



Original Articles

NNMT-driven metabolic reprogramming creates a NAMPT druggable vulnerability and reveals liquid biopsy biomarkers for TKI resistance in EGFR-mutant NSCLC

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ABSTRACT

Lung cancer is the deadliest neoplasia worldwide. Despite the availability of targeted therapies like tyrosine kinase inhibitors (TKIs) for EGFR-driven tumours in Non-Small Cell Lung Cancer (NSCLC), drug resistance remains a major factor that dramatically cuts life expectancy. Here we identify how increased expression of nicotinamide N-methyltransferase (NNMT) in TKI-resistant cancer cells diverts nicotinamide to synthesise 1-Methylnicotinamide (1-MNA) and lowers NAD⁺ levels that generates a druggable nicotinamide phosphoribosyltransferase (NAMPT) metabolic vulnerability. We also report that high blood levels of 1-MNA, the by-product of NNMT activity, are significantly associated with lower survival rates in EGFR TKI-treated NSCLC patients. Taken together, our findings describe a new and highly specific non-genetic metabolic synthetic lethality for mesenchymal-like tumours, which exposes NAMPT as an *in vivo* druggable target and establishes 1-MNA as a novel liquid biopsy biomarker to predict and monitor EGFR TKI resistance in NSCLC.

1. Introduction

Lung carcinoma is the leading cause of worldwide cancer deaths.

Approximately 70 % of all newly diagnosed patients present advanced disease and require systemic drug therapies. Non-Small Cell Lung Cancer (NSCLC) is the most frequent histological subtype and accounts for

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over 80 % of cases. Oncogenic driver alterations, such as EGFR mutations or ALK and ROS1 translocations, have been identified, and EGFR, ALK or ROS and tyrosine kinase inhibitors (TKIs) have significantly improved overall patient survival [1–4]. EGFR mutations are detected in approximately 10–15 % of NSCLC cases in Western populations and up to 40–50 % in East Asian populations. The most common activating mutations -exon 19 deletions (~45 %) and the L858R point mutation (~40 %)- are well-established oncogenic drivers and confer high sensitivity to EGFR TKIs. In contrast, less frequent or atypical EGFR mutations, such as L861Q (~4 %) and G719X (~2 %), are also associated with EGFR TKI sensitivity, albeit with generally lower efficacy compared to classical mutations [5]. While first-line EGFR TKIs such as gefitinib and erlotinib can elicit robust initial responses, treatment failure is inevitable due to the emergence of acquired resistance mechanisms. The most common of these include the secondary *EGFR* T790M mutation (in ~50 % of cases) and *MET* amplification (~5 %), which drive tumour progression despite continued EGFR inhibition [6–11]. Third-generation EGFR TKIs (i.e., osimertinib), which overcome T790M-mediated resistance, have become a first line of treatment for EGFR-mutant NSCLC patients [12,13]. However, a subset of patients acquires resistance through various genetic (e.g., EGFR-C797S mutation and *MET* amplification) and epigenetic mechanisms, including histological transformation from NSCLC to SCLC (small cell lung carcinoma) and the epithelial to mesenchymal transition (EMT) [10,14–18].

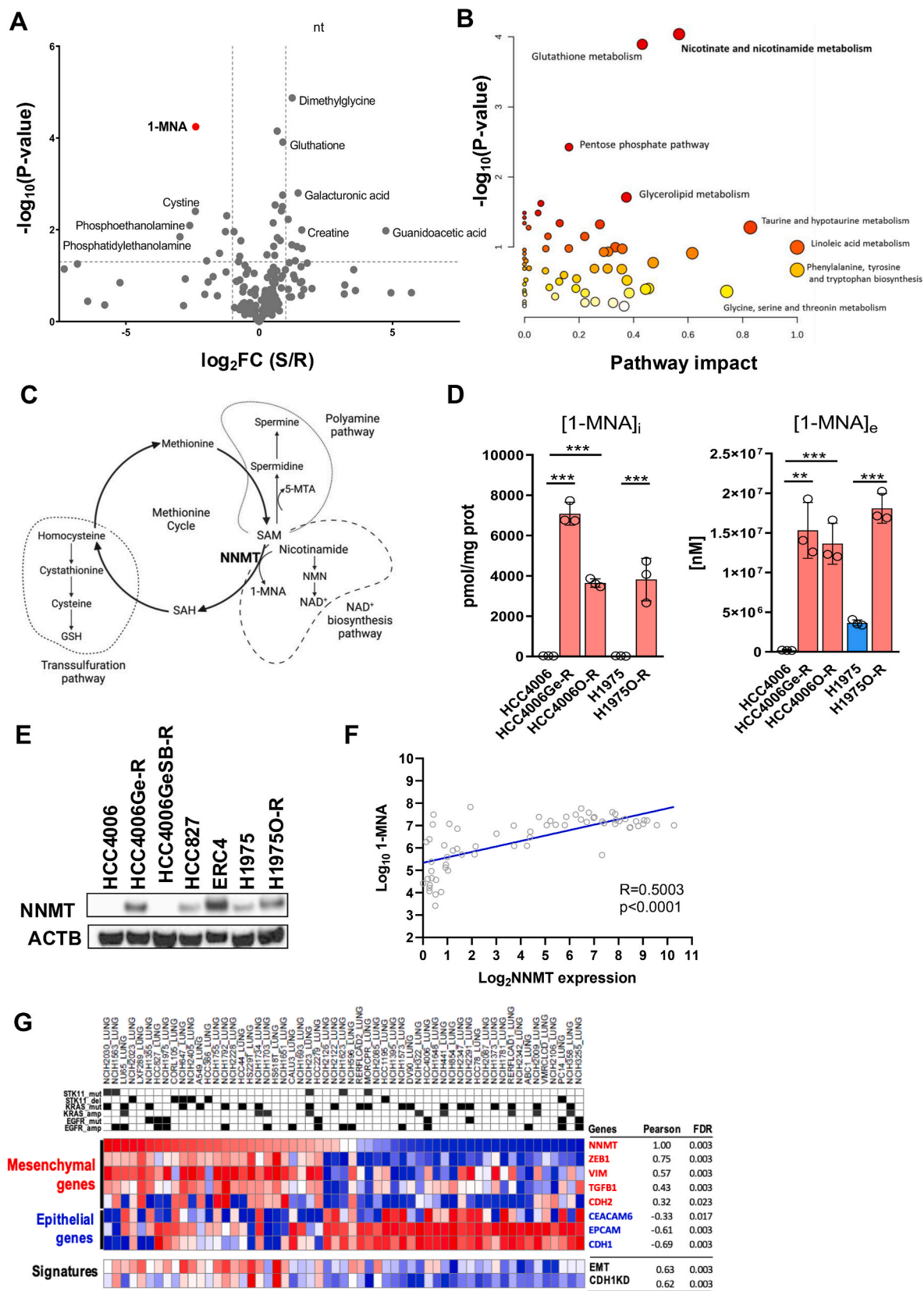
The EMT is a complex epigenetic programme deployed during embryonic development and wound healing and has been also associated with tumour progression and metastasis in many carcinomas, including NSCLC [19,20]. When cancer cells activate aberrantly (and partially) EMT, they acquire a mesenchymal-like phenotype that is poorly differentiated and often refractory to chemotherapy [21–23]. We have previously demonstrated that the inhibition of either the TGF β 1-axis [24] or the atypical G protein-coupled receptor, CXCR7, in the EGFR TKI-resistant cells showing a mesenchymal-like phenotype, promotes mesenchymal to epithelial transition (MET), which results in the re-sensitisation of tumour cells to EGFR inhibition [25]. This EMT-dependent reversible drug-tolerant phenotype involves transcriptomic reprogramming with a downstream affection of the proteome and metabolome, which are the functional drivers of the cancer phenotype [26,27]. Metabolic reprogramming, which is considered a hallmark of cancer, sustains tumour growth and malignant progression in several cancers, including lung carcinomas [28–30]. This metabolic rewiring is not only responsible for fuelling accelerated tumour metabolism: many chromatin modifying-enzymes use metabolic intermediates as cofactors or substrates by connecting epigenomic programmes, such as EMT, with profound metabolic alterations [31]. The interplay between the metabolome and epigenome enhances cellular plasticity, promoting cell survival, growth, and metastatic dissemination; however, the molecular mechanisms governing this crosstalk remain poorly understood [29,30]. Targeting these metabolic adaptations emerges as a potential therapeutic strategy to delay drug resistance or to specifically target resistant tumours [32]. Here the metabolomic analyses of clinically relevant NSCLC cell lines, which are sensitive and resistant to the first and third generations of EGFR TKIs [24,25,33], allowed us to detect a strong dysregulation of nicotinate and nicotinamide metabolism in mesenchymal-like EGFR TKI-resistant cells, which is attributed mainly to the strong up-regulation of NNMT. This metabolic rewiring diverts nicotinamide towards 1-MNA, which drains the NAD⁺ cellular pool and generates a druggable NAMPT-dependent metabolic vulnerability. We also found that 1-MNA levels in liquid biopsies are associated with the EGFR TKI response and *EGFR*-mutant NSCLC patients' poor prognosis.

