**GAI declaration**

I did use generative AI in the production of this report, specifically, I used ChatGPT 3.5 to aid in the learning of how to implement different normalisation techniques like standard normal variate normalisation (SN) and total area normalisation (TAN).

Metabolomic analysis of hypoxic neuroblastoma using a CAM model and NMR spectroscopy

(i) Study Rationale

Metabolomics is a powerful analytical approach for investigating dynamic changes in small-molecule metabolites within biofluids to help understand the biochemical states of a phenotype (Akyol et al., 2023). These changes can reveal perturbations to the genome, transcriptome, and proteome (Guo et al., 2019). Unlike other omics that measure stable molecular entities (DNA and proteins), metabolomics is highly sensitive to environmental changes and experimental design, with minor alterations to sample preparation, handling, or analysis significantly affecting the observed metabolic profile (Emwas et al., 2019). This highlights the critical importance of robust experimental design (Kohler et al., 2016).

Metabolic reprogramming has a pivotal role in the progression of neuroblastoma, an aggressive paediatric cancer that rewires cellular metabolism to increase fuel and growth production (Bansal, Gupta and Ding, 2022). By exposing the neuroblastoma cells to low oxygen (1% O2), hypoxic preconditioning can affect tumour oxygenation and the metabolic signature in vivo. As hypoxia is known to induce cellular pathways that enhance proliferation and survival of cancer cells, a chick embryo chorioallantoic membrane (CAM) model was used to assess the resulting metabolic profile of tumour cells exposed to 1% and 21% oxygen compared to normal control cells exposed to 1% and 21% oxygen (Patiño-Morales et al., 2023). This study aims to reveal metabolic changes in tumour cells under hypoxic conditions, potentially identifying biomarkers or therapeutic targets.

(ii) Study Selection and Experimental Design

Hypoxic preconditioning on tumour oxygenation was measured using fibre-optic oxygen sensors (Presens.de, 2015). Samples consisted of tumours formed by SK-N-AS neuroblastoma cells that were preconditioned in hypoxia or normoxia and implanted into the CAM of a chick embryo. This provides an opportunity to assess the metabolic impact of hypoxia at a cellular level, capturing cellular metabolism and the tumour microenvironment (Ribatti, 2017).

Controlling oxygen is essential for reproducibility (Rogers et al., 2025). This was controlled by culturing the neuroblastoma cells under strictly controlled oxygen conditions, with 1% O2 for hypoxia, and 21% O2 for normoxia. Culture conditions were controlled by using the same cell line (SK-N-AS), passage number, culture medium and incubation periods for all experimental replicates, minimising variability due to external factors. Finally, the metabolite extraction process was controlled by rapidly washing cells with ice-cold PBS, harvesting and immediately freezing with liquid nitrogen to minimise post-harvest metabolic activity to ensure the features reflect their true cellular state (Al-Mutawa et al., 2018). The study’s overall workflow is summarised in Figure 1.

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**Figure 1. Experimental design and data processing to investigate the metabolomic effects of hypoxia on neuroblastoma cells.** Based on research from Al-Mutawa et al., (2018). Created in Biorender.

Additionally, the CAM model creates a cost- and time-effective in vivo system for analysis of direct metabolic responses to hypoxia at a cellular level and in a living organism (Al-Mutawa et al., 2018). By combining in vitro (cell culture) and in vivo (CAM model) approaches, this study can observe how hypoxia directly affects cell metabolism (in vitro) and assess metabolic changes that influence tumour growth in a living system (in vivo). However, this model doesn’t fully replicate the complexity of human tumour biology, and the in vitro cell culture may lack interactions within humans.

Nuclear magnetic resonance (NMR) spectroscopy was used for metabolite profiling. This is non-destructive and allows multiple-dimensional experiments on a single sample (Marion, 2013; Letertre, Giraudeau and de Tullio, 2021). NMR has broad coverage, detecting a wide range of metabolites in a single run, and provides highly reproducible quantitative data with TSP internal standard for chemical calibration. This is beneficial when comparing metabolic profiles across various conditions (Markley et al., 2017). However, NMR is typically less sensitive than mass spectrometry (MS), needing higher concentrations to detect low-abundance metabolites (Gowda and Raftery, 2022). Also, lower resolution means that overlapping chemical shifts are challenging to resolve, making it hard to distinguish similar compounds. Furthermore, NMR spectrometers, like 700 MHz used in this study, are expensive to acquire and maintain (Larive, Barding and Dinges, 2014).

Alternatively, liquid chromatography-mass spectrometry (LC-MS) could have been used for this metabolomic analysis. LC-MS has higher sensitivity, broader coverage, flexible sample preparation techniques, and diverse ionisation methods that can detect polar and non-polar metabolites (Stanislava Rakusanova and Cajka, 2024). However, this requires extensive sample preparation and can be less reproducible due, adding variability between runs. Moreover, ion suppression can occur due to the sample matrix, affecting the metabolite detection sensitivity (Mei et al., 2002).

