoker/nonsmoker).

According to specific embodiments, the control sample comprises a cell of the same type as the cell of the subject.

15 According to specific embodiments, the control sample is from the same type as the biological sample obtained from the subject.

According to specific embodiments, the control sample is a healthy control sample.

According to specific embodiments, the control sample is a non-cancerous tissue of said subject.

20 According to specific embodiments, the control sample is a cancerous cell with a level and/or activity of said urea cycle enzyme, said purine to pyrimidine transversion mutations and/or said CAD enzyme similar to levels and/or activity of same in a healthy cell of the same type.

According to specific embodiments, the control sample is obtained from the scientific 25 literature or from a database, such as the known age matched mean value in a non-cancerous population.

According to specific embodiments, the predetermined threshold is at least 1.1 fold, at 30 least 1.2 fold, at least 1.3 fold, at least 1.4 fold, at least 1.5 fold, at least 2 fold, at least 3 fold, at least 5 fold, at least 10 fold, or at least 20 fold as compared the level and/or activity of the component (e.g. a UC enzyme gene, CAD enzyme, purine to pyrimidine transversion mutations) in a control sample as measured using the same assay such as any DNA (e.g. genome sequencing) RNA (e.g. RNA sequencing, PCR, Northern blot), protein (e.g. western blot, immunocytochemistry, flow cytometry), chromatography and mass spectrometry (e.g. LC-MS), enzymatic and/or chemical assay suitable for measuring level and/or activity of a compound, as 35 further disclosed herein.

According to a specific embodiment, the predetermined threshold is at least 1.1 fold compared to a control sample.

According to specific embodiments, the predetermined threshold is at least 2 %, at least 5 %, at least 10 %, at least 20 %, at least 30 %, at least 40 %, at least 50 %, at least 60 %, at least 5
70 %, at least 80 %, at least 90 %, e.g., 100 %, at least 200 %, at least 300 %, at least 400 %, at least 500 %, at least 600 % as compared the level and/or activity of the component in a control sample.

According to specific embodiment, the shift is determined by a level of purine to pyrimidine transversion mutations.

10 Determining the level of purine to pyrimidine transversion mutation can be effected by any method known in the art, such as but not limited to, whole genome sequencing, DNA sequencing and/or RNA sequencing as further described in the Examples section which follows.

According to specific embodiments, determining the level of purine to pyrimidine transversion mutations is effected at the genomic level.

15 According to specific embodiments, determining the level of purine to pyrimidine transversion mutations is effected at the transcript level.

As noted, the present inventors have shown that the shift from the UC to pyrimidine synthesis in cancer is associated with increased purine to pyrimidine transversion mutations at the DNA, RNA and at times at the protein levels, also referred to as non-synonymous, i.e. they
20 change the encoded amino acid.

Thus, according to specific embodiments, the purine to pyrimidine transversion mutations are non-synonymous purine to pyrimidine transversion mutations.

Hence, according to specific embodiments, the shift from the UC to pyrimidine synthesis can be determined by analyzing at the proteomics of newly formed peptide antigens
25 (peptidomics).

Determining non-synonymous transversion mutations are well known in the art and can be effected by direct analysis of the encoded protein(s) or using *in-silico* proteomics. However, such a determination can also be performed at the RNA or DNA level and using *in-silico* translational tools to test the effect on the translated sequence.

30 To determine purine to pyrimidine transversion mutations, DNA or RNA is first extracted from a biological sample of the tested subject, by methods well known in the art. According to specific embodiments, the DNA or RNA sample is amplified prior to determining sequence alterations, since many genotyping methods require amplification of the region carrying the sequence alteration of interest.

The purine to pyrimidine transversion mutations of some embodiments of the invention can be identified using a variety of methods. For example, one option is to determine the entire gene sequence of a PCR reaction product. Alternatively, a given segment of nucleic acid may be characterized by the size of the molecule as may be determined by e.g. electrophoresis by 5 comparison to a known standard run on the same gel. A more detailed picture of the molecule may be achieved by cleavage with combinations of restriction enzymes prior to electrophoresis, to allow construction of an ordered map. The presence of specific sequences within the fragment can be detected by hybridization of a labeled probe, or the precise nucleotide sequence can be determined by partial chemical degradation or by primer extension in the presence of chain-10 terminating nucleotide analogs.

According to specific embodiments, detecting purine to pyrimidine transversion mutations involves directly determining the identity of the nucleotide at the alteration site by a sequencing assay, an enzyme-based mismatch detection assay, or a hybridization assay. The following is a non-limiting description of methods which can be utilized by some embodiments 15 of the invention:

Sequencing analysis - an isolated DNA is subjected to automated dideoxy terminator sequencing reactions using a dye-terminator (unlabeled primer and labeled di-deoxy nucleotides) or a dye-primer (labeled primers and unlabeled di-deoxy nucleotides) cycle sequencing protocols. For the dye-terminator reaction, a PCR reaction is performed using unlabeled PCR 20 primers followed by a sequencing reaction in the presence of one of the primers, deoxynucleotides and labeled di-deoxy nucleotide mix. For the dye-primer reaction, a PCR reaction is performed using PCR primers conjugated to a universal or reverse primers (one at each direction) followed by a sequencing reaction in the presence of four separate mixes 25 (correspond to the A, G, C, T nucleotides) each containing a labeled primer specific the universal or reverse sequence and the corresponding unlabeled di-deoxy nucleotides.

Hybridization Assay Methods - Hybridization based assays which allow the detection of single base alterations rely on the use of oligonucleotide which can be 10, 15, 20, or 30 to 100 nucleotides long preferably from 10 to 50, more preferably from 40 to 50 nucleotides. Typically, the oligonucleotide includes a central nucleotide complementary to a specific site of a gene and 30 flanking nucleotide sequences spanning on each side of the central nucleotide and substantially complementary to the nucleotide sequences of the gene. Sequence alteration can be detected by hybridization of the oligonucleotide of some embodiments of the invention to the template sequence under stringent hybridization reactions.

The hybridization assay can be effected with oligonucleotide arrays. The chip/array 35 technology has already been applied with success in numerous cases. For example, the

screening of mutations has been undertaken in the BRCA1 gene, in *S. cerevisiae* mutant strains, and in the protease gene of HIV-1 virus [see Hacia et al., (1996) *Nat Genet* 1996;14(4):441-447; Shoemaker et al., (1996) *Nat Genet* 1996;14(4):450-456; Kozal et al., (1996) *Nat Med* 1996;2(7):753-759].

5 For determining purine to pyrimidine transversion mutations, sets of four oligonucleotide probes (one for each base type), preferably sets of two oligonucleotide probes (one for each base type of the biallelic marker) are generally designed that span each position of a portion of the candidate region found in the nucleic acid sample, differing only in the identity of the mutation base. The relative intensity of hybridization to each series of probes at a particular location
10 allows the identification of the base corresponding to the mutation base of the probe.

Single-Strand Conformation Polymorphism (SSCP) - The method (reviewed by Hayashi, *PCR Meth. Appl.*, 1:34-38, 1991) is based on the observation that single strands of nucleic acid can take on characteristic conformations in non-denaturing conditions, and these conformations influence electrophoretic mobility. The complementary strands assume sufficiently different structures that one strand may be resolved from the other. Changes in sequences within the fragment will also change the conformation, consequently altering the mobility and allowing this to be used as an assay for sequence variations (Orita, et al., *Genomics* 5:874-879, 1989; Orita et al. 1989, *Proc. Natl. Acad. Sci. U.S.A.* 86:2776-2770). The SSCP process involves denaturing a DNA segment (e.g., a PCR product) that is labeled on both strands, followed by slow
15 electrophoretic separation on a non-denaturing polyacrylamide gel, so that intra-molecular interactions can form and not be disturbed during the run. This technique is extremely sensitive to variations in gel composition and temperature. A serious limitation of this method is the relative difficulty encountered in comparing data generated in different laboratories, under
20 apparently similar conditions.

25 Dideoxy fingerprinting (ddF) - The ddF technique combines components of Sanger dideoxy sequencing with SSCP (see Liu and Sommer, *PCR Methods Appl.*, 4:97, 1994). A dideoxy sequencing reaction is performed using one dideoxy terminator and then the reaction products are electrophoresed on nondenaturing polyacrylamide gels to detect alterations in mobility of the termination segments as in SSCP analysis.

30 Restriction fragment length polymorphism (RFLP) - This method uses a change in a single nucleotide which modifies a recognition site for a restriction enzyme resulting in the creation or destruction of an RFLP. Single nucleotide mismatches in DNA heteroduplexes are also recognized and cleaved by some chemicals, providing an alternative strategy to detect single base substitutions, generically named the "Mismatch Chemical Cleavage" (MCC) (Gogos et al.,
35 *Nucl. Acids Res.*, 18:6807-6817, 1990). However, this method requires the use of osmium

tetroxide and piperidine, two highly noxious chemicals which are not suited for use in a clinical laboratory.

According to specific embodiments, elevation of at least 2 %, at least 5 %, at least 10 %, at least 20 %, at least 30 %, at least 40 %, at least 50 %, at least 60 %, at least 70 %, at least 80 %, at least 90 %, e.g., 100 %, at least 200 %, at least 300 %, at least 400 %, at least 500 %, at least 600 % in the level of purine to pyrimidine transversion mutations in a genome or RNA sequence as compared to a control sample using e.g. an assay disclosed hereinabove, is indicative of a shift from the UC to pyrimidine synthesis.

According to specific embodiments, elevation of at least 2 %, at least 5 %, at least 10 %, at least 20 %, at least 30 %, at least 40 %, at least 50 %, at least 60 %, at least 70 %, at least 80 %, at least 90 %, e.g., 100 %, at least 200 %, at least 300 %, at least 400 %, at least 500 %, at least 600 % in the level of non-synonymous purine to pyrimidine transversion mutations in a genome sequence, a RNA sequence or in a protein sequence as compared to a control sample is indicative of a shift from the UC to pyrimidine synthesis.

The present inventors have also shown that the shift from the UC to pyrimidine synthesis in cancer is associated with alterations in the expression levels of UC and CAD enzymes.

Thus, according to specific embodiments, the shift is determined by a level and/or activity of a urea cycle enzyme and/or a CAD enzyme.

The urea cycle (UC) is a metabolic process which converts excess nitrogen derived from the breakdown of nitrogen-containing molecules to the excretable nitrogenous compound - urea. The UC enzymes encompassed by specific embodiments of the present invention are selected from the group consisting of ASL, ASS1, OTC, SLC25A15, CPS1 and SLC25A13.

“ASL (argininosuccinate lyase)”, E.C. No. 4.3.2.1, also known as argininosuccinase and ASAL, refers to the polynucleotide or polypeptide expression product of the ASL gene (Gene ID 435). ASL catalyzes the hydrolytic cleavage of argininosuccinate (ASA) into arginine and fumarate. According to a specific embodiment, the ASL refers to the human ASL, such as provided in the following Accession Numbers: NM_000048, NM_001024943, NM_001024944, NM_001024946, NP_000039, NP_001020114, NP_001020115 and NP_001020117 (SEQ ID NO: 8).

“ASS1 (Argininosuccinate synthase 1)”, E.C. No. 6.3.4.5, also known as or Argininosuccinate synthase, Argininosuccinate synthetase, Citrulline-Aspartate Ligase and CTLN1, refers to the polynucleotide or polypeptide expression product of the ASS1 gene (Gene ID 445). ASS1 catalyzes the synthesis of argininosuccinate from citrulline and aspartate. According to a specific embodiment, the ASS1 refers to the human ASS1, such as provided in the following Accession Numbers: NM_000050, NM_054012, XM_005272200,

XM_011518705, XM_017014729, NP_000041, NP_446464, XP_005272257, XP_011517007 and XP_016870218.

“OTC (Ornithine transcarbamylase)” E.C. No. 2.1.3.3, also known as ornithine carbamoyltransferase and OTCase, refers to the polynucleotide or polypeptide expression product of the OTC gene (Gene ID 5009). OTC catalyzes the reaction between carbamoyl phosphate and ornithine to form citrulline and phosphate. According to a specific embodiment, the OTC refers to the human OTC, such as provided in the following Accession Numbers: NM_000531, XM_017029556, NP_000522 and XP_016885045.

“SLC25A15 (Solute Carrier Family 25 Member 15)” or “ORNT1 (Ornithine Transporter 1)”, which are interchangeably used herein, refers to the polynucleotide or polypeptide expression product of the SLC25A15 gene (Gene ID: 10166). SLC25A15, a member of the mitochondrial carrier family, transports ornithine from the cytosol into the mitochondria. According to a specific embodiment, the SLC25A15 refers to the human SLC25A15, such as provided in the following Accession Numbers: NM_014252 and NP_055067.

“CPS1 (Carbamoyl Phosphate Synthetase I)” E.C. No. 6.3.4.16, refers to the polynucleotide or polypeptide expression product of the CPS1 gene (Gene ID 1373). CPS1 catalyzes synthesis of carbamoyl phosphate from ammonia and bicarbonate. The overall reaction that occurs in CPSI is:



According to a specific embodiment, the CPS1 refers to the human CPS1, such as provided in the following Accession Numbers: NM_001122633, NM_001122634, NM_001875, XM_011510640, XM_011510641, NP_001116105, NP_001116106, NP_001866, XP_011508942, XP_011508943, XP_011508944, XP_011508945 and XP_011508946.

“SLC25A13 (Solute Carrier Family 25 Member 13)”, also known as citrin, Mitochondrial Aspartate Glutamate Carrier 2, ARALAR2, CTLN2 and Calcium-Binding Mitochondrial Carrier Protein Aralar2, refers to the polynucleotide or polypeptide expression product of the SLC25A13 gene (Gene ID 10165). SLC25A13, a protein member of the mitochondrial carrier family, catalyzes the exchange of aspartate for glutamate and a proton across the inner mitochondrial membrane, and is stimulated by calcium on the external side of the inner mitochondrial membrane. The SLC25A13 protein contains four EF-hand Ca(2+) binding motifs in the N-terminal domain, and is localized in the mitochondria. According to a specific embodiment, the SLC25A13 refers to the human SLC25A13, such as provided in the following Accession Numbers: NM_001160210, NM_014251, XM_006715831, XM_011515727, XM_017011663, NP_001153682, NP_055066, XP_006715894, XP_011514029, XP_016867152, XP_016867153 and XP_016867154.

“CAD (carbamoyl-phosphate synthetase 2, aspartate transcarbamylase, and dihydroorotase)” refers to the polynucleotide or polypeptide expression product of the CAD gene (Gene ID: 790). CAD is a trifunctional protein which is associated with the enzymatic activities of the first 3 enzymes in the *de-novo* pyrimidine synthesis pathway:

5 - Carbamoyl phosphate synthetase, E.C. No. 6.3.5.5 (CPS II), catalyzes the reaction of 2 ATP + L-glutamine + HCO(3)(-) + H(2)O \rightleftharpoons 2 ADP + phosphate + L-glutamate + carbamoyl phosphate;

- Aspartate transcarbamoylase, E.C. No. 2.1.3.2, catalyzes the reaction of Carbamoyl phosphate + L-aspartate \rightleftharpoons phosphate + N-carbamoyl-L-aspartate; and

10 - Dihydroorotate, E.C. No. 3.5.2.3, catalyses the reaction of (S)-dihydroorotate + H(2)O \rightleftharpoons N-carbamoyl-L-aspartate.

According to a specific embodiment, the CAD refers to the human CAD, such as provided in the following Accession Numbers: NM_001306079, NM_004341, XM_005264555, XM_006712101, NP_001293008, NP_004332, XP_005264612, and XP_006712164.

15 The trifunctional protein encoded by CAD is regulated by the mitogen-activated protein kinase (MAPK) cascade.

According to specific embodiments, CAD is activated CAD. An activated CAD is phosphorylated. Phosphorylation of CAD can be determined by any method known in the art, such as but not limited to, Western blot, Elisa, flow cytometry and mass spectrometry.

20 The terms ASL, ASS1, OTC, SLC25A15, CPS1, SLC25A13 and CAD also encompass functional homologues (naturally occurring or synthetically/recombinantly produced), orthologs (from other species) which exhibit the desired enzymatic activity described hereinabove. The functional homologs also refer to functional portions of ASL, ASS1, OTC, SLC25A15, CPS1, SLC25A13 and CAD which maintain the enzymatic activity of the full length proteins, as 25 described hereinabove.

As used herein, the term “activity” refers to activity of an enzyme per cell. Methods of determining activity of the enzymes disclosed herein are well known in the art and include both enzymatic and chemical assays.

30 As used herein, the phrase “level” when relating to an enzyme (e.g. a UC enzyme, CAD enzyme) refers to the degree of gene expression (e.g. mRNA or protein).

It should be noted that the expression level can be determined in arbitrary absolute units, or in normalized units (relative to known expression levels of a control sample). For example, when using DNA chips, the expression levels are normalized according to internal controls or by using quantile normalization such as RMA.

Expression level can be determined in the cell using any structural, biological or biochemical method which is known in the art for detecting the expression level at the transcript or the protein level.

According to specific embodiments of the invention, the RNA or the protein molecules 5 are extracted from the cell of the subject. Thus, according to specific embodiments, the method further comprises extracting RNA or a protein from the cell prior to the determining.

Methods of extracting RNA or protein molecules from cells of a subject are well known in the art. The extracted RNA can be further processed to a cDNA. Methods of and commercially available kits for converting RNA to cDNA are well known in the art and include 10 e.g. the use of the enzyme reverse transcriptase. Once obtained, the RNA, cDNA or protein molecules can be characterized for the level of various RNA, cDNA and/or protein molecules using methods known in the arts.

According to specific embodiment, determining the level of the enzyme is effected at the transcript level using RNA or DNA detection methods.

15 Thus, according to some embodiments of the invention, detection of the level of the UC enzyme and/or CAD enzyme is performed by contacting the biological sample, the tissue, the cell, or fractions or extracts thereof with a probe (e.g. oligonucleotide probe or primer) which specifically hybridizes to a polynucleotide expressed from the UC gene (e.g. ASL, ASS1, OTC, SLC25A15, CPS1, SLC25A13) and/or CAD gene. Such a probe can be at any size, such as a 20 short polynucleotide (e.g., of 15-200 bases), an intermediate polynucleotide of 100-2000 bases and a long polynucleotide of more than 2000 bases.

The probe used by the present invention can be any directly or indirectly labeled RNA 25 molecule [e.g., RNA oligonucleotide (e.g., of 17-50 bases), an *in-vitro* transcribed RNA molecule], DNA molecule (e.g., oligonucleotide, e.g., 15-50 bases, cDNA molecule, genomic molecule) and/or an analogue thereof [e.g., peptide nucleic acid (PNA)] which is specific to the RNA transcript of the UC and/or CAD gene. According to specific embodiments, the probe is bound to a detectable moiety.

Oligonucleotides designed according to the teachings of the present invention can be generated according to any oligonucleotide synthesis method known in the art such as enzymatic 30 synthesis or solid phase synthesis.

According to specific embodiments, the contacting is effected under conditions which allow the formation of a complex comprising mRNA or cDNA of a UC (e.g. ASL, ASS1, OTC, SLC25A15, CPS1, SLC25A13) and/or CAD gene present in the cell and the probe. The complex can be formed at a variety of temperatures, salt concentration and pH values which may vary

depending on the method and the biological sample used and those of skills in the art are capable of adjusting the conditions suitable for the formation of each nucleotide/probe complex.

Thus, according to an aspect of the present invention there is provided a composition of matter comprising RNA of a cancerous cell of a subject and a probe capable of detecting a polynucleotide expressed from a UC (e.g. ASL, ASS1, OTC, SLC25A15, CPS1, SLC25A13) and/or a CAD gene. According to specific embodiments, the composition further comprises an RNase inhibitor.

Non-limiting examples of methods of detecting RNA and/or cDNA molecules in a cell sample include Northern blot analysis, RT-PCR [e.g., a semi-quantitative RT-PCR, quantitative RT-PCR using e.g., the Light Cycler™ (Roche)], RNA *in-situ* hybridization (using e.g., DNA or RNA probes to hybridize RNA molecules present in the cells or tissue sections), *in-situ* RT-PCR (e.g., as described in Nuovo GJ, et al. Am J Surg Pathol. 1993, 17: 683-90; Komminoth P, et al. Pathol Res Pract. 1994, 190: 1017-25), and oligonucleotide microarray (e.g., by hybridization of polynucleotide sequences derived from a sample to oligonucleotides attached to a solid surface [e.g., a glass wafer] with addressable location, such as Affymetrix microarray (Affymetrix®, Santa Clara, CA)].

As mentioned, according to specific embodiments, determining the level of the enzyme is effected at the protein level using protein detection methods.

Thus, according to some embodiments of the invention, detection of the level of the protein of the UC and/or CAD is performed by contacting the biological sample, the tissue, the cell, or fractions or extracts thereof with an antibody which specifically binds to a UC enzyme (e.g. ASL, ASS1, OTC, SLC25A15, CPS1, SLC25A13) and/or a CAD enzyme. According to specific embodiments, the contacting is effected under conditions which allow the formation of a complex comprising a UC enzyme (e.g. ASL, ASS1, OTC, SLC25A15, CPS1, SLC25A13) and/or a CAD enzyme present in the cell and the antibody (i.e. immunocomplex).

The immunocomplex can be formed at a variety of temperatures, salt concentration and pH values which may vary depending on the method and the biological sample used and those of skills in the art are capable of adjusting the conditions suitable for the formation of each immunocomplex.

Thus, according to an aspect of the present invention there is provided a composition of matter comprising a lysate of a cancerous cell of a subject, and an antibody capable of detecting a UC enzyme (e.g. ASL, ASS1, OTC, SLC25A15, CPS1, SLC25A13) and/or a CAD enzyme. According to a specific embodiment, the composition further comprises a secondary antibody capable of binding the antibody. According to specific embodiments, the composition further comprises a protease inhibitor.

Non-limiting examples of methods of detecting the level of specific protein molecules in a cell sample include Enzyme linked immunosorbent assay (ELISA), Western blot analysis, immunoprecipitation (IP), radio-immunoassay (RIA), Fluorescence activated cell sorting (FACS), immunohistochemical analysis, *in-situ* activity assay (using e.g., a chromogenic substrate applied on the cells containing an active enzyme), *in-vitro* activity assays (in which the activity of a particular enzyme is measured in a protein mixture extracted from the cells) and molecular weight-based approach.

The antibody or probe used by the present invention can be any directly or indirectly labeled antibody or probe. According to specific embodiments, the antibody or probe is bound to a detectable moiety.

The detectable moiety used by some embodiments of the invention can be, but is not limited to a fluorescent chemical (fluorophore), a phosphorescent chemical, a chemiluminescent chemical, a radioactive isotope (such as $[^{125}\text{I}]$ iodine), an enzyme, a fluorescent polypeptide, an affinity tag, and molecules (contrast agents) detectable by Positron Emission Tomography (PET) or Magnetic Resonance Imaging (MRI).

As the teachings of the present invention indicate that a shift from the urea cycle to pyrimidine synthesis in a cancerous cell of subjects indicate higher tumor grade and decreased survival, the methods of the present invention can be used for prognosing and treating cancer and for monitoring efficacy of cancer therapy.

Thus, according to an aspect of the present invention there is provided a method of prognosing cancer in a subject diagnosed with cancer, the method comprising determining a level of purine to pyrimidine transversion mutations in a cancerous cell of the subject as compared to a control sample, wherein said level of said purine to pyrimidine transversion mutations above a predetermined threshold is indicative of poor prognosis, thereby prognosing cancer in the subject.

Thus, an increased level of purine to pyrimidine transversion mutations is indicative of poor prognosis. On the other hand, no change in the purine to pyrimidine transversion mutations levels, or a decreased level of the purine to pyrimidine transversion mutations, indicates better prognosis.

As used herein the term “prognosing” refers to determining the outcome of the disease (cancer).

As used herein “poor prognosis” refers to increased risk of death due to the disease, increased risk of progression of the disease (e.g. cancer grade), and/or increased risk of recurrence of the disease.

According to another aspect of the present invention, there is provided a method of prognosing cancer in a subject diagnosed with cancer, the method comprising determining a level

and/or activity of a urea cycle enzyme SLC25A15 in a cancerous cell of the subject as compared to a control sample, wherein said level and/or activity of said SLC25A15 below a predetermined threshold is indicative of poor prognosis, thereby prognosing cancer in the subject.

According to another aspect of the present invention, there is provided a method of prognosing cancer in a subject diagnosed with cancer, the method comprising determining a level and/or activity of at least two urea cycle enzymes selected from the group consisting of ASL, ASS1, OTC, SLC25A15, CPS1 and SLC25A13 in a cancerous cell of the subject as compared to a control sample, wherein said urea cycle enzymes comprise ASS1 and SLC25A13 said at least two is at least three; wherein said level and/or activity of said ASL, said ASS1, said OTC and/or said SLC25A15 below a predetermined threshold and/or said level and/or activity of said CPS1 and/or said SLC25A13 above a predetermined threshold is indicative of poor prognosis, thereby prognosing cancer in the subject.

According to another aspect of the present invention, there is provided a method of prognosing breast cancer in a subject diagnosed with breast cancer, the method comprising determining a level and/or activity of at least two urea cycle enzymes selected from the group consisting of ASS1, OTC and SLC25A13 in a cancerous cell of the subject as compared to a control sample, wherein said level and/or activity of said ASS1 and/or said OTC below a predetermined threshold and/or said level and/or activity of said SLC25A13 above a predetermined threshold is indicative of poor prognosis, thereby prognosing the breast cancer in the subject.

Thus, a decreased level and/or activity of a UC enzyme selected from the group consisting of ASL, ASS1, OTC and SLC25A15 and/or an increased level a UC enzyme selected from the group consisting of CPS1 and SLC25A13 is indicative of poor prognosis. On the other hand, no change in the UC enzyme selected from the group consisting of ASL, ASS1, OTC, SLC25A15, CPS1 and SLC25A13 or an increased level and/or activity of a UC enzyme selected from the group consisting of ASL, ASS1, OTC and SLC25A15 and/or a decreased level and/or activity of the UC enzyme selected from the group consisting of CPS1 and SLC25A13, indicates better prognosis.

According to specific embodiments, at least 2, at least 3, at least 4, at least 5 or all 6 UC enzymes (ASL, ASS1, OTC, SLC25A15, CPS1 and SLC25A13) are determined, each possibility represent a separate embodiment of the present invention.

According to specific embodiments, the at least two UC enzymes determined are ASL+ASS1, ASL+OTC, ASL+SLC25A15, ASL+CPS1, ASL+SLC25A13, ASS1+OTC, ASS1+SLC25A15, ASS1+CPS1, ASS1+SLC25A13, OTC+SLC25A15, OTC+CPS1,

OTC+SLC25A13, SLC25A15+CPS1, SLC25A15+SLC25A13 or CPS1+SLC25A13, each possibility represent a separate embodiment of the present invention.

According to specific embodiments, the at least three UC enzymes determined are ASL+ASS1+OTC, ASL+ASS1+SLC25A15, ASL+ASS1+CPS1, ASL+ASS1+SLC25A13, 5 ASL+OTC+SLC25A15, ASL+OTC+CPS1, ASL+OTC+SLC25A13, ASL+SLC25A15+CPS1, ASL+SLC25A15+SLC25A13, ASL+CPS1+SLC25A13, ASS1+OTC+SLC25A15, ASS1+OTC+CPS1, ASS1+OTC+SLC25A13, ASS1+SLC25A15+CPS1, ASS1+SLC25A15+SLC25A14, ASS1+CPS1+SLC25A13, OTC+SLC25A15+CPS1, OTC+CPS1+SLC25A13 or SLC25A15+CPS1+SLC25A13, each possibility represent a separate 10 embodiment of the present invention.

