

Metabolic profiling studies of tumour cell phenotypes

**A thesis submitted by
Sophia Kelaini**

**For the degree of Doctor of Philosophy
of Imperial College. London**

**Department of Surgery and Cancer
Faculty of Medicine
Imperial College, London**

2010

Declaration of originality

All work presented in this thesis is the author's own, while everything else that is not is appropriately referenced.

Abstract

Ovarian cancer is a devastating disease affecting millions of women worldwide. Initially successful chemotherapy using platinum-based drugs is often followed by a relapse after which platinum therapy is usually ineffective and prognosis poor. The mechanism by which platinum resistance develops and its reversal to a more sensitive phenotype are of major interest in modern medical oncology, especially for ovarian cancer which has overall low survival rates. In this study, different types of *in vivo*-derived and *in vitro* cell models of platinum resistance were examined within a metabolic context using ^1H NMR spectroscopy and bioinformatics-based approaches to test the hypothesis that a metabolic signature of a platinum resistance phenotype could be defined. The *in vitro*-derived cell models of platinum resistance focused on the study of the *Mlh1* gene, whose loss of expression has been shown to play a role in the development of resistance to platinum drugs and mitochondrial dysfunction. A comparative metabolic analysis of a transient *Mlh1* expression cell model (HEK-293T) involving kidney cell lines of common genetic background and the ovarian cancer cell lines A2780 and its platinum resistant sub-line CP70 of well-established *Mlh1* status was conducted. This resulted in the definition of a metabolic signature associated with *Mlh1* expression, which included metabolites such as glutathione, alanine, *myo*-inositol and phosphocholine. It also achieved to associate the loss of *MLh1* to mitochondria. The *in vivo*-imitating cell model focused on the normoxic & hypoxic baseline metabolic profiling of isogenic ovarian cancer cells that were clinically sensitive (PEA1 and PEO1) or resistant (PEA2 and PEO4) to platinum. The ^1H NMR-generated results showed oxygen level-related changes in glucose, lactate, pyruvate, acetate, and choline-related metabolites. Overall, this study managed to contribute towards the understanding of platinum resistance in ovarian cancer through a metabolic spectrum using a variety of analytical and methodological tools.

Acknowledgments

I would like to thank my supervisors, Dr Hector Keun and Professor Nigel Gooderham, for all their help and support throughout this PhD. I wouldn't have made it this far without your support and guidance. Thank you so much!

I would also like to thank Dr. Euan Stronach at the Ovarian Cancer Centre at Hammersmith Hospital for the provision of the isogenic ovarian cancer cell lines for use in my studies. I would also like to acknowledge Michelle Chen for helping me with the preparations.

Special thanks to Bob Brown and Hani Gabra of OCA and Vincent O'Brien for providing the *in vitro* cellular model samples in this research.

I would like to thank all the people who helped with the project over the years including everyone in the Department of Surgery and Cancer at Imperial College including Professor Jeremy Nicholson, Professor Elaine Holmes, Dr Jasmine K. Sidhu, Dr Toby Athersuch, Dr Olaf Beckonert, James E Ellis, Dr. Jake Pearce, Dr. Orla Teahan, Dr Tsz Tsang, for all their expert advice and for their friendship over the years, as well as Nic Pui Chan, Claire Merrifield, Nor Aini Saidin, Nurul Huda, Dr Volker Behrends, Alexandra Backshall, Corrinne Seal for their help and friendship. I'd also like to thank Nahid Ashby for all the help she has provided in the department over the years. Special thanks to Dr Rachel Cavill for her immensely helpful contribution in the bioinformatics-based analysis.

Last but not least, I would like to thank my very best friends Natasha Strintzou and Andriana Margariti. You've been both so wonderful! Thank you so much for your friendship!

Thank you all for encouraging me and supporting me in everything I do.

Dedications

I would like to dedicate this thesis to my family, mom, and in the memories of my dad, my grandmothers Sophia and Olympia, as well as my aunts Areti and Katina. I miss you all.

Contents

Declaration of originality.....	2
Abstract.....	3
Acknowledgements.....	4
Dedications.....	5
Contents.....	6
List of figures.....	15
List of tables.....	22
List of abbreviations.....	24
Contributions.....	29

1.0 Chapter 1: Introduction

Ovarian cancer and the development of chemotherapeutic resistance

1.1 Cancer & ovarian cancer.....	31
1.1.1 What is ovarian cancer?.....	31
Types of ovarian cancer	
Symptoms and signs	
Staging	
Grading	
Statistics	

	Risk & prevention factors	
	Prognosis	
	Diagnosis & screening	
1.1.2	Techniques in the diagnosis and examination of cancer.....	34
1.1.3	Treatment.....	36
1.1.4	Chemotherapy.....	36
1.1.4.1	Chemotherapeutic agents in ovarian cancer.....	36
1.1.4.1.1	Cisplatin.....	37
	Structure of cisplatin	
	Mechanism of cisplatin action & apoptosis	
1.1.5	Development of resistance to platinum drugs.....	40
1.1.5.1	Mechanisms of platinum resistance.....	40
	Reduced intracellular drug accumulation	
	Increased drug detoxification and inactivation	
	Enhanced DNA repair	
	Evasion of apoptosis	
1.1.6	Hypoxia in cancer and drug resistance.....	47
1.1.7	The role of mitochondria in platinum resistant cells.....	50
	Mitochondrial pathway of apoptosis	
	Mitochondria are important in maintaining normal function and homeostasis	
	Impaired mitochondrial function in cancer	
	Targeting mitochondria as a cancer treatment	
1.2	Metabolism of cancer & the Warburg effect.....	53

1.3	Metabonomics and metabolic footprints.....	56
1.4	Tools and techniques in metabolic analysis.....	57
1.5	The need for metabolic biomarkers.....	58
1.6	NMR-detectable metabolites.....	58
1.7	General aims of this study.....	60

2.0 Chapter 2: Methods

2.1	Molecular biology methods.....	62
2.1.1	Cell culture.....	62
	i) Isogenic ovarian cancer cell lines	
	- Establishment of the isogenic ovarian cancer cell lines	
	ii) Ovarian and kidney cancer cells used in the <i>MLh1</i> study	
2.1.1.2	Cell harvesting.....	64
	Harvesting and passaging of cells using trypsinisation method	
	Harvesting cells by direct methanol extraction method	
2.1.1.3	Cell storage.....	64
2.1.1.4	Cell counting.....	65
	(i) Cell counts using an automatic cell counter	
	(ii) Cell counts using a haemocytometer	
2.1.1.5	Drug treatments and cell proliferation assays.....	65
	(i) alamarBlue® assay	
	(ii) Sulforhodamine B-based (SRB) assay	

2.2 NMR Spectroscopy.....	67
2.2.1 Basic theory.....	67
2.2.1.1 The phenomenon of nuclear magnetic resonance.....	67
2.2.1.2 NMR spectroscopy.....	68
¹ H NMR spectroscopy	
NMR spectrometers	
Nuclear spin and resonance	
The vector model of NMR	
Chemical shifts and couplings	
Free induction decay (FID) & Fourier transformation (FT)	
Pulse sequences	
- Water presaturation	
- NOESY presaturation pulse: D-90°-t ₁ -90°-t _m -90°-acquire	
- Carr-Purcell-Meiboom-Gill (CPMG) pulse: D-90°-t ₁ -90°-t _m -90°-acquire	
2.3 Sample preparation.....	77
2.3.1 NMR sample generation.....	77
i) Preparation of conditioned media from cell culture experiments for ¹ H NMR	
ii) Preparation of cell extracts for ¹ H NMR	
2.4 NMR parameters.....	78
2.5 NMR Spectral data processing.....	79
2.6 Structural assignments.....	80
2.7 Data analysis.....	80

2.7.1 Chemometrics and Pattern Recognition for Statistical Analysis.....	80
2.7.2 Pattern recognition theory.....	81
Principal component analysis	
Data reduction ('binning' or "bucketing") for PCA	
Statistical Total Correlation Spectroscopy (STOCSY)	
2.8 Bioinformatics and statistical tests.....	83
Student's t-test	
Bioinformatics (ORA and NEST-based approach)	
 3.0 Chapter 3: Metabolic examination of platinum resistance in cancer cells using in vitro cell models of platinum resistance.	
 3.1 Introduction & Aims.....	85
3.2 Methods.....	87
3.3 Results.....	90
3.3.1 Metabolic effects of <i>Mlh1</i> expression in HEK-293T cells.....	90
3.3.2 Metabolic effects of <i>MLh1</i> expression in ovarian cells of Platinum Resistance Model.....	101
3.3.3 Model Comparison & Metabolic Signature of <i>Mlh1</i>	110
3.3.3.1 Metabolic ratios.....	113
3.4 Discussion.....	114
3.4.1 Loss of <i>Mlh1</i> and its association with platinum resistance.....	114

3.4.2 In Vitro Metabolic Signature of <i>Mlh1</i> Expression.....	114
---	-----

Glutathione (GSH)

Alanine, Lactate and Branched Chain Amino Acids

Choline-related metabolites

Myo-inositol

3.4.3 Concluding remarks.....	117
-------------------------------	-----

Chapter 4: Over-representation analysis of pathways significant with genes and metabolites selected by correlation to *Mlh1*

4.1 Introduction.....	120
-----------------------	-----

4.2 Methods.....	123
------------------	-----

4.3 Results.....	127
------------------	-----

4.3 Discussion.....	132
---------------------	-----

4.3.1. <i>Mlh1</i> -correlated genes and metabolites.....	132
---	-----

4.3.2 Correlation with previous findings.....	133
---	-----

4.3.3 Concluding Remarks.....	133
-------------------------------	-----

5.0 Chapter 5: Metabolic examination of platinum resistance in cancer cells using in vivo cell models of platinum resistance.

5.1	Introduction.....	136
	5.1.1 Objectives.....	137
5.2	Materials & Methods.....	138
	5.2.1 Cell work.....	138
	5.2.2 Data analysis.....	138
	5.2.3 Visual inspection, metabolic assignments and peak integration.....	139
5.3	Results.....	143
	5.3.1 Testing cisplatin cytotoxicity in hypoxic & normoxic isogenic ovarian cancer cells.....	143
	5.3.2 NMR-based analysis of isogenic ovarian cancer cells.....	145
	5.3.2.1 Examination of intracellular aqueous extracts of isogenic ovarian cancer cells.....	145
	5.3.2.1.1 Examination through pattern recognition analysis.....	145
	5.3.2.1.2 PEA1 vs. PEA2 vs. PEO1 vs. PEO4 isogenic ovarian cancer cells under normoxia or hypoxia.....	146
	5.3.2.1.3 Pattern of change of metabolites under normoxia or hypoxia in intracellular aqueous extracts.....	149
	i) Sensitivity vs. resistance	
	ii) Normoxia vs. Hypoxia	

5.3.2.1.4	Focused comparative examination of metabolic intensities in the intracellular extracts.....	156
5.3.2.1.5	Examination of extracellular media of isogenic ovarian cancer cells.....	159
5.3.2.1.6	Examination of metabolic trends in the media through pattern recognition (PCA) analysis.....	159
5.3.2.1.7	Pattern of change of metabolites under normoxia or hypoxia in extracellular media.....	162
	i) Sensitivity vs. Resistance	
	ii) Normoxia vs. Hypoxia	
5.3.2.1.8	Focused comparative examination of metabolic intensities in the extracellular media.....	168
5.4	Discussion.....	171
5.4.1	Metabolic changes under normoxia and hypoxia.....	171
	Branched chain amino acids	
	Glucose	
	Lactate	
	Glucose & lactate relationship	
	Pyruvate	
	Acetate	
	Myo-inositol	
	Choline-related compounds	
5.4.2	Correlation with previous NMR-based metabolic findings.....	177
5.4.3	Concluding remarks.....	178

6.0 Chapter 6: Conclusions

6.1 Overview.....180

6.2 Clinical relevance and future directions.....181

References..... 183

Appendix.....204

List of Figures

Figure 1.1 - Chemical structure of cisplatin.

Figure 1.2 - An overview of pathways involved in mediating cisplatin-induced cellular effects.

Figure 1.3 - Schematic representation of basic mechanisms of platinum drug resistance

Figure 1.4 - Mechanisms involved in inhibiting the apoptotic signal in cisplatin-resistant tumour cells.

Figure 1.5 - In this figure the structural and functional effects of Hypoxia, HIF-1 and VEGF on tumour microcirculation, cancer metabolism and therapies are illustrated.

Figure 1.6 - Induction of the mitochondrial apoptotic pathway by cisplatin.

Figure 1.7 - Schematic representation of the differences between oxidative phosphorylation, anaerobic glycolysis, and aerobic glycolysis (Warburg effect).

Figure 1.8 - Metabolic processes inside a cancer cell.

Figure 2.1 - A charged spinning particle (i.e. a proton) generates a magnetic field. The direction of the generated magnetic field depends on the particle's rotational direction, which can be predicted by the right hand rule.

Figure 2.2 - A typical NMR spectrometer operating at 600 MHz.

Figure 2.3 - A typical illustration of an NMR spectrometer's internal parts and auxiliary instruments used in detection and acquisition of NMR spectra.

Figure 2.4 - Precessing magnetic moments of nuclei on the x-y-z plane, inside an external static magnetic field (B_0).

Figure 2.5 - A rotating nucleus can take up either of two orientations in an applied electromagnetic field.

Figure 2.6 - Application of a radiofrequency pulse on the bulk magnetisation vector M_0 , inside an oscillating magnetic field B_1 , drives it from its toward the x-y plane from the z- axis, causing its rotation around the x- and y- axes, before returning back to equilibrium.

Figure 2.7 - Example of an FID signal and its transformation from a time domain to a frequency domain signal through Fourier Transformation.

Figure 2.8 - Schematic of a typical Nuclear Overhauser Effect (NOE) pulse sequence.

Figure 2.9 - Schematic of a typical Carr-Purcell-Meiboom-Gill (CPMG) pulse sequence.

Figure 2.10 - Representation of a typical PCA method - a multivariate procedure which rotates the data such that maximum variabilities can be projected onto the axes x, y, and z and, thus, be reduced.

Figure 3.1 - Full resolution ^1H NMR mean spectra of the HEK-293T model, normalized to TSP & Median Fold Change. MutL α + (green), MutL α - (cyan), MutL α + ρ_0 (blue), MutL α - ρ_0 (red).

Figure 3.2 - PC1 & PC2: Scores (A) and corresponding loadings plot (B) of principal component analysis on ^1H NMR CPMG one-dimensional spectra for aqueous intracellular extracts of HEK-293T cells normalized by the Median Fold Change. Each point in A represents an individual cell sample of MutL α + (black), MutL α - (red), MutL α + ρ_0 (blue) and MutL α - ρ_0 (green). The ellipse in A

represents the Hotelling with 95% confidence. Numbers beside data point on the loadings plot in B correspond to specific bins used in the analysis.

Figure 3.3 - PC2 & PC3: Scores (A) and corresponding loadings plot (B) of principal component analysis on ^1H NMR CPMG one-dimensional spectra for aqueous intracellular extracts of HEK-293T cells normalized by the Median Fold Change. Each point in A represents an individual cell sample of MutL α + (black), MutL α - (red), MutL α + p0 (blue) and MutL α - p0 (green). The ellipse in A represents the Hotelling with 95% confidence. Numbers beside data point on the loadings plot in B correspond to specific bins used in the analysis.

Figure 3.4 - The effect of *Mlh1* expression on intracellular glutathione

- A) Median normalised spectra
 - B) Integrated metabolite intensity
 - C) Intensity difference between cell lines of differing *Mlh1* status
- (n = 3. * p<0.05, ** p<0.01, *** p<0.001).

Figure 3.5 - The effect of *Mlh1* expression on intracellular alanine

- A) Median normalised spectra
 - B) Integrated metabolite intensity
 - C) Intensity difference between cell lines of differing *Mlh1* status
- (n = 3. * p<0.05, ** p<0.01, *** p<0.001).

Figure 3.6 - The effect of *Mlh1* expression on intracellular phosphocholine

- A) Median normalised spectra
 - B) Integrated metabolite intensity
 - C) Intensity difference between cell lines of differing *Mlh1* status
- (n = 3. * p<0.05, ** p<0.01, *** p<0.001).

Figure 3.7 – Full resolution ^1H NMR mean spectra of the Platinum Resistance model, normalized to TSP & Median Fold Change. A2780 (black), CP70 (red), CP70 E1 (magenta), CP70 A2 (blue).

Figure 3.8 – PC1 & PC2: Scores (A) and corresponding loadings plot (B) of principal component analysis (PCA) on ^1H NMR CPMG one-dimensional spectra for aqueous intracellular extracts of ovarian cells normalized by the Median Fold Change. Each point in A represents an individual cell sample of A2780 (blue), CP70 (green), CP70/E1 (black) and CP70/A2 (red). The ellipse in A represents the Hotelling with 95% confidence. Numbers beside data point on the loadings plot in B correspond to specific bins used in the analysis.

Figure 3.9 - PC3 & PC4: Scores (A) and corresponding loadings plot (B) of principal component analysis (PCA) on ^1H NMR CPMG one-dimensional spectra for aqueous intracellular extracts of ovarian cells normalized by the Median Fold Change. Each point in A represents an individual cell sample of A2780 (blue), CP70 (green), CP70/E1 (black) and CP70/A2 (red). The ellipse in A represents the Hotelling with 95% confidence. Numbers beside data point on the loadings plot in B correspond to specific bins used in the analysis.

Figure 3.10 - The effect of *Mlh1* expression on intracellular glutathione

- A) Median normalised spectra**
 - B) Integrated metabolite intensity**
 - C) Intensity difference between cell lines of differing *Mlh1* status**
- (n = 3. * p<0.05, ** p<0.01, *** p<0.001).**

Figure 3.11 - The effect of *Mlh1* expression on intracellular alanine

- A) Median normalised spectra**
 - B) Integrated metabolite intensity**
 - C) Intensity difference between cell lines of differing *Mlh1* status**
- (n = 3. * p<0.05, ** p<0.01, *** p<0.001).**

Figure 3.12 - The effect of *Mlh1* expression on intracellular phosphocholine

- A) Median normalised spectra**
 - B) Integrated metabolite intensity**
 - C) Intensity difference between cell lines of differing *Mlh1* status**
- (n = 3. * p<0.05, ** p<0.01, *** p<0.001).**

Figure 3.13 - Visual comparative representation of percentage change in intracellular aqueous extracts of average metabolite integrals in different cell models (n=3).

Figure 3.14 - PC:GPC ratios of average integrated metabolic intensities. Asterisk (*) denotes $p < 0.05$.

Figure 4.1 - Example map of NEST network interactions between metabolites and related genes one step (radius of 1) or two steps away (radius of 2) from a metabolite located at the centre of the network.

Figure 4.2 - Example of a ConsensusPathDB-generated network map of physical entities and complexes related to a metabolite (oleic acid in this instance) which has been previously significantly associated with *Mlh1*. Related proteins (in blue/green colour) correspond to the tested NCI-60 proteins. Out of the 14 proteins appearing in the map, two of them (circled in red) are present in the *Mlh1*-associated panel of 241 proteins.

Figure 5.1 - Full resolution ^1H NMR spectra showing various metabolic assignments for the intracellular aqueous extracts in the isogenic ovarian cancer cells under normoxia (PE1A N, PEA2N, PEO1 N, PEO4 N) and hypoxia (PEA1 H, PEA2 H, PEO1 H, PEO4 H).

Figure 5.2 - Plots showing the effects of cisplatin treatment (5 μM) on the ovarian cancer isogenic lines PEO1 (cisplatin-sensitive) & PEO4 (cisplatin-resistant) in normoxia (21 % O_2) and hypoxia (1% O_2), as measured by the Sulforhodamine B-based proliferation assay after 48 hours of exposure. Error bars indicate standard deviation of the mean (SEM) (n = 16). Asterisk denotes $p < 0.05$ (student's t-test).

Figure 5.3 - Scores plot of principal component analysis (PC2 & PC3) on ^1H NMR CPMG one-dimensional spectra for aqueous intracellular extracts of isogenic ovarian cancer cells normalized to TSP and Median Fold Change. Plot is showing metabolic movements through effect of hypoxia and genetic background of the

cells. Each point represents an individual cell sample. The ellipse represents the Hotelling with 95% confidence.

Figure 5.4 - Loadings plot of principal component analysis (PC2 & PC3) on ^1H NMR CPMG one-dimensional spectra for aqueous intracellular extracts of isogenic ovarian cancer cells normalized to TSP and Median Fold Change. Numbers beside data point correspond to specific bins used in the analysis.

Figure 5.5 - Bar chart showing the metabolic intensities pattern for the intracellular aqueous extracts of PEA1 & PEO1 (cisplatin-sensitive) and PEA2 & PEO4 (cisplatin-resistant) ovarian cancer isogenic cell lines in normoxia (21% O_2) and hypoxia (1% O_2). Changes are expressed in terms of percent-change (n=3).

Figure 5.6 - Bar chart showing the metabolic intensities pattern for the intracellular aqueous extracts of PEA1 & PEO1 (cisplatin-sensitive) and PEA2 & PEO4 (cisplatin-resistant) ovarian cancer isogenic cell lines in normoxia (21% O_2) and hypoxia (1% O_2). Changes are expressed in terms of percent-change (n=3).

Figure 5.7 - Mean absolute values of Median Fold Change-normalized NMR intensities for the intracellular aqueous extracts of A) glucose, B) lactate and C) phosphocholine (PC) between platinum sensitive and resistant isogenic ovarian cancer cells. $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$ (unpaired student's t-test using two-tail distribution). Error bars indicate standard error of the mean (SEM).

Figure 5.8 - PC:GPC ratios of mean absolute values of Median Fold Change-normalized NMR intensities for the intracellular aqueous extracts of PC and GPC between platinum sensitive and resistant isogenic ovarian cancer cells. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$ (unpaired student's t-test using two-tail distribution). Error bars indicate combined differential error of the mean.

Figure 5.9 - Scores plots of principal component analysis on ^1H NMR CPMG one-dimensional spectra for extracellular media of isogenic ovarian cancer cells normalized to TSP. Plot is showing effect of hypoxia on resistant cells across PC4

& PC5. Each scores point represents an individual cell sample while the ellipse represents the Hotelling with 95% confidence.

Figure 5.10 - Loadings plots of principal component analysis (PC4 & PC5) on ^1H NMR CPMG one-dimensional spectra for extracellular media of isogenic ovarian cancer cells normalized to TSP. Numbers beside each data point in the loadings plot correspond to specific bins used in the analysis.

Figure 5.11 - Bar chart showing the metabolic intensities pattern for the extracellular media of PEA1 & PEO1 (cisplatin-sensitive) and PEA2 & PEO4 (cisplatin-resistant) ovarian cancer isogenic cell lines in normoxia (21% O_2) and hypoxia (1% O_2). Changes are expressed in terms of percent-change (n=3).

Figure 5.12 - Bar chart showing the metabolic intensities pattern for the extracellular media of PEA1 & PEO1 (cisplatin-sensitive) and PEA2 & PEO4 (cisplatin-resistant) ovarian cancer isogenic cell lines in normoxia (21% O_2) and hypoxia (1% O_2). Changes are expressed in terms of percent-change (n=3).

Figure 5.13 - Mean absolute values of TSP-normalized NMR intensities for the extracellular media of A) glucose and B) lactate between platinum sensitive and resistant isogenic ovarian cancer cells. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$ (unpaired student's t-test using two-tail distribution). Error bars indicate standard error of the mean (SEM).

Figure 5.14 - Mean absolute values of TSP-normalized NMR intensities for the extracellular media of A) pyruvate, B) acetate and C) myo-inositol between platinum sensitive and resistant isogenic ovarian cancer cells. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$ (unpaired student's t-test using two-tail distribution). Error bars indicate standard error of the mean (SEM).

List of Tables

Table 1.1 – FIGO stages of ovarian cancer (Greene 2002)

Table 1.2 - List of NMR-detectable metabolites

Table 2.1 – *in vivo* and *in vitro* characteristics of patients from whom the isogenic ovarian cancer cell lines were derived. (PE = Pleural Effusion, PA = Peritoneal Ascites)

Table 2.2 – List of cancer cell lines of the models used in the *Mlh1* study

Table 2.3 - Molar extinction coefficients for alamarBlue® at different wavelengths

Table 3.1 – *Mlh1* status and characteristics of the cell lines used in this study

Table 3.2 – Spectral regions integrated for a selection of metabolites that exhibited the biggest changes according to PCA analysis

Table 3.3 – Table showing the metabolites with the biggest changes across the binned spectral kidney metabolome across the 3rd PCA component (PC3)

Table 3.4– Table showing metabolites with the biggest changes across the binned spectral ovarian metabolome across the 4th PCA component (PC4)

Table 3.5 – Percentage change in intracellular aqueous extracts of average metabolite integrals (n=3). Welch's two-sample unequal variance t-test with a two-tailed distribution. * P<0.05, ** P<0.01, * P<0.001**

Table 4.1 – List of the NCI-60 cell lines and their corresponding tissues of origin used in this study

Table 4.2 – Databases and pathways included in the ConsensusPathDB online database

Table 4.3 – Metabolic pathways associated with each metabolite according to CosensusPath database

Table 4.4 - List of significant metabolite pathways using a radius of one (Radius 1) and a radius of two (Radius 2) distance from the metabolic nest after applying a threshold of >1 and $p < 0.05$ in the number of selected proteins.

Table 4.5 –Proteins found to be correlated to metabolites associated with *Mlh1* in the tested protein list. Protein names highlighted in red colour represent the proteins found in the *Mlh1*-correlated NCI-60 20% cut-off list.

Table 5.1 – Spectral regions of the positively confirmed metabolites of the intracellular aqueous extracts samples as observed by NMR.

Table 5.2 – Spectral regions of the positively confirmed metabolites of the extracellular media samples as observed by NMR.

Table 5.3 – Average metabolic ratios (fold-change) in sensitive vs. resistant ovarian cancer isogenic cells in normoxia (21% O₂) & hypoxia (21% O₂) of intracellular aqueous extracts. * $p < 0.05$, ** $p < 0.01$, * $p < 0.001$ (unpaired student's t-test using two-tail distribution).**

Table 5.4 – Average metabolic ratios (fold-change) between normoxic (21% O₂) and hypoxic (5% O₂) ovarian cancer isogenic cells (intracellular aqueous extracts). * $p < 0.05$, ** $p < 0.01$, * $p < 0.001$ (unpaired student's t-test using two-tail distribution).**

Table 5.5 – Average metabolic ratios (fold-change) in sensitive vs. resistant ovarian cancer isogenic cells in normoxia & hypoxia of extracellular media. * $p < 0.05$, ** $p < 0.01$, * $p < 0.001$ (unpaired student's t-test using two-tail distribution).**

Table 5.6 – Average metabolic ratios (fold-change) between normoxic and hypoxic ovarian cancer isogenic cells (extracellular media). * $p < 0.05$, ** $p < 0.01$, * $p < 0.001$ (unpaired student's t-test using two-tail distribution).**

List of Abbreviations

3-BrPA: 3-bromopyruvate

AFP: alpha-fetoprotein

ACSS: acetyl-CoA synthetase

ATP: adenosine triphosphate

BAD: BCL2-associated agonist of cell death

BAX: BCL2-associated X

BBN: *N*-butyl-*N*-(4-hydroxybuty) nitrosamine

Bcl-2: B-cell CLL/lymphoma 2

BER: base excision repair

BMI: body mass index

BRCA: breast cancer gene

BHCG: beta human chorionic gonadotropin

c-Fos: FBJ murine osteosarcoma viral oncogene homolog

c-Myc: myelocytomatosis oncogene

c-Jun: jun oncogene

CA-125: cancer antigen 125

CDD: deoxycytidine deaminase

CPMG: Carr Purcell Meiboom Gill

CT: computerised tomography

DAC: 2'-deoxy-5-azacytidine

DMEM: Dulbecco's modified Eagle's medium

DMSO: dimethyl sulfoxide

DNA: deoxyribonucleic acid

**ERCC1: excision repair cross-complementing rodent repair deficiency,
complementation group 1**

Fas: fatty acid synthase

FBS: foetal bovine serum

FDG-PET: fluorodeoxyglucose – positron emission tomography

FID: free induction decay

GPC: glycerophosphocholine

GSH: glutathione

GST- π : glutathione-s-transferase

GPS: glutamine/penicillin/streptomycin

HIF: hypoxia inducible factor

HEK 293T: human embryonic kidney 293T

HMG: high mobility group

MAS-NMR: magic angle spinning nuclear magnetic resonance

HRT: hormone replacement therapy

JRES: J-resolved

LDH: lactate dehydrogenase

JNK: c-jun N-terminal kinase

MAPK: mitogen-activated protein kinase

MDR: multi-drug resistance

MFC: median fold change

Mlh1: mutL homolog 1

MMR: mismatch repair

mRNA: messenger ribonucleic acid

mtDNA: mitochondrial DNA

MRI: magnetic resonance imaging

NCI: National Cancer Institute

NER: nucleotide excision repair

NMR: nuclear magnetic resonance

NOESY: nuclear Overhauser effect spectroscopy

O-PLS: orthogonal – partial least squares

PBS: phosphate buffer saline

PC: phosphocholine

PCA: principal components analysis

PCOS: polycystic ovary syndrome

PA: peritoneal ascites

PE: pleural effusion

P-gp: p-glycoprotein

pH: potentiometric hydrogen ion concentration

PI3K: phosphoinositide-3-kinase

PLS; partial least squares

PMS2: postmeiotic segregation increased 2

PR: pattern recognition

PRM: platinum resistance model

RPMI: Roswell Park Memorial Institute

RECIST: response evaluation criteria in solid tumours

RNA: ribonucleic acid

ROS: reactive oxygen species

SD: standard deviation

SEM: standard error of the mean

SRB: sulforhodamine-B

STOCSY: statistical total correlation spectroscopy

TCA: trichloroacetic acid

TSP: trimethylsilylpropionate

WHO World Health Organisation

XIAP: X-linked inhibitor of apoptosis

XPA: xeroderma pigmentosum, complementation group A

Contributions

Bob Brown and Vincent O'Brien and their team at OCA (Ovarian Cancer Action) provided the samples for the *Mlh1* study, while Jasmin K. Sidhu helped in the initial *Mlh1* data analysis.

Frozen stock of the isogenic ovarian cancer cell lines was provided by Dr. Euan Stronach at Hammersmith Hospital.

Chapter 1

Introduction

Ovarian cancer and the development of chemotherapeutic resistance

1.1 Cancer and ovarian cancer

1.1.1 What is ovarian cancer?

Ovarian cancer is one of the most common gynaecological malignancies, and, as the name suggests, is a cancer of the ovarian tissues (Melnikov, Scholtens et al. 2009). It's the fourth most common cancer affecting females in England and Wales with the majority of all incidents occurring in women until the age of 85 (Dos Santos Silva, Maclean et al. 2003).

Types of ovarian cancer

Ovarian tumours can be divided into a number of categories depending on the type of cells they are formed from and include: **epithelial carcinomas**, which are the most common, and where the tumours are derived from the surface cells of the ovary (Auersperg, Wong et al. 2001), **germ cell tumours**, where the cancer cells are derived from the egg-producing gonads (ovaries) but are less common than epithelial carcinomas (Auersperg, Wong et al. 2001), as well as **sex cord stromal tumours**, which are relatively rare, and derive from a special type of ovarian tissue, the *sex cords*. (Irving, Carinelli et al. 2005). The above can be further divided into some broader categories like metastatic and miscellaneous (Nucci 2009).

Symptoms and signs

Ovarian cancer has been termed the “silent killer” since most of the signs and symptoms make their appearance late in the tumour's development, when the cancer has reached an advanced stage (Jasen 2009). The most common symptoms include one or more of the following: abdominal pain and increased abdominal size, back pain, bloating, constipation, fatigue, urinary urgency, or a combination of the above (Goff, Mandel et al. 2004).

Staging

Staging in cancer is a way of assessing more effectively the extent to which a cancerous growth has spread in a patient's body from the initial locus of development. According to FIGO (Federation International of Gynaecology and Obstetrics), the main stages in ovarian cancer are four and can be seen in table 1.1 (Greene 2002):

Stage I - growth of tumour is only limited to the ovaries
Stage 1a – limited to one ovary and external capsule is non-raptured, there are also no ascites, while the washings are negative
Stage 1b – similar to stage 1a, but growth is present in both ovaries
Stage 1 - similar to stage 1a or 1b but the capsule has ruptured, while there are ascites and the washings are positive
Stage II - growth of tumour has spread to more than one of the ovaries and may also extend to the pelvic tissues
Stage IIa - extension or metastasis to the uterus or fallopian tubes, the washings are negative and there are also no ascites present
Stage II - similar to stage IIa but with extension of the cancer to more pelvic areas
Stage IIc - similar to stage IIa or IIb but with cancerous growths on the surface of either ovary and may also involve ruptured capsules, ascites with cancerous cells and positive peritoneal washings
Stage III - growth of the tumour in one or both ovaries involving metastases to more organs outside the pelvic area
Stage IIIa - tumour is confined in the pelvic area but also has microscopic implants to abdominal peritoneal areas
Stage IIIb - similar to IIIa but with macroscopic metastases outside the pelvic tissues, with tumours less than 2 cm in size
Stage IIIc - metastases outside the pelvis, with tumours more than 2 cm in diameter. Patients may also be lymph node-positive
Stage IV - metastases in distant tissues such as the liver or extra-peritoneal areas like the brain

Table 1.1 – FIGO stages of ovarian cancer (Greene 2002).

Grading

There are four main grades of ovarian cancer relating to cytological features, based on Broder's classification (Vacher-Lavenu *et al*): grade 1, which involves well differentiated cancerous growths without nuclear atypia, followed by grade 2 with moderately differentiated

growths without nuclear atypia. Grade 3 includes moderately/well differentiated growths with nuclear atypia, while grade 4 involves poorly differentiated or undifferentiated growths with nuclear atypia. It can, thus, be easily deducted that the higher the grade of the tumour, the faster it grows and the least favourable prognosis it has.

Statistics

About 1 to 2% of women in the developed world are affected by ovarian cancer (Emma and Usha 2005) while the risk of a woman developing ovarian cancer in her lifetime is about 1 in 80 in Europe (Tsigkou, Marrelli et al. 2007). The survival rates for ovarian cancer are dependent on the stage of the tumour at the time of diagnosis (Chan, Fuh et al. 2008).

Risk & prevention factors

Ovarian cancer is a multifaceted disease; however there are various factors that may influence its development:

In one collaborative study, reduced ovarian cancer risk was noted in women who had breastfed when compared to women who had never breastfed (Whittemore, Harris et al. 1992). Another collaborative study examining this issue also showed a significant reduction in risk for women who kept breastfeeding for at least 18 months (Danforth, Tworoger et al. 2007). It was also reported that nulligravid women who made attempts to achieve becoming pregnant for over five years were in increased risk for developing ovarian cancer compared to women whose attempts lasted for less than a year (Ness, Cramer et al. 2002). Additionally, in a collective group of 12 case studies which involved 2197 patients, the use of the oral contraceptive pill had been reported to bring about a strong protective effect against the development of ovarian cancer (Whittemore, Harris et al. 1992). The risk reduces even more with increasing years of use, where women who have used the contraceptive pill for more than three years have increased associated benefit compared to women who have never used it (Rosenberg, Palmer et al. 1994). Sterilisation through tubal ligation has also been shown to confer protection against ovarian cancer, and it does not seem to decrease significantly over time, whereas hysterectomy is also linked to a reduced risk (Green, Purdie et al. 1997); (Hankinson, Hunter et al. 1993); (Kjaer, Mellemkjaer et al. 2004). On the contrary, hormone replacement therapy (HRT) has been linked to an increased risk of ovarian cancer. The results of this study indicated a higher risk when administering oestrogen-only replacement therapy when compared to oestrogen-progestin replacement therapy (Zhou, Sun et al. 2008).

Another study by the European Prospective Investigation into Cancer and Nutrition exhibited that obese post-menopausal women, with a BMI more than 30, have an increased risk in relation to women who have a BMI below 25, which is considered to be a normal and healthy weight (Lahmann, Cust et al. 2010). Polycystic ovary syndrome (PCOS), a very common disorder affecting millions of women, had initially been linked to an increased risk of endometrial cancer (Norman, Dewailly et al. 2007). However, even though there are some preliminary data suggesting a link between PCOS and ovarian cancer, the existing scientific data are not sufficient to draw a certain conclusion (Schildkraut, Schwingl et al. 1996); (Solomon 1999). Other meta-analytic studies on analgesic drugs such as paracetamol gave some evidence that frequent use can reduce the ovarian cancer risk (Bonovas, Filioussi et al. 2006). However, findings from other studies have produced inconsistent results ((Merritt, Green et al. 2008); (Wernli, Newcomb et al. 2008); (Schildkraut, Moorman et al. 2006); (Hannibal, Rossing et al. 2008). Furthermore, a recent review of human carcinogens such as tobacco, alcohol, coal smoke, and salted fish as indicated by the International Agency for Research on Cancer, had concluded that there is sufficient evidence to link smoking with ovarian cancer (Secretan, Straif et al. 2009). Finally, women whose relatives have had ovarian cancer are in increased risk of developing the disease (Granstrom, Sundquist et al. 2008) or women with hereditary syndromes such as Lynch syndrome have also been found to be at higher risk ((Ramsoekh, Wagner et al. 2009). At higher risk for epithelial ovarian cancer are also women with mutated alleles for *BRCA1* and *BRCA2* (Antoniou, Pharoah et al. 2003) or *MMR* genes (Peltomaki and Vasen 2004).

Prognosis

Prognosis in ovarian cancer is usually poor and the mortality rate can be very high due to the lack of sensitive early detectors of the disease, which allows for the formation of new tumours through the extensive spreading of cancer cells into distant tissues. However, the good news is that survival trends in England have increased in recent years which could be attributed to cancer care improvements (Rachet, Maringe et al. 2009).

Diagnosis & screening

Early-stage ovarian cancer is difficult to diagnose before it spreads into more advanced disease stages due to the majority of the symptoms being non-specific and therefore of little value in diagnosis (Rossing, Wicklund et al. 2010). Most women (around 70%) are diagnosed with ovarian cancer at stage III or IV with widespread metastases (Rustin, Bast et al. 2004).

Diagnosing cancer in patients may involve the use of various analytical platforms and/or standard wet laboratory tests:

1.1.2 Techniques in the diagnosis and examination of cancer.

These include standard imaging techniques such as X-rays and ultrasound examinations or more advanced techniques such as CT (Computerised Tomography) scans and PET (Positron Emission Tomography). CT scans can help in determining if the cancer has spread into other organs or lymph nodes and involves the injection of a high-contrast dye into the patient followed by multiple three-dimensional scans of the patient's full body (Jung, Lee et al. 2002). In oncology, PET scanning employing the use of tracer fluorodeoxyglucose (FDG), called FDG-PET, is a very widely used method (Weber 2007). FDG is a glucose analogue that is taken up by cells and phosphorylated by hexokinase, with the latter showing enhanced concentrations in aggressive tumours (Andrei, Shutian et al. 2005). The advantage of this method is that FDG is no further metabolized leading to strong radio-labelling in cancer cells (Andrei, Shutian et al. 2005). As a result, FDG-PET can be used not only in cancer diagnosis, but also in staging, and as a monitoring system. There are also other analytical methods that are used not as much for diagnostic purposes at the moment but are very important for the generation of cancer biomarkers or helpful metabolic-related data. Such methodological tools include nuclear magnetic resonance (NMR) which is described in more detail in the methodology chapter (chapter 2).

Other helpful investigations that may help with diagnosis include the detection of the following tumour markers:

Serum Beta Human Chorionic Gonadotropin (BHCG), which is usually present in females where pregnancy is possible but also in females suspected of ovarian cancer (Fishman, Raam et al. 1975). Another important marker is CA-125, which is commonly found on many epithelial tumours, and is useful in the diagnosis and monitoring of the disease, as well as in the early detection in patients with recurrent disease (Panza, Pacilio et al. 1988). Other ovarian cancer markers include TATI, CASA, TPA, or CYFRA21-1a and even genetic markers such as *Ras* oncogenes or *C-erb2* proto-oncogenes (Mazurek, Niklinski et al. 1998).

Physical examinations can also be useful in diagnosis. These can involve bimanual pelvic examination or examination of the liver, spleen, diaphragm, omentum and retroperitoneal

lymph nodes since these are some of the main sites of ovarian cancer metastases (Smith 1979). On the occasions where ovarian cancer is confirmed, a full hysterectomy in the abdominal area may be performed, including omentectomy, para-aortic and lymph node sampling or peritoneal biopsies (Stuart 2003).

Biopsies might also be performed if necessary, and are usually achieved through surgical procedures such as laparotomy or laparoscopy after which suspicious masses are removed and sent for further lab analysis (Spencer, Anderson et al. 2006).

1.1.3 Treatment

For the treatment of ovarian cancer surgery is usually the first-line of approach. It is also used for obtaining tissue samples for histological analysis or for de-bulking purposes. In advanced disease progression, debulking surgery (where as much malignant tissues are removed) is usually necessary, as it can assist towards a better response to subsequent drug treatment (Stuart 2003). Radiation therapy may also be used, but this is mostly applied on smaller areas and more localised tumours during the early stages of the disease, as it is more effective this way rather than on larger cancer areas (Fujiwara, Suzuki et al. 2002). Finally, chemotherapy, which has always been the general standard of care for ovarian cancer through the usage of highly different methods and drug combinations, may also be deemed necessary (McGuire and Markman 2003).

1.1.4 Chemotherapy

1.1.4.1 Chemotherapeutic agents in ovarian cancer

There is a great variety of chemotherapeutic drugs used in the treatment of ovarian cancer. Their use depends on parameters such the grading and staging of the cancer, and more generally, the overall health of the patient. Such drugs include anthracyclines like doxorubicin, alkylating agents like melphalan, spindle poisons like paclitaxel and docetaxel, antimetabolites such as gemcitabine and platinum-based compounds such as cisplatin or carboplatin, as well as the topoisomerase I inhibitor topotecan. (Berkenblit and Cannistra 2005) However, some of the most common treatments against ovarian cancer involve platinum-based drugs:

Platinum anti-cancer drugs have been one of the most important chemotherapy treatments in the treatment of malignancies for many years and some of the most widely used and effective chemotherapeutics agents even today (Boulikas and Vougiouka 2003). Their most important characteristic from which their anticancer activity stems from is their ability to elicit DNA damage.

1.1.4.1.1 Cisplatin

The oldest and perhaps the most famous platinum drug is cisplatin (*cis* diaminodichloroplatinum), a compound that works by inhibiting the production of genetic material in the cell by the formation of specific interactions with the cellular DNA. It was accidentally discovered by Barnett Rosenberg van Camp and his colleagues at Michigan State University in the middle of the 20th century, while examining bacterial growth inhibition with the use of electric currents (Rosenberg, Vancamp et al. 1965). They discovered that electrolysis of the platinum electrodes generated cisplatin, which in turn stopped cell division in *E. coli* bacteria (Rosenberg, Vancamp et al. 1965). A series of experiments was then followed to examine the outcomes of the various platinum complex treatments on human leukemic cells (L1210) and on sarcomas implanted in rats. It was then concluded that *cis*-PtCl₂(NH₃)₂ was the most effective platinum compound, hence commencing the ‘career’ of cisplatin in medical cancer treatments (Rosenberg, VanCamp et al. 1969); (Zwelling, Anderson et al. 1979).

Since its discovery, cisplatin is still a major chemotherapeutic agent in the treatment of ovarian cancers, long after being first-introduced for its treatment in 1973. However, the discovery of various toxicities associated with cisplatin treatment resulted in the development of additional related drugs such as carboplatin, which could be considered as effective as cisplatin, but with fewer side-effects (Kelland 2007) as it causes less neurotoxicity, ototoxicity, and less severe nausea than cisplatin (Adams, Kerby et al. 1989). However it still has other disadvantages since it is more myelotoxic (Kerr, Lewis et al. 1990).

The cytotoxicities associated with platinum drugs like cisplatin and carboplatin are thought to be caused by the generation of platinum-DNA adducts such as mononadducts, intrastrand and interstrand crosslinks, or DNA-protein crosslinks (Fichtinger-Schepman, van Dijk-Knijnenburg et al. 1995). The prevailing notion is that intrastrand crosslinks are the ones most responsible for the cytotoxicity as shown by an experiment on Chinese hamster ovary

cells (CHO), where the majority of such lesion development co-incided with a maximum peak in cytotoxicity (Fichtinger-Schepman, van Dijk-Knijnenburg et al. 1995). Some conflicting opinions exist though, as earlier experiments by Zwelling L.A *et al* had found an *in vitro* correlation between cytotoxicity and interstrand crosslinks in L1210 cells (Zwelling, Anderson et al. 1979). According to McHugh *et al*, however, it's the intrastrand crosslinks that mostly contribute to the platinum drugs' cytotoxic effects (McHugh, Sones et al. 2000). Several more efforts attempting to reduce platinum cytotoxicity as well as to overcome development of resistance to the platinum drugs have resulted in the development of other platinum-based drugs such as oxaliplatin, satraplatin and picoplatin (Kelland 2007); (Boulikas and Vougiouka 2003).

Structure of cisplatin

Cisplatin contains a platinum central atom surrounded by two chlorides and two NH₃ groups in the *cis* position, hence its name, (Jamieson and Lippard 1999) as illustrated in the figure below:

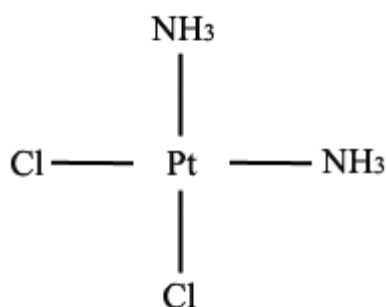


Figure 1.1 - Chemical structure of cisplatin (schematic supplied by author).

Mechanism of cisplatin action & apoptosis

Cisplatin is water-soluble; once inside the cell, its chloride ions dissociate because of the low chloride concentration, resulting in a reactive diamine-platinum complex, which reacts with water molecules during a hydrolytic reaction (Jamieson and Lippard 1999). Following that, cisplatin can bind to the N7 nitrogen atoms of DNA bases, preferentially those of guanine and adenine (Eastman 1987); (Singer 1977). As already mentioned, the cisplatin complex acts through the formation of DNA adducts, most of them intrastrand cross-links (Pinto and Lippard 1985). The DNA damage caused by cisplatin is recognized by the cell's DNA damage recognition machinery, which includes proteins such as high mobility group proteins (HMG) as well as mismatch repair complexes like hMSH2 or hMutS α (Jamieson and Lippard

1999). These proteins are the starting point of a cascade of cell survival or apoptotic signals, which depends on their intensity as well as the crosstalk between the involved effectors (such as the p53 - or the p73- tumour suppressor genes). The protein kinase signal transduction pathway is then activated, which, in turn, activates p53 causing the cell cycle to arrest at the G2/M checkpoint. Whenever the DNA damage to the cell is beyond repair, apoptosis occurs through the Bax and caspase pathway.

Figure 1.2 summarises the sequence of events following cisplatin exposure.

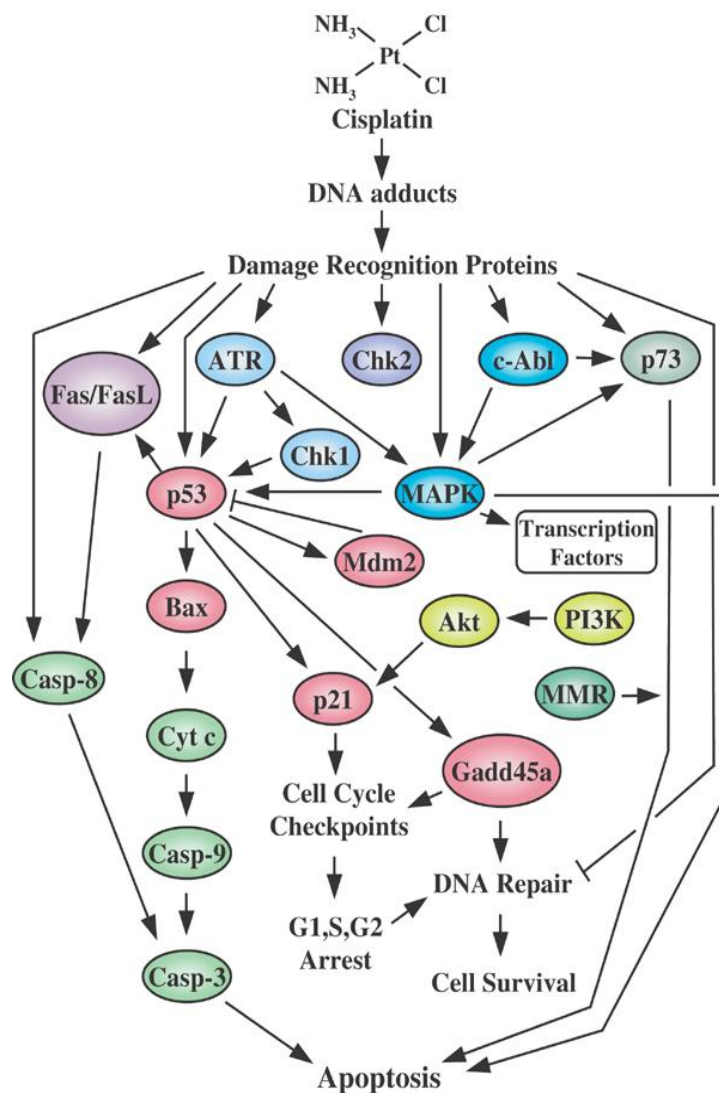


Figure 1.2 - An overview of pathways involved in mediating cisplatin-induced cellular effects. Cell death or cell survival will depend on the relative intensity of the signals generated and the crosstalk between the pathways involved. (Figure and text taken from “Cisplatin: mode of cytotoxic action and molecular basis of resistance” by (Siddik 2003).

1.1.5 Development of resistance to platinum drugs

Women with ovarian cancer may initially have a well-received and effective response to platinum-based chemotherapy, however as time passes, many ovarian carcinomas can become refractory, with the patients dying from progressive chemoresistant disease (Agarwal and Kaye 2003). Cancer tumours are said to be *sensitive* when they appear to readily respond to chemotherapy and *resistant* to platinum when they fail to respond to the treatment either at the start of the treatment or at a later stage (Robinson 1993). Resistance can be either *intrinsic* (already present at the time of diagnosis) or *acquired*, developing after a period of drug administration (Malpas and Rohatiner 1996). According to Response Evaluation Criteria in Solid Tumours (RECIST) group, the progression of disease for the duration of the therapy is defined as a 20% increase in the total sum of the biggest diameter of the target, such as the ovary, or non-target lesion (Therasse, Arbuck et al. 2000).

1.1.5.1 Mechanisms of platinum resistance

Ovarian cancer cells can exhibit resistance to cisplatin (and platinum in general) due to a variety of different factors since there are various fates for platinum once inside the cell. It is still unclear, however, which of these factors contribute most in acquired drug resistance in clinical situations – where progress is many times hindered by the lack of sensitive methods in detecting the crucial drug-induced cellular effects.

A schematic overview of the main mechanisms of platinum resistance is presented in figure 1.3.

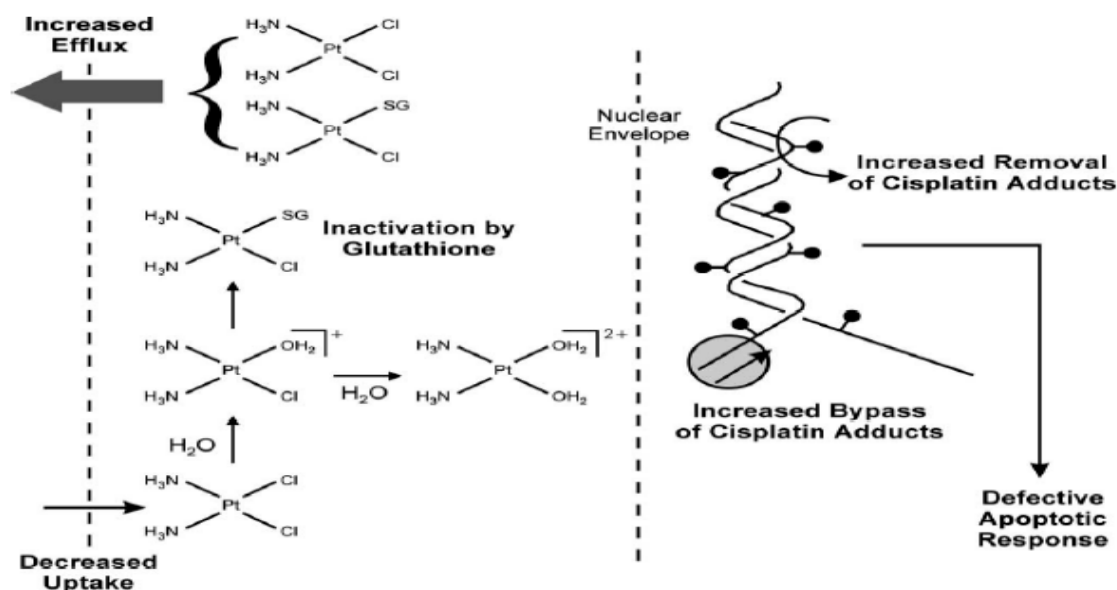


Figure 1.3 – Schematic representation of basic mechanisms of platinum drug resistance (figure and text taken from (Kartalou and Essigmann 2001)).

More specifically, platinum and cisplatin resistance can be linked to:

Reduced intracellular drug accumulation

Reductions in intracellular drug concentrations between 20–70% have been reported in a variety of cisplatin resistant cell lines in the order of 3 to 40-fold (Kelland 1993). However, this is not directly analogous to the strength of resistance (Johnson, Laub et al. 1997).

The reduced intracellular drug accumulations can be attributed to a variety of parameters such as low dose, low bioavailability, insufficient distribution, increased metabolism (high first-pass metabolism), increased excretion, increased plasma protein binding, low binding to target tissues, altered drug diffusion, decreased drug uptake or increased drug efflux or both (Lai, Ozols et al. 1988); (Stewart 2007).

Linked to altered drug concentrations is the development of *multi-drug resistance* (MDR) where tumour cells develop resistance even to drugs functionally and structurally distinct to platinum, such as the anthracyclines. For example, the MDR gene product p-glycoprotein (P-gp) drug efflux pump is involved in the alterations of intracellular drug accumulation, and has been associated with resistance to chemotherapeutic agents (Smith, Carmeli et al. 1993); (Wada, Saikawa et al. 1999); (Wada, Saikawa et al. 1999).

It is noteworthy that many platinum drugs are usually transported into the cells through the hCtr1 transporter in the plasma membrane, which is also a transporter for copper. Apart from hCtr1, other copper-transporting protein transporters include the ATP7A and ATP7B gene products, whose increased expression has been linked to increased resistance and poor survival in ovarian cancer (Samimi, Safaei et al. 2004); (Komatsu, Sumizawa et al. 2000) (Katano, Kondo et al. 2002).

Increased drug detoxification and inactivation

This can take effect as a result of the platinum drug binding to thiol-containing antioxidants like glutathione (Kelland 2007) or metallothioneins (low-molecular weight proteins with a high affinity for heavy metals), which are catalysed by glutathione-S-transferase (GST- π) (Gottesman and Pastan 1993), and whose increased concentrations have been associated with increased cisplatin resistance in various *in vivo* murine & human models (Kelley, Basu et al. 1988); (Kasahara K Fau - Fujiwara, Fujiwara Y Fau - Nishio et al. 1991). There is also a link between high GST- π levels, poor prognosis, and resistance to platinum in ovarian cancer (Green, Robertson et al. 1993); (Sakamoto, Kondo et al. 2001). However, in phase II clinical trials, where attempts to bypass thiol-mediated detoxification were examined, the tested platinum analogue (ZD0473), which does not bind to thiol compounds, did not produce better results than carboplatin (Gore, Atkinson et al. 2002) indicating the pathway might not be a crucial factor in resistance development.

