**Kinetic isotope tracing of glycerol and de novo proteogenic amino acids in Human Lung Carcinoma cells using [$^{13}C_6$]glucose**

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**Abstract:**

In vitro studies involving cell lines or primary cells, provide critical insights into their physiology under normal and perturbed conditions like cancer and infection. Given that there are multiple sources of carbon, nitrogen, and other nutrients available in routinely used standard media (such as DMEM, RPMI), it is vital to quantify their contribution to cellular metabolism. 13C based Isotopic tracers of the media components can be used to kinetically track their oxidation by the cell systems such as Human Lung Carcinoma (A549) cells. In this study, a universally labelled glucose tracer ([13C6]glucose) was used to quantify its metabolic contribution that provided further insights into the central carbon metabolism of A549 cells. Gas chromatography and mass spectrometry (GC-MS) based mass isotopomer analysis (average 13C) of methanolic extracts (glycerol: 5.46±3.53 % and lactate: 74.4±2.65 %), amino acids derived from acid hydrolysates of protein (Serine: 4.51±0.21 %, Glycine: 2.44±0.31 %, Alanine: 24.56±0.59 %, Glutamate: 8.81±0.85 %, Proline: 6.96±0.53 % and Aspartate: 10.72±0.95 %) and the metabolites of the culture filtrate (glycerol: 43.14±1.45 % and lactate: 81.67±0.91 %), allowed to capture the relative contribution of glucose. We observed the Warburg effect and a significant amount of lactate contributed from glucose, was released to the media. 13C glycerol of glucogenic origin was kinetically released to the culture filtrate and might be playing a critical role in metabolic reprogramming of A549 cells. Part of the protein biomass contributed from amino acids were of glucogenic origin. Besides, the workflow adopted for 13C analysis and derived average 13C of each metabolite provided a standard methodology that could be useful in defining the metabolic phenotypes of cells in normal and perturbed conditions. Understanding precisely the altered cellular metabolism to meet the biomass demand under a range of physiological conditions, kinetically, may identify pathways for targeted and effective therapeutic interventions.

Nutrient availability is vital in maintaining a local immune environment in tumour or infected tissues for effective host immune response. Tumour cells and pathogens compete with host immune cells for nutrients as a part of their immune evasion strategies. Resulting nutrition imbalance leads to impaired immune function and compromised outcome in immuno-therapies (Kurmi and Haigis, 2020). Upon infection or chemical signals, cancer cells and activated immune cells adopt metabolic reprogramming to acquire biomass for extensive proliferation by coordinating systemic oxidation of carbon, nitrogen and other nutrients (Kelly and Pearce, 2020; Kurmi and Haigis, 2020). Understanding the altered cellular metabolism to meet the biomass demand, kinetically, will allow in identifying pathways for targeted, effective therapeutic interventions.

The duplication rate of cancerous and immune cells varies widely (17.0-79.5 hours) based on the origin and types (Jain et al., 2012). Moreover, proteins contribute to ~17-26% of the biomass, and its synthesis rate influences the cell duplication period. However, glucose or glutamine and its intermediates contribute <10% of cell mass (Hosios et al., 2016). Like nucleotides, after synthesis of sufficient protein pool, the cells become ready for duplication. Protein synthesis in immortalised and primary cells in culture depends on amino acids present in culture media and de-novo amino acid synthesis. In non-dividing mammalian cells, the ratio of externally supplied versus endogenous amino acids to de novo protein synthesis was about 17:1 (Cambridge et al., 2011).

While, 13C tracers analysis have been a promising tool to define cellular and organ-specific central metabolism (glycolysis, tri-carboxylic acid, pentose phosphate pathways), additional studies to quantify the contribution of nutrients to de novo amino acid synthesis directed towards protein synthesis are essential (Chen et al., 2019; Liu et al., 2020; Hui et al., 2020). Elucidation of the source of amino acids and its incorporation in new protein synthesis will be informative to identify essential steps in cell metabolism to develop interventions for different conditions like cancer and infectious diseases.

In this report, type-II alveolar epithelial (A549) cells were grown in the presence of [13C6]glucose (99.6 %, 17.5 mM). Metabolites isolated from the cell culture filtrate, methanolic cell extracts and acid hydrolysate of protein fractions were analysed using gas chromatography and mass spectrometry (GC-MS) after derivatisation (Figure 1A). A549 cells grew linearly and reached a pseudo isotopic steady-state metabolism by 48 hours, ideal for 13C based metabolic flux analysis (13C-MFA) (Figure 1B).

