Supplementary Information – Pre-clinical rationale for triaging participants to genotype-matched targeted therapies in the NLMT

Previously we have published a paper briefly outlining the pre-clinical rationale for the drug-biomarker combinations being investigated in the NLMT [1]. Here, we significantly extend that data for the large arms B2, C and F where however objective responses were scarce. This represents a much more comprehensive over-view of the pre-clinical data supporting the inclusion of these cohorts. The high levels of activity observed in arm D and G have been clinically confirmed in other studies and the rationale for the choice of FGFR mutations in arm A is discussed in the main text. The rationale for arms E and B1 can be found in our previous summary [1].

Arm B2 – vistusertib (dual mTORC1 and mTORC2 inhibitor) – STK11 loss (B2S), STK11 loss/dual KRAS mutation (B2D)

STK11 loss of function is a common event in non-small cell lung cancer (NSCLC). LKB1 phosphorylates and thereby activates AMPK which activates TSC2 [2]. The TSC1/TSC2 heterodimer is a GTPase activating protein which represses Rheb function, an activator of mTOR. STK11 inactivation was shown to result in mTOR pathway activation in the TCGA lung adenocarcinoma cohort [3]. STK11 mutant NSCLC cell lines fail to inhibit mTOR signalling following glucose deprivation [4]. STK11 loss is associated with upregulation of HIF-1α, which is a direct mTORC1 target and which protects STK11 mutant NSCLC cells from undergoing growth arrest upon withdrawal of glucose [5].

STK11 loss in NSCLC often occurs in association with KRAS mutation [2]. These two molecular aberrations synergise in driving metabolic re-programming with GLUT1 up-regulation (a direct HIF-1α target), increased glucose uptake, enhanced catabolic and anabolic glycolysis and heightened glucose dependency relative to cells with either STK11 (LKB1) loss (L) or KRAS (K) mutation alone [6]. There was significant enrichment of serine/glycine biosynthesis in KL cells, a key anabolic glycolytic process which mediated epigenetic re-programming via an increase in S-adenosyl methionine and an upregulation of DNMT1. KL xenografts were very sensitive to DNMT1 inhibition. Importantly, the metabolic and epigenetic reprogramming was reversible upon dual mTORC1/2 inhibition. These data were obtained using engineered pancreatic epithelial cells but in both dual

STK11/KRAS mutant NSCLC cell lines and KRAS (K) mutant cell lines with STK11 (S) knocked out by CRISPR, KS cells demonstrated enhanced glycolysis and serine/glycine biosynthesis, thus metabolically phenocopying the effect seen in pancreatic epithelial cells [7]. KS cells were more sensitive to glycolysis inhibition using 2-DG and glucose deprivation than K and S cells, as expected.

Vistusertib is a dual mTORC1 and mTORC2 inhibitor. mTORC2 was shown to be a central node in driving enhanced glycolysis across a range of cancer cell lines which included A549 (dual STK11/KRAS mutant NSCLC cell line) [8]. It was demonstrated that Rictor knockdown suppressed glycolytic gene expression, glucose uptake and lactate production. This Rictor-dependent effect of enhancing glycolysis was mediated by c-Myc and importantly was independent of Raptor, HIF-1α and Akt. Rictor up-regulated c-Myc by mediating HDAC phosphorylation which thus drove inhibitory FOXO acetylation. This causes a reduction in miR-34c, a transcriptional target of FOXO, and miR-34c reduces c-Myc RNA stability and translation. These results strongly suggest that inhibition of mTORC2 is a crucial factor in effectively switching off aerobic glycolysis: mTORC1-only inhibition not only has no inhibitory effect on mTORC2 but actually enhances mTORC2 activation [9]. In a direct comparison of vistusertib and rapamycin, the former was more effective at reducing glucose uptake in LKB1/KRAS double mutant A549 cells and it was shown RICTOR knockout reduced glucose uptake to a degree similar to RAPTOR knockdown, thus directly linking mTORC2 activity directly with the induction of glycolysis [10].

Arm C – palbociclib (Cdk4/6 inhibitor) - CDKN2A deletion/termination mutation squamous cell (C1), CDKN2A deletion/termination mutation non-squamous (C2), CDK4 amplification (C3), CDK4 amplification (C4), dual STK11 loss/KRAS mutation (C5), KRAS mutation (C6).