Enhanced DNA repair

Resistance can also be attributed to increased ability to repair DNA often in association with increased removal or increased bypass of DNA-cisplatin adducts (Stewart 2007); (Kartalou and Essigmann 2001); (Ferry, Hamilton et al. 2000).

On occasions of increased DNA adduct repair the apoptotic process may be reduced. For example, in an early study, cell lines derived from patients before (PEO1) and after (PEO4) the clinical appearance of platinum resistance were compared and showed that the PEO4 line had nearly 3-fold more repair synthesis than its more sensitive predecessor (Lai, Ozols et al. 1988). However, as with other mechanisms of resistance, increased DNA repair is not always present in all cells resistant to cisplatin (Schmidt and Chaney 1993).

The nucleotide excision repair (NER) pathway is the pathway responsible for the removal of platinum adducts and the repair of DNA damage (Kelland 2007). Cellular dysfunctions in the NER pathway leads to cisplatin hypersensitivity, whereas its reestablishment restores sensitivity to normal levels (Chaney and Sancar 1996); (Furuta, Ueda et al. 2002). The NER complex consists of at least 17 proteins, but increased expression of even a small number of rate-limiting proteins (like topoisomerase II, ERCC1, XPA, and BRCA1) is enough to increase the repair capacity in resistant cells (Liu 2009).

Topoisomerase II, for example, is associated with repair of DNA crosslinks caused by cisplatin, and it is often the case where its clinical overexpression is linked to the onset of cisplatin resistance (Ali-Osman, Berger et al. 1993); (Hengstler, Lange et al. 1999). Furthermore, increases in *ERCC1* mRNA levels in patient tumours that are no longer sensitive to cisplatin have been noted (Dabholkar, Vionnet et al. 1994). Similarly, overexpression of the *XPA* gene, which contributes to enhanced repair, has been linked to cisplatin resistance (Dabholkar, Vionnet et al. 1994). On the contrary, testicular tumours, which usually are cisplatin-sensitive, produce very low levels of XPA and ERCC1/XPF (Koberle, Masters et al. 1999). Ovarian cancer cells showing BRCA1 overexpression were also shown to be cisplatin resistant and BRCA1-dependent DNA repair is thought to be responsible for this (Husain, He et al. 1998). Additionally, BRCA1 silencing was shown to increase cisplatin sensitivity, while its overexpression in ovarian cancer cells in mice was shown to increase cisplatin resistance (Sylvain, Lafarge et al. 2002).

Decreased DNA mismatch repair (MMR) can also contribute to resistance usually in cells deficient in MMR proteins such as hMLH1, hMSH2, hMSH6, hMutL α (heterodimer of hMLH1 and PMS2) and hMutS α (heterodimer of hMSH2 and hMSH6) (Duckett Dr Fau - Drummond, Drummond Jt Fau - Murchie et al. 1996); (Mello, Acharya et al. 1996; Fink, Nebel et al. 1998); (Stewart 2007); (Vaisman, Varchenko et al. 1998); (Zdraveski, Mello et al. 2000). MMR genes are needed for genome surveillance after replication. Their gene products correct mismatches from DNA replication and evade proofreading (Schofield and Hsieh 2003); (Kunkel and Erie 2005). Most mutations are found in the *Mlh1* gene (50%), and this makes it a highly important gene during carcinogenesis (Tanaka, Zaman et al. 2009). Downregulation of such genes are routinely observed on occasions of cisplatin resistance (Aebi, Kurdi-Haidar et al. 1996); (Drummond, Anthoney et al. 1996); (Fink, Nebel et al. 1996); (Brown, Hirst et al. 1997); (Vaisman, Varchenko et al. 1998). Loss of MMR in

cisplatin resistant cells is also linked to microsatellite instability and decreased apoptosis (Anthoney, McIlwrath et al. 1996); (Mayer, Gillis et al. 2002). Cell lines deficient in the MMR genes *hMLH1* or *hMSH2* were generally found to be resistant to cisplatin in *in vitro* experiments (Brown, Hirst et al. 1997). This is usually caused through the hypermethylation of the *hMLh1* promoter (Plumb, Strathdee et al. 2000) while microsatellite instability in ovarian tumours after treatment with cisplatin was reported to have links to loss of *hMLH1* expression (Watanabe, Koi et al. 2001). Treatment with 2'-deoxy-5-azacytidine (DAC), which works by inhibiting DNA methyltransferase enzymes, re-sensitized the cells to cisplatin (Plumb, Strathdee et al. 2000) by decreasing the hypermethylation of the *hMLH1* promoter, and has also been used in combination with carboplatin (Longley and Johnston 2005).

There are many reports indicating that increased tolerance to cisplatin adducts is also significant in cisplatin resistance ((Johnson, Laub et al. 1997); (Siddik Zh Fau - Mims, Mims B Fau - Lozano et al. 1998); (Yoshida M Fau - Khokhar, Khokhar Ar Fau - Siddik et al. 1998). Loss of apoptotic signalling, as mediated through mutation of genes such as *p53* (Haupt, Berger et al. 2003) is indeed a factor contributing to the lack of cisplatin efficiency in the cells. For example, loss of function of the tumour suppressor *p53* gene through mutation is observed in about a half of all cancers (Hollstein, Sidransky et al. 1991); (Soussi 2000). Tumour cells with defects in *p53*-mediated apoptotic pathway fail to activate cell death, thus, enabling the cells to become tolerant to DNA damage (Kastan, Onyekwere et al. 1991); (Hartwell and Kastan 1994); (Pietenpol, Tokino et al. 1994); (Siddik Zh Fau - Mims, Mims B Fau - Lozano et al. 1998).

MAPK subfamily members (i.e. p38, JNK, and ERK) are also linked to cisplatin function. However, whether MAPK pathway defects mediate cisplatin resistance is still not very clear, as there are various conflicting studies in existence. The MAPK pathway leads to the activation of transcription factors such as c-Myc, c-Fos, and c-Jun (Robinson and Cobb 1997); (Martin-Blanco 2000) which are overexpressed in cisplatin resistant cells, while their decreased expression resensitizes them to the drug (Kartalou and Essigmann 2001); (Pan B Fau - Yao, Yao Ks Fau - Monia et al. 2002).

Other factors can include overexpression of HER-2/neu and the PI3-K/Akt pathway.

Evasion of apoptosis

Overexpressed apoptotic inhibitors, such as survivin and XIAP can also enhance resistance (Asselin, Mills et al. 2001); (Ikeguchi, Liu et al. 2002) by acting directly or indirectly on the function of caspases, which are important effector apoptotic molecules. Activation of caspases 3, 8, and 9 is decreased in cisplatin resistant cells (Henkels Km Fau - Turchi and Jj 1997); (Blanc C Fau - Deveraux, Deveraux Ql Fau - Krajewski et al. 2000); (Asselin, Mills et al. 2001); (Ono Y Fau - Nonomura, Nonomura N Fau - Harada et al. 2001). This inhibition could be due to loss of *Fas* expression after cisplatin treatment, decreasing, thus, the apoptotic signals (Qin and Ng 2002).

Other factors important in the regulation of apoptosis are *Bcl-2* family members, which are located in the mitochondria as homodimers or heterodimers, and can be either proapoptotic (i.e. BAX) or antiapoptotic (i.e. *Bcl-2* and *Bcl-xL*) (Farrow and Brown 1996); (Hanahan and Weinberg 2000); (Schuler and Green 2001); (Gebauer, Mirakhur et al. 2000). Overexpression of *Bcl-2* is associated with cisplatin resistance, sometimes with an increase in glutathione levels (Hockenbery, Oltvai et al. 1993); (Chiao, Carothers et al. 1995). Increased levels of *Bcl-xL* have also been reported in resistant cells (Gebauer, Mirakhur et al. 2000), possibly through inhibition of the negative regulator *Bad* (Hayakawa, Ohmichi et al. 2000). Contradictory results also exist, though; *Bcl-2* overexpression was found to be linked to improved survival of cisplatin-receiving ovarian cancer patients (Herod, Eliopoulos et al. 1996) which is a reminder of our limited knowledge regarding apoptosis and development of resistance.

Figure 1.4 below illustrates the complex multifactorial features of platinum drug resistance.

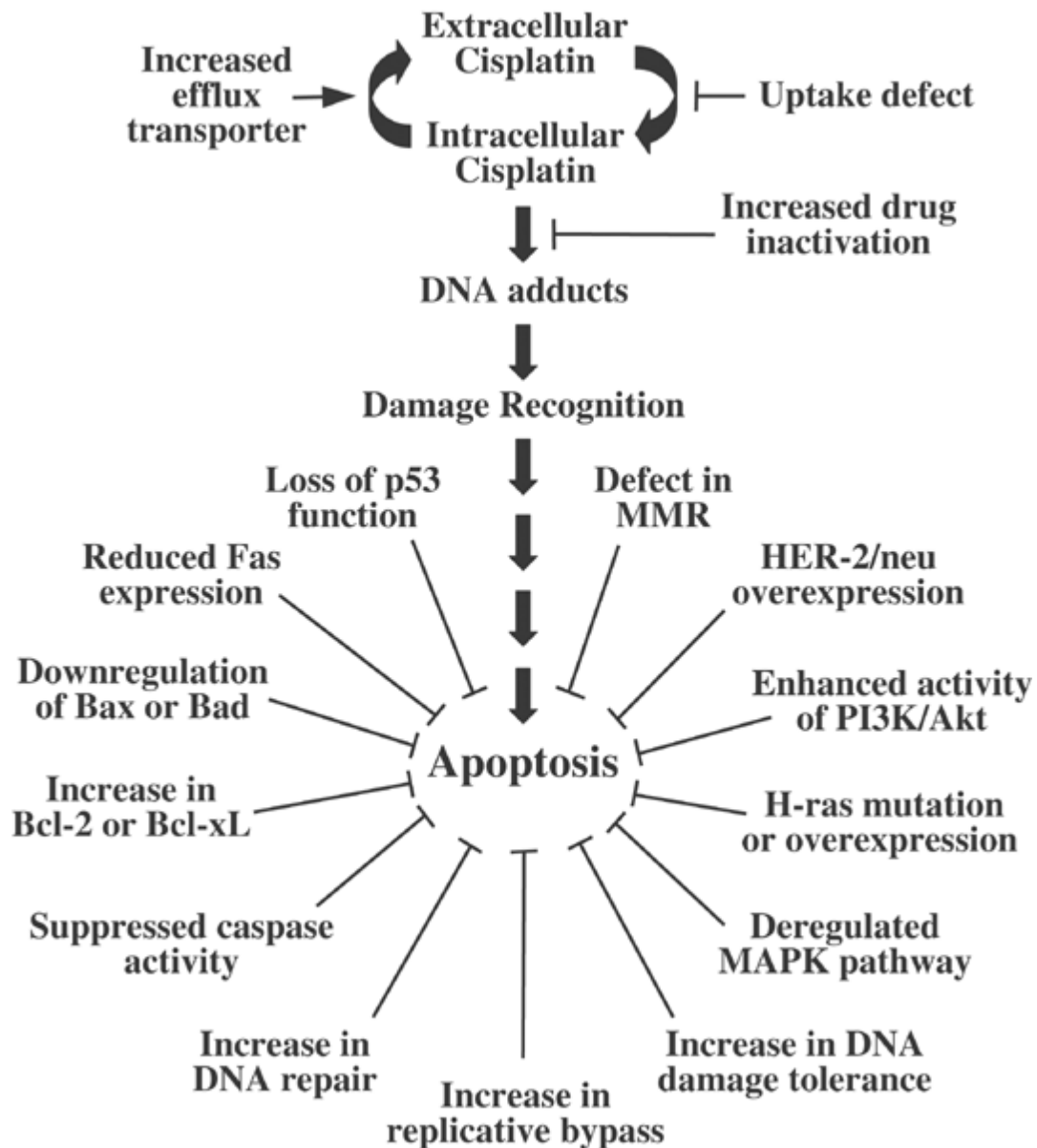


Figure 1.4 - Mechanisms involved in inhibiting the apoptotic signal in cisplatin-resistant tumour cells. More than one mechanism is usually observed in resistant cells, and this contributes to the multifactorial nature of cisplatin resistance (figure and text taken from “Cisplatin: mode of cytotoxic action and molecular basis of resistance” by (Siddik 2003)).

1.1.6 Hypoxia in cancer and drug resistance

Hypoxia is another important factor in the development of platinum resistance. Its presence in tumours, where the oxygen concentration is very low, was first proposed by Thomlinson and Gray while observing the distribution of necrosis in blood vessels (Thomlinson and Gray 1955) even though it was already known that hypoxic cells were resistant to death by ionizing radiation (Gray, Conger et al. 1953).

The rapid expansion of tumour cells usually surpasses vascular growth, leading to an inadequate cancer growth blood supply, thus, resulting in the creation of a hypoxic environment which significantly limits oxygen availability in mitochondrial respiration and ATP synthesis (Xu, Pelicano et al. 2005). This results in the cancer cells up-regulating the glycolytic pathway for energy production (Guppy 2002). Hypoxia is, thus, important in cancer as it has the ability to aggravate the intensity of the Warburg effect (explained further below) and to facilitate tumour survival, thus, resulting in an aggressive phenotype.

The effect of oxygen on regulating glucose metabolism is known as the Pasteur Effect and is mediated through several kinase pathways (Hardie 2000); (Seta, Spicer et al. 2002). The increased glycolysis in cancer cells is not always due to dysfunctional mitochondria but it can also be induced by the tumour microenvironment through overexpression of various glycolytic enzymes (Mathupala, Rempel et al. 2001).

There are many ways by which hypoxia may adversely affect cancer drug treatment, but most commonly hypoxia hinders cancer therapy by promoting tumour cell resistance to conventional chemotherapeutic drugs in a variety of ways:

In hypoxia, the alkylating agents and platinum compounds' function may be reduced due to the enhanced activity of DNA repair enzymes (Ebert, Gunther et al. 2000). Hypoxia may also enhance the production of proteins responsible for the development of drug resistance (i.e. metallothioneins) (Murphy, Laderoute et al. 1994). It can also lead to genome changes such as selection of cells with p53 mutations (Graeber, Peterson et al. 1994) which results in drug resistance through loss of apoptotic potential. Furthermore, the heterogeneous hypoxic tumour blood-flow results in poor and irregular blood supply, leading to restricted drug distribution (Vaupel, Kallinowski et al. 1989); (Vaupel, Thews et al. 2001); (Aoife, David et al. 2003).

In hypoxia, drugs with short half-life in the body may also have trouble reaching the target tumour tissues in time to be effective (Brown 1999). In addition, sometimes the location of viable hypoxic cells very close to the blood vessels may create an obstacle in terms of physical delivery, as bulky chemotherapeutic drugs may need to penetrate many layers to reach the targets (Sutherland 1988); (Tannock 1982); (Durand 1989); (Teicher, Holden et al. 1990).

Low pH is also sometimes found in hypoxic tumour areas. Even though the intracellular pH usually remains at normal levels, the extracellular pH may be quite low (Harris 2002) resulting in a pH gradient which hinders the intracellular drug accumulation creating a problem for drugs that are weak bases (adriamycin) (Skovsgaard 1977). Situations where drugs are dependent on oxygen for maximum function (Teicher, Lazo et al. 1981) may also be problematic. For example, in an early study (Teicher, Holden et al. 1987) normoxic tumour cells showed bigger sensitivity to melphalan, compared to their counterparts that were placed under hypoxic conditions. Since hypoxia causes cells to cycle more slowly or even make them enter cell cycle arrest, drugs like alkylating agents and antimetabolites that act on specific cell-cycle phases or on highly-cycling cells may have reduced effectiveness (Amellem and Pettersen 1991). Finally, hypoxic tumours in general are prone to select for a more malignant and metastatic phenotype (Ivan, Kondo et al. 2001); (Huang, Gu et al. 1998); (Lu, Forbes et al. 2002) by increasing mutations (Jaakkola, Mole et al. 2001) and expression of angiogenetic genes (Huang, Arany et al. 1996), making subsequent attempts at treatment either through surgery radiation or chemotherapy even more difficult. An example is the hypoxia induced transcription factor *HIF-1* gene product (a dimer of HIF-1 α and HIF-1 β subunits). *HIF-1 α* expression is associated with poor prognosis and resistance to therapy in cancers, including ovarian cancer (Sutherland 1988); (Tannock 1982) as it in turn stimulates more transcription genes involved in angiogenesis and glycolysis (Huang, Arany et al. 1996).

Therefore, hypoxia has a crucial adverse role in cancer prognosis not only because of its negative effects on drug resistance but also because it can directly promote a more malignant phenotype. However, the unique features associated with hypoxia, caused by the very low oxygen concentrations and even the presence of necrosis, not normally found in healthy normoxic tissues, could act as potential exploitable targets in cancer therapy.

1.1.7 The role of mitochondria in platinum resistant cells

In recent years, the role of mitochondria in platinum resistance is also considered of importance in the development of platinum resistance as the main cytotoxic activity of cisplatin is mediated by a mitochondria-associated induction of apoptosis. There are conflicting opinions on the mitochondrial association with cisplatin sensitivity (usually involving mitochondrial changes in morphology and function). For example, in a study by Liang and Ulliyatt on mitochondrial-DNA depleted U937 cells, where enhanced sensitivity in cisplatin was reported (Liang and Ulliyatt 1998), it was shown that cells continued to respond to cisplatin and undergo apoptosis. This could mean that mitochondrial DNA-expressed molecules may be important in altering cellular sensitivity to cisplatin-caused apoptosis, but may not be required for the actual apoptotic process itself. On the contrary, in a study by Park *et al* it was shown that SK-Hep1 hepatoma cells that had their mitochondrial DNA-depleted were resistant to cell death through oxidative stress or chemotherapeutic agents (Park, Chang et al. 2004).

Mitochondrial pathway of apoptosis

Generally, it's been known that cisplatin can induce apoptosis by Fas ligand induction on the surface of tumour cells, and killing the Fas-sensitive subpopulations, the direct activation of initiator protease, caspase-8 or cell stress signals via 'activation' of mitochondria (Hirama, Isonishi et al. 2006).

One crucial event in the apoptotic process is the release of cytochrome *c* from the mitochondria into the cytosol. Cytochrome *c* recruits the apoptotic protease-activating factor 1 (Apaf-1) and procaspase-9 forming a complex which then activates the initiator caspase-9. Caspase-9 continues the cascading process with activation of caspase-3, leading to DNA fragmentation and, eventually, apoptosis (Cai, Yang et al. 1998); (Shiozaki, Chai et al. 2002).

Figure 1.6 below illustrates the cisplatin-induced mitochondrial apoptotic pathway:

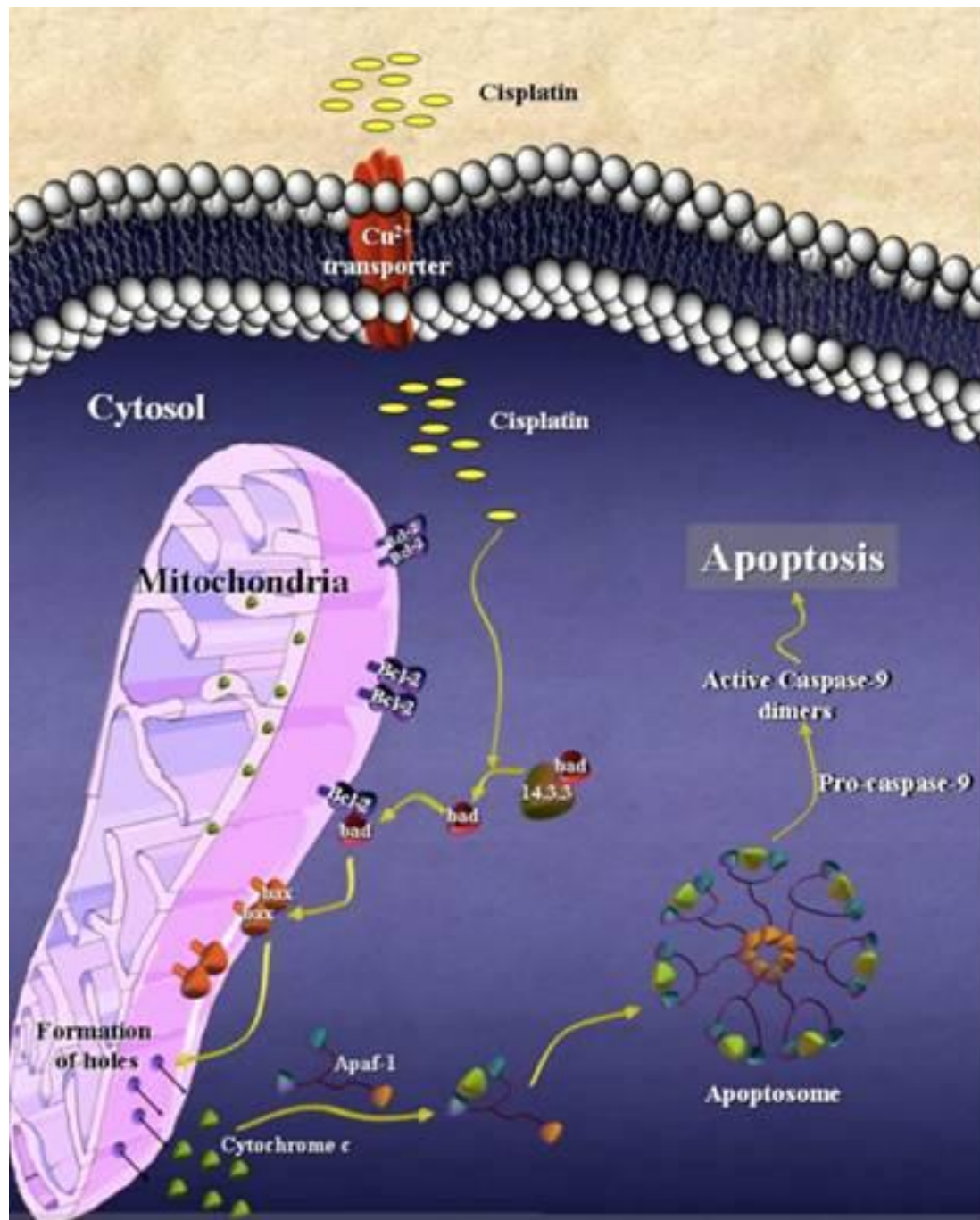


Figure 1.6 - Induction of the mitochondrial apoptotic pathway by cisplatin. A cartoon explaining how cisplatin activates the proapoptotic protein Bax resulting in the release of cytochrome c from the mitochondrial intermembrane space to the cytosol to induce the formation of the apoptosome (Apaf-1 cytochrome c complex); this step is being followed by binding and activation of procaspase-9 to the inner "hub" region of the apoptosome leading to activation of procaspase-3 (taken from “Molecular mechanisms of cisplatin and its liposomally encapsulated form, Lipoplatin™. Lipoplatin™ as a chemotherapy and antiangiogenesis drug” by T. Boulikas).

The role of mitochondria in healthy vs. cancer cells is discussed in more detail below:

Mitochondria are important in maintaining normal function and homeostasis

The role of mitochondria is fundamental in energy production, calcium homeostasis (Clapham 2007), apoptosis and ROS generation (Green and Reed 1998). Their main sources of fuel include glucose, fatty acids, and various amino acids, used for ATP generation through oxidative phosphorylation. The Krebs cycle which takes place in the mitochondria assists in NADH production, which, through the respiratory chain, is an important component of electron transportation and eventually creation of transmembrane potential ($\Delta\Psi$), subsequently used for production of ATP in complex V (Wang, Ogasawara et al. 2010).

Impaired mitochondrial function in cancer

Many cancer cells appear with changes in their mitochondrial structure and function compared to normal cells, including changes in size, shape and number, with fast growing tumour cells appearing to have fewer and smaller mitochondria (Fantin, Berardi et al. 2002); (Toogood 2008); (Modica-Napolitano and Singh 2004) or altered inner mitochondrial membrane proteins (Chang, Schnaitman et al. 1971); (Irwin, Malkin et al. 1978). Mitochondrial dysfunction in the form of altered ATP generation or abnormal increases in ROS has also been observed in cancer cells, as demonstrated by (Chen, Lu et al. 2007) and (Pelicano, Carney et al. 2004). Other examples of impaired mitochondrial function include decreased reparatory complex activities in the mitochondria of hepatoma cells (Chan and Barbour 1983); (Sun, Sepkowitz et al. 1981) and reduced ATPase activity (Pedersen and Morris 1974). Even though mitochondria possess an adequate base excision repair (BER), mutations in the mitochondrial DNA (mtDNA) or decreased activities in the DNA mismatch repair (MMR) system genes, such as *Mlh1*, can also lead to microsatellite instability, and subsequently cancer (Chen, Lu et al. 2007). Increased mitochondrial transmembrane potential (Bernal, Lampidis et al. 1982); (Johnson, Walsh et al. 1981) has also been observed in cancer cells. Since mitochondria are major sources of ROS, increased levels in cancer might mean increased leakage of electrons from the respiratory chain (complexes I & III). This electron leakage may lead to reaction with oxygen molecules forming superoxides and then hydrogen peroxides. Thus, increased ROS in cancer may be a sign of mitochondrial dysfunction - even though other non-mitochondrial sources may also be considered (Pelicano, Lu et al. 2009); (Wang, Ogasawara et al. 2010).

Targeting mitochondria as a cancer treatment

It is possible that the differences in mitochondrial structure and function of cancerous cells may render them susceptible to drug-induced damage. For example, targeting Bcl-2 proteins may force the increase of mitochondrial permeability and release of apoptotic factors. Also, the increased dependence of the cancer cells on glycolysis (due to impaired mitochondrial function) may lead to preferential cell death through the use of glycolytic inhibitors (i.e. 3-bromopyruvate (3-BrPA) or lonidamine) (Chen, Lu et al. 2007) (Armstrong 2006); (Dias and Bailly 2005); (Galluzzi, Larochette et al. 2006); (Morrison, Andera et al. 2009); (Ralph and Neuzil 2009).

However, it should be noted that not all mitochondrial alterations should be generalized for all cancer cells. The alterations may vary depending on the type, origin, stage, metastatic state of the cancer cells, and even specific tumour microenvironments such as hypoxia (Wang, Ogasawara et al. 2010).

1.2 Metabolism of cancer & the Warburg effect

Metabolism is an important aspect of the carcinogenetic process as cancer cells show increased metabolic rates and an extreme demand for energy (Coloff and Rathmell 2006). Cancer cells also exhibit enhanced glucose uptake, which is known as the Warburg effect (Gatenby and Gillies 2004). Paradoxically, glucose is mostly anaerobically metabolized to lactate, providing much less ATP and energy than aerobic respiration; however, cancer cells have selectively developed adaptations to hypoxic conditions (Brahimi-Horn, Chiche et al. 2007). In normal cells apoptosis can occur when the cells are found in an acidic environment. However, cancer cells are able to survive or even thrive in a low pH environment and unfavourable conditions (De Mito and Fais 2005). It has also been reported that the higher the glycolysis rate, the more aggressive the cancer is in terms of invasion and metastasis (Pelicano, Martin et al. 2006). Other metabolic changes in cancer, including ovarian cancer, involve switching from high glycerphosphocholine (GPC) and low phosphocholine (PC) to low GPC and high PC concentrations (Iorio, Mezzanzanica et al. 2005). So, metabolic differences between cancerous and non-cancerous cells could serve as biomarkers of cancer detection (Griffin and Shockcor 2004).

Figure 1.7 illustrates the differences between oxidative phosphorylation, anaerobic glycolysis, and aerobic glycolysis (Warburg effect).

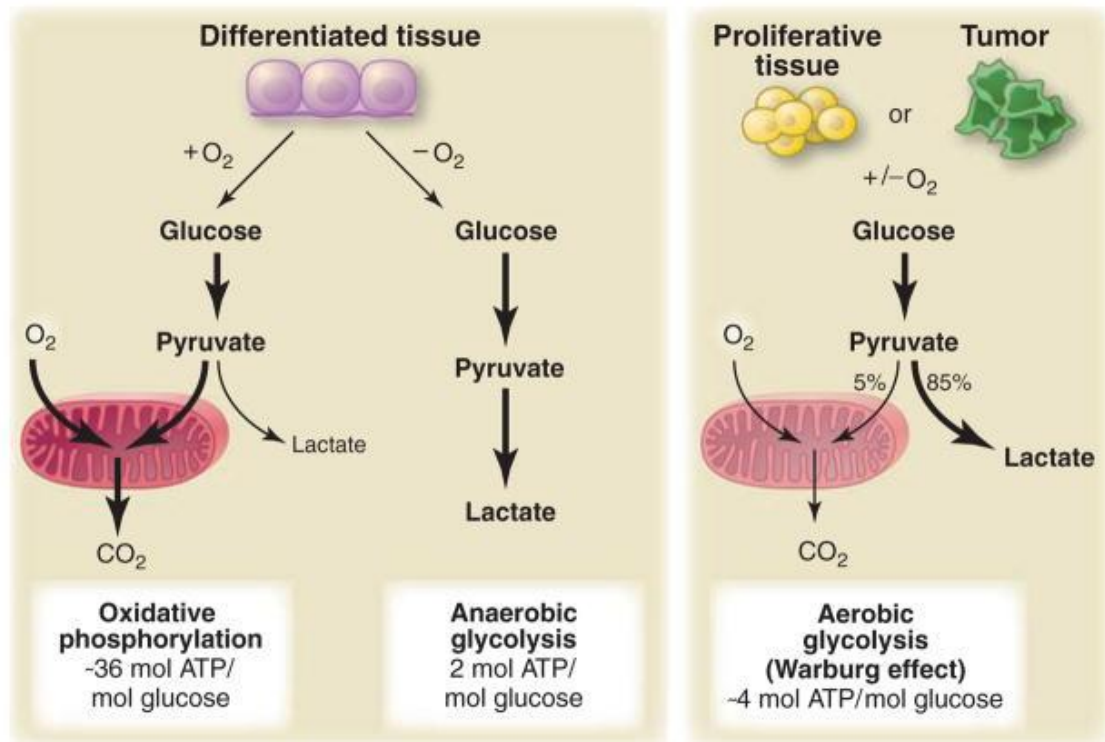


Figure 1.7 - Schematic representation of the differences between oxidative phosphorylation, anaerobic glycolysis, and aerobic glycolysis (Warburg effect). In the presence of oxygen, nonproliferating (differentiated) tissues first metabolize glucose to pyruvate via glycolysis and then completely oxidize most of that pyruvate in the mitochondria to CO₂ during the process of oxidative phosphorylation. Because oxygen is required as the final electron acceptor to completely oxidize the glucose, oxygen is essential for this process. When oxygen is limiting, cells can redirect the pyruvate generated by glycolysis away from mitochondrial oxidative phosphorylation by generating lactate (anaerobic glycolysis). This generation of lactate during anaerobic glycolysis allows glycolysis to continue (by cycling NADH back to NAD⁺), but results in minimal ATP production when compared with oxidative phosphorylation. Warburg observed that cancer cells tend to convert most glucose to lactate regardless of whether oxygen is present (aerobic glycolysis). This property is shared by normal proliferative tissues. Mitochondria remain functional and some oxidative phosphorylation continues in both cancer cells and normal proliferating cells. Nevertheless, aerobic glycolysis is less efficient than oxidative phosphorylation for generating ATP. In proliferating cells, ~10% of the glucose is diverted into biosynthetic pathways upstream of pyruvate production (Vander Heiden, Cantley et al. 2009).

Tumours commonly undergo extreme reprogramming of their basic metabolism, but it is still not very clear if these changes are brought about by tumourigenesis or they are themselves the cause of cancer development. This metabolic switch or transformation can give selective growth advantages and even resistance to apoptosis (Jones and Thompson 2009).

Figure 1.8 below shows a detailed representation of the overall metabolic processes that occur inside a cancer cell.

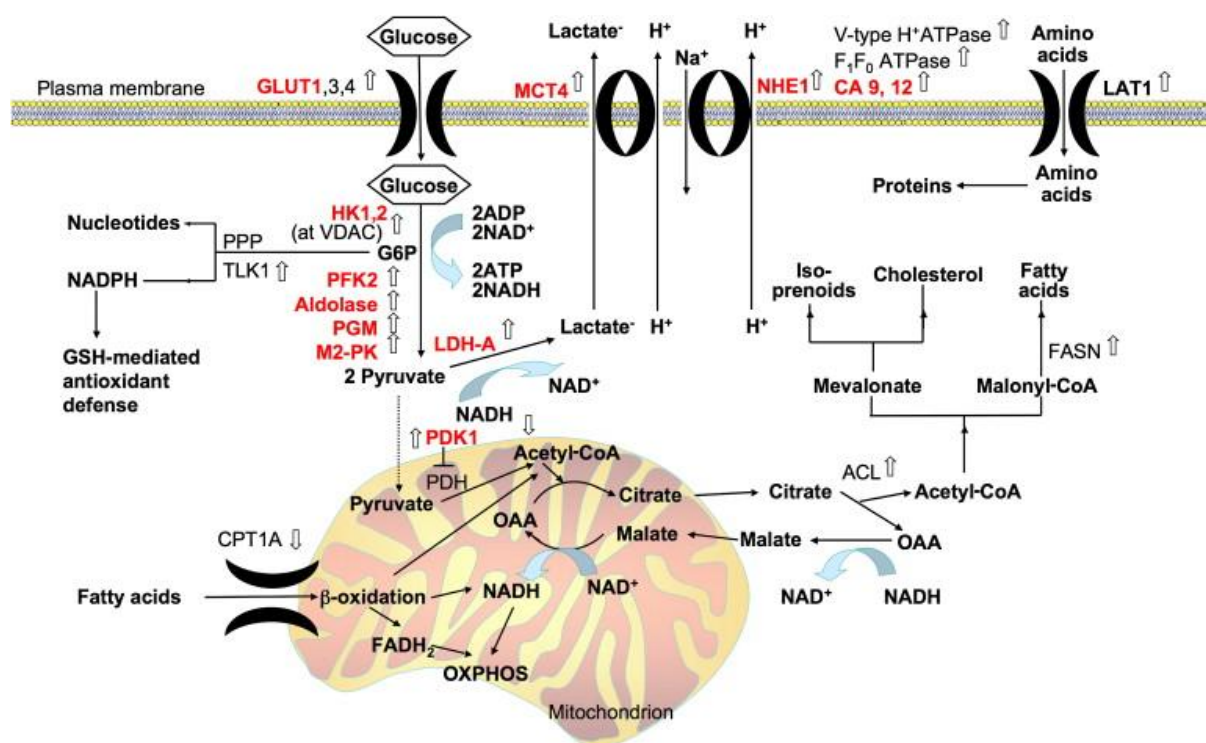


Figure 1.8 - Metabolic processes inside a cancer cell. In normal cells, aerobic glycolysis implies the conversion of glucose via pyruvate into acetyl-CoA and its complete oxidation (through the mitochondrion-localized tricarboxylic acid [TCA] cycle and oxidative phosphorylation) to CO₂ and H₂O (which generates 38 ATP molecules per molecule of glucose). In contrast, in tumour cells, glycolysis tends to be aborted at either of two steps. First, aerobic glycolysis in tumour cells implies conversion of glucose into pyruvate (which generates only two ATP molecules per molecule of glucose) and subsequently into the waste product lactic acid. Second, in tumour cells, acetyl-CoA tends to be introduced into a truncated TCA cycle, with the net result that acetyl-CoA is exported into the cytosol and serves as a building block for cell growth and proliferation. In this truncated TCA cycle, citrate is preferentially exported to the cytosol via the tricarboxylate transporter. Once in the cytosol, citrate is cleaved by ATP citrate lyase (ACL) to generate oxaloacetate and acetyl-CoA. Oxaloacetate is reduced to malate, then reimported into mitochondria and reconverted to oxaloacetate in the matrix (while generating NADH that represses the TCA cycle), and it reacts with acetyl-CoA to complete the substrate cycle. Small arrows pointing up or down indicate cancer-associated upregulation/activation or downregulation/inhibition of enzymes, respectively. Alterations indicated in red can be caused by the activation of HIF-1. CA9 and CA12, carbonic anhydrases 9 and 12; CPT, carnitine palmitoyltransferase; GLUT, glucose transporter; GSH, glutathione; HIF, hypoxia-inducible factor; IDO, indoleamine 2,3-dioxygenase; HK, hexokinase; OXPHOS, oxidative phosphorylation; LAT1, L-type amino acid transporter 1; LDHA, lactate dehydrogenase isoform A; MCT, monocarboxylate transporter; PDH, pyruvate dehydrogenase; PDK, pyruvate dehydrogenase kinase; PFK, phosphofructokinase; PI3K, phosphatidylinositol 3-kinase; PGM, phosphoglycerate mutase; PKM2, pyruvate kinase isoform M2; PPP, pentose phosphate pathway; SCO2, synthesis of cytochrome c oxidase 2; TLK, transketolase; VDAC, voltage-dependent anion channel. (Figure & text taken from “Tumor Cell Metabolism: Cancer's Achilles' Heel” by (Kroemer and Pouyssegur 2008).

1.3 Metabonomics and metabolic footprints

Metabolic profiling includes metabonomics and metabolomics focusing on the analysis of metabolites in entire systems and helping in the understanding of biological mechanisms, including cancer. Metabonomics is the study of metabolic responses to various stimuli such as drugs, environmental changes and diseases (Nicholson, Lindon et al. 1999). Metabonomics is complementary to genomics, which studies the genome, and proteomics, which is the study of the proteins of an organism, and it involves the study of an organism's *metabolome* (Lindon, Nicholson et al. 2000). Metabolomic analysis is often conducted on biofluids, which can be obtained relatively non-invasively and without much discomfort, as with the collection of samples of urine or blood serum. Other fluids can also be used such as cerebrospinal or seminal fluid. It is also possible to use cell culture supernatants or tissue extracts (Lindon, Nicholson et al. 2000).

As cells secrete a large number of metabolites into their external environment, this may reflect metabolic activity in the cells and also the level of transcription and translation of various genes (Kell, Brown et al. 2005). Therefore, a universal measurement of secreted metabolites might be used to analytical advantage. Metabolic footprinting is a recently-developed method for the functional analysis and characterization of cells by defining the extracellular metabolome (Kell, Brown et al. 2005). It is a strategy for the analysis of cells and tissues by studying, in a high-throughput manner, the metabolites that they excrete, take or fail to take from their environment.

1.4 Tools and techniques in metabolic analysis

One of the most useful tools in metabolomics and in the study of cancer metabolism is the phenomenon of nuclear magnetic resonance (NMR), which is exploited in research through NMR spectroscopy. Advantages of NMR-based metabonomics include a relatively quick and easy sample preparation, the use of small sample size (usually less than 0.5 ml of fluid or 20 g of tissue). Also, the fact that it is relatively non-destructive and that the data collection can yield results within a few minutes make NMR-based metabonomics extremely advantageous (Nicholson, Lindon et al. 1999; Lindon, Nicholson et al. 2000). NMR is also employed in the study of intact tissues in magic angle spinning (MAS) NMR spectroscopy.

1.5 The need for metabolic biomarkers

No screening test has yet been shown to effectively reduce the risk of fatality in ovarian cancer and is one of the main reasons that ovarian cancers are not detected early in development. The generation of a routine screen test for the early-stage detection of ovarian cancer could be invaluable in decreasing mortality. Additionally, the likelihood of relapse or developing resistance may also be pre-empted through such screening following successful treatment as well as during treatment to monitor successful response to drugs.

Biomarkers could be a means of allowing clinicians to recognize and detect ovarian cancer at an early stage. That's why the development of new biomarkers for ovarian cancer is needed for the improved detection and monitoring of the disease. Furthermore, metabolic biomarkers have an extra advantage; that of being easy to measure in the clinic by using non-invasive *in vivo* tools such as, for example, PET or MRI.

In addition, platinum biomarkers are essential in optimizing the value of administering a platinum drug to patients by tailoring these to individual patient needs. Biomarkers could help in determining which patients can be managed by such treatment and if the treatment and its continuation are suitable for them, thus, preventing the patients from unnecessary discomfort and distress.

It is not however possible that a single biomarker would be capable of producing sufficient prognostic value, as the intrinsic biology of the tumour and the host response may influence the results. This is where examination of baseline cancer profiles and their metabolic signatures become useful as they can look at the whole cancer metabolome from an elevated point of view, with the ability to get closer looks at specific metabolites that seem to be important in the carcinogenetic process.

1.6 NMR-detectable metabolites

A big variety of metabolites can be identified and quantified with NMR spectroscopy. Key metabolites that may have some kind of association with cancer include the following presented in table 1.2:

NMR-detectable metabolites
alanine
acetate
aspartate
choline
creatine
formate
glucose
glutamate
glutathione
glycerophosphocholine
glycine
isoleucine
histidine
lactate
leucine
<i>myo</i> -inositol
phenylalanine
phosphocholine
pyruvate
succinate
taurine
valine

Table 1.2 - list of NMR-detectable metabolites

1.7 General aims of this study

In this thesis, the main theme of investigation will involve the metabolic examination of platinum resistance in ovarian cancer chemotherapy through the employment of a number of different analytical techniques and platforms, such as NMR, molecular biology and bioinformatics-based approaches, with the aim to generate a much-needed phenotype of platinum resistance.

In the first part of the investigation (chapter 3), this study will deal with the metabolic examination of two *in vitro*-derived cell models of platinum resistance: a) a cell model that consists of kidney cell lines of common genetic background involving selective mitochondrial depletion for examining the association of *Mlh1* to mitochondria and platinum resistance, and b) a model of platinum resistance that consists of well-established ovarian cell lines that are sensitive or resistant to platinum drugs.

The following chapter (chapter 4) will follow a bioinformatics-based approach to compare the metabolic findings of the 3rd chapter to the microarray gene expression data of the publicly available NCI-60 panel which consists of a variety of different cancer cell lines. This approach will take advantage of a newly-developed method by this lab, involving over-representation analysis (ORA) and neighbourhood-based entities (NESTs).

Finally, the examination presented in chapter 5 will focus on the study of a clinically-derived cell model of platinum resistance which will involve the use of isogenic ovarian cancer cells that are either sensitive or resistant to the platinum drug cisplatin after the application of chemotherapy. This examination will involve exposure of the cells to normoxic and hypoxic conditions in order to study the generated intracellular and extracellular metabolic concentration changes and possibly link them to platinum resistance.

Overall, this pre-clinical metabolic-based investigation may assist in the goal towards biomarker identification that has a diagnostic and predictive value in the clinical environment in terms of patient chemotherapy response, and, thus, at times avoid unnecessary side-effect producing treatment.

Chapter 2

Methods

2.1 Molecular biology methods

2.1.1 Cell culture

i) Isogenic ovarian cancer cell lines

All cell lines used in these experiments were grown in sterile tissue culture flasks (Corning[®],UK) and maintained in complete RPMI-1640 (Sigma-Aldrich, UK; cat no: R5886), growth medium with 10% fetal bovine serum (Sigma-Aldrich, UK; cat no: F6178) and 1% L-glutamine, penicillin, streptomycin solution (including 10,000 units of penicillin, 10,000 µg of streptomycin, and 29.2 mg of L-glutamine/ml in 0.85% saline) by Sigma-Aldrich, UK; cat no: G6784. All cells were grown at 37 °C in a SANYO MCO-18M humidified incubator in sterile standard cell culture conditions (5% CO₂ and 21% O₂). For the hypoxic experiments the cell culture incubation conditions were set at 1% O₂, 5% CO₂ at 37 °C, with hypoxia being achieved after approximately 20 minutes from the start of the experiment.

Establishment of the isogenic ovarian cancer cell lines

Two pairs of cell lines were used during the main study, originally established by Langdon and his colleagues (Langdon, Lawrie et al. 1988): PEA1 & PEA2 and PEO1 & PEO4 while an additional pair (PEO14 & PEO23) was used during pilot experiments.

Each pair of cell lines was derived from the ascites or pleural diffusion of patients suffering from ovarian adenocarcinoma before and after the clinical appearance of cisplatin resistance (Langdon, Lawrie et al. 1988). According to Langdon, each set of cell lines was found to be morphologically distinct. Details of their clinical characteristics are given in table 2.1.

Cell line	Morphology	Site	IC50 (µM)
PEA1	Poorly differentiated adenocarcinoma	PE	7.07
PEA2		PA	10.23
PEO1	Poorly differentiated serous adenocarcinoma	PA	1.33
PEO4		PA	11.60
PEO14	Well-differentiated serous adenocarcinoma	PA	1.84
PEO23		PA	10.77

Table 2.1 – *in vivo* and *in vitro* characteristics of patients from whom the isogenic ovarian cancer cell lines were derived. (PE = Pleural Effusion, PA = Peritoneal Ascites)

All isogenic ovarian cancer cell lines used in this study and their corresponding cisplatin IC50 average values were kindly provided by Dr Euan Stronach & Professor Hani Gabra from the Department of Oncology, Faculty of Medicine, Imperial College, London.

ii) Ovarian and kidney cancer cells used in the *MLh1* study

The cells used for the metabolic examination of *Mlh1* involved the following cancer cell lines of ovarian (platinum resistance model) and kidney (HEK-293T model) origin (Table 2.2). All cells in this study were kindly provided by Prof Bob Brown and Dr Vincent O'Brien of Imperial College, London, and Sitemic Scotland Limited, Glasgow, respectively.

HEK-293T Model (Kidney)
HEK-293T MutL α +
HEK-293T MutL α -
HEK-293T MutL α + p0
HEK-293T MutL α - p0
Platinum Resistance Model (Ovarian)
A2780
CP70
CP70 E1
CP70 A2

Table 2.2 – list of cancer cell lines of the models used in the *Mlh1* study

The ovarian cancer cells were cultured in standard conditions as described earlier, and grown in RPMI-1640 media (Gibco, UK; cat no: 31870-025), while the kidney cancer cells were grown in DMEM media (Gibco, UK; cat no: 11960-044). Both media were supplemented with glutamine (Gibco, UK; cat no: 16000-036) and 10% FBS (Autogen Bioclear, UK; cat no: 7.01). All cells were placed in culture for 48 hours before harvesting and counting (5 million cells for each cell line sample). All drug selection exposures imposed on the cells were removed 48 hours before cell harvesting. A detailed description on these cell lines and their characteristics can be found in the methodology section of chapter 3.

2.1.1.2 Cell harvesting

Harvesting of cells using trypsinisation method

Under sterile conditions, and at approximately 70% confluence, cells were detached from their T75 flasks first by removing the media, washing with 5 ml of phosphate buffer saline (PBS) (Sigma-Aldrich, UK; 56064C) and then by adding 5 ml of warmed 10% trypsin in EDTA (Sigma-Aldrich, UK; cat no: 59429C). This reaction was left to continue at 37°C, 5% CO₂ for 5 minutes or until all the cells were detached, and then stopped by the addition of 5ml of fresh culture media. The resulting cell mixture was transferred into sterilized tubes (BD Falcon™, UK) and centrifuged for 5 minutes at 125g in a Beckman C5-6R centrifuge, after which the supernatant was aspirated off. The cell pellet was washed twice by adding 5 ml of cold PBS, followed by resuspension and centrifugation at 125g for 5 minutes. The PBS was suctioned off again and the cells were resuspended in media (15 ml per T75 flask).

Harvesting cells by direct methanol extraction method

For the direct methanol extraction method, the media in the culture vessels were transferred into sterilised tubes. The media samples were then centrifuged (4 °C, 150g, 4 min) to pellet any dead cells and transferred into new sterilised tubes. They were stored at -40 °C until further use. The cells in the flasks were washed twice using 3 ml of cold (4 °C) PBS. The cells were then quenched by adding 3 ml of cold methanol (kept at 4 °C) and allowed to lyse for 2 minutes at room temperature. The cellular material was detached from the culture vessel using a cell scraper. The resulting suspension of cellular material was then transferred from the culture vessels to Eppendorf tubes. The suspensions were dried down overnight using a rotary speedvac at room temperature (RT) and then stored in the -40°C freezer until use.

2.1.1.3 Cell storage

For possible future use, stocks of the cells were frozen down by following the trypsinisation method, except that 1ml of freeze media consisting of 90% FBS and 10% dimethyl sulfoxide (DMSO) (Sigma-Aldrich, UK; cat no: D2650) was used for their resuspension at the final step before their transfer into appropriate cryogenic tubes. The cryogenic tubes containing the cells were immediately transferred to a -80°C freezer inside a polystyrene box in order to prevent a fast rate of freezing and thus reducing cryogenic related cell damage. Within 24 hours, the cells were subsequently transferred into liquid nitrogen for long term storage.

When more cells were needed for growth, the cryogenic vials were removed from the freezer and quickly defrosted in a 37°C water bath. This was followed by centrifugation of the cell solution for 125g for 5 minutes and transfer into a small T25 culture flask with 7 ml of cell culture media. The flasks with the cells were then transferred into the incubator (21% O₂, 5% CO₂ at 37°C).

2.1.1.4 Cell counting

Cell counts using an automatic cell counter

After cell detachment, 0.5 ml of cell suspension was transferred into Beckman-Coulter Accuvette vials containing 9.5 ml of Isoton (1:20 dilution) and counted using a Beckman-Coulter Z2 series cell counter giving the number of cells per ml, which was then adjusted to give the total number of the cells in the suspension.

Cell counts using a haemocytometer

Alternatively, 20 µl of cell suspension was mixed with an equal amount of 0.4% Trypan Blue dye (Sigma-Aldrich, UK; cat no: T8154) in an Eppendorf. 10 µl of this mixture was pipetted onto both sides of the haemocytometer after having placed an appropriate glass cover slip on top of the cell counting chamber. Cells were counted using the middle 25 squares of the cell counting chamber, starting the counting from the top and left of each square. Cells were measured from both sides of the haemocytometer before the procedure was repeated 4 times in total for increased accuracy, while washing the haemocytometer with ethanol in between counts.

The number of cells was calculated by using the following equation:

$$\text{Total No. of cells in suspension} = \text{No. of cells} \times 2 \times 10^4 \times \text{total volume of cell suspension}$$

2.1.1.5 Drug treatments and cell proliferation assays

For the cytotoxicity experiments and confirmation of their IC₅₀ values, a freshly-prepared cisplatin solution (Sigma-Aldrich, UK; cat no: P4394) was added in 96 well plates (Corning®,UK) in concentrations ranging from 0.1 to 1000 µM over a period of 72 hours and tested at frequent time intervals. Other treatments (data not shown) included cell exposure to (50 µM of L-Buthionine-sulfoximine (L-BSO) (Sigma-Aldrich, UK; cat no: B2515) which is

a glutathione-depleting agent, for confirmation purposes when examining glutathione concentrations during NMR-based experiments (used as a proof of principle). Control wells were treated in the same way using the corresponding vehicle agents.

A number of different assays were performed in both pilot and subsequent experiments for optimisation and cross-confirmation purposes and included the following assay kits:

(i) alamarBlue® assay

For the study and measurement of cell growth (or inhibition of cell growth) during drug treatments, cells were plated in 96-well plates, and, where necessary, 100 µl of cell culture media were added with 50 µM of L-BSO and / or varying doses of cisplatin as mentioned above. The alamarBlue® dye (AbD Serotec, UK; cat no: BUF012B) was added after another 24 hours and the absorbance at 570 nm and 600 nm was measured using a Tecan Sunrise™ absorbance reader, after prior calibration of the appropriate incubation time and cell density. The percentage of reduced alamarBlue®, which is linearly correlated to cell number, could then be calculated.

According to manufacturer, to calculate the percent difference in reduction between treated and control cells in cytotoxicity and proliferation assays the following equation was used:

$$\text{Percent difference between treated \& control cells} = \frac{(O2 \times A1) - (O1 \times A2)}{(O2 \times P1) - (O1 \times P2)} \times 100$$

Where: O1 = molar extinction coefficient (E) of oxidized alamarBlue® at 570 nm

- O2= E of oxidized alamarBlue® at 600 nm
- A1 = absorbance of test wells at 570 nm
- A2 = absorbance of test wells at 600 nm
- P1 = absorbance of positive growth control well (cells plus alamarBlue® but no test agent) at 570 nm
- P2 = absorbance of positive growth control well (cells plus alamarBlue® but no test agent at 600 nm

Wavelength	Reduced (R)	Oxidized (O)
540 nm	104395	47619
570 nm	155677	80586
600 nm	14652	117216
630 nm	5494	34798

Table 2.3 - Molar extinction coefficients for alamarBlue® at different wavelengths

(ii) Sulforhodamine B-based (SRB) assay

The SRB assay (Sigma-Aldrich, UK; cat no: TOX6) originally developed by Skehan *et al.* was used for cell density determination of cisplatin cytotoxicity in a 96-well based format, in terms of cellular protein content measurement of viable and non-viable cells. After a 24-hour attachment and the appropriate drug incubation period, cell monolayers were fixed with 10% (wt/vol) trichloroacetic acid (TCA) and incubated for 60 minutes at 4°C, after which the cellular debris was removed by repeated washes with water. The cells were air-dried and stained in 0.4% Sulforhodamine B solution in a volume 50% of the original cell culture media volume. After a staining period of 30 minutes the excess dye was removed by a quick wash using 1% (vol/vol) acetic acid. The cells were air-dried and the protein-bound dye was dissolved in 10 mM Tris base solution for optical density determination at a wavelength of 490 nm using a Tecan Sunrise™ microplate reader after room temperature incubation on a Stuart® Scientific Gyro rocker at 30 rpm for enhanced mixing.

The cell assay data were analysed using the plate-supplied Magellan software.

2.2 NMR Spectroscopy

2.2.1 Basic theory

2.2.1.1 The phenomenon of nuclear magnetic resonance

Nuclear magnetic resonance, or NMR, is a phenomenon occurring when the nuclei of particular atoms are positioned in a static magnetic field and then exposed to an oscillating magnetic field. Not all nuclei experience this phenomenon as this depends on whether these nuclei possess a property called spin (Figure 2.1) (Claridge 2004).

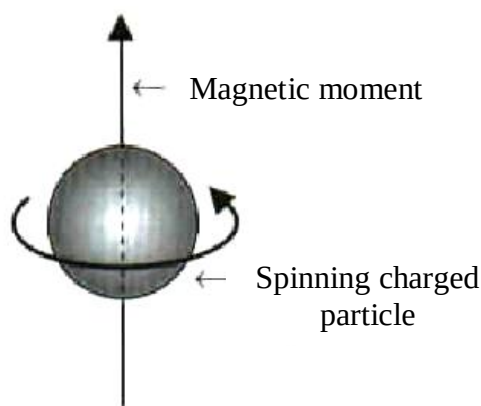


Figure 2.1 - A charged spinning particle (i.e. a proton) generates a magnetic moment. The direction of the generated magnetic moment depends on the particle's rotational direction (taken from Magnetised Nuclear Spin Systems. Last accessed 26 July 2010).

2.2.1.2 NMR spectroscopy

NMR spectroscopy is a technique employed to obtain physical, chemical, electronic and structural information about molecules. There are various types of NMR spectroscopy, such as ^{31}P NMR, ^{19}F NMR, ^{15}N NMR and ^3He NMR, but the most commonly used is ^1H NMR – which also constituted the main technique used in this study (Claridge 2004).