According to specific embodiments, the at least four UC enzymes determined are ASL+ASS1+OTC+SLC25A15, ASL+ASS1+OTC+CPS1, ASL+ASS1+OTC+SLC25A13, ASL+OTC+SLC25A15+CPS1, ASL+OTC+CLC25A15+SLC25A13, ASL+SLC25A15+CPS1+SLC25A13, ASS1+OTC+SLC25A15+CPS1, 15 ASS1+OTC+SLC25A15+SLC25A13, ASS1+SLC25A15+CPS1+SLC25A13 or OTC+SLC25A15+CPS1+SLC25A13, each possibility represent a separate embodiment of the present invention.

According to specific embodiments, the at least five UC enzymes determined are ASL+ASS1+OTC+SLC25A15+CPS1, ASL+ASS1+OTC+SLC25A15+SLC25A13 or ASS1+OTC+SLC25A15+CPS1+SLC25A13, each possibility represent a separate embodiment of the present invention.

According to specific embodiments, the cancer is thyroid, stomach and/or bladder cancer and the at least two UC enzymes are selected from the group consisting of OTC, SLC25A15 and SLC25A13.

According to specific embodiments, the cancer is prostate cancer and the at least two UC enzymes comprise ASS1 and CSP1.

According to specific embodiments, the cancer is lung and/or head and neck cancer and the at least two UC enzymes are selected from the group consisting of ASL, OTC, CPS1 and SLC25A13.

According to specific embodiments, the cancer is hepatic, bile duct and/or kidney cancer and the at least two UC enzymes are selected from the group consisting of ASL, ASS1, OTC and SLC25A15.

According to specific embodiments, the cancer is breast cancer and the at least three UC enzymes comprise ASS1, OTC and SLC25A13.

According to specific embodiments, alteration in level and/or activity in the specified direction of at least 1, at least 2, at least 3, at least 4, at least 5 or all 6 UC enzymes (ASL, ASS1, OTC, SLC25A15, CPS1 and SLC25A13) is indicative of poor prognosis, each possibility represent a separate embodiment of the present invention.

5 According to specific embodiments, alteration in level and/or activity in the specified direction of the at least two UC enzymes ASL+ASS1, ASL+OTC, ASL+SLC25A15, ASL+CPS1, ASL+SLC25A13, ASS1+OTC, ASS1+SLC25A15, ASS1+CPS1, ASS1+SLC25A13, OTC+SLC25A15, OTC+CPS1, OTC+SLC25A13, SLC25A15+CPS1, SLC25A15+SLC25A13 or CPS1+SLC25A13 is indicative of poor prognosis, each possibility represent a separate

10 embodiment of the present invention

According to specific embodiments, alteration in level and/or activity in the specified direction of the least three UC enzymes ASL+ASS1+OTC, ASL+ASS1+SLC25A15, ASL+ASS1+CPS1, ASL+ASS1+SLC25A13, ASL+OTC+SLC25A15, ASL+OTC+CPS1, ASL+OTC+SLC25A13, ASL+SLC25A15+CPS1, ASL+SLC25A15+SLC25A13, 15 ASL+CPS1+SLC25A13, ASS1+OTC+SLC25A15, ASS1+OTC+CPS1, ASS1+OTC+SLC25A13, ASS1+SLC25A15+CPS1, ASS1+SLC25A15+SLC25A14, ASS1+CPS1+SLC25A13, OTC+SLC25A15+CPS1, OTC+CPS1+SLC25A13 or SLC25A15+CPS1+SLC25A13, is indicative of poor prognosis, each possibility represent a separate embodiment of the present invention.

20 According to specific embodiments, alteration in level and/or activity in the specified direction of the at least four UC enzymes ASL+ASS1+OTC+SLC25A15, ASL+ASS1+OTC+CPS1, ASL+ASS1+OTC+SLC25A13, ASL+OTC+SLC25A15+CPS1, ASL+OTC+CLC25A15+SLC25A13, ASL+SLC25A15+CPS1+SLC25A13, ASS1+OTC+SLC25A15+CPS1, ASS1+OTC+SLC25A15+SLC25A13, 25 ASS1+SLC25A15+CPS1+SLC25A13 or OTC+SLC25A15+CPS1+SLC25A13, is indicative of poor prognosis, each possibility represent a separate embodiment of the present invention.

According to specific embodiments, alteration in level and/or activity in the specified direction of the at least five UC enzymes ASL+ASS1+OTC+SLC25A15+CPS1, ASL+ASS1+OTC+SLC25A15+SLC25A13 or ASS1+OTC+SLC25A15+CPS1+ SLC25A13, is indicative of poor prognosis, each possibility represent a separate embodiment of the present invention.

According to specific embodiments, the methods disclosed herein further comprising determining a level and/or activity of a CAD enzyme, wherein said level and/or activity of said CAD enzyme above a predetermined threshold is indicative of poor prognosis.

According to specific embodiments, the methods disclosed herein comprise corroborating the prognosis using a state of the art technique.

Such methods are known in the art and depend on the cancer type and include, but not limited to, complete blood count (CBC), tumor marked tests (also known as biomarkers),
5 imaging (such as MRI, CT scan, PET-CT, ultrasound, mammography and bone scan), endoscopy, colonoscopy, biopsy and bone marrow aspiration.

According to another aspect of the present invention, there is provided a method of monitoring efficacy of cancer therapy in a subject, the method comprising determining a level of purine to pyrimidine transversion mutations in a cancerous cell of the subject undergoing or
10 following the cancer therapy, wherein a decrease in said level of said purine to pyrimidine transversion mutations from a predetermined threshold or in comparison to said level in said subject prior to said cancer therapy, indicates efficacious cancer therapy.

Thus, a decrease in the level of purine to pyrimidine transversion mutations is indicative of the cancer therapy being efficient. On the other hand, if there is no change in the purine to pyrimidine transversion mutations levels, or in case there is an increase in the level of the purine to pyrimidine transversion mutations, then the cancer therapy is not efficient in eliminating (e.g., killing, depleting) the cancerous cells from the treated subject and additional and/or alternative therapies (e.g., treatment regimens) may be used.

According to another aspect of the present invention, there is provided a method of monitoring efficacy of cancer therapy in a subject, the method comprising determining a level and/or activity of a urea cycle enzyme SLC25A15 in a cancerous cell of the subject undergoing or following the cancer therapy, wherein an increase in said level and/or activity of said a urea cycle enzyme SLC25A15 from a predetermined threshold or in comparison to said level and/or activity in said subject prior to said cancer therapy, indicates efficacious cancer therapy.

According to another aspect of the present invention, there is provided a method of monitoring efficacy of cancer therapy in a subject, the method comprising determining a level and/or activity of at least two urea cycle enzymes selected from the group consisting of ASL, ASS1, OTC, SLC25A15, CPS1 and SLC25A13 in a cancerous cell of the subject undergoing or following the cancer therapy, wherein said urea cycle enzymes comprise ASS1 and SLC25A13
25 said at least two is at least three; wherein an increase in said level and/or activity of said ASL, said ASS1, said OTC and/or said SLC25A15 and/or a decrease in said level and/or activity of said CPS1 and/or said SLC25A13 from a predetermined threshold or in comparison to said level and/or activity in said subject prior to said cancer therapy indicates efficacious cancer therapy.

According to another aspect of the present invention, there is provided a method of monitoring efficacy of cancer therapy in a subject diagnosed with breast cancer, the method
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comprising determining a level and/or activity of at least two urea cycle enzymes selected from the group consisting of ASS1, OTC and SLC25A13 in a cancerous cell of the subject undergoing or following the cancer therapy, wherein an increase in said level and/or activity of said ASS1 and/or said OTC and/or a decrease in said level and/or activity of said SLC25A13 from a 5 predetermined threshold or in comparison to said level and/or activity in said subject prior to said cancer therapy, indicates efficacious cancer therapy.

Thus, an increase in the level and/or activity of a UC enzyme selected from the group consisting of ASL, ASS1, OTC and SLC25A15 and/or a decrease in the level and/or activity of a UC enzyme selected from the group consisting of CPS1 and SLC25A13 is indicative of the 10 cancer therapy being efficient. On the other hand, if there is no change in the enzymes level and/or activity, or in case there is a decrease in the level and/or activity of a UC enzyme selected from the group consisting of ASL, ASS1, OTC and SLC25A15 and/or an increase in the level and/or activity a UC enzyme selected from the group consisting of CPS1 and SLC25A13, then the cancer therapy is not efficient in eliminating (e.g., killing, depleting) the cancerous cells from 15 the treated subject and additional and/or alternative therapies (e.g., treatment regimens) may be used.

According to specific embodiments, alteration in level and/or activity in the specified direction of at least 1, at least 2, at least 3, at least 4, at least 5 or all 6 UC enzymes is indicative of efficacious cancer therapy, each possibility represent a separate embodiment of the present 20 invention.

According to specific embodiments, alteration in level and/or activity in the specified direction of the at least two UC enzymes ASL+ASS1, ASL+OTC, ASL+SLC25A15, ASL+CPS1, ASL+SLC25A13, ASS1+OTC, ASS1+SLC25A15, ASS1+CPS1, ASS1+SLC25A13, OTC+SLC25A15, OTC+CPS1, OTC+SLC25A13, SLC25A15+CPS1, SLC25A15+SLC25A13 25 or CPS1+SLC25A13 is of efficacious cancer therapy, each possibility represent a separate embodiment of the present invention.

According to specific embodiments, alteration in level and/or activity in the specified direction of the least three UC enzymes ASL+ASS1+OTC, ASL+ASS1+SLC25A15, ASL+ASS1+CPS1, ASL+ASS1+SLC25A13, ASL+OTC+SLC25A15, ASL+OTC+CPS1, 30 ASL+OTC+SLC25A13, ASL+SLC25A15+CPS1, ASL+SLC25A15+SLC25A13, ASL+CPS1+SLC25A13, ASS1+OTC+SLC25A15, ASS1+OTC+CPS1, ASS1+OTC+SLC25A13, ASS1+OTC+SLC25A14, ASS1+CPS1+SLC25A13, OTC+SLC25A15+CPS1, OTC+CPS1+SLC25A13 or 35 SLC25A15+CPS1+SLC25A13, is indicative of efficacious cancer therapy, each possibility represent a separate embodiment of the present invention.

According to specific embodiments, alteration in level and/or activity in the specified direction of the at least four UC enzymes ASL+ASS1+OTC+SLC25A15, ASL+ASS1+OTC+CPS1, ASL+ASS1+OTC+SLC25A13, ASL+OTC+SLC25A15+CPS1, ASL+OTC+CLC25A15+SLC25A13, ASL+SLC25A15+CPS1+SLC25A13, ASS1+OTC+SLC25A15+CPS1, ASS1+OTC+SLC25A15+SLC25A13, ASS1+SLC25A15+CPS1+SLC25A13 or OTC+SLC25A15+CPS1+SLC25A13, is indicative of efficacious cancer therapy, each possibility represent a separate embodiment of the present invention.

According to specific embodiments, alteration in level and/or activity in the specified direction of the at least five UC enzymes ASL+ASS1+OTC+SLC25A15+CPS1, ASL+ASS1+OTC+SLC25A15+SLC25A13 or ASS1+OTC+SLC25A15+CPS1+ SLC25A13, is indicative of efficacious cancer therapy, each possibility represent a separate embodiment of the present invention.

According to specific embodiments, the methods disclosed herein further comprising determining a level and/or activity of a CAD enzyme, wherein a decrease in the level and/or activity of said CAD enzyme from a predetermined threshold or in comparison to said level in said subject prior to said cancer therapy, indicates efficacious cancer therapy.

According to specific embodiments of the monitoring aspects of the present invention, the predetermined threshold is in comparison to the level in the subject prior to cancer therapy.

According to specific embodiments of the monitoring aspects of the present invention, the predetermined threshold is at least 1.1 fold, at least 1.2 fold, at least 1.3 fold, at least 1.4 fold, at least 1.5 fold, at least 2 fold, at least 3 fold, at least 5 fold, at least 10 fold, or at least 20 fold as compared the level and/or activity of the component (e.g. a UC enzyme, CAD enzyme, purine to pyrimidine transversion mutations) in a control sample or in the subject prior to the cancer therapy as measured using the same assay such as any DNA (e.g. genome sequencing) RNA (e.g. RNA sequencing, PCR, Northern blot), protein (e.g. western blot, immunocytochemistry, flow cytometry), chromatography and mass spectrometry (e.g. LC-MS), enzymatic and/or chemical assay suitable for measuring level and/or activity of a compound, as further disclosed herein.

According to a specific embodiment, the predetermined threshold is at least 1.1 fold as compared the level and/or activity of the component in a control sample or in the subject prior to the cancer therapy.

According to specific embodiments, the predetermined threshold is at least 2 %, at least 5 %, at least 10 %, at least 20 %, at least 30 %, at least 40 %, at least 50 %, at least 60 %, at least 70 %, at least 80 %, at least 90 %, e.g., 100 %, at least 200 %, at least 300 %, at least 400 %, at

least 500 %, at least 600 % as compared the level and/or activity of the component in a control sample or in the subject prior to the cancer therapy.

According to other specific embodiments of this aspect of the present invention, the pre-determined threshold can be determined in a subset of subjects with known outcome of cancer therapy.

According to specific embodiments of the present invention, there is provided a method of treating cancer in a subject in need thereof, the method comprising:

- (a) prognosing the subject according to the methods described herein; and
- (b) treating said subject with a cancer therapy according to the prognosis.

According to specific embodiments of the present invention, there is provided a method of treating cancer in a subject in need thereof, the method comprising:

(a) prognosing the subject according to the methods described herein; and wherein when a poor prognosis is indicated

- (b) treating said subject with a cancer therapy.

According to specific embodiments of the present invention, there is provided a method of treating cancer in a subject in need thereof, the method comprising:

- (a) prognosing the subject according to the methods described herein; and
- (b) selecting a cancer therapy for treating said subject based on the prognosis.

According to specific embodiments of the present invention, there is provided a method of treating cancer in a subject in need thereof, the method comprising:

(a) prognosing the subject according to the methods described herein; and wherein when a poor prognosis is indicated

- (b) selecting a cancer therapy for treating said subject based on the level of said purine to pyrimidine transversion mutations, said urea cycle enzyme and/or said CAD enzyme.

According to specific embodiments, the cancer therapy is selected based on the prognosis of the cancer. That is, a cancer with poor prognosis is treated with a treatment regime suitable for poor prognosis according to e.g. established protocols; while cancer with good prognosis is treated with a treatment regime suitable for good prognosis according to other e.g. established protocols.

As the teachings of the present invention disclose that prognosis of the cancer is indicated by the levels of purine to pyrimidine transversion mutations and/or levels and/or activity of a UC enzyme and/or CAD enzyme; according to specific embodiments, the cancer therapy is selected based on the levels and/or activity of the determined component (e.g. a UC enzyme, CAD enzyme, purine to pyrimidine transversion mutations).

As used herein, the phrase "cancer therapy" refers to any therapy that has an anti-tumor effect including, but not limited to, anti-cancer drugs, radiation therapy, cell transplantation and surgery.

The anti-cancer drugs used with specific embodiments of the present invention include 5 chemotherapy, small molecules, biological drugs, hormonal therapy, antibodies and targeted therapy.

According to specific embodiments, the cancer therapy is selected from the group consisting of radiation therapy, chemotherapy and immunotherapy.

Anti-cancer drugs that can be used with specific embodiments of the invention include, 10 but are not limited to: Acivicin; Aclarubicin; Acodazole Hydrochloride; Acronine; Adriamycin; Adozelesin; Aldesleukin; Altretamine; Ambomycin; Ametantrone Acetate; Aminoglutethimide; Amsacrine; Anastrozole; Anthramycin; Asparaginase; Asperlin; Azacitidine; Azetepa; Azotomycin; Batimastat; Benzodepa; Bicalutamide; Bisantrene Hydrochloride; Bisnafide Dimesylate; Bizelesin; Bleomycin Sulfate; Brequinar Sodium; Bropirimine; Busulfan; 15 Cactinomycin; Calusterone; Caracemide; Carbemper; Carboplatin; Carmustine; Carubicin Hydrochloride; Carzelesin; Cedefingol; Chlorambucil; Cirolemycin; Cisplatin; Cladribine; Crisnatol Mesylate; Cyclophosphamide; Cytarabine; Dacarbazine; Dactinomycin; Daunorubicin Hydrochloride; Decitabine; Dexormaplatin; Dezaguanine; Dezaguanine Mesylate; Diaziquone; Docetaxel; Doxorubicin; Doxorubicin Hydrochloride; Droloxifene; Droloxifene Citrate; 20 Dromostanolone Propionate; Duazomycin; Edatrexate; Eflornithine Hydrochloride; Elsamitruclin; Enloplatin; Enpromate; Epipropidine; Epirubicin Hydrochloride; Erbulozole; Esorubicin Hydrochloride; Estramustine; Estramustine Phosphate Sodium; Etanidazole; Etoposide; Etoposide Phosphate; Etoprine; Fadrozole Hydrochloride; Fazarabine; Fenretinide; Flouxuridine; Fludarabine Phosphate; Fluorouracil; Flurocitabine; Fosquidone; Fostriecin 25 Sodium; Gemcitabine; Gemcitabine Hydrochloride; Hydroxyurea; Idarubicin Hydrochloride; Ifosfamide; Ilmofosine; Interferon Alfa-2a; Interferon Alfa-2b; Interferon Alfa-n1; Interferon Alfa-n3; Interferon Beta- I a; Interferon Gamma- I b; Iproplatin; Irinotecan Hydrochloride; Lanreotide Acetate; Letrozole; Leuprolide Acetate; Liarozole Hydrochloride; Lometrexol Sodium; Lomustine; Losoxantrone Hydrochloride; Masoprocol; Maytansine; Mechlorethamine 30 Hydrochloride; Megestrol Acetate; Melengestrol Acetate; Melphalan; Menogaril; Mercaptopurine; Methotrexate; Methotrexate Sodium; Metoprine; Meturedopa; Mitindomide; Mitocarcin; Mitocromin; Mitogillin; Mitomalcin; Mitomycin; Mitosper; Mitotane; Mitoxantrone Hydrochloride; Mycophenolic Acid; Nocodazole; Nogalamycin; Ormaplatin; Oxisuran; Paclitaxel; Pegaspargase; Peliomycin; Pentamustine; Peplomycin Sulfate; Perfosfamide; 35 Pipobroman; Piposulfan; Piroxantrone Hydrochloride; Plicamycin; Plomestane; Porfimer

Sodium; Porfiromycin; Prednimustine; Procarbazine Hydrochloride; Puromycin; Puromycin Hydrochloride; Pyrazofurin; Riboprine; Rogletimide; Safingol; Safingol Hydrochloride; Semustine; Simtrazene; Sparfosate Sodium; Sparsomycin; Spirogermanium Hydrochloride; Spiromustine; Spiroplatin; Streptonigrin; Streptozocin; Sulofenur; Talisomycin; Taxol;
5 Tecogalan Sodium; Tegafur; Teloxantrone Hydrochloride; Temoporfin; Teniposide; Teroxirone; Testolactone; Thiamiprime; Thioguanine; Thiotepa; Tiazofuirin; Tirapazamine; Topotecan Hydrochloride; Toremifene Citrate; Trestolone Acetate; Triciribine Phosphate; Trimetrexate; Trimetrexate Glucuronate; Triptorelin; Tubulozole Hydrochloride; Uracil Mustard; Uredopa; Vapreotide; Verteporfin; Vinblastine Sulfate; Vincristine Sulfate; Vindesine; Vindesine Sulfate;
10 Vinepidine Sulfate; Vinglycinate Sulfate; Vinleurosine Sulfate; Vinorelbine Tartrate; Vinrosidine Sulfate; Vinzolidine Sulfate; Vorozole; Zeniplatin; Zinostatin; Zorubicin Hydrochloride. Additional antineoplastic agents include those disclosed in Chapter 52, Antineoplastic Agents (Paul Calabresi and Bruce A. Chabner), and the introduction thereto, 1202-1263, of Goodman and Gilman's "The Pharmacological Basis of Therapeutics", Eighth
15 Edition, 1990, McGraw-Hill, Inc. (Health Professions Division).

Non-limiting examples for anti-cancer approved drugs include: abarelix, aldesleukin, aldesleukin, alemtuzumab, alitretinoin, allopurinol, altretamine, amifostine, anastrozole, arsenic trioxide, asparaginase, azacitidine, AZD9291, AZD4547, AZD2281, bevacuzimab, bexarotene, bleomycin, bortezomib, busulfan, calusterone, capecitabine, carboplatin, carmustine, celecoxib,
20 cetuximab, cisplatin, cladribine, clofarabine, cyclophosphamide, cytarabine, dabrafenib, dacarbazine, dactinomycin, actinomycin D, Darbepoetin alfa, Darbepoetin alfa, daunorubicin liposomal, daunorubicin, decitabine, Denileukin diftitox, dextrazoxane, dextrazoxane, docetaxel, doxorubicin, dromostanolone propionate, Elliott's B Solution, epirubicin, Epoetin alfa, erlotinib, estramustine, etoposide, exemestane, Filgrastim, floxuridine, fludarabine, fluorouracil 5-FU,
25 fulvestrant, gefitinib, gemcitabine, gemtuzumab ozogamicin, goserelin acetate, histrelin acetate, hydroxyurea, Ibritumomab Tiuxetan, idarubicin, ifosfamide, imatinib mesylate, interferon alfa 2a, Interferon alfa-2b, irinotecan, lenalidomide, letrozole, leucovorin, Leuprolide Acetate, levamisole, lomustine, CCNU, mecloretamine, nitrogen mustard, megestrol acetate, melphalan, L-PAM, mercaptopurine 6-MP, mesna, methotrexate, mitomycin C, mitotane, mitoxantrone, nandrolone phenpropionate, nelarabine, Nofetumomab, Oprelvekin, Oprelvekin, oxaliplatin, paclitaxel, palbociclib palifermin, pamidronate, pegademase, pegaspargase, Pegfilgrastim, pemetrexed disodium, pentostatin, pipobroman, plicamycin mithramycin, porfimer sodium, procarbazine, quinacrine, Rasburicase, Rituximab, sargramostim, sorafenib, streptozocin, sunitinib maleate, tamoxifen, temozolomide, teniposide VM-26, testolactone,
35 thioguanine 6-TG, thiotepa, thiotepa, topotecan, toremifene, Tositumomab, Trametinib,

Trastuzumab, tretinoin ATRA, Uracil Mustard, valrubicin, vinblastine, vinorelbine, zoledronate and zoledronic acid.

According to specific embodiments, the anti-cancer drug is selected from the group consisting of Gefitinib, Lapatinib, Afatinib, BGJ398, CH5183284, Linsitinib, PHA665752, Crizotinib, Sunitinib, Pazopanib, Imatinib, Ruxolitinib, Dasatinib, BEZ235, Pictilisib, Everolimus, MK-2206, Trametinib / AZD6244, Vemurafenib / Dabrafenib, CCT196969 / CCT241161, Barasertib, VX-680, Nutlin3, Palbociclib, BI 2536, Bardoxolone, Vorinostat, Navitoclax (ABT263), Bortezomib, Vismodegib, Olaparib (AZD2281), Simvastatin, 5-Fluorouracil, Irinotecan, Epirubicin, Cisplatin and Oxaliplatin.

As the present invention discloses that cancer is associated with a shift from the UC to pyrimidine synthesis in the cancerous cells, the present inventors contemplate that cancers prognosed and/or monitored according to some embodiments of the present invention are more susceptible to treatment with agents targeting components associated with these pathways.

Thus, according to specific embodiments, the cancer therapy is selected from the group consisting of L-arginine depletion, glutamine depletion, pyrimidine analogs, thymidylate synthase inhibitor and mammalian target of Rapamycin (mTOR) inhibitor.

Non-limiting examples of L-arginine depletion agents which can be used with specific embodiments of the present invention include arginine deiminase (ADI) polypeptide, arginase I polypeptide, arginase II polypeptide, arginine decarboxylase polypeptide and arginine kinase polypeptide. A pegylated form of the indicated enzymes can also be used, according to specific embodiments, such as ADI-PEG 20 is a formulation of ADI with polyethylene glycol (PEG) having an average molecular weight of 20 kilodaltons (PEG 20) and a pegylated form of the catabolic enzyme arginase I (peg-ArgI, such as disclosed in Fletcher M et al., (2015) Cancer Res. 75(2):275-83). According to other specific embodiments, a cobalt-containing arginase polypeptide such as described in WO2010/051533 can be used.

Glutamine depletion agents that can be used with specific embodiments of the invention can act on intracellular and/or extracellular glutamine, e.g., on the glutamine present in the cytosol and/or the mitochondria, and/or on the glutamine present in the peripheral blood. Non-limiting examples of glutamine depleting agents include, inhibitors of glutamate-oxaloacetate-transaminase (GOT), carbamoyl-phosphate synthase, glutamine-pyruvate transaminase, glutamine-tRNA ligase, glutaminase, D-glutaminase, glutamine N-acetyltransferase, glutaminase-asparaginase Aminooxyacetate (AOA, an inhibitor of glutamate-dependent transaminase), phenylbutyrate and phenylacetate.

Non-limiting examples of pyrimidine analogs which can be used with specific embodiments of the invention include arabinosylcytosine, gemcitabine and decitabine.

Non-limiting examples of thymidilate synthase inhibitor that can be used according to specific embodiments of the present invention include fluorouracil (5-FU), capecitabine (an oral 5-FU pro-drug) and pemetrexed.

Another cancer therapy that can be used according to specific embodiments of the present invention include inhibitors of the mammalian target of Rapamycin (mTOR) pathway. Non-limiting Examples of mTOR inhibitors include Rapamycin and rapalogs [rapamycin derivatives e.g. temsirolimus (CCI-779), everolimus (RAD001), and ridaforolimus (AP-23573), deforolimus (AP23573), everolimus (RAD001), and temsirolimus (CCI-779)].

Alternatively or additionally, according to specific embodiments, the cancer therapy comprises any agent which downregulates expression and/or activity of the upregulated enzyme (e.g. CPS1, SLC25A13, CAD).

Downregulation can be at the genomic (e.g., homologous recombination, genome editing and/or site specific endonucleases), the transcript level using a variety of molecules which interfere with transcription and/or translation [e.g., RNA silencing agents (e.g., antisense, siRNA, shRNA, micro-RNA), Ribozyme, DNAzyme, TFO] or at the protein level (e.g., aptamers, small molecules and inhibitory peptides, antagonists, enzymes that cleave the polypeptide, antibodies and the like).

According to specific embodiments, the cancer therapy comprises over-expressing within tumor cells of the subject the downregulated (e.g. ASL, ASS1, OTC, SLC25A15).

