Prognostic and therapeutic impact of argininosuccinate synthetase-1 control in bladder cancer as monitored longitudinally by PET imaging

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**Running title:** Prognostic and predictive role of ASS1 loss in bladder cancer

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**Conflicts of Interest**
Dr Bomalaski and Dr Bor-Wen Wu are employees of Polaris Group, manufacturer of ADI-PEG20. The remaining authors have no conflict of interest.
Abstract

Targeted therapies have yet to have significant impact on the survival of patients with bladder cancer. In this study, we focused on the urea cycle enzyme argininosuccinate synthetase (ASS1) as a therapeutic target in bladder cancer, based on our discovery of the prognostic and functional import of ASS1 in this setting. ASS1 expression status in bladder tumors from 183 Caucasian and 295 Asian patients was analyzed, along with its hypothesized prognostic impact and association with clinicopathological features including tumor size and invasion. Further, the genetics, biology and therapeutic implications of ASS1 loss were investigated in urothelial cancer cells. We detected ASS1 negativity in 40% of bladder cancers, where multivariate analysis indicated worse disease-specific and metastasis-free survival. ASS1 loss secondary to epigenetic silencing was accompanied by increased tumor cell proliferation and invasion, consistent with a tumor suppressor role for ASS1. In developing a treatment approach, we identified a novel targeted antimetabolite strategy to exploit arginine deprivation with pegylated arginine deiminase (ADI-PEG20) as a therapeutic. ADI-PEG20 was synthetically lethal in ASS1-methylated bladder cells and its exposure was associated with a marked reduction in intracellular levels of thymidine, due to suppression of both uptake and de novo synthesis. We found that thymidine uptake correlated with thymidine kinase-1 protein levels, and that thymidine levels were imageable with [18F]-fluoro-L-thymidine (FLT)-positron emission tomography (PET). In contrast, inhibition of de novo synthesis was linked to decreased expression of thymidylate synthase and dihydrofolate reductase. Notably, inhibition of de novo synthesis was associated with potentiation of ADI-PEG20 activity by the antifolate drug pemetrexed. Taken together, our findings argue that arginine deprivation combined with antifolates warrants clinical investigation in ASS1-negative urothelial and related cancers, using FLT-PET as an early surrogate marker of response.
Edited Precis

This important study offers a preclinical proof-of-concept for a targeted strategy to treat bladder cancers, where targeted therapies have yet to have any significant impact on patient survival.
Introduction

Urothelial carcinoma is the fourth most common malignancy diagnosed in men, and the seventh commonest cause of solid cancer-related death in the US, accounting for 15,000 deaths per annum (1). The 5-year overall survival rate of 40-60% for muscle invasive bladder cancer has plateaued over the last three decades, in part, due to the frequent comorbidities in this patient group (2). Moreover, despite $4.0 billion spent each year on bladder cancer treatment in the US alone – one of the highest expenditures in oncology – targeted therapies have been ineffective to date in this disease (3).

Recently, arginine auxotrophy or the absolute requirement for exogenous arginine for cellular growth has been detected in several chemoresistant solid cancers including hepatocellular carcinoma, melanoma, mesothelioma, and prostate cancer (4-6). Arginine fuels an array of biosynthetic reactions including proteins, nitric oxide (NO), polyamines, agmatine, and the amino acids proline and glutamate and therefore may modulate tumorigenesis at multiple levels (7). Inactivation of the pleiotropic enzyme, argininosuccinate synthetase 1 (ASS1), with key roles in the urea cycle, citrulline-NO cycle and arginine biosynthesis has emerged as a principal driver of tumoral arginine auxotrophy, with evidence for epigenetic silencing and HIF-1alpha-mediated transcriptional repression of ASS1 (5, 8, 9). Significantly, ASS1 loss has been associated with decreased overall survival in ovarian cancer and myxofibrosarcoma, and reduced metastasis-free survival in osteosarcoma implicating a tumor suppressor function for this metabolic gene (10-12). Thus, arginine deprivation is being tested increasingly in the clinic, modelled on the successful introduction of another amino acid depletor, namely asparaginase, in the management of acute lymphoblastic leukemia five decades ago (13). Specifically,
several early phase trials of the arginine-degrading mycoplasmal enzyme, pegylated arginine deiminase (ADI-PEG20) have led to phase II/III randomized studies in melanoma, hepatocellular cancer and mesothelioma (14-16).