^1H NMR spectroscopy

High resolution ^1H NMR spectroscopy is the application of nuclear magnetic resonance in spectroscopy with respect to hydrogen-1 protons found in the molecules of a sample (Silverstein 2005). It has been shown to be one of the best techniques for the analysis of biofluids and for determining the structure of molecules, due to the fact that hydrogen is very abundant in biological systems and its nucleus is very sensitive to NMR signals (Claridge 2004).

NMR spectrometers

A typical ^1H NMR spectrometer (NMR magnet) is seen in Figure 2.2 while figure 2.3 illustrates its main internal parts, as well as the instruments used for spectral detection and acquisition.



Figure 2.2 - A typical NMR spectrometer operating at 600 MHz (taken from Bruker BioSpin, UltraShield Plus Magnets Delivering Ultimate Shielding Performance. Last accessed 15 August 2010).

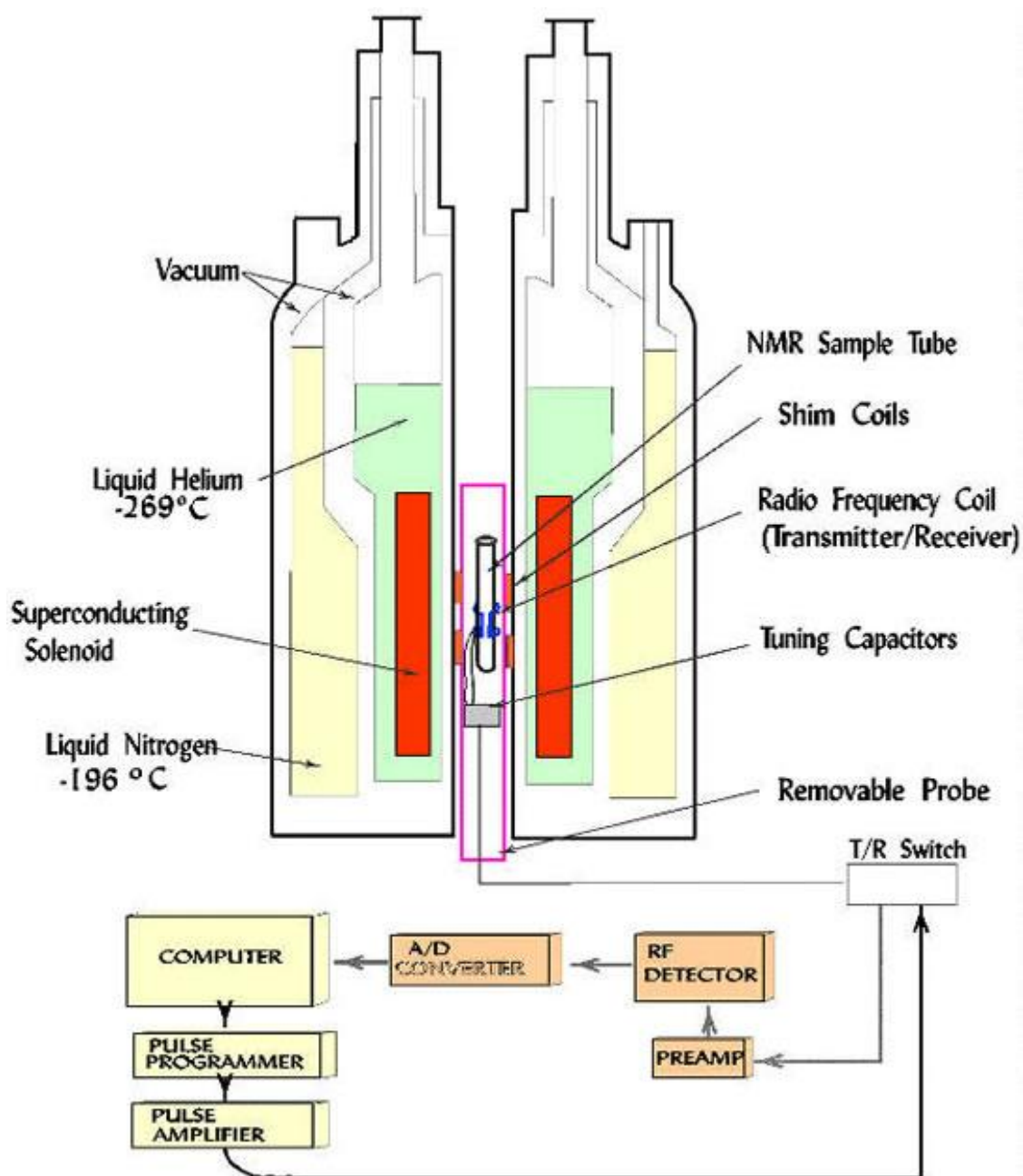


Figure 2.3 - A typical illustration of an NMR spectrometer's internal parts and auxiliary instruments used in detection and acquisition of NMR spectra (text and figure taken from Lakehead University Instrumentation Laboratory, *NMR Theory of Operations*. Last accessed 04 September 2010).

Nuclear spin and resonance

The property of spin is fundamental in NMR. All atoms have nuclei with a characteristic nuclear spin quantum number, I , with values multiples of $\frac{1}{2}$ that can be positive or negative. The nuclei that have an 'I' value of zero ($I = 0$) do not possess a nuclear spin and, thus, cannot exhibit a nuclear magnetic resonance (NMR-silent) (Claridge 2004). Some elements

like carbon-12 do not have a nuclear spin as their atomic mass and number are even; however the majority of isotopes possess a nuclear spin that can in principle be observed by NMR (Claridge 2004). The spinning nucleus carries charge and while spinning it possesses a magnetic moment, μ . The magnetic moment can be calculated by the formula:

$$\mu = \gamma P,$$

where γ is the *magnetogyric ratio* (which is constant for any nuclide and is an indication of how magnetically strong a nucleus is) and P is the *angular momentum* (caused by a non-zero I number). Both of the magnetic moment and angular momentum have magnitude and direction and can be represented as vectors (Claridge 2004).

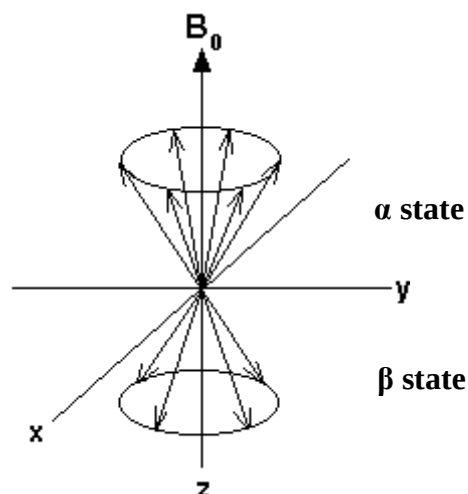


Figure 2.4 – precessing magnetic moments of nuclei on the x-y-z plane, inside an external static magnetic field (B_0). The field is conventionally applied along the z- axis of the Cartesian co-ordinate frame. The nuclei motions are represented as vectors moving across the surface of a cone (figure and text taken from John C. Edwards, *Principles of NMR*. Last accessed 23 July 2010).

Inside an external static magnetic field (B_0) all the microscopic magnetic moments align themselves in the direction of the field as their energy states become quantised. The nuclei are able to take $2I+1$ possible orientation inside the magnetic field. The half-spin nuclei can take two possible orientations with respect to the static magnetic field, α and β (Figure 2.5). The α state has a component of the magnetic moment parallel to the field and is of lower energy, while in the β state is anti-parallel to it. This difference in energy is termed as ΔE ,

and is dependent on the strength of the interaction between the magnetic field and the nucleus (Claridge 2004). It is calculated by the formula:

$$\Delta E = h\nu = h\gamma B_0/2\pi,$$

where ν is the frequency of the absorbed radiation and h is Planck's constant (Claridge 2004).

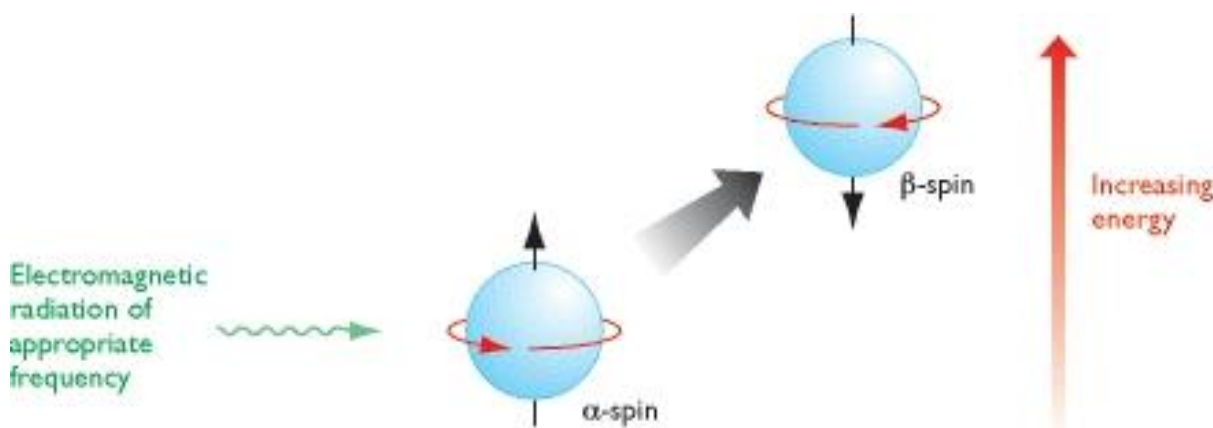


Figure 2.5 - A rotating nucleus can take up either of two orientations in an applied electromagnetic field. The energy separation between the α and β spin states is determined by measuring the frequency of electromagnetic radiation needed to induce an $\alpha \rightarrow \beta$ transition (figure and text taken from Bios Scientific Publishers, *Assembly of the Transcription Initiation Complex*. Last accessed 18 August 2010).

When the nucleus is placed inside a static magnetic field it is forced to *precess*. This precession depends on the strength of the field and the magnetogyric ratio of the spin and is referred to as the *Larmor precession*. Related to the Larmor precession is the *Larmor frequency* of the nucleus, which is the resonant frequency of its spin. Irradiation at the Larmor frequency can cause a ΔE transition in the spin state of the nuclei. When irradiating the nuclei at the Larmor frequency they interchange between the lower energy and the higher energy state. When this is achieved, they nuclei are said to be ‘in resonance’ with the applied radiation (Claridge 2004).

Modern NMR spectrometers employ very strong field strengths with operating frequencies as high as 800 MHz or more, which, for protons, correspond to their resonant frequencies. The intensity of the NMR signal is measured by integrating the area under the generated peak's

resonance line and is proportional to the number of nuclei that give rise to that signal (Claridge 2004).

The vector model of NMR

In order for the nuclear magnetic resonance to occur by inducing transitions between energy states, the application of a time-dependent oscillating magnetic field at the Larmor frequency of the spin is necessary. This field is designated B_1 to distinguish it from the static B_0 field. When the field is static (B_0), nuclei will precess at a random distribution of orientations, with only a small excess being in the lower energy state (parallel to the applied field). This results in the creation of various *magnetic moment vectors* (Claridge 2004). When an oscillating magnetic field (B_1) is applied through a short intense pulse of radiofrequency (passed around a coil placed around the sample in the magnet), the nuclei in the sample become excited. This way, transitions between spin states are brought about and phase coherence is imposed on the randomly distributed molecular magnetic moment vectors. For example, following a typical 90° pulse, the individual spin vectors cluster together along the y-axis of the x-y plane, and are said to be in *phase coherence* (Claridge 2004). To simplify the representation of all the magnetic vectors, the bulk magnetisation vector, M_z or M_0 , term is used, which is the sum of all the individual vectors together (Figure 2.6).

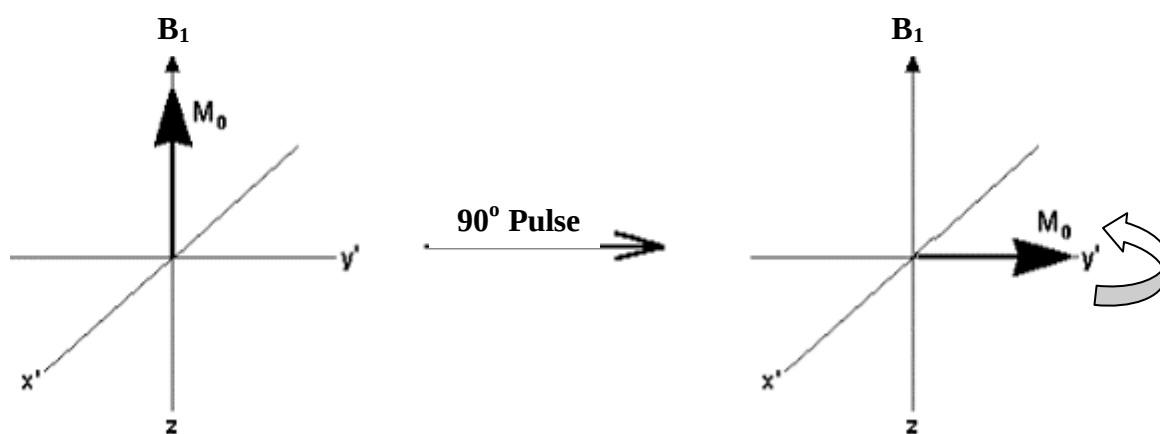


Figure 2.6 - Application of a radiofrequency pulse in an oscillating magnetic field B_1 on the bulk magnetisation vector M_0 , drives it from its equilibrium position toward the x-y plane from the z- axis, causing its rotation around the x- and y- axes, before returning back to equilibrium (taken from John C. Edwards, *Principles of NMR*. Last accessed 23 July 2010).

Chemical shifts and couplings

Nuclei are surrounded by circulating electrons whose movements are induced by the externally-applied magnetic field. These moving electrons create their own magnetic field, which in turn results in the “shielding” of the various nuclei from the external magnetic B_0 field. As a result, nuclei found in different chemical environments give rise to signals of different resonance frequencies. The highly-shielded nuclei will emit at lower frequencies, whereas the nuclei with less electron-generated shielding will give rise to higher frequencies (Claridge 2004). The term “*chemical shift*” is given to the shift in resonance frequency caused by the chemical environment in which the nucleus is found and is denoted as δ . Its position is not absolute but measured relatively to standard compounds, one of which is trimethylsilylpropionate (TSP). It is measured in parts per million (ppm) by the following formula:

$$\delta = (\nu - \nu_{\text{ref}}) / \nu_{\text{ref}} \times 10^6,$$

where δ is the chemical shift, ν is the frequency of the signal of interest, and ν_{ref} is the frequency of the standard (Claridge 2004).

Chemical shift is a very important parameter in NMR as it allows observation of nuclei either on their own or in groups and it very helpful in drawing conclusions about their chemical environment and even molecule structure. In a molecule, it's not uncommon for magnetic interactions to occur between nuclei of neighbouring atoms, thus, giving rise to NMR signals which provide useful information on their structural anatomy. It is also important to note that individual signals may be split into several more lines in the spectra, as a result of the interaction with neighbouring nuclei. This phenomenon is known as spin-spin or scalar coupling (Claridge 2004).

Free induction decay (FID) & Fourier transformation (FT)

A free induction decay (FID) is generated as irradiated excited nuclei relax (Figure 2.7). The signal amplitude of the FID decreases over time as the net magnetization returns back to equilibrium. The generated decaying signal is the free induction decay and contains the sum of all the frequencies from the targeted nuclei. Due to its high frequency, the signal cannot be

recorded directly but is mixed with a lower frequency signal to produce a digitised interferogram of low frequency, the FID (Claridge 2004).

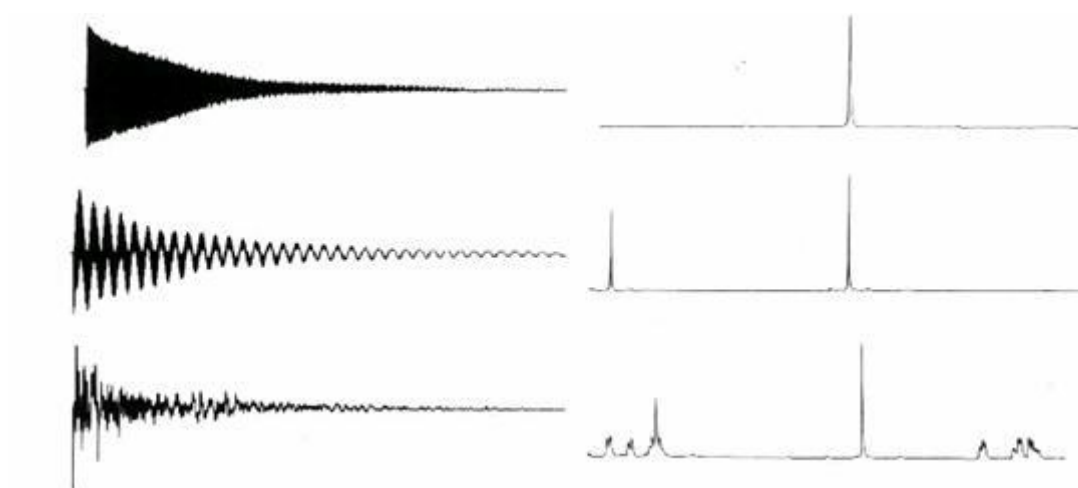


Figure 2.7 – Example of an FID signal and its transformation from a time domain to a frequency domain signal through Fourier Transformation (taken from John C. Edwards, *Principles of NMR*. Last accessed 23 July 2010).

Because in NMR the intensity of the FID signal is presented as a function of frequency, Fourier Transformation (FT) of the FID is applied in order to yield a frequency domain spectrum (Claridge 2004).

Pulse sequences

The pulse sequences used in this study included the following:

Water presaturation

Before the application of a 90° pulse, the sample is irradiated with a long pulse of low energy using the exact frequency at which the protons of water precess. This results in the saturation of the typically highly increased water signal leading to its removal from the resulting NMR spectrum. This is very helpful in the observation of the smaller signals in the spectrum coming from the less abundant molecules in the sample or the ones with relatively low concentrations (Claridge 2004).

The main pulse sequences used in this study were the “NOESY” and “CPMG” pulses both of them including presaturation pulses applied during the relaxation delay, D, for the removal of water proton signals from the spectra.

NOESY presaturation pulse: $D-90^\circ-t_1-90^\circ-t_m-90^\circ$ -acquire

NOESY stands for Nuclear Overhauser Effect Spectroscopy and the Nuclear Overhauser Effect (NOE) between nuclear spins is used for establishing the corresponding correlations. It can be used either in 1-dimensional (1D) or 2-dimensional (2D) experiments. In the 1D experiments of this study, the NOESY pulse sequence (Figure 2.8) involved the radiation of the sample with three separate radiofrequency pulses, which resulted in the magnetisation vector to move into the x - y plane, then into the z axis, and eventually back to the x - y plane. This generally leads to suppression of all the signals not currently in the 90° orientation right before the acquisition of the FID. As previously mentioned, the water presaturation pulse was applied in between the 90° pulses (Claridge 2004).

The pattern followed was $D-90^\circ-t_1-90^\circ-t_m-90^\circ$ -acquire.

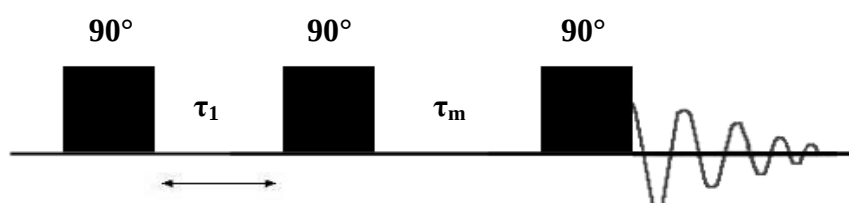


Figure 2.8 - Schematic of a typical Nuclear Overhauser Effect (NOE) pulse sequence (supplied from author’s own schematic diagrams).

Usually the applied magnetic field (B_1) exhibits some heterogeneity, with the edges of the sample volume not experiencing the field fully and, thus, not experiencing the full 90° pulses. There is a strong correlation between those regions and the residual solvent and therefore, the suppression of these regions leads to a better suppression of solvent signals coming from the water resonance (Claridge 2004).

Carr-Purcell-Meiboom-Gill (CPMG) pulse: $D-90^\circ-\{\tau-180^\circ-\tau\}_n$ -acquire

The CPMG pulse comprises of a single initial 90° pulse followed by a repeated 180° pulse. This results in the rotation of the magnetisation vector into the x - y plane causing phase coherence in all the magnetisation vectors within the sample. This is followed by a 180° pulse, which re-phases the magnetisation vectors into coherence but permits a T_2 relaxation to occur. The effect that is achieved by using this particular pulse sequence is an elimination of the larger molecular weight compounds that have shorter T_2 relaxation times due to the delay in the FID acquisition. This pulse sequence also helps in eliminating resonances that have been broadened by chemical exchanges (Claridge 2004).

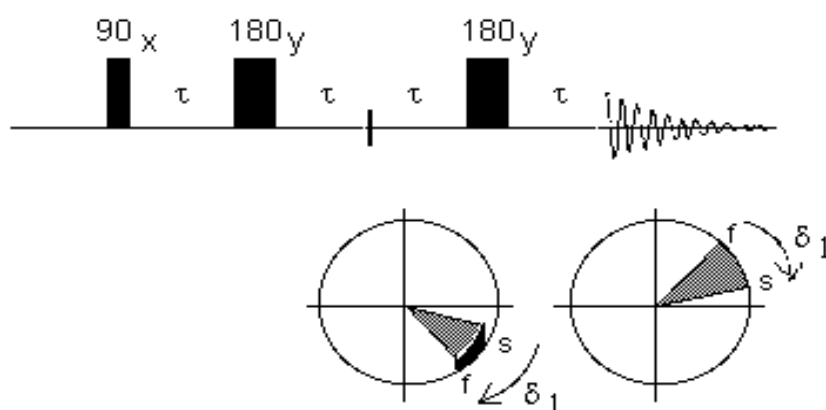


Figure 2.9 – Schematic of a typical Carr-Purcell-Meiboom-Gill (CPMG) pulse sequence (taken from *Basic Concepts T2 - Relaxation time*. Last accessed 25 July 2010).

2.3 Sample preparation

2.3.1 NMR sample generation

Prior to NMR analysis, the sample replicates were harvested from their culture flasks and the cells were detached from the surface either with the standard trypsinisation method (*Mlh1* study) or the optimised direct methanol extraction method (isogenic study) as previously mentioned on page 64.

i) Preparation of extracellular media for ^1H NMR

The culture media samples were defrosted at room temperature and 550 μl of each was mixed with 50 μl of 0.2% (w/v) Trimethylsilyl-2,2,3,3-tetradeuteriopropionic acid (TSP) in deuterium oxide (D_2O) (GOSS Scientific Instruments Ltd, UK) in D_2O and added into 5 mm NMR

tubes before analysis by ^1H NMR. Prior to NMR analysis, tubes were inverted twice to ensure samples were adequately mixed.

(ii) Preparation of intracellular cell extracts for ^1H NMR

The cells were extracted using the methanol-chloroform-water extraction method as previously recommended elsewhere (Le Belle, Harris et al. 2002). The cells were placed on ice and a mixture of 200 μl of CHCl_3 and 100 μl of methanol was added. The cells were vortexed and an additional 300 μl H_2O of HPLC-grade was then added. The cells were vortexed again to create a uniform suspension and then centrifuged for 10 minutes at 16000g. After carefully removing the Eppendorfs from the centrifuge, the resulting upper aqueous layers were removed and placed into new Eppendorfs, while the lower organic layers were transferred into glass vials. The whole procedure of extracting the cells was repeated on the remaining sample in order to enhance metabolic recovery. The layers were placed in the fume hood overnight for evaporation of methanol. The aqueous cell extracts were subsequently freeze-dried and reconstituted in 600 μl of phosphate buffer (0.2 M Na_2HPO_4 , 0.043 M NaH_2PO_4 , 100 μM TSP, 3 mM NaN_3 , 100% D_2O). They were then vortexed and 550 μl of the aqueous extract solution was transferred into a 5 mm NMR tube for NMR analysis.

2.4 NMR parameters

The intracellular aqueous extracts and media samples from repeat experiments were analyzed using ^1H NMR spectroscopy and involved the following general parameters using minor modifications where necessary: NMR spectral data were acquired on a Bruker DRX600 spectrometer which was operating at 600.13 MHz ^1H NMR frequency and 300 K. Prior to the spectral acquisition gradient shimming was applied. ^1H NMR spectra of the samples were acquired using a 1D NOESY pulse sequence ($\text{RD}-90^\circ-\text{t}_1-90^\circ-\text{t}_m-90^\circ\text{-acquire}$) and a Carr-Purcell-Meiboom-Gill (CPMG) pulse sequence ($\text{RD}-90^\circ-\{\tau-180^\circ-\tau\}_n\text{-acquire}$). The CPMG sequence generated spectra edited by T_2 relaxation times, *i.e.* with reduced signals from high molecular weight species or systems in intermediate chemical exchange. The 1D NOESY experiment generated a corresponding unedited spectrum with improved solvent peak suppression. For all spectra 128 free induction decays (FIDs) were collected into 32K complex data points, using a spectral width of 12,019 Hz (20 ppm), with a 2 s relaxation delay between pulses. A water presaturation pulse was applied throughout the relaxation delay. For the 1D NOESY experiment an additional presaturation pulse was applied during

the mixing time (t_m , 100 ms) and t_1 was held constant at 3 μ s. For the CPMG experiment $n = 300$ and $\tau = 400$ μ s, for a total T_2 relaxation time of 240 ms. The CPMG spectral editing method was used for helping in decreasing the content generated by high molecular weight entities in the spectra.

2.5 NMR Spectral data processing

The spectral data were zero-filled by a factor of 2 and the FIDs were multiplied by an exponential weighting function that was equivalent to a line broadening of 1 Hz prior to Fourier transformation (FT) using XWin-NMR (Bruker Biospin Ltd.) software. Further data processing & analysis, unless otherwise stated, was achieved using NMRProc in-house software (used in baseline corrections and referencing spectra) & MetaSpectra (used in the import of NMR data into Matlab), written by Dr T.M.D. Ebbels, Dr. H C. Keun, Mr J.T.Pearce, Dr Rachel Cavill & Dr. Olivier Cloarec (Cloarec, Dumas et al. 2005); (Pearce, Athersuch et al. 2008). The acquired NMR spectra for both intracellular extracts and media samples were phased and baseline-corrected before being referenced to an internal TSP standard at δ 0.0. All spectra were scaled to TSP and the intracellular aqueous extract additionally normalized by the median fold change (MFC) to the median spectrum; i.e. during MFC normalization each of the spectra was divided by a reference spectrum which was the median of all the samples in the NMR dataset providing a fold change for each spectral point (Dieterle, Ross et al. 2006). Only analysis of the 1D CPMG spectra was conducted, as the signals from residual protein in the sample were suppressed. Metabolites that were clearly resolved and appeared to alter concentration by either visual inspection of the spectra or after multivariate analysis were selected for quantitative analysis. Metabolic peaks were, thus, subsequently integrated for semi-quantitative evaluation comparison; this was achieved by computing the area under each desired resonance peak of each spectrum using Matlab-based code in Matlab version 7.6.0.324 R2008a (MathWorks, UK) written and compiled by Dr Rachel Cavill and Dr Hector Keun. The data were then imported into SIMCA-P+ version 11.5 (Umetrics, UK) for comparative analysis.

2.6 Structural assignments

The various ^1H NMR metabolic assignments were achieved as follows:

- by consulting pre-existing knowledge of metabolic spectra in the databases of online NMR libraries, such as BioMagResBank (BMRB) (Ulrich, Akutsu et al. 2008) and the Human Metabolome Project (HMDB) (Wishart, Knox et al. 2009),
- by comparing the generated chemical shifts with known published compound values of similar pH (Fan, Higashi et al. 1986); (Nicholson, Foxall et al. 1995),
- by using the Chenomx[®] NMR Suite software including Chenomx[®] Processor and Chenomx[®] Profiler (Chenomx Inc, Canada) with its supplemented 600 MHz internal metabolite library at neutral or near-neutral pH,
- by applying STOCSY: On the occasions where metabolic assignments could not be positively confirmed, the substrate identities were confirmed using computational spectral correlation analysis such as STOCSY (Cloarec, Dumas et al. 2005),
- by spiking samples with known standards for further confirmation of specific metabolic assignments using an *in-house* spike calculator. 1D ^1H NMR spectra were recorded using the same parameters as previously mentioned. If the newly-recorded resonance of the standard compound matched the frequency of the unknown metabolite, a positive identification was made. Some of the compounds that were spiked into NMR samples included creatine, phosphocreatine, pyruvate and acetate.

2.7 Data analysis

2.7.1 Chemometrics and Pattern Recognition for Statistical Analysis

Once the NMR spectra are acquired, there are various chemometric methods and tools in existence for facilitating and simplifying extraction of information from the complex datasets, which usually deal with large amounts of data, making it difficult to identify meaningful differences. Such tools include multivariate statistical analysis, or pattern recognition (PR), which are able to identify and interpret concealed patterns of observations and variables in the spectral data (Lindon, Holmes et al. 2001).

2.7.2 Pattern recognition theory

Pattern recognition can be divided into two categories, supervised and unsupervised. An example of unsupervised PR is Principal Component Analysis (PCA) (Figure 2.10). Principal component 1 (PC1) is a combination of variables that explains the greatest variation. The second principal component (PC2) defines the next largest amount of variation. There is no limit to the number of possible principal components. Supervised methods include soft independent modelling of class analogy (SIMCA) and Partial Least Squares (PLS) methods. Supervised methods use the class information given for a set of sample data to optimise the separation between two or more sample classes. Supervised methods need a second independent data set to test class predictions made using the training set (Lindon, Holmes et al. 2001); (Spencer, Bonnin et al. 2009).

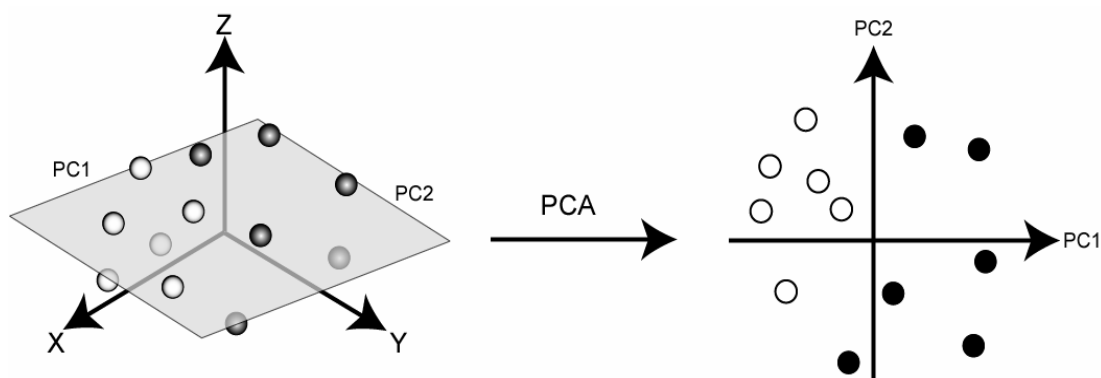


Figure 2.10 - Representation of a typical PCA method - a multivariate procedure which rotates the data such that maximum variabilities can be projected onto the axes x, y, and z and, thus, be reduced (figure courtesy of Dr. H. Keun, Imperial College, London).

Principal component analysis

PCA is multivariate method of analysis used widely when dealing with large datasets. Its use enables the large numbers of variables in the multivariate and multidimensional datasets to be reduced into a small amount of dimensional axes while at the same time managing to retain the variations in the data. This reduction in dimensionality is achieved through the use of p variables $X_1, X_2, \dots, X_n$ by finding their combinations to generate uncorrelated principal components (PCs) $PC_1, PC_2, \dots, P_n$, also termed *eigenvectors*. PCs separate the samples according to the greatest variation in the dataset and are ordered in a way so that PC1 exhibits the highest amount of the variation, PC2 exhibits the second greatest amount of variation, PC3 exhibits the third greatest amount of the variation, and so on:

$$(PC_1) \geq (PC_2) \geq (PC_3) \geq \dots \geq (PC_n)$$

Visually, this results in the generation of a *scores plot* and a *loadings plot* allowing easy visualization of trends in the data. The scores plot represents the position of each sample in the new co-ordinate space. It helps in correlating spectral regions to specific metabolites responsible for the observed trends, as well as identifying metabolic outliers. Each point on a scores plot represents one sample from its corresponding NMR spectra. Points close to each other have similar metabolic profiles while the further away they are located from each other they more dissimilar their profiles are. The loadings plots complement the scores plots and visualise the contribution of the spectral regions to the calculation of the scores. Each loadings plot point represents a specific region of the NMR spectra and thus a potential metabolite. Points found at the circumference of the plot are the most significant in the calculating process of the scores (Maltese and Verpoorte 2009).

Data reduction ('binning' or "bucketing") for PCA

During pattern recognition analysis the NMR spectra were divided and the signal integrals calculated in $\delta 0.04$ intervals across the chemical shift range ($\delta 0$ to 9.96). The water regions ($\delta 4.5$ to $\delta 5$) were excluded to remove variation in the pre-saturation coming from the water resonance. The TSP regions were also removed from the data prior to further analysis. The integrated NMR spectral regions were divided by the total spectral intensities for normalization purposes. The resulting reduced and normalized NMR spectral data were then imported into an Excel sheet and were mean-centered prior to PCA analysis using the SIMCA-P software. SIMCA-P was used for the generation of all the models of PCA loadings and scores plots in this study. During this process, Matlab was also used for visualization of the spectra.

Statistical Total Correlation Spectroscopy (STOCSY)

STOCSY stands for statistical total correlation spectroscopy and helps in identifying peak intensities that are highly correlated leading in turn to identification of peaks belonging to the same molecule and, thus, aiding in spectral assignment (Cloarec, Dumas et al. 2005). The usefulness of this method is not limited in peaks from the same entity but can also lead to identification of molecules that are interdependent on each other but belong to the same metabolic pathway. Graphical representation of the correlation matrix in the sample dataset

can be done in Matlab providing an easy visualisation of the correlated resonances potentially belonging to the same molecule. Maintaining the spectrometer conditions constant, the resonance intensities from a specific molecule always have the same ratio and they should be fully correlated between samples (correlation coefficient $r=1$). In practice, though r is usually less than 1 due to the various peak overlaps and the spectral noise, which are almost always present in the ^1H NMR of bio-samples. Despite that however, very high correlations are usually achieved between the corresponding resonances of the variables in the same molecule.

2.8 Bioinformatics and statistical tests

Statistical tests that were used in this study included the following:

Student's t-test

Student's t-test can be used to determine if the average values for each set of groups is significantly different by comparing the actual difference between two means in relation to the variation in the data and producing a 'p' (probability) value. There are two versions of the t-test. The *one-sample Student's t-test* tests a hypothesis by comparing a sample mean with a hypothesized true mean, and the *two-sample Student's t-test* which uses a similar technique to compare a two sample mean using standard error of the difference.

Bioinformatics (ORA and NEST-based approach)

This thesis also included some bioinformatics analysis involving over-representation analysis (ORA) and neighbourhood-based entities (NESTs). For this part of the study (chapter 4) the detailed methodology as well as the statistical tests that were used during this analysis are presented in the relevant section of chapter 4 (section 4.2).

Chapter 3

Metabolic examination of platinum resistance in cancer cells using *in vitro* cell models of platinum resistance.

3.1 Introduction & Aims

The *Mlh1* gene (MutL gene of *E. coli* or MutL homolog) is a member of the Mismatch Repair (MMR) gene family (Cooper, Kohonen-Corish et al. 2008) and is critical in maintaining genomic stability. *hMlh1* is the human homolog of the gene which usually forms active complexes with another protein, PMS2 (Schafmayer, Buch et al. 2007).

Cells that lack *Mlh1* are thought to have a reduced capacity of transmitting nuclear DNA damage signals to the apoptotic machinery. *Mlh1* loss has also been associated with Lynch syndrome (hereditary nonpolyposis colon cancer) (Balmana, Stockwell et al. 2006) and Muir-Torre syndrome (Ponti, Losi et al. 2006). Lack of *Mlh1* expression also leads to microsatellite instability, which is found to be crucial in the mechanism of cancer development, as experiments in *Mlh1*-deficient mice demonstrate (Prolla, Baker et al. 1998). Attempts on re-introducing the gene in *Mlh1*-null mice however have been found to increase sensitivity (Buermeyer, Wilson-Van Patten et al. 1999). Reduced *Mlh1* expression has been found to be implicated in contributing to increased resistance to DNA-damaging compounds (Hardman, Afshari et al. 2001) and decreased sensitivity of various cancer cell lines to platinum-based drugs such as cisplatin (Balch, Huang et al. 2004). Since MMR-deficient cells are much less sensitive to cisplatin (Papouli, Cejka et al. 2004) than those with fully functional MMR gene mechanisms, this has been argued to play a role in the development of platinum resistance after chemotherapy (Plumb, Strathdee et al. 2000).

The precise way that loss of *Mlh1* expression directly affects response to DNA damage caused by platinum cytotoxic drugs remains unclear. However, further insight on the role of *Mlh1* loss and its relationship with cancer has been shown by a recent study by Tessem et al, where a metabolic signature associated with *Mlh1* in the gut tissue was produced. According to this study, a type of genetic instability involving MSI-H (high-frequency microsatellite instabilities) in a large percentage of patients with hereditary nonpolyposis colorectal cancer frequently occurs, and stems from epigenetic silencing of *Mlh1* through methylation of its promoter sequences. This study managed to produce an *MLh1* metabolic signature which included metabolites such as glucose, lactate, phosphocholine and myo-inositol (Tessem, Selnæs et al. 2010). On a separate note, other studies examining *Mlh1* have additionally reported alterations in the mitochondrial membrane potential and mitochondrial distribution within cancerous cells (Isonishi, Saitou et al. 2001).

Most importantly, it has also been suggested that platinum resistance in cancer chemotherapy also involves mitochondria. For example, it was found that mitochondrial DNA-depleted U937 cells exhibited increased cisplatin sensitivity, demonstrating that these cells continued to respond to death by cisplatin through apoptosis (Liang and Ulliyatt 1998). Other experiments with mitochondrial DNA-depleted SK-Hep1 hepatoma cells, have shown that carrying mitochondrial DNA mutations confers resistant to various forms of cell death, such as oxidative stress and chemotherapeutic agents like cisplatin (Park, Chang et al. 2004). These findings suggest that an important cisplatin-resistance mechanism lies in the protection of mitochondria from DNA damaging agents.

Here, we further investigate this association by the study of *Mlh1*-specific metabolic signatures *in vitro* models: a transient *Mlh1* expression cell model in the kidney epithelial cell line HEK-293T, in which the effect of mitochondrial depletion was also examined, and a Platinum Resistance Model (PRM) with ovarian cell lines (A2780 & CP70) of well-established *Mlh1* status.

3.2 Methods

Spectra were acquired and analysed for intracellular aqueous extracts from four groups of kidney cell lines and four groups of ovarian cell lines. These cell lines represent different models of *Mlh1*-controlled states with differences in their origin, genetic background and selective process. All cell samples were kindly provided by Dr Vincent O'Brien (formerly of the Beatson Inst. Glasgow) & Prof Bob Brown (Imperial College London / Royal Marsden Hospital) and his group.

The HEK-293T MutL α cells are derived from human embryonic kidney HEK 293T cells lacking *MLh1*, after transfection with an *hMLH1* cDNA vector. More specifically, HEK-293T cells are a derivative of HEK-293 kidney cells that stably express the large T-antigen of SV40, and are more easily transfectable. Furthermore, the HEK-293T MutL α cell lines are under the control of the inducible Tet-Off expression system (Cejka, Stojic et al. 2003). The MutL α - ρ 0 cell lines were selected for depletion of mitochondrial DNA after exposing them in 50 ng/ml ethidium bromide and 50 ng/ml uridine.

The two ovarian cancer cell lines, A2780 and CP70, are well-characterized models for cisplatin sensitive and cisplatin-resistant lines, respectively, and have been originally described by Behrens and his team as useful tools in studying platinum resistance mechanisms (Behrens, Hamilton et al. 1987). CP70 is a sub-line of A2780 derived by prolonged *in vitro* exposure to cisplatin that lacks *Mlh1* expression. Clonal sub-populations of CP70 expressing *Mlh1* (CP70 E1) or not (CP70 A2) after chromosome 3 microcell transfer were also used in this study to allow more specific examination of the *Mlh1* to phenotypic variation in this model.

Table 3.1 summarises the cell lines and their relevant characteristics.

Cell Lines	<i>Mlh1</i> Expression	Details
HEK-293T Model (Kidney)		
HEK-293T MutL α +	+	<i>Mlh1</i> restored by plasmid transfection – <i>Mlh1</i> is expressed.
HEK-293T MutL α -	-	<i>Mlh1</i> restored by plasmid transfection – <i>Mlh1</i> expression switched OFF by Doxocycline exposure
HEK-293T MutL α + ρ 0	+	<i>Mlh1</i> restored by plasmid transfection – <i>Mlh1</i> is expressed - Mitochondrial DNA depletion by ethidium bromide and uridine exposure
HEK-293T MutL α - ρ 0	-	<i>Mlh1</i> restored by plasmid transfection – <i>Mlh1</i> expression switched off by Doxocycline exposure - Mitochondrial DNA depletion by ethidium bromide and uridine exposure
Platinum Resistance Model (Ovarian)		
A2780	+	Cisplatin-sensitive
CP70	-	Cisplatin-resistant
CP70 E1	+	<i>Mlh1</i> restored by chromosome 3 microcell transfer
CP70 A2	-	Only contains chromosome 3 regions without <i>Mlh1</i> expression

Table 3.1 – *Mlh1* status and characteristics of the cell lines used in this study.

Harvested cells were extracted and ^1H NMR spectra were obtained as described in chapter 2. The data were also processed and normalized as previously described and then visually inspected to characterize metabolic effects of *Mlh1* status. Their mean intracellular metabolic profiles were compared within the same model as well as across both models.

Multivariate analysis in the form of Principal Component Analysis (PCA) was performed as described in chapter 2, to visualize patterns and differences between the cell groups as well as the variables (metabolites) that contributed to these differences.

Intensities of positively assigned metabolites which have been previously associated with cancer in the literature (i.e. alanine, phosphocholine) or contribution to development of elevated platinum resistance (i.e. glutathione) were chosen for further quantitative analysis using in-house software as described in chapter 2.

For the relative quantification of each of the selected metabolites the area under the spectral peak was calculated using the ppm regions presented in Table 3.2. Their relative metabolite intensities were then compared and tested for significance.

Aqueous Cell Extracts Metabolites	Start (ppm)	End (ppm)
Alanine	1.47	1.5
Choline	3.205	3.2134
Phosphocholine	3.2134	3.2335
Glycerophosphocholine	3.2335	3.2435
Glutathione	2.485	2.61
Lactate (1)	1.32	1.35
Lactate (2)	4.095	4.142
Myo-inositol	4.065	4.085

Table 3.2 – Spectral regions integrated for a selection of metabolites that exhibited the biggest changes according to PCA analysis.

3.3 Results

In this study, the two different cell models were examined both individually (sections 3.3.1 & 3.3.2 for the HEK-293T and PRM, respectively) and together as a pair (section 3.3.3).

3.3.1 Metabolic effects of *Mlh1* expression in HEK-293T cells

Figure 3.1 shows full resolution mean ^1H NMR spectra of intracellular aqueous extracts for the cell lines constituting the HEK-293T model, referenced to TSP and normalized by Median Fold Change: MutL α + (green), MutL α - (cyan), MutL α + p0 (blue), MutL α - p0 (red). Metabolites were visually examined across the aliphatic spectral region ($\delta 0 - \delta 4.1$) and the aromatic spectral region ($\delta 6 - \delta 9.5$) region. The metabolic changes included differences in intracellular alanine, choline, phosphocholine, glycerophosphocholine, glutathione *myo*-inositol, and lactate intensities as well as branched-chain amino acids (valine, leucine, isoleucine). However, consistent changes across the cell lines on the basis of *Mlh1* expression were observed mostly on the levels of intracellular choline, phosphocholine and glutathione.

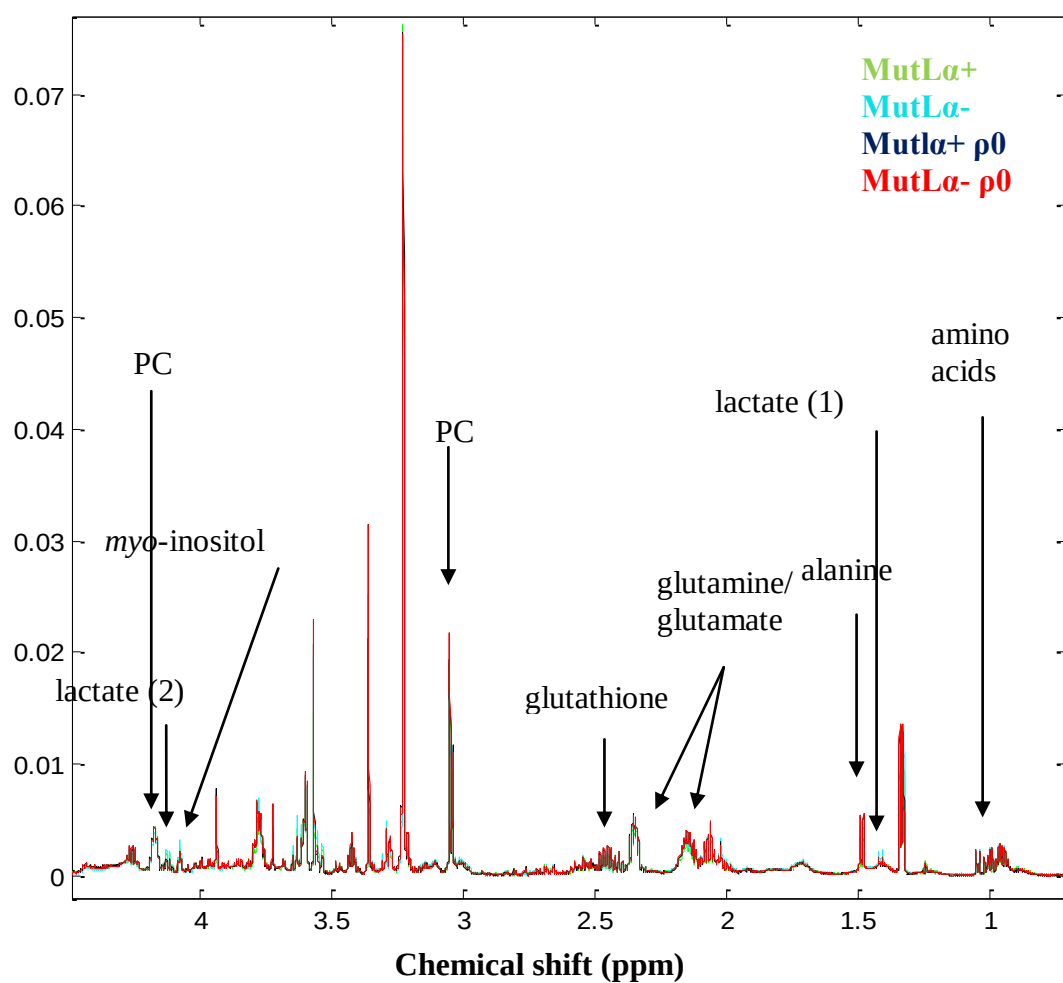


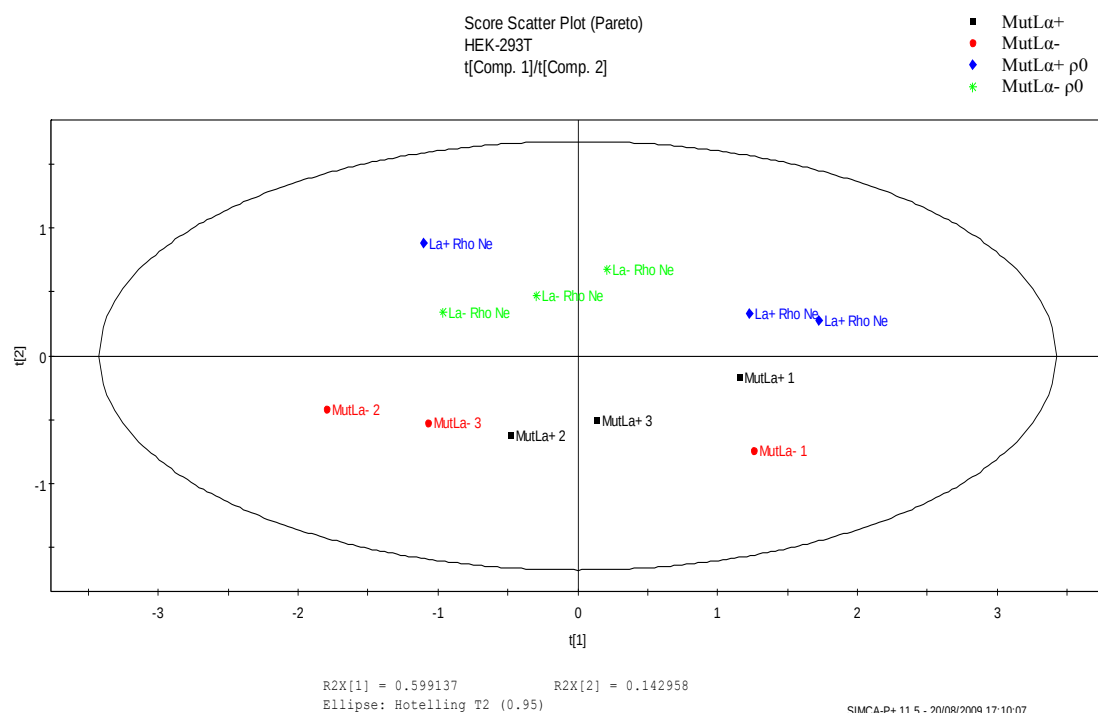
Figure 3.1 - Full resolution ^1H NMR mean spectra of the HEK-293T model, normalized to TSP & Median Fold Change. MutLa+ (green), MutLa- (cyan), MutLa+ p0 (blue), MutLa- p0 (red)

In order to systematically and more objectively define a metabolic signature associated with *Mlh1* status, the bucketed CPMG spectral data were imported into SIMCA, and pattern recognition was used to identify discriminatory metabolites between the groups as described in chapter 2. We then investigated the global metabolic differences in an unsupervised approach by performing principal component analysis (PCA). This approach was chosen because PCA methods can help in reducing the dimensionality of the data, so the original variables were reduced to a smaller number of variables termed principal components (PCs). Before multivariate statistical analysis was performed all spectral data were bucketed followed by the use of “Pareto scaling”, which is defined as the mean-centered data divided by the square root of the standard deviation. This type of scaling is able to provide a reasonable balance of contributions from high and low signals

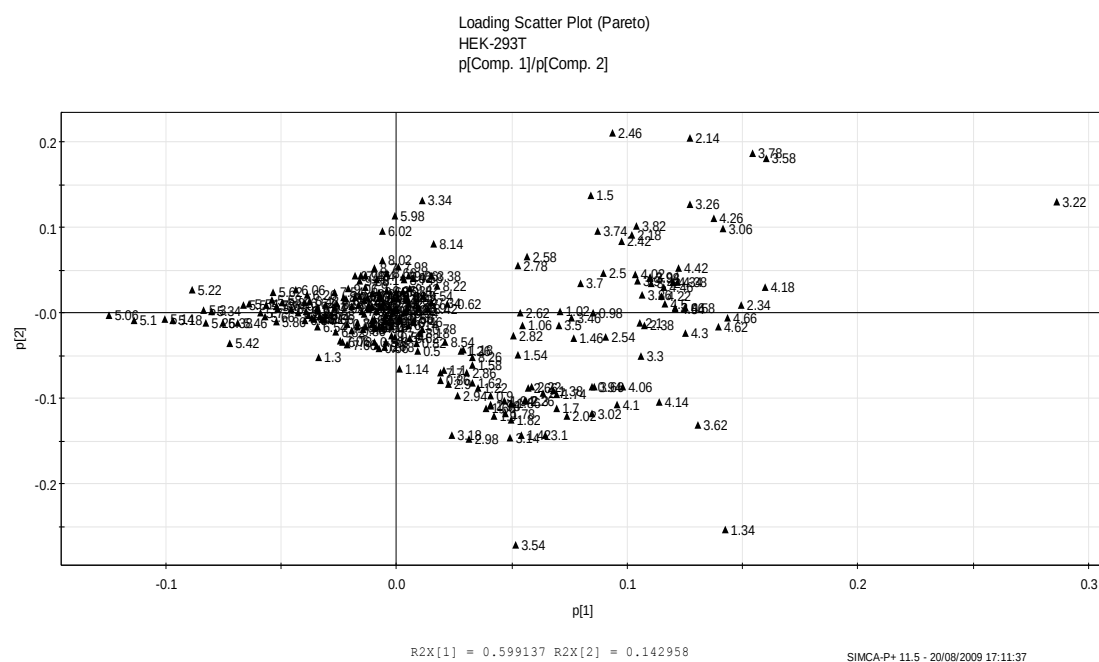
For the HEK-293T cell model data, PCA extracted three principal components (PC1, PC2, and PC3) which corresponded to three dimensions of greatest variance in the data. This approach identified the NMR spectra-derived metabolic differences across all three components. In particular, the first principal component (PC1) did not appear to separate the cell lines into distinct groups (Figure 3.2); PC2 however specifically identified differences between intracellular aqueous extracts of groups of cells with functional mitochondria and cells which had their mitochondrial DNA depleted (Figures 3.2 & 3.3). In addition, PC3 was able to differentiate between the two groups of cells that possess functional mitochondria into two separate clusters on the basis of *Mlh1* expression status (Figure 3.3). A separation on the basis of *Mlh1* expression was not observed for mtDNA depleted cells. Since the HEK-293T model acted as a model of transient *Mlh1* gene expression, the observed cell group clusterings may indicate that the metabolic effects of *Mlh1* were mediated by mitochondria.

For the HEK-293T PCA scores plots (Figures 3.2 A and 3.3 A) there are corresponding loadings plots (Figures 3.2B and 3.3B) representing metabolites at specific NMR spectral regions, where peaks that may contribute extensively to the variance between the different cell groups can be visualized.

For example, some of the metabolic resonances that were found to differentiate samples into groups across PC2 are seen at δ 1.5, 2.46, 3.22, 3.78 and 5.22 for the mitochondrial-DNA depleted cells (MutLa + ρ 0 and MutLa - ρ 0), whereas resonances exhibiting the opposite trend for non-mitochondrial-DNA cels across PC2 can be seen at δ 1.34, 1.54 3.54 and 3.62 (Figure 3.2 B).