Over-expression can be at the genomic level [*i.e.*, activation of transcription via promoters, enhancers, regulatory elements, genome editing e.g., using homology directed repair (HDR), and/or by small molecules which can activate expression], at the transcript level (*i.e.*, correct splicing, polyadenylation, activation of translation) or at the protein level (*i.e.*, post-translational modifications, interaction with substrates and the like, and/or delivery of the protein itself or of a functional portion thereof into the cells). Upregulation can be also achieved using vectors (nucleic acid constructs) comprising an exogenous polynucleotide encoding the desired expression product or a functional portion thereof, which are designed and constructed to express the desired expression product in the mammalian cells (preferably in tumor cells).

As noted the present inventors contemplate that cancers prognosed and/or monitored according to some embodiments of the present invention are more susceptible to treatment with immune modulating agent.

Hence, according to specific embodiments, the cancer therapy comprises an immune modulation agent.

Immune modulating agents are typically targeting an immune-check point protein.

As used herein the term "immune-check point protein" refers to an antigen independent protein that modulates an immune cell response (i.e. activation or function). Immune-check point proteins can be either co-stimulatory proteins [i.e. positively regulating an immune cell activation or function by transmitting a co-stimulatory secondary signal resulting in activation of an immune cell] or inhibitory proteins (i.e. negatively regulating an immune cell activation or function by transmitting an inhibitory signal resulting in suppressing activity of an immune cell).

Numerous check-point proteins are known in the art and include, but not limited to, PD1, PDL-1, B7H2, B7H3, B7H4, BTLA-4, HVEM, CTLA-4, CD80, CD86, LAG-3, TIM-3, KIR, IDO, CD19, OX40, OX40L, 4-1BB (CD137), 4-1BBL, CD27, CD70, CD40, CD40L, GITR, 10 CD28, ICOS (CD278), ICOSL, VISTA and adenosine A2a receptor.

According to specific embodiments, the immune modulating agent is a PD1 antagonist, such as, but not limited to an anti-PD1 antibody.

PD1 (Programmed Death 1), gene symbol PDCD1, is also known as CD279. According to a specific embodiment, the PD1 protein refers to the human protein, such as provided in the 15 following GenBank Number NP_005009.

Anti-PD1 antibodies suitable for use in the invention can be generated using methods well known in the art. Alternatively, art recognized anti-PD1 antibodies can be used. Examples of anti-PD1 antibodies are disclosed for example in Topalian, et al. NEJM 2012, US Patent Nos. US 7,488,802; US 8,008,449; US 8,609,089; US 6,808,710; US 7,521,051; and US 8168757, US 20 Patent Application Publication Nos. US20140227262; US20100151492; US20060210567; and US20060034826 and International Patent Application Publication Nos. WO2008156712; WO2010089411; WO2010036959; WO2011159877; WO2013/019906; WO 2014159562; WO 2011109789; WO 01/14557; WO 2004/004771; and WO 2004/056875, which are hereby incorporated by reference in their entirety.

Specific anti-PD1 antibodies that can be used according to some embodiments of the present invention include, but are not limited to, Nivolumab (also known as MDX1106, BMS-936558, ONO-4538, marketed by BMY as Opdivo); Pembrolizumab (also known as MK-3475, Keytruda, SCH 900475, produced by Merck); Pidilizumab (also known as CT-011, hBAT, hBAT-1, produced by CureTech); AMP-514 (also known as MEDI-0680, produced by AZY and MedImmune); and Humanized antibodies h409A11, h409A16 and h409A17, which are described in PCT Patent Application No. WO2008/156712.

According to specific embodiments, the immune modulating agent is a CTLA4 antagonist, such as, but not limited to an anti-CTLA4 antibody.

CTLA4 (cytotoxic T-lymphocyte-associated protein 4), is also known as CD152. According to a specific embodiment the CTLA-4 protein refers to the human protein, such as provided in the following GenBank Number NP_001032720.

Anti-CTLA4 antibodies suitable for use in the invention can be generated using methods well known in the art. Alternatively, art recognized anti-CTLA4 antibodies can be used. Examples of anti-CTLA4 antibodies are disclosed for example in Hurwitz et al. (1998) Proc. Natl. Acad. Sci. USA 95(17): 10067-10071; Camacho et al. (2004) J. Clin. Oncology 22(145); Abstract No. 2505 (antibody CP-675206); and Mokyr et al. (1998) Cancer Res. 58:5301-5304; US Patent Nos. US 5,811,097; US 5,855,887; US 6,051,227; US 6,207,157; US 6,207,156; US 6,682,736; US 6,984,720; US 5,977,318; US 7,109,003; US 7,132,281; US 8,993,524 and US 7,605,238, US Patent Application Publication Nos. 09/644,668; 2005/0201994; 2002/086014, International Application Publication Nos. WO2014066834; WO 01/14424 and WO 00/37504; WO2002/0039581; WO 98/42752; WO 00/37504; WO 2004/035607; and WO 01/14424, and European Patent No. EP1212422B1, which are hereby incorporated by reference in their entirety.

Specific anti-CTLA4 antibodies that can be used according to some embodiments of the present invention include, but are not limited to Ipilimumab (also known as 10D1, MDX-D010), marketed by BMS as Yervoy™; and Tremelimumab, (ticilimumab, CP-675,206, produced by MedImmune and Pfizer).

As the present invention discloses that the a shift from the UC to pyrimidine synthesis and the pyrimidine-rich transversion mutational bias enhance the response to immune-modulation therapy independently of mutational load both in mouse models and in patient correlative studies, the present inventors contemplate that cancers diagnosed, prognosis and/or monitored according to some embodiments of the present invention are more susceptible to treatment with immune-modulation therapy in combination with agents that specifically promote pyrimidines to purines nucleotide imbalance.

Thus, according to specific embodiments, the cancer therapy comprises an agent which induces a pyrimidines to purines nucleotide imbalance.

According to a specific embodiment, the cancer therapy comprises an immune modulation agent and an agent which induces a pyrimidines to purines nucleotide imbalance.

Additionally or alternatively, according to an aspect of the present invention, there is provided a method of potentiating cancer treatment with an immune modulating agent in a subject in need thereof, the method comprising:

(a) determining a shift from the urea cycle to pyrimidine synthesis in a cancerous cell of the subject as compared to a control sample; and

(b) treating said subject with an agent which induces a pyrimidines to purines nucleotide imbalance when said shift is indicated.

As used herein the term "induces a pyrimidines to purines nucleotide imbalance" refers to an increase in the ratio of pyrimidines to purines in a cell in the presence of the agent as compared to same in the absence of the agent, which may be manifested in e.g. increased levels of pyrimidines, decreased levels of purines and/or increased level of purine to pyrimidine transversion mutations.

According to specific embodiments, the increase is at least 1.1 fold, at least 1.2 fold, at least 1.3 fold, at least 1.4 fold, at least 1.5 fold, at least 2 fold, at least 3 fold, at least 5 fold, at least 10 fold, or at least 20 fold in the ratio of pyrimidines to purines in a cell in the presence of the agent as compared to same in the absence of the agent, which may be determined by e.g. chromatography and mass spectrometry (e.g. LC-MS), whole genome sequencing, DNA sequencing and/or RNA sequencing.

According to specific embodiments, the predetermined threshold is at least 2 %, at least 5 %, at least 10 %, at least 20 %, at least 30 %, at least 40 %, at least 50 %, at least 60 %, at least 70 %, at least 80 %, at least 90 %, e.g., 100 %, at least 200 %, at least 300 %, at least 400 %, at least 500 %, at least 600 % in the ratio of pyrimidines to purines in a cell in the presence of the agent as compared to same in the absence of the agent.

According to specific embodiments, the agent which induces a pyrimidines to purines nucleotide imbalance comprises an anti-folate agent.

Anti-folate agents which can be used with specific embodiments of the invention are known in the art and include, but not limited to, methotrexate, pemetrexed, proguanil, pyrimethamine, trimethoprim, aminopterin, trimetrexate, edatrexate, piritrexim, ZD1694, lometrexol, AG337, LY231514 and 1843U89.

According to specific embodiments, the anti-folate agent comprises methotrexate.

As used herein the term "about" refers to $\pm 10\%$

The terms "comprises", "comprising", "includes", "including", "having" and their conjugates mean "including but not limited to".

The term "consisting of" means "including and limited to".

The term "consisting essentially of" means that the composition, method or structure may include additional ingredients, steps and/or parts, but only if the additional ingredients, steps and/or parts do not materially alter the basic and novel characteristics of the claimed composition, method or structure.

As used herein, the singular form "a", "an" and "the" include plural references unless the context clearly dictates otherwise. For example, the term "a compound" or "at least one compound" may include a plurality of compounds, including mixtures thereof.

Throughout this application, various embodiments of this invention may be presented in 5 a range format. It should be understood that the description in range format is merely for convenience and brevity and should not be construed as an inflexible limitation on the scope of the invention. Accordingly, the description of a range should be considered to have specifically disclosed all the possible subranges as well as individual numerical values within that range. For example, description of a range such as from 1 to 6 should be considered to have specifically 10 disclosed subranges such as from 1 to 3, from 1 to 4, from 1 to 5, from 2 to 4, from 2 to 6, from 3 to 6 etc., as well as individual numbers within that range, for example, 1, 2, 3, 4, 5, and 6. This applies regardless of the breadth of the range.

Whenever a numerical range is indicated herein, it is meant to include any cited numeral (fractional or integral) within the indicated range. The phrases "ranging/ranges between" a first 15 indicate number and a second indicate number and "ranging/ranges from" a first indicate number "to" a second indicate number are used herein interchangeably and are meant to include the first and second indicated numbers and all the fractional and integral numerals therebetween.

As used herein the term "method" refers to manners, means, techniques and procedures for accomplishing a given task including, but not limited to, those manners, means, techniques 20 and procedures either known to, or readily developed from known manners, means, techniques and procedures by practitioners of the chemical, pharmacological, biological, biochemical and medical arts.

When reference is made to particular sequence listings, such reference is to be understood to also encompass sequences that substantially correspond to its complementary sequence as 25 including minor sequence variations, resulting from, e.g., sequencing errors, cloning errors, or other alterations resulting in base substitution, base deletion or base addition, provided that the frequency of such variations is less than 1 in 50 nucleotides, alternatively, less than 1 in 100 nucleotides, alternatively, less than 1 in 200 nucleotides, alternatively, less than 1 in 500 nucleotides, alternatively, less than 1 in 1000 nucleotides, alternatively, less than 1 in 5,000 30 nucleotides, alternatively, less than 1 in 10,000 nucleotides.

It is appreciated that certain features of the invention, which are, for clarity, described in the context of separate embodiments, may also be provided in combination in a single embodiment. Conversely, various features of the invention, which are, for brevity, described in the context of a single embodiment, may also be provided separately or in any suitable 35 subcombination or as suitable in any other described embodiment of the invention. Certain

features described in the context of various embodiments are not to be considered essential features of those embodiments, unless the embodiment is inoperative without those elements.

Various embodiments and aspects of the present invention as delineated hereinabove and as claimed in the claims section below find experimental support in the following examples.

5

EXAMPLES

Reference is now made to the following examples, which together with the above descriptions illustrate some embodiments of the invention in a non-limiting fashion.

Generally, the nomenclature used herein and the laboratory procedures utilized in the present invention include molecular, biochemical, microbiological and recombinant DNA techniques. Such techniques are thoroughly explained in the literature. See, for example, "Molecular Cloning: A laboratory Manual" Sambrook et al., (1989); "Current Protocols in Molecular Biology" Volumes I-III Ausubel, R. M., ed. (1994); Ausubel et al., "Current Protocols in Molecular Biology", John Wiley and Sons, Baltimore, Maryland (1989); Perbal, "A Practical Guide to Molecular Cloning", John Wiley & Sons, New York (1988); Watson et al., "Recombinant DNA", Scientific American Books, New York; Birren et al. (eds) "Genome Analysis: A Laboratory Manual Series", Vols. 1-4, Cold Spring Harbor Laboratory Press, New York (1998); methodologies as set forth in U.S. Pat. Nos. 4,666,828; 4,683,202; 4,801,531; 5,192,659 and 5,272,057; "Cell Biology: A Laboratory Handbook", Volumes I-III Cellis, J. E., ed. (1994); "Culture of Animal Cells - A Manual of Basic Technique" by Freshney, Wiley-Liss, N. Y. (1994), Third Edition; "Current Protocols in Immunology" Volumes I-III Coligan J. E., ed. (1994); Stites et al. (eds), "Basic and Clinical Immunology" (8th Edition), Appleton & Lange, Norwalk, CT (1994); Mishell and Shiigi (eds), "Selected Methods in Cellular Immunology", W. H. Freeman and Co., New York (1980); available immunoassays are extensively described in the patent and scientific literature, see, for example, U.S. Pat. Nos. 3,791,932; 3,839,153; 3,850,752; 3,850,578; 3,853,987; 3,867,517; 3,879,262; 3,901,654; 3,935,074; 3,984,533; 3,996,345; 4,034,074; 4,098,876; 4,879,219; 5,011,771 and 5,281,521; "Oligonucleotide Synthesis" Gait, M. J., ed. (1984); "Nucleic Acid Hybridization" Hames, B. D., and Higgins S. J., eds. (1985); "Transcription and Translation" Hames, B. D., and Higgins S. J., eds. (1984); "Animal Cell Culture" Freshney, R. I., ed. (1986); "Immobilized Cells and Enzymes" IRL Press, (1986); "A Practical Guide to Molecular Cloning" Perbal, B., (1984) and "Methods in Enzymology" Vol. 1-317, Academic Press; "PCR Protocols: A Guide To Methods And Applications", Academic Press, San Diego, CA (1990); Marshak et al., "Strategies for Protein Purification and Characterization - A Laboratory Course Manual" CSHL Press (1996); all of which are incorporated by reference as if fully set forth herein. Other general references are provided

throughout this document. The procedures therein are believed to be well known in the art and are provided for the convenience of the reader. All the information contained therein is incorporated herein by reference.

5

MATERIALS AND METHODS

Determination of the urea cycle genes dysregulation score (UCD-score) - The UCD-score is a weighted sum of rank-normalized expression of the 6 urea cycle (UC) genes - ASL, ASS1, CPS1, OTC, SLC25A13 and SLC25A15; wherein:

+1 was assigned as weight for the genes CPS1 and SLC25A13;

-1 was assigned as weight for the genes ASL, ASS1, OTC and SLC25A15.

Evaluation of UC genes expression in patient samples from “The Cancer Genome Atlas (TCGA)” - TCGA gene expression profiles of 5,645 patients samples (comprising 629 normal samples) encompassing samples from 13 cancer types and a substantial number of healthy control samples (>10 for each cancer type) were downloaded from the Broad Firehose resources on Jan 28, 2016, doi:10.7908/C11G0KM9). Following, expression levels of 6 genes involved in the UC (i.e. ASL, ASS1, CPS1, OTC, SLC25A13 and SLC25A15) in the cancer patients were compared to their expression in the healthy controls using the Student’s T-test and the UCD-score was calculated. Components with significant fold changes in specific tumor types are presented in Figure 3A. The differences remain significant vs. random shuffling of cancer/normal labels in each cancer type ($P<1E-6$) and random choice of sets of metabolites of similar size ($P<2.67E-3$). Based on the UCD-score, tumor samples were divided equally into 5 bins; and CAD expression (rank-normalized across the samples in each cancer type) was compared across these bins using a Wilcoxon rank sum test (Figure 3D).

TCGA DNA mutation analysis - TCGA mutation profiles of 7,462 tumor samples encompassing 18 cancer types were downloaded from cBioPortal¹⁸ on Feb 1, 2017. The data from cBioPortal does not include healthy control samples but integrates the mutation analysis from different TCGA centers to avoid center specific bias in mutation calls. Samples with less than 5 mutation events were excluded from further analysis. For analyses that involved comparison within each cancer type, the 13 cancer types that had sufficient sample size ($N>150$), which results in 983,404 single point mutation events (including 745,712 non-synonymous mutations) in 4963 samples, were used. The fraction of transversions from purines (R) to pyrimidines (Y), denoted herein as $f(R\rightarrow Y)$, was determined per each sample and was defined as the fraction of R->Y point mutations over all point mutations occurring in a given sample. In order to study the downstream effects of purine to pyrimidine mutations the transversion rates were quantified based on the coding (sense) strand (i.e. the TCGA mutation data was converted

to its complementary sequences in genes transcribed from the (-)-strand of the genomic DNA). The fraction of transversions from pyrimidines to purines, denoted herein as $f(Y \rightarrow R)$, was determined and defined in an analogous manner. Following, the association between UC dysregulation and $R \rightarrow Y$ transverse mutations was analyzed using four different approaches:

- 5 1. The $R \rightarrow Y$ mutation rates in UC dysregulated samples (top 30% of UCD-score, denoted herein as UC-dys) was compared to the $R \rightarrow Y$ mutation rates in UC intact samples (bottom 30% of UCD-score, denoted herein as UC-WT) at the pancancer level and in each cancer type individually (Figure 6B) using a Wilcoxon rank sum test.
- 10 2. The difference between $R \rightarrow Y$ and $Y \rightarrow R$ mutation rates in UC dysregulated samples (top 30 %) was compared to the difference between $R \rightarrow Y$ and $Y \rightarrow R$ mutation rates in UC intact samples (bottom 30 %) at the pancancer level and in each cancer type individually (Figure 7B) using a Wilcoxon rank sum test.
- 15 3. The correlation across cancer types between median UCD-score and median pyrimidine mutation bias ($f(R \rightarrow Y) - f(Y \rightarrow R)$) of each cancer type was analyzed using Spearman correlation analysis (Figure 7C).
- 20 4. Assessing whether purine to pyrimidine mutations associated with UC dysregulation was positively selected, based on the premise that a greater rate of non-synonymous mutations relative to synonymous mutations is indicative of positive selection. To this end, the normalized fraction of nonsynonymous purine to pyrimidine mutations in UC dysregulated vs. UC intact samples was determined (Figure 7C). Specifically, the selective advantage (S) of $R \rightarrow Y$ mutation was estimated by the formula:

$$S = \frac{N_{R \rightarrow Y}}{N_{R \rightarrow Y} + S_{R \rightarrow Y}} / \frac{N_{all}}{N_{all} + S_{all}} \quad (1)$$

- 25 Where $N_{R \rightarrow Y}$ denotes nonsynonymous mutation level of purine to pyrimidine transversions; $S_{R \rightarrow Y}$ denotes synonymous mutation level of purine to pyrimidine transversions; N_{all} denotes nonsynonymous mutation level of all mutation events; and S_{all} denotes synonymous mutation level of all mutation events.

For this specific analysis, additional TCGA samples which had less than 5 mutation events either for synonymous or nonsynonymous mutations were filtered out, leading to 4817 samples in 13 cancer types.

Patient survival analysis - Kaplan Meier analysis and Cox proportional hazard model were performed to identify the association of UCD-score with patient survival (according to the TCGA cBioPortal data described above). The survival of patients with high-UCD score (top 30

(%) and low-UCD score (bottom 30 %) were compared using the logrank test¹⁹, and the effect size was quantified by the difference in the area under the curves (ΔAUC). To control for potential confounders, a Cox regression analysis was performed, while controlling for patients' age, sex, race, and cancer types, as follows:

5
$$h_s(t, \text{patient}) \sim h_{0s}(t) \exp(\beta_{UCD} UCD + \beta_{age} \text{age}), \quad (2)$$

Where s is an indicator variable over all possible combinations of patients' stratifications based on race, sex and cancer type;

h_s is the hazard function (defined as the risk of death of patients per time unit); and $h_{0s}(t)$ is the baseline-hazard function at time t of the s^{th} stratification.

The model contains two covariates: (i) *UCD*: UCD-score based on the urea cycle deregulation signatures, and (ii) *age*: age of the patient. The β s are the regression coefficients of the covariates, which quantify the effect of covariates survival, determined by standard likelihood maximization of the model¹⁹. The results of this analysis are presented in (Figure 3E).

15 **Detection of somatic mutations in DNA and RNA** - To capture variants in the coding region, exome-seq data of 18 individual cancer and matched normal cohorts was downloaded from TCGA portal. For each BAM file of normal and cancer variants were called using the GATK (V. 3.6) '*HaplotypeCaller*'^{20,21} utility with same hg38 assembly that the TCGA used for exome-seq mapping and applying '-ERC GVCF' mode to produce a comprehensive record of 20 genotype likelihoods for every position in the genome regardless of whether a variant was detected at that site or not. The purpose of using the GVCF mode was to capture confidence score for every site represented in a paired normal and cancer cohort for detecting somatic mutation in cancer. Following, the paired GVCFs from each paired cohorts was combined using GATK's '*GenotypeGVCFs*' utility yielding genotype likelihood scores for every variant in cancer 25 and the paired normal sample. In the next step GATK's '*VariantRecalibrator*' utility using dbSNP VCF (v146: ftp://ftp.ncbi.nlm.nih.gov/snp/organisms/human_9606_b146_GRCh38p2/VCF) file was used by selecting annotation criteria of QD;MQ;MQRankSum;ReadPosRankSum;FS;SOR, followed by GATK's '*ApplyRecalibration*' utility with 'SNP' mode. Using GATK's '*VariantFiltration*' utility 30 the variants with VQSLOD ≥ 4.0 were selected. Finally, somatic mutations were defined as the loci whose genotype [1/1, 0/1, or 0/0 with 'PL' (Phred-scaled likelihood of the genotype) score = 0, i.e., highest confidence] in cancer was distinct from that in the paired normal. The final somatic mutations were mapped on an exonic site of a transcript by '*bcftools*' tool (V. 1.3)²¹ using BED file of coding region in hg38 assembly.

To capture variants in RNA, BAM files of RNA-Seq data was downloaded for the same normal and cancer cohorts as described above. GATK's '*SplitNCigarReads*' utility was used to split the reads into exon segments and hard-clipped to any sequence overhanging into the intronic regions. Following, GATK's '*HaplotypeCaller*' utility was used with the same hg38 assembly
5 that the TCGA used for RNA-Seq mapping. To reduce false positive and false negative calls the '*dontUseSoftClippedBases*' argument with the '*HaplotypeCaller*' with minimum phred-scaled confidence threshold was used for calling variants set to be 20. Following, the variants were filtered using '*VariantFiltration*' utility based on Fisher Strand values (FS > 30) and Qual By Depth values (QD < 2.0). Each of the output VCF files was used for annotation of coding
10 regions on the transcripts to which the variants were mapped by using '*bcftools*' with BED file of coding region in hg38 assembly. Based on this data, the overall R->Y mutation bias, f(R->Y)-f(Y->R) was compared between UC dysregulated vs. UC intact samples using Wilcoxon rank sum test.

Detection of somatic mutations in the proteome - To map the DNA variants to protein sequence, peptide spectrum (PSM) data was downloaded for 42 breast cancer samples, out of which only 4 samples overlapped with the samples analyzed for DNA mutations calls above. For each transcript in the somatic variant VCF file, complete coding sequence of RNA was constructed using the GATK's '*FastaAlternateReferenceMaker*' utility. On this variant incorporated coding sequence, a codon affected by this variant site was captured and *in-silico* translated into an amino acid. A change was considered as a '*non-synonymous*' change if the translated amino acid differed from the reference amino acid; and otherwise '*synonymous*'. Based on this data, the overall R->Y mutation-mapped amino acid changes we compared between UC dysregulated vs. UC intact samples using the Wilcoxon rank sum test.

Genome-scale metabolic network modeling - genome-scale metabolic modeling was used to study the stoichiometric balance of nitrogen metabolism between urea production and pyrimidine synthesis. For a metabolic network with m metabolites and n reactions, the stoichiometric constraints can be represented by a stoichiometric matrix S, as follows:

$$\sum_j S_{ij} v_j = 0, \quad (3)$$

where the entry S_{ij} represents the stoichiometric coefficients of metabolite i in reaction j , and v_j stands for the metabolic flux vector for all reactions in the model. The model assumes steady metabolic state, as represented in equation (3) above, constraining the production rate of each metabolite to be equal to its consumption rate. In addition to the mass balance, a

constraint-based model limits the space of possible fluxes in the metabolic network's reactions through a set of (in)equalities imposed by thermodynamic constraints, substrate availability and the maximum reaction rates supported by the catalyzing enzymes and transporting proteins, as follows:

$$5 \quad \alpha_j \leq v_j \leq \beta_j, \quad (4)$$

where α_j and β_j defines the lower and upper bounds of the metabolic fluxes for different types of metabolic fluxes. (i) The exchange fluxes model the metabolite exchange of a cell with the surrounding environment via transport reactions, enabling a pre-defined set of metabolites to 10 be either taken up or secreted from the growth media. (ii) Enzymatic directionality and flux capacity constraints define lower and upper bounds on the fluxes as represented in equation (4) above. The human metabolic network model²⁴ was used with biomass function introduced in Folger et al.²⁵ under the Roswell Park Memorial Institute Medium (RPMI)-1640.

To study the metabolic alterations occurring in UC dysregulated cancer cells (having increased growth and biomass production rates, and increase CAD activity versus healthy cells), 15 a flux-balance-based analysis²³ was performed. The maximal production rate of urea was computed while gradually increasing the demand constraints for biomass production rates and the flux via the three enzymatic reactions of CAD – Carbamoyl-phosphate synthetase 2 (CPS2), Aspartate transcarbamylase (ATC) and Dihydroorotate - up to their maximal feasible values in 20 the model (Figure 5A, right). In addition, the nitrogen utilization in each of the conditions sampled in the procedure above was computed, by subtracting the total amount of nitrogen excreted from the amount of nitrogen uptake, while taking into account the nitrogen's stoichiometry in all nitrogen-containing metabolites (Figure 5A, left).

Joint transcriptomic and metabolomic analysis of tumor samples - Recently published 25 data of joint transcriptomic and metabolomic measurements across 58 breast cancer (BC) tumors vs. healthy controls²³ and 29 hepatocellular carcinoma (HCC) samples vs. healthy controls²⁴ was analyzed, to further study the association between UC dysregulation and metabolites levels in clinical samples. For each sample, a score denoting the ratio of pyrimidine to purine 30 metabolite levels in the given sample was computed. Following, the samples were divided into two groups based on their UCD-scores and the two groups were compared using Wilcoxon rank-sum, in each of the two cancer types (Figure 5C).

Patient samples - Plasma urea levels measurements were taken from Hemato- Oncology patients' medical record excluding patients' identifiers and with approval by the ethic committee (TLV 0016-17). Prostate specimens were obtained upon informed consent and with evaluation

and approval from the corresponding ethics committee (CEIC code OHEUN11-12 and OHEUN14-14). Blood samples were taken from patients diagnosed with benign prostate hyperplasia (BPH) with normal PSA levels or with prostate adenocarcinoma (PCa); and a scheduled surgery as anticancer treatment (PCa) or to alleviate disease-related symptoms (BPH) 5 served as an inclusion criteria. The biopsy-based diagnosis was corroborated in the surgical piece. The blood was collected following overnight fasting and prior to surgery. Plasma was extracted from the blood samples and analysed for urea concentration, following standard clinical procedures. Following urea concentration analysis, outliers were removed using the ROUT method ($Q = 1\%$).

10 ***Cell and cell cultures*** - Patients fibroblast studies were performed anonymously on cells devoid of all patient identifiers. Punch biopsies were taken from UC deficient patients to generate fibroblast cell line. HepG2 cell line was purchased from ATTC. OTC and CPS1 deficient cell lines as well as control fibroblasts were purchased from Coriell Institute for Medical Research (GM06902; GM12604). Cells were cultured using standard procedures in a 15 37 °C humidified incubator with 5 % CO₂ in Dulbecco's Modified Eagle's Medium (DMEM, sigma-aldrich) supplemented with 10-20 % heat-inactivated fetal bovine serum, 10 % pen-strep and 2 mM glutamine. All cells were tested routinely for Mycoplasma using Mycoplasma EZ-PCR test kit (#20-700-20, Biological Industries, Kibbutz Beit Ha'emek).