2. Results

2.1. Untargeted metabolomics identifies nicotinate and nicotinamide metabolism dysregulation in mesenchymal-like EGFR TKI-resistant NSCLC cells

In order to unravel any potential metabolic changes associated with EGFR TKI resistance in NSCLC, we analysed the EGFR TKI-sensitive HCC4006 (*EGFR*^{E19del}) and H1975 (*EGFR*^{L858R/T790M}) cell lines, and their respective gefitinib- or osimertinib-resistant counterparts HCC4006Ge-R, HCC4006O-R and H1975O-R (Fig. S1A) [24]. These EGFR TKI resistant models developed in our laboratory show a strong mesenchymal-like phenotype based on epithelial (CDH1) and mesenchymal (CDH2 and VIM) protein expression (Fig. S1B) and *Gene Set Enrichment Analysis* of the RNA-Seq data showing a significant enrichment of the *Hallmark Epithelial to Mesenchymal Transition signature* in EGFR TKI-resistant cells (Fig. S1C). Importantly, no evidence of *MET* amplification (assessed by qPCR, Fig. S1D) or secondary *EGFR* mutations such as C797S (analysed by Sanger sequencing, data not shown) was detected. Using an untargeted LC-MS-based metabolomics platform in these mesenchymal-like EGFR TKI-resistant cell models [28,29], we detected over 3800 features, of which 183 metabolites were confidently identified and relatively quantified. Consistent alterations were observed across the cell models, with each cell line (sensitive or resistant) treated as a biological replicates and analysed in triplicate to ensure technical reproducibility (Fig. 1A). To further investigate which metabolic pathways sustain this metabolic rewiring, a *Metabolite Set Enrichment Analysis* was performed using MetaboAnalyst [34], which unveiled shared significant dysregulation of the nicotinate, nicotinamide and glutathione pathways, among others (Fig. 1B). Subsequently, we validated these metabolic changes by using a targeted LC-MS/MS method which quantitatively determined metabolites comprised in the nicotinamide, transsulfuration, polyamine pathways and methionine cycle (Fig. 1C). Among the analysed metabolites, 1-MNA appeared as the most significantly altered in all the EGFR TKI-resistant cell models (Table S1). 1-MNA is the metabolic by-product generated by NNMT, which catalyses the methylation of nicotinamide using S-adenosylmethionine (SAM) as the universal methyl donor. This enzymatic activity establishes a direct connection among one-carbon metabolism, the regulation of cellular methylation homeostasis and NAD⁺ levels [30]. 1-MNA accumulates intracellularly in all mesenchymal-like EGFR TKI-resistant cell models, and it is also exported to extracellular media (Fig. 1D). As shown in Fig. 1E, an increase in NNMT protein levels was clearly detected across gefitinib- (Ge-R), erlotinib- (ERC4, a subclone of the HCC827 cell line described in Ref. [24]) and osimertinib- (O-R) resistant mesenchymal-like cells, which links its enzymatic activity to 1-MNA production. Interestingly, NNMT was not induced in the HCC4006GeSB-R cells, which grow and resist gefitinib in the presence of the TGF β R inhibitor, SB-431542, to maintain the epithelial phenotype, while cells acquire resistance to gefitinib due to the *EGFR* T790M secondary mutation [24]. To investigate if a correlation exists between NNMT and 1-MNA beyond the EGFR TKI-resistant phenotype and in the broader NSCLC context, and regardless of the EGFR mutation status, we analysed the multi-omic data from *Cancer Cell Line Encyclopedia* (CCLE) [35], which integrates genomic, transcriptomic and metabolomic data [35]. Our findings revealed a significant positive correlation between the 1-MNA levels and *NNMT* mRNA expression (Fig. 1F). They also revealed that the *NNMT* mRNA levels correlated positively with canonical mesenchymal markers (VIM, ZEB1) and inversely with epithelial-specific markers (CDH1, EPCAM) (Fig. 1G, top). NNMT expression also exhibited a strong correlation with the enrichment scores of EMT from the MSigDB database and our previously published CDH1-knockout signatures [20,21], analysed by a *single sample Gene Set Enrichment Analysis* (ssGSEA) (Fig. 1G, bottom) [36,37].

Given previous evidence suggesting NNMT's involvement in EGFR TKI resistance [38,39], we evaluated whether inhibiting NNMT either



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Fig. 1. 1-MNA is a biomarker of NNMT activity in mesenchymal-like lung cancer cells. (A) Volcano plot showing the common deregulated metabolites comparing the EGFR TKI-sensitive (HCC4006 and H1975) *versus* -resistant NSCLC (HCC4006Ge-R, HCC4006O-R, H1975O-R) cell lines. Pooled data are represented as the mean log2 fold changes based on $n = 3$ independent assays. (B) Metabolite Set Enrichment Analysis based on the identified common 183 metabolites. (C) Scheme showing the nicotinamide metabolism and the linked metabolic pathways. (D) Quantification of intracellular and extracellular 1-MNA by LC-MS/MS. Data are presented as the mean $\pm$ s.d. with $n = 3$ independent assays analysed by a two-tailed unpaired *t*-test. * $P < 0.05$, ** $P < 0.01$ and *** $P < 0.001$. Ge-R: gefitinib-resistant. O-R: osimertinib-resistant. (E) NNMT protein levels in the EGFR TKI-sensitive and TKI-resistant NSCLC cell lines analysed by western blotting. Ge-R: gefitinib-resistant. GeSB-R: gefitinib and SB431542 resistant. ERC4: erlotinib-resistant HCC827. O-R: osimertinib-resistant. (F) Pearson correlation analysis between the NNMT transcript levels with the mesenchymal (in red), epithelial (in blue) markers and curated transcriptional signatures of EMT and CDH1 repressions (CDH1KD) in the LUAD cell lines from the CCLE dataset [35]. (G) Correlation between NNMT mRNA expression and intracellular 1-MNA levels in the LUAD cell lines from the CCLE dataset [35]. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

genetically through shRNA or pharmacologically would reverse the mesenchymal-like TKI-resistant phenotype in our cell models. The genetic suppression of NNMT in the mesenchymal-like EGFR TKI-resistant cells restored drug sensitivity, as demonstrated by cytotoxicity and colony formation assays, and reduced mesenchymal markers, while reactivated epithelial markers (Fig. S1E). Besides, NNMT ectopic overexpression or repression increased and decreased NNMT activity in terms of intracellular and extracellular 1-MNA levels, respectively (Fig. S1F and S1G). Using 1-methylquinolinium (1-MQ), which is a pharmacological NNMT inhibitor that interacts with the active site of nicotinamide [38,40], resulted in a marked decrease in NNMT activity in the mesenchymal-like EGFR TKI-resistant cells (Fig. S1H). The proliferation curves shown in Fig. S1I demonstrate that 1-MQ restores sensitivity to osimertinib in OR cells. Consistently, the colony formation assay revealed that the percentage of reduction in colony number induced by osimertinib improved significantly with the addition of the NNMT inhibitor, changing from $84 \pm 11\%$ to $24 \pm 6\%$ ($p < 0.05$). Finally, 1-MQ partially reversed the mesenchymal-like phenotype, as indicated by an increase in the proportion of CDH1-positive cells (from $2 \pm 1\%$ to $7 \pm 2\%$) and a decrease in VIM-positive cells (from $62 \pm 3\%$ to $29 \pm 4\%$, $p < 0.05$) (Fig. S1I).

In summary, these results indicate that NNMT is a key factor in mesenchymal-like phenotype and EGFR TKI resistance in NSCLC cells and, more importantly, increased NNMT activity diverts nicotinamide towards the 1-MNA production, emerging as a potential surrogate biomarker for NNMT activity.