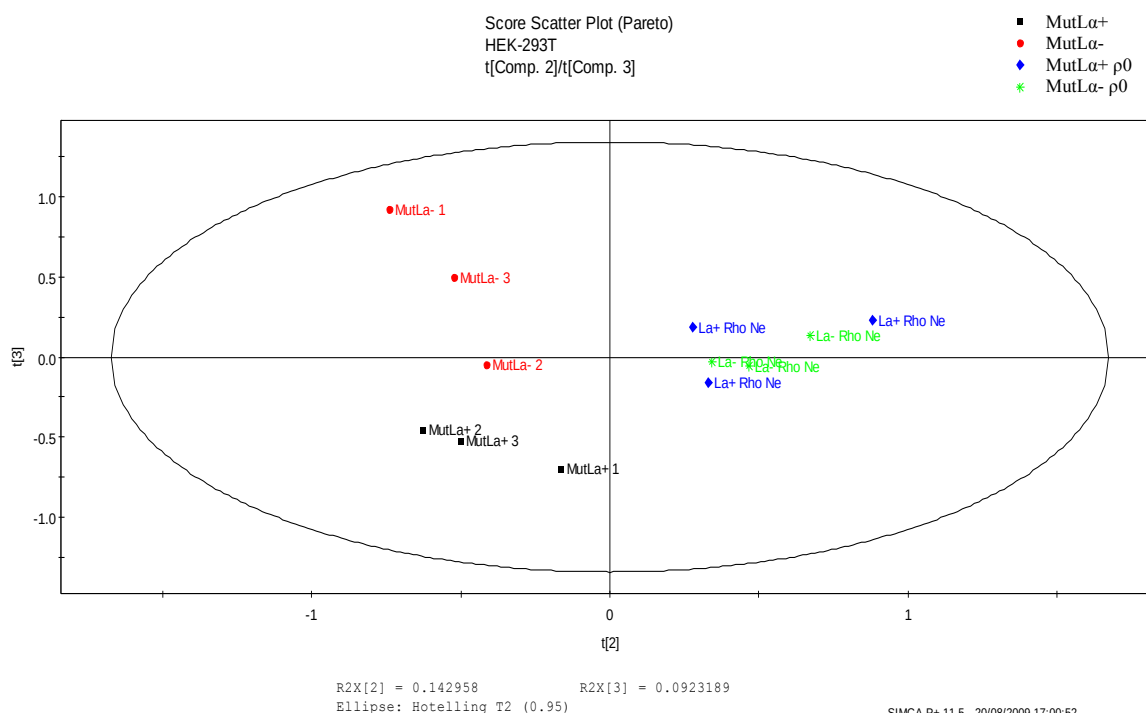


A

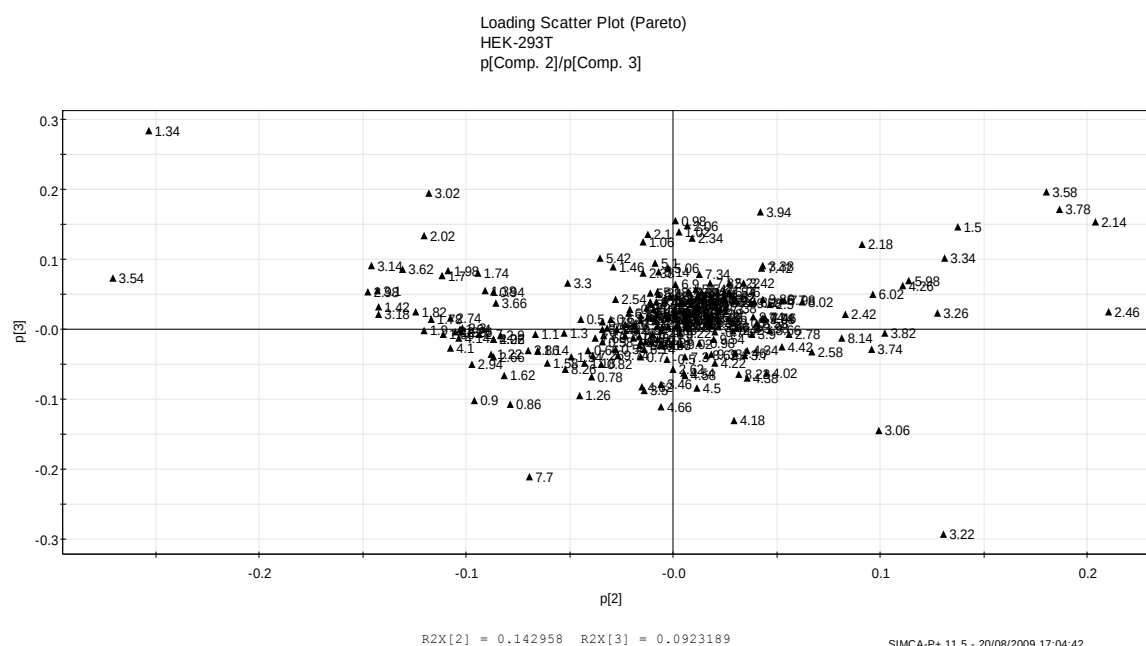


B

Figure 3.2 - PC1 & PC2: Scores (A) and corresponding loadings plot (B) of principal component analysis on ^1H NMR CPMG one-dimensional spectra for aqueous intracellular extracts of HEK-293T cells normalized by the Median Fold Change. Each point in A represents an individual cell sample of MutLa+ (black), MutLa- (red), MutLa+ p0 (blue) and MutLa- p0 (green). The ellipse in A represents the Hotelling with 95% confidence. Numbers beside data point on the loadings plot in B correspond to specific bins used in the analysis.



A



B

Figure 3.3 - PC2 & PC3: Scores (A) and corresponding loadings plot (B) of principal component analysis on ^1H NMR CPMG one-dimensional spectra for aqueous intracellular extracts of HEK-293T cells normalized by the Median Fold Change. Each point in A represents an individual cell sample of MutLa+ (black), MutLa- (red), MutLa+ p0 (blue) and MutLa- p0 (green). The ellipse in A represents the Hotelling with 95% confidence. Numbers beside data point on the loadings plot in B correspond to specific bins used in the analysis.

The third component (PC3) was the component that showed a clear *Mlh1* expression status separation between the cell groups (Table 3.3) in this model. The metabolites, the resonances of which constitute approximately 20% of the spectra, are presented below in descending ppm value order.

The PCA-derived data combined with full spectral resolution of the one-dimensional spectra and reported data from the literature allowed identification and assignment of specific metabolites for both components. The metabolite assignments along with their corresponding ppm and loading values are presented in table 3.3.

PC3		
ppm	Loading Value	Metabolite
7.7	- 0.21058	Unassigned (1)
5.42	+ 0.101489	Unassigned (2)
4.18	- 0.12893	Phosphocholine
3.94	+ 0.16842	Creatine
3.78	+ 0.172373	Glutathione/Glutamate
3.58	+ 0.196177	<i>Myo</i> -inositol
3.34	+ 0.10211	Glycerophosphocholine
3.22	- 0.291832	Choline/Phosphocholine
3.06	- 0.144356	Unassigned (3)
3.02	+ 0.195801	Creatine
2.34	+ 0.130972	Glutamate
2.18	+ 0.120939	Glutathione/glutamine/glutamate
2.14	+ 0.153524	Glutathione/glutamine/glutamate
2.1	+ 0.136284	Glutathione/glutamine/glutamate
2.06	+ 0.149316	Glutamate
2.02	+ 0.134372	Glutamate
1.5	+ 0.146588	Alanine
1.34	+ 0.283922	Lactate (1)
1.06	+ 0.126024	Amino acids (isoleucine, leucine, valine)
1.02	+ 0.138837	Amino acids (isoleucine, leucine, valine)

Table 3.3 – Table showing the metabolites with the biggest changes across the binned spectral kidney metabolome across the 3rd PCA component (PC3)

Integration data of some of the most notable metabolites observed after NMR and PCA analyses in the HEK-293T cells are presented below (with all intracellular aqueous extracts normalized to the median spectrum). These metabolites include glutathione, alanine and phosphocholine as a representation of the most meaningful changes observed in the spectral data.

In this particular model, intracellular glutathione levels were higher in the *Mlh1*-negative lines as Figure 3.4 A illustrates. However, the difference was not significant in the mitochondrial DNA-depleted cells (Figure 3.4 B and C). A similar situation was observed with alanine (Figure 3.5 A), whose levels were also increased in the *Mlh1*-negative cells (MutL α -). However, the difference was negligible (Figure 3.5 B) between *Mlh1* positive (MutL α + ρ 0) and *Mlh1*-negative cells (MutL α - ρ 0) whose mitochondrial DNA was depleted. Phosphocholine levels were significantly decreased in the *Mlh1*-negative cells (MutL α -) and slightly decreased in the MutL α - ρ 0 cells compared to their *Mlh1*-positive parent cell lines (Figure 3.6 A); similarly to alanine though, the difference in the phosphocholine levels between *Mlh1*-positive and *Mlh1*-negative cells was much less pronounced in the mitochondrial DNA-depleted cell lines (Figure 3.6 B).

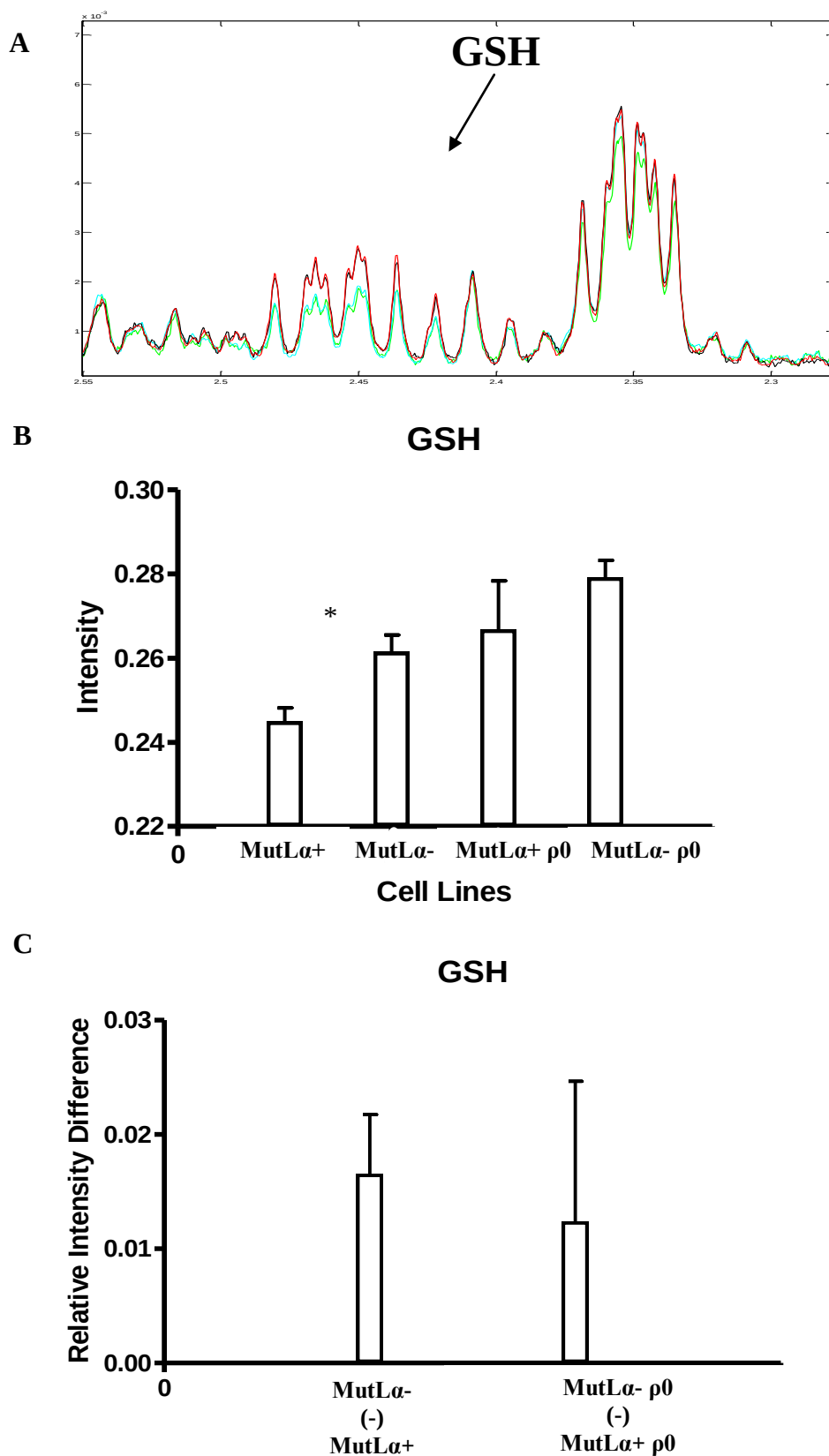


Figure 3.4 - The effect of *Mlh1* expression on intracellular glutathione

A) Median normalized spectra

B) Integrated metabolite intensity

C) Intensity difference between cell lines of differing *Mlh1* status

n = 3. * p<0.05, ** p<0.01, * p<0.001**

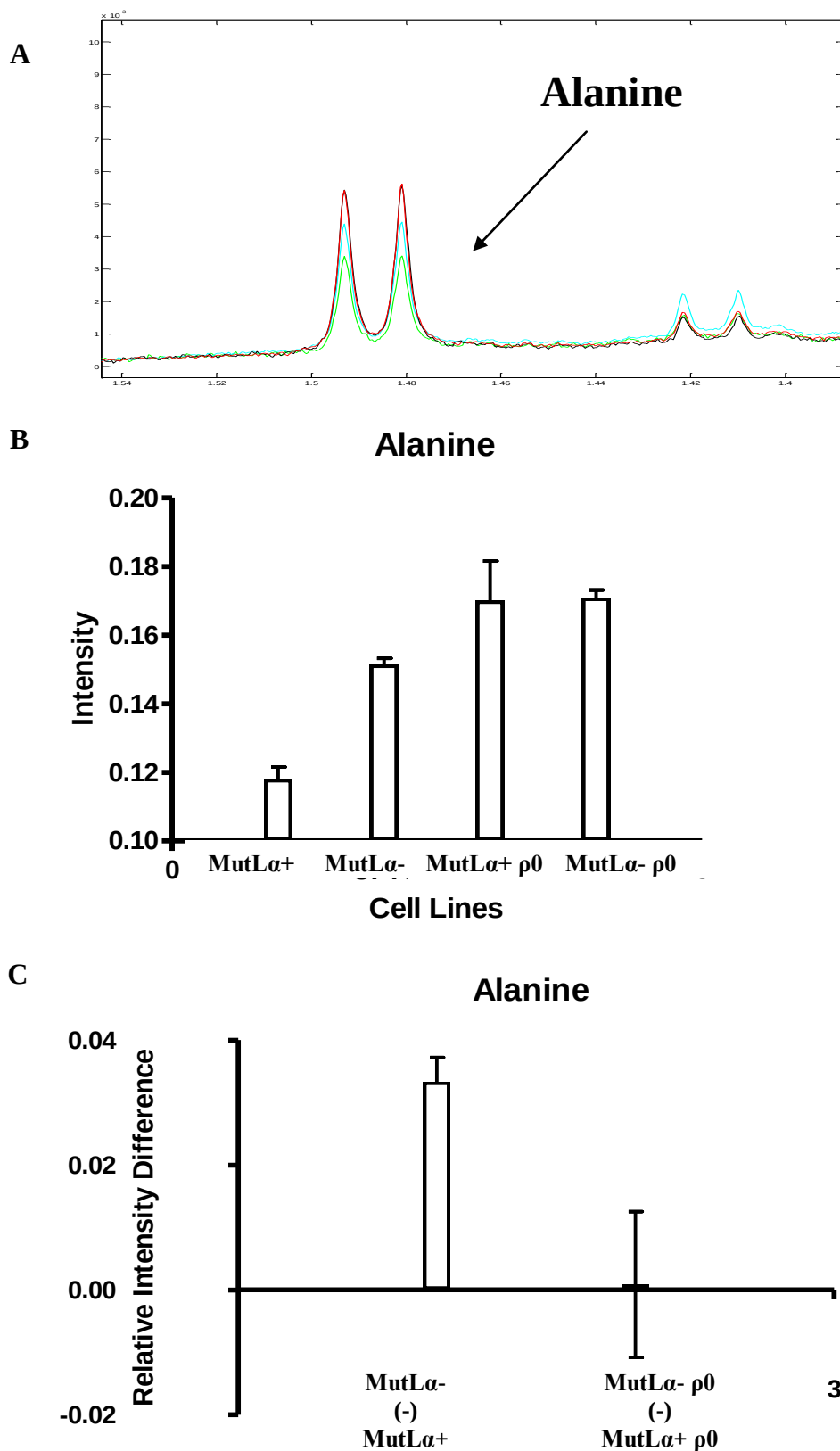


Figure 3.5 - The effect of *Mlh1* expression on intracellular alanine
A) Median normalized spectra
B) Integrated metabolite intensity
C) Intensity difference between cell lines of differing *Mlh1* status
n = 3. * p<0.05, ** p<0.01, * p<0.001**

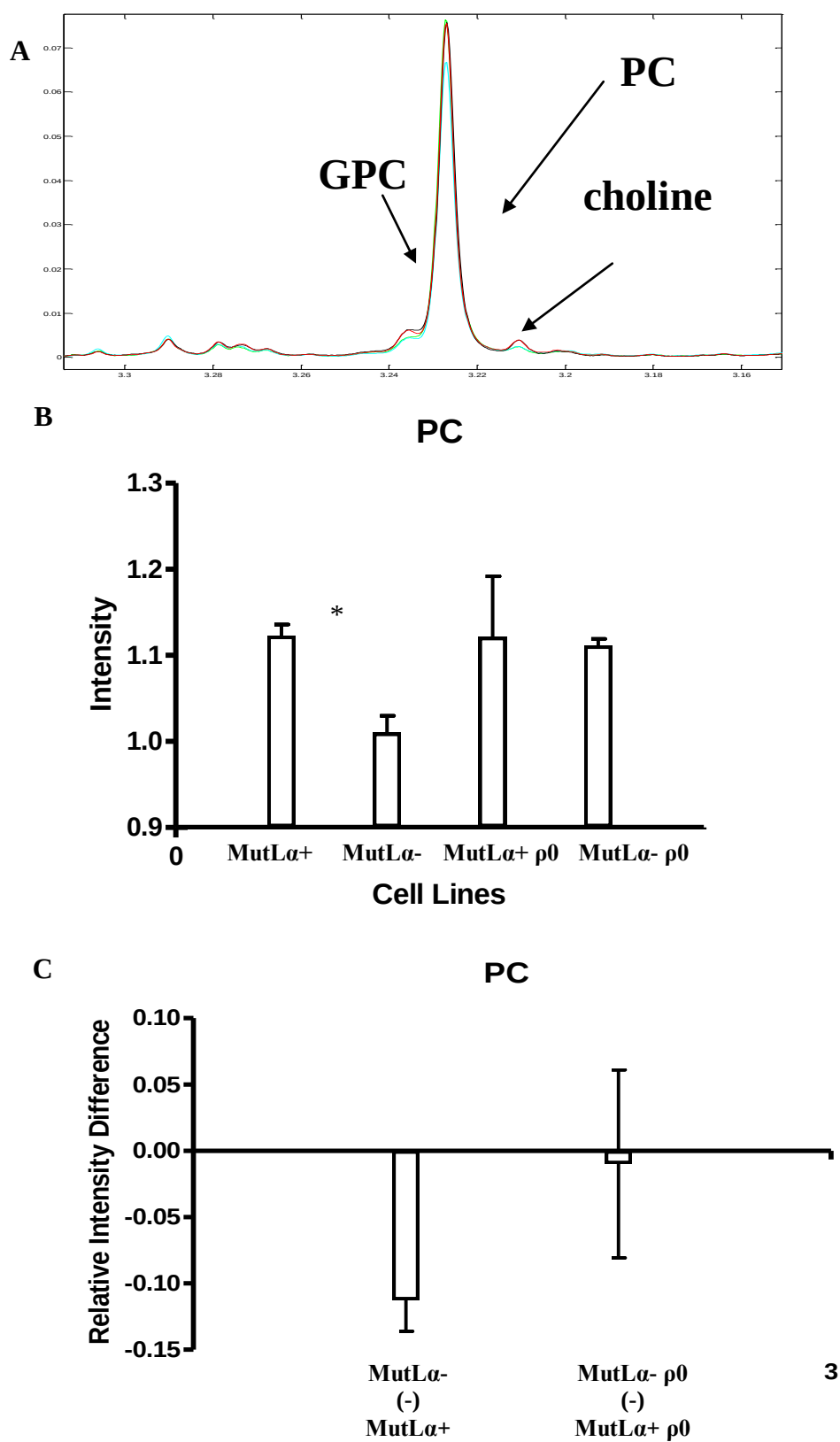


Figure 3.6 - The effect of *Mlh1* expression on intracellular phosphocholine

A) Median normalized spectra

B) Integrated metabolite intensity

C) Intensity difference between cell lines of differing *Mlh1* status

n = 3. * p<0.05, ** p<0.01, *** p<0.001

3.3.2 Metabolic effects of *Mlh1* expression in ovarian cells of Platinum Resistance Model

Representative full resolution CPMG ^1H NMR mean spectra of the ovarian cells of the Platinum Resistance Model, referenced to TSP and normalized to Median Fold Change are presented below in Figure 3.7 and revealed changes in the intracellular levels of lactate, *myo*-inositol, glutathione, alanine, phosphocholine and branched chain amino acids. Spectra of A2780, CP70, CP70 E1, and CP70 A2 cells are represented in black, red, green and blue, respectively.

Inspection of the scores and loadings plots (Figures 3.8 and 3.9) from a PCA analysis model of these spectra identified a more specific panel of metabolites that were altered in response to *Mlh1* expression status. Metabolites that were significantly altered on the presence or absence of *Mlh1*, across the different cell conditions, are described further below. The integral intensities for the selected metabolites were calculated as previously described in chapter 2.

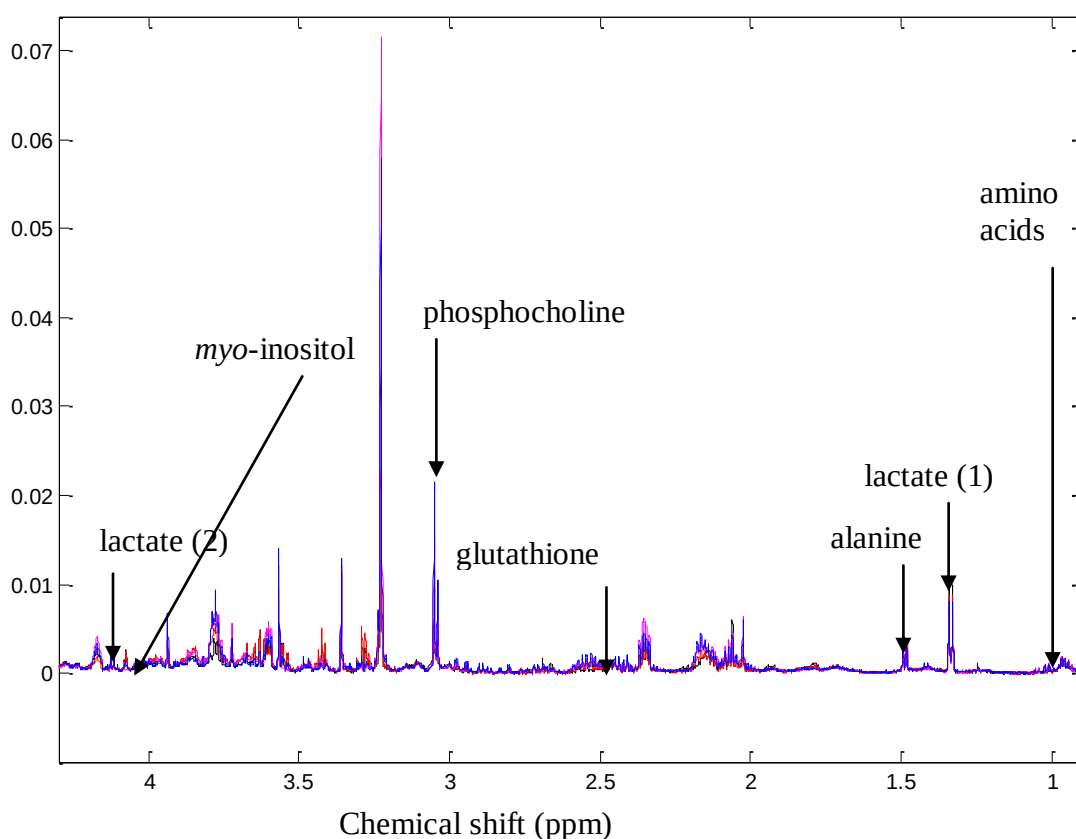


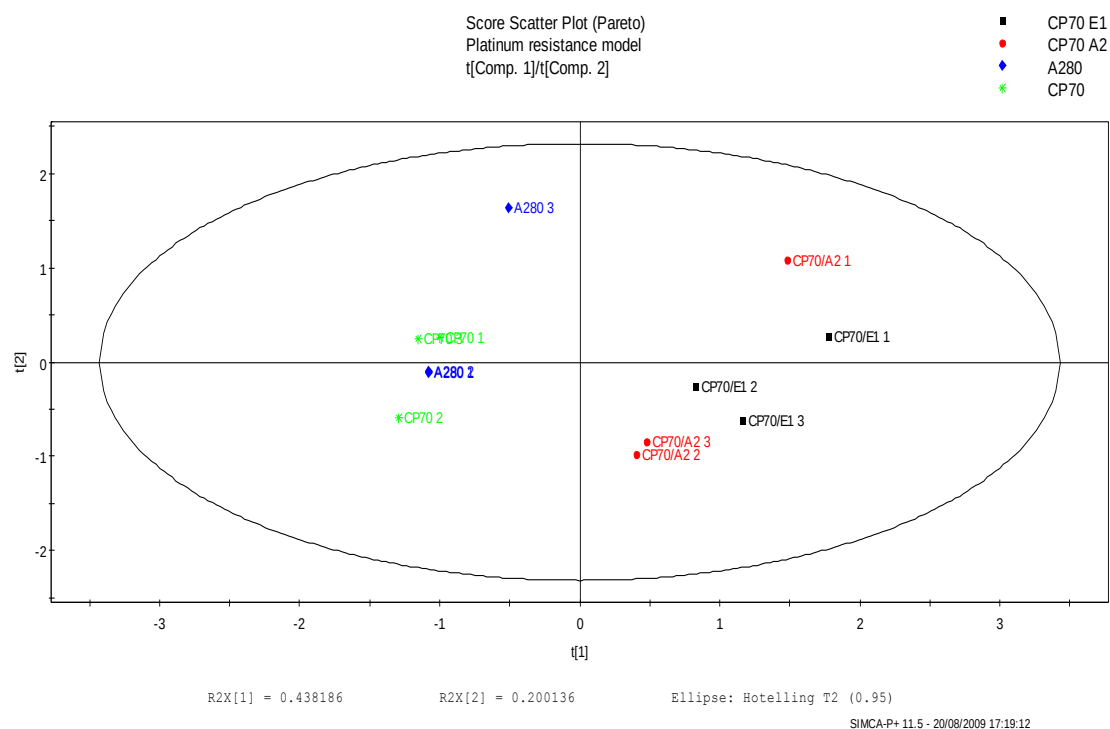
Figure 3.7 – Full resolution ^1H NMR mean spectra of the Platinum Resistance model, normalized to TSP & Median Fold Change. A2780 (black), CP70 (red), CP70 E1 (magenta), CP70 A2 (blue)

Similarly to the HEK-293T cell model, PCA scores plots of the reduced ovarian spectra showed a trend toward separation of the *Mlh1*-negative and *Mlh1*-positive cell groups (Figures 3.8 A and 3.9 A).

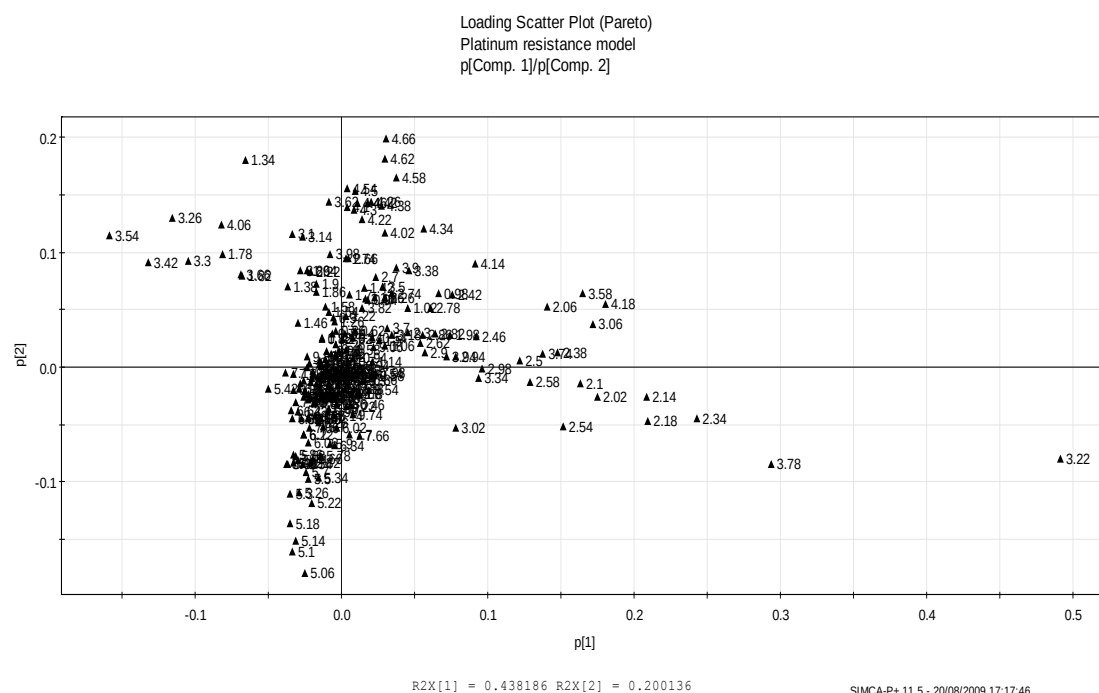
Of the four principal components for the Platinum Resistance model, PC1 separated the cell lines into two groups: one consisting of the platinum resistant lines A2780 and CP70, and one consisting of the clonal sub-population cells of the CP70 E1 and CP70 A2 lines. PC2 did not achieve to separate any of the lines into clear and distinct groups. The third (PC3) as well as the fourth (PC4) turned out to produce the clearest separation between ovarian cells of different *Mlh1* status and genetic background. In particular, each of the four ovarian cell groups was clustered into a separate quadrant created by both PC3 and PC4. This denotes a separation based not only on their *Mlh1* status but possibly other independent factors. Those may be determined by the genetic background of those cells as well as their subsequent genetic manipulation as in the occasion of CP70 E1 and CP70 A2 where specific targeting of *Mlh1* occurred, using complete or partial chromosomal transfer. The *Mlh1* gene expression effect can still be seen across PC4 where *Mlh1*-negative cells are located in the top two quadrants whereas the *Mlh1*-positive cells are clustered in the bottom two quadrants of the ellipse.

Figures 3.8 B and 3.9 B of the corresponding PCA loadings plot show the specific NMR spectral regions of various metabolites responsible for the variation in PC1 & PC2 and PC3 & PC4 of the Platinum Resistance model.

Resonances that separated the *Mlh1*-positive from the *Mlh1*-negative cell lines across PC4 can be seen in Figure 3.9 B. Higher resonances for the *Mlh1*-negative cells were seen at δ 1.34, 1.5, 2.18, 2.54, 3.54, 3.78 and 7.7, while lower ones could be seen at δ 2.34, 2.7, 3.22, 3.62, 3.86 and 4.18. The opposite was true for the *Mlh1*-positive cells.

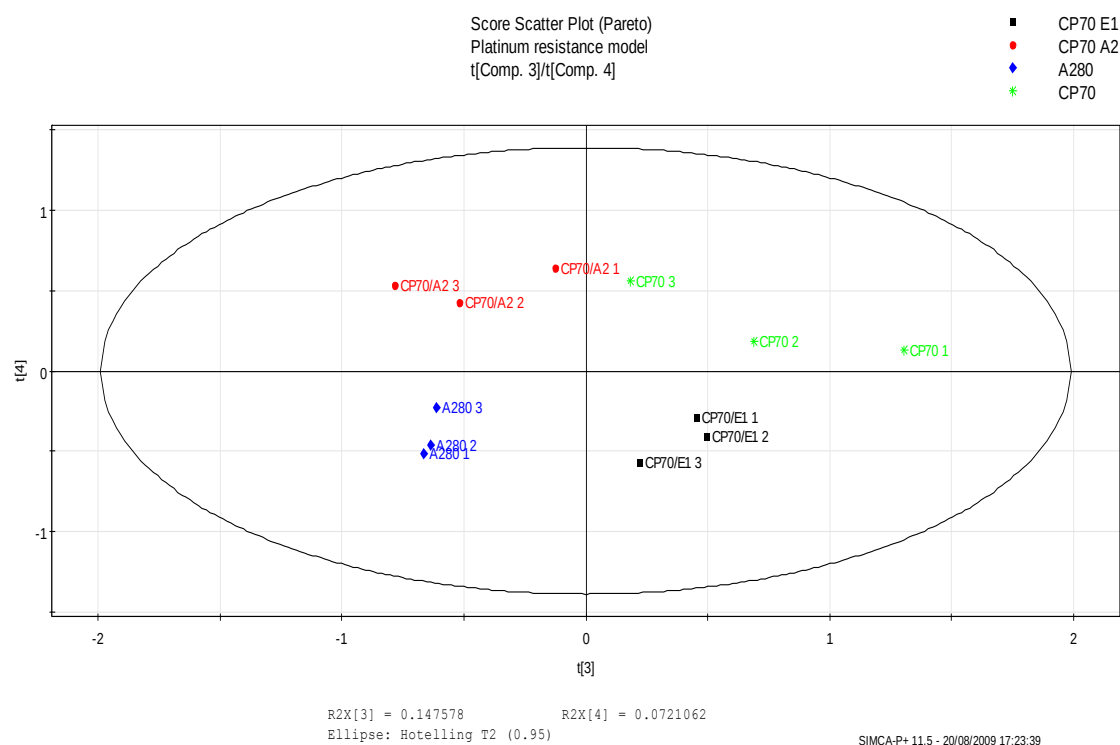


A

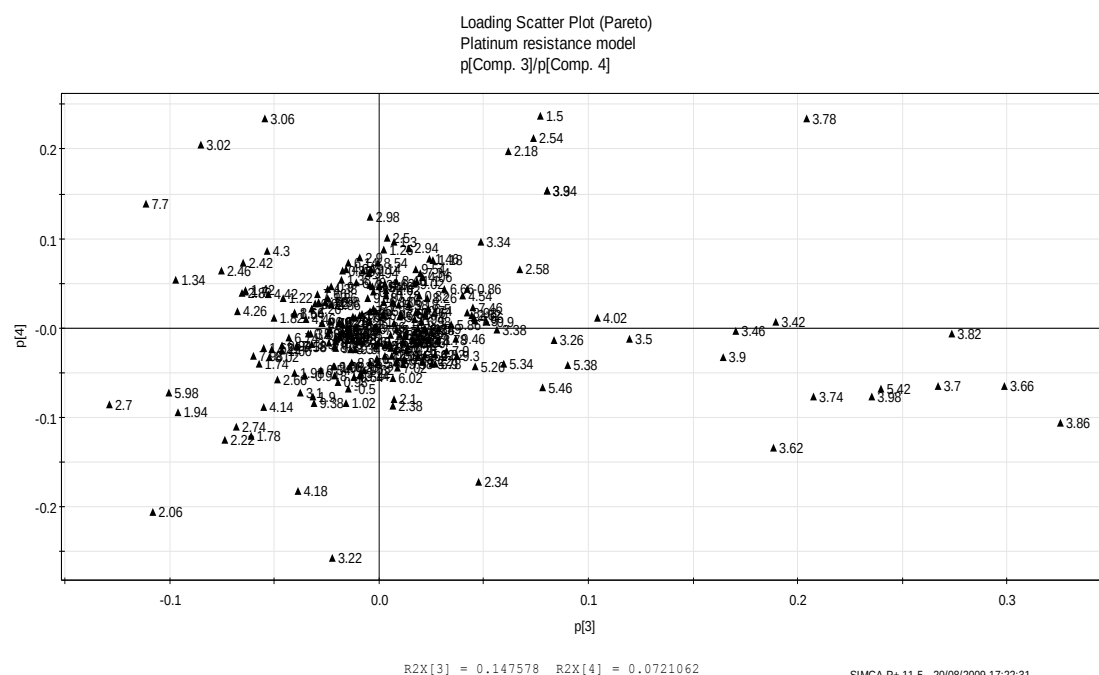


B

Figure 3.8 – PC1 & PC2: Scores (A) and corresponding loadings plot (B) of principal component analysis (PCA) on ^1H NMR CPMG one-dimensional spectra for aqueous intracellular extracts of ovarian cells normalized by the Median Fold Change. Each point in A represents an individual cell sample of A280 (blue), CP70 (green), CP70 E1 (black) and CP70 A2 (red). The ellipse in A represents the Hotelling with 95% confidence. Numbers beside data point on the loadings plot in B correspond to specific bins used in the analysis.



A



B

Figure 3.9 – PC3 & PC4: Scores (A) and corresponding loadings plot (B) of principal component analysis (PCA) on ^1H NMR CPMG one-dimensional spectra for aqueous intracellular extracts of ovarian cells normalized by the Median Fold Change. Each point in A represents an individual cell sample of A280 (blue), CP70 (green), CP70 E1 (black) and CP70 A2 (red). The ellipse in A represents the Hotelling with 95% confidence. Numbers beside data point on the loadings plot in B correspond to specific bins used in the analysis.

In this model, the component that showed a clear separation between the cell groups, based on *Mlh1* expression status, was the fourth component (PC4). After analysis of the PC3 and PC4 components in the loadings column plots, the ovarian metabolites that showed the biggest changes across the fourth component (PC4) and which constituted approximately 20% of the total spectral features are presented in Table 3.4 in descending ppm value order. PC4 shows separation of the cell groups on their *Mlh1* basis alone.

PC4		
ppm	Loading Value	Metabolite
7.7	+ 0.138482	Unassigned (1)
4.18	- 0.182966	Phosphocholine
3.94	+ 0.154029	Creatine
3.86	- 0.106708	Methionine
3.78	+ 0.235156	Alanine
3.62	- 0.133823	Ethanol
3.34	+ 0.096839	Glycerophosphocholine
3.3	+ 0.152956	Myo-inositol
3.22	- 0.258136	Choline/ Phosphocholine
3.06	+ 0.234367	Unassigned (2)
3.02	+ 0.205264	Creatine
2.98	+ 0.124512	Unassigned (3)
2.74	- 0.110324	Unassigned (4)
2.54	+ 0.212563	Glutathione
2.5	+ 0.101446	Glutamate/ Glutamine
2.34	- 0.172459	Glutamate
2.22	- 0.124986	Glutamate
2.18	+ 0.197899	Glutamate
2.06	- 0.206247	Glycoprotein's
1.94	- 0.094926	Acetate / Arginine
1.78	- 0.121272	Unassigned (5)
1.5	+ 0.237684	Alanine
1.3	+ 0.096177	Lactate / Heroine

Table 3.4 – Table showing metabolites with the biggest changes across the binned spectral ovarian metabolome across the 4th PCA component (PC4)

It is important to note that some bins can contain more than one metabolite, meaning that the metabolite directly responsible for the observed variance cannot be clearly defined without compound-specific profiling. However, the clear separation PCA results that were obtained indicate a high potential value of metabolic profiles reflecting important biochemical differences between the different cell groups.

Some of the most notable metabolic differences observed after NMR and PCA analyses in the Platinum Resistance model are presented below in a semi-quantitative manner and, as with the HEK-293T cell model, also include glutathione, alanine and phosphocholine.

All intracellular aqueous extracts were normalized to Median Fold Change. The ^1H NMR spectra of the selected intracellular metabolites were quantified by integrating the peak areas between their corresponding ppm ranges (Table 3.2). After integration, their metabolic relative intensities were generated by subtracting the quantified intensity values of the *Mlh1*-positive cells from those of the *Mlh1*-negative cells.

As it would be expected intracellular glutathione levels were again higher in the *Mlh1*-negative cell lines (Figure 3.10). The difference was less pronounced between the plasmid-transfected cells CP70 E1 and CP70 A2 cell lines as illustrated more clearly by Figure 3.10 B. Intracellular alanine levels were higher in the *Mlh1*-negative cells (Figure 3.11 A) and the relative difference was more pronounced between the A2780 and CP70 cells (Figure 3.11 B). Phosphocholine levels (Figure 3.12 A) were decreased in the *Mlh1*-negative cells. The relative difference in phosphocholine is similar between each cell pair in the ovarian Platinum Resistance Model.

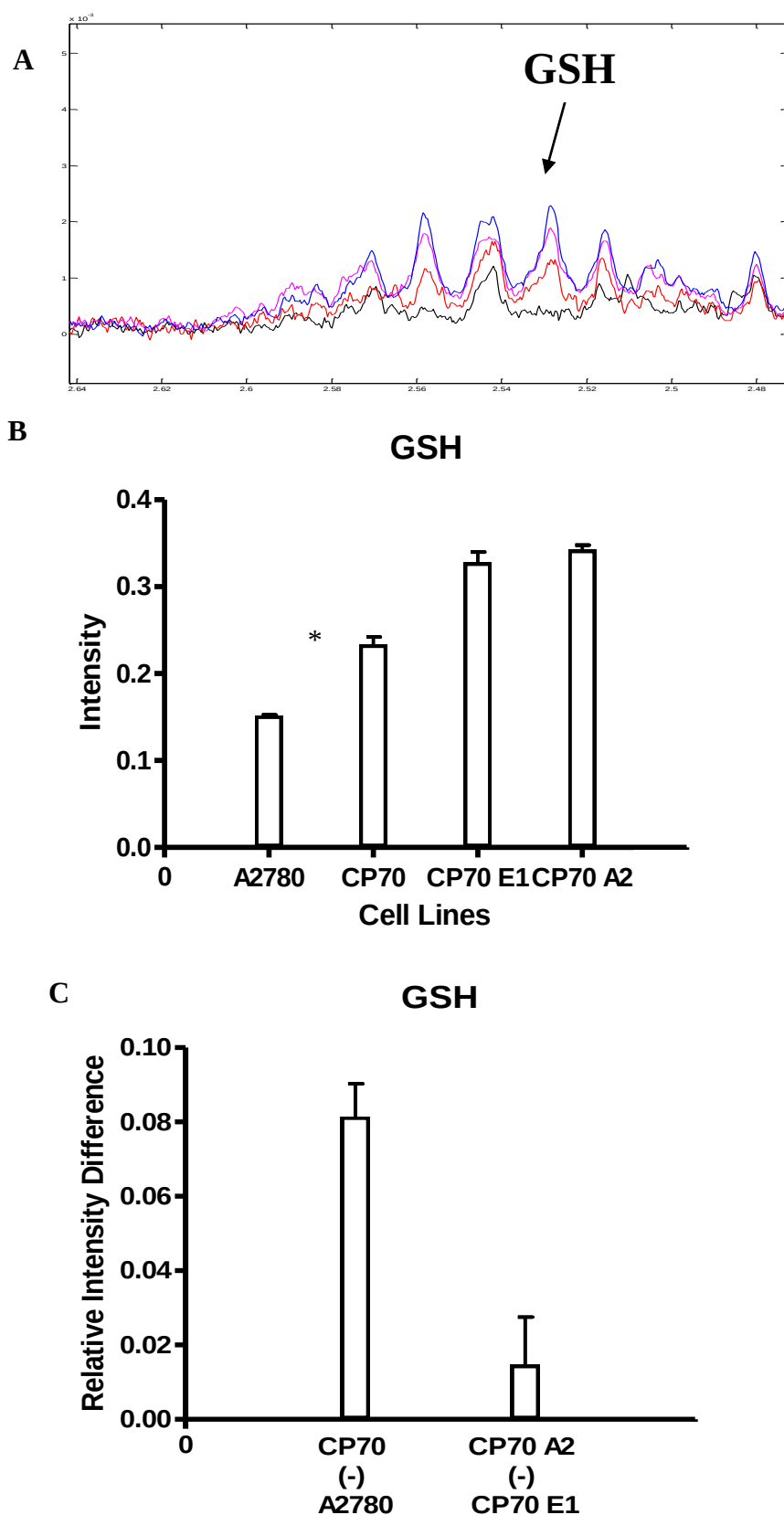


Figure 3.10 - The effect of *Mlh1* expression on intracellular glutathione
 A) Median normalised spectra
 B) Integrated metabolite intensity
 C) Intensity difference between cell lines of differing *Mlh1* status
 n = 3. * p<0.05, ** p<0.01, *** p<0.001

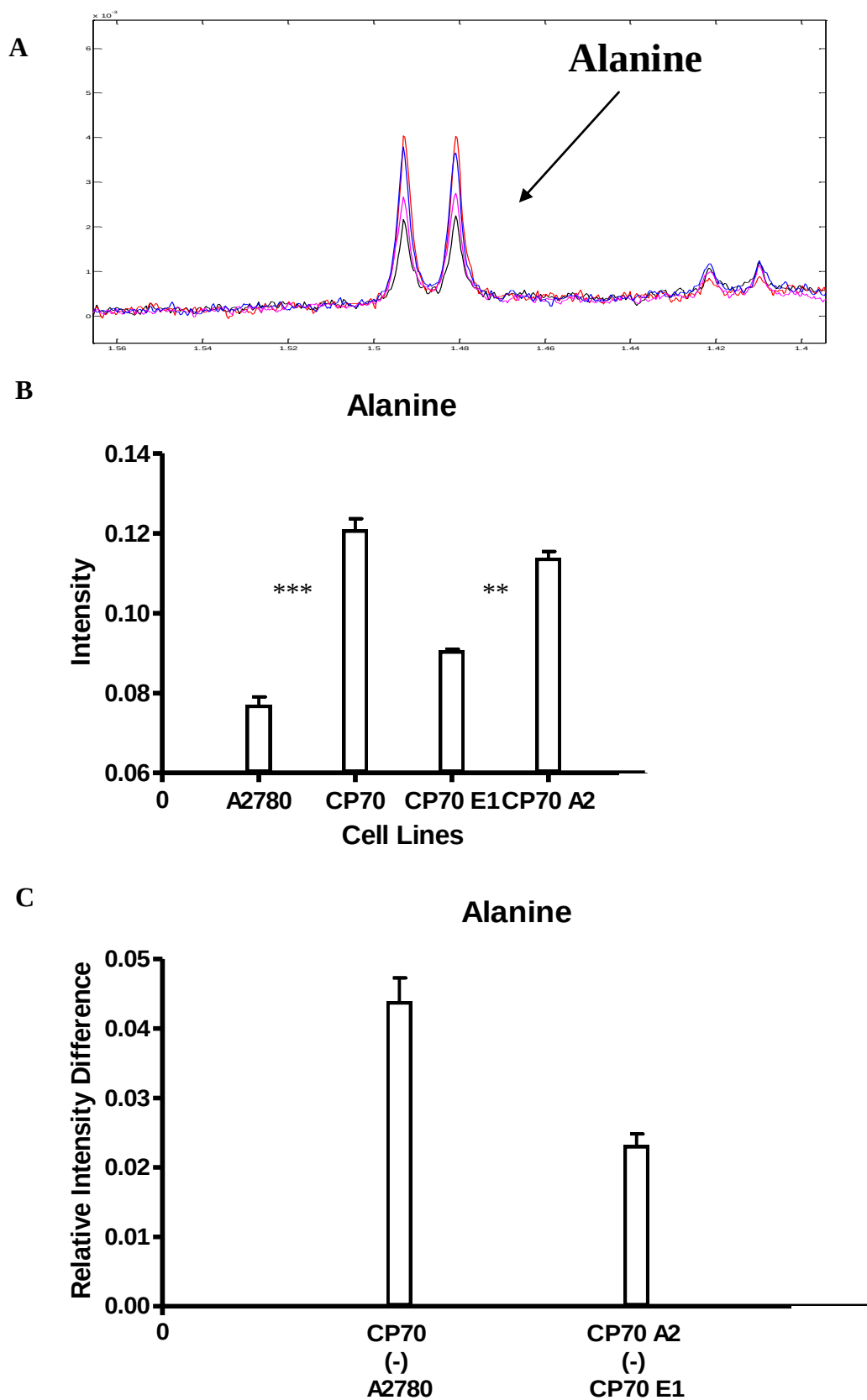


Figure 3.11 - The effect of *Mlh1* expression on intracellular alanine
 A) Median normalised spectra
 B) Integrated metabolite intensity
 C) Intensity difference between cell lines of differing *Mlh1* status
 n = 3. * p<0.05, ** p<0.01, *** p<0.001

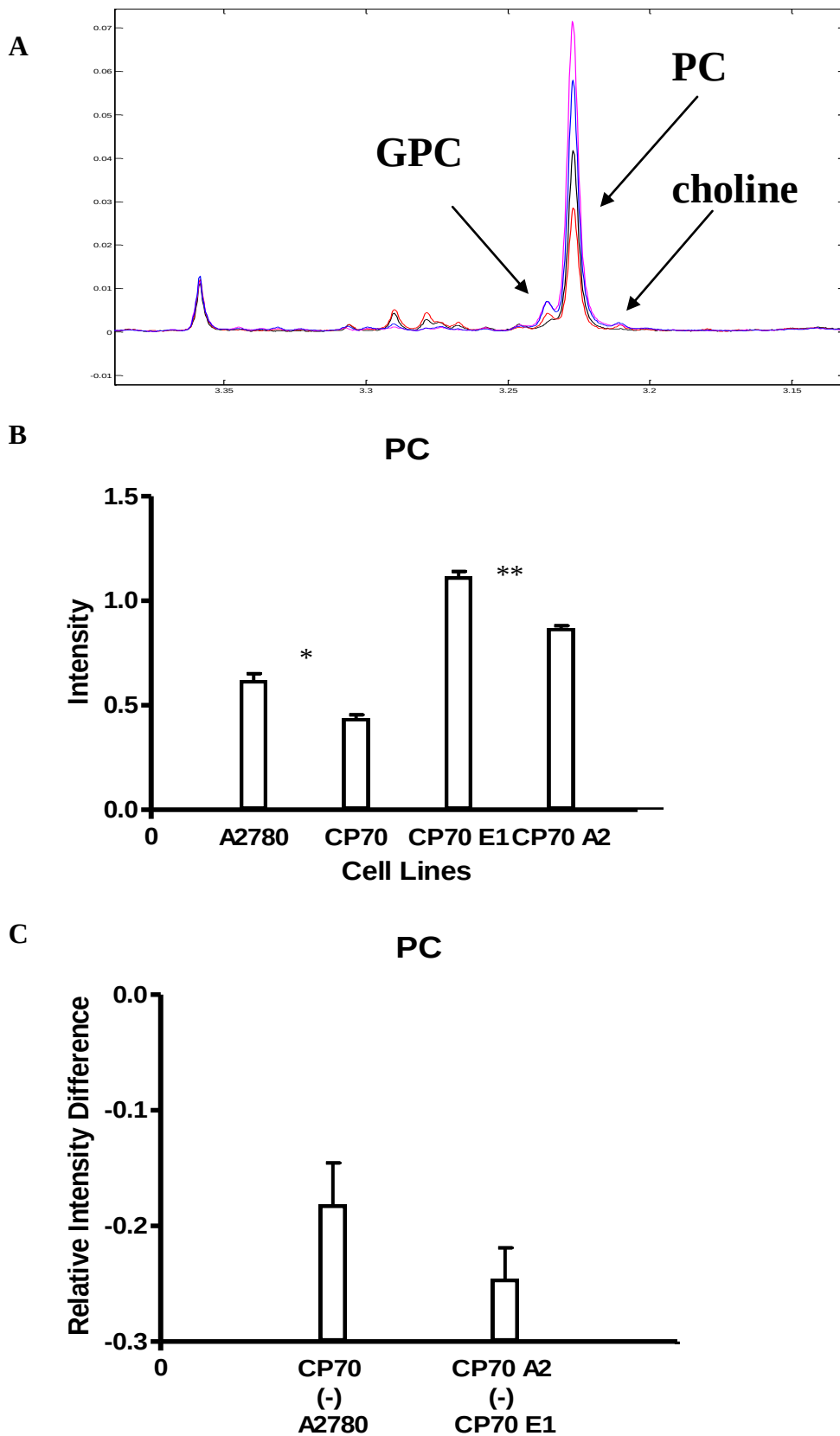


Figure 3.12 - The effect of *Mlh1* expression on intracellular phosphocholine

A) Median normalised spectra

B) Integrated metabolite intensity

C) Intensity difference between cell lines of differing *Mlh1* status (shown in negative scale)

n = 3. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$

3.3.3 Model Comparison & Metabolic Signature of *Mlh1*

Comparison of the different cell models allowed us to specifically identify a putative metabolic signature unique to the expression of the *Mlh1* gene. Metabolites that were consistently altered across the different conditions are described further below for all models and an explanation is given on why this identification was possible.

Table 3.5 describes the alterations in the panel of the most important metabolites identified from analysis of *Mlh1* expression across all cell lines in both the HEK-293T and Platinum Resistance models in terms of metabolic percentage change. The values for the intracellular aqueous extracts were based upon the average metabolite changes (n=3) with the *Mlh1*-negative cells referenced to their corresponding parental *Mlh1*-positive cell lines.

Ratios (% change)	CP70:A2780	CP70 A2:CP70 E1	MutLα ⁻ :MutLα ⁺	MutLα ⁻ ρ0:MutLα ⁺ ρ0
alanine	+57 ***	+26 **	+28 **	0
GSH	+54 *	+04	+07 *	+05
lactate (2)	-15	-01	+06	-10
myo-inositol	-03	+65 ***	+17 *	-02
choline	+72 *	+11 **	+07 *	+04
PC	-29 *	-22	-10	-01
GPC	+47	-07	-06	-04
creatine	+05	+52	+21	-07
unknown B	+08	+25 *	-10	+07
unknown A	+19	+24	+28	+12
glycine	-16	+48	+34	+5
PC (2)	-30*	-20*	-10*	-2

Table 3.5 - Percentage change in intracellular aqueous extracts of average metabolite integrals (n=3). Welch's two-sample unequal variance t-test with a two-tailed distribution. * p<0.05, ** p<0.01, * p<0.001**

The results showed that two models of different genetic origin exhibited similar metabolic behaviour. More specifically, the majority of the metabolic alterations, as displayed by the CP70 A2:CP70 E1 and MutLα⁻:MutLα⁺ ratio comparisons, showed similar trends in terms of both directionality and levels of change. This was made apparent despite the obvious differences in cellular origin between the two models, where the HEK-293T consists of kidney cell lines whereas the Platinum Resistance model consists of ovarian cell lines. A conclusion that could be extracted after seeing these metabolic similarities, is the fact that the

metabolic changes observed in this instance could only be originating from the *Mlh1* gene and its subsequent protein expression.

The reason for reaching this conclusion stands behind the actual genetic constitution of the different cell models employed in this study. On this particular experiment, the ovarian lines CP70 E1 and CP70 A2 differed only in terms of *Mlh1* possession; the CP70 E1 cell line is *Mlh1*-positive after *Mlh1* gene restoration, while CP70 A2 is *Mlh1*-negative. Both of these cell lines have chromosome 3 fragments (after the transfection procedure as described earlier) and both originate from CP70 cells, which constitute their parental cell line, and are *Mlh1*-negative. This model system was important in helping with the elucidation of the *Mlh1* metabolic signature as it acts in a very specific way, i.e. it allowed us to observe the effect of *Mlh1* in isolation from other genetic factors that might affect the resulting metabolic phenotype. In turn, the CP70 cell line was itself compared to a known standard cell line of known *Mlh1* status (A2780, which is *Mlh1*-positive). The CP70 and A2780 sub-model comparison was initially able to give us an indication of the metabolic levels of change *within* the Platinum Resistance model as both lines could act as controls of Platinum Resistance behaviour. In the same way, comparison of the MutL α - and MutL α + sub-model *within* the HEK-293T model (a system which allows transient *Mlh1* expression through doxocycline exposure by acting as an on/off *Mlh1* switch), could more accurately pinpoint metabolic changes in response to *Mlh1* expression being turned on or off.