Palbociclib is a potent and selective inhibitor of Cdk4 and Cdk6 [11]. Cyclin D-CDK4/6 heterodimers monophosphorylate Rb to allow subsequent hyperphosphorylation of Rb by cyclin E-Cdk2, [12] which thus prevents its binding to and inhibition of E2F transcription factors. This allows DNA synthesis and passage through the G1-S checkpoint. *In vitro* palbociclib has potent anti-proliferative effects on Rb-proficient cancer cells including lung cancer cells but has no effect on growth of Rb null cells [11]. It exhibits significant anti-

tumour effects in vivo in Rb proficient xenografts but not in Rb null models. The two most sensitive models were both CDKN2A deleted. Inactivation of CDKN2A is seen in 72% of squamous cell cancers [13] and in 43% lung adenocarcinoma [3]. Two isogenic lung cancer cell lines with knockdown of Rb were generated [14]. Palbociclib significantly decreased cell growth only in the Rb proficient cell lines and significantly reduced growth of Rb-proficient xenografts with no effect on Rb null tumours. Palbociclib activated a senescence programme in Rb proficient cells but importantly also mediated an Rb-dependant apoptotic response in these cells. Apoptosis was induced by SMAC activation: palbociclib led to an Rb-mediated repression of FOXM1 and survivin thus allowing the up-regulation of SMAC resulting in cell death. Thus, palbociclib as a single agent, in a Ras- and p53-independent fashion, mediates the induction of apoptosis in Rb-proficient NSCLC. The data suggested that monotherapy with palbociclib could be cytotoxic rather than just cytostatic in Rb-proficient lung cancer.

The sensitivity of Rb proficient cells to palbociclib (and resistance of Rb null lines) and also of cells that lack functional p16 encoded by CDKN2A has been confirmed in multiple cell line series including melanoma [15], breast cancer (where high levels of cyclin D1 was also seen in the group of cell lines sensitive to palbociclib) [16], glioblastoma (both in vitro and in orthotopic xenografts) [17], renal cell cancer [18] and ovarian cancer [19]. The cancers of all participants eligible for palbociclib treatment in the NLMT had to be proven as Rb wild type by NGS: if the Rb read failed then they were not eligible without repeat biopsy. We treated two cohorts of patients with cancers harbouring Ras mutations with palbociclib. Cdk4 is crucial in the bypass of Ras-induced senescence specifically in lung adenocarcinoma cells: its loss significantly reduced the development and the progression of cancer in a conditional KRAS G12V driven mouse model [20]. In this model palbociclib also significantly reduced the development of Ras-driven cancers and the growth of established cancers. A much greater growth inhibitory effect of cdk4 inhibition in Ras mutant lung cancer using abemaciclib has also been pre-clinically demonstrated using xenograft models [21]. Any aberration activating Akt was an exclusion criteria for this KRAS mutant arm C6. Akt activation inhibits Ras-mediated senescence via inhibitory phosphorylation of GSK3β which is activated by RAS and which phosphorylates the histone chaperone HIRA facilitating its localisation to PML bodies and thus the formation of senescence-associated heterochromatin foci [22]. Finally, we treated dual STK11 loss/KRAS mutation bearing cancers with palbociclib based on data demonstrating that

palbociclib-induced cell cycle arrest could be converted to senescence (the process of geroconversion) by the presence of active mTOR signalling (cohort C5) [23]. Given the impact of STK11 loss on inactivation of AMPK in situations of cellular energy stress [2] we expected this to result in activation of mTOR signalling via de-repression of Rheb and subsequent inactivation of TSC1/2 as discussed above for cohort B2.

Arm F - capivasertib (AKT inhibitor) – PIK3CA mutant SCC (cohort F1), PIK3CA amplified SCC (cohort F2), PIK3CA mutant/PTEN deleted non-squamous NSCLC and all AKT mutations (cohort F3), PTEN deleted SCC (cohort F4)