2.2. Increased NNMT activity redirects nicotinamide and depletes NAD^+ , generating a novel NAMPT-dependent metabolic vulnerability in EGFR TKI-resistant NSCLC

As we have described earlier, we developed a quantitative LC-MS/MS method to analyze nicotinamide metabolism and its associated methionine and NAD^+ pathways. Increased 1-MNA production reported in Fig. 1D is consistently associated with low nicotinamide and NAD^+ levels in NNMT^{high} mesenchymal-like EGFR TKI resistant cell models (Fig. 2A), which underscores the pivotal role of NNMT in this profound metabolic rewiring. Although no differences in SAM levels were detected (data not shown), the SAM/SAH (S-adenosylhomocysteine) ratio was significantly lower compared to the EGFR TKI-sensitive epithelial cells, suggesting that the intracellular SAM pool might be replenished by methionine despite its increased demand. Nicotinamide can be converted into NAD^+ through the salvage pathway by nicotinamide phosphoribosyltransferase (NAMPT) or be alternatively converted into 1-MNA by NNMT [41]. Having observed the association between EGFR TKI resistance and lowered intracellular NAD^+ levels, we hypothesised that high NNMT activity, which diverts nicotinamide to 1-MNA, could significantly deplete the NAD^+ pool. In line with this, the ectopic NNMT overexpression in the parental HCC4006 cells substantially increased both the intracellular and extracellular 1-MNA levels (Fig. S1F), which led to a significant drop in nicotinamide, NAD^+ and the SAM/SAH ratio (Fig. S2A), resembling the metabolic changes seen in the mesenchymal-like EGFR TKI-resistant cells. Accordingly, NNMT depletion using lentiviral shRNA in the EGFR TKI-resistant HCC4006Ge-R cells shifted the metabolite profiles towards those of their parental

counterparts (Fig. S2A). Interestingly, ectopic overexpression of NNMT not only recapitulates the metabolic adaptations affecting nicotinamide and NAD metabolism but also induces partial activation of the EMT program, characterized by subtle changes in EMT marker expression, disruption and reduction of CDH1-mediated cell-cell junctions, and increased resistance to EGFR TKIs (Fig. S2B).

The low NAD^+ levels observed in the mesenchymal-like EGFR TKI-resistant cells might compromise their survival when a critical NAD^+ pool threshold is reached. NAMPT catalyses the conversion of nicotinamide into nicotinamide mononucleotide (NMN), the first- and rate-limiting step of the NAD^+ salvage pathway [41]. Thus, reducing NAMPT activity could impair NAD^+ synthesis and potentially affect cell survival. Indeed, transient NAMPT depletion using siRNA reduced viability by nearly 50 % in the mesenchymal-like HCC4006Ge-R cells, an effect mitigated by NNMT depletion using siRNA (Fig. 2B). Furthermore, daporinad (DAP, also known as FK866/APO866), a specific NAMPT inhibitor used in preclinical cancer models and clinical trials [42], significantly attenuated growth of NNMT^{high} mesenchymal-like EGFR TKI-resistant cells (Fig. 2C), which denotes their exceptional sensitivity to NAMPT inhibition. This sensitivity to DAP resulted from a substantial decrease in NAD^+ synthesis due to reduced substrate availability, which is rescued with NMN, the NAMPT product and a NAD^+ precursor capable of restoring the NAD^+ pool (Fig. 2D and S2C). Additionally, depleting NNMT using lentiviral shRNA in mesenchymal-like EGFR-TKI-resistant HCC4006Ge-R cells significantly attenuated DAP-induced autophagy, apoptotic cell death, and NAD^+ depletion, while the NNMT overexpression in epithelial-like counterparts enhanced DAP effects (Fig. 2E and F and S2D). Interestingly, pharmacological inhibition of NNMT with 1-MQ used in the previous section abrogated DAP's cytotoxicity (Fig. 2G) by preventing NAD^+ depletion (Fig. S2E). Finally, we verified that the NNMT-driven, NAMPT-dependent metabolic vulnerability is specific to lung cancer cells and does not affect normal cells, such as normal lung fibroblasts isolated from pneumothorax surgical specimens. Neither lentiviral shRNA-mediated knockdown of NNMT nor its pharmacological inhibition with 1MQ altered the NAD^+ depletion pattern induced by DAP and, more importantly, these interventions were unable to reverse the antiproliferative effects of the NAMPT inhibitor in normal fibroblasts (Fig. S2F).

In summary, NNMT overexpression in mesenchymal-like EGFR TKI-resistant NSCLC cells exclusively diverged nicotinamide to 1-MNA, decreasing the intracellular NAD^+ pool and revealing a novel metabolic vulnerability which can be exploited by pharmacologically inhibiting NAMPT activity.

2.3. NAMPT inhibition selectively targets the mesenchymal-like NSCLC cells *in vivo*

Motivated by the results whereby the NNMT^{high} lung cancer cells demonstrated an exquisite sensitivity to DAP treatment as a single-agent *in vitro*, we ran a proof-of-concept experiment *in vivo*. Our prior work has established that the EGFR TKI-resistant NSCLC cell lines, such as those deriving from HCC827 and HCC4006, exhibit a significant growth disadvantage when engrafted onto immunocompromised nude mice [33] due to the EMT-associated induction of vasoconstrictor factor endothelin 1 (EDN1), which reduces the blood supply to tumours by