By comparing these models and sub-models it was possible to draw a general picture of the possible metabolic effects attributed to *Mlh1* only. Figure 3.13 below assists in the comparative visualization of the directionality of the metabolic percent-change ratios between the different cell models and sub-models. A metabolic directionality was observed when comparing CP70 A2:A2780 vs. CP70 A2:CP70 E1 (Figure 3.13 A) and CP70:A2780 vs. MutL α -:MutL α + (Figure 3.13 B) sub-models, with metabolites such as GSH and alanine both showing increasing levels. The CP70 A2:CP70 E1 vs. MutL α - :MutL α (Figure 3.13 C) comparison though also produced a directionality (observed for the majority of metabolites, n=10) despite the fact that the sub-models belonged in different cell models. Some metabolites in particular such as alanine, GPC, and choline) showed close similarities not only in directionality but also on the level of metabolic change.

Metabolic signature of MLH1 (model and sub-model percentage change comparison)

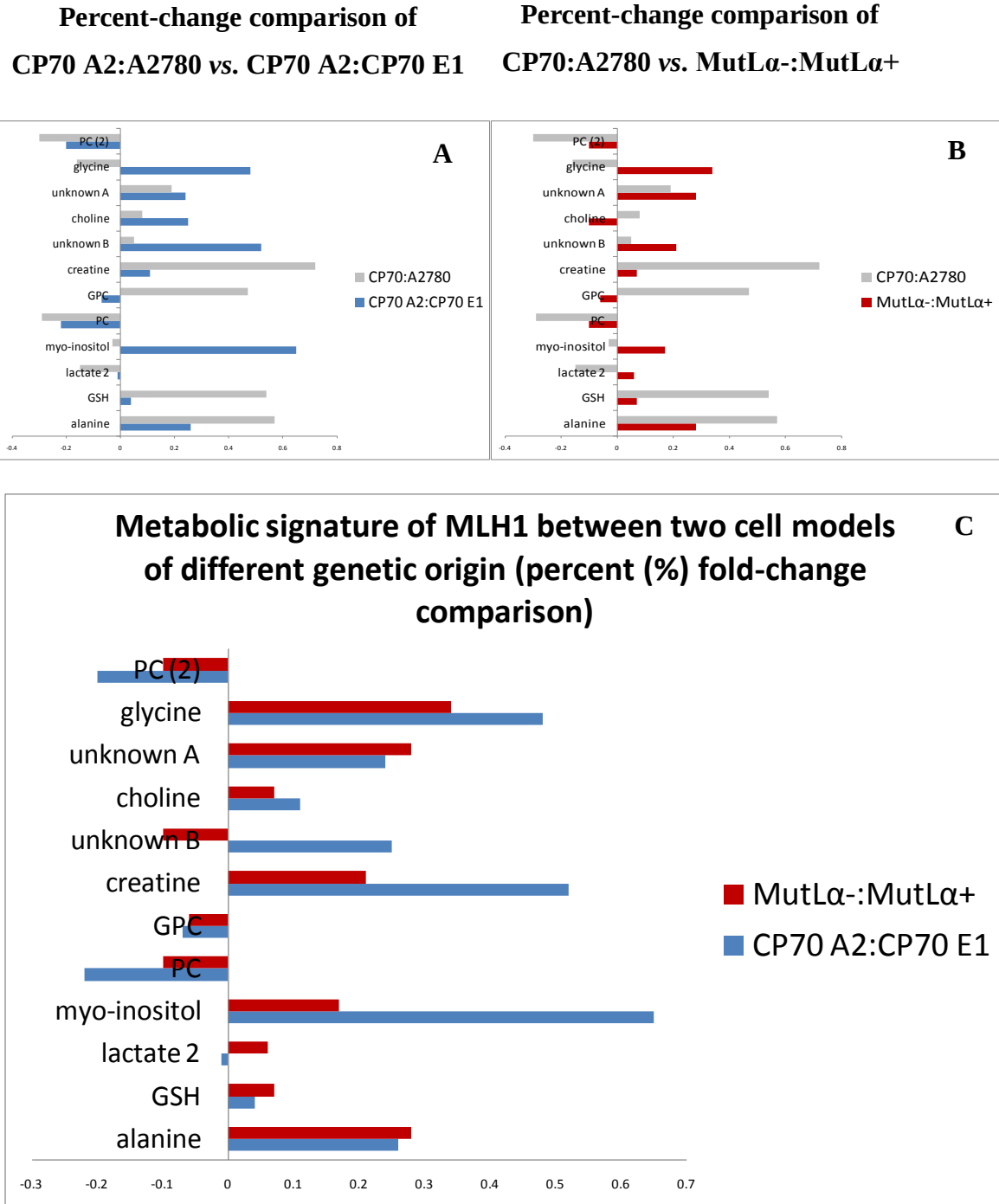


Figure 3.13 – Visual comparative representation of percentage change in intracellular aqueous extracts of average metabolite integrals in different cell models (n=3).

3.3.3.1 Metabolic ratios

Calculating metabolic ratios is a good way of examining relationships between metabolites, in this particular case, PC & GPC since changes in such a ratio could be associated with cell growth, cell cycling and, thus, cancer.

The ratio of PC to GPC can be important in terms of cell proliferation and cancer aggressiveness. The significance of the [PC]:[GPC] ratio in cell transformation and malignancy has been confirmed by various studies on tumour cell lines. In particular, it has been found that increased [PC]:[GPC] values may serve as indicators of malignancy in NMR tumour investigations (Bhakoo *et al.*, 1996).

Interestingly, there was a consistent decrease in the [PC]:[GPC] ratios in the *Mlh1*-negative cell lines (Figure 3.14) in this study, with the difference more apparent in the *Mlh1* cell lines of the platinum resistance model. In addition, the mitochondrial DNA-depleted cells exhibit the smallest change in ratio concentration.

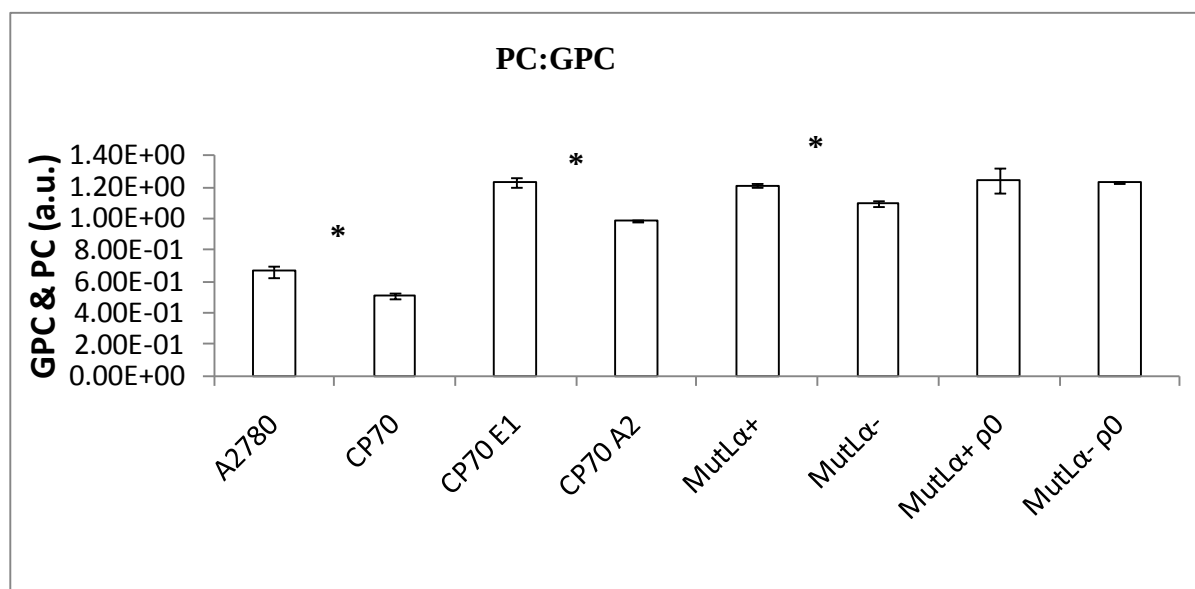


Figure 3.14 - PC:GPC ratios of average integrated metabolic intensities. Asterisk (*) denotes $p < 0.05$.

3.4 Discussion

In this study, pairs of kidney (HEK-293T derived) and ovarian cancer (A2780-derived) cell lines, either positive or negative for the MMR protein *Mlh1*, were assessed for metabolic changes correlating to loss of *Mlh1*, platinum resistance and mitochondrial status and function. Here, the association of loss of *Mlh1* with impaired mitochondria and platinum resistance within a metabolic context between two models of different cell origin *in vitro* was investigated.

3.4.1 Loss of *Mlh1* and its association with platinum resistance

Earlier reports from the clinical and preclinical setting regarding alterations in MMR proteins such as *Mlh1* suggested that they play a role in platinum chemotherapy resistance, and that the effect of the loss of *Mlh1* could be apparent even in cell lines of different origin (ovarian, lung, breast or colorectal). *In vitro* studies (Aebi, Kurdi-Haidar et al. 1996); (Brown, Hirst et al. 1997); (Fink, Aebi et al. 1998); (O'Brien and Brown 2006) have indeed reported a relationship between MMR loss and platinum resistance. Furthermore, clinical studies have linked MMR deficiency with decreased response to platinum-based chemotherapy (Fink, Nebel et al. 1998); (Strathdee, Appleton et al. 2001); (Gifford, Paul et al. 2004).

3.4.2 *In Vitro* Metabolic Signature of *Mlh1* Expression

In this study, the availability of two different cell models (HEK-293T & PRM) with differential *Mlh1* expression provided an ideal opportunity to study platinum drug resistance within a metabolic context. Here, the NMR-generated metabolic signature of *Mlh1* and its likely contribution to platinum resistance and impaired mitochondrial status is discussed:

The main metabolic changes in the NMR spectral data included changes in metabolites known to be important in cancer progression (i.e. phosphocholine) and increased platinum resistance (i.e. glutathione). These NMR-observed metabolites were found to be part of the overall *Mlh1* metabolic signature (described in section 3.3.3). A more comprehensive description of these metabolites is presented below and intends to discuss the meaning of these findings in more detail:

Glutathione (GSH)

GSH levels were consistently higher for all *Mlh1*-negative cells. This increase is interesting since reduced expression of *Mlh1* is associated with increased resistance to platinum (Ding,

Mohd et al. 2009) and at the same time, resistance to platinum has been linked to increased detoxification by thiol containing groups (Doz, Roosen et al. 1993). These results are indeed consistent with the changes observed here. The greatest percent-change increase was observed in the CP70 cells when compared to the A2780 cells, (suggesting the possibility that *Mlh1* is not the only relevant gene causing these changes between the cell groups). The rest of the cells showed a small-scale increase in GSH in both the PRM and the HEK-293T cell models. Since mitochondria are responsible for oxidative phosphorylation and the mitochondrial respiratory chain can be an important source of increased reactive oxygen species (ROS) in cancer (Pelicano, Carney et al. 2004), increases in GSH may be a compensation mechanism for the higher levels of oxidative stress resulting from improper metabolism of oxygen in the mitochondria. This is relevant to one of this study's main hypotheses, which theorised that *Mlh1* is somehow responsible for regulating mitochondrial function.

Alanine, Lactate and Branched Chain Amino Acids

There was a significant increase in intracellular alanine in all cells with *Mlh1* loss except in the case of the mitochondrial DNA-depleted cells (both MutL α ⁺ ρ 0 and MutL α ⁻ ρ 0) where the change was 0%. More specifically, alanine was found to be increased in CP70 (*Mlh1*-negative) cells as well as in CP70 A2 cells, which are also *Mlh1*-negative. Significantly higher alanine levels have been observed in other cancers like prostate cancer, consistent with a need for increased protein synthesis in tumour cells (Tessem, Swanson et al. 2008). Of course, increases in alanine could also be attributed to catabolism of proteins within the cancer cell. Another explanation could be the metabolism of lactate. As lactate is also known to be converted to alanine via pyruvate by ALT (alanine transaminase) and lactate dehydrogenase (LDH), this may be a possible source of the significant increase in alanine. In our study however, the levels of intracellular lactate appear to be slightly decreased in all *Mlh1*-negative cells across both models with the exception of MutL α ⁻ which shows a slight increase in the HEK-293T model. These decreases are mostly in contrast to the generally accepted idea of increased glycolysis and enhanced lactate production in cancerous cells as compared to non-cancerous ones - a typical feature of tumour cell metabolism – thus, at this instance lactate does not seem to follow the expected glycolytic pattern or be directly linked to the alanine increases. Interestingly though, reports regarding alanine formation had shown that it can also be produced through the oxidation and catabolism of branched-chain amino acids, namely valine, leucine, and isoleucine (Odessey, Khairallah et al. 1974). Along with

alanine, all three of these amino acids were identified as some of the biggest separators of *Mlh1* status in the HEK-293T model. Such an observation was not made in the PRM though, possibly due to additional genetic factors coming into play. Cells use leucine, isoleucine, and valine for protein synthesis or alternatively these amino acids are catabolized into sources for glucose and lipid generation (Doering and Danner 2000). Preservation of these amino acids is important in cell survival. It may be possible that the HEK-293T cell model revealed some increased need of the cancer cells for amino acids for the production of alanine.

Choline-related metabolites

Choline and its metabolites are generally needed for maintaining the structural integrity and signalling functions of cellular membranes. However, choline-related metabolism has been previously identified to be altered in tumour cells and tissues (Podo 1999); (Glunde, Jacobs et al. 2006) - with emphasis given on three main molecules: choline, phosphocholine (PC) and glycerophosphocholine (GPC).

In our study, intracellular PC levels were seen to decrease in all the *Mlh1*-negative cells. However the decrease in the mitochondrial DNA-depleted cells was miniscule at just 1%. Changes in GPC levels were not consistent across the *Mlh1*-negative cells, with a great increase observed in the ovarian *Mlh1*-negative CP70 cell line at 47%. The rest of the *Mlh1* negative cells exhibited a small decrease in their GPC concentration levels. Increased PC levels have been observed in tumours (Eliyahu, Kreizman et al. 2007) and in cells with loss of tumour suppressor genes such as p53 (Mori, Delsite et al. 2004), as well as in cells with higher growth rates and increasing tumorigenic potential in general. Increases in PC have also been observed in ovarian cell lines (Iorio, Mezzanzanica et al. 2005). In our study such an observation could not be made, and thus, it was not currently possible to associate *Mlh1* loss and PC with increased cancer aggressiveness or metastasis. The reason why PC changed in the non mitochondrial DNA-depleted cells but remained stable in the *Mlh1*-negative ones who had their mitochondrial DNA depleted could be perhaps explained by the fact that PC is involved in cell membrane synthesis by acting as a precursor of phosphatidylcholine in phospholipid membranes (Attard, Jean et al. 1999). The decrease in PC levels observed in our study for the *Mlh1*-negative cells could be consistent with decreased utilization for cell membrane synthesis implying some possible defect in membrane structure. The other possibility is an increased hydrolysis of PC into choline, hence its increased levels (see below). In addition, the fact that mitochondria-depleted cells in the HEK-293T kidney cell

model show only a very small difference in PC levels may also support a connection between *Mlh1* and mitochondria, since in the absence of mitochondria, PC would not be utilized any further. Since PC is catalysed by choline kinase converting ATP + choline into PC and ADP, the stable PC levels in the mitochondria-depleted cells may reflect a type of oxidative phosphorylation dysfunction in the mitochondria.

Choline levels were increased intracellularly in all *Mlh1*-negative cells. The greatest percent-change increase was observed in the CP70 cells at 72% followed by CP70 A2. The differences in the HEK-293T model were less pronounced compared to the ovarian cells, and were increased by 7% for the MutL α - cells and 4% for the MutL α - p0 cells. The increased choline levels could perhaps be explained in association with the PC findings discussed above; it might be possible that at the specific time point of the experiment, phosphorylation and conversion of choline into phosphocholine by choline kinase had not occurred yet, hence the decreased PC but increased choline concentrations in the *Mlh1*-negative cells. This could be confirmed in future experiments through monitoring the enzyme activity of choline kinase.

Myo-inositol

Myo-inositol increased the most in the *Mlh1*-negative CP70 A2 cells from the PRM model and the MutL α - cells from the HEK-293T cell model. It also decreased slightly for both CP70 and MutL α - p0 cells, however this difference was minuscule. These changes are probably mostly indicative of the background genetic variability in the tested cell lines. The increases observed in some of the *Mlh1*-negative cells, may also reflect differences caused by the genetic manipulation (transfection/microcell transfer) that was part of the experimental process. It might also be possible that, with myo-inositol being an osmolyte, increased levels were needed to accommodate for a possible increase in osmotic stress for these particular cell lines - which is usually achieved through downregulation of intracellular sodium and potassium (Kultz and Csonka 1999).

3.4.3 Concluding remarks

Our findings offer a range of possible explanations on the role of specific metabolites and their association with the *Mlh1* gene, platinum resistance and mitochondria. It has been found that cells exposed to cisplatin exhibit certain degrees of inhibition in respiration (Tacka, Dabrowiak et al. 2004) possibly due to direct conjugation to DNA and proteins of

mitochondria (Cullen, Yang et al. 2007). It is possible that cells lacking *Mlh1* are more capable in maintaining their homeostasis and energy balance than cells possessing the fully functional copy of the gene, thus rendering them more resistant to chemotherapeutic treatments. Overall, this study has managed to isolate and identify a unique *Mlh1* metabolic signature, which includes metabolites with an important role in cancer development, as well as to connect mitochondrial metabolism to *Mlh1* loss of expression.

Chapter 4

Over-representation analysis of pathways significant with genes and metabolites selected by correlation to *Mlh1*

4.1 Introduction

As a continuation of the *Mlh1* study presented in chapter 3, a novel bioinformatics approach, which is described in section 4.2, was applied in order to compare our previous metabolic findings with specific published NCI-60 cell line microarray gene expression profiles and check for any possible associations between them.

The NCI-60 is a gene expression panel consisting of 60 cancer cell lines displaying patterns according to their tissue of origin. The tissues involved include types of hematopoietic, epithelial, mesenchymal and melanocytic origin (Ross *et al.* 2000). It was developed by the US National Cancer Institute (NCI) as a drug discovery tool and cellular mechanics information source and was eventually developed to serve as an anticancer drug screen support (Shoemaker, 2006).

Table 4.1 lists the cell lines included in the NCI-60 panel and whose publicly-available gene expression profiles were used in this study.

The analysis presented later in this chapter was made possible through the use of a continually updated online database system, called ConsensusPathDB:

ConsensusPathDB is a database focusing on interactions between entities such as genes, RNA proteins, metabolites and various types of complexes in humans. This can make possible the study of heterogeneous interactions through their comprehensive integration into the system, and thus, increase the chances of the analysis being closer to the biological reality. More specifically, the analysis of biological systems and their functions through interaction networks has been steadily increasing over recent years, facilitating the generation of knowledge and understanding of biological processes either in a molecular or a wider systemic level. However, a considerable amount of these interactions are interspersed in a variety of different databases, which either focus on different sets of reactions, or face problems with incomplete overlaps and differing information and formatting details, even between similar databases. Thus, a question arises about the ability of such interaction networks to represent the “biological truth” in a more wholesome way. For the time being, ConsensusPathDB seems to be one of the best answers in addressing this issue.

NCI-60 Cell Lines			
Cell Line Name	Panel Name	Cell Line Name	Panel Name
CCRF-CEM	Leukemia	M14	Melanoma
HL-60(TB)	Leukemia	MDA-MB-435	Melanoma
K-562	Leukemia	SK-MEL-28	Melanoma
MOLT-4	Leukemia	SK-MEL-5	Melanoma
RPMI-8226	Leukemia	UACC-257	Melanoma
SR	Leukemia	UACC-62	Melanoma
A549/ATCC	Non-Small Cell Lung	IGR-OV1	Ovarian
EKVX	Non-Small Cell Lung	OVCAR-3	Ovarian
HOP-62	Non-Small Cell Lung	OVCAR-4	Ovarian
HOP-92	Non-Small Cell Lung	OVCAR-5	Ovarian
NCI-H226	Non-Small Cell Lung	OVCAR-8	Ovarian
NCI-H23	Non-Small Cell Lung	NCI/ADR-RES	Ovarian
NCI-H322M	Non-Small Cell Lung	SK-OV-3	Ovarian
NCI-H460	Non-Small Cell Lung	786-0	Renal
NCI-H522	Non-Small Cell Lung	A498	Renal
COLO 205	Colon	ACHN	Renal
HCC-2998	Colon	CAKI-1	Renal
HCT-116	Colon	RXF 393	Renal
HCT-15	Colon	SN12C	Renal
HT29	Colon	TK-10	Renal
KM12	Colon	UO-31	Renal
SW-620	Colon	PC-3	Prostate
SF-268	CNS	DU-145	Prostate
SF-295	CNS	MCF7	Breast
SF-539	CNS	MDA-MB-231/ATCC	Breast
SNB-19	CNS	HS 578T	Breast
SNB-75	CNS	BT-549	Breast
U251	CNS	T-47D	Breast
LOX IMVI	Melanoma		
MALME-3M	Melanoma		

Table 4.1 – List of the NCI-60 cell lines and their corresponding tissues of origin used in this study (sourced from NCI <http://dtp.cancer.gov/mtargets/mt_index.html> Accessed 24 June 2010).

So far the whole ConsensusPathDB database includes interactions from 12 separate databases (Table 4.2) at the time of writing. Because not all of these databases have the same name for their housed interactions, the way all these different entities are linked to each other is through the use of common identifiers such as UniProt in the case of proteins (Uniprot Consortium, 2010), Ensembl in the case of genes or transcripts (Flicek *et al.*, 2008) and KEGG/ChEBI in the case of metabolites (Degtyarenko *et al.*, 2008).

Integrated Databases in ConsensusPathDB.
BioCarta (signalling)
BioGRID (protein)
DIP (protein)
HPRD (protein)
HumanCyc (metabolic)
IntAct (protein)
KEGG (metabolic)
MINT (protein)
Netpath (signalling)
PID - Pathway Interaction Database (signalling)
PIG - Pathogen Interaction Gateway (host-pathogenic & host-host protein)
Reactome (metabolic and signalling)
SPIKE (protein interactions, signalling)

Table 4.2 – Databases and pathways included in the ConsensusPathDB online database (data supplied by (Kamburov, Wierling et al. 2009).

The general methodology and results of this chapter's analysis are presented in the following sections:

4.2 Methods

One of the biggest problems with traditional biochemical pathway networks is that the construction and “packaging” of these molecular interactions are arbitrary, and at times it can be based on the personal choices of the map-constructing individuals. As previously mentioned, this can sometimes lead to a biased network representation of the biological reality. It can also lead to various biochemical interactions being overlooked. This study has tried to overcome this problem by employing a novel bioinformatics-based methodology, explained in more detail below, while looking at the same time for *Mlh1*-based associations with the results of the previous chapter.

In particular, a novel method involving the microarray gene expression profiles of the NCI-60 panel using the ConsensusPathDB database was tested for the first time, focusing on the search for gene and metabolic pathway associations involving *Mlh1*. This procedure, aimed at overcoming the problem posed by the traditional biochemical pathway networks, by employing the use of Over-Representation Analysis (ORA) and Neighbourhood-based entity sets (NESTs).

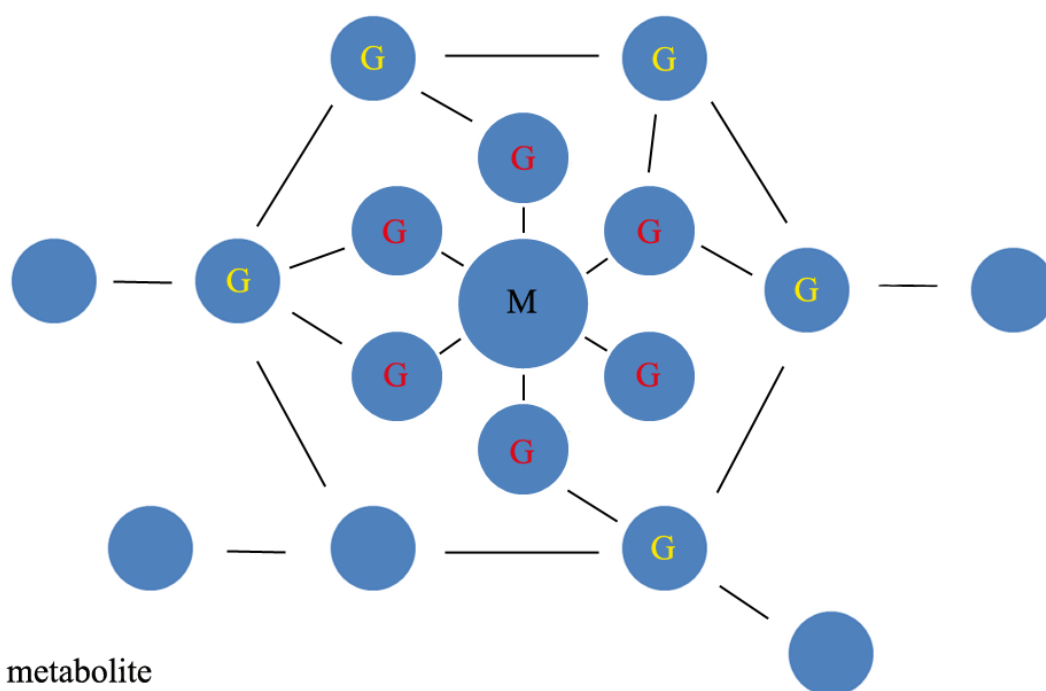
Over-representation analysis is an effective way of addressing the challenge of interpreting gene lists generated from microarray data. It uses a cumulative hypergeometric test to define whether a set of genes in a list is statistically represented more than what would be expected by chance (Leong, 2009).

The NEST-based approach was also chosen because of the fact that it can overcome single-database boundaries, as it can continue even further beyond and display cross-talking between pathways.

Figure 4.1 illustrates the general principle in the neighbourhood-based approach. The NESTs form sub-networks that contain all the physical entities around a central sub-network, which has a “radius”. This is defined as the number of interactions from the centre. When there is a single interaction the radius is said to be a radius of one (radius 1), whilst with two interactions, the radius is two (radius 2). The area including the metabolite and the genes for each radius will be referred to as a “module”.

In general, the genes in the NCI-60 panel can be measured for significant correlations with another gene (such as *Mlh1* in this study), by searching for physical entities (i.e. metabolites) that have a predefined number of genes surrounding them. For example, in this chapter, the number of surrounding genes was set to 2, for reasons explained later on in this section.

NEST INTERACTION NETWORK



M = metabolite

G = gene located 1 step (radius of 1) away from metabolite

G = gene located two steps (radius of 2) away from metabolite

Figure 4.1 – Example map of NEST network interactions between metabolites and related genes one step (radius of 1) or two steps away (radius of 2) from a metabolite located at the centre of the network.

The basic steps that were taken during the NEST-based approach in this study are presented below:

- 1) Firstly, the publicly available microarray gene expression data from the NCI-60 cancer cell line were downloaded (Bussey, 2006) and, subsequently, the total number of the genes present in the NCI-60 panel was translated into Uniprot protein identifiers for use with the ConsensusPathDB database.

***NOTE:** From this point onwards, the term “proteins” will be used to refer to the corresponding genes, as each of the genes is now considered to be linked to the Uniprot label of the protein it codes for.*

- 2) This was followed by the generation of a panel with 241 protein names significantly correlated with *Mlh1*. This was generated through specific statistical tests involving the Pearson correlation and the subsequent application of a False Discovery Rate (FDR) using a 20% cut-off, which was applied to the set of p values on the total panel of proteins to determine the level of false positives.
- 3) As a separate step, the input lists consisting of the list of genes measured in the transcriptomic data on the NCI-60 panel (31,122 proteins with 15,506 unique IDs) and the generated gene panel of the *Mlh1*-associated proteins (241 proteins with 225 unique IDs) were entered into *in-house* software written in Python (by Rachel Cavill, 2009) for comparison with the modules (NESTs) present in the ConsensusPath DB database. Subsequently, the total number of proteins in the NCI-60 panel was compared with the ConsensusPathDB data, and the effective size of modules (including any identifier repeats) was calculated. This effective size was in turn compared to the *Mlh1*-associated panel of 241 proteins, which counted the number of selected proteins in each module. The probability (p-values) of this amount of overlap was then calculated through the application of a hypergeometric distribution test, as mentioned earlier. The above procedure focused on a unique module each time before moving on to the next one in line. Thus, for the “radius 1” analysis a total of 2,021 modules were examined, while for the “radius 2” one, 1973 modules were examined in total. Finally, a filtering process was also applied, where NESTs were selected based on if they had more than 1 protein around a metabolite (>1), as well as a probability value less than 0.05 ($p < 0.05$). This filtering

tried to achieve a strengthening of the significance of the resulting data and, thus, increase the confidence on the results. (As already explained, the p-values reflected how significant the overlap between the input list and the members of the pathways were as compared to random, with small p-values indicating that the input list contained more members than as expected by chance).

4.3 Results

The results of the NEST-based analysis are presented in this section of the chapter:

The list of the 241 protein names generated at the beginning of the analysis, which were found to be associated with *Mlh1*, can be seen in the appendix section at the end of this thesis, while the results of the NEST analysis are summarised in Table 4.4. The following table (Table 4.3) lists some of the metabolic pathways associated with each metabolite:

Metabolic Pathways			
oleic acid	palmitate	stearate	sialyl-3-paragloboside
fatty acid amide hydrolase	Interaction of Hexadecanoic acid	Stearoyl-CoA hydrolase	GDP-L-fucose + Sialyl-3-paragloboside $\rightleftharpoons$ GDP + IV3NeuAc,III3Fuc-nLc4Cer
Oleoyl-[acyl-carrier protein] hydrolase	Interaction of Hexadecanoic acid and Protein	Octadecanoyl-[acyl-carrier protein] + H ₂ O $\rightleftharpoons$ Acyl-carrier protein + Octadecanoic acid	CMP-N-acetylneuraminate + Paragloboside $\rightleftharpoons$ CMP + Sialyl-3-paragloboside
Oleoyl-[acyl-carrier protein] + H ₂ O $\rightleftharpoons$ Acyl-carrier protein + (9Z)-Octadecenoic acid	Palmitoyl-CoA hydrolase	ceramide + H ₂ O $\rightleftharpoons$ stearate + sphingosine [endoplasmic reticulum]	CMP-N-acetylneuraminate + Sialyl-3-paragloboside $\rightleftharpoons$ CMP + 3',8'-LD1
Interaction of Oleoyl-[acyl-carrier protein] and H ₂ O	depalmitoylation of eNOS	Stearoyl-CoA + H ₂ O $\rightleftharpoons$ CoA + Octadecanoic acid	Interaction of GDP-L-fucose and alpha-N-
Interaction of H ₂ O and Oleoyl-CoA	ATP + Hexadecanoic acid + CoA $\rightleftharpoons$ AMP + Palmitoyl-CoA + Diphosphate	Interaction of H ₂ O and Stearoyl-CoA	Acetylneuraminy-2,3-beta-D-galactosyl-1,4-N-acetyl-beta-D-
RXN-9644	Interaction of Palmitoyl-CoA and H ₂ O	Interaction of Octadecanoic acid	Interaction of CMP-N-acetylneuraminate and beta-D-Galactosyl-1,4-N-acetyl-beta-D-
Oleoyl-CoA + H ₂ O $\rightleftharpoons$ CoA + (9Z)-Octadecenoic acid	1-palmitoylglycerone phosphate + hexadecanol $\Rightarrow$ O-hexadecylglycerone phosphate + palmitate	phytoceramide + H ₂ O $\Rightarrow$ stearate + phytosphingosine	glucosaminy-1,3-beta-D-Neolactotetraosylceramide α -2,3-sialyltransferase
	Interaction of Hexadecanoyl-[acp] and H ₂ O	ceramide + H ₂ O $\Rightarrow$ stearate + sphingosine [Golgi]	

Table 4.3 – Metabolic pathways associated with each metabolite according to CosensusPath database

As already mentioned, a part of this procedure involved ignoring those pathways that had only a single picked entity (since the p-value on these occasions may be misleading) and focusing on metabolites that had at least two significant proteins around them in each module. By looking at the generated table (Table 4.4) with the results of the analysis, there were only four metabolites which were found at the centres of the modules, and which had more proteins from the *Mlh1* 20% FDR cut-off list, than one would expect at random.

Looking at all the proteins one step away from the metabolites, there are 9 proteins one step (one radius) away from oleic acid with two of them associated with *Mlh1* ($p = 0.00207$). There are also 37 proteins one step away from palmitate (palmic acid), with two of them associated with *Mlh1* ($p = 0.03330$).

Looking out further, i.e. two steps away from the metabolite (radius of two), two more significant modules can be found; those for sialyl-3-paragloboside (2 out of 38 proteins, $p = 0.03497$) and stearate (2 out of 40 proteins, $p = 0.03841$).

The table below (Table 4.4) displays the list of the top significant metabolites for each radius, respectively. As already mentioned, those metabolites consisted of oleic acid, palmitate, sialyl-3-paragloboside and stearate.

Metabolites	Number of modules	Proteins in pathway	Proteins selected	Protein P-value
Radius 1	2021			
Oleic acid		9	2	0.00207
Palmitate		37	2	0.0333
Radius 2	1973			
sialyl-3-paragloboside		38	2	0.03497
stearate		40	2	0.03841

Table 4.4 - List of significant metabolite pathways using a radius of one (Radius 1) and a radius of two (Radius 2) distance from the metabolic nest after applying a threshold of >1 and $p < 0.05$ in the number of selected proteins.

After these metabolites were identified, it was also possible to cross-check which proteins from each of these modules were in the *Mlh1*-correlated list of 241 proteins. The cross-examination generated the results seen in table 4.5.

These protein names included ACOT4_HUMAN and FAS_HUMAN in the case of oleic acid, palmitate and stearate, and FUT9_HUMAN and NCAM1_HUMAN in the case of sialyl-3-paragloboside.

The online-generated ConsensusPathDB network map of oleic acid in Figure 4.2 is a visualisation example of these interactions.

Oleic acid (radius 1)

ACOT1_HUMAN, ACOT2_HUMAN, **ACOT4_HUMAN**, BAAT_HUMAN, BACH_HUMAN,
FAS_HUMAN, FFAR1_HUMAN, SAST_HUMAN

Palmitate (radius 1)

A8K2J7_HUMAN, ACOT1_HUMAN, ACOT2_HUMAN, **ACOT4_HUMAN**, ACOT8_HUMAN,
ACSL1_HUMAN, ACSL3_HUMAN, ACSL4_HUMAN, ACSL5_HUMAN, ACSL6_HUMAN, BAAT_HUMAN,
BACH_HUMAN, **FAS_HUMAN**, FFAR1_HUMAN, PPARA_HUMAN, PPT1_HUMAN, PPT2_HUMAN,
RXRA_HUMAN, SAST_HUMAN, TBL1R_HUMAN, TBL1X_HUMAN

Stearate (radius 2)

ABCB_B_HUMAN, ACOT1_HUMAN, ACOT2_HUMAN, **ACOT4_HUMAN**, ACOT8_HUMAN,
APHC_HUMAN, ASA2C_HUMAN, ASA3L_HUMAN, ASAH2_HUMAN, ASAH1_HUMAN, ASAH3_HUMAN,
ATX1_HUMAN, BAAT_HUMAN, BACH_HUMAN, **FAS_HUMAN**, LAT2_HUMAN, NLTP_HUMAN,
PPT1_HUMAN, PPT2_HUMAN, Q96EA2_HUMAN, S27A5_HUMAN, SAST_HUMAN, XCT_HUMAN, ZN277_
HUMAN

Sialyl-3-paragloboside (radius 2)

CF015_HUMAN, FUT4_HUMAN, FUT5_HUMAN, FUT6_HUMAN, FUT7_HUMAN, **FUT9_HUMAN**,
FUT10_HUMAN, **NCAM1_HUMAN**, SIA8A_HUMAN, SIA8B_HUMAN, SIA8C_HUMAN,
SIA8D_HUMAN, SIA8E_HUMAN, SIA8F_HUMAN, SIA10_HUMAN

Table 4.5 - Proteins found to be correlated to metabolites associated with *Mlh1* in the tested protein list. Protein names highlighted in red colour represent the proteins found in the *Mlh1*-correlated NCI-60 20% cut-off list.

During the ConsensusPathDB analysis, proteins not present in the list of the tested proteins, can still be seen in the network map (Figure 4.2) around each metabolite mentioned. This is due to a name-spacing mapping problem, so no test could be applied to check for their significance. For example in the case of oleic acid (Figure 4.2) some genes, namely ACBG1_HUMAN, ACBG2_HUMAN, FAAH1_HUMAN and FAAH2_HUMAN, were absent from the total protein list.

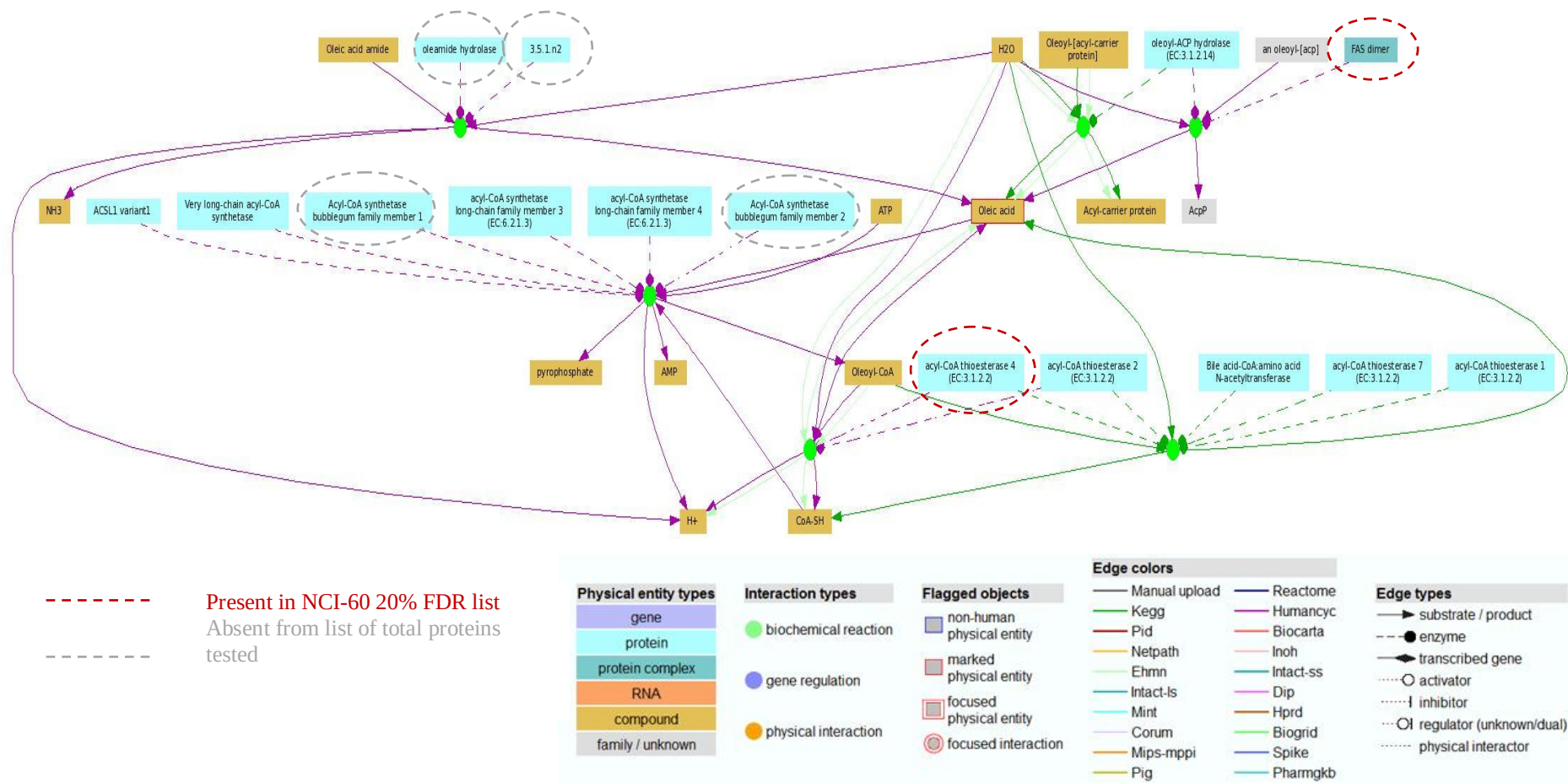


Figure 4.2 - Example of a ConsensusPathDB-generated network map of physical entities and complexes related to a metabolite (oleic acid in this instance) which has been previously significantly associated with *Mlh1*. Related proteins (in blue/green colour) correspond to the tested NCI-60 proteins. Out of the 14 proteins appearing in the map, two of them (circled in red) are present in the *Mlh1*-associated panel of 241 proteins.

4.3 Discussion

4.3.1. *Mlh1*-correlated genes and metabolites

The *Mlh1*-associated genes that were identified as significant using the bioinformatics-based methodology described in this chapter were ACOT4, FAS, FUT9 and NCAM1 while their related metabolites were oleic acid, palmitate, stearate and sialyl-3-paragloboside. Here, we take a more detailed look at these entities as a way of confirming a possible connection with platinum resistance in cancer and, wherever possible, an association with the metabolic findings of the previous chapter (chapter 3).

One of the genes that came up as significant in this study was ACOT4. ACOT4 encodes for Acyl-coenzyme A thioesterase 4. These thioesterases are enzymes responsible for the hydrolysis of acyl-CoAs to the free fatty acid and coenzyme A (CoASH), thus, being able to regulate intracellular concentrations of acyl-CoAs, free fatty acids and CoASH (Hunt and Alexson 2002). In particular, according to GeneRIF, ACOT4 is responsible for the termination of beta-oxidation of medium-chain dicarboxylic acids releasing the corresponding free acids (Westin, Hunt et al. 2005). The significant metabolites associated with ACOT4 were three closely related metabolites, namely oleic acid, palmitate and stearate.

Palmitate (hexadecanoate) is a typical fatty acid the anionic form of saturated palmitic acid. It is involved in various metabolic pathways including beta-oxidation, Krebs cycle and the respiratory chain for the production of energy in many tissues, including muscle tissues. It is formed from tripalmitin and its subsequent oxidation reactions involve catalysis of palmitoyl CoA by a long chain acyl CoA synthetase. Palmitate oxidation for the production of energy is impressive as it can provide a net yield of 129 ATP molecules per molecule (Salway 2004). At the moment however, there is insufficient data to provide a link between palmitate, cancer or platinum resistance; there have been reports however that have linked its retinyl form (retinyl palmitate) to genotoxicity and photo-clastogenicity in mouse lymphoma cells and in Jurkat T-cells (Dufour, Whitwell et al. 2009).

The other ACOT4 associated metabolite that appeared important in the correlation with *Mlh1* was oleic acid. In animals, oleic acid is generated by the dehydrogenation of stearate (Annison, Linzell et al. 1967), which was also associated with ACOT4 in this study. Past reports have shown that in breast cancer cells, oleic acid induced migration and proliferation of the cells as well as increased

survival and invasion. It also involved activation of MEK1/2, ERK1/2, FAK and Src, which also have been associated with cancer (Soto-Guzman *et al.*, 2009).

Fas was another important gene identified in this study, which according to published data in the literature, is one of the strongest candidates for conferring platinum drug resistance in cancer cells. *Fas* is a 45 KDa transmembrane polypeptide found on the cell membranes of normal and cancerous cells (Watanabe-Fukunaga *et al.*, 1992; Nagata, 1994). Together with its ligand, *FasL*, the *Fas/FasL* system can regulate the process of apoptosis. Cancer cells can avoid apoptosis by down-regulating the expression of *Fas*. For example, lung cancer cells that were cisplatin-resistant showed low *Fas* protein levels with a significant decrease in apoptosis. Additional reports showed that *Fas* over-expression could reverse drug resistance in H446/CDDP cells. The *Fas* mechanisms by which cisplatin resistance is regulated still remain unclear though. Perhaps, *Fas*-resistant cells can be resistant to chemotherapy, due to the disruption of pathways involved in the apoptotic induction by chemotherapeutic drugs (Los, Herr *et al.* 1997); (Wu, Wang *et al.* 2010).

FUT9 and NCAM1 were also *Mlh1*-related genes that were identified as significant, while their related metabolite was Sialyl-3-paragloboside. FUT9 is one of the genes encoding for a group of enzymes called alpha-3-fucosyltransferases which can catalyze the reaction in the synthesis of Lewis antigens (Hittelet, Camby *et al.* 2003). FUT9 also synthesizes the LeX oligosaccharide, a sugar expressed in organ buds during human embryogenesis. This antigen has been mentioned to be aberrantly expressed in association with various malignant tissues (Cailleau-Thomas, Coullin *et al.* 2000); (Hakomori 1998); (Orntoft and Vestergaard 1999) and perhaps may give some validity in the association between FUT9 and platinum resistance; however, for the time being, knowledge regarding this connection is very limited. NCAMs (neural cell adhesion molecules) are antigens expressed in normal tissues but also in cancers such as neuroblastomas, myelomas and acute myeloid leukaemias. Current experimental regimens involve anti-NCAM target therapy (Jensen and Berthold 2007) in cancer patients but there are no data presently available regarding its association with resistance to platinum drugs, especially in ovarian cancer. However, recent studies have connected various immunoglobulin-like cell adhesion molecules with ovarian cancer and receptor tyrosine kinases. In addition, proteolytic processing of these molecules produces fragments whose activities that can support ovarian cancer development (Bombardelli and Cavallaro 2010). Overall, it would be prudent that some consideration was given to these genes and their associated metabolites for possible implications with platinum resistance in the future.

4.3.2 Correlation with previous findings

Apart from the above correlations of the aforementioned *Mlh1*-associated genes and metabolites with cancer and/or platinum resistance, the previous metabolic findings in Chapter 3 were also compared with the findings presented in the current chapter.

In the previous chapter, the metabolites whose levels showed significant changes and that were found to be correlated with *Mlh1* expression and platinum resistance included glutathione, alanine, lactate, branched chain amino acids, *myo*-inositol and choline-related metabolites such as phosphocholine.

It was not currently possible to find a direct connection between the findings in the two chapters (chapters 3 and 4), as none of the metabolites in the bioinformatics study and the cell-cultured based study appear to be associated at a first glance. However, while looking for indirect relationships an interesting fact seemed to appear in the case of phosphocholine. In the previous chapter it was mentioned that phosphocholine is involved in cell membrane synthesis by acting as a precursor of phosphatidylcholine in phospholipid membranes (Attard, Jean et al. 1999). In the past, there was a study on the mechanism through which certain fatty acids could enhance the biosynthesis of phosphatidylcholine in rat hepatocytes in culture. In particular, long-chained fatty acids increased incorporation of [methyl-(3)H] choline from phosphocholine into phosphatidylcholine, while oleate and palmitate supplementation enhanced phosphatidylcholine production by 3- and 2.2-fold, respectively (Pelech, Pritchard et al. 1983). The mechanism by which these events occurred is still unclear but it could for example, involve the utilisation of ACOT4 as a means of driving these events forward, perhaps by indirectly promoting phosphocholine utilisation in the cells.

4.3.3 Concluding Remarks

In conclusion, this study successfully tested in practice a novel way for a NEST-based examination of complex pathways involving genes and metabolites, using *Mlh1* as a representative example to showcase its effectiveness. It also managed to identify candidate genes that, through their relation to *Mlh1* as well as various cancers, may make them a good starting point for basing platinum resistance-related experiments in the future.

Chapter 5

Metabolic examination of platinum resistance in cancer cells using *in vivo* cell models of platinum resistance.

5.1 Introduction

Ovarian cancer is a devastating disease affecting millions of women worldwide (Tsigkou, Marrelli et al. 2007). Despite successful initial therapy with platinum drugs in women with advanced ovarian cancer, a relapse often follows, after which platinum therapy is mostly ineffective resulting in a poor outcome (Hartmann, Lu et al. 2005). This is due to the fact that like many other cancers, ovarian cancer cells can exhibit increased platinum resistance – which can be either intrinsic or acquired – caused by a variety of different factors, such as, for example, increased DNA repair (Parker, Eastman et al. 1991). However, platinum anti-cancer drugs, such as cisplatin, are still some of the most important and effective chemotherapy agents in the treatment of malignancies (Boulikas 2004) and despite the advent of less toxic and less side effect-producing drugs, they are still very widely used.

It has been postulated that one of the many possible reasons adding to the appearance of resistance to platinum drugs, is the effect of hypoxia. It is well known that oxygen concentrations in solid tumours can reach very low levels compared to normal tissues, making the tumour area quite heterogeneous in terms of oxygenation status. In general, hypoxic environments are commonly seen in many human cancers and are often associated with poor prognosis (Brown and Wilson 2004). These hostile microenvironments are usually created due to tumours having a distant and inadequate blood-vessel supply (Tannock 1998), thus, leading to ineffective drug exposure. Other reasons accounting for the increased hindering of cytotoxic drugs in hypoxic tumours include: a decreased cell proliferation rate, which is conversely analogous to the distance from the blood vessels, as well as a decrease of sensitivity to apoptosis i.e. mediated by p53 through hypoxic selection of cells with p53 loss (Brown and Wilson 2004). In addition, some anticancer agents require increased oxygen conditions in order to bring about a significant cytotoxic effect (Batchelder, Wilson et al. 1996). Lastly, upregulation of genes (i.e. p-glycoprotein) known to be involved in drug resistance has also been associated with an increased resistance in hypoxia (Wartenberg, Ling et al. 2003).

Additionally, there is growing evidence that not only hypoxia contributes to resistance in standard drug regimes, but it is also associated with a more aggressive phenotype, increased angiogenesis (Harris 2002), high number of mutations (Yuan and Glazer 1998), invasion (Pennacchietti, Michieli et al. 2003) and metastasis (Subarsky and Hill 2003).

Current strategies aiming at increasing the efficacy of cancer treatments involve hypoxia-activated prodrugs, hypoxia-selective gene therapy, HIF-1 targeting, and even recruitment of specifically recombinant anaerobic bacteria (Brown and Wilson 2004). However, not enough focus has been given in the study of the metabolic profile of cancer cells, either *in vivo* or *in vitro*, that are resistant to platinum under either normoxic or hypoxic conditions.

5.1.1 Objectives

Taking into account the above considerations, the main goals of the experiments presented in this chapter were to:

- Attempt to use an *in vivo* platinum resistance metabolic model of cancer *in vitro*, by observing and comparing the metabolic behaviour of isogenic ovarian cancer cell line pairs, namely PEA1 & PEA2, and PEO1 & PEO4. The metabolic baseline differences between platinum-sensitive (PEA1 and PEO1) and platinum-resistant cells (PEA2 and PEO4) were measured under both conditions of high (normoxia) and low (hypoxia) oxygen levels, in order to achieve *in vivo* conditions that would most resemble real-life situations.
- Possibly identify platinum drug resistance phenotype biomarkers of cancer metabolism with the help of NMR spectroscopy.

5.2 Materials & Methods

Spectral metabolic profiles of the selected PEA1 & PEA2, and PEO1 & PEO4 isogenic ovarian cancer cells under both normoxic (21 % O₂) and hypoxic (1% O₂) conditions were measured using ¹H NMR spectroscopy and then analysed using pattern recognition tools.

The main methodology is summarised below, while more details on the methods employed in this study have been presented in chapter 2. Normoxic cells are identified by an 'N' suffix (i.e. PEA1 N) while hypoxic cells with an 'H' (i.e. PEA1 H).

5.2.1 Cell work

A more detailed description of the methodology is presented in chapter 2, but in summary for the NMR-based work, the isogenic pairs of the ovarian cancer cells were split 48 hours before seeding equal numbers into T25 flasks (approx. 1.3 million cells each). They were then left to attach for at least 24 hours under standard normoxic conditions (21 % O₂ and 5% CO₂ at 37 °C) followed by exposure to hypoxic conditions (1% O₂ and 5% CO₂ at 37 °C) for 24 or 48 hours. Immediately prior to cell harvesting, media aliquots were collected, centrifuged and transferred to tubes for removal of pelleted dead cells. The cell monolayers in the flasks were then washed twice with cold PBS before lysing with cold methanol (MeOH). The resulting cell suspensions were transferred to new tubes and dried down using a rotary speedvac machine overnight. The aqueous metabolites of the cell extracts were prepared for NMR analysis using the CHCl₃:MeOH extraction method. Eventually, the media and aqueous cell extract samples were transferred into NMR tubes for ¹H NMR spectral acquisition using CPMG pulse programme sequences. For the cell assay-based work, the SRB assay was used on the cells in a 96-well plate format to test for cytotoxicity effects after 24 or 48 hour cisplatin exposure (5 µM). More details for the above can be seen in methodology chapter 2.

5.2.2 Data analysis

As a first step, intracellular aqueous extracts and extracellular media were examined and compared under either or both normoxic and hypoxic conditions using pattern recognition (PCA) analysis (Simca-P software) and comparative signal integration of the data (Matlab and Excel software).

In preparation for the PCA analysis, the CPMG NMR spectra of the intracellular aqueous data were referenced to TSP and normalized to median fold change, and divided into 0.04 bins,

excluding the TSP and water regions (as previously described in chapter 2). Similarly, the data from the media samples were processed in the same way but were only scaled to TSP.

5.2.3 Visual inspection, metabolic assignments and peak integration

Firstly, the NMR spectra were visually inspected and an example full resolution spectrum from the intracellular extracts of the isogenic pairs under normoxia and hypoxia can be seen in Figure 5.1. Metabolic assignments were then conducted as previously described in chapter 2 and achieved using published literature data, online metabolic databases, spiking experiments and use of appropriate software (i.e. ChenomX). The areas between the chemical shift ppm values (noted as ‘Start’ and ‘End’) located underneath the spectral peaks of the various metabolic resonances were used for peak integration and subsequent relative quantification of their intra- and extracellular concentrations.

The positively confirmed spectral regions and their corresponding metabolic assignments for the intracellular extracts and the media can be seen in Tables 5.1 and 5.2, respectively.