20 ***Crystal Violet Staining*** - Cells were seeded in 12-wells plates at 75,000-150,000 cells / well in triplicates. Time 0 was determined as the time the cells adhered to the culture plate, which was about 10 hours following seeding. For each time point, cells were washed with PBS X1 and fixed in 4 % PFA (in PBS). Following, cells were stained with 0.5 % Crystal Violet (Catalog number C0775, Sigma-Aldrich) for 20 minutes (1 ml per well) and washed with water. The cells were then incubated with 10 % acetic acid for 20 minutes with shaking. The extract 25 was diluted 1 : 1 - 1 : 4 in water and absorbance was measured for each time point at 595 nm every 24 hours.

30 ***Immunohistochemistry*** - Four micrometer paraffin embedded tissue sections were deparaffinized and rehydrated. Endogenous peroxidase was blocked with three percent H₂O₂ in methanol. For ASL, ASS1 and ORNT1 (*SLC25A15*) staining, antigen retrieval was performed in citric acid (pH 6), for 10 minutes, using a low boiling program in the microwave to break protein cross-links and unmask antigens. Following, the sections were pre-incubated with 20 % normal horse serum and 0.2 % Triton X-100 for 1 hour at RT, biotin block via Avidin/Biotin Blocking Kit (SP-2001, Vector Laboratories, Ca, USA). The blocked sections were incubated overnight at room temperature followed by 48 hours at 4 °C with the following primary antibodies: ASL (1 : 35 50, Abcam, ab97370, CA, USA); ASS1 (1 : 50, Abcam, ab124465, CA, USA), ORNT1 (1 : 200,

NBP2-20387, novus biologicals, CO, USA), OTC (1 : 3-1 : 200, HPA000570, Sigma-aldrich). All antibodies were diluted in PBS containing 2 % normal horse serum and 0.2 % Triton. Following, the sections were washed three times with PBS and incubated with secondary biotinylated IgG antibody at for 1.5 hour at room temperature, washed three times in PBS and 5 incubated with avidin-biotin Complex (Elite-ABC kit, Vector Lab, CA, USA) for additional 90 minutes at room temperature, followed by DAB (Sigma) reaction. Stained sections were examined and photographed by a bright field microscope (E600, Tokyo, Japan) equipped with Plan Fluor objectives (10x) connected to a CCd camera (DS-Fi2, Nikon). Digital images were collected and analyzed using Image Pro+ software. Images were assembled using Adobe 10 Photoshop (Adobe Systems, San Jose, CA).

15 **Viral infection** - Primary fibroblasts were infected with HCMV and harvested at different times points following infection for ribosome footprints (deep sequencing of ribosome-protected mRNA fragments) as previously described²⁵. Briefly, human foreskin fibroblasts (HFF) were infected with the Merlin HCMV strain and the cells were harvested at 5, 12, 24 and 72 hours post infection. Cells were pre-treated with Cylcoheximide and ribosome protected fragments were then generated and sequenced. Bowtie v0.12.7 (allowing up to 2 mismatches) was used to perform the alignments. Reads with unique alignments were used to compute footprints densities in units of reads per kilobase per million (RPKM).

20 **Metabolomics analysis** - HepG2 cell lines were seeded at $3 - 5 \times 10^6$ cells per 10 cm plate and incubated with 4 mM L-glutamine (α -15N, 98 %, Cambridge Isotope Laboratories) for 24 hours. Subsequently, cells were washed with ice-cold saline, lysed with a mixture of 50 % methanol in water added with 2 μ g / ml ribitol as an internal standard and quickly scraped followed by three freeze-thaw cycles in liquid nitrogen. Following, the sample was centrifuged in a 4 °C cooled centrifuge and the supernatant was collected for consequent GC-MS analysis. 25 The pellets were dried under air flow at 42 °C using a Techne Dry-Block Heater with sample concentrator (Bibby Scientific) and the dried samples were treated with 40 μ l of a methoxyamine hydrochloride solution (20 mg ml⁻¹ in pyridine) for 90 minutes while shaking at 37 °C followed by incubation with 70 μ l N,O-bis (trimethylsilyl) trifluoroacetamide (Sigma) for additional 30 minutes at 37 °C.

30 **Isotopic labeling** - Hepatocellular and ovarian carcinoma cells were seeded in 10 cm plates and once cell confluence reached 80 % cells were incubated with 4 mM L-GLUTAMINE, (ALPHA-15N, 98 %, Cambridge Isotope Laboratories, Inc.) for 24 hours.

35 **GC-MS analysis** - GC-MS analysis used a gas chromatograph (7820AN, Agilent Technologies) interfaced with a mass spectrometer (5975 Agilent Technologies). An HP-5ms capillary column 30 m \times 250 μ m \times 0.25 μ m (19091S-433, Agilent Technologies) was used.

Helium carrier gas was maintained at a constant flow r°C via a ramp of $4\text{ }^\circ\text{C min}^{-1}$, $250\text{--}215\text{ }^\circ\text{C}$ via a ramp of $9\text{ }^\circ\text{C min}^{-1}$, $215\text{--}300\text{ }^\circ\text{C}$ via a ramp of $25\text{ }^\circ\text{C min}^{-1}$ and maintained at $300\text{ }^\circ\text{C}$ for additional 5 minutes. The MS was effected by electron impact ionization and operated in full-scan mode from $m/z = 30\text{--}500$. The inlet and MS transfer line temperatures were maintained at 5 $280\text{ }^\circ\text{C}$, and the ion source temperature was $250\text{ }^\circ\text{C}$. Sample injection ($1\text{--}3\text{ }\mu\text{l}$) was in split less mode.

10 **Nucleotide analysis – Materials:** Ammonium acetate (Fisher Scientific) and ammonium bicarbonate (Fluka) of LC-MS grade; Sodium salts of AMP, CMP, GMP, TMP and UMP (Sigma-Aldrich); Acetonitrile of LC grade (Merck); water with resistivity $18.2\text{ M}\Omega$ obtained using Direct 3-Q UV system (Millipore).

15 **Extract preparation:** Samples were concentrated in speedvac to eliminate methanol, and then lyophilized to dryness, re-suspended in $200\text{ }\mu\text{l}$ of water and purified on polymeric weak anion columns [Strata-XL-AW $100\text{ }\mu\text{m}$ (30 mg ml^{-1} , Phenomenex)] as follows: each column was conditioned by passing 1 ml of methanol followed by 1 ml of formic acid/methanol/water (2/25/73) and equilibrated with 1 ml of water. The samples were loaded, and each column was washed with 1 ml of water and 1 ml of 50 % methanol. The purified samples were eluted with 1 ml of ammonia/methanol/water (2/25/73) followed by 1 ml of ammonia/methanol/water (2/50/50) and then collected, concentrated in speedvac to remove methanol and lyophilized. Following, the obtained residues were re-dissolved in $100\text{ }\mu\text{l}$ of water and centrifuged for 20 5 minutes at $21,000\text{ g}$ to remove insoluble material.

25 **LC-MS analysis:** The LC-MS/MS instrument used for analysis of nucleoside monophosphates was an Acquity I-class UPLC system (Waters) and Xevo TQ-S triple quadrupole mass spectrometer (Waters) equipped with an electrospray ion source and operated in positive ion mode. MassLynx and TargetLynx software (version 4.1, Waters) were applied for data acquisition and analysis. Chromatographic separation was done on a $100\text{ mm} \times 2.1\text{ mm}$ internal diameter, $1.8\text{ }\mu\text{m}$ UPLC HSS T3 column equipped with $50\text{ mm} \times 2.1\text{ mm}$ internal diameter, $1.8\text{ }\mu\text{m}$ UPLC HSS T3 pre-column (both Waters Acquity) with mobile phases A (10 mM ammonium acetate and 5 mM ammonium hydrocarbonate buffer, pH 7.0 adjusted with 10 % acetic acid) and B (acetonitrile) at a flow rate of 0.3 ml min^{-1} and column temperature $35\text{ }^\circ\text{C}$. A gradient was used as follows: for 0–3 min the column was held at 0 % B, 3–3.5 min a linear increase to 100 % B, 3.5–4.0 min held at 100 % B, 4.0–4.5 min back to 0 % B and equilibration at 0 % B for 2.5 min. Samples kept at $8\text{ }^\circ\text{C}$ were automatically injected in a volume of $3\text{ }\mu\text{l}$. For mass spectrometry, argon was used as the collision gas with a flow of 0.15 ml min^{-1} . The capillary voltage was set to 2.90 kV , source temperature $150\text{ }^\circ\text{C}$, desolvation temperature $350\text{ }^\circ\text{C}$, cone gas flow 150 l hr^{-1} , desolvation gas flow 650 l hr^{-1} .

Downregulation of OTC - HEPG2 Cells were infected with pLKO-based lentiviral vector with or without the human OTC short hairpin RNA (shRNA) encoding one or two separate sequences combined (RHS4533-EG5009, GE Healthcare, Dharmacon). Transduced cells were selected with 4 µg ml⁻¹ puromycin.

5 **Virus infection** - Primary fibroblasts were infected with HCMV and harvested at different time points following infection for ribosome footprints (deep sequencing of ribosome-protected mRNA fragments) as previously described (Tirosh et al., 2015). Briefly human foreskin fibroblasts (HFF) were infected with the Merlin HCMV strain and harvested cells at 5, 10 12, 24 and 72 hours post infection. Cells were pre-treated with Cycloheximide and ribosome protected fragments were then generated and sequenced. Bowtie v0.12.7 (allowing up to 2 mismatches) was used to perform the alignments. Reads with unique alignments were used to compute footprints densities in units of reads per kilobase per million (RPKM).

15 Cancer cells were infected with pLKO-based lentiviral vector with or without the human OTC and SLC25A15, ASS1 short hairpin RNA (shRNA) (Dharmacon). Transduced cells were selected with 2-4 µg ml⁻¹ puromycin.

20 **Transient transfection** - LOX-IMVI melanoma cells were seeded in 6-well plates at 70,000cells/ well, or in 12-well plates at 100,000cells/ plate. At the following day, cells were transfected with either 700 pmol or 350 pmol siRNA siGenome SMARTpool targeted to human SLC25A13 mRNA (#M-007472-01, Dharmacon), respectively. Hepatocellular and ovarian carcinoma cells were seeded in 6-well plate at 10⁶ or 70,000 cells/ well respectively, transfected with 2-3 µg of the OTC (EXa3688-LV207 GENECOPOEIA) or ORNT1 (EXu0560-LV207 GENECOPOEIA) plasmids. Transfection was effected with Lipofectamine® 2000 Reagent (#11668027, ThermoFisher Scientific), in the presence of Opti-MEM® I Reduced Serum Medium (#11058021, ThermoFisher Scientific). Four hours following transfection, medium was replaced and the experiments were performed 48-108 hours post transfection.

25 **Over expression** - LOX-IMVI melanoma cells were transduced with pLEX307-based lenti-viral vector with or without the human SLC25A13 transcript, encoding for Citrin. Transduced cells were selected with 2 µg / ml Puromycin.

30 **In-vivo experiments** - 8 weeks old Balb/c or C57BL mice were injected with 4T1 breast cancer cells (in the mammary fat fad) or with CT26 colon cancer cells (sub-cutaneous). 3 weeks following injection an advanced tumor was observed and palpated. Urine was collected from mice presenting adverse tumors. Pyrimidine pathway related metabolites were assessed by LC-MS at Baylor College of medicine. Control urine was obtained from Balb/c or C57BL mice similar in age which were not injected. Samples below 100 µl were excluded from the analysis.

35 All animal experiments were approved by the Weizmann Institute Animal Care and Use

Committee Following US National Institute of Health, European Commission and the Israeli guidelines (IACUC 21131015-4).

Syngeneic mouse models - 8 weeks old C57BL/6 male mice were injected sub-cutaneous in the right flank with MC38 mouse colon cancer cells infected with either an empty vector (EV) or with shASS1. For each injection, 5×10^5 tumor cells were suspended in 200 μl DMEM containing 5 % matrigel. Following injection, on days 8, 13, 17, 20, mice were treated with 250 μg of anti PD-1 antibody (Clones 29F.1A12, RPM114, Bio Cell) or PBS (control) as control. On day 22, mice were euthanized and tumors were removed and incubated in 1 ml of PBS containing Ca²⁺, Mg²⁺ (Sigma D8662) with 2.5 mg / ml Collagenase D (Roche) and 1 mg / ml DNase I (Roche). Following 20 minutes incubation at 37 °c, the tumors were processed into a single cell suspension by mechanically grinding on top of wire mesh and repeated washing and filtering onto 70 μM filter (Falcon). Single cell suspensions from tumors were stained for flow cytometry analysis with CD3-FITC (clone 17A2), CD4-PE (clone GK15) and CD8a-APC (clone 53-6.7) all from Biolegend. Next, the cells were fixed using BD cytofix/cytoperm solution (BD Biosciences) and acquired on LSRII flow cytometer at the Weizmann FACS facility and analyzed with FlowJo software (Tree Star). The tumor volume was quantified by the formula, $(l \times w \times h) \pi/6$, and normalized by their volume on day 11 when the mean tumor volume reached around 100 mm³. The response to anti-PD1 therapy (and empty vector) was quantified by the tumor volume change at time t , $\Delta V_t = (V_t - V_0)/V_0$, where V_t denotes the normalized tumor volume at a given time t , and V_0 denotes the tumor volume on day 11. The overall response of treated and control groups was compared by Wilcoxon ranksum test of ΔV_t on day 21, and the sequential tumor growth was compared using ANOVA over the whole period (where the internal tumor volume was measured on day 9, 13,17, and 19).

Western blotting - Cells were lysed in RIPA (Sigma-Aldrich) and 0.5 % protease inhibitor cocktail (Calbiochem), 1 % phosphatase inhibitor cocktail (P5726, sigma-aldrich). Following centrifugation, the supernatant was collected and protein content was evaluated by the Bradford assay. 100 μg from each sample under reducing conditions were loaded into each lane on a 10 % SDS polyacrylamide gel and separated by electrophoresis. Following electrophoresis, proteins were transferred to Immobilon transfer membranes (Tamar, Jerusalem, Israel). Nonspecific binding was blocked by incubation with TBST [10 mM Tris-HCl (pH 8.0), 150 mM NaCl, 0.1 % Tween 20] containing 5 % skim milk or BSA 3 % (Sigma catalog no: A7906) for 1 hour at room temperature. Membranes were subsequently incubated with primary antibodies against: p97 (1 : 10,000, PA5-22257, Thermo Scientific), GAPDH (1 : 1000, 14C10, #2118, Cell Signaling), CAD (1 : 1000, ab40800, abcam), phospho-CAD (Ser1859) (1 : 1000, #12662, Cell Signaling), ASL (1 : 1000, ab97370, Abcam), MAP2K1 (1 : 10000, MFCD00239713, Sigma-

Aldrich), OTC (1 : 1000, ab203859, Abcam). Following, the membranes were incubated with the secondary antibodies used were: using peroxidase-conjugated AffiniPure goat anti-rabbit IgG or goat anti-mouse IgG (Jackson ImmunoResearch, West Grove, PA) and detected by enhanced chemiluminescence western blotting detection reagents (EZ-Gel, Biological Industries). The 5 bands were quantified by Gel Doc™ XR+ (BioRad) and analyzed by ImageLab 5.1 software (BioRad).

Predicting the success of immune checkpoint inhibitors therapy - Three different melanoma ICT datasets (Van Allen et al., 2015, Hugo et al., 2016 and Roh et al., 2017) treated with anti-CTLA4 therapy and anti-PD1 therapy were analyzed. The third dataset includes both 10 anti-CTLA4 and anti-PD1, however the anti-PD1 arm was analyzed because it has a larger sample size. The definition of responders determined by the combination of RECIST criteria (treating complete response (CR) and partial response (PR) as responders and the progressive disease (PD) as non-responders) was followed. UCD-score between responders and non-responders were compared in two datasets (Van Allen et al., 2015 and Hugo et al., 2016) where 15 UC enzymes are available using Wilcoxon ranksum test; the third dataset (Roh et al., 2017) has nanostring data, where not all of the expression of 6 UC genes are available. The association of CAD expression and ICD response was evaluated in an analogous manner. The predictive power of mutational load and PTMB for the success of anti-PD1 was evaluated in connection with the *in vivo* anti-PD1 experiment using ROC analysis in the datasets where the processed 20 mutation data was available (Roh et al., 2017).

Production and purification of membrane HLA molecules - Cell line pellets were collected from 2×10^8 cells. Cell pellets were homogenized through a cell strainer on ice with lysis buffer containing 0.25 % sodium deoxycholate, 0.2 mM iodoacetamide, 1 mM EDTA, 1:300 Protease Inhibitors Cocktail (Sigma-Aldrich, P8340), 1 mM PMSF and 1 % octyl-β-D 25 glucopyranoside in PBS. Samples were then incubated at 4 °C for 1 hour. The lysates were cleared by centrifugation at 48,000g for 60 minutes at 4 °C, and then were passed through a pre-clearing column containing Protein-A Sepharose beads. HLA-I molecules were immunoaffinity purified from cleared lysate with the pan-HLA-I antibody (W6/32 antibody purified from HB95 hybridoma cells) covalently bound to Protein-A Sepharose beads. Affinity column was washed 30 first with 10 column volumes of 400 mM NaCl, 20 mM Tris-HCl followed by 10 volumes of 20 mM Tris-HCl, pH 8.0. The HLA peptides and HLA molecules were then eluted with 1 % trifluoracetic acid followed by separation of the peptides from the proteins by binding the eluted fraction to disposable reversed-phase C18 columns (Harvard Apparatus). Elution of the peptides 35 was effected with 30 % acetonitrile in 0.1 % trifluoracetic acid (Milner et al., 2013). The eluted peptides were cleaned using C18 stage tips as described previously (Rappaport et al., 2003).

Identification of eluted HLA peptides - The HLA peptides were dried by vacuum centrifugation, solubilized with 0.1 % formic acid, and resolved on capillary reversed phase chromatography on 0.075x300 mm laser-pulled capillaries, self-packed with C18 reversed-phase 3.5 μ m beads (Reprosil-C18-Aqua, Dr. Maisch GmbH, Ammerbuch-Entringen, Germany) (Ishihama et al., 2002). Chromatography was performed with the UltiMate 3000 RSLCnano-capillary UHPLC system (Thermo Fisher Scientific), which was coupled by electrospray to tandem mass spectrometry on Q-Exactive-Plus (Thermo Fisher Scientific). The HLA peptides were eluted with a linear gradient over 2 hours from 5 to 28 % acetonitrile with 0.1 % formic acid at a flow rate of 0.15 μ l / minute. Data was acquired using a data-dependent “top 10” method, fragmenting the peptides by higher-energy collisional dissociation. Full scan MS spectra was acquired at a resolution of 70,000 at 200 m/z with a target value of 3×10^6 ions. Ions accumulated to an AGC target value of 105 with a maximum injection time of generally 100 milliseconds. The peptide match option was set to Preferred. Normalized collision energy was set to 25 % and MS/MS resolution was 17,500 at 200 m/z. Fragmented m/z values were dynamically excluded from further selection for 20 seconds. The MS data were analyzed using MaxQuant (Cox and Mann, 2008) version 1.5.3.8, with 5 % false discovery rate (FDR). Peptides were searched against the UniProt human database (July 2015) and customized reference databases that contained the mutated sequences identified in the sample by WES. N-terminal acetylation (42.010565 Da) and methionine oxidation (15.994915 Da) were set as variable modifications. Enzyme specificity was set as unspecific and peptides FDR was set to 0.05. The match between runs option was enabled to allow matching of identifications across the samples belonging the same patient.

HLA typing was determined from the WES data by POLYSOLVER version 1.0 (Shukla et al., 2015); and the HLA allele to which the identified peptides match to was determined using the NetMHCpan version 4.0 (Hoof et al., 2009; Nielsen and Andreatta, 2016). The abundance of the peptides was quantified by the MS/MS intensity values, following normalization with the summed intensity of both UC-perturbed and control cell lines. The hydrophobicity of a peptide was determined by the fraction of hydrophobic amino acid in the peptide, which we termed *hydrophobic score*. The abundance of the peptides of top 20 % hydrophobic score vs bottom 20 % of hydrophobic score was compared using Wilcoxon ranksum test in UCD cell lines and control cell lines.

Peptidomics analysis - To identify the neo-antigens, nonsynonymous mutations in UCD perturbed cells to the mass-spec data from the un-perturbed and perturbed cells were mapped. The raw mass-spec data was transformed to mzML format using MSConvertGUI tool, integrated in ProteoWizard 3.0 (Chambers et al., 2012). The mzML files from cell lines, each from

with/without UC perturbation conditions, were used as an input to RAId_DbS tool, with all default parameters and recommended settings for our application (Alves et al., 2007). 2 missed cleavage sites at most were allowed. For terminal group molecular weight (Da), the default 1.0078 and 17.0027 were chosen respectively for N-terminal and C-terminal attached chemical group, which accounts for the Hydrogen signal and -COOH group respectively. The default mass tolerance (Da) of 1.0 in precursor ion and 0.2 in product ion parameters were used. Finally, the "RAId score" was used to identify peptides using P-value threshold of 0.05 (and E-value <= 1). Following, the reference protein sequence database from NCBI (Refseq release 82) was used to map the peptides to protein IDs. In identifying single amino acid polymorphisms (SAPs) all amino acids were allowed for. The RAId_DbS outputs, each from the paired cell lines, were used to map the amino-acid change to non-synonymous mutations on genes, separately for R->Y and Y->R cases, reported in VCF files, using in-house python script.

Statistics - Statistical analyses were performed using one-way ANOVA, dependent and independent-samples Student's T-test or Wilcoxon rank sum test of multiple or two groups, with Dunnett's correction when required. Log-transformed data were used where differences in variance were significant and variances were correlated with means. The sample size was chosen in advance based on common practice of the described experiment and is indicated. Each experiment was conducted with biological and technical replicates and repeated at least three times unless specified otherwise. When samples were distributed non-normally, Mann-Whitney analysis was performed. Statistical tests were done using Statsoft's STATISTICA, ver. 10. All error bars represent statistical error (SER). P<0.05 was considered significant in all analyses (*P<0.05, **P<0.005, ***P<0.0005, **** P<0.0001).

EXAMPLE 1

ASSOCIATION BETWEEN UC DYSREGULATION, CAD AND PYRIMIDINE SYNTHESIS

Patients with inborn deficiency in the UC components ornithine transcarbamylase (OTC), argininosuccinate lyase (ASL), argininosuccinate synthase (ASS1) or the transporter ornithine translocase (SLC25A15 or ORNT1) have increased pyrimidine-related metabolites in plasma or urine whereas patients with inborn carbamoyl phosphate synthetase I (CPS1) deficiency do not⁷⁻¹¹. These findings raise the possibility that a block in ureagenesis in non-cancerous settings is associated with increased pyrimidine synthesis and that a specific rewiring of the UC components is required for this association (Figure 1A). Hence, to assess the direct implications of UC dysregulation, fibroblasts from OTC deficient (OTCD) and ORNT1 deficient (ORNT1D) patients were studied. As shown in Figures 1B-C, these fibroblasts were significantly more

proliferative (as evident by the crystal violet stain) and exhibited elevated levels of activated CAD protein as compared to fibroblasts from healthy controls. On the contrary, fibroblasts from CPS1 deficient patients proliferated to the same extent and exhibited the same levels of activated CAD protein as fibroblasts from healthy controls (data not shown). Additionally, 5 cytomegalovirus infection which has been reported to cause activation of CAD and expansion of pyrimidine pools¹², leads to time dependent reduction in ASS1 expression and elevation in the UC transporter SLC25A13 levels in concordance with CAD elevation (Figure 1D). These findings suggest a metabolic link between specific changes in UC components' expression, CAD activation, nucleotide synthesis and proliferation. To assess the potential mechanism underlying 10 this metabolic association an online free NCBI protein alignment and BLAST tools were utilized, revealing high structural homology and high identity between the proximal UC enzymes, CPS1 and OTC; and between the components of the CAD - CPS2 and ATC, respectively (Figure 1E). These findings together with the reported increased nitrogen flux through the UC over pyrimidine synthesis¹³, suggest that in multiple circumstances, diversion of 15 metabolites from the UC enzymes to the CAD enzyme would decrease ureagenesis and substantially enhance pyrimidine synthesis and proliferation.

EXAMPLE 2

UC DYSREGULATION CORRELATES WITH CANCER PROGNOSIS

20 Metabolic redirection from the UC towards CAD (denoted herein as UCD) is expected from down-regulation of ASS1, ASL, OTC, or SLC25A15 (ORNT1), or from up-regulation of CPS1 or SLC25A13 (citrin). Thus, for example, as shown in Figures 2A-D, downregulation of ASS1 or OTC in cancer cells using shRNA resulted in increased proliferation and pyrimidine synthesis. To further substantiate this notion, in addition to downregulation of OTC in the 25 hepatocellular carcinoma (HepG2), SLC25A15 (ORNT1) was downregulated in ovarian carcinoma (SKOV), and SLC25A13 (citrin) was overexpressed in melanoma cells (LOX IMVI). Following each specific perturbation, CAD activation was measured through its phosphorylation on serine 1859. Importantly, each of these separate perturbations led to an increase in CAD phosphorylation and enhanced cellular proliferation *in vitro* (Figures 2F-G). Furthermore, 30 downregulation of OTC and SLC25A15 (ORNT1), resulted in increased ¹⁵N labelling of uracil from glutamine *in vitro* and increased tumor growth *in vivo* (Figure 2H).

Taken together, UC dysregulation and the consequent flux of nitrogen towards CAD can be achieved through specific alterations in expression of different enzymes in the cycle (Figure 1A).

To quantify the total extent of expression dysregulation in the above described 6 UC enzymes [i.e. ASS1, ASL, OTC, SLC25A15 (ORNT1), CPS1, SLC25A13 (citrin)] a UCD-score was computed. The UCD-score takes the aggregate expression of the 6 enzymes in the direction that supports metabolic redirection toward CAD. Specifically, it is a weighted sum of rank-normalized expression of the six genes across tumor samples, where ASS1, ASL, OTC, and SLC25A15 (ORNT1) take the weight of -1 and CPS1 or SLC25A13 (citrin) take the weight of +1.

By analyzing the human tumor transcriptomics data from the cancer genome atlas (TCGA) collection, the expression levels of the 6 UC genes show the alteration that supports metabolic redirection toward CAD in most TCGA tumor samples compared to their normal controls. Moreover, a majority of tumors harbour expression alterations in at least two UC components in the direction that enhances CAD activity (Figure 3A, Table 1 hereinbelow). As shown in Figures 3B-C and 4B, UCD was also evident at the protein level. Beyond its association with CAD activity (Figures 3A, 3C and 4A), UCD (and the UCD-score) was associated with higher tumor grade (Figure 4A). Importantly, both the specific changes in UC components' expression and independently, high CAD phosphorylation representing high CAD activity, were significantly associated with decreased cancer patients' survival (Figures 3E and 4D-E).