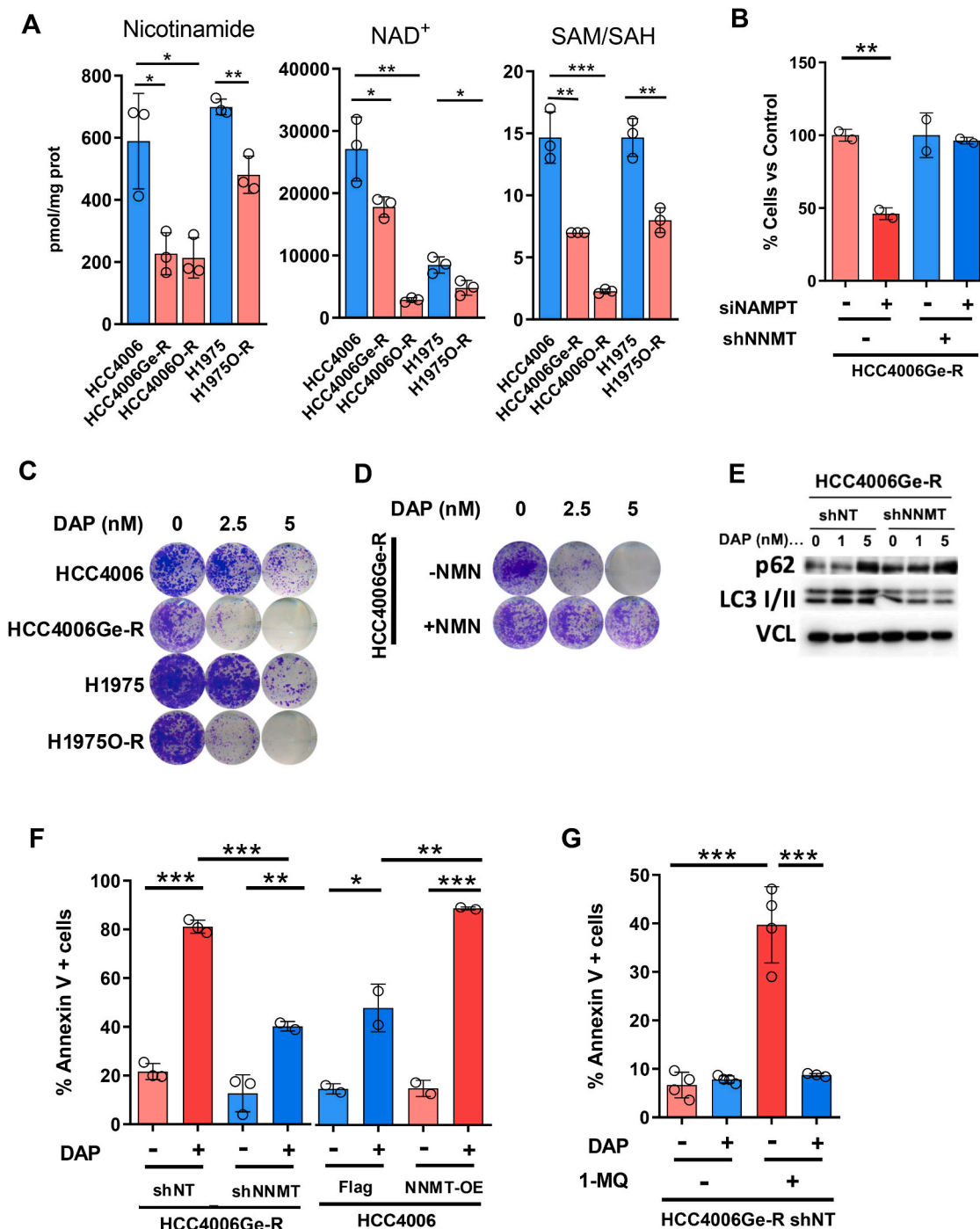


Fig. 2. NNMT overexpression rewires NAD⁺ metabolism and generates a new metabolic vulnerability. **(A)** Quantification of nicotinamide, NAD⁺ and SAM/SAH ratio by LC-MS/MS. Data are presented as the mean $\pm$ s.d. with $n = 3$ independent assays analysed by a two-tailed unpaired t -test. * $P < 0.05$, ** $P < 0.01$ and *** $P < 0.001$. Ge-R: gefitinib-resistant. O-R: osimertinib-resistant. **(B)** HCC4006Ge-R cells that stably expressed shRNA by targeting NT or NNMT were 72-h transfected with siRNA targeting NAMPT or the control and the % of surviving cells was assessed. Data are presented as the mean $\pm$ s.d. based on $n = 2$ replicates analysed by a one-way ANOVA. ** $P < 0.01$. **(C)** Cells HCC4006/HCC4006Ge-R and H1975/H1975O-R were treated with DAP for 10 days. Surviving cells were stained with crystal violet. The displayed image is representative of $n = 2$ independent experiments. **(D)** HCC4006Ge-R cells were treated with DAP and with DAP with NMN (100 μ M) for 10 days. Surviving cells were stained with crystal violet. The displayed image is representative of $n = 2$ independent experiments. **(E)** LC3 lipidation (which reflects autophagosome formation), and p62 (a selective autophagy marker whose accumulation indicates impaired autophagic flux, while its reduction reflects active and functional autophagy) were analysed by western blotting. **(F)** Percentages of Annexin-V-positive (apoptotic) cells after the 72-h DAP treatment of TKI-resistant HCC4006Ge-R cells stably expressing shRNA by targeting non-target (shNT) or shRNA against NNMT (shNNMT), or TKI-sensitive HCC4006 cells stably expressing the control (empty vector) or ectopic NNMT. Data are presented as the mean $\pm$ s.d. based on $n = 3$ independent assays analysed by a one-way ANOVA * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$. **(G)** Percentages of Annexin-V-positive cells assay in HCC4006Ge-R shNT cells after the 72-h treatment with DAP (5 nM) and DAP + 1-MQ (100 μ M) combination. Data are presented as the mean $\pm$ s.d. based on $n = 3$ independent assays analysed by a one-way ANOVA *** $P < 0.001$. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

contributing to induce tumour cell dormancy [43]. We initially attempted to establish H1975O-R xenografts in nude mice, but none of the injected animals developed tumours (data not shown). Even using NSG mice, which have a higher level of immunodeficiency, the growth of H1975O-R xenografts was extremely delayed, taking over 100 days to reach measurable size in only a subset of mice (Fig. S3B). Considering these limitations of xenograft models using EGFR TKI-resistant mesenchymal-like cells, we opted to explore an alternative experimental design. We revealed that NNMT was highly expressed in not only EGFR-TKI-resistant cells (Fig. 1E) but also in other NSCLC cells with a mesenchymal-like phenotype. Furthermore, a meta-analysis of drug sensitivity data from the GDSC (*Genomics of Drug Sensitivity in Cancer*) project [44] shows a positive correlation between NNMT expression in CCLE cell lines and sensitivity to DAP (Fig. S3A). Driven by these *in silico* findings, we conclude that the mesenchymal-like, NNMT^{high}, KRAS-mutant A549 cell line, characterized by its robust *in vivo* growth and limited therapeutic options, is an ideal candidate for the *in vivo* study. Before proceeding, we confirmed that the stable NNMT depletion in A549 cells induced an epithelial-like phenotype with high CDH1 and low VIM expression (Fig. S3C), which led to lower 1-MNA levels (Fig. S3D) and resistance to the anti-tumoral effects of DAP (Fig. S3E) because cells retained the ability to maintain NAD⁺ levels (Fig. S3F). Next, we subcutaneously injected the epithelial-like NNMT^{low} (PC9 and HCC4006) and mesenchymal-like NNMT^{high} (A549) cell lines into nude mice. When tumour size reached approximately 100 mm³, animals were randomised and administered with vehicle or DAP (Fig. 3A), which was well-tolerated and had no adverse effects on mouse body weight (Fig. S3G). The mesenchymal-like NNMT^{high} tumours demonstrated substantial sensitivity to DAP by displaying pronounced tumour volume

reduction compared to epithelial-like NNMT^{low} (483.7 ± 281 vs. 128.9 ± 71.8 mm³) (Fig. S3H) and marked NAD⁺ pool depletion (Fig. 3B). The specific anti-tumoral effect of DAP on NNMT^{high} tumours was confirmed with extensive necrosis without tumour cells (H&E staining) and reduced proliferation (Fig. 3C and D).

In conclusion, these *in vivo* experiments demonstrate that mesenchymal-like NNMT^{high} tumours' increased susceptibility to the NAMPT inhibitor DAP, underscoring a strategic approach to exploit NAD⁺ biosynthesis inhibition in treating NSCLC, particularly targeting those tumours showing the mesenchymal-like phenotype.

2.4. Increased tumour NNMT expression and blood 1-MNA levels correlate with poor prognosis in NSCLC patients

Over the last few years, we have collected clinical samples from patients diagnosed with advanced EGFR-mutant lung adenocarcinoma (LUAD) on first-line TKI therapy (Table S2). Of them, 24 patients with documented EGFR TKI sensitising mutations (either exon 19 deletion or L858R mutation), treated with first-generation TKIs and clinically confirmed EGFR-T790M status post-relapse were selected for the NNMT level analysis. Immunohistochemistry (IHC) assays on paired pre- and post-TKI treatment FFPE tissue samples revealed that the re-biopsies from the EGFR-T790M negative patients exhibited significantly high NNMT protein levels compared to those patients harbouring the EGFR-T790M mutation, the most common mechanism of resistance to erlotinib and gefitinib ($p < 0.05$, Fisher's test) (Fig. 4A–B). Consistent with our *in vitro* findings in the extracellular medium, patients with higher tumoral NNMT expression exhibited significantly increased levels of 1-MNA in peripheral blood serum (Fig. 4C). Importantly, and in line