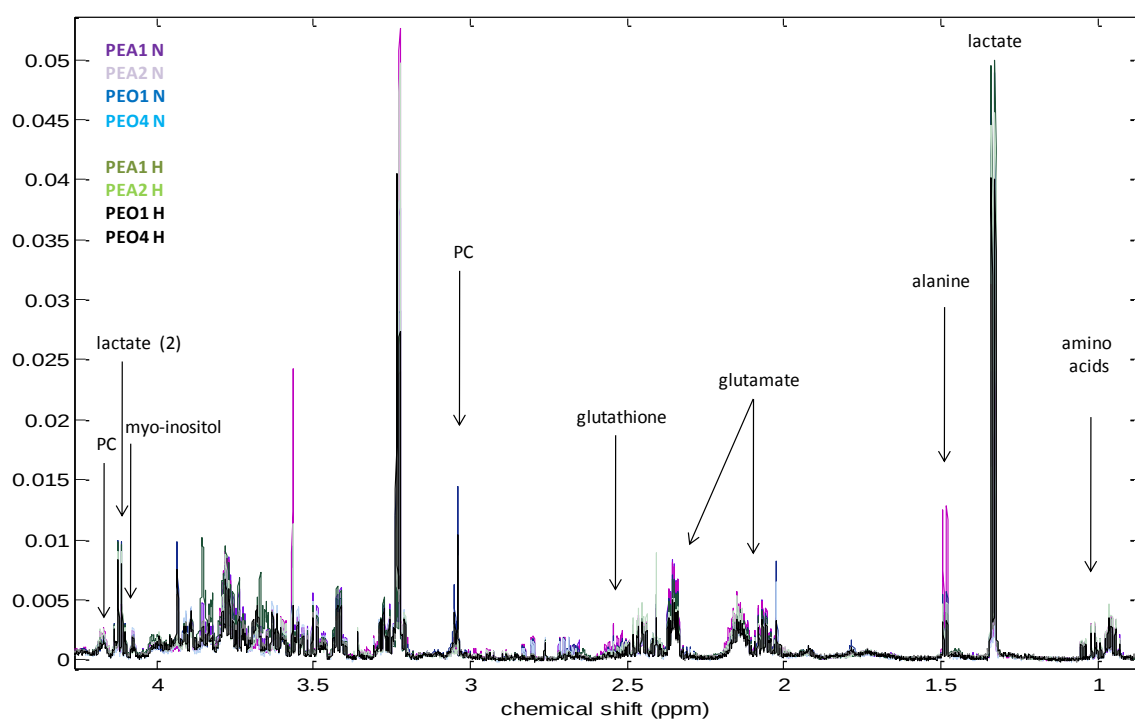


Figure 5.1 - Full resolution ^1H NMR spectra showing various metabolic assignments for the intracellular aqueous extracts in the isogenic ovarian cancer cells under normoxia (PEA1 N, PEA2N, PEO1 N, PEO4 N) and hypoxia (PEA1 H, PEA2 H, PEO1 H, PEO4 H).

Metabolic assignments (intracellular aqueous extracts)		
Start (ppm)	End (ppm)	Metabolites
0.9	0.95	isoleucine
0.95	0.96	isoleucine + leucine
0.96	0.985	leucine
0.985	1.005	valine
1.005	1.03	isoleucine
1.03	1.07	valine
1.31	1.355	lactate
1.47	1.51	alanine
2.01	2.09	glutamate
2.09	2.2	glutamate
2.33	2.375	glutamate
2.4	2.415	succinate
2.43	2.485	glutamine/glutamate
2.485	2.6	glutathione
2.661	2.695	aspartate
2.695	2.73	aspartate
2.785	2.815	aspartate
3.03	3.045	creatine
3.205	3.2125	choline
3.215	3.23	phosphocholine
3.23	3.241	glycerophosphocholine
3.241	3.295	taurine + <i>myo</i> -inositol + glucose
3.295	3.331	taurine + <i>myo</i> -inositol + glucose
3.335	3.365	methanol
3.45	3.51	glucose
3.51	3.56	<i>myo</i> -inositol
3.56	3.575	glycine
3.7	3.8	glutathione + glutamate + glutamine
3.92	3.95	creatine
4.06	4.09	<i>myo</i> -inositol
4.09	4.14	lactate
4.145	4.21	phosphocholine
4.62	4.63	glucose
5.23	5.26	glucose
8.4	8.42	formate

Table 5.1 - Spectral regions of the positively confirmed metabolites of the intracellular aqueous extracts samples as observed by NMR.

Metabolic assignments (extracellular media)		
Start (ppm)	End (ppm)	Metabolites
0.92	0.948	isoleucine
0.948	0.9595	isoleucine + leucine
0.9595	0.9815	leucine
0.9815	1.002	valine
1.002	1.028	isoleucine
1.028	1.06	valine
1.16	1.21	ethanol
1.3	1.36	lactate + threonine
1.468	1.4935	alanine
1.6	1.78	leucine
1.9	1.94	acetate
2	2.08	glutamate
2.08	2.2	glutamine
2.371	2.384	pyruvate
2.4	2.416	succinate
3.64	3.692	ethanol
3.805	3.88	glucose
3.88	3.94	glucose
4.05	4.09	<i>myo</i> -inositol
4.08	4.16	lactate
4.62	4.68	glucose
5.2	5.27	glucose
6.8	6.94	tyrosine
7.02	7.12	histidine
7.13	7.26	tyrosine
7.3	7.5	phenylalanine
7.7	7.9	histidine
8.45	8.47	formate

Table 5.2 - Spectral regions of the positively confirmed metabolites of the extracellular media samples as observed by NMR.

5.3 Results

The main results from the metabolic investigation in platinum resistance in the selected isogenic ovarian cancer cell pairs under two different oxygen conditions are presented in the following sections.

As a first step, cytotoxicity-based cellular assays were conducted, whilst the intracellular and extracellular content of the isogenic ovarian cancer cell lines was subsequently inspected using both spectral and multivariate analysis. This analysis led to the generation of a specific cellular baseline metabolic profile and the identification of several known and unknown metabolites. The positively confirmed metabolites that showed the most meaningful changes were then selected to undergo further analysis.

5.3.1 Testing cisplatin cytotoxicity in hypoxic & normoxic isogenic ovarian cancer cells

Before commencing the NMR analysis, preliminary *in vitro* cytotoxicity assays were repeatedly performed to test whether hypoxia had any effects a) on the growth of the selected isogenic cell lines and b) in platinum (cisplatin) resistance.

The generated plots (Figure 4.1) from the results of the cytotoxicity assay (SRB) performed on one of the cell pairs (PEO1 & PEO4) revealed that:

Hypoxia hindered growth of both sensitive and resistant cells compared to normoxia-exposed cells. In addition, cisplatin-treated cells in hypoxia showed greater survival than similarly treated cells in normoxia, with the effect more pronounced in the hypoxic resistant lines (PEA2 H and PEO4 H); whilst especially for the PEO4 cells the difference between treated and untreated cells was minimal.

The PEO1 and PEO4 cell pair was chosen for testing the above, as it was the one previously shown to have a bigger difference in LD50 values (see chapter 2) between the isogenic cell lines, compared to the PEA1 and PEA2 cell pair.

These results give more support to the theory of hypoxia playing a role in the resistance against platinum drugs like cisplatin, and also prepared the ground for the subsequent NMR-based analysis of the selected isogenic pairs.

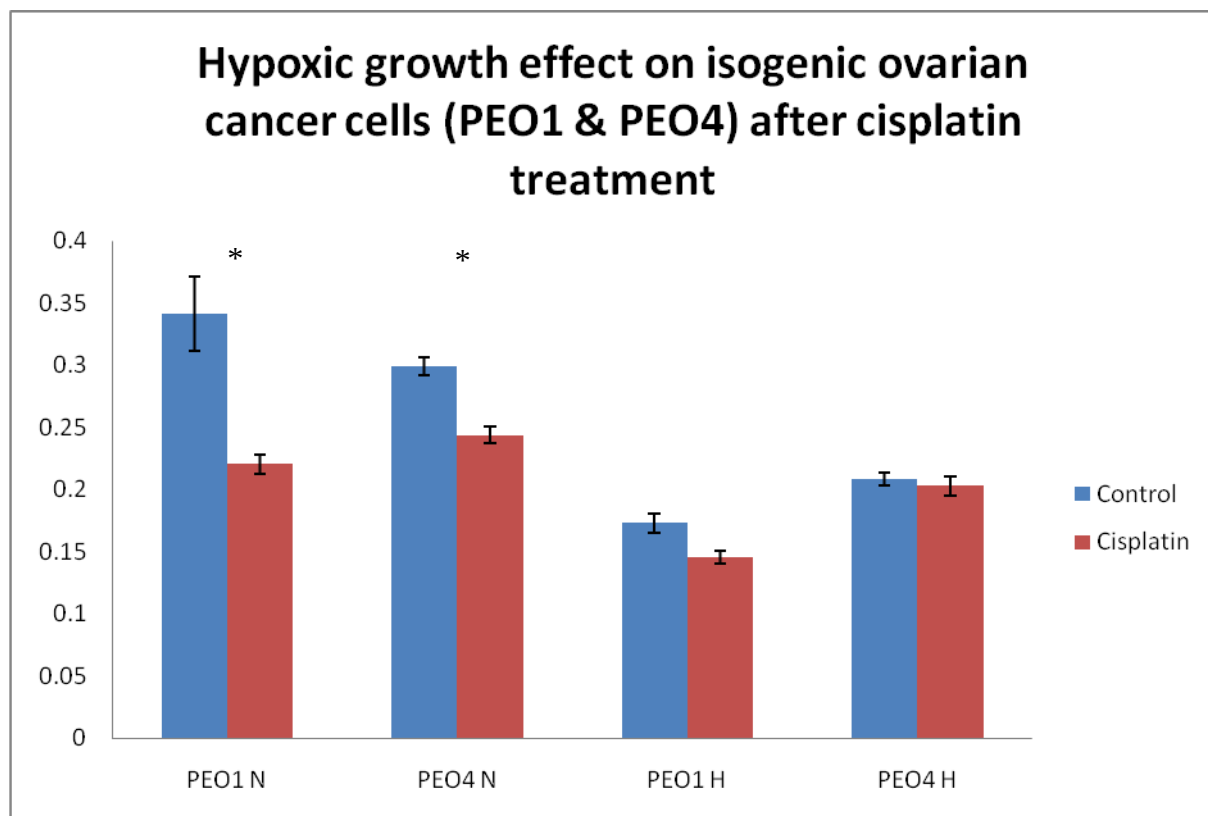
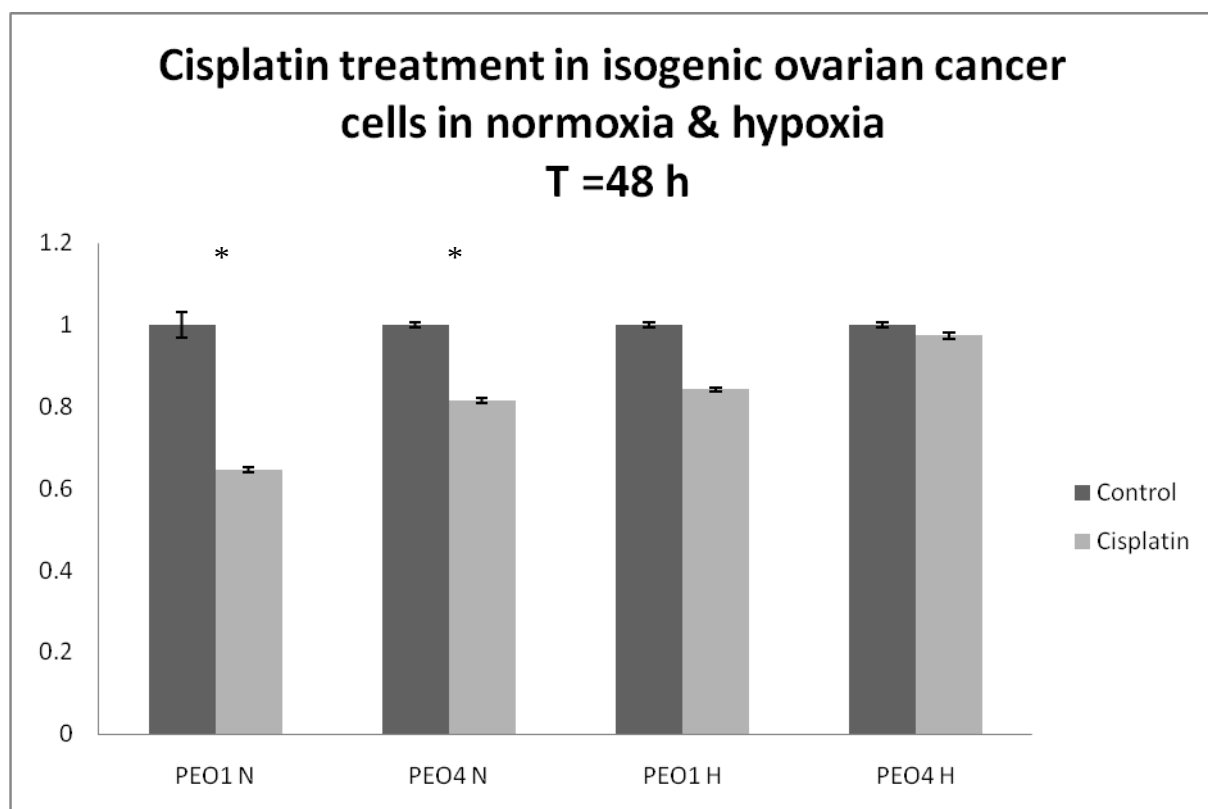


Figure 5.2 – Plots showing the effects of cisplatin treatment (5 μ M) on the ovarian cancer isogenic lines PEO1 (cisplatin-sensitive) & PEO4 (cisplatin-resistant) in normoxia (21 % O_2) and hypoxia (1% O_2), as measured by the Sulforhodamine B-based proliferation assay after 48 hours of exposure. Error bars indicate standard deviation of the mean (SEM) (n = 16). Asterisk denotes $p < 0.05$ (student's t-test).

5.3.2 NMR-based analysis of isogenic ovarian cancer cells

The baseline NMR investigation that followed immediately after the cell proliferation assays, revealed a variety of changes in the metabolic phenotype of all the cell lines examined. The results on the intracellular and extracellular content of the cells are presented below:

5.3.2.1 Examination of intracellular aqueous extracts of isogenic ovarian cancer cells

While looking at the intracellular extracts for clues on their behaviour in regards to platinum resistance, it was obvious that their metabolic phenotype was affected by hypoxia.

A straight-forward visualisation of these effects was achieved through pattern recognition analysis, and is presented in the next section:

5.3.2.1.1 Examination through pattern recognition analysis

In order to make the relationship between the cell lines visually clearer, a specific method of pattern recognition (PCA analysis) was performed in all of the cell pairs together, under both normoxia and hypoxia.

Visual inspection through PCA seemed to reveal two distinct effects induced by exposure of the cells to the two oxygen conditions. These included movement of the cell clusters in the PCA plots (Figures 5.3 and 5.4) for both sensitive and resistant cells towards a hypoxic metabolic phenotype, as well as an additional effect, which seemed to be based on the background genetic differences between the cell lines.

These results are presented in more detail below:

5.3.2.1.2 PEA1 vs. PEA2 vs. PEO1 vs. PEO4 isogenic ovarian cancer cells under normoxia or hypoxia

Multivariate analysis on the NMR-generated data of the two cell pairs revealed the following:

When the isogenic cell lines were examined together under both normoxia and hypoxia, the principal components that were able to produce the clearest separation between the profiles of the intracellular extracts in all cell lines were PC2 and PC3. In particular, PC2 accounted for about 19% of the variance, whereas PC3 accounted for approximately 14%.

The general idea that was deduced after PCA analysis (Figure 5.3) was that the normoxic phenotype seemed to shift across the PC2 axis, i.e. towards the right side of the plot, where most of the hypoxia-exposed lines were located. The corresponding loadings plot (Figure 5.4) revealed a shift towards glycolytic compounds (such as lactate at δ 1.34), under hypoxia for all cells (both sensitive and resistant) regardless of their resistance status.

The loadings plot in Figure 5.4 shows the bins responsible for the metabolic movements of the sensitive cells and resistant cells in these normoxic and hypoxic conditions. For example, the metabolic bin belonging to lactate (δ 1.34) seemed to be increased in hypoxic cells (PEO1 H and PEO4 H), whilst resonances such as those found at δ 1.5, 2.18, 2.34, 2.54, 3.58, and 3.78 seemed to be increased in normoxic cells mostly. On the other hand, examples of metabolic resonances differentiating resistant from sensitive cells could be found at 1.5 and 3.58 (PEA2 N cells) as well as 2.34 and 3.78 (PEA1 N cells).

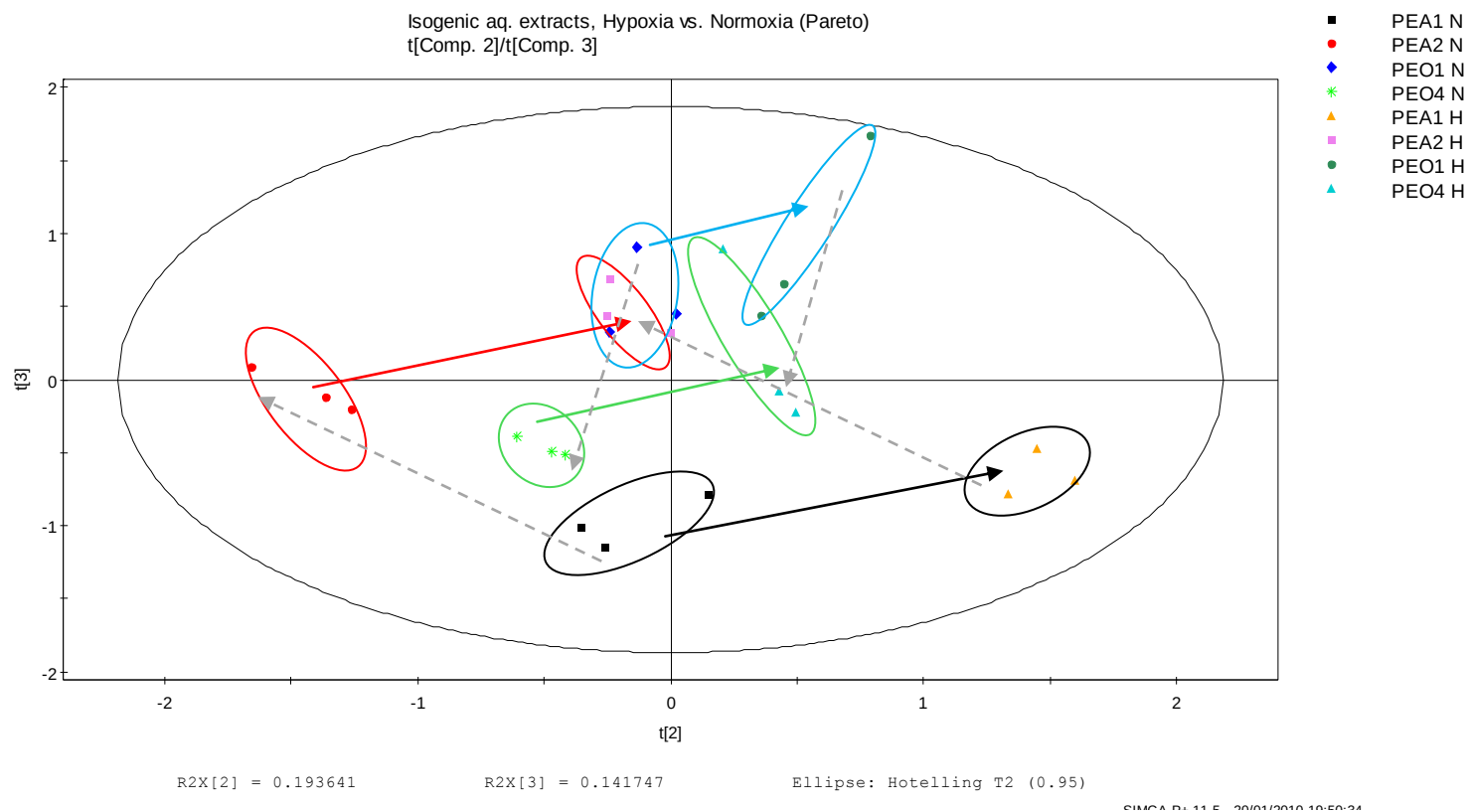


Figure 5.3 - Scores plot of principal component analysis (PC2 & PC3) on ^1H NMR CPMG one-dimensional spectra for aqueous intracellular extracts of isogenic ovarian cancer cells normalized to TSP and Median Fold Change. Plot is showing metabolic movements through effect of hypoxia and genetic background of the cells. Each point represents an individual cell sample. The ellipse represents the Hotelling with 95% confidence.

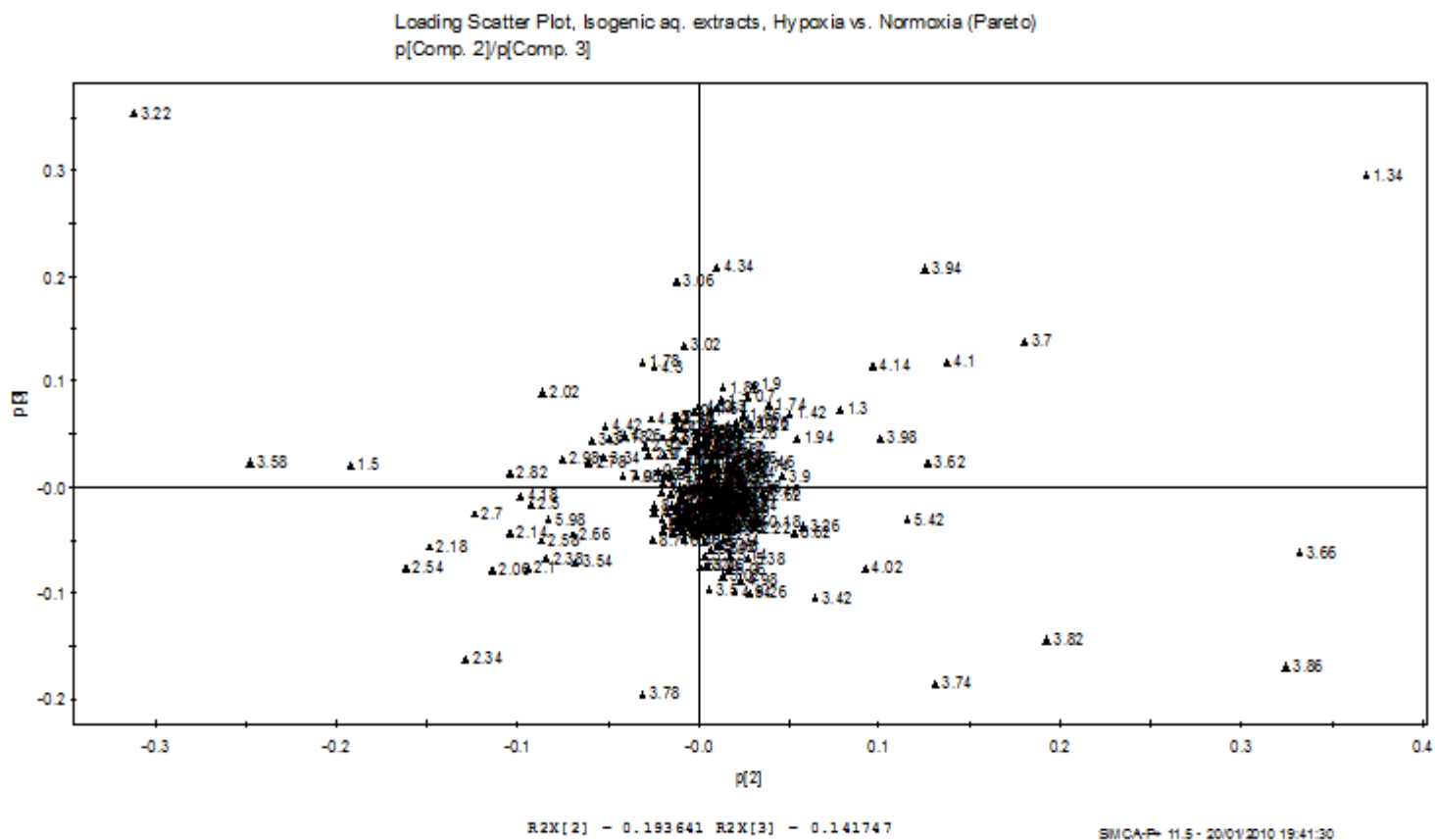


Figure 5.4- Loadings plot of principal component analysis (PC2 & PC3) on ^1H NMR CPMG one-dimensional spectra for aqueous intracellular extracts of isogenic ovarian cancer cells normalized to TSP and Median Fold Change. Numbers beside data point correspond to specific bins used in the analysis.

5.3.2.1.3 Pattern of change of metabolites under normoxia or hypoxia in intracellular aqueous extracts

At this stage, it is important to note that in order to understand how hypoxia may affect resistance to platinum, at least in these particular cell models, it is also necessary to observe the relative changes in intensity levels between them. This is presented in more detail in a later section of the chapter (section 5.3.2.1.5) where a closer and more individual look is taken; however, the general pattern as well as the levels of the various intracellular metabolic changes between all of the cell lines can still be seen in Table 5.3 and the bar chart in Figure 5.5, where a percent-change based comparison was conducted and is presented here for illustrative and comparative purposes.

i) Sensitivity vs. resistance

As a first step, a percent-change comparison was made between intracellular intensities of isogenic cell lines in either normoxia or hypoxia. This meant comparing the cell lines in the following ratio-based way:

- PEA2 N vs. PEA1 N (normoxia)
- PEO4 N vs. PEO1 N (normoxia)

and

- PEA2 H vs. PEA1 H (hypoxia)
- PEO4 H vs. PEO1 H (hypoxia)

The percent-change calculations of the metabolic ratios (Table 5.3 and Figure 5.5) revealed the following intracellular events between isogenic cell lines in normoxia or hypoxia, in terms of directionality (i.e. increased or decreased levels as compared to their isogenically-paired cell line in the same oxygen condition).

(Significant differences are marked with the relevant asterisks).

INTRACELLULAR AQUEOUS EXTRACTS				
METABOLITES	PEA2 N/PEA1 N	PEO4 N/PEO1 N	PEA2 H/PEA1 H	PEO4 H/PEO1 H
GPC	55***	-14*	136**	38*
Glucose	-18	4	-26	-21
<i>Myo</i> -inositol (2)	11	6	-14**	-15
Isoleucine	-5	-9	7	1
Aspartate (2)	137***	-5	-13	-38
Glutamate	6	-19**	-2	9
Glutathione	19*	-19***	8	-7
Glycine	267***	-15***	106***	3
Leucine	51*	-13*	11	5
Valine	42	-15	9*	11
PC	37*	30**	82***	10
Taurine	-17	-11	-26*	-3
Choline	-13	19	18	0
Alanine	171***	-45***	16*	12
Creatine	17*	-38**	11	29
Lactate (2)	-2	-57***	-8	16
Succinate	20	-24**	44*	0

Table 5.3 – Average metabolic ratios (percent-change) in sensitive vs. resistant ovarian cancer isogenic cells in normoxia (21% O₂) & hypoxia (21% O₂) of intracellular aqueous extracts. * p<0.05, ** p<0.01, * p<0.001 (unpaired student's t-test using two-tail distribution).**

More specifically the concentrations of the metabolites were altered as follows:

Lactate decreased in all resistant cell lines except in the case of PEO4 H, where it increased. Creatine, alanine, valine, leucine, glycine and glycerophosphocholine increased in all resistant cell lines except in the case of PEO4 N where they decreased. Phosphocholine increased in all resistant cell lines. Taurine decreased in all resistant cell lines whilst glutathione increased in PEA2 but decreased in PEO4 cell lines. Glucose increased in all resistant cells except in the case of PEO4 N, where it showed a slight increase. Glutamate increased in PEO4 H and PEA2 N but decreased in PEO4 N and PEA2 H cell lines. Isoleucine decreased in normoxic resistant cells but increased in the hypoxic resistant cell lines while *myo*-inositol increased in the normoxic resistant cells but decreased in the hypoxic resistant ones. Aspartate decreased in all resistant cells but increased in PEA2 N cell lines. Succinate increased in PEA2 cells but decreased in PEO4 N, while it showed no significant changes between PEO4 H and PEO1 H. Finally, choline increased in PEO4 N and PEA2 H but decreased in PEA2 N. It also showed no significant changes between PEO4 H and PEO1 H.

From the above, it could be gathered that some of the most interesting changes metabolites, involved phosphocholine and taurine, which increased and decreased, respectively, in all resistant cell lines regardless of oxygen exposure, as well as isoleucine and *myo*-inositol which respectively increased and decreased consistently but only in the hypoxia-exposed resistant cell lines. Again, it's important to note that genetic variability between the particular cell lines seems to come into play each time the intracellular metabolic intensities are examined and compared.

Figure 5.5 below visualises all these patterns:

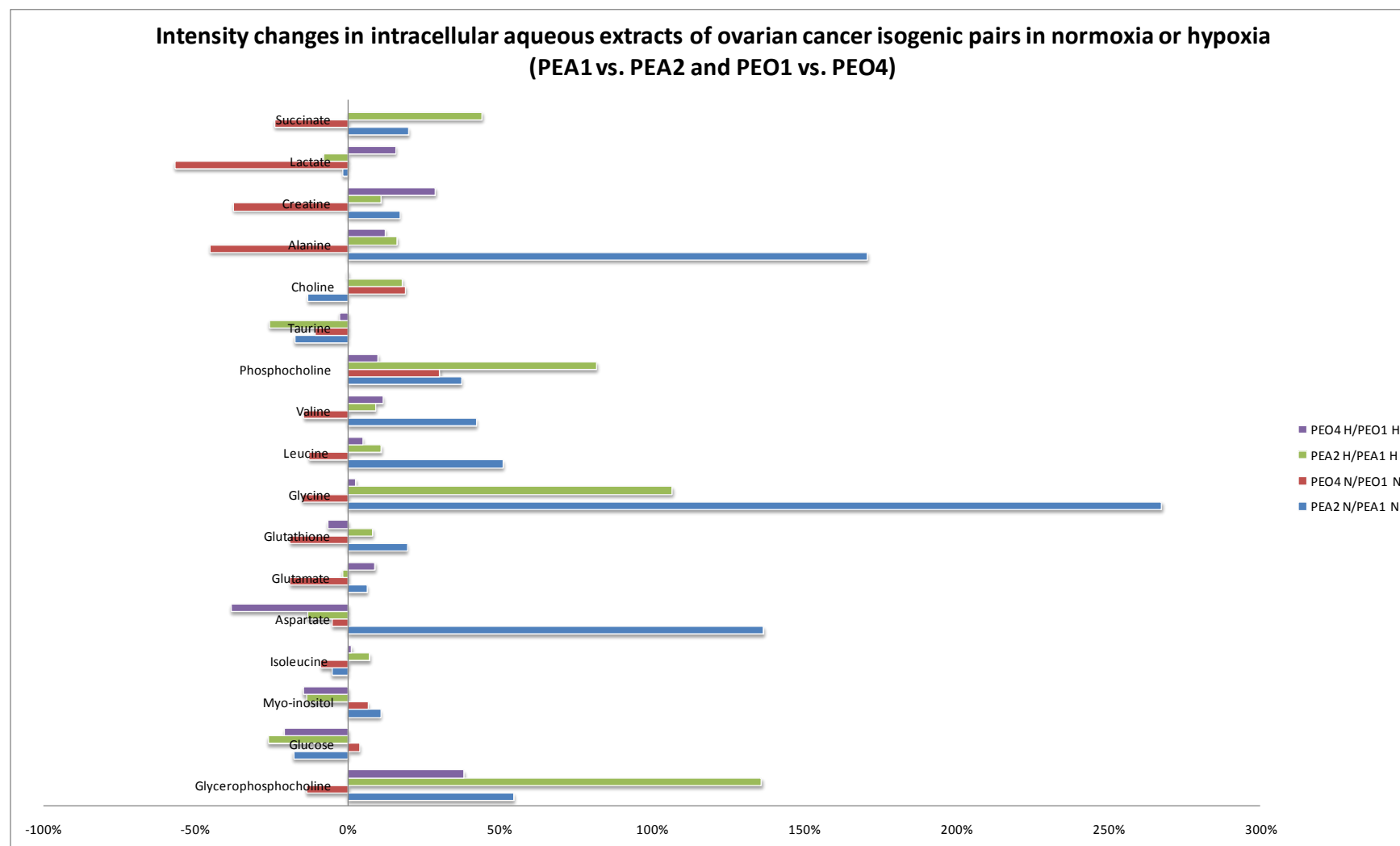


Figure 5.5 - Bar chart showing the metabolic intensities pattern for the intracellular aqueous extracts of PEA1 & PEO1 (cisplatin-sensitive) and PEA2 & PEO4 (cisplatin-resistant) ovarian cancer isogenic cell lines in normoxia (21% O₂) and hypoxia (1% O₂). Changes are expressed in terms of percent-change (n=3).

ii) Normoxia vs. Hypoxia

The cell lines were also individually compared in terms of normoxic effect vs. hypoxic effect since changes in the metabolic phenotype can also affect the way the cells respond to platinum. In this instance, cell lines were compared in the following ratio-based way in order to individually, and more effectively, isolate the hypoxia-induced changes:

PEA1 N vs. PEA1 H

PEA2 N vs. PEA2 H

PEO1 N vs. PEO1 H

PEO4 N vs. PEO4 H

Table 5.4 below provides the actual percentage change values for the intracellular aqueous extracts in normoxia- vs. hypoxia-exposed cell lines. Each normoxic cell line was compared to its hypoxic equivalent, in order to assess the level of metabolic change in response to hypoxia. The pattern and levels of change are presented below (Table 5.4 and Figure 5.6).

INTRACELLULAR AQUEOUS EXTRACTS				
METABOLITES	PEA1 N/PEA1 H	PEA2 N/PEA2 H	PEO1 N/PEO1 H	PEO4 N/PEO4 H
GPC	5	-31*	10	-32**
Glucose	22	36*	5	37*
Myo-inositol (2)	4	34***	1	26*
Isoleucine	9	-3	-8	-17
Aspartate	36	271***	125***	247**
Glutamate	22	32**	48**	10*
Glutathione	46**	61**	68**	46**
Glycine	16	107***	71***	42**
Leucine	-29	-4	-5	-21**
Valine	-29	-8	-8	-29
PC	38	5	9	29**
Taurine	4	16*	12	3
Choline	5	-23*	-2	16
Alanine	-3	125***	74***	-15*
Creatine	12*	18*	68*	-19**
Lactate (2)	-39*	-34***	29	-52***
Succinate	-25	-37**	23	-7

Table 5.4 – Average metabolic ratios (percent-change) between normoxic (21% O₂) and hypoxic (5% O₂) ovarian cancer isogenic cells (intracellular aqueous extracts). * p<0.05, ** p<0.01, * p<0.001 (unpaired student's t-test using two-tail distribution).**

More specifically, the metabolic changes that were observed involved the following:

Taurine, phosphocholine, glycine, glutathione, glutamate, aspartate, *myo*-inositol and glucose decreased in all hypoxia-exposed cell lines. Valine and leucine increased in all hypoxic cells compared to their corresponding normoxic ones while succinate and lactate increased only in the case of PEO1 N. Creatine decreased only in the case of PEO4 N and alanine decreased in the PEA1 N and PEO4 N cell lines. Choline decreased in PEA2 N and PEO1 N cell lines whilst isoleucine only increased in PEA1 N cells. Lactate decreased in all hypoxic cells except PEO1 H. Finally, glycerophosphocholine increased in the hypoxic resistant cell lines but not in the normoxic ones.

Figure 5.6 helps in visualising these patterns and shows the metabolic changes of the intracellular extracts in response to hypoxia.

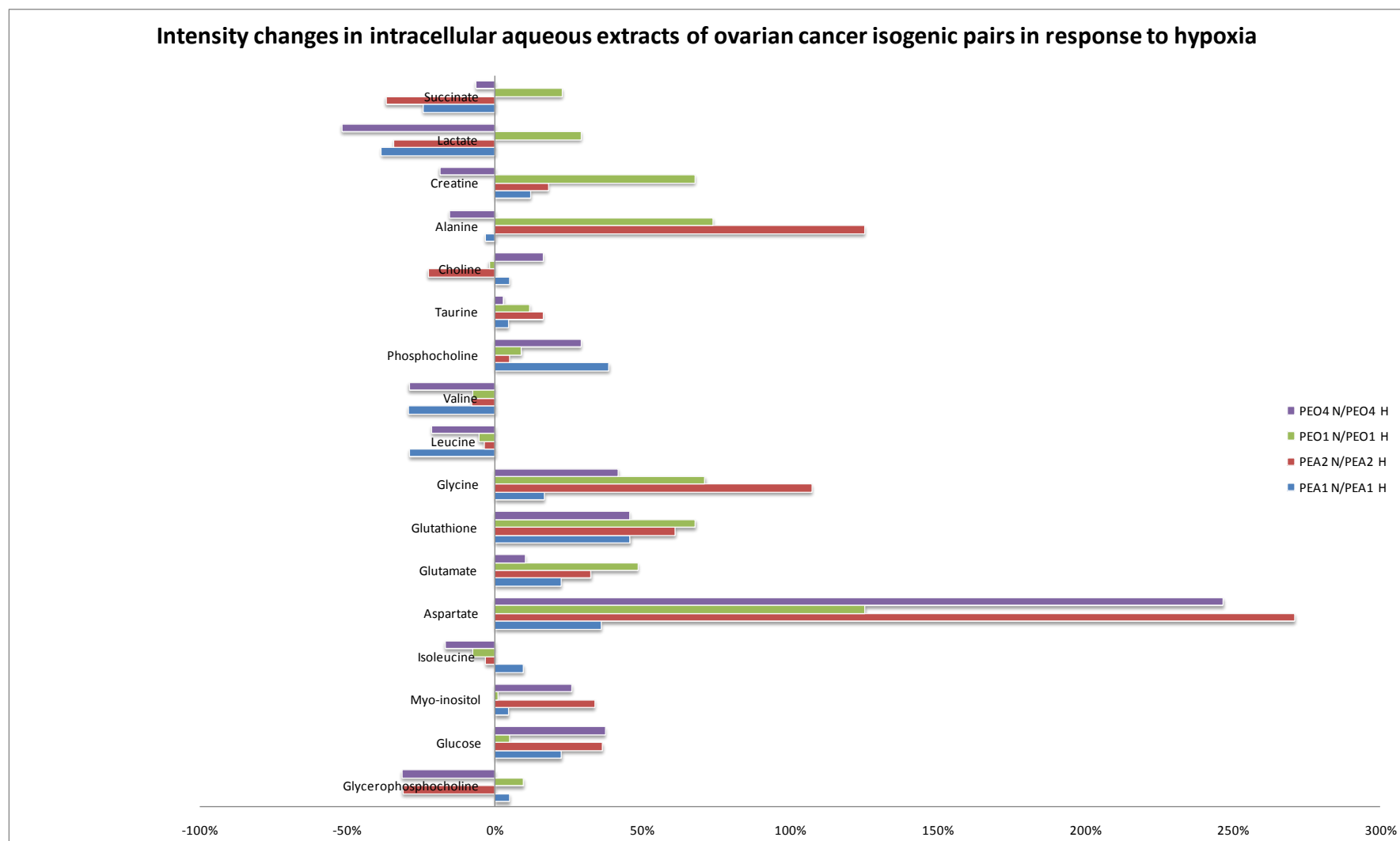
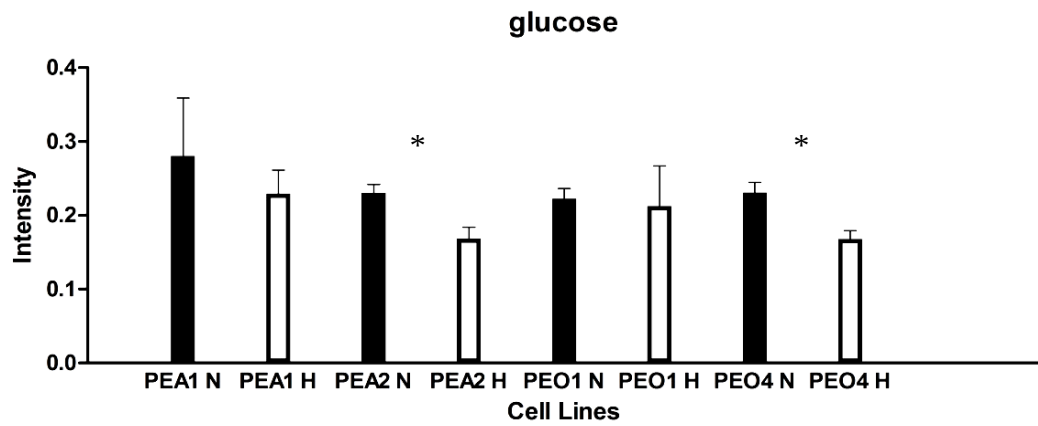


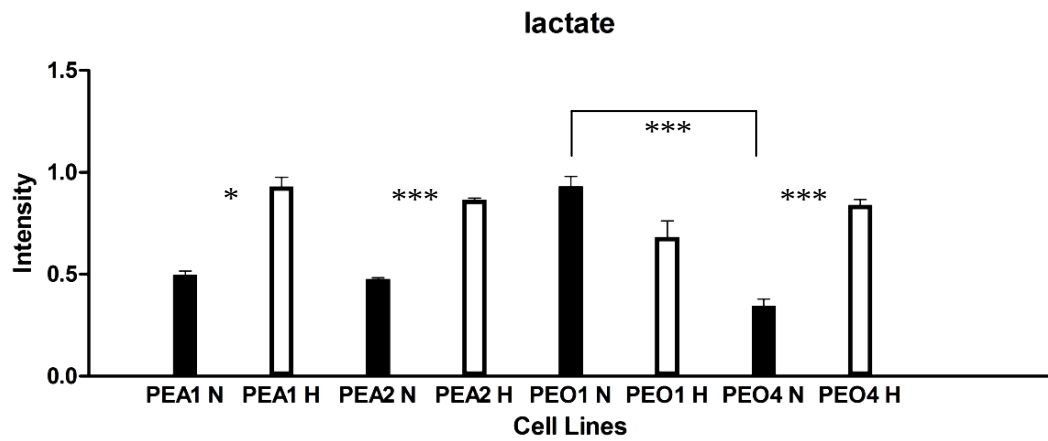
Figure 5.6 - Bar chart showing the metabolic intensities pattern for the intracellular aqueous extracts of PEA1 & PEO1 (cisplatin-sensitive) and PEA2 & PEO4 (cisplatin-resistant) ovarian cancer isogenic cell lines in normoxia (21% O₂) and hypoxia (1% O₂). Changes are expressed in terms of percent-change (n=3).

5.3.2.1.4 Focused comparative examination of metabolic intensities in the intracellular extracts

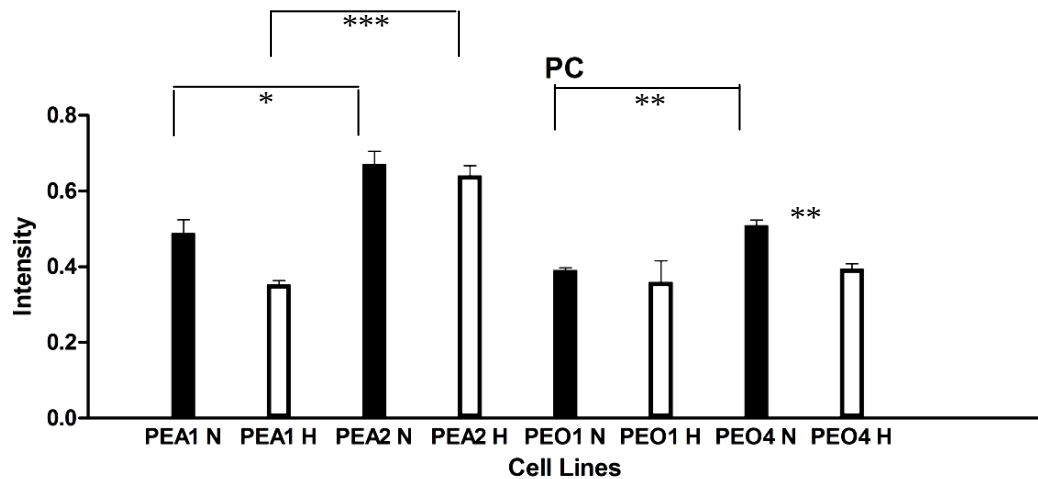
A representative selection of the previously analysed and relatively quantified NMR-observed intracellular metabolites, which exhibited the most consistent changes and/or had a more direct association with the Warburg effect (such as glucose and lactate) are presented in the following pages. This section aims at aiding in the visualisation of these particular metabolites and the discussion of the results in section 5.5. As previously mentioned, the intracellular aqueous extracts were relatively quantified (Figure 5.7) by integrating the peak areas between their corresponding ppm ranges (Table 5.1). The metabolic intensities were compared and tested for significance using the student's t-test, as described earlier.



A



B



C

Figure 5.7 - Mean absolute values of Median Fold Change-normalized NMR intensities for the intracellular aqueous extracts of A) glucose, B) lactate and C) phosphocholine (PC) between platinum sensitive and resistant isogenic ovarian cancer cells. $p < 0.05$, ** $p < 0.01$, * $p < 0.001$ (unpaired student's t-test using two-tail distribution). Error bars indicate standard error of the mean (SEM).**

The ratios of phosphocholine and glycerophosphocholine were also examined (Figure 5.8) in this study and appeared to have low intracellular PC:GPC values.

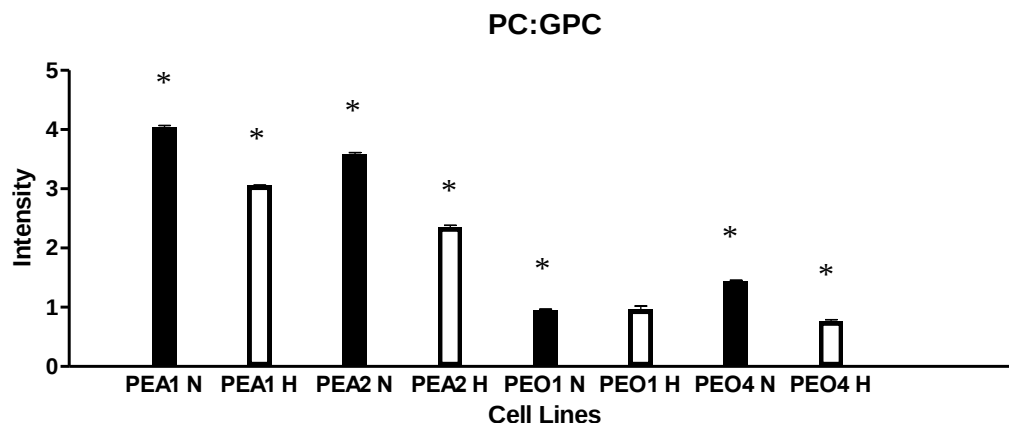


Figure 5.8 - PC:GPC ratios of mean absolute values of Median Fold Change-normalized NMR intensities for the intracellular aqueous extracts of PC and GPC between platinum sensitive and resistant isogenic ovarian cancer cells. * $p < 0.05$, ** $p < 0.01$, * $p < 0.001$ (unpaired student's t-test using two-tail distribution). Error bars indicate combined differential error of the mean.**

5.3.2.1.5 Examination of extracellular media of isogenic ovarian cancer cells

Similarly to the intracellular extracts, the extracellular media were also examined and compared under either or both normoxic and hypoxic conditions. PCA and percentage-change examinations were also performed as previously described.

As before, the hypoxic conditions seemed to have an effect on the metabolic phenotype in the media, as they had in the extracts, allowing the possibility of some kind of association with increased or decreased platinum resistance.

5.3.2.1.6 Examination of metabolic trends in the media through pattern recognition (PCA) analysis

While looking at the top principal components (i.e. PC1, PC2...) in the media, PCA analysis seemed to initially present a bigger challenge in finding clear metabolic clusters of separation for either sensitive or resistant lines in normoxia or hypoxia. This could be due to the high abundance of nutrients already present in the cell culture media, “drowning” the important metabolic changes and, thus, rendering them more difficult to examine through PCA. Looking further down in the hierarchy of components (i.e. PC4 & PC5) though, a separation started to become a little clearer. The variance for PC4 is about 58% and for PC5 around 47%. As shown in figure 5.9 below, some hypoxia-induced metabolic changes that were restricted to resistance status only became apparent: when examining all cell lines together under normoxia and hypoxia, the majority of the normoxic resistant cells (in green) seemed to show a very obvious separation from their hypoxic counterparts (in red), whereas, the sensitive cells (in gray) under the same conditions did not appear to show any separation but rather a much wider and more interspersed distribution throughout the plot area. The loadings plot in Figure 5.10 shows the resonances of the metabolic bins responsible for the scores in Figure 5.9. For example, resonances at δ 2.34 and 3.78 for the resistant cells seemed to separate them from the sensitive cells with resonances found at δ 2.46 and 4.1.

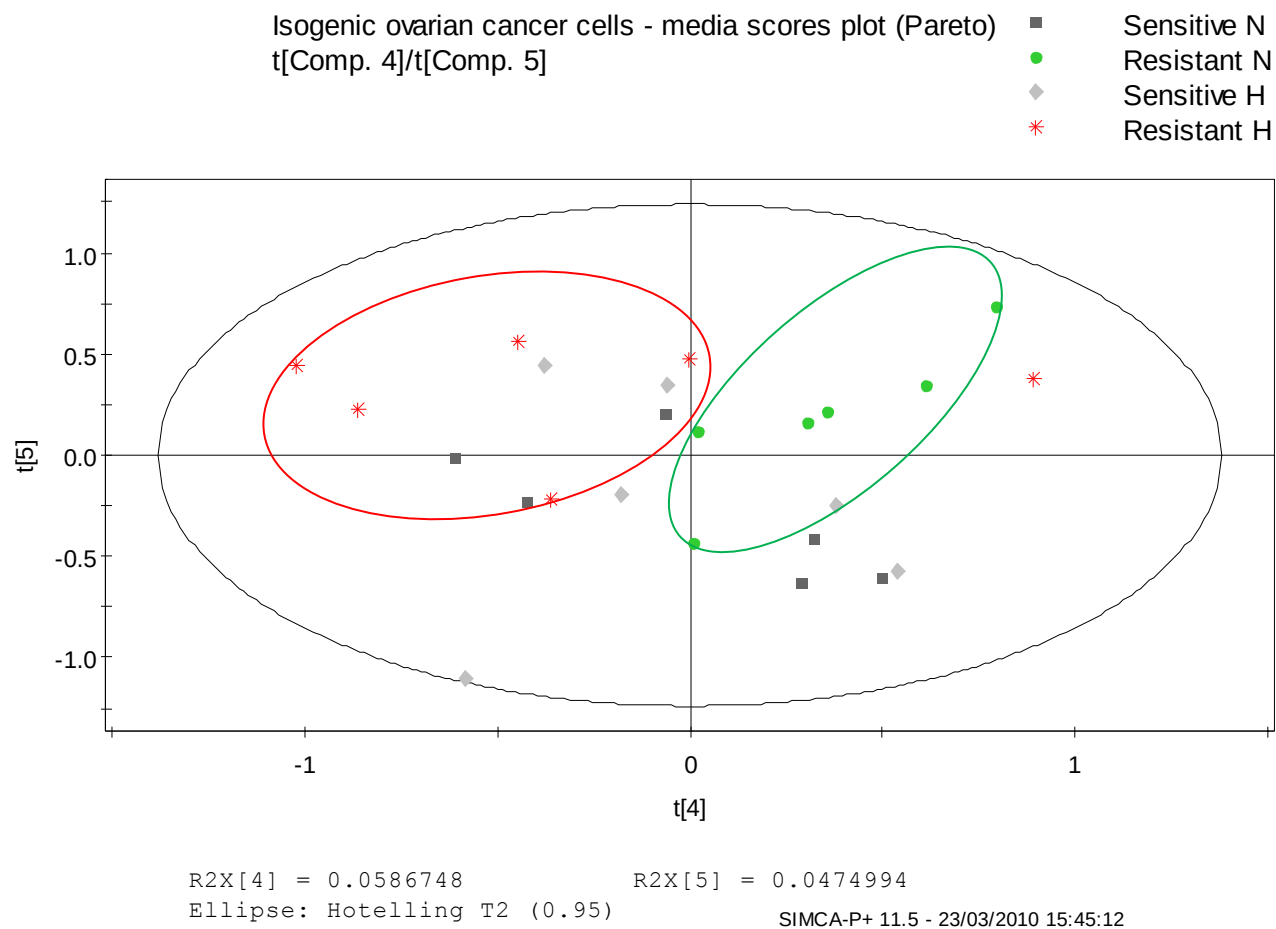


Figure 5.9 - Scores plots of principal component analysis on ^1H NMR CPMG one-dimensional spectra for extracellular media of isogenic ovarian cancer cells scaled to TSP. Plot is showing effect of hypoxia on resistant cells across PC4 & PC5. Each scores point represents an individual cell sample while the ellipse represents the Hotelling with 95% confidence.

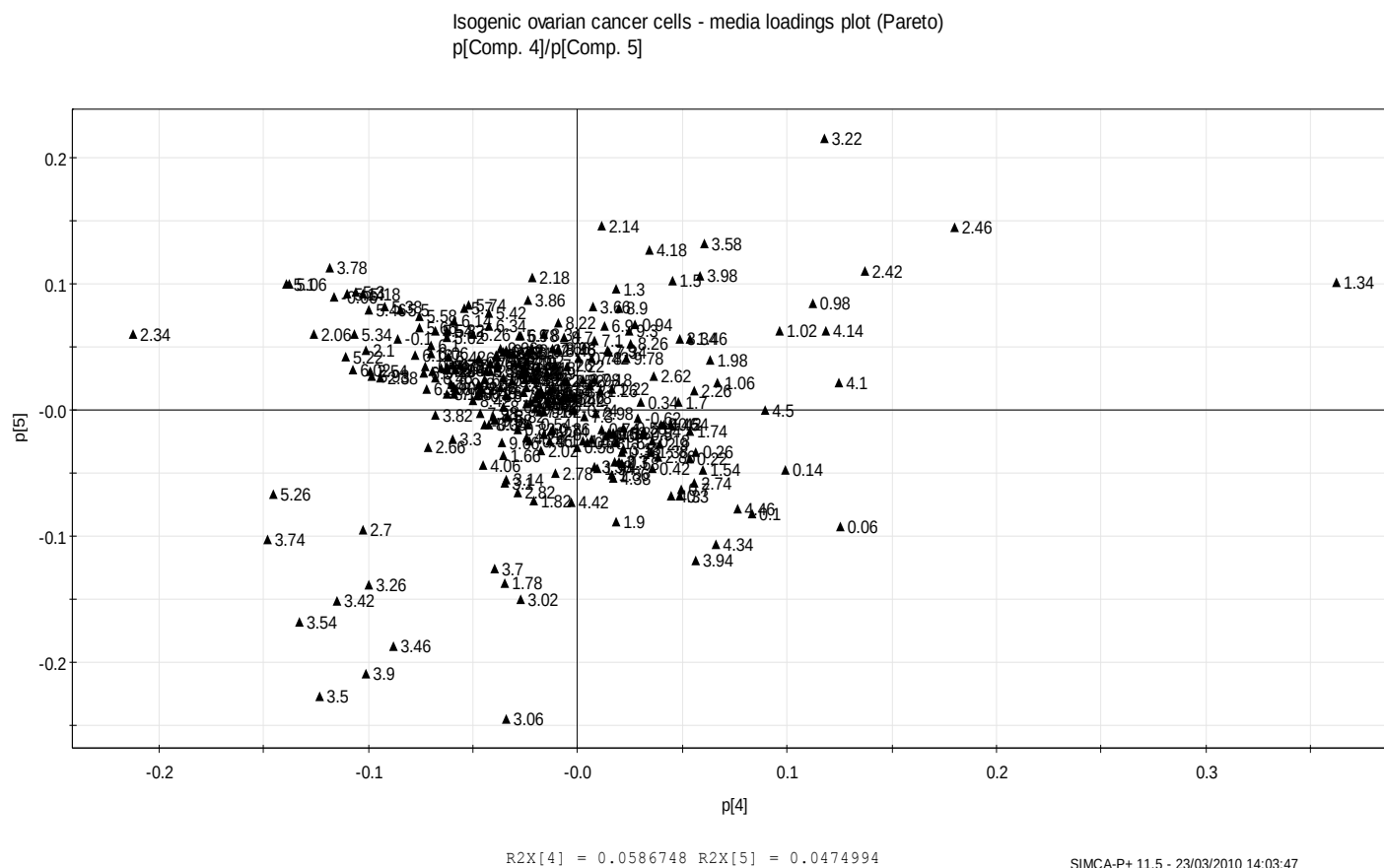


Figure 5.10 - Loadings plots of principal component analysis (PC4 & PC5) on ^1H NMR CPMG one-dimensional spectra for extracellular media of isogenic ovarian cancer cells scaled to TSP. Numbers beside each data point in the loadings plot correspond to specific bins used in the analysis.