Here, we characterized ASS1 in bladder cancer based on the fact that loss of chromosome 9, including q34 the locus of ASS1, is a known early event in urothelial tumorigenesis (17). Previously, Linnenbach et al provided evidence for a truncation mutation at the ASS1 locus in a ureteric carcinoma sample, while searching for tumor suppressors at 9q34 (18). Here, we show that ASS1 negativity is a common event in bladder cancer detected in ~40% of tumors by immunohistochemistry. Furthermore, to address a putative tumor suppressor role, we performed additional genetic and epigenetic studies of ASS1 using a panel of bladder cancer cell lines. Finally, we studied the therapeutic consequences of ASS1 loss in bladder cancer, identifying a novel targeted antimetabolite strategy that combines arginine deprivation with the antifolate, pemetrexed. These studies were reinforced by metabolomic and positron emission tomography (PET) tracer analyses, which identify intracellular thymidine levels as a novel biomarker of early treatment response.
Materials and Methods

Primary tumor characteristics

Tissue microarrays (TMAs) were constructed using a training set of biopsy samples, comprising 183 urothelial carcinomas collected at the Cancer Research UK Cambridge Research Institute (19), and an independent test set of 295 urothelial carcinomas, which were resected consecutively with curative intent in Chi Mei Medical Center, Taiwan, between January, 1996 and March, 2004. Institutional review boards in the UK (Cambridgeshire Local Research Ethics Committee) and Taiwan (IRB971006) ratified the tissue procurement. The diagnostic criteria were based on the updated American Joint Committee on Cancer Staging.

Immunohistochemistry

ASS1 immunohistochemistry was performed using liver control sections as described previously (5). The scoring of TS (1:100, Clone EPR4545, Epitomics, CA) and DHFR (1:100, Clone: EPR5285, Epitomics, CA) was performed using an H-score, determined by the percentage and intensity of positively stained tumor cells with cases stratified according to high (no less than the median score) or low expression (less than the median score) (20).

Cell lines and culture

Six bladder cancer cell lines were grown in a humidified atmosphere at 37°C and 5% CO₂ in endotoxin-free RPMI medium with 10% fetal bovine serum (RT112, 5637, SCaBER, 253J, T24) or DMEM with 10% fetal bovine serum (UMUC3). The arginine concentration was ~10-fold higher in the culture medium compared to that
found in human serum (i.e. 1mM versus 0.06-0.1mM respectively). Experiments were performed using cells at 70% confluency, which were harvested for analysis of ASS1 promoter methylation, constitutive ASS1 mRNA and protein. Corroborative cell lines studies in a non-urothelial solid cancer (mesothelioma) were performed and all cell lines were authenticated by STR profiling (LGC Standards, Middlsx, UK).

Methylation specific PCR (MSP)

1μg of genomic DNA was modified with Sodium Bisulphite using the EZ DNA methylation kit (Zymo, CA, USA) according to the manufacturer’s instructions. 50ng of modified DNA was amplified using the following primers designed using the ASS1 promoter sequence: UF (5’ GTAGGAGGGAAGGGGTTTTT); UR (5’ ACAAAAAACAAATAACCCAAA); MF (5’ GTAGGAGGGAGGGTGGTTTT); and MR (5’ GCAAAAAACAAATAACCCGAA), where U=unmethylated and M=methylated. Control CpGenome universal methylated and unmethylated DNA were obtained from Merck Millipore (Watford, UK).

Pyrosequencing

Methylation in the CpG islands of the ASS1 gene in the urothelial cell line panel was also analyzed using pyrosequencing technology. For pyrosequencing of the paraffin primary bladder cancer samples, PCR amplification and sequencing primers were optimized using PyroMark Assay Design Software 2.0. Details of PCR primers and conditions are available on request.

Sequencing analysis

The coding exons and flanking sequences of ASS1 were analyzed by Sanger
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sequencing in forward and reverse in the panel of bladder cancer cell lines. Details of PCR primers and conditions are available on request.

\textbf{Quantitative Real-time RT-PCR}

Total RNA was extracted using the RNeasy Mini kit (Qiagen) and reverse-transcribed with the High Capacity cDNA Reverse Transcription kit (Applied Biosystems) according to the manufacturers’ instructions. Multiplex PCR was performed on an ABI Prism 7500 Real-Time PCR Instrument (Applied Biosystems). Primers and TaqMan probes for \textit{ASS1} (FAM) and 18S (VIC) were obtained from Applied Biosystems (TaqMan® Gene Expression Assays).

\textbf{ASS1 overexpression and knockdown}

\textit{ASS1} overexpression was performed in 253J, UMUC-3 and T24 bladder cancer cells using \textit{ASS1}pIREShyg3 (or control) vector under hygromycin (200 $\mu$g/ml) selection, as described previously (10). \textit{ASS1} knockdown in 5637 bladder cancer cells was performed using the pSilencer 4.1-CMVpuro vector (Ambion, Paisley, UK) containing short hairpin RNA (shRNA) sequences targeting \textit{ASS1} (SMARTpool, Dhharmacon) or a non-targeting control shRNA under puromycin (1 $\mu$g/ml) selection.