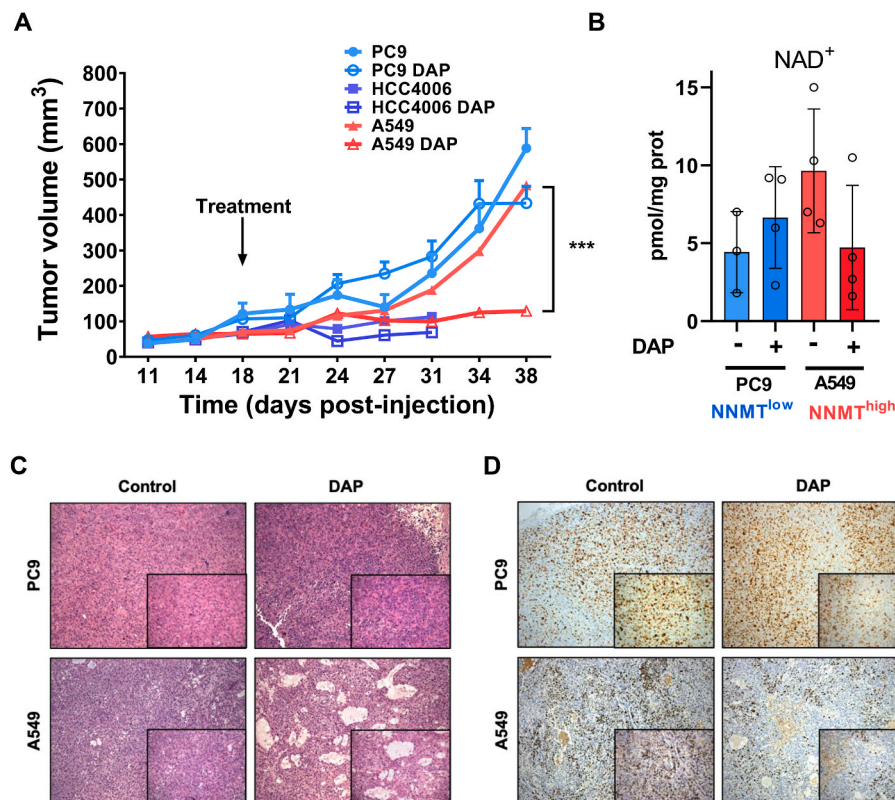


Fig. 3. Mesenchymal-like NSCLC tumours are vulnerable to NAMPT inhibition *in vivo*. (A) $2 \cdot 10^6$ of PC9, HCC4006 and A549 cells were grafted on nude mouse flanks and allowed to grow for 18 days to then be treated with DAP (10 mg/kg). Data are presented as the mean ± s.d. based on $n = 12$ tumours per group analysed by a two-tailed unpaired *t*-test. *** $P < 0.0001$. (B) Quantification of the NAD⁺ levels in the PC9 and A549 xenograft tumours after DAP treatment. Data are presented as the mean ± s.d. based on $n = 3$ in the PC9 control and 4 in the other groups analysed by the two-tailed unpaired *t*-test. (C) Representative H&E staining of A549 and PC9, vehicle or DAP-treated xenografts. Inset, x40 and x20. (D) IHC staining of ki67 of the A549 and PC9, vehicle or DAP-treated xenografts. The displayed images are representative of $n = 3$ mice (10x magnification, insets 20x).

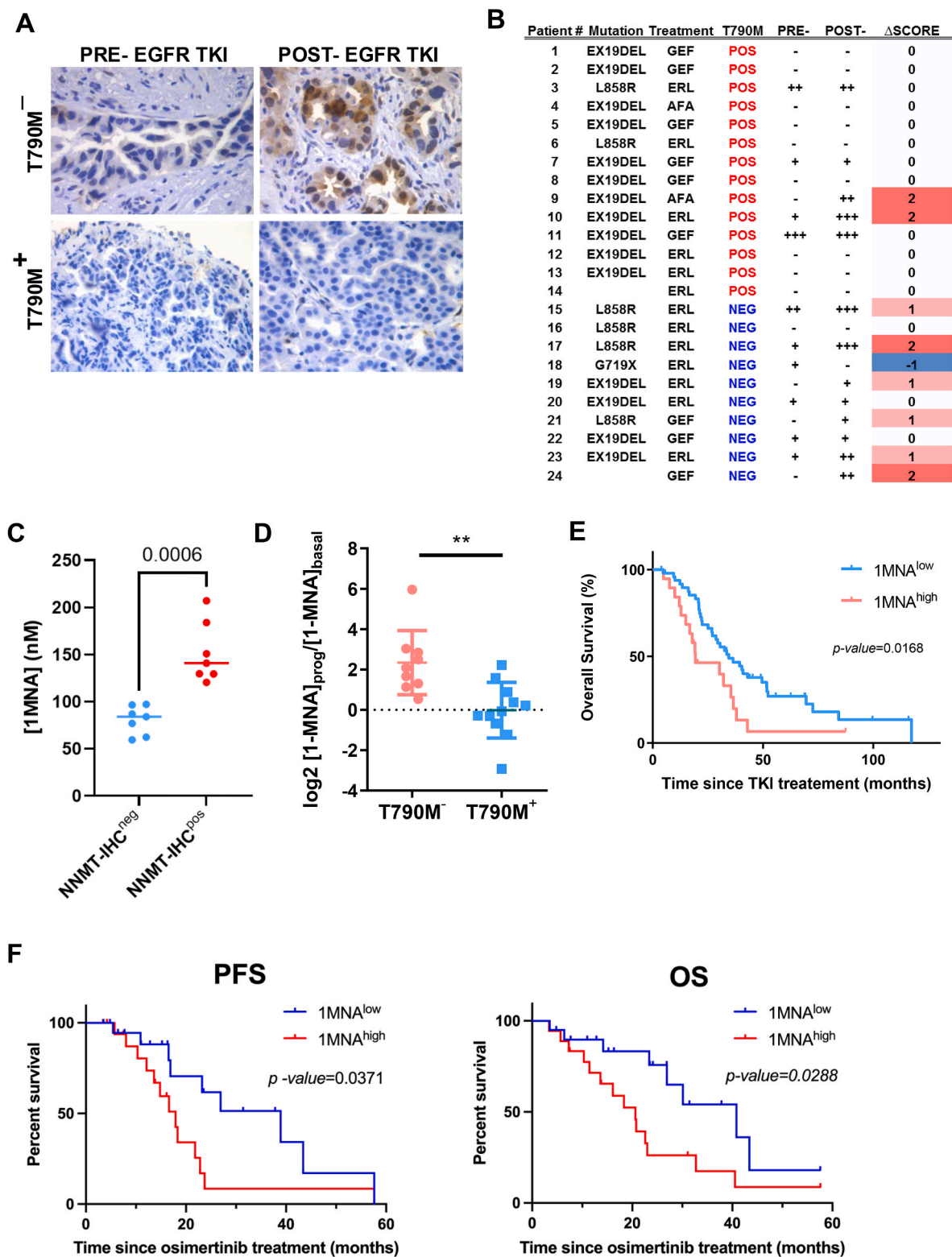


Fig. 4. High NNMT expression and activity are linked to poorer survival outcomes in EGFR-mutant LUAD patients undergoing EGFR TKI treatment. (A) (B) Representative NNMT staining of tissue biopsy and clinicopathologic features of 24 EGFR-mutant LUAD patients analysed by IHC, before first-generation EGFR TKIs (PRE-TKI) and at the time of RECIST-confirmed progression (POST-TKI) with or without an acquired secondary EGFR-T790M mutation. (C) Correlation analysis between baseline 1-MNA levels in liquid biopsies and *in situ* protein expression analysed by IHC in matched tissue samples from (A). (D) 1-MNA serum levels of pre- and post-first-generation TKI at the disease progression ratio for EGFR-mutant LUAD patients (*n* = 21) classified as positive or negative for the EGFR-T790M mutation and analysed by the Mann-Whitney test ***P* < 0.01. (E) Kaplan-Meier curve for OS of the first-generation TKI-treated EGFR-mutant LUAD patients (*n* = 69) classified by low and high serum 1-MNA levels, either above or below the median value, respectively. (F) Kaplan-Meier curves for PFS and OS of the first-line osimertinib-treated EGFR-mutant LUAD patients (*n* = 38) classified by low and high serum 1-MNA levels, either above or below the median value, respectively. Censored data are indicated by tick marks. Data from the patients who had not relapsed/died at the time of the analysis were censored based on the last recorded date on which the patient was known to be alive.

with the IHC findings, the progression-to-baseline ratio of serum 1-MNA levels was significantly higher ($p = 0.0012$) in *EGFR*-T790M-negative patients from a cohort of 21 advanced *EGFR*-mutant LUAD cases following relapse on first-generation *EGFR* TKIs (Fig. 4D).

These observations led us to hypothesize that NNMT overexpression in mesenchymal-like tumours may increase 1-MNA secretion into the bloodstream, potentially correlating with clinical outcomes in *EGFR*-mutant LUAD patients treated with TKIs. To test this, we measured baseline serum 1-MNA levels in pre-treatment blood samples from 69 patients receiving first-generation *EGFR* TKIs. Patients were stratified into high and low NNMT activity groups using the median 1-MNA level as a cutoff. Kaplan–Meier analysis revealed that higher baseline 1-MNA levels were significantly associated with shorter overall survival (OS) compared to those with lower levels (18.3 vs. 34.47 months; $p = 0.0168$) (Fig. 4E). We had previously shown in tumour tissue that patients lacking the *EGFR*-T790M resistance mutation to first-generation TKIs exhibited higher NNMT expression and greater 1-MNA production than those with the mutation at progression (Fig. 4A–D). Based on this, we further evaluated the impact of *EGFR* T790M status on OS. Patients were stratified according to *EGFR*-T790M mutation status and 1-MNA levels (above or below the group-specific median). Kaplan–Meier analysis showed that elevated baseline 1-MNA levels were significantly associated with shorter OS only in patients without the T790M mutation (Fig. S4A). Finally, we next analysed an independent cohort of *EGFR*-mutant LUAD patients treated with first-line osimertinib ($n = 38$). Pre-treatment liquid biopsies revealed a clear and statistically significant association between serum 1-MNA levels and patient prognosis, as demonstrated by Kaplan–Meier analyses of progression-free survival (PFS) and OS (Fig. 4F).