Taken together, UCD in cancer is a result of coordinated alterations in UC enzyme activities, where CPS1 and SLC25A13 tend to be up-regulated, while ASL, ASS1, OTC and SLC25A15 tend to be down-regulated to increase substrate supply to CAD and enhance pyrimidine synthesis (see Figure 4A); and most importantly UCD correlates with cancer prognosis and patient's survival.

Table 1: Fraction of the samples of UC dysregulated and PTMB in different cancer types.

Tumor types	UCD samples	PTMB samples
LIHC	95.5%	79.8%
BLCA	79.5%	92.9%
LUSC	72.1%	98.8%
CESC	69.6%	83.2%
STAD	66.7%	76.5%
SARC	66.1%	65.6%
KIRC	63.5%	63.0%
KIRP	61.3%	60.9%
LUAD	59.8%	89.5%
HNSC	58.5%	81.0%
BRCA	55.3%	63.8%
UCEC	42.9%	85.7%
PRAD	35.9%	51.5%

LGG	34.7%	43.9%
OV	30.8%	67.9%
SKCM	11.8%	48.7%

* The table lists the fraction of the TCGA samples where UCD-score is higher than the mean UCD score of corresponding healthy tissues (2nd column), and the fraction of the samples where PTMB is higher than expected (3rd column) in 15 different cancer types (1st column).

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EXAMPLE 3

NITROGEN METABOLITES CAN SERVE AS CANCER BIOMARKERS

Metabolic modelling of the network wide effects supports the notion that UCD would result in a diversion of nitrogenous metabolites from catabolic to anabolic processes, leading to increased synthesis of nitrogen rich metabolites, such as pyrimidines, and decreased ureagenesis 10 (Figures 5A and 5F). This modelling along with the experimental results described above suggests that changes in nitrogen metabolites in cancer may be detectable in biofluids, thereby allowing non-invasive cancer monitoring. To this end, the urine nitrogenous pyrimidine metabolites of mice bearing tumors *vs.* disease-free animals were compared. Interestingly, increased pyrimidine synthesis related metabolites were detected in the urine of mice bearing 15 breast or colon tumors as compared to control mice (Figure 5B) which was accompanied with UCD (Figure 5G). Furthermore, the analysis of purine and pyrimidine metabolites in patients with hepatocellular carcinoma and breast cancer showed a significant correlation between the UCD-score and the increase in pyrimidines (Figure 5C).

Following, a proof-of-principle analysis in biofluids from individuals with cancer was 20 conducted. A significantly elevated levels of pyrimidines in urine of patients with prostate cancer was found, compared to healthy controls (Figure 5G). In addition, the medical records of cancer patients in a large medical center in Israel was surveyed and the results demonstrated that in comparison to the established age- matched mean urea values in health¹⁴, children across a broad array of cancer types have significantly decreased plasma urea levels at the day of 25 admission (Figure 5D). In concordance, a significant decrease in plasma urea levels we observed in 519 patients with prostate cancer when compared to 257 individuals diagnosed with benign prostate hyperplasia (Figure 5E).

Taken together, these findings support the global dysregulation of nitrogen metabolism especially in advanced cancer that favours nitrogen utilization for pyrimidine synthesis over 30 systemic urea disposal, resulting in identifiable nitrogen metabolites alterations in mice and cancer patients' bio-fluids and suggest monitoring these changes as cancer biomarkers.

EXAMPLE 4**UCD IS ASSOCAITED WITH INCREASED PURINE TO PYRIMIFINE
TRASNVERSION MUTATIONS IN CANCER**

The data shows dysregulation of UC enzyme(s) in cancer resulting in increased CAD activity that leads to increased pyrimidine levels. To test this effect directly, the equilibrium between purines and pyrimidines in osteosarcoma and hepatic cancer cells upon downregulation of ASS1 and OTC, respectively, was determined. As predicted, perturbed UC enzyme activity increased pyrimidine levels and significantly altered the ratio between purines and pyrimidines (Figures 6A and 7A). Similarly, a cellular increase in the ratio of pyrimidine to purine metabolites was also found in the other UCD induced cancer cells generated (Figure 2F and 8).

As nucleotide imbalance has been reported to promote carcinogenesis by increasing mutagenesis^{15,16}, the genome of the UCD induced cellular cancer models was sequenced to uncover the genomic ramifications of UCD. An overall specific pyrimidine bias toward purines to pyrimidines (R->Y) compared to pyrimidines to purines (Y->R) point mutations on the DNA coding strand, was detected (Figure 9). Furthermore, the TCGA data was interrogated and demonstrated that altered expression of genes encoding UC proteins was significantly associated with increased purine to pyrimidine transversion mutations in the DNA coding strand in many cancer types, denoted herein as (PTMB) (Figure 6B, Table 1 hereinabove). Importantly, this association remained significant by controlling for the complementary pyrimidine to purine mutation (on the coding strand) in all cancer samples combined (Figure 7B); and across individual cancer types (Figure 7C). Interestingly, relative to samples with normal UC activity, in UCD samples the purine to pyrimidine mutations have a greater tendency to be non-synonymous, i.e. they change the encoded amino acid (Figure 6C), suggesting that a shift toward pyrimidine mutation in UCD samples may confer a fitness advantage to the tumor. Indeed, the elevated purine to pyrimidine mutations associated with UCD persisted also at the mRNA level, as observed via the analysis of DNA and RNA sequences of 18 breast cancers samples (Figure 7D). Furthermore, proteomic analysis of 18 breast cancer tumors¹⁷ showed that all non-synonymous mutations identified at the DNA level persisted at the protein level, affirming that these mutations indeed induce the respective amino acid changes (Figure 7E). Of note, the expression levels of the UC genes SLC25A13, SLC25A15 and CAD were among the top 10 % of genes associated with the purine to pyrimidine mutation rates in cancer (Figure 7F). Finally, the increased purine to pyrimidine mutation rate was associated with patient survival, independent of the rate of overall mutations (Figure 6D).

Together, these results demonstrate that UCD induces a specific pyrimidine-rich transversion mutational bias signature in cancer that propagates from the DNA to mRNA to protein levels and is associated with patients' survival.

EXAMPLE 5

UCD IS ASSOCAITED WITH BETTER RESPONE TO IMMUNE MODULATING THERAPIES

UCD-elicited pyrimidine-rich transversion mutational bias (PTMB) could result in the presentation of neo-antigens in tumor cells. Due to the outstanding relevance of this phenomenon for immunotherapy (Topalian et al., 2016), UCD and PTMB effects on the efficacy of immune checkpoint therapy (ICT) was evaluated. To this end, the transcriptomics of published data of melanoma patients treated with ICT (Van Allen et al., 2015),(Hugo et al., 2016) was analyzed and the UCD scores of the tumors were computed (where the gene expression of the 6 UC genes were available). Interestingly, responders to both anti-PD1 (Hugo et al., 2016) and anti-CTLA4 (Van Allen et al., 2015) therapy, had significantly higher UCD-scores than non-responders (Figure 10A), and interestingly, this separation was higher than that seen using CAD expression levels (Figure 11A). Following, a large exome sequencing cohort of patients treated with anti-PD1 (Roh et al., 2017) was analyzed, and indeed PTMB was found to be a stronger predictor of response to anti-PD1 therapy than mutational load (Figure 10B).

To learn more about the potential mechanisms underlying the increased ICT response associated with UCD and PTMB, an HLA peptidomics analysis was performed on the genetically engineered UCD cancer cells having high PTMB levels (shown in Figures 2F and 8). It was found that the presentation of more neo-antigens with PTMB may be one factor that contributes to immunogenicity (Table 2 hereinbelow). Additionally, UCD could contribute to the immunogenicity through the presentation of more abundant and hydrophobic peptides (Figures 11B-C), which are known to incur stronger immunogenicity (Chowell et al., 2015); and highly hydrophobic peptides were found to be significantly more abundant than expected in UCD but not in control cells (Figure 11D). Notably in this context, analysing the codon table of amino acids, revealed that R->Y mutations are significantly more likely to generate hydrophobic amino acids than other types of point mutations (Fisher exact test P<9.5E-5, odd ratio=2.67).

Taken together, these findings testify that the association of UCD to higher ICT efficacy is likely due to its combined effects of potentially generating PTMB-linked neo-antigens and perhaps more importantly, by generating more abundant and hydrophobic HLA-bound peptides.

Following, UCD and PTMB was induced in a syngeneic mouse model of colon cancer by knocking down ASS1. This UC perturbation resulted in larger tumors *in vivo* (Figures 12A-C),

as was expected given the increased proliferation observed in UCD induced cancer cell-lines. Notably, the ASS1 perturbed tumors were significantly more sensitive to anti-PD1-based ICT than the unperturbed ones (Figure 10C). This increased therapeutic response was associated with enhanced specific infiltration of CD8 cytotoxic T-cells and not CD4 helper-T cells, as found in other studies (Wei et al., 2017) (Figures 10D and 12D). Notably, the response to anti-PD1 treatment was more efficient in mice bearing the ASS1 knockdown tumors compared to mice bearing unperturbed control tumors, reflected by a significantly attenuated progression of the tumor (Figures 10E and 12E).

Table 2: Identities of neo-antigens in UC-perturbed cancer cell lines

UC Perturbation	Line	Gene	Transcript ID	R>Y		Y>R							
				Peptides in vector control	SEQ ID NO	Peptides in UCD cells	SEQ ID NO	Gene	Transcript ID	Untreated Peptide	SEQ ID NO	Treated Peptide	SEQ ID NO
Citrin OE	HLA-DRB5	NM_002125	GRPDAEY	1	GRPDDEY	9	IVL	NM_005547	ELSEQQEGQL	24	ELSEQQEGQL	26	
	PNPLA3	NM_025225	VCSCFIPF	2	VCSCFMPF	10	CALR	NM_004343	KEEEAAEDK	25	KEEEAAEDK	27	
	TPSD1	NM_012217	ALPVLASPAY	3	VLPVLASLAY	11							
OTC KO	HLA-DRB1	NM_002124	QPKRECHF	4	QLKRECQF	12							
	HepG2	HLA-B	NM_005514	TAADTAAQQTQR	5	QPMWECQF	13						
	TPSD1	NM_012217	ALPVLASPAY	6	QHKMECQF	14							
ASS1 KO	HLA-DRB1	NM_002124	AVTELGRPDAEY	7	TAADRAAQVTPG	15							
	U2os	HLA-DRB5	NM_002125	EDRRAAVDT	8	TAADTAAQVTPG	16						
					TAADTGQAQVTPG	17							
					VLPVLASLAY	18							
					AATELGRPDAEH	19							
					AAIKLGRPDAEH	20							
					AATELGRPNAEH	21							
					AATELGRPDAQH	22							

*Three different human cancer cell lines, melanoma (LOX), hepatocellular carcinoma (HEPG2) and osteosarcoma (U2OS), induced with different UCD generated more neo-antigens. The neo-antigens pulled down with HLA following specific UC perturbation in different cancers show they are enriched with R>Y mutation.

Taken together, the data reveals an oncogenic metabolic rewiring that maximizes the use of nitrogen by cancer cells and has diagnostic and prognostic values. Specifically, UCD was shown to be a common event in cancer which enhances nitrogen anabolism to pyrimidines by supplementing CAD with the three substrates needed for its function, supporting cell 5 proliferation and mutagenesis, and correlating with survival risk. Moreover, the data reveals the hitherto unknown direct link between metabolic alterations in cancer, changes in nitrogen composition in biofluids and a genome-wide shift in mutational bias toward pyrimidines, generating metabolic and mutational signatures which encompass a persistent disruption in purine to pyrimidine nucleotide balance. The pyrimidine-rich transversion mutational bias 10 propagates from the DNA to RNA and protein levels, leading to the generation of peptides with increased predicted immunogenicity, enhancing the response to immune-modulation therapy independently of mutational load both in mouse models and in patient correlative studies (Figure 10F).

Although the invention has been described in conjunction with specific embodiments 15 thereof, it is evident that many alternatives, modifications and variations will be apparent to those skilled in the art. Accordingly, it is intended to embrace all such alternatives, modifications and variations that fall within the spirit and broad scope of the appended claims.

All publications, patents and patent applications mentioned in this specification are herein 20 incorporated in their entirety by reference into the specification, to the same extent as if each individual publication, patent or patent application was specifically and individually indicated to be incorporated herein by reference. In addition, citation or identification of any reference in this application shall not be construed as an admission that such reference is available as prior art to the present invention. To the extent that section headings are used, they should not be construed as necessarily limiting.

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WHAT IS CLAIMED IS:

1. A method of treating cancer in a subject in need thereof, the method comprising:
 - (a) determining a shift from the urea cycle to pyrimidine synthesis in a cancerous cell of the subject as compared to a control sample; and
 - (b) treating said subject with an immune modulating agent when said shift is indicated.
2. A method of potentiating cancer treatment with an immune modulating agent in a subject in need thereof, the method comprising:
 - (a) determining a shift from the urea cycle to pyrimidine synthesis in a cancerous cell of the subject as compared to a control sample; and
 - (b) treating said subject with an agent which induces a pyrimidines to purines nucleotide imbalance when said shift is indicated.
3. The method of any one of claims 1-2, wherein said shift is determined by level of purine to pyrimidine transversion mutations.
4. The method of any one of claims 1-2, wherein said shift is determined by a level and/or activity of a urea cycle enzyme and/or a CAD enzyme.
5. The method of claim 4, wherein said urea cycle enzyme is selected from the group consisting of ASL, ASS1, OTC, SLC25A15, CPS1 and SLC25A13.
6. A method of prognosing cancer in a subject diagnosed with cancer, the method comprising determining a level of purine to pyrimidine transversion mutations in a cancerous cell of the subject as compared to a control sample, wherein said level of said purine to pyrimidine transversion mutations above a predetermined threshold is indicative of poor prognosis, thereby prognosing cancer in the subject.
7. A method of monitoring efficacy of cancer therapy in a subject, the method comprising determining a level of purine to pyrimidine transversion mutations in a cancerous cell of the subject undergoing or following the cancer therapy, wherein a decrease in said level of said purine to pyrimidine transversion mutations from a predetermined threshold or in comparison to said level in said subject prior to said cancer therapy, indicates efficacious cancer therapy.

8. A method of prognosing cancer in a subject diagnosed with cancer, the method comprising determining a level and/or activity of a urea cycle enzyme SLC25A15 in a cancerous cell of the subject as compared to a control sample, wherein said level and/or activity of said SLC25A15 below a predetermined threshold is indicative of poor prognosis, thereby prognosing cancer in the subject.

9. A method of monitoring efficacy of cancer therapy in a subject, the method comprising determining a level and/or activity of a urea cycle enzyme SLC25A15 in a cancerous cell of the subject undergoing or following the cancer therapy, wherein an increase in said level and/or activity of said a urea cycle enzyme SLC25A15 from a predetermined threshold or in comparison to said level and/or activity in said subject prior to said cancer therapy, indicates efficacious cancer therapy.

10. The method of any one of claims 8-9, wherein said cancer is selected from the group consisting of thyroid cancer, hepatic cancer, bile duct cancer and kidney cancer.

11. A method of prognosing cancer in a subject diagnosed with cancer, the method comprising determining a level and/or activity of at least two urea cycle enzymes selected from the group consisting of ASL, ASS1, OTC, SLC25A15, CPS1 and SLC25A13 in a cancerous cell of the subject as compared to a control sample, wherein said urea cycle enzymes comprise ASS1 and SLC25A13 said at least two is at least three;

wherein said level and/or activity of said ASL, said ASS1, said OTC and/or said SLC25A15 below a predetermined threshold and/or said level and/or activity of said CPS1 and/or said SLC25A13 above a predetermined threshold is indicative of poor prognosis, thereby prognosing cancer in the subject.

12. A method of monitoring efficacy of cancer therapy in a subject, the method comprising determining a level and/or activity of at least two urea cycle enzymes selected from the group consisting of ASL, ASS1, OTC, SLC25A15, CPS1 and SLC25A13 in a cancerous cell of the subject undergoing or following the cancer therapy;

wherein said urea cycle enzymes comprise ASS1 and SLC25A13 said at least two is at least three; wherein an increase in said level and/or activity of said ASL, said ASS1, said OTC and/or said SLC25A15 and/or a decrease in said level and/or activity of said CPS1 and/or said

SLC25A13 from a predetermined threshold or in comparison to said level and/or activity in said subject prior to said cancer therapy indicates efficacious cancer therapy.

13. The method of any one of claims 11-12, wherein said cancer is thyroid, stomach and/or bladder cancer and said at least two urea cycle enzymes are selected from the group consisting of OTC, SLC25A15 and SLC25A13.

14. The method of any one of claims 11-12, wherein said cancer is prostate cancer and said at least two urea cycle enzymes comprise ASS1 and CSP1.

15. The method of any one of claims 11-12, wherein said cancer is lung and/or head and neck cancer and said at least two urea cycle enzymes are selected from the group consisting of ASL, OTC, CPS1 and SLC25A13.

16. The method of any one of claims 11-12, wherein said cancer is hepatic, bile duct and/or kidney cancer and said at least two urea cycle enzymes are selected from the group consisting of ASL, ASS1, OTC and SLC25A15.

17. The method of any one of claims 11-12, wherein said cancer is breast cancer and said at least three urea cycle enzymes comprise ASS1, OTC and SLC25A13.

18. A method of prognosing breast cancer in a subject diagnosed with breast cancer, the method comprising determining a level and/or activity of at least two urea cycle enzymes selected from the group consisting of ASS1, OTC and SLC25A13 in a cancerous cell of the subject as compared to a control sample,

wherein said level and/or activity of said ASS1 and/or said OTC below a predetermined threshold and/or said level and/or activity of said SLC25A13 above a predetermined threshold is indicative of poor prognosis, thereby prognosing the breast cancer in the subject.

19. A method of monitoring efficacy of cancer therapy in a subject diagnosed with breast cancer, the method comprising determining a level and/or activity of at least two urea cycle enzymes selected from the group consisting of ASS1, OTC and SLC25A13 in a cancerous cell of the subject undergoing or following the cancer therapy;

wherein an increase in said level and/or activity of said ASS1 and/or said OTC and/or a decrease in said level and/or activity of said SLC25A13 from a predetermined threshold or in

comparison to said level and/or activity in said subject prior to said cancer therapy, indicates efficacious cancer therapy.

20. The method of any one of claims 6, 8, 10-11 and 13-18, further comprising determining a level and/or activity of a CAD enzyme, wherein said level and/or activity of said CAD enzyme above a predetermined threshold is indicative of poor prognosis.

21. The method of any one of claims 7, 9-10, 12-17 and 19, further comprising determining a level and/or activity of a CAD enzyme, wherein a decrease in the level and/or activity of said CAD enzyme from a predetermined threshold or in comparison to said level in said subject prior to said cancer therapy, indicates efficacious cancer therapy.

22. The method of any one of claims 4-5, 20 and 21, wherein said CAD is activated CAD.

23. The method of any one of claims 6, 8, 10-11, 13-18, 20 and 22, comprising corroborating the prognosis using a state of the art technique.

24. A method of treating cancer in a subject in need thereof, the method comprising:

(a) prognosing the subject according to the method of any one of claims 6, 8, 10-11, 13-18, 20 and 22-23; and
(b) treating said subject with a cancer therapy according to the prognosis.

25. A method of treating cancer in a subject in need thereof, the method comprising:

(a) prognosing the subject according to the method of any one of claims 6, 8, 10-11, 13-18, 20 and 22-23; and wherein when a poor prognosis is indicated
(b) treating said subject with a cancer therapy.

26. A method of treating cancer in a subject in need thereof, the method comprising:

(a) prognosing the subject according to the method of any one of claims 6, 8, 10-11, 13-18, 20 and 22-23; and
(b) selecting a cancer therapy for treating said subject based on the prognosis.

27. A method of treating cancer in a subject in need thereof, the method comprising:

(a) prognosing the subject according to the method of any one of claims 6, 8, 10-11, 13-18, 20 and 22-23; and wherein when a poor prognosis is indicated

(b) selecting a cancer therapy for treating said subject based on the level of said purine to pyrimidine transversion mutations, said urea cycle enzyme and/or said CAD enzyme.

28. The method of any one of claims 6-27, wherein said predetermined threshold is at least 1.1 fold.

29. The method of any one of claims 1-6, 8, 10-11, 13-18, 20 and 22-28, wherein said control sample is a non-cancerous cell of said subject.

30. The method of any one of claims 1-6, 8, 10-11, 13-18, 20 and 22-28, wherein said control sample is a cancerous cell with a level and/or activity of said urea cycle enzyme, said purine to pyrimidine transversion mutations and/or said CAD enzyme similar to levels and/or activity of same in a healthy cell of the same type.

31. The method of any one of claims 1-9, 11-12 and 20-30, wherein said cancer is selected from the group consisting of hepatic cancer, osteosarcoma, breast cancer, colon cancer, thyroid cancer, stomach cancer, lung cancer, kidney cancer, prostate cancer, head and neck cancer, bile duct cancer and bladder cancer.

32. The method of any one of claims 1-9, 11-12 and 20-30, wherein said cancer is selected from the group consisting of hepatic cancer, osteosarcoma, breast cancer and colon cancer.

33. The method of any one of claims 7, 9-10, 12-17, 19 and 21-32, wherein said cancer therapy comprises a therapy selected from the group consisting of L-arginine depletion, glutamine depletion, pyrimidine analogs, thymidylate synthase inhibitor and mammalian target of Rapamycin (mTOR) inhibitor.

34. The method of any one of claims 7, 9-10, 12-17, 19 and 21-32, wherein said cancer therapy comprises an immune modulation agent.

35. The method of claim 34, wherein said cancer therapy further comprises an agent which induces a pyrimidines to purines nucleotide imbalance.

36. The method of any one of claims 1 and 3-5, further comprising treating said subject with an agent which induces a pyrimidines to purines nucleotide imbalance when said shift is indicated.

37. The method of claim 2, further comprising treating said subject with the immune modulation agent.

38. The method of any one of claims 1-5 and 34-37, wherein said immune modulation agent comprises anti-PD1.

39. The method of any one of claims 1-5 and 34-37, wherein said immune modulation agent comprises anti-CTLA4.

40. The method of any one of claims 2 and 35-39, wherein said agent which induces a pyrimidines to purines nucleotide imbalance comprises an anti-folate agent.

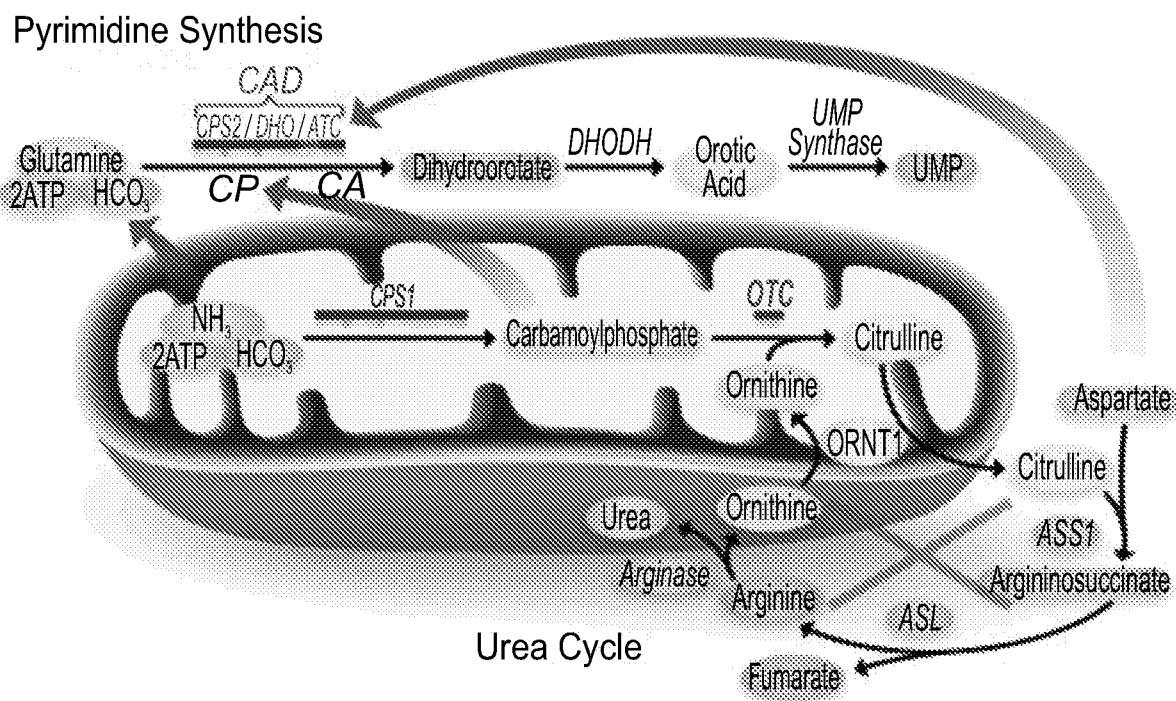
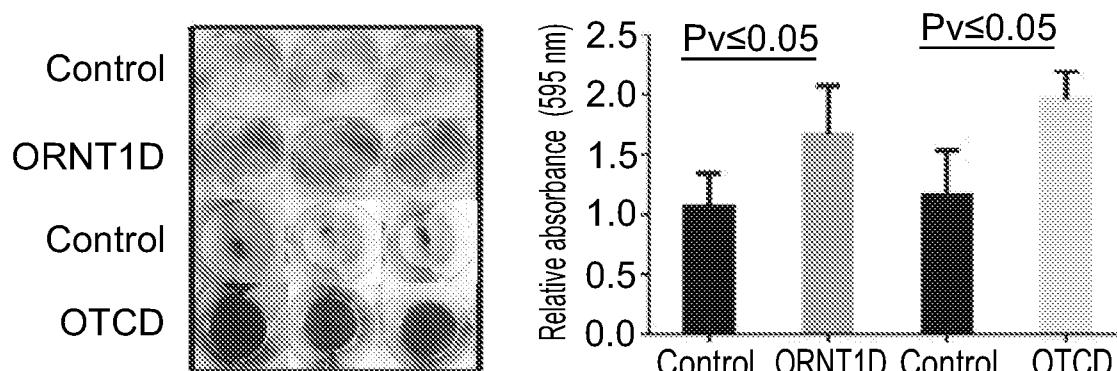
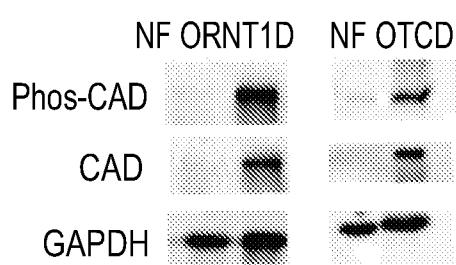
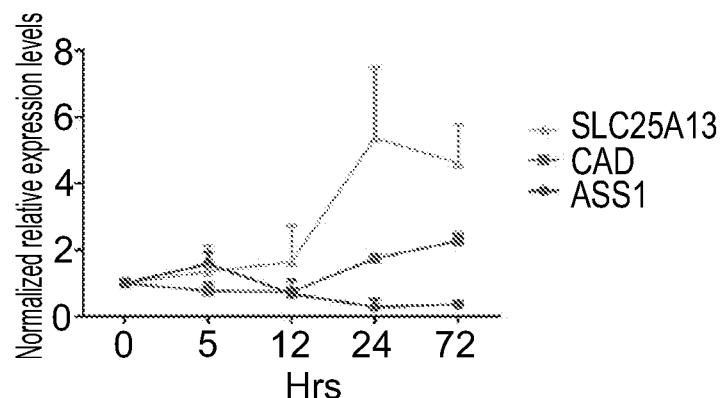
41. The method of claim 40, wherein said anti-folate agent comprises methotrexate.