\textbf{Proliferation assays}

Briefly, 3x10$^4$ cells were suspended in 200 l of serum-free DMEM, plated into a 96 well plate and incubated for 48h. Subsequently 40 l of CellTitre Aqueous One solution (Promega, G3580) was added to each well and the plate was incubated for between 15 min to 1h before reading at 450nm (Labtech, LT4000MS).
Invasion assays

Briefly transwells (Corning, 3422) were coated with Matrigel (BD Bioscience, 354234) diluted 1:3 with serum free DMEM and allowed to set for 1h at 37°C. Transwells were placed in the wells of a 24 well plate containing 500μl of DMEM (PAA) with 10% serum. 3 x 10^4 cells in 200μl of serum free DMEM were plated into the top of the transwell, and incubated at 37°C for 48hr. Transwells were removed from the DMEM + 10% FCS and placed into 500μl of Trypsin/EDTA (PAA) for 1hr at 37°C. The undersides of transwells were then washed with trypsin and the cell suspension transferred to 9.5ml of Isoton and the number of cells quantified on a CASY counter. All experiments were repeated at least three times using quintuplicate readings.

Immunoblotting

Western blotting for ASS1 (BD Biosciences, UK) was performed as described previously (5). Membranes were also probed for TS (Sigma-Aldrich, UK), DHFR, TK1 (Abcam, UK), p53R2 (Santa Cruz Biotechnology, US), PARP (Signal Transduction), β-actin and Hsc70.

Drug treatments

Arginine depletion experiments were performed using ADI-PEG20 (Polaris Group, San Diego, US) and 2% dialysed FBS (>10KDa, Autogen Bioclear, Wilts, UK). Cell viability was measured using the MTT assay according to manufacturer’s instructions (Roche Diagnostics, UK). Pemetrexed (Eli Lilly and Company, IN, USA) was purchased from Barts Chemopharmacy. Combination drug treatments of ADI-PEG20 and pemetrexed were studied using Chou-Talalay methodology and the effect on tumor cell apoptosis was detected using PARP cleavage (Cell Signaling...
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Technology) and annexin V staining (21). Proteosome inhibition and demethylation experiments were performed using MG132 (1 μM, Sigma-Aldrich, UK) and 5-aza-2’-deoxycytidine (5-Aza-dC, 0.1-1 μM, Sigma-Aldrich, UK), respectively.

**Liquid chromatography-mass spectrometry (LC/MS)**

PBS control and ADI-PEG20 treated cells (3 \times 10^6) were washed twice with phosphate buffer saline and then harvested by adding 10 mL of ice cold 80% methanol containing 0.1% formic acid (all reagents from Fisher Scientific, UK). Cell extracts were prepared using a standard protocol and injected into the liquid chromatography-mass spectrometry (LC-MS) system. LC was performed using the nanoACQUITY UltraPerformance (UP) LC system (Waters, UK) and MS was executed using a Waters Q-ToF Premier operated in positive and negative electrospray (ES) ionization modes. Data Acquisition and analysis was performed using Water Masslynx software (V 4.1) and MZmine software (version 2.2), respectively. Peak lists including retention, M/Z and peak intensities were exported from MZmine and imported into Microsoft Office Excel 2007, revealing volcano plots and the fold change and p-value for individual metabolites. Subsequent targeted metabolomic analysis was performed in the positive ion mode.

**\textit{In vitro} tracer uptake assays**

Cells were plated at 1 \times 10^5 cells per well the evening before the experiment. The next day cells were incubated with ADI-PEG20 (750ng/ml). On the day of the assay, the drug media was removed and incubated in media supplemented with 2% dialyzed fetal bovine serum for 1 hr. \[^3\text{H}\] thymidine was then added at 1 μci/ml to each well and incubated for a further 1 hr. Cells were then washed twice in ice cold PBS, and
harvested with trypsin. The cell lysates were collected and added to tubes containing 2 ml of scintillation fluid and the cell associated $^3$H radioactivity was measured using the $\beta$ scintillation counter (LKB Instruments). The radioactivity uptake in nmoles, was normalized to the number of viable cells to give pmole uptake per 10000 live cells and expressed relative to untreated controls. All experiments were done in triplicate and repeated 3 times.