Efflux of labelled 3C units (glycerol: 43.14±1.45 % and lactate: 81.67±0.91 %) in normoxic A549 cells were observed (Figure 1C, 1D). A marked glycerogenic flow provided glycerol (5.46±3.53 %) as a final 3C export substrate together with lactate (74.4±2.65 %) (Figure 1E). Both lactate and glycerol production is assumed to contribute significantly to convert glucose to 3C units, thus lowering the adverse effects of excess glucose. Lactate and glycerol also act as signalling molecules and provide the metabolic phenotype of cancer and immune cells by regulating multiple gene expressions (Hu et al., 2017).

To an extent, there is de novo synthesis of Serine (4.51±0.21 %) and Glycine (2.44±0.31 %) with a significant contribution from the media, which might support the 1-Carbon metabolism needed for cell proliferation (Figure 1F). Pyruvate from C13 glucose contributes to labelled lactate (74.4±2.65 %) and alanine (24.56±0.59 %). Subsequent, mitochondria metabolism lead to the synthesis of labelled Glutamate (8.81±0.85 %), Proline (6.96±0.53 %) and Aspartate (10.72±0.95 %)(Figure 1F). These amino acids are utilised in metabolic pathways and also get incorporated into the synthesis of new proteins. Essential amino acids (threonine, lysine, valine, tyrosine, phenylalanine) in the protein hydrolysate were unlabelled and contributed from the culture media. GC-MS based mass isotopomer analysis of the metabolites from the culture filtrate, methanolic fraction, amino acids derived from acid hydrolysates of protein allowed us to capture i) the relative contribution of glucose vs other carbon sources on metabolism, ii) the rate of C13 incorporation in amino acids shedding light on potential pathway activities, by employing an iii) optimised analytical conditions concerning ideal derivatising reagents, concentrations of extracts and amount of substrate.

One of the limitations of this study is the lack of monitoring gluconeogenesis from labelled C3 metabolites that might have contributed to a fraction of the C13 glucose molecules, which needs additional follow up experiment.

Overall, the study quantified the contribution of amino acids originated from glucose metabolism and used in protein biosynthesis of A549 cells kinetically supporting the cancerous metabolic phenotype. This study highlights the feasibility of adopting measurements from protein-derived amino acids, which are stable and straightforward to analyse using GC-MS, to undertake steady-state 13C MFA using various 13C tracers to define the metabolic phenotypes of A549 cells better.

In perturbed conditions like cancer and infectious diseases, metabolic hyperactivity with increased protein catabolism and negative nitrogen balance is commonly observed. So,

understanding the dynamic changes in amino acids and primarily the pathways that contribute to their synthesis or their precursor molecules could be useful as a nutritional supplement for disease management and improving its' outcomes. Pathogens, like *Mycobacterium leprae*, uses host glucose pool to synthesize the majority of amino acids, and these related pathways are useful to target for anti-leprosy drug development (Borah et al., 2019). Exogenous alanine in T cells is not used in metabolism but utilised for protein synthesis and its normal activation (Ron-Harel et al., 2019). So, targeting the evolutionarily conserved phenomenon of protein synthesis by modulating amino acid metabolism could influence immune responses in cancer, infection and autoimmunity.

In summary, the workflow adopted to monitor the fate of 13C glucose analysis and deriving average 13C of labelled metabolite provides an optimised tool that could be useful in metabolic phenotypes of cells exposed to perturbed conditions, including viral and bacterial infection. It may also be helpful to understand the anterograde and retrograde signalling in mitochondria that provides cues for uncontrolled cell proliferation.

## SUPPLEMENTAL INFORMATION

Supplemental information includes the methodology adopted in this study. The datasets could be previewed using this DOI URL (http://dx.doi.org/10.17632/gc3ss3s4jx.1)

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## AUTHOR CONTRIBUTIONS

S.A., S.M. and R.K.N. designed the study; S.A. carried out all the experiments and data acquisition; S.A., J.T., M.S., S.K.M. analyzed the data; S.A., J.T., M.S., S.K.M. and R.K.N. contributed in data interpretation and R.K.N. and S.K.M. wrote the first version of the

manuscript, which was read and approved by all co-authors. R.K.N. is the guarantor and lead author of this work.

## DECLARATION OF INTERESTS

The authors declare no competing interests.