42. The method of any one of claims 3, 6-7 and 20-41, wherein said purine to pyrimidine transversion mutations are non-synonymous purine to pyrimidine transversion mutations.

43. The method of any one of claims 3, 6-7 and 20-42, wherein said determining said level of purine to pyrimidine transversion mutations is effected at the genomic level.

44. The method of any one of claims 4-5 and 8-42, wherein said determining said level of said enzyme is effected at the transcript level.

45. The method of any one of claims 4-5 and 8-42, wherein said determining said level of said enzyme is effected at the protein level.

Figure 1A**Figure 1B****Figure 1C****Figure 1D**

2/17

Figure 1E

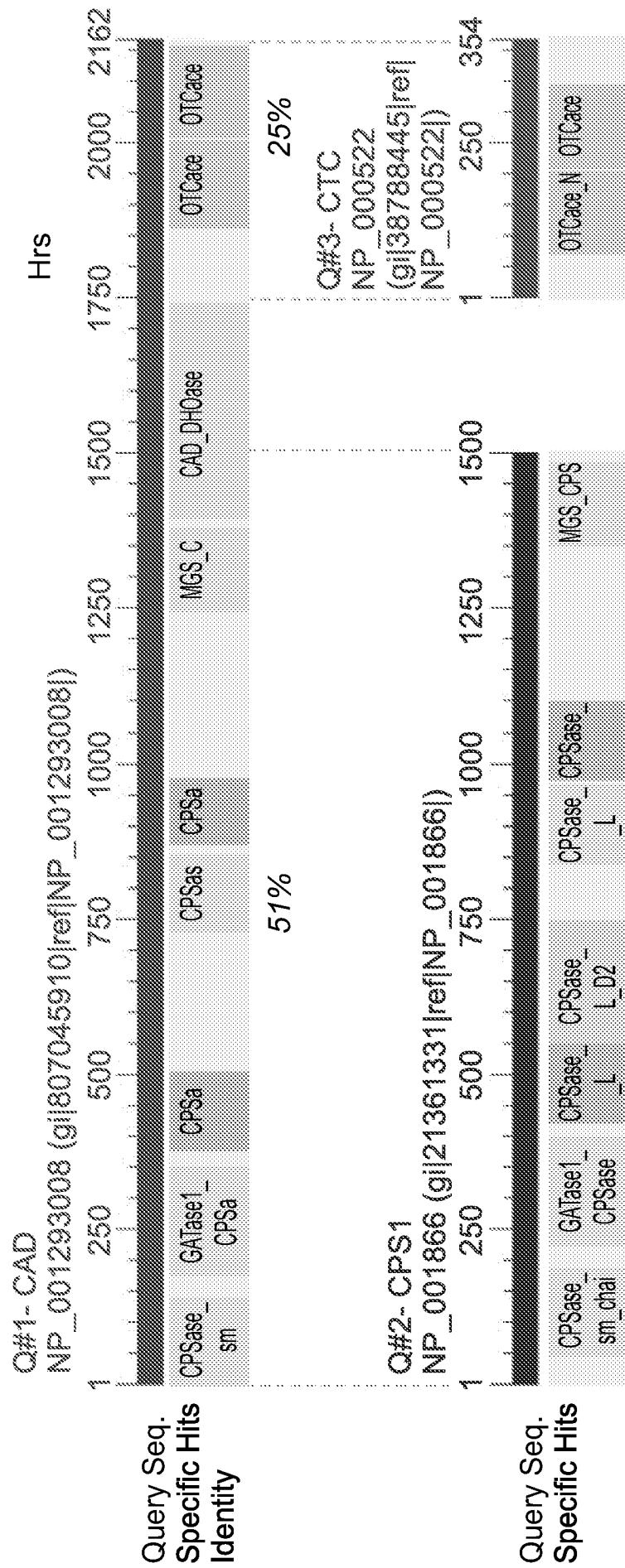


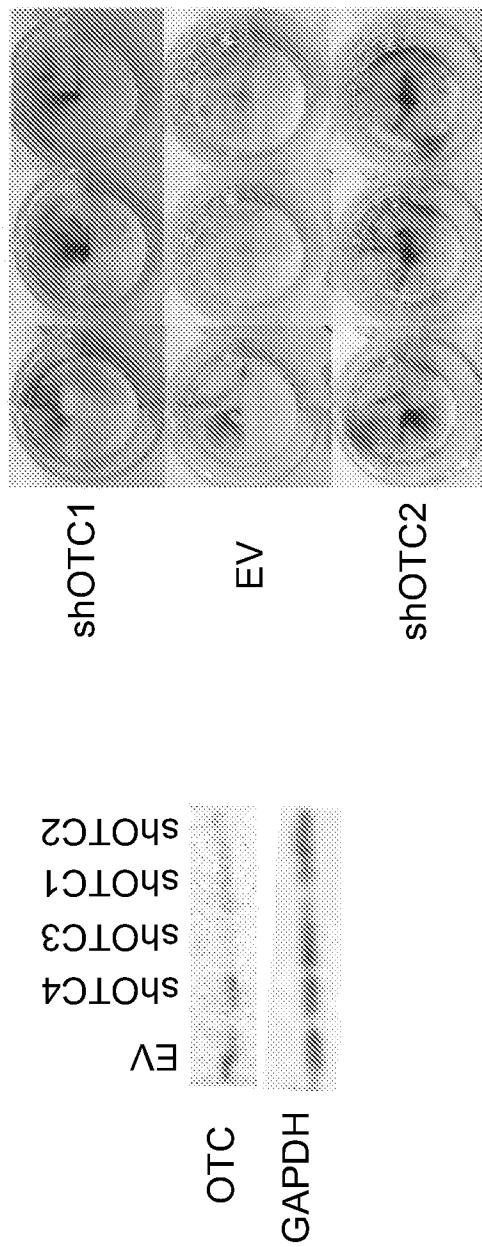
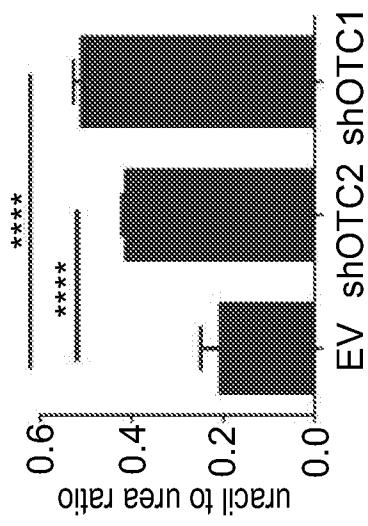
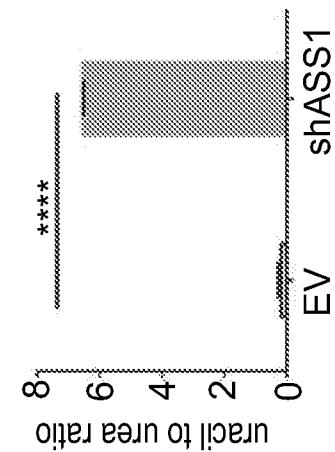
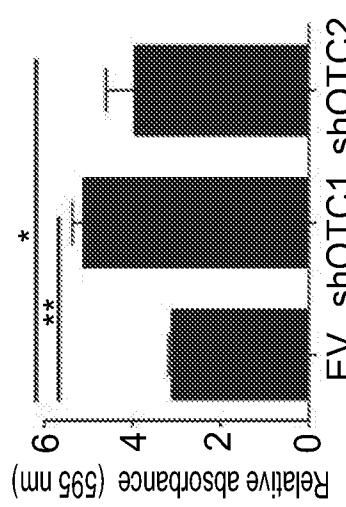
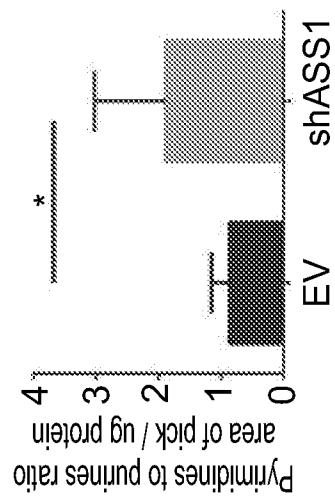
Figure 2A**Figure 2C****Figure 2D****Figure 2E**

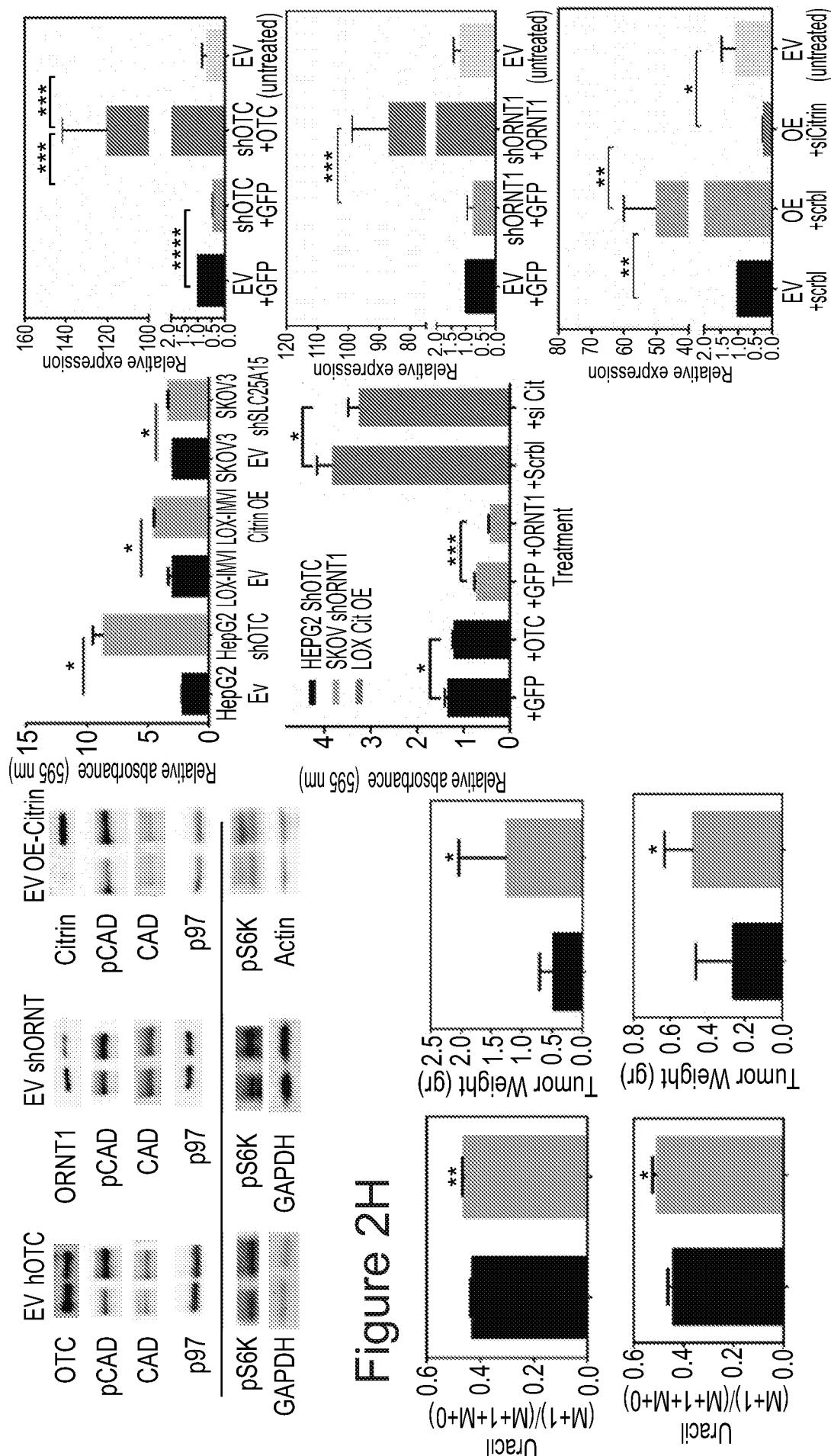
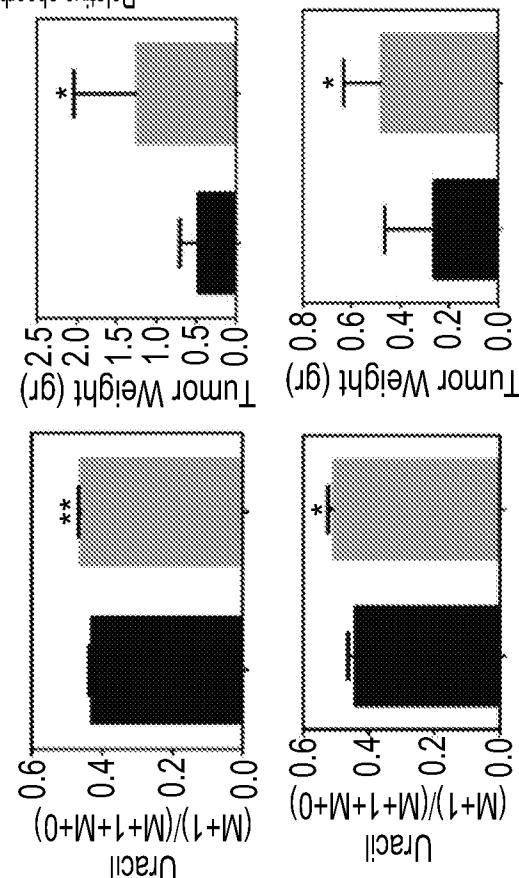
Figure 2F**Figure 2H**

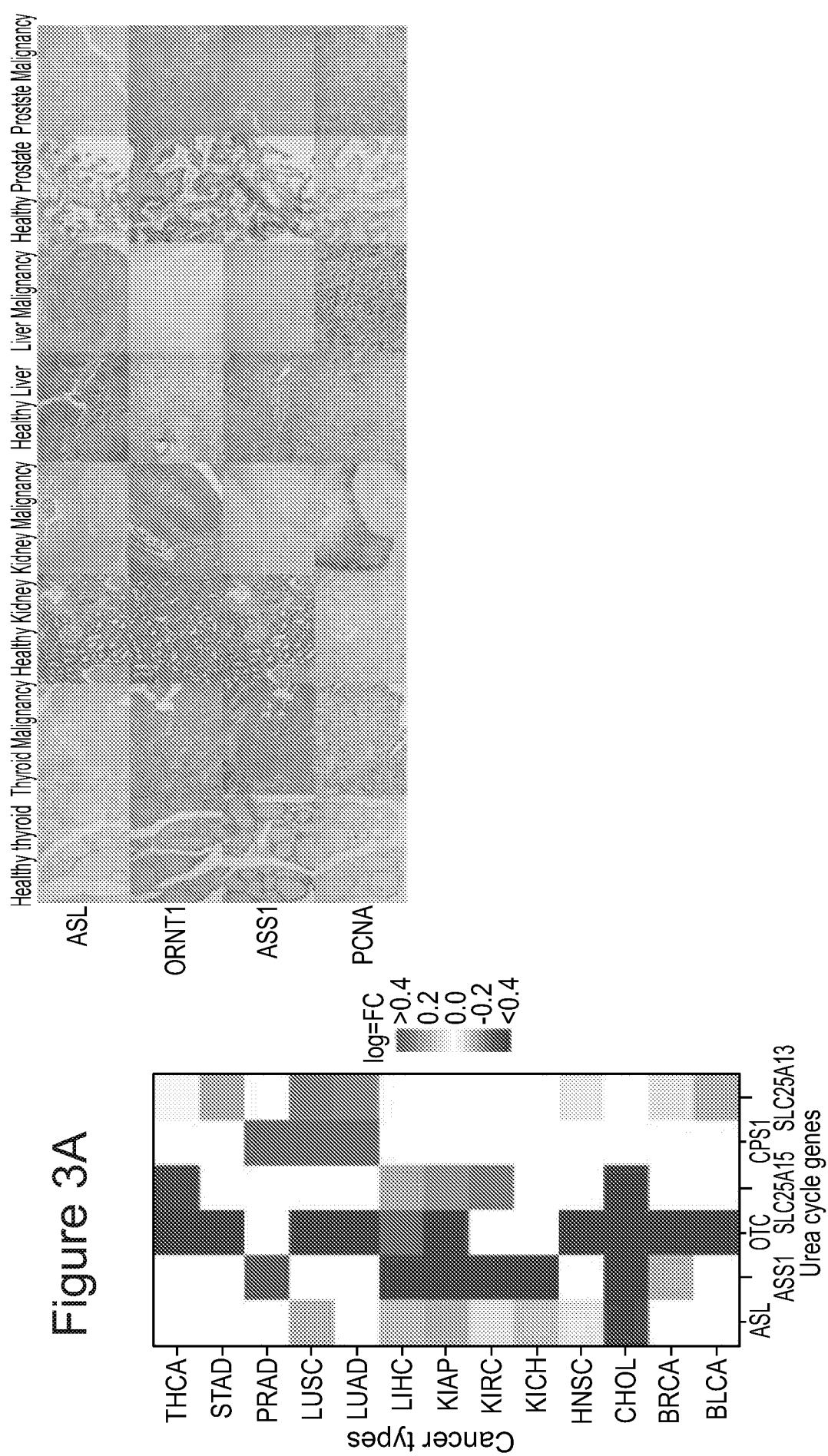
Figure 3B

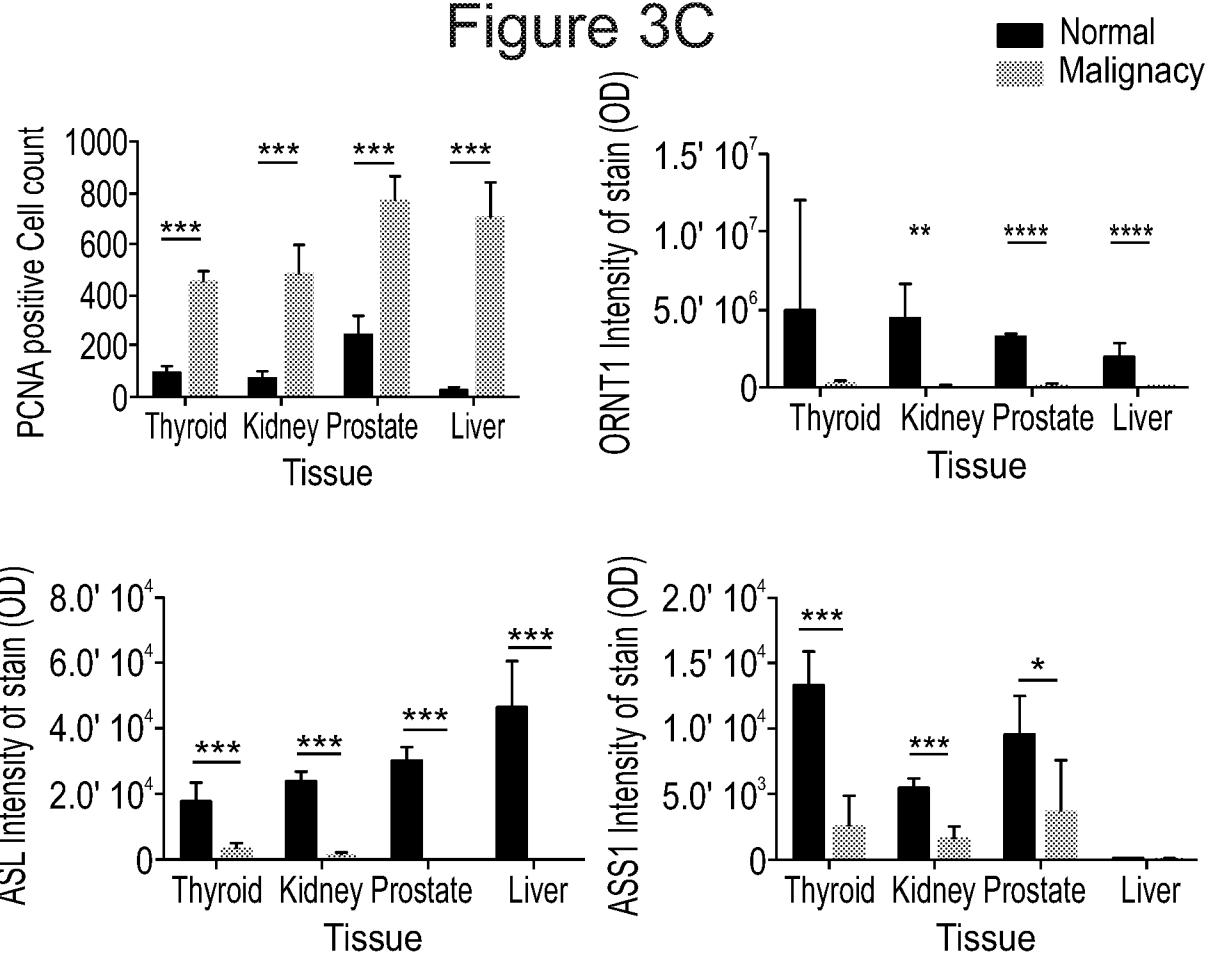
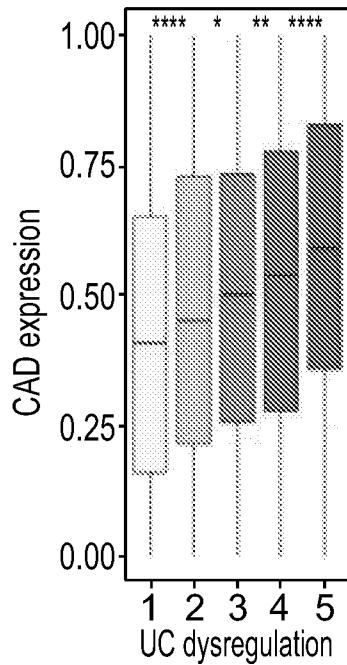
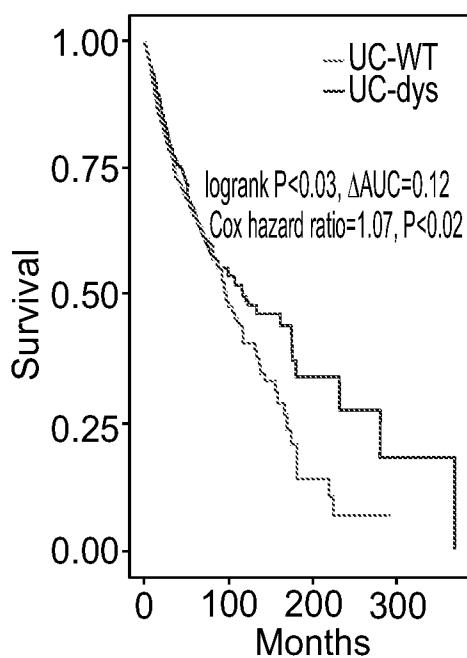
Figure 3C**Figure 3D****Figure 3E**

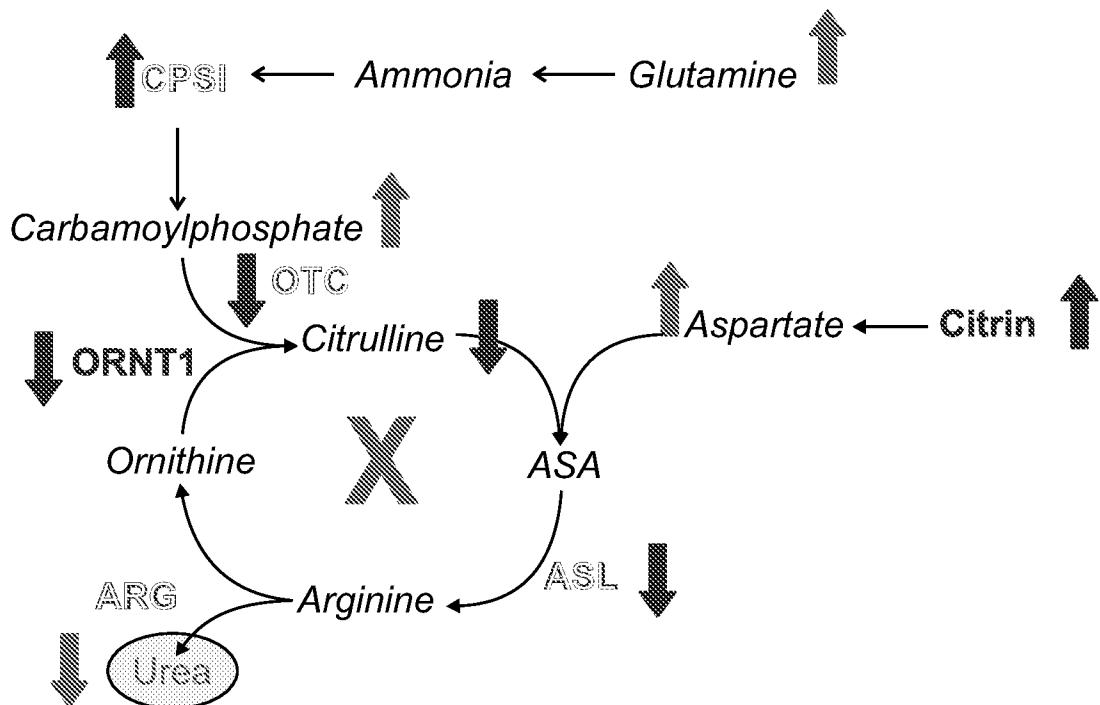
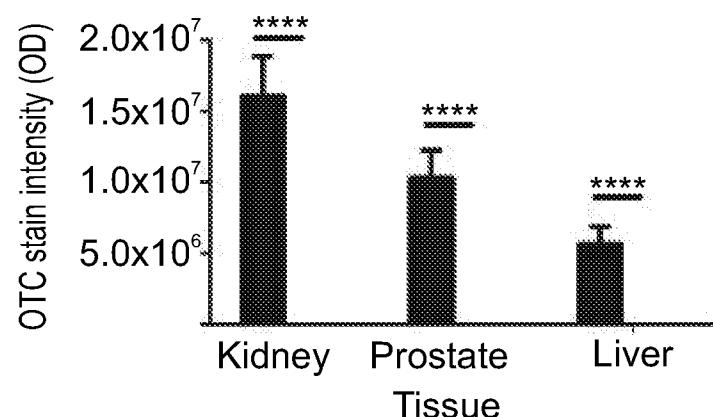
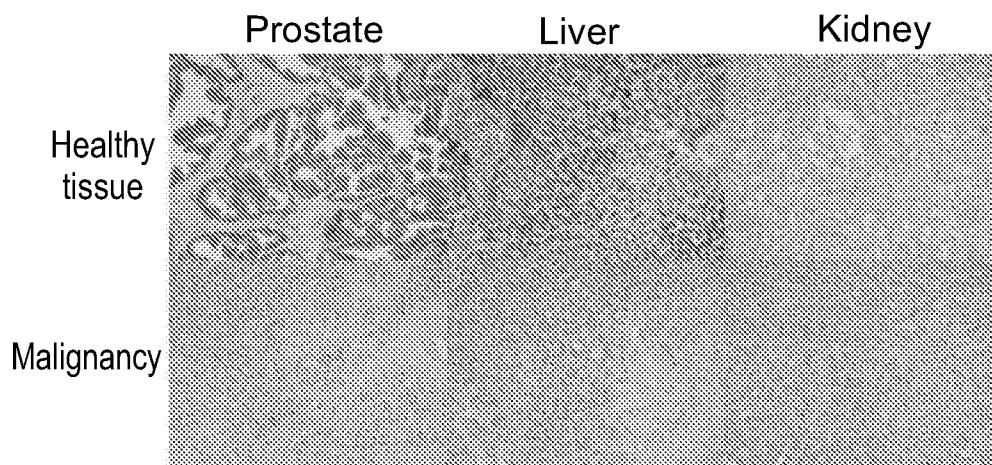
Figure 4A**Figure 4B**