**In vivo experiments**

UMUC-3 cells ($1 \times 10^7$ in 0.1ml) were implanted into the flank of CD1 nu/nu mice and developed into visible tumors by 11 days. For the in vivo $[^{18}F]$-fluorothymidine (FLT)-PET studies, animals (n=5) were injected i.v. via the tail vein with 15-18 MBq of $[^{18}F]$-FLT and imaged using an Inveon microPET/CT (Siemens, Knoxville, TN) before and 24 hours after treatment with ADI-PEG20 (5IU). Standardized uptake values (SUV) were calculated by dividing the activity concentration in each voxel by the injected dose and dividing by the animal weight. For drug combination studies mice were separated into four i.p. treatment groups (n=11 per group): PBS control (day 1 and 8), ADI-PEG20 (5IU on day 1 and 8), pemetrexed (PEM; 100mg/kg on days 2-4 and 9-11); and ADI-PEG20 (5IU on D1 and 8) plus PEM (100mg/kg on days 2-4 and 9-11). Tumor volume and animal weights were recorded and all mice were sacrificed on day 11; tumors were collected and stored for immunohistochemical analyses. Additional $^{18}$F-FLT microPET/CT imaging was performed in female UMUC3 bladder cancer tumor-bearing CD1 nude mice (n=5 per group) assessing the drug combination. Animals were imaged at baseline immediately before i.p. treatment with 5IU ADI (ADI and PEM+ADI treatment groups) or PBS (PBS and PEM treatment groups) and imaged again 24 h later. Immediately after the 24h imaging timepoint, the PEM
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and PEM+ADI treatment groups received 100mg/kg PEM i.p. for 5 days while the others received PBS, with $^{18}$F-FLT imaging carried out on day 7.

**Statistical analysis**

GraphPad Prism version 5.0 and SPSS 14.0 were used to test results for statistical significance. The associations of ASS1 expression level with clinicopathological factors were evaluated using the Chi-square, Fisher exact, or Wilcoxon rank-sum test as appropriate. For all analyses, two-sided tests of significance were used with $p<0.05$ considered significant.
Results

Loss of ASS1 confers poor survival in bladder cancer

First, we screened for ASS1 expression in a training set bladder cancer TMA derived from a European population revealing that 45% (83/183) of biopsies were ASS1-negative. The validation TMA (Fig. 1A-F) from an Asian population yielded similar results with 37% of bladder cancer cases (108/295) being ASS1-negative. ASS1 negativity conferred a poor disease-specific (p=0.011) and metastasis-free survival (p=0.001) on multivariate analysis (Fig. 1G-H and Table 1). Furthermore, ASS1 loss was linked to several adverse clinicopathological features, including increased tumor size and grade, lymph node involvement and vascular invasion (Supplementary Table 1 and 2).

Methylated ASS1 is linked to increased proliferation and invasion of bladder cancer cells

To understand the regulation of ASS1 expression and its role in urothelial malignancy we screened six bladder carcinoma cell lines for genomic and epigenetic alterations. FISH analysis confirmed all lines were either triploid or tetraploid with the ASS1 locus intact (data not shown). There were no mutations in ASS1 and no loss of heterozygosity in the region immediately surrounding ASS1 (data not shown). Instead, the UMUC-3 and 253J cell lines were fully methylated while the T24 line was partially methylated at the ASS1 promoter, with negligible ASS1 mRNA and no ASS1 protein. In contrast, all the ASS1-unmethylated cell lines (SCaBER, RT112, and 5637) expressed ASS1 mRNA and protein (Fig. 2A). Notably, ASS1 promoter methylation was greatest in UMUC-3 and 253J compared to the T24 cell line by
pyrosequencing of six CpG islands within the \textit{ASS1} promoter. In contrast, the \textit{ASS1}-positive bladder cancer cell lines were unmethylated by pyrosequencing validating the results obtained with MS-PCR (Fig. 2B). The demethylating agent decitabine reexpressed \textit{ASS1} protein in 253J, UMUC-3 and T24 cells, confirming epigenetic silencing as a key mechanism involved in \textit{ASS1} inactivation in bladder cancer cells (data not shown) (22).

To determine the correlation between immunohistochemical expression of \textit{ASS1} and methylation status, tissue blocks of an independent set of 30 urothelial carcinomas with a high tumor cell content (>70%) were selected for pyrosequencing. Following optimization of primers for paraffin-embedded tissue, only one \textit{ASS1}-positive tumor had an average methylation value of 45% that was greater than the threshold value of 40% (Supplementary Fig. S1). The remaining tumors with methylation values > 40% were all negative for \textit{ASS1} immunoexpression (p<0.001), validating the role of promoter methylation in \textit{ASS1} silencing in primary bladder cancer (Supplementary Table 3).

Next, we addressed the significance of epigenetic loss of \textit{ASS1} in bladder cancer by manipulating \textit{ASS1} in our panel of urothelial cancer cell lines. While \textit{ASS1} overexpression decreased the proliferation of 253J and UMUC-3 cells, \textit{ASS1} siRNA increased the proliferation of 5637 cells, consistent with a tumor suppressor role for \textit{ASS1} in urothelium (Fig. 2C). We also observed a reciprocal relationship between \textit{ASS1} expression and cell invasion by performing matrigel invasion assays, confirming that \textit{ASS1} loss increased the invasiveness of urothelial tumor cells \textit{in vitro} (Fig. 2D).
ASS1-methylated bladder cancer cell lines are sensitive to ADI-PEG20

To test the effect of arginine deprivation on bladder cancer cell line viability, we treated the panel of bladder cancer cell lines with the arginine depletor, ADI-PEG20. All ASS1-negative cell lines were sensitive to ADI-PEG20 with the highly methylated lines 253J and UMUC-3 displaying the greatest sensitivity to arginine depletion; in contrast, the ASS1-positive cell lines 5637, RT112 and SCaBER were resistant to ADI-PEG20 (Fig. 3A). Moreover, forced ASS1 overexpression in ASS1-negative cell lines reduced their sensitivity to ADI-PEG20, validating the role of ASS1 loss in mediating the synthetic lethal effect to arginine deprivation (Fig. 3B).