In summary, NNMT overexpression is not only strongly linked to mesenchymal-like features in *EGFR*-mutant NSCLC tumours but also emerges as a potential predictive biomarker in LUAD patients lacking the *EGFR*-T790M mutation who progress on first-generation *EGFR* TKIs, as well as in those receiving first-line osimertinib, which has replaced first-generation TKIs as the current standard of care.

3. Discussion

The persistence of acquired resistance to *EGFR* TKIs substantially shortens NSCLC patients' life expectancy, which accentuates the imperative to develop advanced therapeutic modalities [45,46]. Secondary *EGFR*-T790M mutations are well-documented for conferring first-generation *EGFR* TKIs resistance, and the advent of third-generation *EGFR* TKIs like osimertinib has been essential, particularly as a frontline intervention for advanced *EGFR*-mutant NSCLC [12, 13]. Nevertheless, resistance mechanisms are not limited to genetic variations that arise through selection or *de novo* mutations. This underscores the complexity of the drug resistance landscape, especially when resistance emerges against osimertinib, recently approved as first treatment line, presenting a substantial therapeutic dilemma for cases not driven by genetic alterations such as *EGFR*-C797S mutations or *MET* amplification [47].

Our search for novel non-genetic vulnerabilities led us to run a comprehensive metabolomic analysis of our well-characterised and clinically relevant *EGFR* TKI-sensitive and -resistant cell models [24,25, 33], by unveiling marked dysregulation in the nicotinate and nicotinamide metabolic pathways in the mesenchymal-like *EGFR* TKI-resistant cells. Remarkably, consistent and significant 1-MNA accumulation, linked with NNMT overexpression, remained elevated across all the tested cell lines. This scenario aligns with several studies showing how increased NNMT protein expression, as measured by IHC, correlates with shorter PFS in *EGFR* TKI-resistant NSCLC patients [39,48]. Intriguingly, our elaborate clinical study based on the IHC analysis of paraffin-embedded tumour pre- and post-first-generation TKI biopsies discovered that an increased NNMT expression was predominantly associated with the *EGFR*-T790M negative subpopulation, which has

previously gone unnoticed. Consequently, our findings support the incorporation of NNMT IHC into the panel of histopathological tools for more precise NSCLC subtyping and personalized guidance of anti-*EGFR* therapies.

EGFR-mutant tumours dynamically adapt to the pressure exerted by TKI treatments. Consequently, optimal patient specimens for thoroughly investigating these changes are sequential needle biopsy samples from the same patient. Nonetheless, they are scarce and rarely available. Conversely, liquid biopsies offer a less invasive and more readily available sample that is capable of reliably mirroring the metabolic adaptations of tumours to treatments [49,50]. With this premise, we decided to evaluate if a correlation existed between serum levels of 1-MNA, a surrogate marker of tumour NNMT activity, and clinical outcomes in the *EGFR*-mutant NSCLC patients on first line of *EGFR* TKI from first to third generation. We have also identified a significant interaction between blood 1-MNA levels and the *EGFR*-T790M mutation status in the cohort of first-generation *EGFR* TKI-treated patients, which serve as predictor for OS. Although further sample analyses are required, our findings substantiate a pronounced augmentation of NNMT in *EGFR*-T790M negative tumours, along with an increase in circulating 1-MNA levels in the *EGFR*-T790M negative NSCLC patients who have progressed on *EGFR* TKIs. Even more importantly, circulating 1-MNA also holds prognostic value in another cohort of LUAD patients receiving first-line osimertinib, which has now replaced first-generation TKIs as the preferred first-line treatment [12]. Measuring circulating 1-MNA levels may thus serve as a relevant biomarker for detecting the emergence of mesenchymal-like, *EGFR* TKI-resistant clones characterized by elevated NNMT activity. Based on current knowledge and existing literature, our findings open a novel avenue for exploring metabolic biomarkers in NSCLC through liquid biopsy.

Having acknowledged that cell plasticity of mesenchymal-like tumours is a mechanism for chemoresistance [51], the transition back to a more drug-sensitive epithelial state via *MET* could improve therapeutic responses. To date, the pharmacological inhibition of TGF β 1, ZEB1 [52, 53] or miR-200 family members to promote *MET* is still challenging because it may inadvertently affect normal cells or promote the dedifferentiation and proliferation of dormant metastatic cells in distant niches [54]. To overcome such problems, our previous research has demonstrated that depleting EMT-specific targets, such as CXCR7, can promote *MET* in *EGFR*-mutant tumours [25]. Similarly, targeting NNMT, as identified in the *EGFR* TKI-resistant cells with a mesenchymal-like phenotype, emerges as a viable strategy. Prior studies have shown that NNMT depletion using shRNA enhances the sensitivity of the *EGFR*-mutant NSCLCs to *EGFR* TKIs, which suggests NNMT targeting to be an effective method to overcome resistance and to enhance the efficacy of anti-*EGFR* therapies [39,48,55,56]. Our findings further corroborate that pharmacological NNMT inhibition using 1-MQ enhances *EGFR* TKI responsiveness in the subset of NSCLC with acquired resistance to the *EGFR* TKIs with a mesenchymal-like phenotype, suggesting that 1-MQ would delay the emergence of *EGFR* TKI resistance in the clinical setting [57–59].

Apart from oncogenic mutations impacting signalling pathways, metabolic alterations also significantly contribute to tumorigenesis [39]. Tumour cells depend on steady nutrient supply from their environment to fuel growth, with changes in specific metabolites influencing gene expression profiles, cellular differentiation programmes or the interaction with their microenvironment [60]. Metabolic alterations such as IDH1 mutations in gliomas are necessary for malignant transformation [61]. Similarly, the extensive metabolic rewiring herein reported in the methionine cycle and on the NAD⁺ salvage pathway is a direct outcome of NNMT overexpression. This process involves the transfer of methyl groups from SAM to nicotinamide, which results in 1-MNA production that, in turn, reduces nicotinamide availability and impacts the NAD⁺ pool in the NNMT^{high} mesenchymal-like *EGFR* TKI-resistant NSCLC cells. As NAD⁺ is an essential co-enzyme for energy metabolism, DNA repair and cell growth, tumour cells elevate NAD⁺ biosynthesis to meet

their increased demands and limiting its availability may undermine the metabolic adaptations that facilitate tumour progression [62,63]. Clinical trials utilising NAMPT inhibitors like DAP for melanoma and lymphoma treatment demonstrate that their efficacy is enhanced when combined with additional treatments as we propose here for NSCLC treatment, which highlights the need to discern biomarkers to identify which tumours are susceptible to such strategies [64,65]. The remarkable drop in the NAD^+ levels in the EGFR TKI-resistant cells with a mesenchymal-like phenotype can be mitigated by suppressing NNMT, which indicates potential metabolic vulnerability. Following this research line, we discovered that DAP selectively affects mesenchymal-like tumour cells (and not normal cells such as fibroblasts) with increased NNMT levels, with apoptotic cell death and increased autophagic flux directly linked with significant NAD^+ reduction, which is reversible only through either NNMT down-regulation or inhibition by 1-MQ [66,67]. Therefore, the interaction among NNMT activity, nicotinamide availability and NAMPT-mediated NAD^+ biosynthesis is crucial for cellular NAD^+ homeostasis and the response to DAP treatment exclusively in tumour cells [68]. Overall, our findings underscore that deciphering metabolic control mechanisms in the mesenchymal-like EGFR TKI-resistant cells allows for their targeted disruption *in vivo*, as demonstrated by increased mesenchymal-like NNMT^{high} tumour xenografts' sensitivity to DAP treatment compared to their epithelial NNMT^{low} counterparts in nude mice. This establishes a relevant metabolic synthetic lethality, which could have significant clinical implications.

In conclusion, our research demonstrates that NNMT plays an essential role in enabling NSCLC cancer cells to adapt to anti-EGFR therapies by unveiling a highly specific non-genetic metabolic vulnerability that can be pharmacologically targeted. Moreover, the presence of 1-MNA, the metabolic by-product of NNMT activity, in liquid biopsies from patients with the EGFR mutant NSCLC could serve as a valuable predictive biomarker with the potential to identify which patients are likely to respond to EGFR TKIs, and may inform about strategies to inhibit NNMT pharmacologically, thus, preventing the emergence of resistance to EGFR TKI therapy. Nonetheless, additional studies are imperative to reinforce these results and to determine the definitive clinical relevance of NNMT as both a biomarker and a therapeutic target in this NSCLC population.