Despite the advantages of LC-MS, NMR was chosen due to its superior reproducibility, non-destructive workflow and ability to compare relative metabolite concentrations across samples.

(iii) Data Preparation

The raw NMR spectra were initially normalised to the internal standard trimethylsilyl propionate (TSP), ensuring chemical shift alignment across samples to improve the reliability of peak identification and quantification (Gowda and Raftery, 2022). However, despite TSP normalisation being essential for peak consistency and spectral alignment, it doesn’t correct for differences in dilution effects, sample concentration, or complete signal intensity among samples (Grasso et al., 2022). Therefore, further normalisation techniques were required to confirm that the observed metabolic variations reflected true biological differences.

Median normalisation (MN) adjusts the intensity of each sample by dividing each value by the median intensity of the sample, ensuring comparable data without changing the variance structure between metabolites. Unlike methods such as PQN or TAN, which adjust values relative to a reference, causing proportional intensity scaling, MN keeps the relative distribution of metabolite intensities within each sample. This preserves the natural variability of each metabolite, which is crucial for univariate analysis in detecting statistically significant differences between groups, e.g. normoxia vs. hypoxia (Jauhiainen et al., 2014).

In contrast, PQN accounts for dilution effects and sample concentration differences by scaling samples based on the reference spectrum (control). This method divides each intensity by a sample-specific quotient, the median ratio of all sample intensities to the reference, correcting systematic dilution effects and does not assume a balanced total signal across samples (Dieterle et al., 2006). However, this requires a good reference sample and may not be ideal if many metabolites vary dramatically between conditions. Given that the study hypothesises that metabolism will differ significantly between hypoxic and normoxic cells, PQN may not be suitable. MN with Glog transformation was chosen as it had the lowest RSD (Figure 2) and reduced dimensionality across features (Figure 3).

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**Figure 2. The effect of normalisation techniques on the RSD with metabolomic data of neuroblastoma across hypoxic and normoxic conditions.** Boxplots comparing the relative standard deviation (RSD) of metabolite intensities across the raw spectra and different normalisation methods: median, median with Glog, probabilistic quotient normalisation (PQN), PQN with Glog, standard normal variate normalisation (SN), SN with Glog, total area normalisation (TAN), TAN with Glog.

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**Figure 3. Scatter graphs of normalisation techniques with metabolomic data of neuroblastoma across hypoxic and normoxic conditions.** The raw data is compared to median normalisation, Probabilistic Quotient Normalisation (PQN), and median normalisation + GLOG transformation.

(iv) Univariate Data Analysis

The dataset consisted of 354 spectra bins and 72 unpaired samples with no missing values. The Shapiro-Wilk test (Shapiro and Francia, 1972) was applied to each metabolite for normality interpretation. This revealed that the majority (81.92%) of metabolites were non-normal (p ≤ 0.05; Table 1).

**Table 1. Metabolite distribution in metabolomic data of neuroblastoma across hypoxic and normoxic conditions.**

|  |  |  |
| --- | --- | --- |
| Distribution Category | Count | Percentage (%) |
| Normal | 64 | 18.08 |
| Non-normal | 290 | 81.92 |

Given the high proportion of non-normally distributed metabolites, a non-parametric analysis of variance (ANOVA) test was chosen to determine the presence of statistically significant differences in the means of a continuous variable across more than two groups. The assumptions made were that the data was normalised and transformed correctly, with a homogeneity of variances. The Benjamini-Hochberg (FDR) method was also used to adjust p-values for multiple testing correction and false discovery rate control. There were 341 significant DAMs with FDR-adjusted p-values less than 0.05 (Figure 4). A screen shot of a graph

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**Figure 4. Non-parametric ANOVA Scatter plot of metabolite features found in neuroblastoma cells across different oxygen conditions**. The plot presents the results of a one-way ANOVA analysis integrated in MetaboAnalyst. The x-axis indexes the metabolites, and the y-axis represents the -log(p-value) of their abundance differences across experimental conditions (control, normal hypoxic, tumour normoxic, tumour hypoxic). Created with MetaboAnalyst (Xia et al., 2009).

Glutamate reoccured multiple times and was the most significant DAM (p-value=5.1138e-14). The distribution of glutamate, glucose, and choline display these metabolites at generally higher levels in hypoxic tumour conditions compared to normoxic tumour conditions, with clear seperation between the cells and tumours (Figure 5). However, the post-hoc Tukey’s HSD could not confirm the specifics of group seperation within this non-parametric framework (Table 2). This highlights a limitation of MetaboAnalyst (Xia et al., 2009), as it lacks non-parametric post-hoc tests. Methods such as Dunn’s test should be integrated to maintain non-parametric integrity while allowing pairwise group comparison.