ADI-PEG20 inhibits pyrimidine metabolism in ASS1-negative tumor cells

We proceeded to analyse the metabolic effects of ADI-PEG20 and confirmed a significant decrease in intracellular arginine with a corresponding increase in citrulline in ASS1-negative bladder cancer cells. Marked reductions in thymidine and increases in thymine and glutamine were also detected by LC/MS (Fig. 4A). In contrast, ADI-PEG20 reduced arginine levels to a minor extent in the ASS1-positive RT112 control cell line while intracellular levels of thymidine, thymine, and glutamine were unaffected indicating a non-essential role for exogenous arginine. Due to these differential effects on intracellular thymidine, we interrogated gene pathways modulated by ADI-PEG20 identifying downregulation of the folate-dependent nucleotide synthesizing enzymes thymidylate synthase (TS) and dihydrofolate reductase (DHFR) specifically in ASS1-negative tumor cells (data not shown). Marked suppression of DHFR and TS mRNA and protein was detected in all three ADI-sensitive bladder cancer cell lines whereas no effect was seen in the RT112...
control cell line exposed to ADI-PEG20 (Fig. 4B-C). We also analysed the thymidine salvage pathway, as a compensatory source of cellular thymidine upon suppression of \textit{de novo} pyrimidine synthesis. Unexpectedly, ADI-PEG20 also reduced $^3$H-thymidine uptake in the arginine-auxotrophic cell lines associated with downregulation of thymidine kinase 1 (TK1) (23) with no effect in RT112 control cells (Fig. 4D-E). Notably, co-treatment with MG132, a proteosome inhibitor that blocks protein degradation, abrogated the ADI-PEG20-induced suppression of TS and TK1 levels (Fig. 4F). More generally, similar data on thymidine salvage and \textit{de novo} synthesis pathways were obtained in a panel of ADI-treated ASS1-negative (methylated) mesothelioma cell lines (Supplementary Fig. S2). Finally, we assessed whether \textit{in vivo} imaging of thymidine uptake could provide an early biomarker of response to ADI-PEG20 therapy using UMUC-3 xenografts. Consistent with our \textit{in vitro} studies, there was a 66% decrease in the maximum standardized uptake value (SUVmax) on $^{[18}F]FLT$-PET after 24h of ADI-PEG20 therapy (Fig. 4G).

**Enhanced effect of ADI-PEG20 and pemetrexed in bladder cancer**

To exploit our findings in the clinic we employed the multitargeted antifolate pemetrexed, which blocks enzymes including TS and DHFR and is reported to have modest activity in bladder cancer (24). High TS expression, in particular, has been linked to poor clinical outcome and resistance to antifolates in cancers of the bladder, lung, mesothelium and colon (25-29). Moreover, bladder TMA analysis revealed that ASS1 loss was associated with a reciprocal increase in TS and DHFR expression – both linked to poor outcome for disease-specific and metastasis-free survival – emphasizing the interdependence between arginine auxotrophy and modulation of nucleotide biosynthesis (Supplementary Fig. S3 and S4; and Supplementary Table 1). Thus, we hypothesized that ADI-PEG20 may potentiate the
cytotoxicity of pemetrexed in ASS1-negative tumor cells via modulation of folate-dependent enzymes. First, we showed that pemetrexed and ADI-PEG20 enhanced apoptosis compared with either agent alone by increasing PARP cleavage and annexin V binding in vitro in ASS1-methylated tumor cell lines (Fig. 5A-B). Secondly, ADI-PEG20 significantly reduced tumor growth of the UMUC-3 cell line (p<0.001) in xenografted nude mice. While pemetrexed alone had no effect, the combination was more effective than ADI-PEG20 alone (p<0.05) (Fig. 5C-D). Analysis of tumors from the xenograft studies confirmed reduced TS, DHFR and TK1 levels in ADI-PEG20-treated animals (Fig. 5E). Finally, to validate our results we executed an [18F]-FLT microPET/CT imaging study of the combination therapy in the UMUC-3 xenograft tumor model. Our SUVmax data showing the superior efficacy of the ADI-PEG20 and pemetrexed drug combination over that of ADI-PEG20 alone are consistent with our earlier tumor volumetric measurements (Fig. 5F). Notably, there was a lack of effect on SUVmax of pemetrexed in animals that had not been pre-treated with ADI-PEG20.
Discussion