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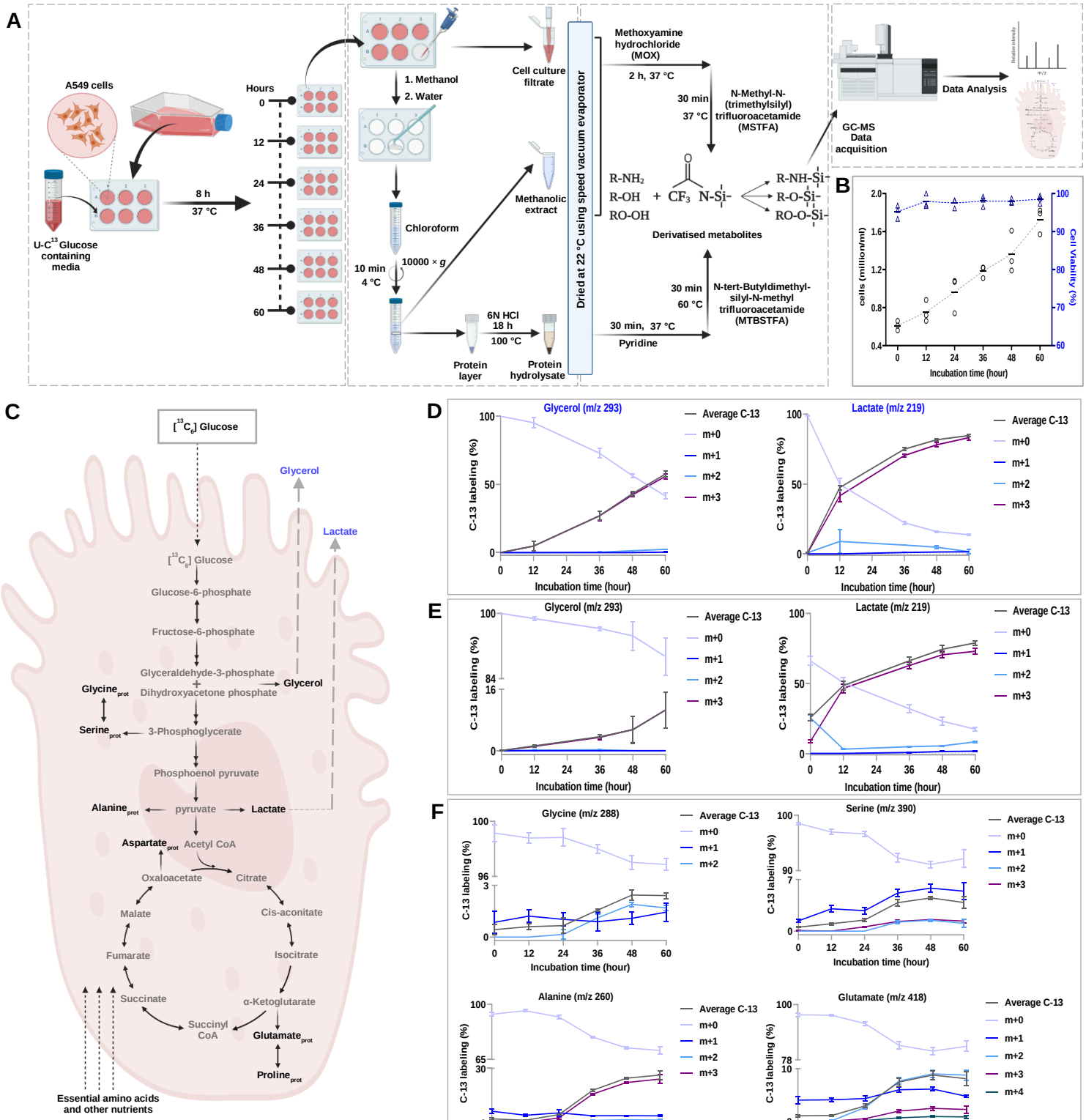


**Figure 1. Kinetic isotopic labelling of A549 cell metabolites with [U-13C] glucose.** (A) Workflow design of 13C labelling experiment. (B) Growth kinetics and viability of A549 cells cultured in vitro (C) Incorporation of 13C from [$^{13}C_6$] glucose through glycolysis and Krebs cycle. (D, E and F) Average 13C and individual mass isotopomer distributions (MIDs) in % of metabolites from cell culture filtrate, methanolic extract and acid hydrolysate protein (Amino acid$_{pro}$) samples. The MIDs were corrected for natural isotope abundances. Data represents n = 3 replicates ± SD.