**Table 2. Results of non-parametric ANOVA (Kruskal-Wallis) of metabolite levels between groups in MetaboAnalyst.** Created with MetaboAnalyst (Xia et al., 2009).

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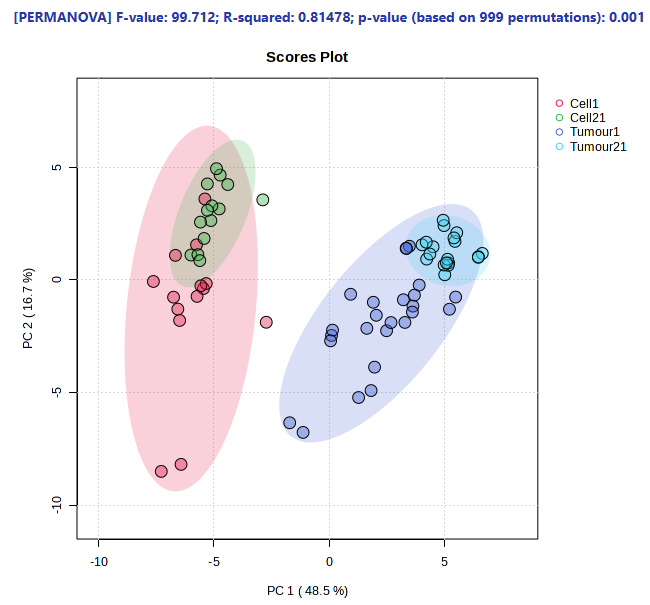
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**Figure 5. Violin plots of significant metabolites in neuroblastoma across hypoxic and normoxic conditions.** The violin plots display the distribution of glutamate, glucose, and choline across cell and tumour samples in hypoxic (Cell1, Tumour1) and normoxic (Cell21, Tumour21) conditions. Created with MetaboAnalyst (Xia et al., 2009). Formatted with BioRender.

(v) Multivariate Data Analysis

Principal coordinate analysis (PCA) was performed to explore the clustering and variance of metabolite abundance across the four experimental conditions. PCA, an unsupervised multivariate analysis technique, reduces data dimensionality while retaining the data’s most important variance. The PCA plot (Figure 6) illustrates the distribution of samples across the first two principal components. Hypoxic conditions displayed more variation compared with normoxic conditions, which clustered tightly near the hypoxic samples. Cells clustered separately from tumours with the most separation across PC1 (48.4%). A PERMANOVA test was performed to validate the observed separation between groups, yielding an F value of 46.864, R-squared of 0.674, and a p-value of 0.001 (based on 999 permutations).



**Figure 6. PCA plot of normal cells and cancer cells across hypoxic and normoxic conditions.** The principle coordinate analysis (PCA) plot shows the separation of groups across component 1 (48.5%) and component 2 (16.7%). Created with MetaboAnalyst (Xia et al., 2009).

PLSDA

**A graph with colored circles and numbers

AI-generated content may be incorrect.**The supervised multivariate method, partial least squares discriminant analysis (PLS-DA), maximises the separation between predefined groups, identifying metabolites most responsible for intergroup separation. Although distinct from the unsupervised PCA, the PLS-DA scores plot (Figure 7) displays consistent clustering of the four groups, but with greater separation between hypoxic conditions across component 2 (12.6%). Five-fold cross-validation suggests that five components are the optimal, with 100% accuracy, explaining 97.8% of total variance (R2), and excellent predictive performance (Q2=0.9521; Table 3; Figure 8). The driving forces of separation were visualised as a biplot (Figure 9), revealing that glutamate and glucose are most strongly associated with tumour samples, particularly under hypoxic conditions.

**Figure 7. PLSDA plot of normal cells and cancer cells across hypoxic and normoxic conditions.** Partial least squares discriminatory analysis (PLS-DA) score plot shows the separation of groups across component 1 (48.5%) and component 2 (12.6%). The ellipses represent 95% confidence intervals. Created with MetaboAnalyst (Xia et al., 2009).

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**Figure 8. PLS-DA cross-validation results measured by Q2.** The performance of partial least squares discriminatory analysis (PLS-DA) shows explained variance (R2), predictive ability (Q2), and accuracy across different model components. The optimal model is marked by a red star. Created with MetaboAnalyst (Xia et al., 2009).

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AI-generated content may be incorrect.**Table 3. PLS-DA cross-validation results details.** Created with MetaboAnalyst (Xia et al., 2009).