Progress in the targeted therapy of advanced urothelial carcinoma has lagged behind other urological malignancies, especially prostate and renal cancer. For the first time we have identified ASS1 as a novel bladder cancer biomarker with wider implications for the therapy and monitoring of cancers dependent on exogenous arginine for survival. We show that despite exhibiting a poorer prognosis, ASS1-negative bladder cancer is amenable to dual targeting with ADI-PEG20 and antifolates with early treatment response monitoring by $^{[18}\text{F}]$FLT-PET.

Our bladder cancer cell line studies reveal that unmethylated ASS1 functions as a bona fide tumor suppressor and is consistent with recent studies in osteosarcomas and myxofibrosarcomas (11, 12). Exactly how ASS1 promoter methylation leads to a requirement for exogenous arginine, that promotes increased tumor cell proliferation and invasion is unclear. Interestingly, previous studies have associated exogenous arginine with DNA synthesis in Burkitt lymphoma cells and to increased proliferation of caco-2 colon cancer cells via a glutamine-dependent effect. (30, 31) Yamauchi et al proposed that under conditions of limited arginine supply intracellular glutamine is diverted for arginine synthesis since supplying exogenous arginine was shown to promote nucleotide synthesis. We found for the first time that upon depletion of arginine in ASS1-negative tumor cells, glutamine levels increased indicative of a block on nucleotide synthesis. Thus, our metabolomic experiments with ADI-PEG20 support the hypothesis that the ASS1 epimutation reprograms extracellular arginine so that it is sparing for glutamine in nucleotide metabolism. Additional metabolomic flux studies are underway to explore further the interdependence of arginine and glutamine in the context of tumoral ASS1-
deficiency. Exogenous arginine has also been shown to stimulate intestinal cell motility via activation of NO and focal adhesion kinase (FAK) signalling (32, 33). In particular, arginine is a dominant amino acid modulator of mTOR, a nodal pathway that integrates cell growth, proliferation, invasion and metastasis of various cancers, including urothelial malignancy (34-36) ENREF 42; moreover, arginine deprivation has been shown to repress mTOR and its downstream substrates p70 S6 kinase and 4E-BP1 in ASS1-negative cancer cells (6, 37). Indeed, it is becoming clear that arginine deprivation has multiple effects on gene expression and that this is likely to be determined not only by cell type but also by the status of the ASS1 gene (38).

The ADI-PEG20-induced suppression of the folate-dependent enzymes TS and DHFR and sensitization of ASS1-negative tumor cells to the cytotoxic effects of pemetrexed, provides a novel metabolic approach to retargeting cytotoxic chemotherapy (39). Our results also validate the work of Cheng et al, who showed that the irreversible TS inhibitor 5-fluorouracil was more effective when combined with pegylated human arginase in a hepatocellular carcinoma xenograft (40). It is noteworthy that the ADI-PEG20 and antifolate doublet has a wide therapeutic window in the xenograft studies. Moreover, our experience from an ongoing clinical trial of arginine depletion in mesothelioma (NCT01279967), includes a patient treated concurrently with ADI-PEG20 and the antifolate methotrexate for an unrelated condition; the combination was safe and led to a prolonged period of disease control (manuscript in preparation).

Furthermore, the decreased uptake of thymidine following ADI-PEG20 treatment as shown by the $^3$H-thymidine and FLT tracer studies may be exploited as an early therapeutic biomarker for imaging ASS1-methylated tumors. This is in contrast to recent work with ADI-PEG20 in ASS1-negative melanoma xenografts in
which there was no effect on TK1 expression and FLT-PET metabolic responses were not detected (41). These and previous results using FDG tracers may be explained in part by the differential regulation of ASS1 promoter with HIF-1alpha-mediated repression in melanoma compared with epigenetic silencing identified in several other arginine auxotrophic cancers (9, 16, 42).

Thus, arginine deprivation with ADI-PEG20 suppresses both the de novo and salvage pathways of nucleotide synthesis in ASS1-negative bladder cancer cells, which may be exploited for therapy and imaging, respectively (Fig. 6). In addition to ADI-PEG20 modulating protein turnover as indicated by the MG132-mediated recovery of TS and TK1 enzymes, several other mechanisms may underlie the reduced levels of target proteins, including chemical attack by peroxynitrite, a potent oxidant and nitrating species generated by arginine deprivation (43-45). More generally, the reciprocal relationship between thymidine and glutamine in ASS1-negative tumors reinforces several other preclinical approaches targeting amino acid utilization in cancer, including methionine, glycine and serine (46-49). Significantly, we observed that ASS1 downregulation was linked to the reciprocal upregulation of the serine biosynthetic pathway enzymes, PHGDH, PSAT1, and PSPH in bladder cancer (data not shown) and this warrants further evaluation in view of the important roles of serine in tumor cell proliferation and bioenergetics.