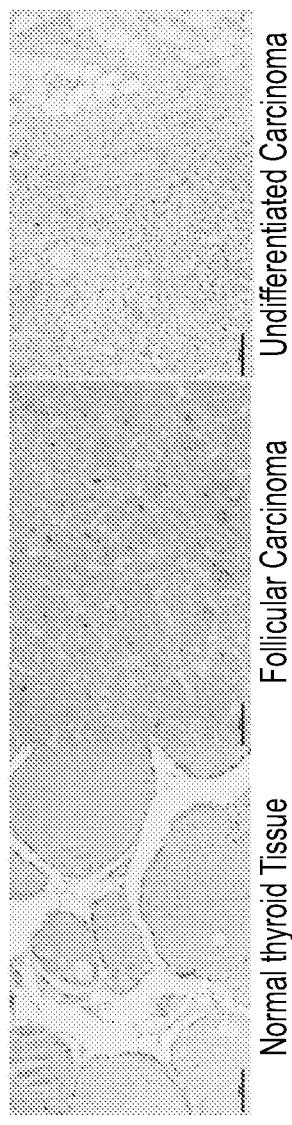
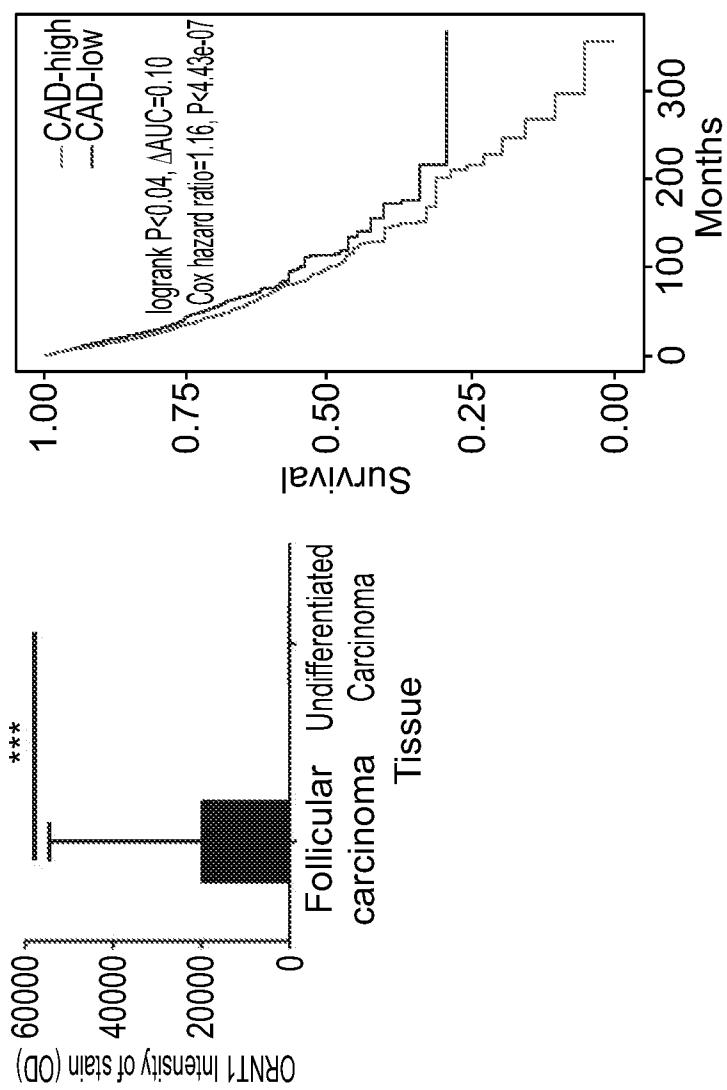
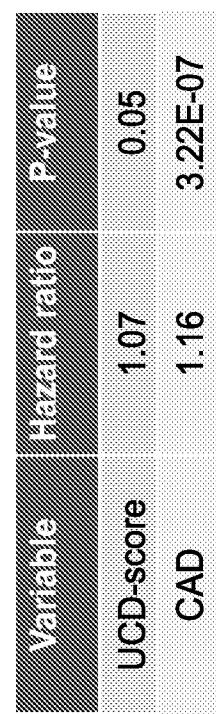
Figure 4C**Figure 4D****Figure 4E**

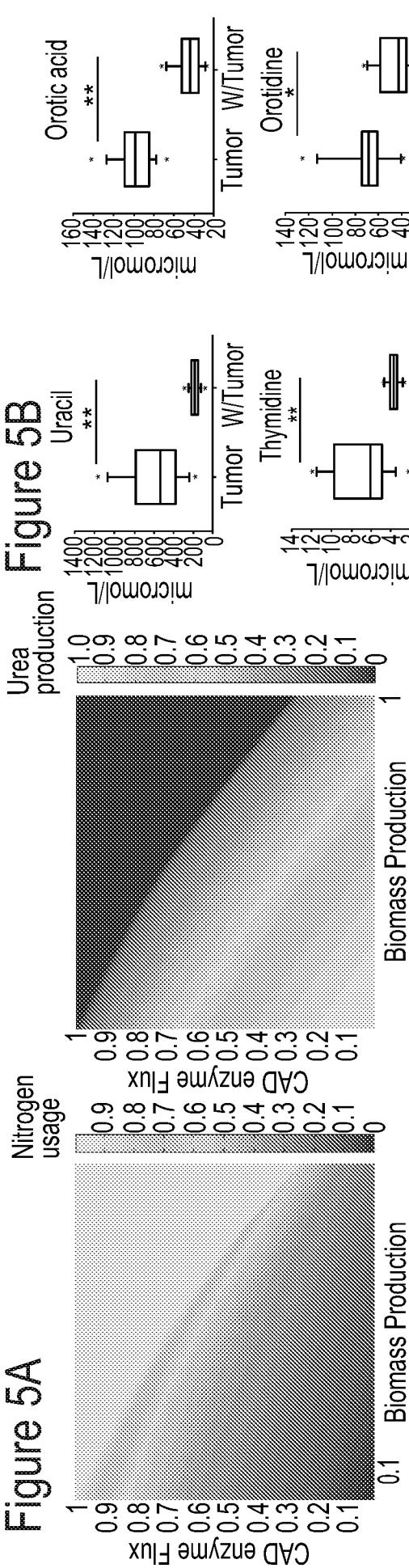
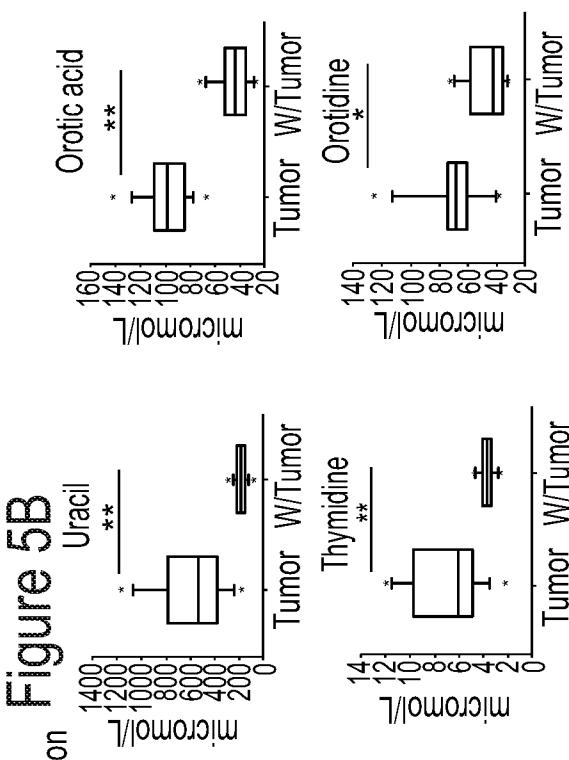
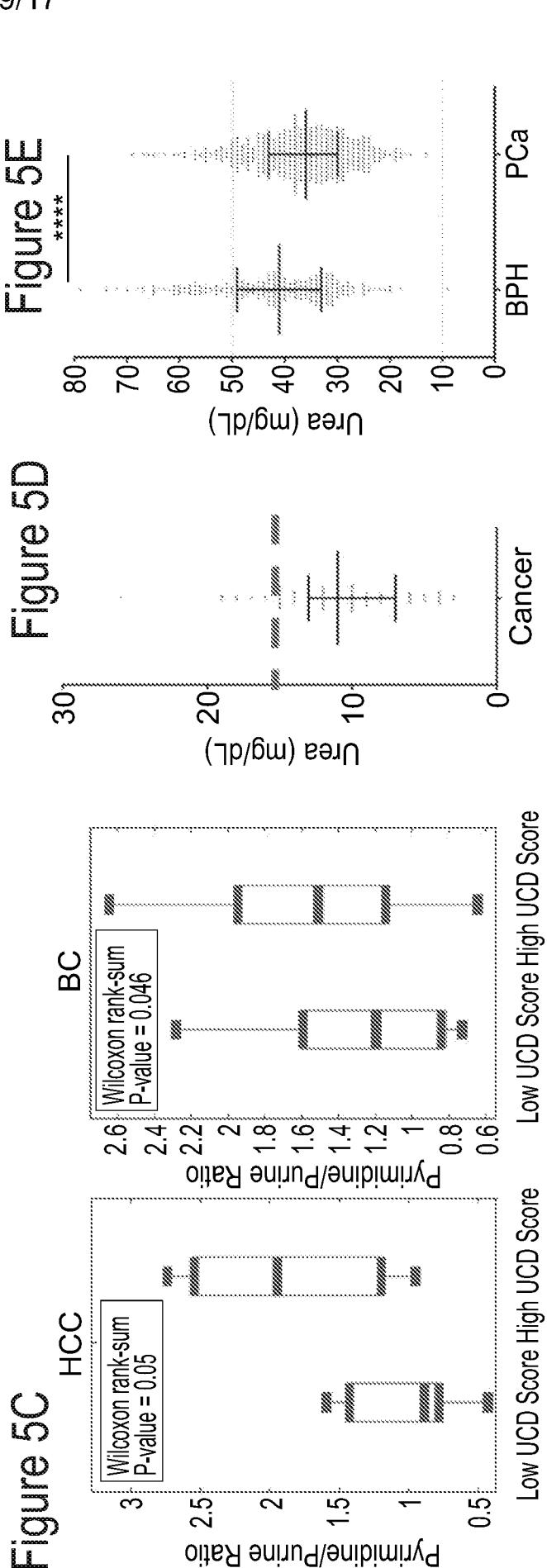
Figure 5A**Figure 5B****Figure 5C**

Figure 5F

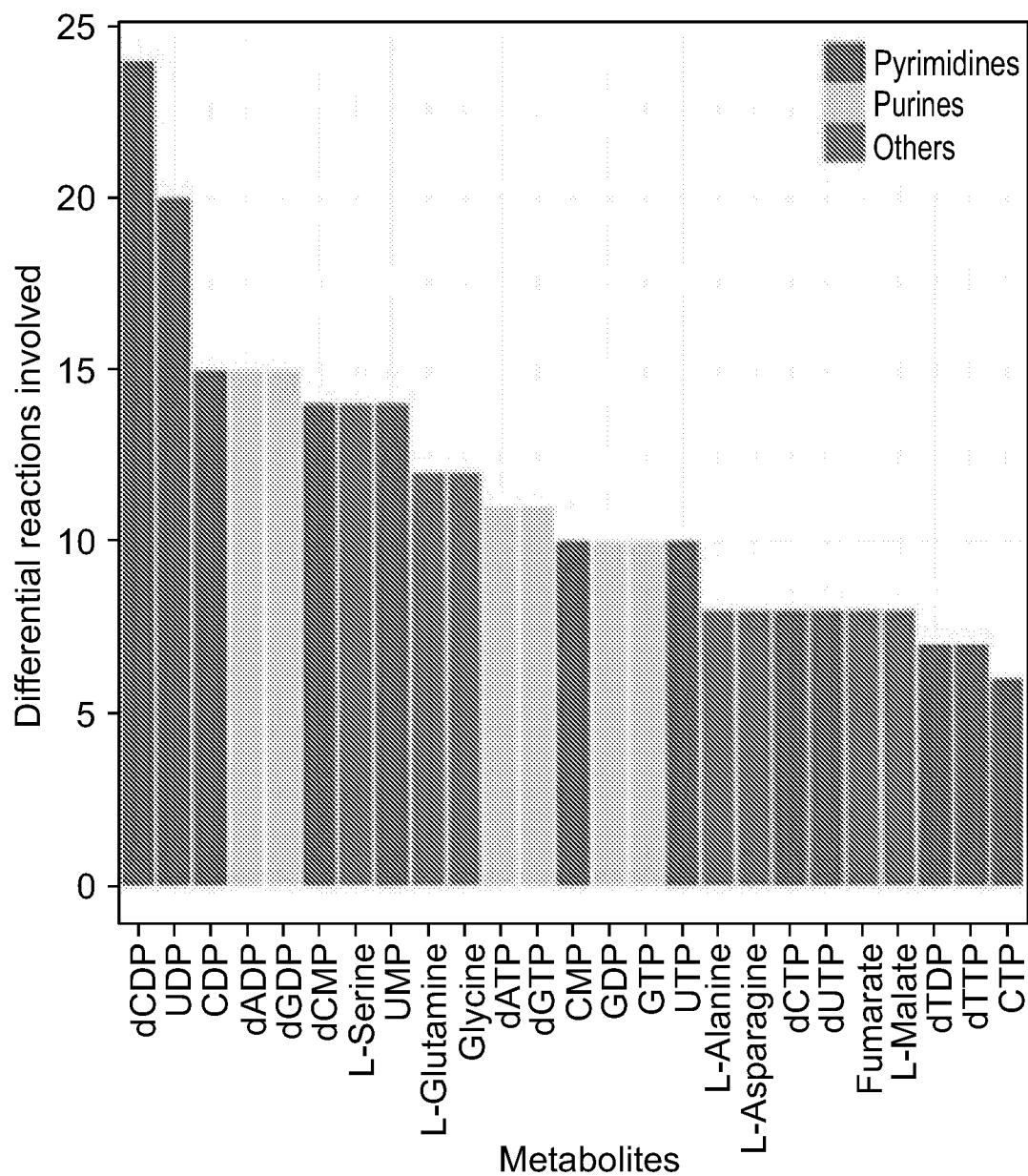


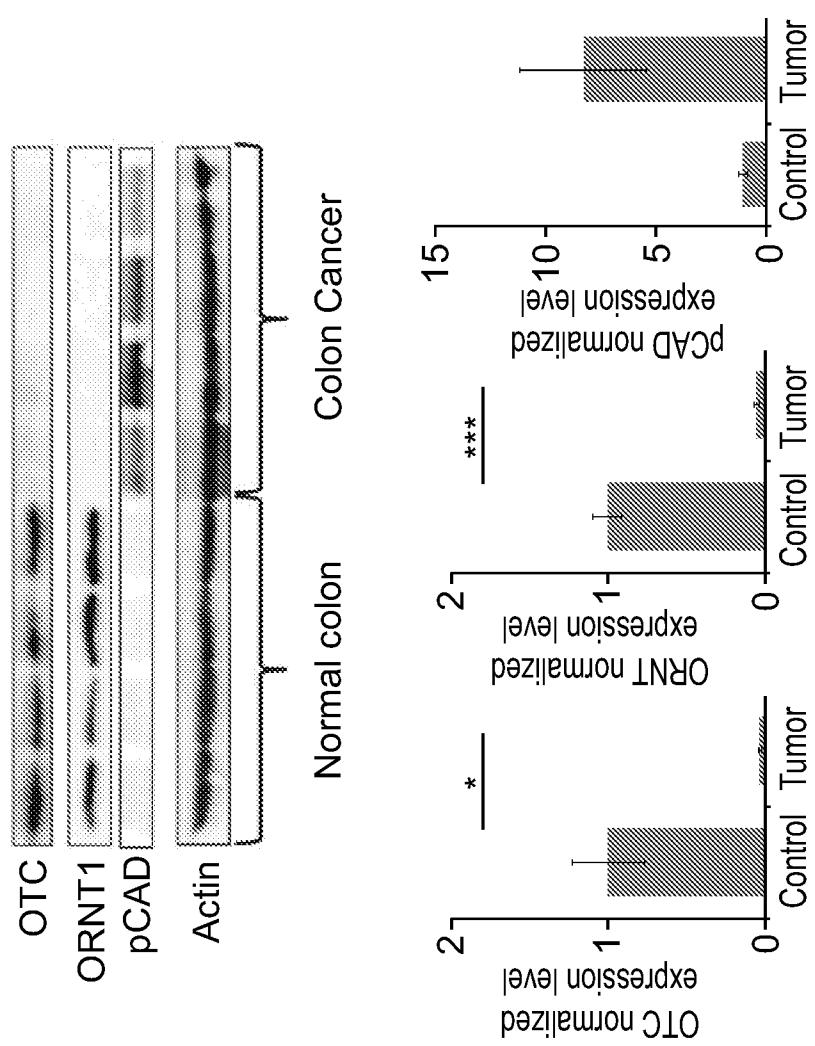
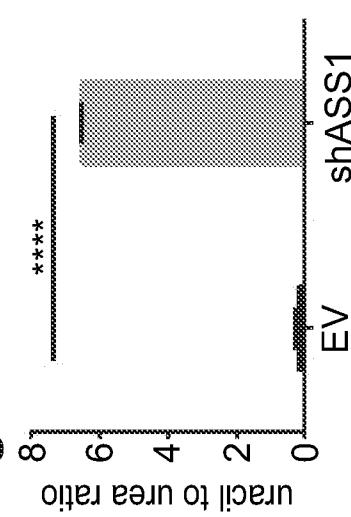
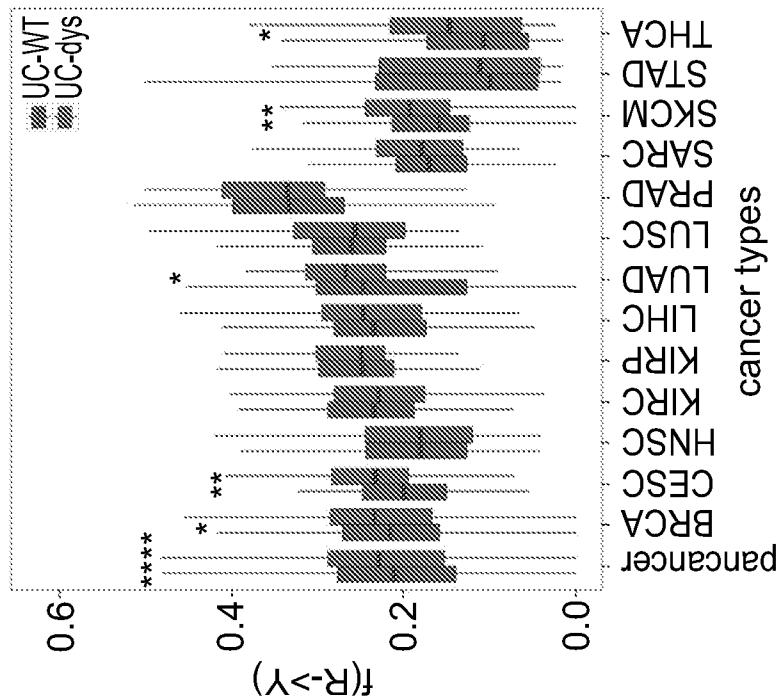
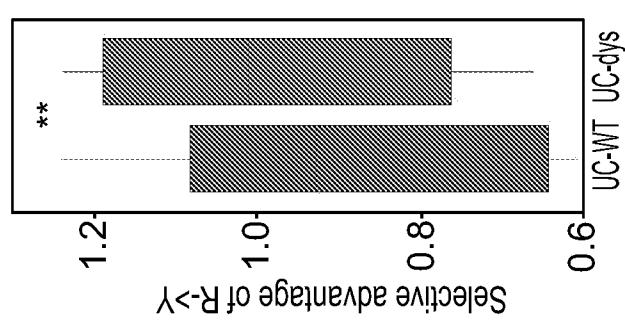
Figure 5G

Figure 6A**Figure 6B****Figure 6C****Figure 6D**

Variants	Median ratio	p-value
$R \rightarrow Y$	1.08	0.05
overall mutation	0.94	0.16
$Y \rightarrow R$	1.44	0.43

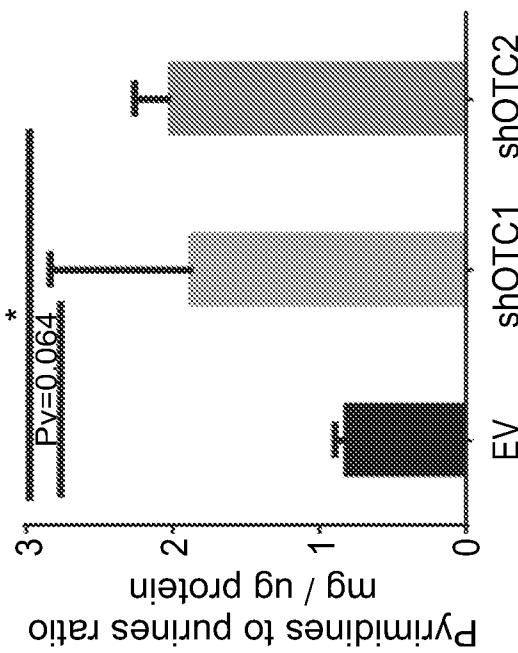
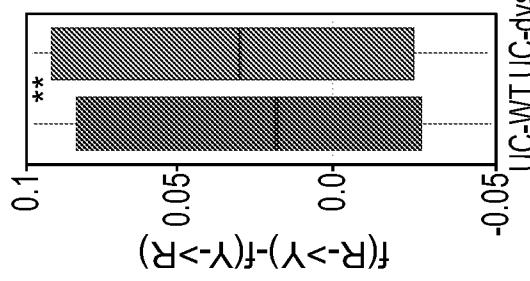
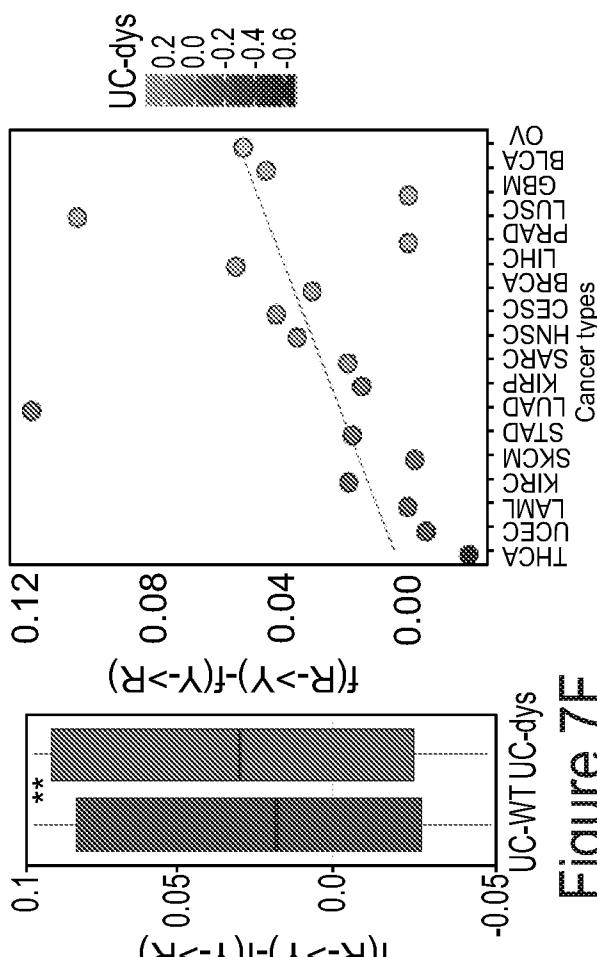
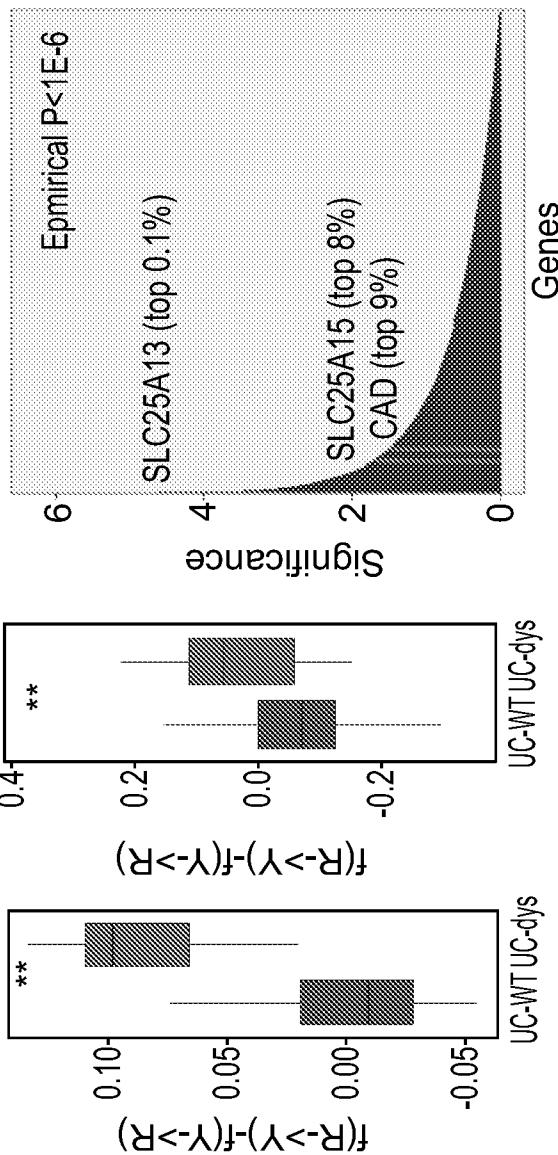
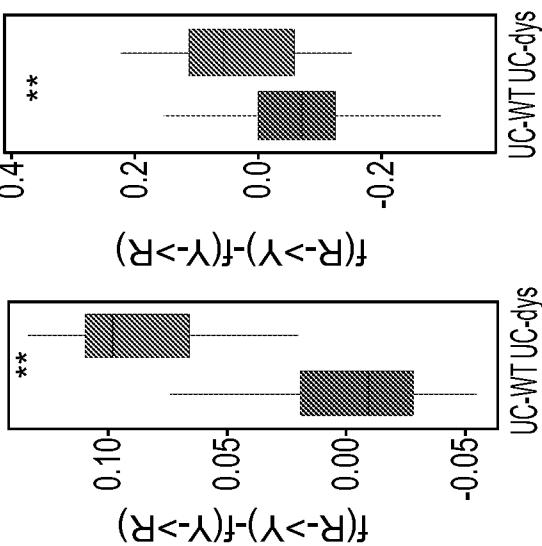
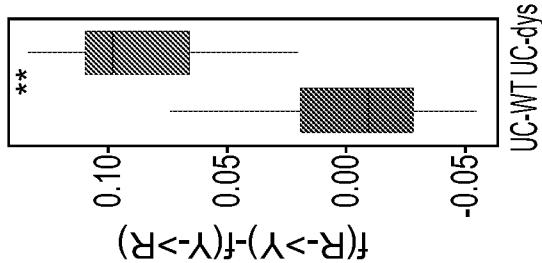
Figure 7A**Figure 7B****Figure 7C****Figure 7F****Figure 7E****Figure 7D**