4. Methods

4.1. NSCLC cell lines and STR assays

HCC4006, PC9, NCI-H1975 (referred to as H1975), H1648 and A549 NSCLC cells were obtained from the ATCC and maintained as specified. EGFR-mutant cell lines were exposed to increasing concentrations of EGFR TKIs for 6 months as previously reported (24). All the resistant cell lines proliferate normally in the presence of 10 μM EGFR TKIs. Having confirmed resistance, cells were cultured without drugs and their resistance to EGFR TKIs was periodically examined. Cell lines' identities were confirmed by the STR analysis (Extended Data STR assay).

4.2. Patient population

This study was carried out in accordance with the Declaration of Helsinki, including all the amendments, and the participating hospitals required Institutional Research Ethics Board approval (reference number 2020-174-1). All the patients provided their written informed consent before being included in the study and before study procedures, sampling and analyses. Following routine clinical practice, patients' data were retrospectively obtained from their medical records. The mutational status of EGFR (both primary and secondary T790M mutations) was determined by the institutions as part of their diagnostic routine. The patients ($n = 69$) diagnosed with LUAD (stage IV) and who harboured EGFR sensitising mutations (either exon 19 deletion or L858R

mutation) were recruited between January 2012 and February 2021 at the Hospital Clínico and the Hospital General, both in Valencia (Spain). Tumour stage was classified according to the Revisions in the International System for Staging Lung Cancer. To determine 1-MNA levels, 69 pre-TKI and 20 post-TKI serum samples at disease progression, as confirmed by Response Evaluation Criteria in Solid Tumours (RECIST), were used. Of the 69 enrolled patients who progressed on first-generation EGFR TKI therapy, 24 underwent a second biopsy for routine clinical evaluation and had leftover tissues to evaluate NNMT levels by IHC. The second cohort of first-line osimertinib-treated patients diagnosed with LUAD (stage IV) and who harboured EGFR sensitising mutations (either exon 19 deletion or L858R mutation) were recruited between January 2019 and September 2024 at the Hospital General in Valencia (Spain).

4.3. Histology and immunohistochemistry

All the IHC procedures were performed in the Department of Pathology at the Hospital Quirónsalud (Valencia, Spain). The slides from the pre- and post-TKI FFPE biopsies were stained with haematoxylin and eosin (H&E) and tested with the NNMT antibody (Sigma Aldrich HPA059180). The NNMT staining scores were given by board-certified pathologists. They were designated as follows: -, negative expression; +, weak expression; ++, moderate expression; +++, strong expression.

Mouse xenograft tumours were fixed in 4 % formalin overnight at room temperature. The specimen was then embedded in paraffin, from which 5- μm sections were generated. Then paraffin sections were deparaffinised in xylene and were rehydrated in a graded alcohol series and boiled with citrate buffer, pH 6. Sections were incubated with the ki67 primary antibody. For antibody detection, Envision Detection System Peroxidase/DAB (Dako) was used. The procedure was performed on a Dako Autostainer Link 48. Image acquisition and analyses were carried out with a Leica DMD108 microscope.

4.4. Untargeted mass spectrometry metabolomics analysis

LC separation was performed inside a XBridge BEH Amide column (2.1 mm $\times$ 150 mm, 2.5 μm particle size; Waters) using a gradient of solvent A (20 mM ammonium acetate, 20 mM ammonium hydroxide in 95:5 water:acetonitrile, pH 9.45) and solvent B (acetonitrile). The flow rate was 150 $\mu\text{L}/\text{min}$, the column temperature was 25 $^{\circ}\text{C}$, the autosampler temperature was 5 $^{\circ}\text{C}$ and the injection volume was 10 μL . The LC gradient was as follows: 0 min, 90 % B; 2 min, 85 % B; 3 min, 75 % B; 7 min, 75 % B; 8 min, 70 % B; 9 min, 70 % B; 10 min, 50 % B; 12 min, 50 % B; 13 min, 25 % B; 14 min, 25 % B; 16 min, 0 % B; 21 min, 0 % B; 22 min, 90 % B; 25 min, 90 % B. The autosampler temperature was 5 $^{\circ}\text{C}$ and the injection volume was 10 μL . The mass spectrometer was operated in either the positive or negative ion modes to scan from m/z 70 to 700 at 1 Hz at the resolving power of 140,000, AGC target 3E6 and the maximum injection time was 200 ms; from m/z 650 to 1200 at 1 Hz and at a resolving power of 140,000, AGC target 5E5 and the maximum injection time was 500 ms. Data were acquired in the centroid mode. Other MS parameters were as follows: sheath gas flow rate, 30 (arbitrary units); aux gas flow rate, 5 (arbitrary units); sweep gas flow rate, 0 (arbitrary units); spray voltage, 3.4 kV; capillary temperature, 320 $^{\circ}\text{C}$; S-lens RF level, 50. For the data analysis, LC-MS raw data files were converted into the mzXML format using msconvert (3.0.20315, ProteoWizard). Peaks were integrated with El-Maven (v0.0.12.1, Elucidata) using windows of 10 ppm and the retention time as previously determined employing a library of standards.

4.5. Targeted mass spectrometry-based analysis

UPLC separation was performed in an Acquity UPLC system (Waters, UK) equipped with an Acquity UPLC BEH C18 (1.7 μm , 2.1 $\times$ 100 mm; Waters) column. The column and the autosampler temperatures were set

at 40 °C and 4 °C, respectively. The sample injection volume was 4 µL. Eluents consisted in 0.5 % formic acid and 0.3 % heptafluorobutyric acid in water (eluent A) and acetonitrile (eluent B). The flow rate was set at 0.3 mL/min. A 9-min elution gradient was performed as follows: the initial eluent composition was set at 97 % A and 3 % B, which was linearly changed to 90 % A and 10 % B in 2 min. Then the proportion of B was increased to 30 % in the next 3 min, followed by a further increase to 100 % B, which was reached at min 6 and kept for 1.5 min. Finally, the initial conditions were recovered and maintained for 2 min for column conditioning purposes.

The MS analysis was performed by a Waters Xevo TQ-S mass spectrometer (Waters) equipped with an ESI source working in the positive-ion mode multiple reaction monitoring (MRM) mode. A capillary voltage of 2 kV, a source temperature of 120 °C and a desolvation temperature of 380 °C were applied. Desolvation and the cone gas flow were set as 800 L/h and 150 L/h, respectively, and the collision gas was 0.25 mL/min. Transitions, cone voltages and collision energies were automatically tuned for each metabolite with the QuanOptimizer software (Waters). The data station operating software was MassLynx 4.1 (Waters). The MS conditions (including quantification and qualification transitions, cone voltage, collision energy, retention time and the internal standard used for normalisation purposes) for each metabolite and internal standard are detailed in the Supplementary Methods.

Calibration curves were generated by plotting the peak area ratio of the respective compound to the corresponding internal standard *versus* the nominal concentration. The line of best fit was determined by linear-weighted ($1/\times$) least-squares regression. The linearity acceptance criterion for the correlation coefficient was 0.99 or better. Each back-calculated standard concentration should be within ± 15 % deviation from the nominal value, except for the LLOQ, for which the maximum acceptable deviation was ± 20 %. Standard calibration curves, with concentrations falling within the 0.62–20,000 nM range, were prepared by serial half dilutions. Internal standard concentrations were kept constant at all the calibration points at 3000 nM for 3Aminobenzamide and CystineD6, 300 nM for MetD3 and 5MTA, 200 nM for MNAD3 and 100 nM for theanine and PheD5.

4.6. Sample preparation for the LC-MS analysis

Serum samples. Firstly, 100 µL of serum sample were allowed to thaw on ice and then 25 µL of 40 mM N-ethylmaleimide were added to derivatise free thiols. Protein precipitation was performed by adding three volumes of cold methanol. After vortexing, samples were incubated at -20 °C for 30 min. To remove protein precipitates, samples were centrifuged twice at 10,000 g for 10 min at 4 °C, and supernatants were transferred to clean tubes. A 400 µL aliquot was evaporated to dryness and resuspended in 80 µL of eluent A that contained internal standards. Another aliquot was diluted at 1/20 into eluent A that contained internal standards.