In conclusion, our studies linking the biology of arginine auxotrophic bladder cancer to adverse clinicopathological variables accentuate ASS1 loss as a novel biomarker for evaluation in patients with urothelial cancer. Specifically, targeted modulation of pyrimidine synthesis – via suppression of the de novo and salvage nucleotide pathways – using arginine-depleting drugs combined with antifolates has
the potential to improve the management of urothelial and related cancers with methylated ASS1.

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Table-1. Multivariate analysis for disease-specific and metastasis-free survival in bladder cancer

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<td>1 - 0.003*</td>
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<td>1 - 0.004*</td>
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<td>High grade</td>
<td>1.058 0.192-4.641</td>
<td>1.238 0.425-3.607</td>
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*, Statistically significant
Figure Legends

Figure 1. ASS1 immunohistochemistry and clinical correlation in bladder cancer

Representative H&E and ASS1 staining of normal urothelium (A-B), low-grade (C-D) and high-grade (E-F) urothelial carcinomas (X400). ASS1 expression is present in normal urothelium and the low-grade lesion but absent in the high-grade lesion with vascular endothelial cells serving as the positive internal control. ASS1 deficiency is highly predictive of worse disease-specific (G) and metastasis-free survival (H) by log-rank tests.

Figure 2: Epigenetic silencing of ASS1 in bladder cancer linked to increased tumor cell proliferation and invasion

(A) ASS1 mRNA, protein and methylation status of a panel of six bladder cancer cell lines
(B) Pyrosequencing revealed more ASS1 methylation in 253J and UMUC-3 cells than T24 cells, whereas 5637, SCaBER and RT112 cells were unmethylated consistent with the MS-PCR data. (C) Overexpressing (O/E; inset panel) ASS1 in UMUC-3 and 253J cell lines resulted in a significant (5%-20%) reduction in proliferation, respectively; in contrast, knockdown of endogenous ASS1 (inset panel) in 5637 cells caused a significant 20% increase in proliferation. *p<0.0001 (3 independent experiments). (D) Overexpression of ASS1 in 253J and UMUC-3 cell lines caused a significant (50% and 75%, respectively) reduction of invasion, while knockdown of endogenous ASS1 in 5637 cells resulted in a significant (75%) increase in invasion. *p<0.0001 (3 independent experiments). Con = control empty vector cells.

Figure 3: ADI-PEG20 is cytotoxic to ASS1-methylated bladder cancer cell lines

(A) Bladder cancer cell line viability following exposure to varying doses of ADI-PEG20 (0-20,000ng/ml) was assessed by MTT assay at 6 days. (B) Overexpression of ASS1 in
ASS1-methylated cell lines induced resistance to ADI-PEG20 by 6 days of ADI-PEG20 treatment, most marked in the T24 cell line, validating ASS1 loss as a biomarker of sensitivity to arginine deprivation.

Figure 4: ADI-PEG20 modulates pyrimidine metabolism in ASS1-methylated cancer cells.

(A) Cell extracts from ASS1-negative bladder cancer lines and the RT112 control treated with ADI-PEG20 (750ng/ml) for 24h were subjected to metabolomic analysis by LC/MS. Arginine decreased in all cell lines however the reduction was at least one-log-fold greater in the ASS1-negative tumor cells. Glutamine and thymine increased while thymidine decreased specifically in the ADI-treated ASS1-negative bladder cell lines with no effect in RT112 control cells. Data represents mean and SE (n=6). ADI-PEG20 downregulated TS and DHFR mRNA (B) and protein (C) by 24h specifically in the ASS1-methylated cell lines. In contrast, the DNA synthetic enzyme ribonucleotide reductase subunit p53R2 was unaltered, confirming the specific downregulatory effect of ADI-PEG20 on TS and DHFR proteins; β-actin as the loading control (50). (D) [3H]thymidine uptake in bladder cancer cells after treatment with ADI-PEG20 (750 ng/ml). The cells remaining after ADI treatment decrease their uptake of thymidine by 24h in all ASS1-negative cell lines except the T24 bladder cell line, which showed a reduction by 72h (*p<0.05; **p<0.005). In contrast [3H] thymidine uptake was unchanged in the control RT112 cell line after ADI treatment. Data represents mean and SE (n=9). (E) ADI-PEG20 reduced TK1 protein levels by 24hrs specifically in ASS1-negative bladder cancer cell lines. β-actin as the loading control. (F) ADI-PEG20-mediated loss of TS and TK1 can be abrogated by inhibiting the proteasome with MG132. UMUC-3 cells were plated and incubated overnight; subsequently cells were exposed to DMSO (Con), ADI-PEG20 (750ng/ml), ADI-PEG20 and MG132 or MG132
Prognostic and predictive role of ASS1 in bladder cancer