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**Figure 9. Biplot normal cells and cancer cells across hypoxic and normoxic conditions.** Partial least squares discriminatory analysis (PLS-DA) score plot shows the separation of groups across component 1 (48.5%) and component 2 (12.6%). Key metabolites that drive separation are labelled with arrows orientating the separation. The ellipses represent 95% confidence intervals. Created with MetaboAnalyst (Xia et al., 2009).

(vi) Metabolite Contextualisation

Variable importance in projection (VIP) scores identified the metabolites most responsible for group separation, with scores above 1.0 considered significant (Figure 10).

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**Figure 10. VIP plot of normal cells and cancer cells across hypoxic and normoxic conditions.** Variance importance in projection (VIP) scores from PLS-DA are on the x-axis and rank metabolites based on their contribution to group separation. The heatmap on the right depicts the metabolite abundances across conditions, with red as high and blue as low. Created with MetaboAnalyst (Xia et al., 2009).

Glutamate, glucose, and choline were selected for pathway analysis (Figure 11) due to high VIP scores, statistical significance, and higher levels in hypoxic tumour samples (Figure 10). Neomycin, kanamycin and gentamicin biosynthesis were most significant (p = 0.0040268). Via HIF1α upregulation, gentamicin exposure is known to induce a hypoxia-like state in tumour cells (Elliott and Jiang, 2019). Nitrogen metabolism was associated with glutamate, reflecting evidence of an increased demand for nitrogen in tumours (Pavlova and Thompson, 2016). However, these pathways had an impact value of 0.0. This low impact was common across most mapped pathways (Table 4).

In contrast, starch and sucrose metabolism was the most impacted pathway due to glucose presence (impact=0.4207). This mirrors the Warburg effect, where tumour cells have altered glucose metabolism (Pavlova and Thompson, 2016). Arginine biosynthesis was also associated with these metabolites, with some impact (0.07483). This is a precursor to nitric oxide, which may influence cellular hypoxia responses. Arginine is essential for cellular growth and can become limited in rapid growth phases, like malignancy (Albaugh, Pinzon-Guzman and Barbul, 2017).

The pathways are all limited by the number of metabolites present, with a maximum of 1 metabolite present per pathway.

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**Figure 11. Scatter plot of glutamate, glucose, and choline pathway analysis in hypoxic tumour cells.** The x-axis is the pathway impact and the y-axis represents -log10(p) values, indicating significance. Point size and colour reflect the impact and significance. Created with MetaboAnalyst (Xia et al., 2009).

**Table 4. Pathway enrichment scores of glucoses, glutamate and choline.** Created with MetaboAnalyst (Xia et al., 2009).

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(vii) Summary and Conclusions

This study explored the metabolic impact of hypoxia on neuroblastoma cells and tumours using a chick embryo CAM model. PCA and PLS-DA highlighted key discriminatory metabolites between conditions, including glucose, 3-hydroxybutyrate, glutamate, and betaine/myo-inositol. Pathway analysis revealed alterations in butanoate, nitrogen, galactose, starch and sucrose metabolism, reflecting a trend of hypoxia-induced metabolic reprogramming to deal with rapid growth and limited resources. However, the pathways were limited by a limited number of metabolites per pathway and low impact scores, affecting the robustness of these findings.

Although NMR provides reproducible data and is non-destructive, low-abundance metabolites may have been missed, compared to MS, which is more sensitive and detects a broader range of metabolites. This may lead to an underestimation of the metabolic diversity, particularly for low-abundance biologically important metabolites.

Additionally, while the CAM model provides an efficient in vivo system, it does not fully replicate the complexity of human tumour biology, limiting the application of findings.

Metabolomics helps define metabolic changes linked to genetic differences, environmental influences, disease, or drug influences (Dunn et al., 2011).

Changes to the low-molecular-weight metabolites in a sample can indicate early biological changes, as major changes can be seen after minor stimuli, due to perturbations in metabolic pathways (Dunn et al., 2011). Metabolomics provides insights into cellular metabolism, compared to proteomics which measures protein expression and post-translational modifications. This makes metabolomics more sensitive to environmental changes, such as hypoxia. However, this also makes metabolomics vulnerable to technical variability, increasing the demand for careful experimental design, normalising and processing. In contrast, proteomics is generally more robust to experimental validation. Future studies within the field of multi-omics would be valuable, integrating metabolomics, proteomics and transcriptomics to provide a comprehensive overview of active pathways. This omics can be integrated in bioinformatic tools like PathIntegrate (Wieder et al., 2024).

Future studies could also utilise a targeted metabolomics approach using liquid chromatography-mass spectrometry (LC-MS), offering greater sensitivity and detection capabilities.

Appendix:

Normalisation code available: <https://github.com/ricefarmer2002/Normalisation-method-comparisons/tree/main>

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Word count: 2018

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