5.3.2.1.7 Pattern of change of metabolites under normoxia or hypoxia in extracellular media

The percent-change inspection of the metabolic ratios revealed the following extracellular events between sensitive and resistant isogenic cell lines in normoxia and hypoxia. The visualisation of the levels and patterns of the various metabolic changes between the cell lines can be seen in the tables and bar charts below (Table 5.5 & Figure 5.11 for ‘sensitivity vs. resistance’, as well as Table 5.6 & Figure 5.12 for the ‘hypoxia vs. normoxia’ comparisons).⁷

i) Sensitivity vs. Resistance

The table below (Table 5.5) provides the actual percentage change values for each metabolic comparison as well as the relevant statistical significance, marked with the relevant asterisks.

METABOLITES	MEDIA			
	PEA2 N/PEA1 N	PEO4 N/PEO1 N	PEA2 H/PEA1 H	PEO4 H/PEO1 H
Acetate	-34***	-51*	-7	-2
Glutamine	-1*	23*	5*	15*
Myo-inositol	-6	0	7	26
Pyruvate	-32***	-30	6	2
Glucose	13***	58*	19*	86***
Glutamate	2	4	1	17*
Isoleucine	5**	10	5*	12*
Leucine	3*	19*	6*	19**
Lactate (2)	-17***	-43*	-10*	-32**
Phenylalanine	1	5	4*	10
Succinate	7**	16*	8**	14*
Valine	1*	19**	6*	19**
Alanine	29***	-49**	13**	-15*
Histidine	2	7	21	-1
Tyrosine	-3	11	10***	9

Table 5.5 - Average metabolic ratios (percent-change) in sensitive vs. resistant ovarian cancer isogenic cells in normoxia & hypoxia of extracellular media. * p<0.05, ** p<0.01, * p<0.001 (unpaired student’s t-test using two-tail distribution).**

By looking at the relevant tables and figures it could be deduced that:

Tyrosine and glutamine increased in all resistant cells but decreased in PEA2 N. (The decrease in tyrosine and glutamine between PEA2 N and PEA2 H cells was negligent at 3% and 1%, respectively). Histidine increased in all resistant cells but decreased in PEO4 H. This

decrease was also minuscule at 1%. Alanine increased in PEA2 cells but decreased in PEO4 cell lines. Valine, leucine, isoleucine, succinate, phenylalanine, glutamate, and glucose increased in all resistant cell lines. Lactate and acetate decreased in all resistant cell lines. (The acetate decrease was less steep in the case of the hypoxic cells compared to the normoxic ones). Pyruvate increased in hypoxic resistant cells while it decreased in the normoxic resistant ones. This change was less pronounced for the hypoxic cells. *Myo*-inositol followed a similar pattern; however there was no significant change in the PEO4 N cell line as in the case of pyruvate when compared to PEO1 N.

Summarising the above observations, the extracellular metabolites that showed consistent changes included valine, leucine, isoleucine, glutamate, succinate, phenylalanine and glucose whose intensities were higher in all resistant cell lines, as well as acetate and lactate which were decreased in the same lines. Interestingly, pyruvate and *myo*-inositol seemed to respond to the hypoxic conditions by showing an increasing trend in their concentrations in the hypoxic media but not in the normoxic. Another notable difference in behaviour was also observed in the case of acetate where the decrease observed in all resistant cells was less pronounced for the hypoxic pairs.

Figure 5.11 below visualises these changes:

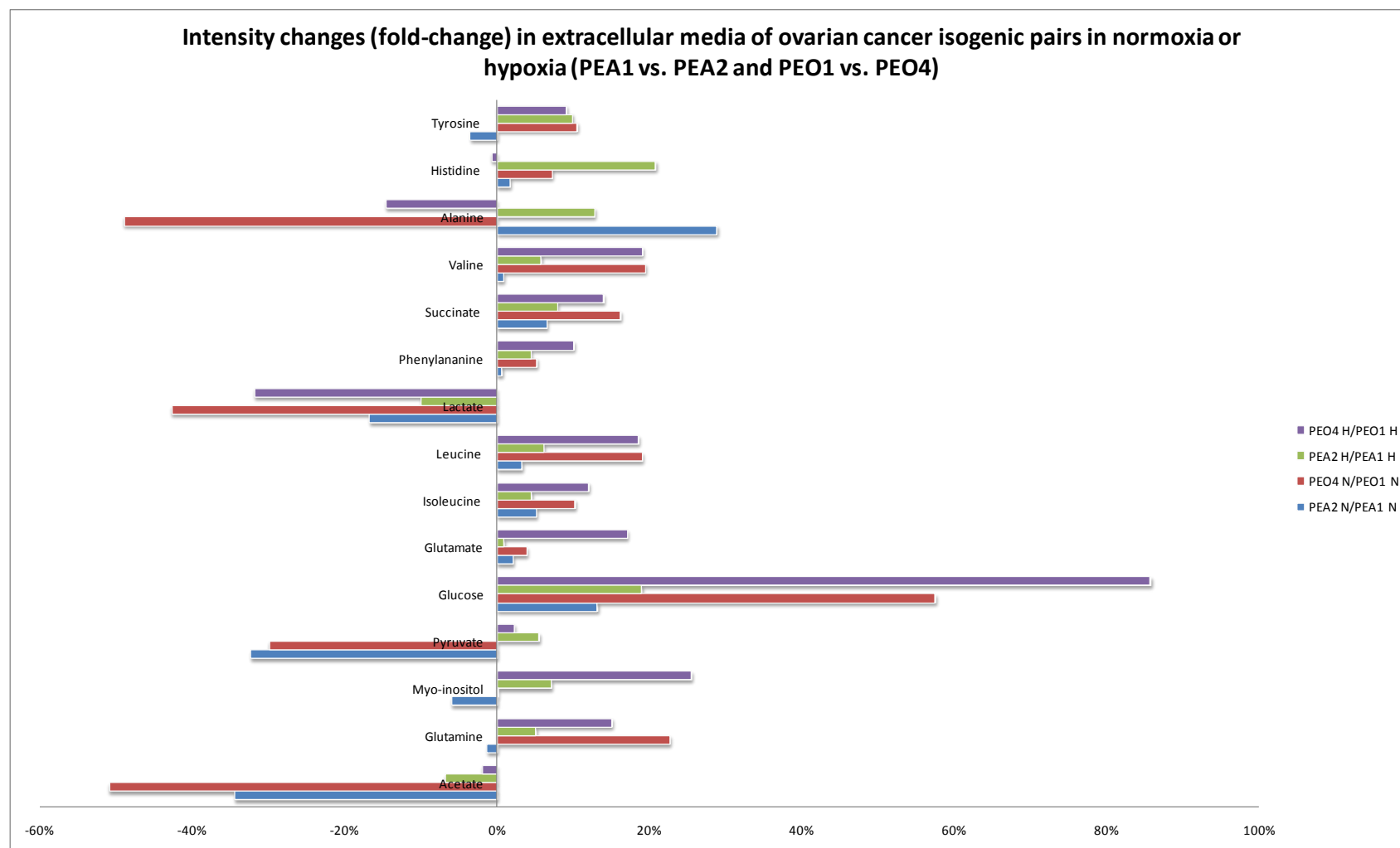


Figure 5.11 - Bar chart showing the metabolic intensities pattern for the extracellular media of PEA1 & PEO1 (cisplatin-sensitive) and PEA2 & PEO4 (cisplatin-resistant) ovarian cancer isogenic cell lines in normoxia (21% O₂) and hypoxia (1% O₂). Changes are expressed in terms of percent-change (n=3).

ii) Normoxia vs. Hypoxia

Similarly, the metabolic ratios between extracellular media samples of the same cell line when exposed to either normoxia or hypoxia revealed the following events.

Table 5.6 below provides the actual percentage change values for the normoxic vs. hypoxic metabolic comparisons.

METABOLITES	MEDIA			
	PEA1 N/PEA1 H	PEA2 N/PEA2 H	PEO1 N/PEO1 H	PEO4 N/PEO4 H
Acetate	114***	51**	190***	45
Glutamine	-9*	-15***	-3	3
Myo-inositol	-2	-14**	19	-5
Pyruvate	191***	87***	192***	100
Glucose	43***	36**	54***	31
Glutamate	6*	7**	18*	5
Isoleucine	-1	-1	7	5
Leucine	-2	-5*	6	7
Lactate	-41***	-45***	-31**	-42*
Phenylalanine	0	-3**	8	3
Succinate	2	0	4	6
Valine	-1	-6*	5	5*
Alanine	84***	110***	107***	23
Histidine	3	-13*	0	8
Tyrosine	1	-12**	2	3

Table 5.6 - Average metabolic ratios (percent-change) between normoxic and hypoxic ovarian cancer isogenic cells (extracellular media). * p<0.05, ** p<0.01, *p<0.001 (unpaired student's t-test using two-tail distribution).**

Particular changes that could be observed from the corresponding tables and figures involved the following extracellular events in terms of concentration change:

Acetate, pyruvate, glucose, glutamate and alanine decreased in all hypoxic cells. Acetate and pyruvate showed the biggest levels of change in the hypoxic sensitive cells compared to the hypoxic resistant ones. This pattern was also observed in the case of glucose. Succinate also decreased in all hypoxic cells, however there was 0% change in the case of resistant cell line PEA2. Lactate increased in all hypoxic cells, regardless of resistance status. (Also, its levels of change were mostly similar for the majority of cell pairs). Tyrosine, histidine and phenylalanine increased in PEA2 H cells only, with the majority of the remaining hypoxic

cells decreasing. The changes for these, however, were miniscule. Valine, leucine and isoleucine showed an increase in PEA1 H and PEA2 H cells, whereas the rest of the resistant cells slightly increased. On this occasion, glutamine and *myo*-inositol followed a more random pattern

Figure 5.12 below shows the visualised pattern of these changes.

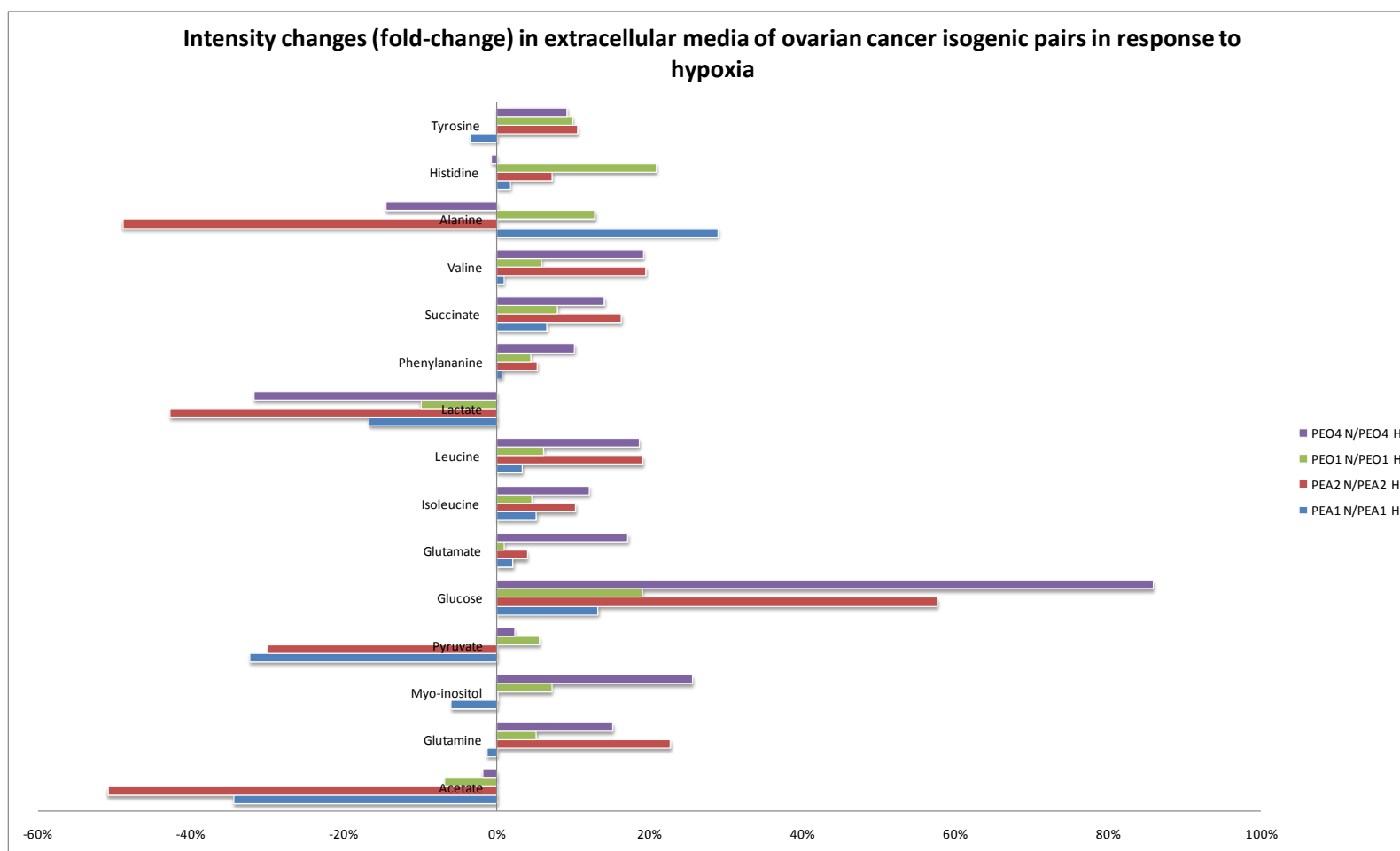


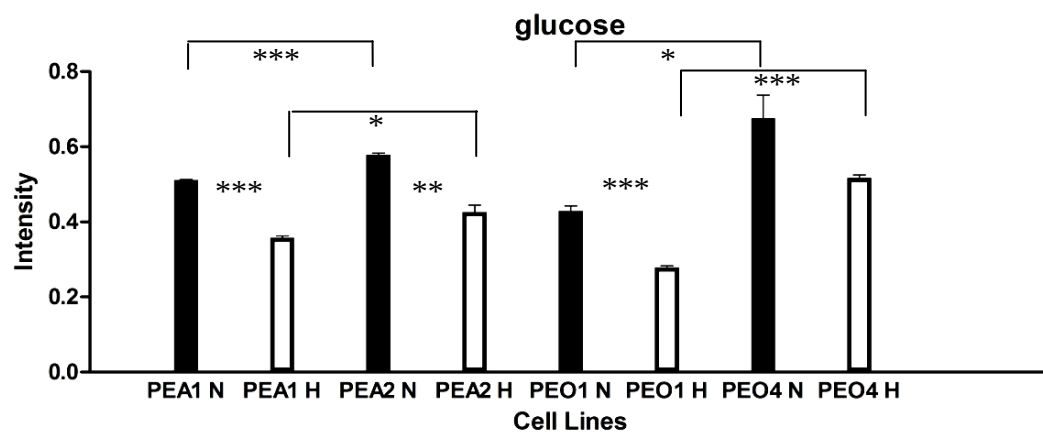
Figure 5.12 – bar chart showing the metabolic intensities pattern for the extracellular media of PEA1 & PEO1 (cisplatin-sensitive) and PEA2 & PEO4 (cisplatin-resistant) ovarian cancer isogenic cell lines in normoxia (21% O₂) and hypoxia (1% O₂). Changes are expressed in terms of percent-change (n=3).

5.3.2.1.8 Focused comparative examination of metabolic intensities in the extracellular media

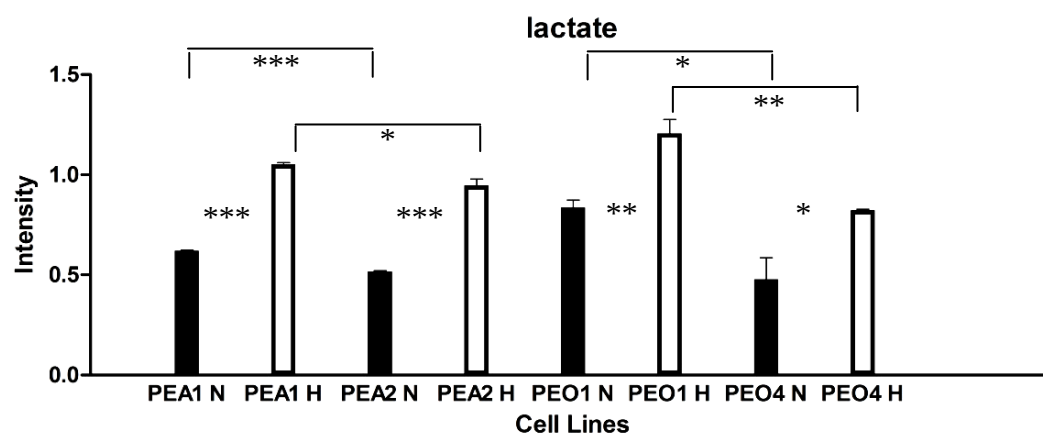
Similarly to the intracellular extracts examinations, an individual look at the extracellular intensities of the media metabolites that appeared to be consistently-patterned and/or associated with glycolysis and the Warburg effect, helped in highlighting their behavioural similarities and differences in more detail.

A few representative plots of these metabolites are presented below and discussed in more detail in section 5.5. They include glucose, lactate, pyruvate, acetate and *myo*-inositol.

As previously mentioned, the extracellular media were scaled to TSP and the ^1H NMR spectra of the selected intracellular metabolites were relatively quantified (Figures 5.15 and 5.16) by integrating the peak areas between their corresponding ppm ranges (Table 5.2). Asterisks denote the relevant values of significance.

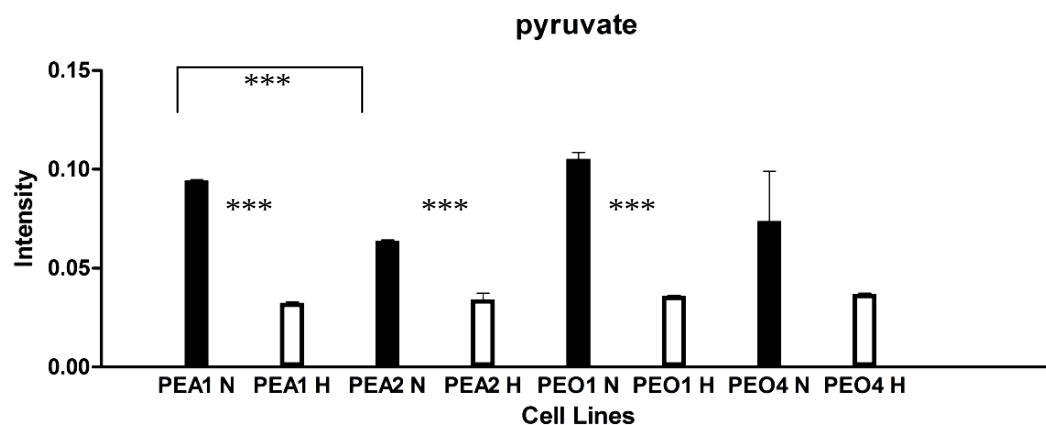


A

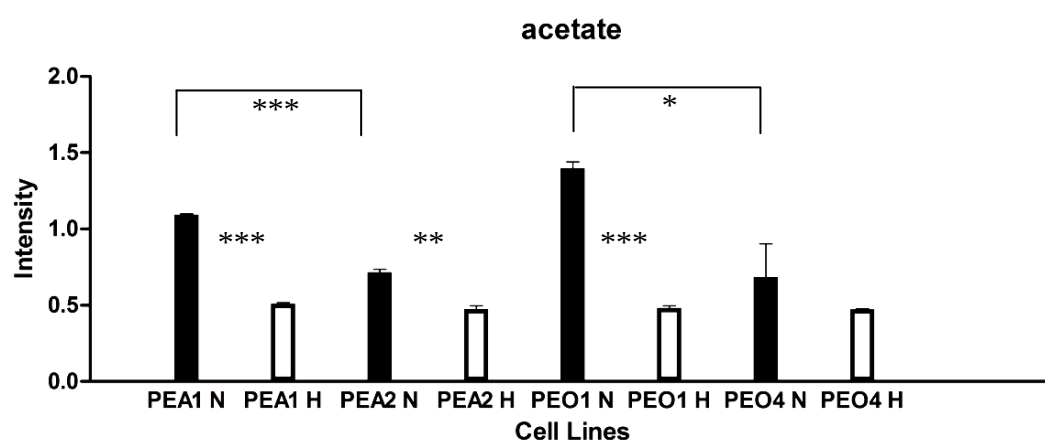


B

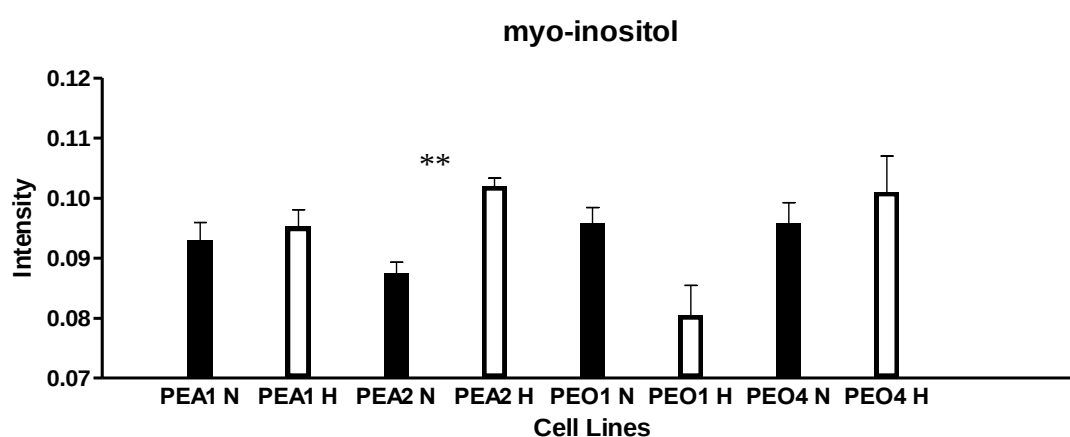
Figure 5.13 - Mean absolute values of TSP-scaled NMR intensities for the extracellular media of A) glucose and B) lactate between platinum sensitive and resistant isogenic ovarian cancer cells. * $p < 0.05$, ** $p < 0.01$, * $p < 0.001$ (unpaired student's t-test using two-tail distribution). Error bars indicate standard error of the mean (SEM).**



A



B



C

Figure 5.14 - Mean absolute values of TSP-scaled NMR intensities for the extracellular media of A) pyruvate, B) acetate and C) *myo*-inositol between platinum sensitive and resistant isogenic ovarian cancer cells. * $p < 0.05$, ** $p < 0.01$, * $p < 0.001$ (unpaired student's t-test using two-tail distribution). Error bars indicate standard error of the mean (SEM).**

5.5 Discussion

Cancer cells exhibit increased glycolysis for generation of ATP (the Warburg effect) (Christofk, Vander Heiden et al. 2008). Among the biggest factors considered to contribute to this effect are mitochondrial respiration dysfunction and hypoxia, which are frequently linked to resistance to chemotherapeutic agents in the tumour microenvironment (Xu, Pelicano et al. 2005). However, it still remains unclear whether this increased glycolytic activity in cancer cells is caused by intrinsic metabolic changes or by the anaerobic conditions within the tumour (Zu and Guppy 2004). Our study focused on examining metabolic alterations both under standard oxygen conditions and in response to hypoxia.

Taking into account the declining levels for a considerable amount of metabolites, hypoxia seemed to have a considerable effect on nutrient consumption. Cancer cell proliferation requires synthesis of various macromolecules such as lipids, proteins, and nucleotides (DeBerardinis, Mancuso et al. 2007). Under hypoxia this need for both growth and survival could lead to utilisation of additional sources for energy production apart than the first choice molecule, which is usually glucose. At the same time, decreased levels may also reflect impediments in cell growth. Based on the metabolic observations, nutrients like glucose, taurine, phosphocholine, glycine, glutathione, glutamate and aspartate in the intracellular extracts seemed to be declining for all hypoxic cells, regardless of their resistance status, with possibilities including either the cells' attempt to protect themselves against hypoxic injury and/or compensate for any possible energy deficits.

5.4.1 Metabolic changes under normoxia and hypoxia

Branch-chained amino acids

Some exceptions occurred, though, in the case of the essential amino acids leucine, isoleucine and valine. Both valine and leucine increased intracellularly under hypoxia, regardless of their resistance status. The reason why this happens may be explained by a number of possibilities. First, it may be possible that hypoxia may promote the uptake of these essential amino acids from the media into the cell for various cellular functions including cytoprotection. A study by Nishimura and colleagues has shown that valine can contribute to the generation of high-energy phosphates in mitochondria and protect heart cells from hypoxia (Nishimura, Muragaki et al. 1989). Additionally, leucine levels inside the cells may be higher under hypoxia, since it has been shown to play a role in HIF-1 α -mediated transcription whose interaction with the

necessary p300 co-activator relies upon a hydrophobic interface that is leucine-rich (Gu, Milligan et al. 2001). Isoleucine also increased intracellularly under hypoxia, but interestingly only for the hypoxic resistant samples, indicating a possible contribution to the resistance effect in hypoxia with an as yet unknown mechanism. *In vitro* studies in the past, though, have speculated that leucine and isoleucine might promote carcinogenic activity *in vivo* in the bladder of rats (Kakizoe, Komatsu et al. 1982). Both of these amino acids were also later shown to cause bladder tumorigenicity in a rat model of carcinogenesis using the *N*-butyl-*N*-(4-hydroxybutyl) nitrosamine (BBN) initiator (Nishio, Kakizoe et al. 1986).

Glucose

Looking at some more metabolites in detail, intracellular glucose in resistant cells showed a declining tendency for most lines when compared to their isogenic sensitive lines which, according to our initial hypothesis, it was something to be expected for resistant cells. In addition, intracellular glucose exhibited declining concentration levels in all hypoxic samples when compared to normoxic ones; it is notable however that the decrease was only significant in the hypoxic cisplatin-resistant cells, which is on its own interesting as it may signify a tendency of the resistant cells to consume more glucose than the cisplatin-sensitive lines in hypoxia. In turn, extracellular glucose in the hypoxic media also exhibited lower concentrations when compared to the normoxic ones, whilst when compared to their isogenic sensitive lines, it was found to be higher in the resistant cells. First of all, the decreased glucose levels in the hypoxic samples could point towards a number of possibilities. One possibility could be that intracellular glucose was consumed universally by all cells; however, since this decrease was more significant in the resistant cells under hypoxia, this could support the theory for the existence of an exaggerated Warburg effect in such a low oxygen environment, leading, this way perhaps to increased resistance in hypoxic conditions. From another standpoint, since extracellular glucose was higher in the resistant cells, compared to the sensitive ones, this might also mean decreased glucose uptake in the resistant cells. In conjunction with the lactate observations discussed below, these findings might reveal a mechanism by which resistant cells behave under hypoxia, which is also related to the use of lactate as a substrate for energy production in hypoxia (see paragraph below).

Lactate

Lactate is a molecule closely associated to glucose. In this study, intracellular lactate levels increased for all hypoxic cells when compared to normoxic ones, with the exception of

cisplatin-sensitive cell line PEO1 H (The reason for the different behaviour in PEO1 H might lie in the actual genetic composition of the cell line or due to an experimental artefact). The above events may indicate that at least for the rest of cells there is a possible trend towards increased lactate production in hypoxia. The lactate production in cells is usually driven from glucose consumption. It is also known that in the absence of oxygen, glucose undergoes fermentation to lactate, indicating that the majority of cells increased their anaerobic respiration by converting more glucose to lactate. The situation became a little clearer while examining the media samples, as all of the cell lines followed a more consistent pattern. Extracellular lactate levels increased for all hypoxic cells compared to the normoxic samples, indicating lactate production in hypoxia (with the excess lactate being secreted out of the cell). Indeed, because the majority of cancer cells cannot store carbon as neither glycogen nor triglycerides, the excess carbon atoms generated from the glycolytic process are usually secreted as lactate (Bui and Thompson 2006).

Glucose & lactate relationship

The study and comparison of the metabolic behaviour of glucose and lactate under normoxia and hypoxia, seemed to reveal a possible mechanism for the resistant cells: since, all resistant cells (in both oxygen conditions) seemed to have lower lactate concentrations than their isogenic sensitive cells intracellularly, this could imply the possibility of lactate being used as an alternative means of energy production for these cells. This can also be supported by the glucose findings while examining the hypoxic resistant cells: there, significantly higher intracellular glucose consumption occurred, coupled with increased extracellular glucose levels. This may mean that hypoxia may have led to an increased lactate production as a preferential energy reserve for the cells under such conditions. This is also supported by the observation of increased glucose levels in the media of the resistant samples, possibly indicating less glucose (but higher lactate) uptake.

Summarising the above, three conclusions can be reached so far:

- Hypoxia affects the levels of glycolytic entities such glucose and lactate. For the majority of cell lines, concentrations were lower for glucose (both intra- and extracellularly) and higher for lactate (both intra- and extracellularly, compared to normoxic.

- Resistant cells seemed to consume more glucose (with the effect exaggerated in hypoxia) as demonstrated by their decreased intracellular glucose levels in the resistant cells.
- Since extracellular glucose levels in the media were higher in resistant cells whilst their intracellular glucose levels were declining (or remained relatively stable) this might mean that resistant cells may also prefer lactate as an additional energy source after lactate becomes available through the glycolytic process. Indeed, intracellular and extracellular lactate levels in resistant cells are declining (with the difference more apparent in the PEO1/PEO4 pair). On some occasions, hypoxic conditions seemed to make these changes more apparent.

Pyruvate

Pyruvate is another candidate molecule associated with the development of platinum resistance. Pyruvate is usually produced from glucose (glycolysis), supplying energy to living cells during the citric acid cycle (Alberts, Johnson et al. 2002). Examining the pyruvate levels in the media, sensitive and resistant cells showed decreased levels in all hypoxic lines, regardless of resistance status. However, under hypoxic conditions, pyruvate levels showed very similar concentrations between resistant and sensitive cells whereas under normoxia, the resistant lines exhibited a much greater difference in their levels compared to the sensitive ones. As in the case of acetate, the concentration gap between hypoxic sensitive and resistant cells was minimal. There are various possibilities for this behaviour in hypoxic pyruvate levels.

First of all, if the increased levels of lactate were compared with the very low pyruvate levels in the hypoxic media (for both resistant and sensitive cells) this could indicate increased consumption of pyruvate for the formation of lactate. Indeed, it has been found that when there is insufficient delivery of oxygen to the cells, there can be a shift towards lactate production as an alternative way for energy production (Andrews, Chen et al. 1999). Metabolic alterations involving pyruvate have been previously observed in cancer (i.e. increased glycolysis and glutaminolysis) which are known to increase lactate in tumour cells (Sonveaux, Vegran et al. 2008); (DeBerardinis, Mancuso et al. 2007) which are consistent with the increased lactate production/secretion discussed earlier.

It is also possible that pyruvate plays a part in protecting the cancer cell during hypoxia; pyruvate can stabilize HIF-1 α and inhibit the pyruvate dehydrogenase complex. This has been shown to promote malignancy (McFate, Mohyeldin et al. 2008). Also, by allowing this HIF stabilization, pyruvate could promote resistance to oxidative stress and anti-cancer therapy (Martinive, Defresne et al. 2006); (Dewhirst, Cao et al. 2008). Additionally, it is known that pyruvate can reduce the ability of chemotherapeutic agents to induce apoptosis. For example, it has been shown to reduce the cytotoxicity of cisplatin in renal proximal tubular epithelial cells (Baek, Kwon et al. 2003) and, in general, of apoptosis-inducing drugs, working through oxidative stress (Frederiksen, Siemens et al. 2003); (Sanna and Rofstad 1994); (Song, Liu et al. 2006). Besides its metabolic role, pyruvate can act as a strong anti-oxidant (O'Donnell-Tormey, Nathan et al. 1987); (Andrae, Singh et al. 1985); (Lee, Kang et al. 2003); (Martin 1996), interfering with ROS-induced intracellular signals, necessary for cellular functions such as cell proliferation or apoptosis (Dewhirst, Cao et al. 2008); (Pelicano, Carney et al. 2004) and, thus, protecting the cancer cells from oxidative stress. Additionally, pyruvate has been shown to decrease hypoxia-induced DNA damage and increase DNA repair in liver tumours (Roudier, Bachelet et al. 2007) possibly by maintaining PARP activity, in hypoxic cancer cells (Roudier and Perrin 2009).

Acetate

In the case of acetate there seemed to be a difference in behaviour between normoxic and hypoxic lines (see tables 5.5 and 5.6). Looking closer at its extracellular levels, all hypoxic cell lines had decreased acetate in the media (possibly due to effects in growth). However, this decline might also reveal an intrinsic metabolic mechanism, which only becomes apparent in hypoxia, and implicates the regulation of concentration of compounds like acetic acid. For example, acetate uptake in tumour cells is thought to be mediated by acetyl-CoA synthetase (ACSS). Studies have shown that ACSS expression is upregulated in hypoxia, suggesting that this may play an important role in controlling the metabolism of acetate in cancer cells (Yoshii, Furukawa et al. 2007). In addition to that, the acetate levels in all resistant cell lines appeared to have significantly decreased concentration levels compared to their sensitive counterparts. However, under hypoxia, the difference between resistant and sensitive cell lines was relatively minimal. It is not entirely clear why this behaviour was observed. Normally, though, the concentration of acetate in cells is kept at low levels so as to avoid disrupting the physiological pH. Since on this occasion the overall change in acetate levels was smaller for the resistant cells but bigger for the sensitive cells, one could theorise that for some reason resistant cells did

not need to alter greatly their pH in hypoxia compared to sensitive cells, and have in general a better-adapted ability to withstand low pH-inflicted pressure, contributing, thus, to the overall effect of resistance.

Myo-inositol

In the case of *myo*-inositol a unique pattern was observed. As with other molecules, intracellular *myo*-inositol decreased in all hypoxic cell lines. However, this decrease was much more pronounced in the case of the resistant cells (Table 5.4). This might indicate either enhanced *myo*-inositol consumption or less production from glucose in hypoxia. This also became more apparent when comparing changes between the isogenic pairs; intracellular *myo*-inositol decreased in all hypoxia-exposed resistant cell lines but not in the normoxia-exposed ones (Table 5.3). Conversely, extracellular *myo*-inositol exhibited increases in the hypoxic resistant cells, while the normoxic resistant either exhibited a small decrease or remained stable. This might indicate reduced uptake of *myo*-inositol from the extracellular media in the case of hypoxic resistant cells. Reports on *myo*-inositol in ovarian cancer cells have showed increased levels under standard culture conditions and since it has been speculated that *myo*-inositol may act as a kind of anti-oxidant (Muraoka and Miura 2004) it could be assumed possible to confer resistance against drugs. In our study, if there is indeed decreased *myo*-inositol utilisation in hypoxic resistant cells, this may mean that resistant cells in hypoxia have different ways of dealing with chemotherapeutic drugs, by-passing the need for increased *myo*-inositol and, thus, stimulating the interest of further investigating its role.

Choline-related compounds

The phospholipid glycerophosphocholine (GPC) is another intracellular metabolite that showed a notable response regarding its hypoxic concentration levels when compared to the normoxic ones. Under hypoxia, GPC levels increased for the resistant cells but decreased for the sensitive cells. The pattern was more random when comparing sensitive vs. resistant cells. A previous study had shown that GPC in hypoxia had declined to non-detectable levels (following an initial increase), while genes that encoded related phosphatidylcholine enzymes were downregulated (Ackerstaff, Pflug et al. 2001). It was speculated that such processes could have an effect on the changes observed in GPC levels under hypoxia. However, how this is linked to phosphatidylcholine metabolism and their expression levels, especially under hypoxia, still remains relatively unexplored (Glunde, Jie et al. 2004). Phosphocholine decreased in all hypoxic cells but also increased in all resistant cells. This may indicate that this particular

metabolite is not greatly affected by the presence of oxygen. However, just the fact that phosphocholine was increased in the resistant cells compared to their isogenic sensitive ones, gives some support to existing data that link its increased levels to cancer. For example, previous studies have demonstrated that loss of p53 lead to increased phosphocholine in colon cancer cells and may suggest that on this occasion at least, the observed increased malignancy may be partly mediated through choline-related pathways (Mori, Delsite et al. 2004). Other studies have also shown that phosphocholine can act as a biomarker in breast cancer expressing upregulation in various choline transporters or choline kinase genes (Eliyahu, Kreizman et al. 2007).

5.4.2 Correlation with previous NMR-based metabolic findings

While trying to compare the metabolic lab findings of this chapter with those presented in chapter 3, it quickly became obvious that a metabolic comparison between cancer cell lines of different genetic orientation and, thus, different phenotypic behaviour, can be challenging at times.

The first part of the study involved pairs of kidney (HEK-293T derived) and ovarian cancer (A2780-derived) cell lines, either positive or negative for the MMR protein *Mlh1*. The metabolic changes correlating to platinum resistance included metabolites such as glutathione whose levels were consistently higher for all *Mlh1*-negative cells. Compared to the last part of this study (chapter 5) the glutathione changes seemed more random, with only the PEA1 & PEA2 isogenic pair “agreeing” with the first part of the study. Interestingly, though, hypoxia seemed to try and “correct” to a certain degree this anomaly created by PEO1 & PEO4 by reducing the concentration differences in glutathione between the isogenic lines. In the first part of the study there was a significant increase in intracellular alanine in all cells with *Mlh1* loss (except in the mitochondrial DNA-depleted cells). In the isogenic study, alanine increased in all cells except PEO4 N. Interestingly again, hypoxia seemed to “correct” this discrepancy of the normoxic cells, with both resistant hypoxic resistant lines showing increased alanine concentrations. Intracellular glucose in resistant cells showed declining levels for most lines compared to their isogenic sensitive ones (with the effect more exaggerated in hypoxic conditions while once again “correcting” the levels of PEO4 cells when compared to normoxia). In the *Mlh1* study, the levels of intracellular lactate slightly decreased in all *Mlh1*-negative cells across both models (with the exception of MutL α - which showed a small increase in the HEK-293T model). In the isogenic study, lactate levels in resistant cells also

declined compared to their isogenic sensitive ones (with the exception of one line, PEO4 H). Intracellular PC levels were seen to decrease in all the *Mlh1*-negative cells (but with the decrease in the mitochondrial DNA-depleted cells being miniscule at just 1%). On the contrary, phosphocholine increased in all resistant cells of the isogenic study, when compared to the sensitive ones. In the *Mlh1* study *Mlh1*-negative cells exhibited a small decrease in their GPC concentration levels (with the exception of the ovarian *Mlh1*-negative CP70 cell line). In the isogenic study, GPC levels increased for the resistant cells (with the exception of PEO4 N). However, hypoxia apparently catered for this discrepancy once more by increasing the levels of said cell line in low oxygen conditions. Choline levels were increased intracellularly in all *Mlh1*-negative cells, however in the isogenic study resistant vs. sensitive its levels were more random. In the *Mlh1* study, *myo*-inositol increased in the *Mlh1*-negative CP70 A2 cells from the PRM model and the MutL α - cells from the HEK-293T cell model. But it didn't change for the CP70 and the MutL α - ρ 0 cells, where there was a miniscule decrease. In the isogenic study though, intracellular *myo*-inositol increased in the normoxic resistant but decreased in the hypoxic resistant ones as compared to the sensitive cells.

All the above changes reveal some consistencies and some discrepancies between the two NMR-based studies. On some occasions however, hypoxia appeared to correct these disagreements as with the cases of alanine or GPC. Of course, this “correction” was not universal throughout the metabolic data, and on certain occasions as in the case of *myo*-inositol, some of the changes appeared to be non-agreeable between the two studies or even within them. However, on such instances, it is important to mention that other known and unknown factors seem to come into play, and which include significant parameters such as the background genetic variability of the tested cancer cell lines.

5.4.3 Concluding remarks

In summary, this part of the study examined the baseline metabolic phenotype of platinum resistance cells under two different oxygen conditions and, as a result, it managed to observe meaningful concentration changes of metabolites in relation to cancer and platinum resistance. This way, further insight was added in the fight against chemotherapeutic resistance which was made possible through the study and identification of metabolic entities that seem to contribute to it.

Chapter 6

Conclusions

6.1 Overview

Cancer chemotherapy has developed to become the standard of patient care. However, the frequently arising clinical problems of acquired resistance to platinum based cancer drugs is a crucial determining factor and is usually associated with poor prognostic outcome. Studying and understanding the mechanisms of resistance, and especially its reversal, was therefore of critical significance in offering better chances of improvement in both treatment and outcome for the cancer patient.

In general, metabolic profiling like the one presented in this study, could prove useful in delivering non-invasive biomarkers of resistance to assist with cancer detection as early as possible in the development of the disease. In other words, it could potentially identify patients who are either suitable or unsuitable to give a successful response to platinum-based chemotherapy.

The general theme of this PhD thesis was the metabolic investigation of platinum resistance and cancer using a number of different approaches, employing NMR, cell culture- and bioinformatics-based methods, as approaching this multifactorial problem from different angles was deemed essential in assisting towards the general understanding of it.

The first part of this investigation initiated with the study of *Mlh1*-specific metabolic signatures of two cell models *in vitro*: a transient *Mlh1* expression cell model involving kidney cell lines of common genetic background after selective mitochondrial depletion, and a model of platinum resistance consisting of well-established ovarian cell lines. In this part of the study, the metabolites whose levels showed significant correlation with *Mlh1* expression and platinum resistance included glutathione, alanine, lactate, branch-chained amino acids, *myo*-inositol and choline-related metabolites such as phosphocholine. Our findings offered a range of interpretations on the role of these metabolites and their association with *Mlh1*, platinum resistance and mitochondria, suggesting that cells with *Mlh1* loss of expression can render cells more resistant to chemotherapeutic treatments. It also managed to identify a unique *Mlh1* metabolic signature as well as to connect mitochondrial metabolism to *Mlh1* loss of expression.

As a continuation of this study, a bioinformatics based approach was also applied for the comparison of the above metabolic findings to the results published in the NCI-60 gene expression database as well as to crosscheck possible correlations. The *Mlh1*-related genes that

were identified as important, using a bioinformatics-based methodology by exploiting the ConsensusPath online database, were ACOT4, FAS and FUT9 and NCAM1. At the same time, their significantly associated metabolites that helped towards their identification were oleic acid, palmitate, sialyl-3-paragloboside, and stearate. In general, this part of the study managed to test in practice a newly-developed NEST-based examination that involved complex pathways of genes and metabolites, using *Mlh1* as the centre of investigation.

The final part of this study explored platinum resistance in a different set of cancer cell lines, and involved isogenic ovarian cancer cells that offered to give a more accurate representation of in vivo situations and platinum resistance. The use of isogenetically matched cell lines which have been derived directly from cancer patient before and after the development of platinum resistance helped immensely in identifying and confirming possible causative or influencing factors of this phenomenon from a metabolic point of view. In addition, taking into account that hypoxia on many clinical occasions confers cancer aggressiveness, the experiments that took place also focused on examining metabolic alterations not only under standard oxygen conditions but also in response to reduced oxygen availability (hypoxia). At the same time, particular focus was given on metabolic changes that could be connected to increased platinum resistance under hypoxia. The results gave a variety of metabolic responses in both intracellular extracts and extracellular media, and included concentration changes in glucose, lactate, pyruvate, acetate, and choline-related metabolites.

6.2 Clinical relevance and future directions

From a clinical point of view, metabonomics is able to generate biomarkers that have been proven useful in detection, prediction of response as well as identification of new therapeutic avenues. Its predictive potential is considered a key parameter in translational research, because the methods employed (most commonly, NMR spectroscopy) provide minimal invasiveness, are non-destructive and frequently use miniscule amounts of sample fluids or tissues. The relatively small size of the number of metabolic entities when compared to genes, transcripts and proteins, in theory at least, also offer the ability to capture a snapshot of the cellular environment. And since, metabolomics is among the fastest systems to react to any changes in an organism's system it can reflect metabolic changes in response to stimuli such as platinum drugs.

In fact, the metabolic response to platinum drugs is actually one of the possible future investigations that can be considered when examining platinum resistance in cancer. Thus, apart from the baseline profiles generated in this study, a continuation of this investigation could involve a variety of isogenic cell lines being tested for a metabolic response to platinum drugs such as cisplatin.

In addition, treatment of such cell lines with a type of modulator of resistance (i.e. aphidicolin) that, on some occasions, are known to effectively “reverse” resistance by acting on metabolic entities/pathways would complete the metabolic picture between predictive biomarkers and biomarkers of response.

In summary the current study as well as any related future investigations could:

- Remove from consideration false positive or negative results in assays involving biomarkers for the disease.
- Lead towards the development of less toxic and side-effect producing but more effective salvage chemotherapy regimens.
- Identify early on the onset of the disease the patients that suffer from premalignant forms before they develop into active cancer so that curative treatment can be started without delay.
- Identify causative gene(s) that could not be directly associated with resistance due to lack of insight into the wider picture, but could only be identified through multianalytical platforms and complex computational methods. Such a development would be possibly beneficial in producing gene-specific treatments, perhaps also finding potential future applications in gene therapy.

As a conclusion, this study managed to give added insight into the metabolic universe of cancer and platinum resistance with the assistance of analytical tools such as NMR, molecular-based techniques and computational approaches. At the same time it may also help to pave the way for further *in vitro* and translational metabolic-based studies and assist in the fight towards better management and treatment of the deleterious disease that is cancer.

References

- Ackerstaff, E., B. R. Pflug, et al. (2001). "Detection of increased choline compounds with proton nuclear magnetic resonance spectroscopy subsequent to malignant transformation of human prostatic epithelial cells." Cancer Res **61**(9): 3599-3603.
- Adams, M., I. J. Kerby, et al. (1989). "A comparison of the toxicity and efficacy of cisplatin and carboplatin in advanced ovarian cancer. The Swons Gynaecological Cancer Group." Acta Oncol **28**(1): 57-60.
- Aebi, S., B. Kurdi-Haidar, et al. (1996). "Loss of DNA mismatch repair in acquired resistance to cisplatin." Cancer Res **56**(13): 3087-3090.
- Agarwal, R. and S. B. Kaye (2003). "Ovarian cancer: strategies for overcoming resistance to chemotherapy." Nat Rev Cancer **3**(7): 502-516.
- Alberts, B., A. Johnson, et al. (2002). Molecular Biology of the Cell, Fourth Edition, Garland Science.
- Ali-Osman, F., M. S. Berger, et al. (1993). "Topoisomerase II inhibition and altered kinetics of formation and repair of nitrosourea and cisplatin-induced DNA interstrand cross-links and cytotoxicity in human glioblastoma cells." Cancer Res **53**(23): 5663-5668.
- Amellem, O. and E. O. Pettersen (1991). "Cell inactivation and cell cycle inhibition as induced by extreme hypoxia: the possible role of cell cycle arrest as a protection against hypoxia-induced lethal damage." Cell Prolif **24**(2): 127-141.
- Andrae, U., J. Singh, et al. (1985). "Pyruvate and related alpha-ketoacids protect mammalian cells in culture against hydrogen peroxide-induced cytotoxicity." Toxicol Lett **28**(2-3): 93-98.
- Andrei, P., R. Shutian, et al. (2005). "Dependence of FDG uptake on tumor microenvironment." Int J Radiat Oncol Biol Phys. **62**(2): 545-553.
- Andrews, D. L., W. J. A. Chen, et al. (1999). "Ethanol Attenuates Lactate Production in Hypoxic Postnatal Day 4 Rat Cerebella." Alcohol (Fayetteville, N.Y.) **19**(1): 31-35.
- Annison, E. F., J. L. Linzell, et al. (1967). "The oxidation and utilization of palmitate, stearate, oleate and acetate by the mammary gland of the fed goat in relation to their overall metabolism, and the role of plasma phospholipids and neutral lipids in milk-fat synthesis." Biochem J **102**(3): 637-647.
- Anthony, D. A., A. J. McIlwrath, et al. (1996). "Microsatellite instability, apoptosis, and loss of p53 function in drug-resistant tumor cells." Cancer Res **56**(6): 1374-1381.

- Antoniou, A., P. D. Pharoah, et al. (2003). "Average risks of breast and ovarian cancer associated with BRCA1 or BRCA2 mutations detected in case Series unselected for family history: a combined analysis of 22 studies." Am J Hum Genet **72**(5): 1117-1130.
- Aoife, M. S., J. B.-H. David, et al. (2003). "Tumour hypoxia, chemotherapeutic resistance and hypoxia-related therapies." Cancer Treat Rev **29**(4): 297-307.
- Armstrong, J. S. (2006). "Mitochondria: a target for cancer therapy." Br J Pharmacol **147**(3): 239-248.
- Asselin, E., G. B. Mills, et al. (2001). "XIAP regulates Akt activity and caspase-3-dependent cleavage during cisplatin-induced apoptosis in human ovarian epithelial cancer cells." Cancer Res **61**(5): 1862-1868.
- Attard, M., G. Jean, et al. (1999). "Severity of phenotype in cystinosis varies with mutations in the CTNS gene: predicted effect on the model of cystinosis." Hum Mol Genet **8**(13): 2507-2514.
- Auersperg, N., A. S. Wong, et al. (2001). "Ovarian surface epithelium: biology, endocrinology, and pathology." Endocr Rev **22**(2): 255-288.
- Baek, S. M., C. H. Kwon, et al. (2003). "Differential roles of hydrogen peroxide and hydroxyl radical in cisplatin-induced cell death in renal proximal tubular epithelial cells." J Lab Clin Med **142**(3): 178-186.
- Balch, C., T. H. Huang, et al. (2004). "The epigenetics of ovarian cancer drug resistance and resensitization." Am J Obstet Gynecol **191**(5): 1552-1572.
- Balmana, J., D. H. Stockwell, et al. (2006). "Prediction of MLH1 and MSH2 mutations in Lynch syndrome." JAMA **296**(12): 1469-1478.
- Baronzio, G. F., E. D. Hager, et al. (2006). "Influence of Tumor Microenvironment on Thermoresponse." Hyperthermia in Cancer Treatment: A Primer, Springer US: 67-91.
- Batchelder, R. M., W. R. Wilson, et al. (1996). "Oxygen dependence of the cytotoxicity of the enediyne anti-tumour antibiotic esperamicin A1." Br J Cancer Suppl **27**: S52-56.
- Behrens, B. C., T. C. Hamilton, et al. (1987). "Characterization of a cis-diamminedichloroplatinum(II)-resistant human ovarian cancer cell line and its use in evaluation of platinum analogues." Cancer Res **47**(2): 414-418.
- Berkenblit, A. and S. A. Cannistra (2005). "Advances in the management of epithelial ovarian cancer." J Reprod Med **50**(6): 426-438.
- Bernal, S. D., T. J. Lampidis, et al. (1982). "Rhodamine-123 selectively reduces clonal growth of carcinoma cells in vitro." Science **218**(4577): 1117-1119.

- Blanc C Fau - Deveraux, Q. L., S. Deveraux Ql Fau - Krajewski, et al. (2000). "Caspase-3 is essential for procaspase-9 processing and cisplatin-induced." Cancer Res **60**(16).
- Bombardelli, L. and U. Cavallaro (2010). "Immunoglobulin-like cell adhesion molecules: novel signaling players in epithelial ovarian cancer." Int J Biochem Cell Biol **42**(5): 590-594.
- Bonovas, S., K. Filioussi, et al. (2006). "Paracetamol use and risk of ovarian cancer: a meta-analysis." Br J Clin Pharmacol **62**(1): 113-121.
- Boulikas, T. (2004). "Low toxicity and anticancer activity of a novel liposomal cisplatin (Lipoplatin) in mouse xenografts." Oncol Rep **12**(1): 3-12.
- Boulikas, T. and M. Vougiouka (2003). "Cisplatin and platinum drugs at the molecular level. (Review)." Oncol Rep **10**(6): 1663-1682.
- Brahimi-Horn, M. C., J. Chiche, et al. (2007). "Hypoxia signalling controls metabolic demand." Curr Opin Cell Biol **19**(2): 223-229.
- Brown, J. M. (1999). "The hypoxic cell: a target for selective cancer therapy--eighteenth Bruce F. Cain Memorial Award lecture." Cancer Res **59**(23): 5863-5870.
- Brown, J. M. and W. R. Wilson (2004). "Exploiting tumour hypoxia in cancer treatment." Nat Rev Cancer **4**(6): 437-447.
- Brown, R., G. L. Hirst, et al. (1997). "hMLH1 expression and cellular responses of ovarian tumour cells to treatment with cytotoxic anticancer agents." Oncogene **15**(1): 45-52.
- Buermeyer, A. B., C. Wilson-Van Patten, et al. (1999). "The human MLH1 cDNA complements DNA mismatch repair defects in Mlh1-deficient mouse embryonic fibroblasts." Cancer Res **59**(3): 538-541.
- Bui, T. and C. B. Thompson (2006). "Cancer's sweet tooth." Cancer Cell **9**(6): 419-420.
- Cai, J., J. Yang, et al. (1998). "Mitochondrial control of apoptosis: the role of cytochrome c." Biochim Biophys Acta **1366**(1-2): 139-149.
- Cailleau-Thomas, A., P. Coullin, et al. (2000). "FUT4 and FUT9 genes are expressed early in human embryogenesis." Glycobiology **10**(8): 789-802.
- Cejka, P., L. Stojic, et al. (2003). "Methylation-induced G(2)/M arrest requires a full complement of the mismatch repair protein hMLH1." EMBO J **22**(9): 2245-2254.
- Chan, J. K., K. Fuh, et al. (2008). "The treatment and outcomes of early-stage epithelial ovarian cancer: have we made any progress?" Br J Cancer **98**(7): 1191-1196.
- Chan, S. H. and R. L. Barbour (1983). "Adenine nucleotide transport in hepatoma mitochondria. Characterization of factors influencing the kinetics of ADP and ATP uptake." Biochim Biophys Acta **723**(1): 104-113.