(1μM) for 24 hours with immunoblotting as shown (G) $[^{18}F]$-FLT microPET/CT imaging in a UMUC-3 bladder tumor-bearing CD1 nude mouse (i) before treatment showing uptake in tumor (red arrow), and increased tracer concentration in intestine (white arrow) and also gall bladder. Transverse views before (ii) and 24h after (iii) treatment with 5 IU ADI-PEG20 show a reduction in tumour SUVmax after treatment (3.2 vs 1.1 after treatment) indicating a decrease in tumour cell proliferation.

Figure 5: ADI-PEG20 sensitises ASS1-methylated cancer cells to pemetrexed

ASS1-negative bladder cancer cells (UMUC-3) and malignant mesothelioma cells (MSTO) were treated with fixed doses of ADI-PEG20 and pemetrexed (PEM; Supplementary Table 4) with enhanced cell killing in vitro and in vivo: (A) increased PARP cleavage by western blotting and (B) increased annexin V staining by FACS was observed in the combination drug treatment compared to either drug alone by 96h (n=6). (C and D) Xenograft experiment showing enhanced control of UMUC-3 cells with the combination compared to either drug alone (#, p<0.05). ADI-PEG20, but not pemetrexed, had significant single agent activity compared to vehicle control (*, p<0.001). (E) Representative TS, DHFR and TK1 immunohistochemistry (X400) showing reduced expression of all three enzymes in ADI-treated UMUC-3 xenograft tumors; residual strong DHFR staining in stromal cells compared to negative tumor cells with ADI-PEG20 treatment. Pemetrexed and ADI-PEG20 treatments reduce and relocate TK1 expression from the nucleus to the cytoplasm; the drug combination further reduces TK1 expression compared with either drug alone. (F) $[^{18}F]$-FLT microPET/CT imaging response to treatment of ASS1-negative UMUC-3 tumors to ADI-PEG20 combined with pemetrexed. The ADI-treated animals showed a decrease in $^{18}$F-FLT SUVmax compared to PBS as seen previously (p<0.05; Fig 4G). Imaging at 7 days showed
a significant decrease in SUVmax for the PEM+ADI treatment group vs PBS control (p<0.01) as well as PEM+ADI vs ADI alone (p<0.05).

**Figure 6: Schematic of the inhibitory effects of ADI-PEG20 on *de novo* thymidylate biosynthesis and salvage pathways in bladder cancer.** ADI-PEG20 suppresses TS and DHFR protein and sensitises ASS1-negative bladder cancer cells to the cytotoxic effects of pemetrexed. In addition, ADI-PEG20 blocks thymidine uptake linked to reduced TK1 protein, which is detectable using $[^{18}\text{F}]$FLT-PET. RFC= reduced folate carrier; THF=tetrahydrofolate; PEM=pemetrexed.
**Figure 4**

**A**

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<tr>
<th></th>
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<tr>
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<td>200</td>
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</tr>
<tr>
<td>T24</td>
<td></td>
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**B**

<table>
<thead>
<tr>
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<tbody>
<tr>
<td>0.75 J</td>
<td>*</td>
<td>*</td>
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<td>0.75 J</td>
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<td>0.75 J</td>
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<td>0.75 J</td>
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Relative mRNA units:
- 253J
- UMUC-3
- T24
- RT112 (control)

*p<0.05
**p<0.01

**C**

<table>
<thead>
<tr>
<th></th>
<th>253J</th>
<th>UMUC-3</th>
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<th>RT112</th>
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<tr>
<td>36 kDa</td>
<td>*</td>
<td>*</td>
<td>*</td>
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</tr>
<tr>
<td>21 kDa</td>
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<td>45 kDa</td>
<td>*</td>
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**ADI** (750ng/ml)

**TS**

**DHFR**

**D**

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<tr>
<td>% over control</td>
<td>*</td>
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</table>

**[H]Thymidine uptake**

**E**

- 253J
- UMUC-3
- T24
- RT112

**ADI** (750ng/ml)

**TK1** (25 kDa)

**β-actin**

**F**

- **ADI** (750ng/ml)
- **Con**

**36 kDa**

**25 kDa**

**TK1**

**Hsc70**

**G**

- **i)**
- **ii)**
- **iii)**

**ADI**

**MG132**

**TS**

**TK1**

**Hsc70**

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Figure 6

* Downregulation of TS and TK1 protein via modulation of protein turnover.
Prognostic and therapeutic impact of argininosuccinate synthase-1 control in bladder cancer as monitored longitudinally by PET imaging

Michael Allen, Phuong Luong, Chantelle Hudson, et al.

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