Figure 8

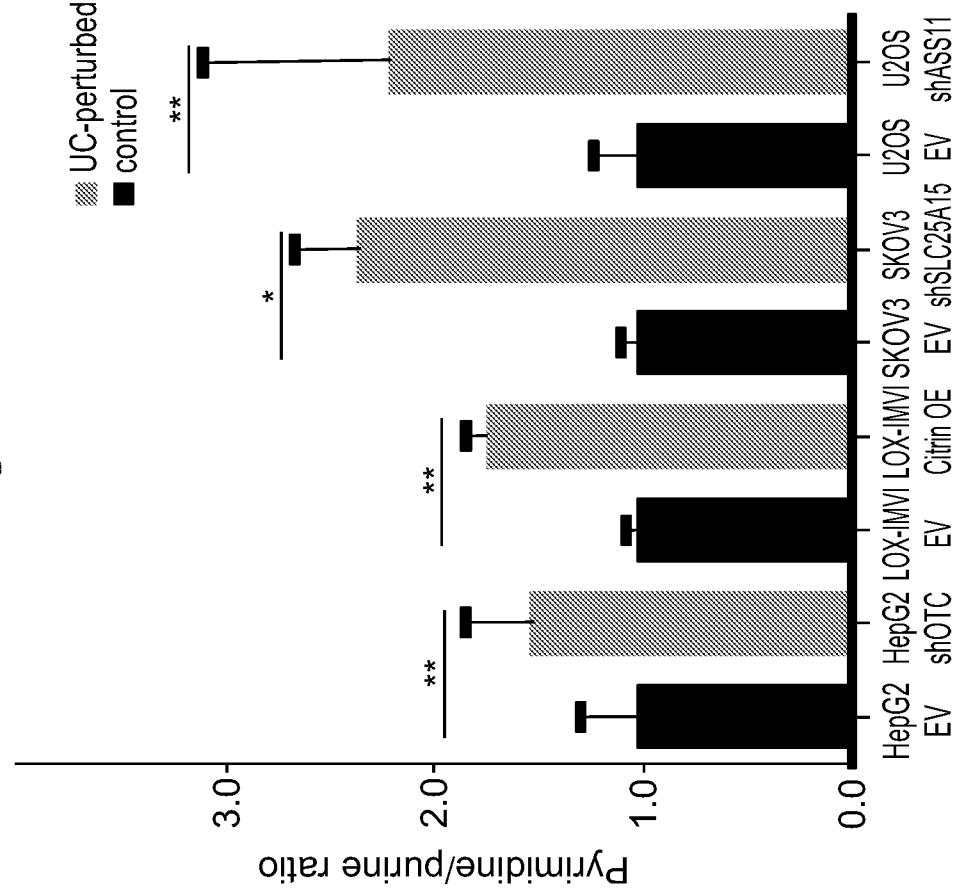


Figure 9

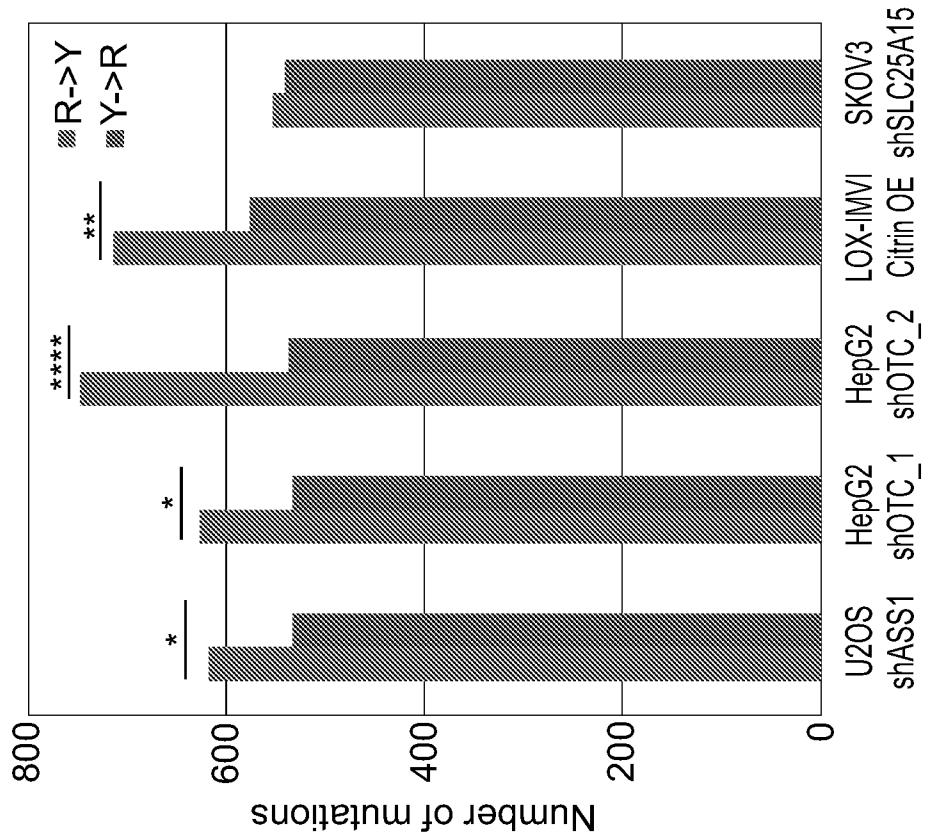


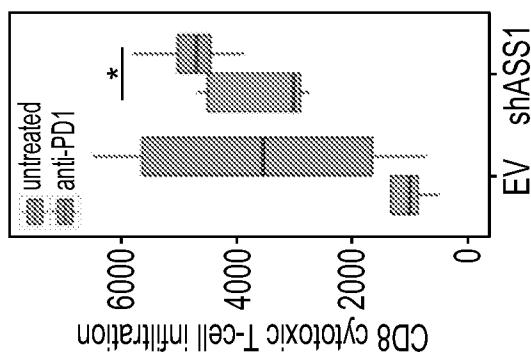
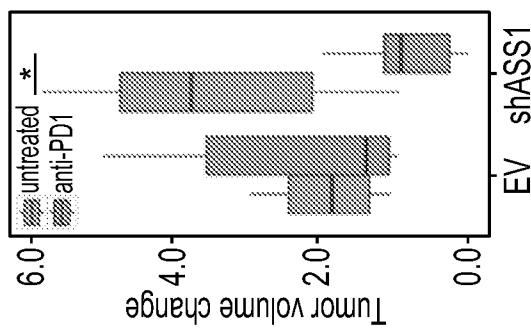
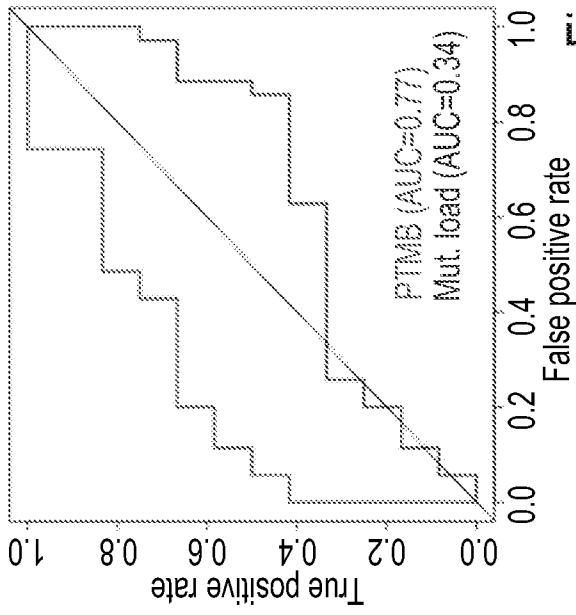
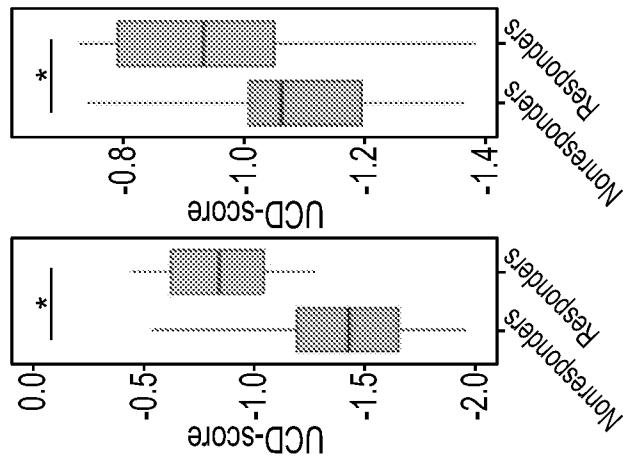
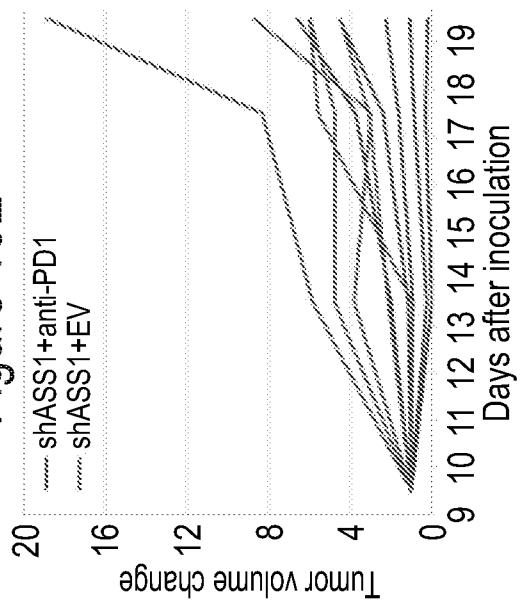
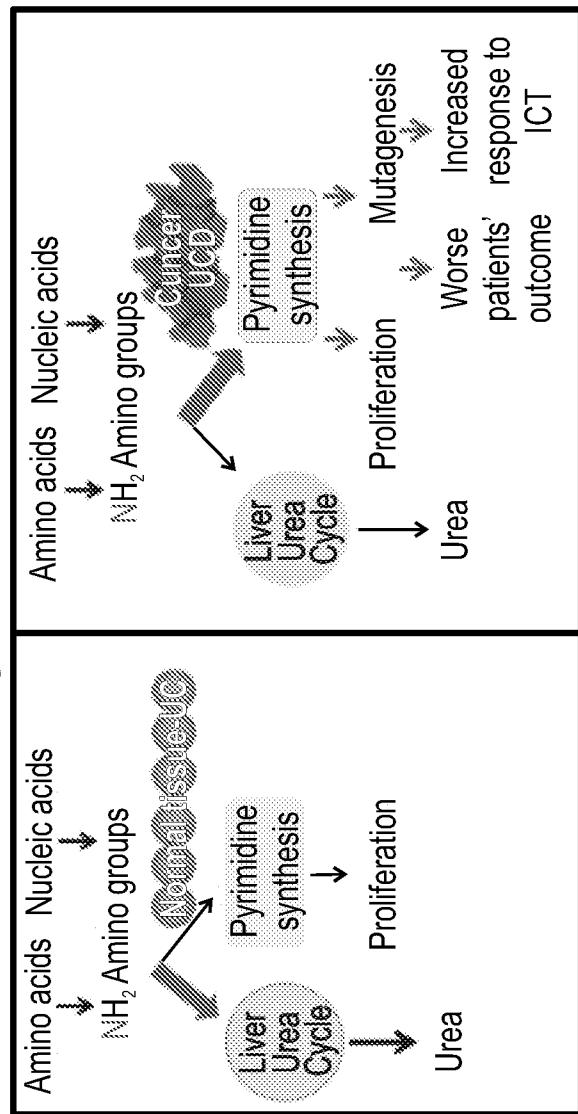
Figure 10D**Figure 10C****Figure 10B****Figure 10A****Figure 10E****Figure 10F**

Figure 11D

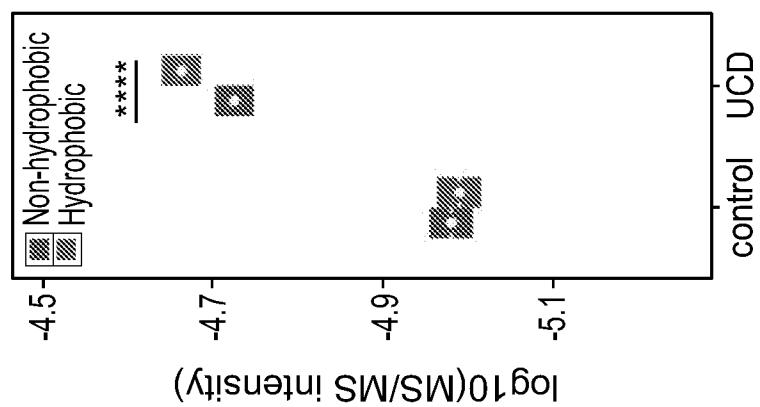


Figure 11C

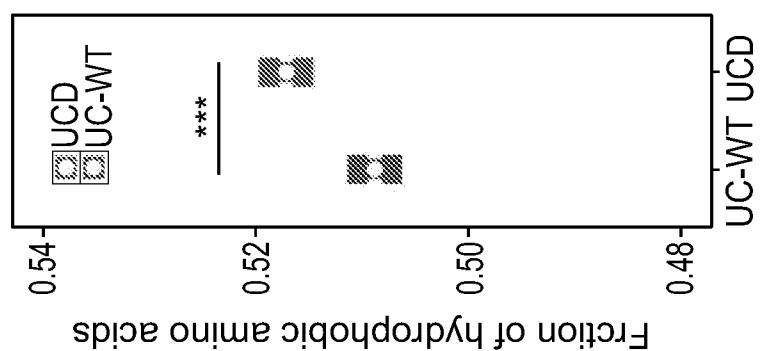


Figure 11B

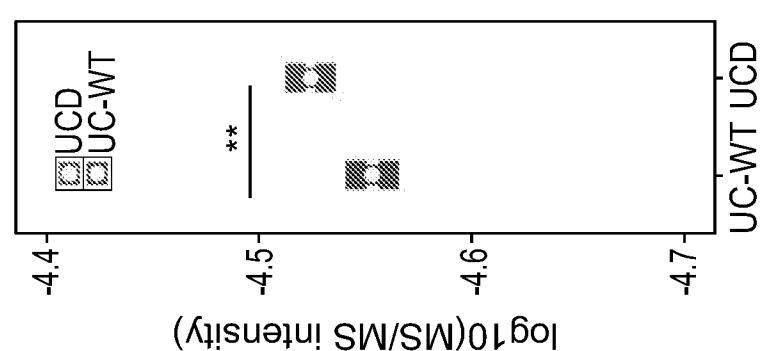


Figure 11A

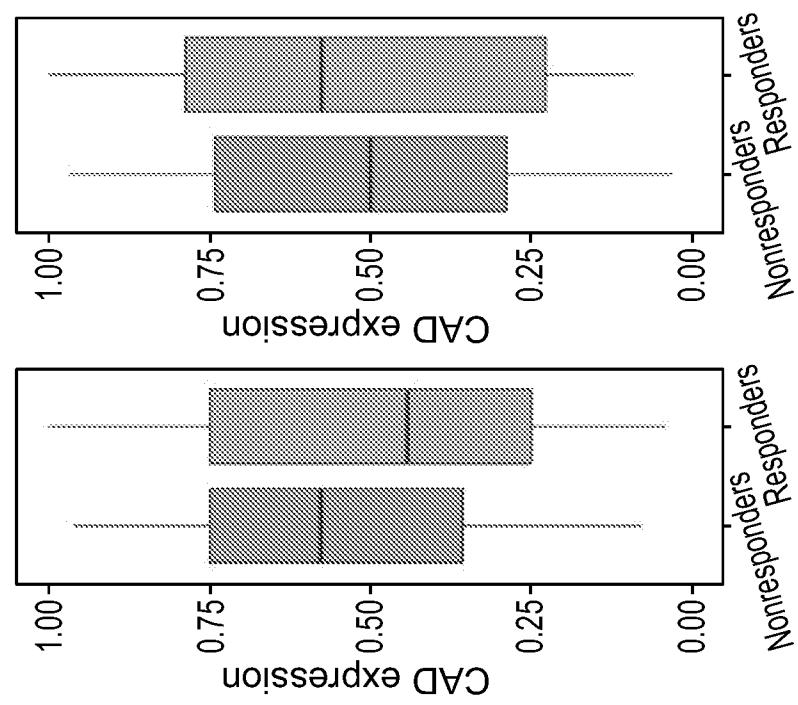
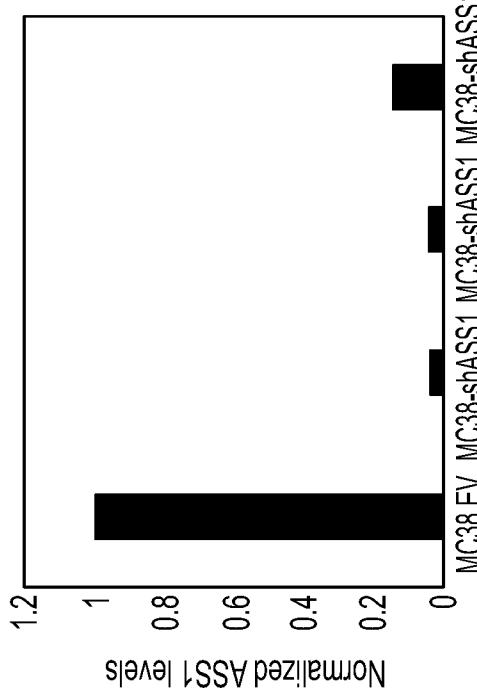
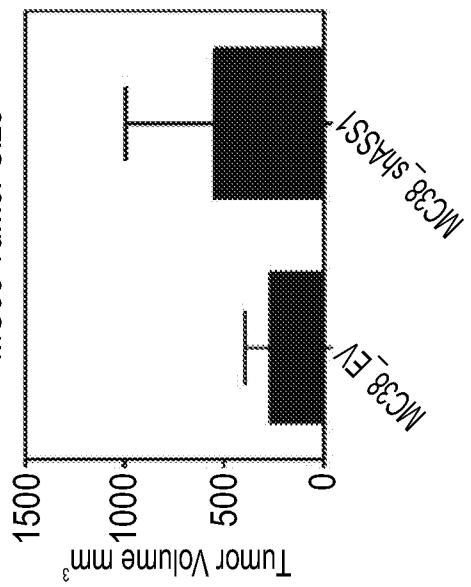
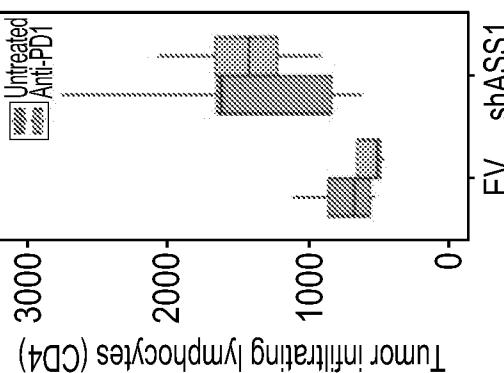
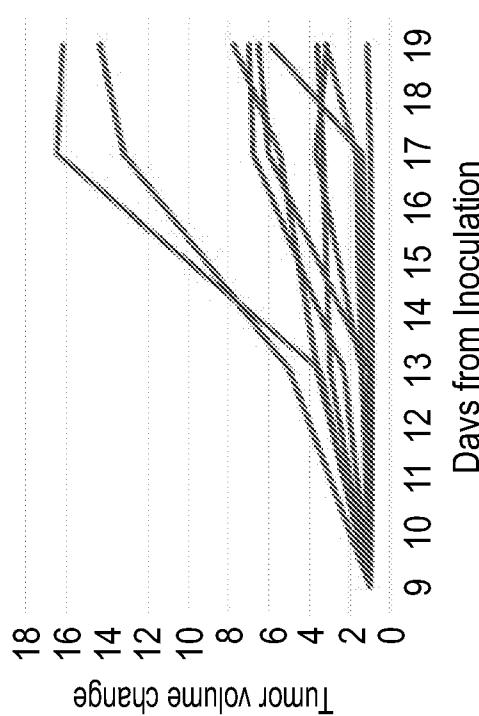
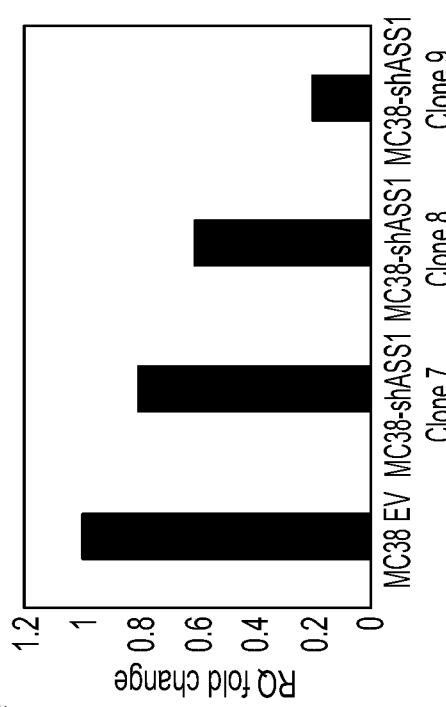


Figure 12A**Figure 12C****Figure 12D****Figure 12E****Figure 12B**

ASS1
p97
MC38 EV
MC38-shASS1 Clone 7
MC38-shASS1 Clone 8
MC38-shASS1 Clone 9

INTERNATIONAL SEARCH REPORT

International application No
PCT/IL2018/050289

A. CLASSIFICATION OF SUBJECT MATTER
 INV. C12Q1/25 G01N33/574
 ADD.

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
C12Q G01N

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

EPO-Internal, WPI Data, BIOSIS, EMBASE

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	WO 2016/181393 A1 (YEDA RES & DEV [IL]) 17 November 2016 (2016-11-17) cited in the application claims 2,21 ----- A WO 2016/007625 A1 (TDW GROUP; HE WEI [US]) 14 January 2016 (2016-01-14) claim 23 ----- A WO 2014/151982 A2 (POLARIS GROUP; ALMASSY ROBERT [US]) 25 September 2014 (2014-09-25) page 41, line 29 - page 42, line 20 ----- A WO 2006/048749 A1 (PFIZER PROD INC [US]; GOMEZ-NAVARRO JESUS [US]) 11 May 2006 (2006-05-11) claim 1 ----- -/-	1-5,22, 29-45 1-5,22, 29-45 1-5,22, 29-45 1-5,22, 29-45

Further documents are listed in the continuation of Box C.

See patent family annex.

* Special categories of cited documents :

- "A" document defining the general state of the art which is not considered to be of particular relevance
- "E" earlier application or patent but published on or after the international filing date
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- "O" document referring to an oral disclosure, use, exhibition or other means
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Date of the actual completion of the international search

Date of mailing of the international search report

6 June 2018

22/08/2018

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Authorized officer

Gundlach, Björn

INTERNATIONAL SEARCH REPORT

International application No PCT/IL2018/050289

C(Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X,P	WO 2018/032020 A1 (AEGLEA BIO THERAPEUTICS LLC [US]) 15 February 2018 (2018-02-15) the whole document ----- AGNELLO G ET AL: "Depleting blood arginine with AEB1102 (Pegylarginase) exerts additive anti-tumor and synergistic survival benefits when combined with immunomodulators of the PD-1 pathway", JOURNAL FOR IMMUNOTHERAPY OF CANCER 20171101 BIOMED CENTRAL LTD. NLD, vol. 5, no. Supplement 2, 1 November 2017 (2017-11-01), XP009505820, ISSN: 2051-1426 abstract -----	1-5,22, 29-45 1-5,22, 29-45
X,P	KHADEIR R S ET AL: "The impact of ADI-PEG20 on PDL1 expression in ASS1 deficient uveal melanoma", CANCER RESEARCH 20170701 AMERICAN ASSOCIATION FOR CANCER RESEARCH INC. NLD, vol. 77, no. 13, Supplement 1, 1 July 2017 (2017-07-01), XP009505821, ISSN: 1538-7445 abstract -----	1-5,22, 29-45
A	SHIRAN RABINOVICH ET AL: "Diversion of aspartate in ASS1-deficient tumours fosters de novo pyrimidine synthesis", NATURE, vol. 527, no. 7578, 11 November 2015 (2015-11-11), pages 379-383, XP055294215, GB ISSN: 0028-0836, DOI: 10.1038/nature15529 abstract; figure 4 -----	1
1		

INTERNATIONAL SEARCH REPORT

International application No.
PCT/IL2018/050289

Box No. II Observations where certain claims were found unsearchable (Continuation of item 2 of first sheet)

This international search report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. Claims Nos.:
because they relate to subject matter not required to be searched by this Authority, namely:

2. Claims Nos.:
because they relate to parts of the international application that do not comply with the prescribed requirements to such an extent that no meaningful international search can be carried out, specifically:

3. Claims Nos.:
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

Box No. III Observations where unity of invention is lacking (Continuation of item 3 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:

see additional sheet

1. As all required additional search fees were timely paid by the applicant, this international search report covers all searchable claims.

2. As all searchable claims could be searched without effort justifying an additional fees, this Authority did not invite payment of additional fees.

3. As only some of the required additional search fees were timely paid by the applicant, this international search report covers only those claims for which fees were paid, specifically claims Nos.:

4. No required additional search fees were timely paid by the applicant. Consequently, this international search report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:

1-5(completely); 22, 29-45(partially)

Remark on Protest

- The additional search fees were accompanied by the applicant's protest and, where applicable, the payment of a protest fee.
- The additional search fees were accompanied by the applicant's protest but the applicable protest fee was not paid within the time limit specified in the invitation.
- No protest accompanied the payment of additional search fees.

FURTHER INFORMATION CONTINUED FROM PCT/ISA/ 210

This International Searching Authority found multiple (groups of) inventions in this international application, as follows:

1. claims: 1-5(completely); 22, 29-45(partially)

A method of treating cancer in a subject in need thereof, the method comprising:(a) determining a shift from the urea cycle to pyrimidine synthesis in a cancerous cell of the subject as compared to a control sample; and(b) treating said subject with an immune modulating agent when said shift is indicated.

2. claims: 6-21, 23-28(completely); 22, 29-45(partially)

A method of prognosing cancer in a subject diagnosed with cancer, the method comprising determining a level of purine to pyrimidine transversion mutations in a cancerous cell of the subject as compared to a control sample, wherein said level of said purine to pyrimidine transversion mutations above a predetermined threshold is indicative of poor prognosis, thereby prognosing cancer in the subject, whereby the marker of which the level is determined is ASL, ASS1, OTC, etc. and related methods of monitoring efficacy of tumor treatment using the same markers.

INTERNATIONAL SEARCH REPORT

Information on patent family members

International application No
PCT/IL2018/050289

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