Capivasertib is a potent inhibitor of all 3 AKT isoforms with sub-10 nanomolar IC_{50} [24]. In a large cell line panel there was a significant relationship between PIK3CA mutations or PTEN loss and capivasertib sensitivity. There was negative correlation with RAS mutation and for arm F the cancers of all participants eligible for capivasertib treatment in the NLMT had to be proven as RAS wild type by NGS: if the RAS read failed then patients were not eligible without repeat biopsy. Dose dependent inhibition of growth and tumour regressions were seen in PIK3CA mutant and PTEN null xenografts. In a separate series of NSCLC cell lines 13/14 lines with activated AKT had PIK3CA mutations, PTEN loss or RAS/EGFR mutations/Her2 amplifications [25]. Wild type PIK3CA (to phenocopy amplified PIK3CA) and mutant PIK3CA were over-expressed in transformed bronchial epithelial cells resulting in consistent hyperactivation of AKT [26]. Overexpression of both mutant and wild type PIK3CA resulted in enhanced anchorage-independent growth and cellular migration. In an independent study, lung cancer cell lines with PIK3CA copy number gain or mutation were strongly correlated with activation of AKT [27]. Knockdown of PIK3CA inhibited anchorage-dependant and -independent colony formation but had no effect on wild type cells. Transgenic mice with doxycycline inducible mutant PIK3CA targeted to alveolar epithelial cells rapidly developed lung adenocarcinomas [28]. Doxycycline withdrawal led to rapid complete regression of tumours. Pharmacological PIK3CA inhibition suppressed pAKT levels and led to dramatic reductions in tumour burden. In a series of NSCLC cell lines, PIK3CA mutant, PIK3CA amplified or PTEN null cells (and those with altered RTKs) lines were significantly more sensitive to PI3Kinase inhibition than those lines without these aberrations [29]. PI3Kinase inhibition resulted in dose dependant tumour regressions in PIK3CA or PTEN altered xenograft models.

Human LUSC cell lines engineered to over-express wild type (to recapitulate PIK3CA amplification) or mutant PIK3CA showed significantly elevated levels of pAKT compared with cells transfected with empty vector and demonstrated enhanced proliferation that could be reversed with PI3K inhibition [30]. The PIK3CA transfected clones had enhanced motility and invasiveness together with enhanced activity of MMP2 and 9, effects again significantly reduced by PI3K inhibition. Over-expressed wild type or mutant PIK3CA also mediated epithelial mesenchymal transformation. Cell release from parental line spheroids was unaffected by PI3K inhibition but release from the PIK3CA amplified or mutated clones was significantly reduced. Migration and invasion could also be significantly inhibited by PI3K inhibition in human SCC cell lines harbouring mutant PIK3CA. In vivo, the parental un-transfected SCC cell line grew at the same rate as the PIK3CA mutant transfected variant and PIK3CA inhibition caused a similar volumetric reduction in growth. However, importantly there was a doubling of tumour necrosis and a decrease in tumour tissue in the treated PIK3CA mutant transfectant. This demonstrates the limitations of the commonly used method of simple measurement of tumour volume to assess therapeutic impact in vivo and also demonstrated the greater sensitivity of PIK3CA mutant cells to PI3K inhibition compared to wild type squamous carcinoma lung cancer cells. PI3K inhibitor treatment also reduced the proportion of vimentin positive cells to that seen in parental xenografts.

Tumour initiating cells (TICs) are highly clonogenic and tumourogenic and can grow as spheroids in serum-free conditions. Immortalised human bronchial epithelial cell expressing mutant PIK3CA or that were PTEN null generated significantly more and larger lung cancer spheroids (LCSs) which had higher levels of the TIC markers Oct-4 and Nanog [31]. Akt knockdown in a PIK3CA mutant cell line significantly reduced the number and size of LCSs, reduced the expression of stem cell markers and reduced the expansion of tumours in immunodeficient mice. Pharmacological inhibition of AKT reduced Akt phosphorylation in PIK3CA mutant NSCLC cells and significantly and dose-dependently reduced the ability of the cells to form LCSs. It was subsequently demonstrated that Akt activation supported the self-renewal and tumorogenicity of NSCLC ILCs through activation of IkK which activated NFkB causing the induction of an autocrine IL-6 loop and STAT3 activation.

Finally, squamous lung cancers are heavily reliant on glycolysis and PI3K/AKT/mTOR/HIF-1α activation drives high GLUT1 expression [32]. Higher Akt activation is associated with greater HIF-1α and GLUT1 expression. Squamous lung cancers show significant enhancement of serine biosynthesis and are therapeutically vulnerable to the inhibition of glycolysis. Importantly, GLUT1 expression is positively correlated with PIK3CA copy number and the highest expression of GLUT1 is in cancers with PTEN loss.

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