Cultured cell samples. For the 6-well plates, media were removed, and cells were washed twice with cold PBS. Cells were scraped using 1000 µL of 8 mM NEM in water. Cells were lysed in three freeze-thaw cycles. Then a 250 µL aliquot was extracted using 750 µL of cold methanol. After vortexing, samples were incubated at -20 °C for 30 min. To remove protein precipitates, samples were centrifuged twice at 10,000 g for 10 min at 4 °C, and supernatants were transferred to clean tubes. An 800 µL aliquot was evaporated to dryness and resuspended in 80 µL of eluent A that contained internal standards. Another aliquot was diluted at 1/20 into eluent A that contained internal standards.

Cell culture media. Firstly, 20 µL of spent media were allowed to thaw on ice and then 5 µL of 40 mM NEM were added. Protein precipitation was performed by adding three volumes of cold methanol. After vortexing, samples were incubated at -20 °C for 30 min. To remove protein precipitates, samples were centrifuged twice at 10,000 g for 10 min at 4 °C, and supernatants were transferred to clean tubes. An aliquot was diluted at 1/20 into eluent A that contained internal standards.

4.7. Western blot analysis

Lysate preparation and Western blotting were performed as previously described [33]. A list of antibodies is listed in the Supplementary Methods.

4.8. NAD^+/NADH levels quantification

The NAD^+/NADH -Glo Assay kit (Promega) was used by taking the manufacturer's advice as a faster method to quantify NAD^+/NADH than UPLC-MS/MS. Briefly, $3.5 \cdot 10^3$ cells were seeded on 96-well plates and left for 24 h to adhere before DAP addition. NAD^+/NADH detection reagent was added after a 24 h drug incubation period. The 1-h post-incubation supernatant was transferred to a white-well plate to evaluate luminescence in an Infinite M200pro (Tecan).

4.9. Cellular viability assays

Live cells were counted using Countess II (Thermo Fisher Scientific) and $5 \cdot 10^3$ cells per well were seeded on 96-well plates to compare cell growth kinetics by running the sulphorhodamine B assay as described by the NCI. For the crystal violet cell viability assay, cells were seeded at low density ($5 \cdot 10^3$ cells per well) on 6-well plates and were left for 24 h to adhere. Then the medium was replaced with vehicle and the appropriate drug (DAP or EGFR TKIs) and was renewed every 3 days until approximately 70 % confluency was reached. The supernatant was replaced with fixing solution and cells were stained with crystal violet 0.4 % (v/v). Plates were then washed under running water, allowed to dry and were scanned for imaging purposes.

4.10. Apoptotic cell death studies with Annexin-V staining

Adherent cells were trypsinised and pooled with the supernatant. Apoptosis was assessed using an Annexin-V-FITC conjugate (ThermoFisher Scientific) and Propidium Iodide (ThermoFisher Scientific) staining according to the manufacturer's instructions. After incubation, samples were placed on ice and run using a BD LSRFortessa. Data were analysed with the FlowJo software. Appropriate unstained and single-stained samples were used for the controls and compensation.

INCell Analyzer 2000 (Ge Healthcare Life Sciences) was used to systematically quantify cell death. Briefly, $5 \cdot 10^3$ cells were seeded in black-wall 96-well plates and left for 24 h to adhere. Appropriate siRNA was added to wells and, 72 h after treatment, cell death was evaluated *in situ* with Annexin-V, propidium iodide and Hoechst 33342 staining. A 20x/0.45, Plan Fluor ELWD objective was used and the INCell Investigator Image Analysis 1000 Workstation software was employed to analyze the acquired images.

4.11. cDNA/shRNA constructs and lentiviral infection

pLX304 was a gift from David Root (Addgene plasmid # 25890; <http://n2t.net/addgene:25890>; RRID: Addgene_25890) (24). The cDNA for NNMT (HsCD00442343) and NAMPT (HsCD00442891) were cloned to the pLX304 vector and obtained from DNASU, the Arizona State University Biodesign Institute.

The shRNA lentiviral vector construct, designed by the RNAi Consortium, was used as previously described [24]. The non-target sequence (5' – GCGCGATAGCGCTAATAATTT – 3') was employed as a control for either CDH1 shRNA (5' – AATGCCATCGTTGTTCACTGG – 3') or shNNMT (5' – ACCCTCGGGATTACC TAGAAA – 3'). With the TransIT-LT1 transfection reagent (Mirus), the 293LT cell line (Cell Biolabs) was transfected with the pLKO.1 constructs and packaging plasmids, pCMV-ΔR8.2dvpr and pCMV-VSV-G. Lentiviral supernatants were collected, and viral supernatant quality was routinely tested with Lenti-X Go Stix (Clontech). Supernatants were applied to the target cells. Following puromycin or blasticidin selection, cell viability assays were

performed 7 days post-infection.

4.12. siRNA transfection

siRNAs (pooled oligos), the control siRNA-A (Santa Cruz Biotechnology sc-37007) and NAMPT siRNA (Santa Cruz Biotechnology sc-45843) were transfected into the target cells using TransIT-siQUEST reagents (Mirus) following the manufacturer's instructions.

4.13. Murine drug treatment studies

All the animal treatment studies were reviewed and approved by the Institutional Animal Care and Use Committee (reference number A1516219851895) of the University of Valencia (Spain). Approximately $2 \cdot 10^6$ total cells were injected subcutaneously into the flanks and centre backs of Nu/Nu mice (Envigo). The size of the engrafted tumours was measured with digital callipers and grown until approximately 100 mm³. Tumours were then randomised, and mice were treated (intraperitoneally) daily for 20 days with either vehicle or daporinad (10 mg/kg). Immunohistological analyses of mouse xenografts were performed as described above. Sections were stained with H&E and ki67.

4.14. Immunofluorescence

Briefly, $0.1 \cdot 10^6$ cells were plated on 12-well plates and left for 24 h to adhere. After this time, cells were fixed in 4 % PFA. Cells were blocked after permeabilization with 0.2 % Triton X-100. Primary antibodies were incubated overnight in a wet chamber and the conjugated secondary antibody was incubated at room temperature for 1h. Nuclei were labelled with Hoechst 33342 (Molecular Probes, Life Technologies). Image acquisition and analyses were performed with the Olympus FV1000 confocal microscope and a 40X objective.

4.15. Statistical analysis

Unless otherwise stated, comparisons of statistical significance were performed using an ANOVA and Tukey's multiple comparison test or the *t*-test whenever applicable. $P < 0.05$ was considered statistically significant.

CRedit authorship contribution statement

I. Pulido: Writing – original draft, Visualization, Validation, Methodology, Funding acquisition, Formal analysis, Data curation. **J.C. García-Cañaveras:** Visualization, Validation, Investigation, Formal analysis, Data curation. **M.L. Rodríguez:** Methodology, Investigation, Formal analysis, Data curation. **J.H. Becker:** Investigation, Formal analysis, Data curation, Conceptualization. **A. López:** Methodology, Investigation, Formal analysis, Data curation. **M. Aupí:** Investigation, Formal analysis, Data curation. **S. Aparisi:** Investigation, Formal analysis, Data curation. **L. Chuliá-Peris:** Methodology, Investigation, Data curation. **E. Tamayo-Torres:** Methodology, Investigation, Data curation. **C. Carreres-Rey:** Methodology, Investigation, Data curation. **J. Alcácer Fernández-Coronado:** Methodology, Data curation. **M. Benet:** Validation, Investigation, Data curation. **S. Montero:** Validation, Methodology, Data curation. **S. Mena:** Validation, Investigation, Data curation. **J. Sandoval:** Validation, Methodology, Data curation. **J. Pereda:** Validation, Investigation, Formal analysis. **J. Alcácer:** Validation, Methodology, Formal analysis. **S. Calabuig-Fariñas:** Validation, Investigation, Data curation. **E. Jantus-Lewintre:** Validation, Investigation, Data curation. **A. Cremades:** Validation, Investigation, Formal analysis. **J.M. Galbis-Caravajal:** Validation, Methodology, Investigation. **P. Martín-Martorell:** Validation, Methodology, Investigation, Formal analysis, Data curation. **A. Insa:** Validation, Methodology, Investigation. **Malek G. Massad:** Validation, Investigation, Formal analysis. **O. Juan-Vidal:** Validation, Methodology, Investigation,

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Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at <https://doi.org/10.1016/j.canlet.2025.218032>.

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