- Chaney, S. G. and A. Sancar (1996). "DNA repair: enzymatic mechanisms and relevance to drug response." J Natl Cancer Inst **88**(19): 1346-1360.
- Chang, L. O., C. A. Schnaitman, et al. (1971). "Comparison of the mitochondrial membrane proteins in rat liver and hepatomas." Cancer Res **31**(2): 108-113.
- Chen, Z., W. Lu, et al. (2007). "The Warburg effect and its cancer therapeutic implications." J Bioenerg Biomembr **39**(3): 267-274.
- Chiao, C., A. M. Carothers, et al. (1995). "Apoptosis and altered redox state induced by caffeic acid phenethyl ester (CAPE) in transformed rat fibroblast cells." Cancer Res **55**(16): 3576-3583.
- Christofk, H. R., M. G. Vander Heiden, et al. (2008). "Pyruvate kinase M2 is a phosphotyrosine-binding protein." Nature **452**(7184): 181-186.
- Clapham, D. E. (2007). "Calcium signaling." Cell **131**(6): 1047-1058.
- Claridge, D. W. T. (2004). "Nuclear magnetic resonance in chemical engineering: Principles and applications." Oxford: elsevier.
- Cloarec, O., M. E. Dumas, et al. (2005). "Statistical total correlation spectroscopy: an exploratory approach for latent biomarker identification from metabolic ¹H NMR data sets." Anal Chem **77**(5): 1282-1289.
- Coloff, J. L. and J. C. Rathmell (2006). "Metabolic regulation of Akt: roles reversed." J Cell Biol **175**(6): 845-847.
- Cooper, W. A., M. R. Kohonen-Corish, et al. (2008). "Prognostic significance of DNA repair proteins MLH1, MSH2 and MGMT expression in non-small-cell lung cancer and precursor lesions." Histopathology **52**(5): 613-622.
- Cullen, K. J., Z. Yang, et al. (2007). "Mitochondria as a critical target of the chemotherapeutic agent cisplatin in head and neck cancer." J Bioenerg Biomembr **39**(1): 43-50.
- Dabholkar, M., J. Vionnet, et al. (1994). "Messenger RNA levels of XPAC and ERCC1 in ovarian cancer tissue correlate with response to platinum-based chemotherapy." J Clin Invest **94**(2): 703-708.
- Danforth, K. N., S. S. Tworoger, et al. (2007). "Breastfeeding and risk of ovarian cancer in two prospective cohorts." Cancer Causes Control **18**(5): 517-523.
- De Mito, A. and S. Fais (2005). "Tumor acidity, chemoresistance and proton pump inhibitors." Future Oncol **1**(6): 779-786.
- DeBerardinis, R. J., A. Mancuso, et al. (2007). "Beyond aerobic glycolysis: transformed cells can engage in glutamine metabolism that exceeds the requirement for protein and nucleotide synthesis." Proc Natl Acad Sci U S A **104**(49): 19345-19350.

- Dewhirst, M. W., Y. Cao, et al. (2008). "Cycling hypoxia and free radicals regulate angiogenesis and radiotherapy response." Nat Rev Cancer **8**(6): 425-437.
- Dias, N. and C. Bailly (2005). "Drugs targeting mitochondrial functions to control tumor cell growth." Biochem Pharmacol **70**(1): 1-12.
- Dieterle, F., A. Ross, et al. (2006). "Probabilistic quotient normalization as robust method to account for dilution of complex biological mixtures. Application in ¹H NMR metabonomics." Anal Chem **78**(13): 4281-4290.
- Ding, X., A. B. Mohd, et al. (2009). "MLH1 expression sensitises ovarian cancer cells to cell death mediated by XIAP inhibition." Br J Cancer **101**(2): 269-277.
- Doering, C. B. and D. J. Danner (2000). "Amino acid deprivation induces translation of branched-chain alpha-ketoacid dehydrogenase kinase." Am J Physiol Cell Physiol **279**(5): C1587-1594.
- Dos Santos Silva, I., A. Maclean, et al. (2003). "Does ovarian stimulation increase the risk of ovarian cancer?" Reprod Med Rev **11**(01): 57-66.
- Doz, F., N. Roosen, et al. (1993). "Metallothionein and anticancer agents: the role of metallothionein in cancer chemotherapy." J Neurooncol **17**(2): 123-129.
- Drummond, J. T., A. Anthoney, et al. (1996). "Cisplatin and adriamycin resistance are associated with MutLalpha and mismatch repair deficiency in an ovarian tumor cell line." J Biol Chem **271**(33): 19645-19648.
- Duckett Dr Fau - Drummond, J. T., A. I. Drummond Jt Fau - Murchie, et al. (1996). "Human MutSalpha recognizes damaged DNA base pairs containing O6-methylguanine, O4-methylthymine, or the cisplatin-d(GpG) adduct." Proc Natl Acad Sci U S A **93**(13): 6443-6447.
- Dufour, E. K., J. Whitwell, et al. (2009). "Retinyl palmitate is non-genotoxic in Chinese hamster ovary cells in the dark or after pre-irradiation or simultaneous irradiation with UV light." Mutat Res **672**(1): 21-26.
- Durand, R. E. (1989). "Distribution and activity of antineoplastic drugs in a tumor model." J Natl Cancer Inst **81**(2): 146-152.
- Eastman, A. (1987). "The formation, isolation and characterization of DNA adducts produced by anticancer platinum complexes." Pharmacol Ther **34**(2): 155-166.
- Ebert, M. P., T. Gunther, et al. (2000). "Expression of metallothionein II in intestinal metaplasia, dysplasia, and gastric cancer." Cancer Res **60**(7): 1995-2001.
- Eliyahu, G., T. Kreizman, et al. (2007). "Phosphocholine as a biomarker of breast cancer: Molecular and biochemical studies." Int J Cancer **120**(8): 1721-1730.

- Emma, J. C. and M. Usha (2005). "Epithelial ovarian cancer and induction of ovulation." Rev Gynaecol Pract **5**(3): 131-138.
- Fan, T. W., R. M. Higashi, et al. (1986). "Combined use of ¹H-NMR and GC-MS for metabolite monitoring and in vivo ¹H-NMR assignments." Biochim Biophys Acta **882**(2): 154-167.
- Fantin, V. R., M. J. Berardi, et al. (2002). "A novel mitochondriotoxic small molecule that selectively inhibits tumor cell growth." Cancer Cell **2**(1): 29-42.
- Farrow, S. N. and R. Brown (1996). "New members of the Bcl-2 family and their protein partners." Curr Opin Genet Dev **6**(1): 45-49.
- Ferry, K. V., T. C. Hamilton, et al. (2000). "Increased nucleotide excision repair in cisplatin-resistant ovarian cancer cells: role of ERCC1-XPF." Biochem Pharmacol **60**(9): 1305-1313.
- Fichtinger-Schepman, A. M., H. C. van Dijk-Knijnenburg, et al. (1995). "Cisplatin- and carboplatin-DNA adducts: is PT-AG the cytotoxic lesion?" Carcinogenesis **16**(10): 2447-2453.
- Fink, D., S. Aebi, et al. (1998). "The role of DNA mismatch repair in drug resistance." Clin Cancer Res **4**(1): 1-6.
- Fink, D., S. Nebel, et al. (1996). "The role of DNA mismatch repair in platinum drug resistance." Cancer Res **56**(21): 4881-4886.
- Fink, D., S. Nebel, et al. (1998). "Enrichment for DNA mismatch repair-deficient cells during treatment with cisplatin." Int J Cancer **77**(5): 741-746.
- Fishman, W. H., S. Raam, et al. (1975). "Markers for ovarian cancer: regan isoenzyme and other glycoproteins." Semin Oncol **2**(3): 211-216.
- Frederiksen, L. J., D. R. Siemens, et al. (2003). "Hypoxia induced resistance to doxorubicin in prostate cancer cells is inhibited by low concentrations of glyceryl trinitrate." J Urol **170**(3): 1003-1007.
- Fujiwara, K., S. Suzuki, et al. (2002). "Local radiation therapy for localized relapsed or refractory ovarian cancer patients with or without symptoms after chemotherapy." Int J Gynecol Cancer **12**(3): 250-256.
- Furuta, T., T. Ueda, et al. (2002). "Transcription-coupled nucleotide excision repair as a determinant of cisplatin sensitivity of human cells." Cancer Res **62**(17): 4899-4902.
- Galluzzi, L., N. Larochette, et al. (2006). "Mitochondria as therapeutic targets for cancer chemotherapy." Oncogene **25**(34): 4812-4830.

- Gatenby, R. A. and R. J. Gillies (2004). "Why do cancers have high aerobic glycolysis?" Nat Rev Cancer **4**(11): 891-899.
- Gebauer, G., B. Mirakhur, et al. (2000). "Cisplatin-resistance involves the defective processing of MEKK1 in human ovarian adenocarcinoma 2008/C13 cells." Int J Oncol **16**(2): 321-325.
- Gifford, G., J. Paul, et al. (2004). "The acquisition of hMLH1 methylation in plasma DNA after chemotherapy predicts poor survival for ovarian cancer patients." Clin Cancer Res **10**(13): 4420-4426.
- Glunde, K., M. A. Jacobs, et al. (2006). "Choline metabolism in cancer: implications for diagnosis and therapy." Expert Rev Mol Diagn **6**(6): 821-829.
- Glunde, K., C. Jie, et al. (2004). "Molecular causes of the aberrant choline phospholipid metabolism in breast cancer." Cancer Res **64**(12): 4270-4276.
- Goff, B. A., L. S. Mandel, et al. (2004). "Frequency of symptoms of ovarian cancer in women presenting to primary care clinics." JAMA **291**(22): 2705-2712.
- Gore, M. E., R. J. Atkinson, et al. (2002). "A phase II trial of ZD0473 in platinum-pretreated ovarian cancer." Eur J Cancer **38**(18): 2416-2420.
- Gottesman, M. M. and I. Pastan (1993). "Biochemistry of multidrug resistance mediated by the multidrug transporter." Annu Rev Biochem **62**: 385-427.
- Graeber, T. G., J. F. Peterson, et al. (1994). "Hypoxia induces accumulation of p53 protein, but activation of a G1-phase checkpoint by low-oxygen conditions is independent of p53 status." Mol Cell Biol **14**(9): 6264-6277.
- Granstrom, C., J. Sundquist, et al. (2008). "Population attributable fractions for ovarian cancer in Swedish women by morphological type." Br J Cancer **98**(1): 199-205.
- Gray, L. H., A. D. Conger, et al. (1953). "The concentration of oxygen dissolved in tissues at the time of irradiation as a factor in radiotherapy." Br J Radiol **26**(312): 638-648.
- Green, A., D. Purdie, et al. (1997). "Tubal sterilisation, hysterectomy and decreased risk of ovarian cancer. Survey of Women's Health Study Group." Int J Cancer **71**(6): 948-951.
- Green, D. R. and J. C. Reed (1998). "Mitochondria and apoptosis." Science **281**(5381): 1309-1312.
- Green, J. A., L. J. Robertson, et al. (1993). "Glutathione S-transferase expression in benign and malignant ovarian tumours." Br J Cancer **68**(2): 235-239.
- Greene, L. F. (2002). AJCC Cancer Staging Manual
- Griffin, J. L. and J. P. Shockcor (2004). "Metabolic profiles of cancer cells." Nat Rev Cancer **4**(7): 551-561.

- Gu, J., J. Milligan, et al. (2001). "Molecular mechanism of hypoxia-inducible factor 1 α - p300 interaction. A leucine-rich interface regulated by a single cysteine." J Biol Chem **276**(5): 3550-3554.
- Guppy, M. (2002). "The hypoxic core: a possible answer to the cancer paradox." Biochem Biophys Res Commun **299**(4): 676-680.
- Hakomori, S. (1998). "Cancer-associated glycosphingolipid antigens: their structure, organization, and function." Acta Anat (Basel) **161**(1-4): 79-90.
- Hanahan, D. and R. A. Weinberg (2000). "The hallmarks of cancer." Cell **100**(1): 57-70.
- Hankinson, S. E., D. J. Hunter, et al. (1993). "Tubal ligation, hysterectomy, and risk of ovarian cancer. A prospective study." JAMA **270**(23): 2813-2818.
- Hannibal, C. G., M. A. Rossing, et al. (2008). "Analgesic drug use and risk of epithelial ovarian cancer." Am J Epidemiol **167**(12): 1430-1437.
- Hardie, D. G. (2000). "Metabolic control: a new solution to an old problem." Curr Biol **10**(20): R757-759.
- Hardman, R. A., C. A. Afshari, et al. (2001). "Involvement of mammalian MLH1 in the apoptotic response to peroxide-induced oxidative stress." Cancer Res **61**(4): 1392-1397.
- Harris, A. L. (2002). "Hypoxia--a key regulatory factor in tumour growth." Nat Rev Cancer **2**(1): 38-47.
- Hartmann, L. C., K. H. Lu, et al. (2005). "Gene expression profiles predict early relapse in ovarian cancer after platinum-paclitaxel chemotherapy." Clin Cancer Res **11**(6): 2149-2155.
- Hartwell, L. H. and M. B. Kastan (1994). "Cell cycle control and cancer." Science **266**(5192): 1821-1828.
- Haupt, S., M. Berger, et al. (2003). "Apoptosis - the p53 network." J Cell Sci **116**(Pt 20): 4077-4085.
- Hayakawa, J., M. Ohmichi, et al. (2000). "Inhibition of BAD phosphorylation either at serine 112 via extracellular signal-regulated protein kinase cascade or at serine 136 via Akt cascade sensitizes human ovarian cancer cells to cisplatin." Cancer Res **60**(21): 5988-5994.
- Hengstler, J. G., J. Lange, et al. (1999). "Contribution of c-erbB-2 and topoisomerase II α to chemoresistance in ovarian cancer." Cancer Res **59**(13): 3206-3214.
- Henkels Km Fau - Turchi, J. J. and T. Jj (1997). "Induction of apoptosis in cisplatin-sensitive and -resistant human ovarian cancer." Cancer Res **57**(20).

- Herod, J. J., A. G. Eliopoulos, et al. (1996). "The prognostic significance of Bcl-2 and p53 expression in ovarian carcinoma." Cancer Res **56**(9): 2178-2184.
- Hirama, M., S. Isonishi, et al. (2006). "Characterization of mitochondria in cisplatin-resistant human ovarian carcinoma cells." Oncol Rep **16**(5): 997-1002.
- Hittelet, A., I. Camby, et al. (2003). "Binding Sites for Lewis Antigens Are Expressed by Human Colon Cancer Cells and Negatively Affect Their Migration." Lab Invest **83**(6): 777-787.
- Hockenbery, D. M., Z. N. Oltvai, et al. (1993). "Bcl-2 functions in an antioxidant pathway to prevent apoptosis." Cell **75**(2): 241-251.
- Hollstein, M., D. Sidransky, et al. (1991). "p53 mutations in human cancers." Science **253**(5015): 49-53.
- Huang, L. E., Z. Arany, et al. (1996). "Activation of hypoxia-inducible transcription factor depends primarily upon redox-sensitive stabilization of its alpha subunit." J Biol Chem **271**(50): 32253-32259.
- Huang, L. E., J. Gu, et al. (1998). "Regulation of hypoxia-inducible factor 1alpha is mediated by an O2-dependent degradation domain via the ubiquitin-proteasome pathway." Proc Natl Acad Sci U S A **95**(14): 7987-7992.
- Hunt, M. C. and S. E. H. Alexson (2002). "The role Acyl-CoA thioesterases play in mediating intracellular lipid metabolism." Progress in Lipid Research **41**(2): 99-130.
- Husain, A., G. He, et al. (1998). "BRCA1 up-regulation is associated with repair-mediated resistance to cis-diamminedichloroplatinum(II)." Cancer Res **58**(6): 1120-1123.
- Ikeguchi, M., J. Liu, et al. (2002). "Expression of survivin mRNA and protein in gastric cancer cell line (MKN-45) during cisplatin treatment." Apoptosis **7**(1): 23-29.
- Iorio, E., D. Mezzanzanica, et al. (2005). "Alterations of choline phospholipid metabolism in ovarian tumor progression." Cancer Res **65**(20): 9369-9376.
- Irving, J. A., S. Carinelli, et al. (2005). "Uterine tumors resembling ovarian sex cord tumors are polyphenotypic neoplasms with true sex cord differentiation." Mod Pathol **19**(1): 17-24.
- Irwin, C. C., L. I. Malkin, et al. (1978). "Differences in total mitochondrial proteins and proteins synthesized by mitochondria from rat liver and Morris hepatomas 9618A, 5123C, and 5123tc." Cancer Res **38**(6): 1584-1588.
- Isonishi, S., M. Saitou, et al. (2001). "Mitochondria in platinum resistant cells." Hum Cell **14**(3): 203-210.
- Ivan, M., K. Kondo, et al. (2001). "HIFalpha targeted for VHL-mediated destruction by proline hydroxylation: implications for O2 sensing." Science **292**(5516): 464-468.

- Jaakkola, P., D. R. Mole, et al. (2001). "Targeting of HIF-alpha to the von Hippel-Lindau ubiquitylation complex by O2-regulated prolyl hydroxylation." Science **292**(5516): 468-472.
- Jamieson, E. R. and S. J. Lippard (1999). "Structure, Recognition, and Processing of Cisplatin-DNA Adducts." Chem Rev **99**(9): 2467-2498.
- Jasen, P. (2009). "From the "silent killer" to the "whispering disease": ovarian cancer and the uses of metaphor." Med Hist **53**(4): 489-512.
- Jensen, M. and F. Berthold (2007). "Targeting the neural cell adhesion molecule in cancer." Cancer Lett **258**(1): 9-21.
- Johnson, L. V., M. L. Walsh, et al. (1981). "Monitoring of relative mitochondrial membrane potential in living cells by fluorescence microscopy." J Cell Biol **88**(3): 526-535.
- Johnson, S. W., P. B. Laub, et al. (1997). "Increased platinum-DNA damage tolerance is associated with cisplatin resistance and cross-resistance to various chemotherapeutic agents in unrelated human ovarian cancer cell lines." Cancer Res **57**(5): 850-856.
- Jones, R. G. and C. B. Thompson (2009). "Tumor suppressors and cell metabolism: a recipe for cancer growth." Genes Dev **23**(5): 537-548.
- Jung, S. E., J. M. Lee, et al. (2002). "CT and MR Imaging of Ovarian Tumors with Emphasis on Differential Diagnosis1." Radiographics **22**(6): 1305-1325.
- Kakizoe, T., H. Komatsu, et al. (1982). "Detection of amino acids as possible promoters of bladder cancer in rats by measuring their enhancement of agglutination of bladder cells by concanavalin A." Gann **73**(6): 870-873.
- Kamburov, A., C. Wierling, et al. (2009). "ConsensusPathDB--a database for integrating human functional interaction networks." Nucleic Acids Res **37**(Database issue): D623-628.
- Kartalou, M. and J. M. Essigmann (2001). "Mechanisms of resistance to cisplatin." Mutat Res **478**(1-2): 23-43.
- Kasahara K Fau - Fujiwara, Y., K. Fujiwara Y Fau - Nishio, et al. (1991). "Metallothionein content correlates with the sensitivity of human small cell lung cancer cell lines to cisplatin." Cancer Res **51**(12): 6304-6311.
- Kastan, M. B., O. Onyekwere, et al. (1991). "Participation of p53 protein in the cellular response to DNA damage." Cancer Res **51**(23 Pt 1): 6304-6311.
- Katano, K., A. Kondo, et al. (2002). "Acquisition of resistance to cisplatin is accompanied by changes in the cellular pharmacology of copper." Cancer Res. **62**(22): 6559-6565.

- Kell, D. B., M. Brown, et al. (2005). "Metabolic footprinting and systems biology: the medium is the message." Nat Rev Microbiol **3**(7): 557-565.
- Kelland, L. (2007). "The resurgence of platinum-based cancer chemotherapy." Nat Rev Cancer **7**(8): 573-584.
- Kelland, L. R. (1993). "New platinum antitumor complexes." Crit Rev Oncol Hematol **15**(3): 191-219.
- Kelley, S. L., A. Basu, et al. (1988). "Overexpression of metallothionein confers resistance to anticancer drugs." Science. **241**(4874): 1813-1815.
- Kerr, D. J., C. Lewis, et al. (1990). "The myelotoxicity of carboplatin is influenced by the time of its administration." Hematol Oncol **8**(1): 59-63.
- Kjaer, S. K., L. Mellemkjaer, et al. (2004). "Tubal sterilization and risk of ovarian, endometrial and cervical cancer. A Danish population-based follow-up study of more than 65 000 sterilized women." Int J Epidemiol **33**(3): 596-602.
- Koberle, B., J. R. Masters, et al. (1999). "Defective repair of cisplatin-induced DNA damage caused by reduced XPA protein in testicular germ cell tumours." Curr Biol **9**(5): 273-276.
- Komatsu, M., T. Sumizawa, et al. (2000). "Copper-transporting P-type adenosine triphosphatase (ATP7B) is associated with cisplatin resistance." Cancer Res **60**(5): 1312-1316.
- Kroemer, G. and J. Pouyssegur (2008). "Tumor Cell Metabolism: Cancer's Achilles' Heel." Cancer Cell **13**(6): 472-482.
- Kultz, D. and L. Csonka (1999). "What sets the TonE during osmotic stress?" Proceedings of the National Academy of Sciences of the United States of America **96**(5): 1814-1816.
- Kunkel, T. A. and D. A. Erie (2005). "DNA mismatch repair." Annu Rev Biochem **74**: 681-710.
- Lahmann, P. H., A. E. Cust, et al. (2010). "Anthropometric measures and epithelial ovarian cancer risk in the European Prospective Investigation into Cancer and Nutrition." Int J Cancer **126**(10): 2404-2415.
- Lai, G. M., R. F. Ozols, et al. (1988). "Enhanced DNA repair and resistance to cisplatin in human ovarian cancer." Biochem Pharmacol **37**(24): 4597-4600.
- Langdon, S. P., S. S. Lawrie, et al. (1988). "Characterization and properties of nine human ovarian adenocarcinoma cell lines." Cancer Res **48**(21): 6166-6172.
- Le Belle, J. E., N. G. Harris, et al. (2002). "A comparison of cell and tissue extraction techniques using high-resolution ¹H-NMR spectroscopy." NMR Biomed **15**(1): 37-44.

- Lee, Y. J., I. J. Kang, et al. (2003). "Mechanisms of pyruvate inhibition of oxidant-induced apoptosis in human endothelial cells." Microvasc Res **66**(2): 91-101.
- Liang, B. C. and E. Ulyatt (1998). "Increased sensitivity to cis-diamminedichloroplatinum induced apoptosis with mitochondrial DNA depletion." Cell Death Differ **5**(8): 694-701.
- Lindon, J. C., E. Holmes, et al. (2001). "Pattern recognition methods and applications in biomedical magnetic resonance." Prog Nucl Magn Reson Spectroscop **39**(1).
- Lindon, J. C., J. K. Nicholson, et al. (2000). "Metabonomics: Metabolic processes studied by NMR spectroscopy of biofluids." Concepts in Magnetic Resonance **12**(5): 289-320.
- Liu, F. S. (2009). "Mechanisms of chemotherapeutic drug resistance in cancer therapy--a quick review." Taiwan J Obstet Gynecol **48**(3): 239-244.
- Longley, D. B. and P. G. Johnston (2005). "Molecular mechanisms of drug resistance." J Pathol **205**(2): 275-292.
- Los, M., I. Herr, et al. (1997). "Cross-resistance of CD95- and drug-induced apoptosis as a consequence of deficient activation of caspases (ICE/Ced-3 proteases)." Blood **90**(8): 3118-3129.
- Lu, H., R. A. Forbes, et al. (2002). "Hypoxia-inducible factor 1 activation by aerobic glycolysis implicates the Warburg effect in carcinogenesis." J Biol Chem **277**(26): 23111-23115.
- Malpas, J. S. and A. Rohatiner (1996). Chapter 15 Principles of cancer chemotherapy. Advances in Oncobiology. H. Gloria and E. E. Bittar, Elsevier. **Volume 1**: 317-350.
- Maltese, F. and R. Verpoorte (2009). Metabolomics: Novel Tool for Studying Complex Biological Systems. Molecular Techniques in Crop Improvement. S. M. Jain and D. S. Brar, Springer Netherlands: 493-510.
- Martin-Blanco (2000). "p38 MAPK signalling cascades: ancient roles and new functions." Bioassays **22**(7).
- Martin, A. (1996). "The use of antioxidants in healing." Dermatol Surg **22**(2): 156-160.
- Martinive, P., F. Defresne, et al. (2006). "Preconditioning of the tumor vasculature and tumor cells by intermittent hypoxia: implications for anticancer therapies." Cancer Res **66**(24): 11736-11744.
- Mathupala, S. P., A. Rempel, et al. (2001). "Glucose catabolism in cancer cells: identification and characterization of a marked activation response of the type II hexokinase gene to hypoxic conditions." J Biol Chem **276**(46): 43407-43412.
- Mayer, F., A. J. Gillis, et al. (2002). "Microsatellite instability of germ cell tumors is associated with resistance to systemic treatment." Cancer Res **62**(10): 2758-2760.

- Mazurek, A., J. Niklinski, et al. (1998). "Clinical tumour markers in ovarian cancer." Eur J Cancer Prev **7**(1): 23-35.
- McFate, T., A. Mohyeldin, et al. (2008). "Pyruvate dehydrogenase complex activity controls metabolic and malignant phenotype in cancer cells." J Biol Chem **283**(33): 22700-22708.
- McGuire, W. P., 3rd and M. Markman (2003). "Primary ovarian cancer chemotherapy: current standards of care." Br J Cancer **89 Suppl 3**: S3-8.
- McHugh, P. J., W. R. Sones, et al. (2000). "Repair of intermediate structures produced at DNA interstrand cross-links in *Saccharomyces cerevisiae*." Mol Cell Biol **20**(11): 3425-3433.
- Mello, J. A., S. Acharya, et al. (1996). "The mismatch-repair protein hMSH2 binds selectively to DNA adducts of the anticancer drug cisplatin." Chem Biol **3**(7): 579-589.
- Melnikov, A., D. Scholtens, et al. (2009). "Differential methylation profile of ovarian cancer in tissues and plasma." J Mol Diagn **11**(1): 60-65.
- Merritt, M. A., A. C. Green, et al. (2008). "Talcum powder, chronic pelvic inflammation and NSAIDs in relation to risk of epithelial ovarian cancer." Int J Cancer **122**(1): 170-176.
- Modica-Napolitano, J. S. and K. K. Singh (2004). "Mitochondrial dysfunction in cancer." Mitochondrion **4**(5-6): 755-762.
- Mori, N., R. Delsite, et al. (2004). "Loss of p53 function in colon cancer cells results in increased phosphocholine and total choline." Mol Imaging **3**(4): 319-323.
- Morrison, B. J., L. Andera, et al. (2009). "Future use of mitocans against tumour-initiating cells?" Mol Nutr Food Res **53**(1): 147-153.
- Muraoka, S. and T. Miura (2004). "Inhibition of xanthine oxidase by phytic acid and its antioxidative action." Life Sci **74**(13): 1691-1700.
- Murphy, B. J., K. R. Laderoute, et al. (1994). "Metallothionein IIA is up-regulated by hypoxia in human A431 squamous carcinoma cells." Cancer Res **54**(22): 5808-5810.
- Ness, R. B., D. W. Cramer, et al. (2002). "Infertility, fertility drugs, and ovarian cancer: a pooled analysis of case-control studies." Am J Epidemiol **155**(3): 217-224.
- Nicholson, J. K., P. J. Foxall, et al. (1995). "750 MHz ¹H and ¹H-¹³C NMR spectroscopy of human blood plasma." Anal Chem **67**(5): 793-811.
- Nicholson, J. K., J. C. Lindon, et al. (1999). "'Metabonomics': understanding the metabolic responses of living systems to pathophysiological stimuli via multivariate statistical analysis of biological NMR spectroscopic data." Xenobiotica **29**(11): 1181-1189.

- Nishimura, I., Y. Muragaki, et al. (1989). "Tissue-specific forms of type IX collagen-proteoglycan arise from the use of two widely separated promoters." J Biol Chem **264**(33): 20033-20041.
- Nishio, Y., T. Kakizoe, et al. (1986). "L-isoleucine and L-leucine: tumor promoters of bladder cancer in rats." Science **231**(4740): 843-845.
- Norman, R. J., D. Dewailly, et al. (2007). "Polycystic ovary syndrome." Lancet **370**(9588): 685-697.
- Nucci, M. R. (2009). Gynecologic Pathology: A Volume in Foundations in Diagnostic Pathology Series Churchill Livingstone
- O'Brien, V. and R. Brown (2006). "Signalling cell cycle arrest and cell death through the MMR System." Carcinogenesis **27**(4): 682-692.
- O'Donnell-Tormey, J., C. F. Nathan, et al. (1987). "Secretion of pyruvate. An antioxidant defense of mammalian cells." J Exp Med **165**(2): 500-514.
- Odessey, R., E. A. Khairallah, et al. (1974). "Origin and possible significance of alanine production by skeletal muscle." J Biol Chem **249**(23): 7623-7629.
- Ono Y Fau - Nonomura, N., Y. Nonomura N Fau - Harada, et al. (2001). "Loss of p73 induction in a cisplatin-resistant bladder cancer cell line." Mol Urol **5**(1).
- Orntoft, T. F. and E. M. Vestergaard (1999). "Clinical aspects of altered glycosylation of glycoproteins in cancer." Electrophoresis **20**(2): 362-371.
- Pan B Fau - Yao, K. S., B. P. Yao Ks Fau - Monia, et al. (2002). "Reversal of cisplatin resistance in human ovarian cancer cell lines by a c-jun." Biochem Pharmacol **63**(9).
- Panza, N., G. Pacilio, et al. (1988). "Cancer antigen 125, tissue polypeptide antigen, carcinoembryonic antigen, and beta-chain human chorionic gonadotropin as serum markers of epithelial ovarian carcinoma." Cancer **61**(1): 76-83.
- Papouli, E., P. Cejka, et al. (2004). "Dependence of the cytotoxicity of DNA-damaging agents on the mismatch repair status of human cells." Cancer Res **64**(10): 3391-3394.
- Park, S. Y., I. Chang, et al. (2004). "Resistance of mitochondrial DNA-depleted cells against cell death: role of mitochondrial superoxide dismutase." J Biol Chem **279**(9): 7512-7520.
- Parker, R. J., A. Eastman, et al. (1991). "Acquired cisplatin resistance in human ovarian cancer cells is associated with enhanced repair of cisplatin-DNA lesions and reduced drug accumulation." J Clin Invest **87**(3): 772-777.
- Pearce, J. T., T. J. Athersuch, et al. (2008). "Robust algorithms for automated chemical shift calibration of 1D ¹H NMR spectra of blood serum." Anal Chem **80**(18): 7158-7162.

- Pedersen, P. L. and H. P. Morris (1974). "Uncoupler-stimulated adenosine triphosphatase activity. Deficiency in intact mitochondria from Morris hepatomas and ascites tumor cells." J Biol Chem **249**(11): 3327-3334.
- Pelech, S. L., P. H. Pritchard, et al. (1983). "Fatty acids promote translocation of CTP:phosphocholine cytidyltransferase to the endoplasmic reticulum and stimulate rat hepatic phosphatidylcholine synthesis." J Biol Chem **258**(11): 6782-6788.
- Pelicano, H., D. Carney, et al. (2004). "ROS stress in cancer cells and therapeutic implications." Drug Resist Updat **7**(2): 97-110.
- Pelicano, H., W. Lu, et al. (2009). "Mitochondrial dysfunction and reactive oxygen species imbalance promote breast cancer cell motility through a CXCL14-mediated mechanism." Cancer Res **69**(6): 2375-2383.
- Pelicano, H., D. S. Martin, et al. (2006). "Glycolysis inhibition for anticancer treatment." Oncogene **25**(34): 4633-4646.
- Peltomaki, P. and H. Vasen (2004). "Mutations associated with HNPCC predisposition -- Update of ICG-HNPCC/INSiGHT mutation database." Dis Markers **20**(4-5): 269-276.
- Pennacchietti, S., P. Michieli, et al. (2003). "Hypoxia promotes invasive growth by transcriptional activation of the met protooncogene." Cancer Cell **3**(4): 347-361.
- Pietenpol, J. A., T. Tokino, et al. (1994). "Sequence-specific transcriptional activation is essential for growth suppression by p53." Proc Natl Acad Sci U S A **91**(6): 1998-2002.
- Pinto, A. L. and S. J. Lippard (1985). "Binding of the antitumor drug cis-diamminedichloroplatinum(II) (cisplatin) to DNA." Biochim Biophys Acta **780**(3): 167-180.
- Plumb, J. A., G. Strathdee, et al. (2000). "Reversal of drug resistance in human tumor xenografts by 2'-deoxy-5-azacytidine-induced demethylation of the hMLH1 gene promoter." Cancer Res **60**(21): 6039-6044.
- Podo, F. (1999). "Tumour phospholipid metabolism." NMR Biomed **12**(7): 413-439.
- Ponti, G., L. Losi, et al. (2006). "Value of MLH1 and MSH2 mutations in the appearance of Muir-Torre syndrome phenotype in HNPCC patients presenting sebaceous gland tumors or keratoacanthomas." J Invest Dermatol **126**(10): 2302-2307.
- Prolla, T. A., S. M. Baker, et al. (1998). "Tumour susceptibility and spontaneous mutation in mice deficient in Mlh1, Pms1 and Pms2 DNA mismatch repair." Nat Genet **18**(3): 276-279.
- Qin, L. F. and I. O. Ng (2002). "Induction of apoptosis by cisplatin and its effect on cell cycle-related proteins and cell cycle changes in hepatoma cells." Cancer Lett **175**(1): 27-38.

- Rachet, B., C. Maringe, et al. (2009). "Population-based cancer survival trends in England and Wales up to 2007: an assessment of the NHS cancer plan for England." Lancet Oncol **10**(4): 351-369.
- Ralph, S. J. and J. Neuzil (2009). "Mitochondria as targets for cancer therapy." Mol Nutr Food Res **53**(1): 9-28.
- Ramsoekh, D., A. Wagner, et al. (2009). "Cancer risk in MLH1, MSH2 and MSH6 mutation carriers; different risk profiles may influence clinical management." Hered Cancer Clin Pract **7**(1): 17.
- Robinson, M. J. and M. H. Cobb (1997). "Mitogen-activated protein kinase pathways." Curr Opin Cell Biol **9**(2): 180-186.
- Robinson, S. (1993). "Principles of Chemotherapy." Eur J Cancer Care **2**(2): 55-65.
- Rosenberg, B., L. Vancamp, et al. (1965). "Inhibition of Cell Division in Escherichia coli by Electrolysis Products from a Platinum Electrode." Nature **205**: 698-699.
- Rosenberg, B., L. VanCamp, et al. (1969). "Platinum compounds: a new class of potent antitumour agents." Nature **222**(5191): 385-386.
- Rosenberg, L., J. R. Palmer, et al. (1994). "A case-control study of oral contraceptive use and invasive epithelial ovarian cancer." Am J Epidemiol **139**(7): 654-661.
- Rossing, M. A., K. G. Wicklund, et al. (2010). "Predictive value of symptoms for early detection of ovarian cancer." J Natl Cancer Inst **102**(4): 222-229.
- Roudier, E., C. Bachelet, et al. (2007). "Pyruvate reduces DNA damage during hypoxia and after reoxygenation in hepatocellular carcinoma cells." FEBS J **274**(19): 5188-5198.
- Roudier, E. and A. Perrin (2009). "Considering the role of pyruvate in tumor cells during hypoxia." Biochim Biophys Acta **1796**(2): 55-62.
- Rustin, G. J., R. C. Bast, Jr., et al. (2004). "Use of CA-125 in clinical trial evaluation of new therapeutic drugs for ovarian cancer." Clin Cancer Res **10**(11): 3919-3926.
- Sakamoto, M., A. Kondo, et al. (2001). "Analysis of gene expression profiles associated with cisplatin resistance in human ovarian cancer cell lines and tissues using cDNA microarray." Hum Cell **14**(4): 305-315.
- Salway, J. E. (2004). Metabolism at a Glance, Third Edition. Oxford: Blackwell Publishing.
- Samimi, G., R. Safaei, et al. (2004). "Increased expression of the copper efflux transporter ATP7A mediates resistance to cisplatin, carboplatin, and oxaliplatin in ovarian cancer cells." Clin Cancer Res **10**(14): 4661-4669.
- Sanna, K. and E. K. Rofstad (1994). "Hypoxia-induced resistance to doxorubicin and methotrexate in human melanoma cell lines in vitro." Int J Cancer **58**(2): 258-262.

- Schafmayer, C., S. Buch, et al. (2007). "Genetic investigation of DNA-repair pathway genes PMS2, MLH1, MSH2, MSH6, MUTYH, OGG1 and MTH1 in sporadic colon cancer." Int J Cancer **121**(3): 555-558.
- Schildkraut, J. M., P. G. Moorman, et al. (2006). "Analgesic drug use and risk of ovarian cancer." Epidemiology **17**(1): 104-107.
- Schildkraut, J. M., P. J. Schwingl, et al. (1996). "Epithelial ovarian cancer risk among women with polycystic ovary syndrome." Obstet Gynecol **88**(4 Pt 1): 554-559.
- Schmidt, W. and S. G. Chaney (1993). "Role of carrier ligand in platinum resistance of human carcinoma cell lines." Cancer Res **53**(4): 799-805.
- Schofield, M. J. and P. Hsieh (2003). "DNA mismatch repair: molecular mechanisms and biological function." Annu Rev Microbiol **57**: 579-608.
- Schuler, M. and D. R. Green (2001). "Mechanisms of p53-dependent apoptosis." Biochem Soc Trans **29**(Pt 6): 684-688.
- Secretan, B., K. Straif, et al. (2009). "A review of human carcinogens--Part E: tobacco, areca nut, alcohol, coal smoke, and salted fish." Lancet Oncol **10**(11): 1033-1034.
- Seta, K. A., Z. Spicer, et al. (2002). "Responding to hypoxia: lessons from a model cell line." Sci STKE **2002**(146): re11.
- Shiozaki, E. N., J. Chai, et al. (2002). "Oligomerization and activation of caspase-9, induced by Apaf-1 CARD." Proc Natl Acad Sci U S A **99**(7): 4197-4202.
- Siddik, Z. H. (2003). "Cisplatin: mode of cytotoxic action and molecular basis of resistance." Oncogene **22**(47): 7265-7279.
- Siddik Zh Fau - Mims, B., G. Mims B Fau - Lozano, et al. (1998). "Independent pathways of p53 induction by cisplatin and X-rays in a." Cancer Res **58**(14).
- Silverstein (2005). Spectrometric Identification of Organic Compounds, 7th Edition. New York, Wiley
- Singer, B. (1977). "Sites in nucleic acids reacting with alkylating agents of differing carcinogenicity of mutagenicity." J Toxicol Environ Health **2**(6): 1279-1295.
- Skovsgaard, T. (1977). "Transport and binding of daunorubicin, adriamycin, and rubidazone in Ehrlich ascites tumour cells." Biochem Pharmacol **26**(3): 215-222.
- Smith, C. D., S. Carmeli, et al. (1993). "Scytophycins, novel microfilament-depolymerizing agents which circumvent P-glycoprotein-mediated multidrug resistance." Cancer Res **53**(6): 1343-1347.
- Smith, W. G. (1979). "Surgical Treatment of Epithelial Ovarian Carcinoma." Clin Obstet Gynecol **22**(4): 939-956.

- Solomon, C. G. (1999). "The epidemiology of polycystic ovary syndrome. Prevalence and associated disease risks." Endocrinol Metab Clin North Am **28**(2): 247-263.
- Song, X., X. Liu, et al. (2006). "Hypoxia-induced resistance to cisplatin and doxorubicin in non-small cell lung cancer is inhibited by silencing of HIF-1alpha gene." Cancer Chemother Pharmacol **58**(6): 776-784.
- Sonveaux, P., F. Vegran, et al. (2008). "Targeting lactate-fueled respiration selectively kills hypoxic tumor cells in mice." J Clin Invest **118**(12): 3930-3942.
- Soussi, T. (2000). "p53 Antibodies in the sera of patients with various types of cancer: a review." Cancer Res **60**(7): 1777-1788.
- Spencer, J. A., K. Anderson, et al. (2006). "Image guided biopsy in the management of cancer of the ovary." Cancer Imaging **6**: 144-147.
- Spencer, S. J., D. A. Bonnin, et al. (2009). "Bioinformatics methods for learning radiation-induced lung inflammation from heterogeneous retrospective and prospective data." J Biomed Biotechnol **2009**: 892863.
- Stewart, D. J. (2007). "Mechanisms of resistance to cisplatin and carboplatin." Crit Rev Oncol Hematol **63**(1): 12-31.
- Strathdee, G., K. Appleton, et al. (2001). "Primary ovarian carcinomas display multiple methylator phenotypes involving known tumor suppressor genes." Am J Pathol **158**(3): 1121-1127.
- Stuart, G. C. (2003). "First-line treatment regimens and the role of consolidation therapy in advanced ovarian cancer." Gynecol Oncol **90**(3 Pt 2): S8-15.
- Subarsky, P. and R. P. Hill (2003). "The hypoxic tumour microenvironment and metastatic progression." Clin Exp Metastasis **20**(3): 237-250.
- Sun, A. S., K. Sepkowitz, et al. (1981). "A study of some mitochondrial and peroxisomal enzymes in human colonic adenocarcinoma." Lab Invest **44**(1): 13-17.
- Sutherland, R. M. (1988). "Cell and environment interactions in tumor microregions: the multicell spheroid model." Science **240**(4849): 177-184.
- Sylvain, V., S. Lafarge, et al. (2002). "Dominant-negative activity of a Brca1 truncation mutant: effects on proliferation, tumorigenicity in vivo, and chemosensitivity in a mouse ovarian cancer cell line." Int J Oncol **20**(4): 845-853.
- Tacka, K. A., J. C. Dabrowiak, et al. (2004). "Effects of cisplatin on mitochondrial function in Jurkat cells." Chem Res Toxicol **17**(8): 1102-1111.

- Tanaka, Y., M. S. Zaman, et al. (2009). "Polymorphisms of MLH1 in benign prostatic hyperplasia and sporadic prostate cancer." Biochem Biophys Res Commun **383**(4): 440-444.
- Tannock, I. (1982). "Response of aerobic and hypoxic cells in a solid tumor to adriamycin and cyclophosphamide and interaction of the drugs with radiation." Cancer Res **42**(12): 4921-4926.
- Tannock, I. F. (1998). "Conventional cancer therapy: promise broken or promise delayed?" Lancet **351 Suppl 2**: SII9-16.
- Teicher, B. A., S. A. Holden, et al. (1990). "Classification of antineoplastic treatments by their differential toxicity toward putative oxygenated and hypoxic tumor subpopulations in vivo in the FSaIIC murine fibrosarcoma." Cancer Res **50**(11): 3339-3344.
- Teicher, B. A., S. A. Holden, et al. (1987). "Approaches to defining the mechanism of enhancement by Fluosol-DA 20% with carbogen of melphalan antitumor activity." Cancer Res **47**(2): 513-518.
- Teicher, B. A., J. S. Lazo, et al. (1981). "Classification of antineoplastic agents by their selective toxicities toward oxygenated and hypoxic tumor cells." Cancer Res **41**(1): 73-81.
- Tessem, M. B., M. G. Swanson, et al. (2008). "Evaluation of lactate and alanine as metabolic biomarkers of prostate cancer using ¹H HR-MAS spectroscopy of biopsy tissues." Magn Reson Med **60**(3): 510-516.
- Tessem, M.-B., K. M. Selnæs, et al. (2010). "Discrimination of Patients with Microsatellite Instability Colon Cancer using ¹H HR MAS MR Spectroscopy and Chemometric Analysis." J Proteome Res **9**(7): 3664-3670.
- Therasse, P., S. G. Arbuck, et al. (2000). "New guidelines to evaluate the response to treatment in solid tumors. European Organization for Research and Treatment of Cancer, National Cancer Institute of the United States, National Cancer Institute of Canada." J Natl Cancer Inst **92**(3): 205-216.
- Thomlinson, R. H. and L. H. Gray (1955). "The histological structure of some human lung cancers and the possible implications for radiotherapy." Br J Cancer **9**(4): 539-549.
- Toogood, P. L. (2008). "Mitochondrial drugs." Curr Opin Chem Biol **12**(4): 457-463.
- Tsigkou, A., D. Marrelli, et al. (2007). "Total inhibin is a potential serum marker for epithelial ovarian cancer." J Clin Endocrinol Metab **92**(7): 2526-2531.
- Ulrich, E. L., H. Akutsu, et al. (2008). "BioMagResBank." Nucleic Acids Research **36**(suppl 1): D402-D408.

- Vaisman, A., M. Varchenko, et al. (1998). "The role of hMLH1, hMSH3, and hMSH6 defects in cisplatin and oxaliplatin resistance: correlation with replicative bypass of platinum-DNA adducts." Cancer Res **58**(16): 3579-3585.
- van den Berg, R. A., H. C. Hoefsloot, et al. (2006). "Centering, scaling, and transformations: improving the biological information content of metabolomics data." BMC Genomics **7**: 142.
- Vander Heiden, M. G., L. C. Cantley, et al. (2009). "Understanding the Warburg effect: the metabolic requirements of cell proliferation." Science **324**(5930): 1029-1033.
- Vaupel, P., F. Kallinowski, et al. (1989). "Blood flow, oxygen and nutrient supply, and metabolic microenvironment of human tumors: a review." Cancer Res **49**(23): 6449-6465.
- Vaupel, P., O. Thews, et al. (2001). "Treatment resistance of solid tumors: role of hypoxia and anemia." Med Oncol **18**(4): 243-259.
- Wada, H., Y. Saikawa, et al. (1999). "Selectively induced high MRP gene expression in multidrug-resistant human HL60 leukemia cells." Exp Hematol **27**(1): 99-109.
- Wang, F., M. A. Ogasawara, et al. (2010). "Small mitochondria-targeting molecules as anti-cancer agents." Mol Aspects Med **31**(1): 75-92.
- Wartenberg, M., F. C. Ling, et al. (2003). "Regulation of the multidrug resistance transporter P-glycoprotein in multicellular tumor spheroids by hypoxia-inducible factor (HIF-1) and reactive oxygen species." FASEB J **17**(3): 503-505.
- Watanabe, Y., M. Koi, et al. (2001). "A change in microsatellite instability caused by cisplatin-based chemotherapy of ovarian cancer." Br J Cancer **85**(7): 1064-1069.
- Weber, W. A. (2007). "18F-FDG PET in Non-Hodgkin's Lymphoma: Qualitative or Quantitative?" J Nucl Med **48**(10): 1580-1582.
- Wernli, K. J., P. A. Newcomb, et al. (2008). "Inverse association of NSAID use and ovarian cancer in relation to oral contraceptive use and parity." Br J Cancer **98**(11): 1781-1783.
- Westin, M. A., M. C. Hunt, et al. (2005). "The identification of a succinyl-CoA thioesterase suggests a novel pathway for succinate production in peroxisomes." J Biol Chem **280**(46): 38125-38132.
- Whittemore, A. S., R. Harris, et al. (1992). "Characteristics relating to ovarian cancer risk: collaborative analysis of 12 US case-control studies. IV. The pathogenesis of epithelial ovarian cancer. Collaborative Ovarian Cancer Group." Am J Epidemiol **136**(10): 1212-1220.

- Wishart, D. S., C. Knox, et al. (2009). "HMDB: a knowledgebase for the human metabolome." Nucleic Acids Res **37**(suppl 1): D603-D610.
- Wu, W., H.-d. Wang, et al. (2010). "Up-regulation of fas reverses cisplatin resistance of human small cell lung cancer cells." J Exp Clin Cancer Res **29**(1): 49.
- Xu, R. H., H. Pelicano, et al. (2005). "Inhibition of glycolysis in cancer cells: a novel strategy to overcome drug resistance associated with mitochondrial respiratory defect and hypoxia." Cancer Res **65**(2): 613-621.
- Yoshida M Fau - Khokhar, A. R., Z. H. Khokhar Ar Fau - Siddik, et al. (1998). "Cytotoxicity and tolerance to DNA adducts of alicyclic mixed amine platinum(II) homologs in tumor models sensitive and resistant to cisplatin or tetraplatin." Oncol Rep **5**(5).
- Yoshii, Y., T. Furukawa, et al. (2007). "Acceleration of tumor acetate uptake under hypoxia: A new perception of tumor imaging by acetate." J Nucl Med **48** (Supplement 2):75P.
- Yuan, J. and P. M. Glazer (1998). "Mutagenesis induced by the tumor microenvironment." Mutat Res **400**(1-2): 439-446.
- Zdraveski, Z. Z., J. A. Mello, et al. (2000). "Multiple pathways of recombination define cellular responses to cisplatin." Chem Biol **7**(1): 39-50.
- Zhou, B., Q. Sun, et al. (2008). "Hormone replacement therapy and ovarian cancer risk: a meta-analysis." Gynecol Oncol **108**(3): 641-651.
- Zu, X. L. and M. Guppy (2004). "Cancer metabolism: facts, fantasy, and fiction." Biochem Biophys Res Commun **313**(3): 459-465.
- Zwelling, L. A., T. Anderson, et al. (1979). "DNA-protein and DNA interstrand cross-linking by cis- and trans-platinum(II) diamminedichloride in L1210 mouse leukemia cells and relation to cytotoxicity." Cancer Res **39**(2 Pt 1): 365-369.

Appendix

39784_HUMAN	CAMK2D_HUMAN	FBXO3_HUMAN	KIAA0774_HUMAN	LSM4_HUMAN	PCDHGC3_HUMAN	SIGIRR_HUMAN	TRIM35_HUMAN
AADACL2_HUMAN	CAMSAP1_HUMAN	FBXO45_HUMAN	KIAA0999_HUMAN	LUC7L2_HUMAN	PCDHGC3_HUMAN	SLC35F5_HUMAN	TRIOBP_HUMAN
ABCA6_HUMAN	CCDC17_HUMAN	FGF18_HUMAN	KIAA0999_HUMAN	LYRM5_HUMAN	PCDHGC3_HUMAN	SLC44A5_HUMAN	TLL11_HUMAN
ABI2_HUMAN	CCNI_HUMAN	FLII_HUMAN	KIAA1147_HUMAN	MAN2A1_HUMAN	PCDHGC3_HUMAN	SLC8A1_HUMAN	TUBGCP2_HUMAN
ACOT4_HUMAN	CDC42BPA_HUMAN	FLJ13137_HUMAN	KILLIN_HUMAN	MAP3K7IP1_HUMAN	PDLIM7_HUMAN	SLC9A9_HUMAN	TUG1_HUMAN
AGBL5_HUMAN	CGB_HUMAN	FLT1_HUMAN	KIR2DS2_HUMAN	MAPK1_HUMAN	PDLIM7_HUMAN	SLMO2_HUMAN	TWISTNB_HUMAN
AGPS_HUMAN	CHRNA10_HUMAN	FSTL5_HUMAN	KIR2DS5_HUMAN	MATR3_HUMAN	PGK1_HUMAN	SMCR7L_HUMAN	UBASH3B_HUMAN
AGTPBP1_HUMAN	CHST14_HUMAN	FTH1_HUMAN	KLF12_HUMAN	MET_HUMAN	PKD1_HUMAN	SOBP_HUMAN	UQCC_HUMAN
AKR1C1_HUMAN	CIZ1_HUMAN	FUT9_HUMAN	KLF3_HUMAN	MGST1_HUMAN	PNKD_HUMAN	SPTLC3_HUMAN	USP24_HUMAN
AKR1C1_HUMAN	CIZ1_HUMAN	GAB3_HUMAN	L3MBTL3_HUMAN	MLH1_HUMAN	PNPO_HUMAN	SREBF2_HUMAN	USP31_HUMAN
AKR1C2_HUMAN	CLCN4_HUMAN	GAS5_HUMAN	LAGE3_HUMAN	MLL3_HUMAN	PQLC1_HUMAN	STAG3L4_HUMAN	USP40_HUMAN
AKR1C2_HUMAN	CMIP_HUMAN	GEMIN8_HUMAN	LBA1_HUMAN	MS4A6A_HUMAN	PRKACB_HUMAN	STARD13_HUMAN	USP48_HUMAN
ALDH3B2_HUMAN	COG1_HUMAN	GLCCI1_HUMAN	LEO1_HUMAN	MSL-1_HUMAN	PRODH_HUMAN	STK35_HUMAN	UTP15_HUMAN
ANGPT2_HUMAN	CRTAP_HUMAN	GLS_HUMAN	LIPH_HUMAN	MTERFD2_HUMAN	PTCD3_HUMAN	STK35_HUMAN	VPS26A_HUMAN
APLP2_HUMAN	CSNK1A1_HUMAN	GMEB2_HUMAN	LMCD1_HUMAN	NCAM1_HUMAN	PVRL3_HUMAN	SUOX_HUMAN	WWTR1_HUMAN
APBP2_HUMAN	CTBP1_HUMAN	GRM7_HUMAN	LMNA_HUMAN	NDFIP2_HUMAN	RAB12_HUMAN	TAF1B_HUMAN	XIST_HUMAN
AZIN1_HUMAN	CTDSPL_HUMAN	HBE1_HUMAN	LMO7_HUMAN	NEGR1_HUMAN	RABGGTB_HUMAN	TBCD_HUMAN	ZNF138_HUMAN
BAZ1A_HUMAN	CTSB_HUMAN	hCG_1806964_HUMAN	LOC100126784_HUMAN	NFKBIB_HUMAN	RDH13_HUMAN	TEX9_HUMAN	ZNF236_HUMAN
C10orf46_HUMAN	DAGLB_HUMAN	HEG1_HUMAN	LOC100130360_HUMAN	NFKBID_HUMAN	RHBDD3_HUMAN	TM2D1_HUMAN	ZNF404_HUMAN
C10orf57_HUMAN	DDHD1_HUMAN	HNRNPC_HUMAN	LOC100147773_HUMAN	NLRC5_HUMAN	RHEB_HUMAN	TM2D1_HUMAN	ZNF540_HUMAN
C11orf24_HUMAN	DENND2A_HUMAN	HSPA4_HUMAN	LOC116412_HUMAN	NRP2_HUMAN	RHO_HUMAN	TMEM127_HUMAN	ZNF546_HUMAN
C11orf57_HUMAN	DHX34_HUMAN	ICAM2_HUMAN	LOC129293_HUMAN	NRP2_HUMAN	RNF213_HUMAN	TMEM173_HUMAN	ZNF618_HUMAN
C18orf54_HUMAN	DHX57_HUMAN	INSM2_HUMAN	LOC201164_HUMAN	NSMCE4A_HUMAN	RPS15A_HUMAN	TMEM178_HUMAN	ZNF766_HUMAN
C19orf54_HUMAN	DLEU2_HUMAN	INTS10_HUMAN	LOC283666_HUMAN	NUP153_HUMAN	RRAD_HUMAN	TMEM196_HUMAN	ZNF787_HUMAN
C1orf142_HUMAN	DLG2_HUMAN	INTS10_HUMAN	LOC285908_HUMAN	NY-SAR-48_HUMAN	RSPRY1_HUMAN	TMEM200A_HUMAN	
C2orf64_HUMAN	EDIL3_HUMAN	IRF2BP2_HUMAN	LOC388610_HUMAN	OSBPL11_HUMAN	S100A16_HUMAN	TMEM43_HUMAN	
C4orf30_HUMAN	EPB41L2_HUMAN	IVD_HUMAN	LOC399884_HUMAN	PARP14_HUMAN	SAMD10_HUMAN	TMEM56_HUMAN	
C6orf176_HUMAN	FAM109A_HUMAN	JMJD1C_HUMAN	LOC440894_HUMAN	PCDH20_HUMAN	SEC22C_HUMAN	TPPP_HUMAN	
C6orf201_HUMAN	FAM80A_HUMAN	JMJD1C_HUMAN	LOC440934_HUMAN	PCDHA6_HUMAN	SERAC1_HUMAN	TRIM22_HUMAN	
C6orf89_HUMAN	FAM83A_HUMAN	KCTD1_HUMAN	LOC643733_HUMAN	PCDHA6_HUMAN	SFRS1_HUMAN	TRIM3_HUMAN	
C7orf20_HUMAN	FAS_HUMAN	KIAA0391_HUMAN	LOC644656_HUMAN	PCDHA6_HUMAN	SFT2D1_HUMAN	TRIM3_HUMAN	

Table A1 - List of all the *Mlh1*-associated NCI-60 protein names (Unirpot IDs) from chapter 4