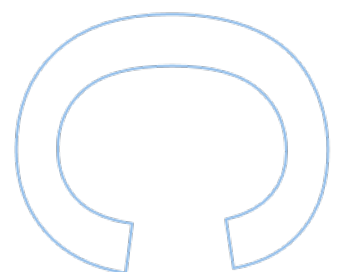
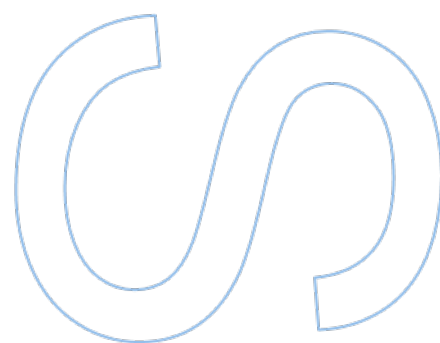
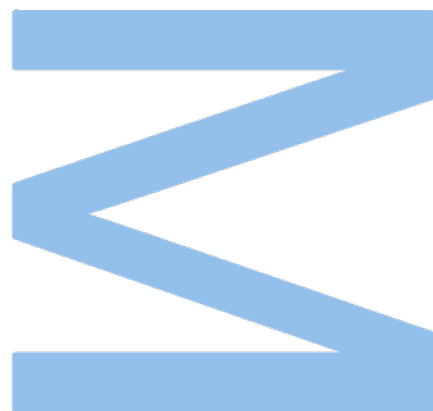


Exploring the mechanisms of resistance in ovarian cancer: A metabolomic approach

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Resumo

O carcinoma seroso de alto grau (HGSC) representa o subtipo predominante no cancro do ovário, sendo responsável por cerca de 75% de todos os casos de cancro epitelial do ovário. A elevada taxa de mortalidade observada em doentes com HGSC pode ser atribuída aos desafios da resistência ao tratamento. Os protocolos terapêuticos atuais envolvem cirurgia citorrredutora seguida de um regime de tratamento farmacológico que combina Paclitaxel (PTX) e Carboplatina (CARB). Esta quimioterapia tem por objetivo alterar diversos mecanismos celulares que orquestram o ciclo celular, induzindo, em última análise, a morte das células tumorais. Apesar destes esforços, um número significativo de doentes com HGSC sofre uma recaída da doença, o que resulta numa taxa de sobrevivência global de aproximadamente 12 meses.

Para obter um maior conhecimento sobre os processos de quimiorresistência em HGSC, esta dissertação de Mestrado teve como objetivo investigar as alterações metabólicas existentes na resistência ao Paclitaxel (PTX) num modelo *in vitro*. Foi utilizada uma abordagem metabolómica para realizar uma análise abrangente de uma miríade de pequenas moléculas (metabolitos) presentes nas células, tais como, aminoácidos, ácidos orgânicos, açúcares, lípidos, entre outros.

A metodologia de investigação envolveu a utilização de um modelo de linha celular com resistência intrínseca à Carboplatina (CARB), que foi posteriormente desenvolvida numa variante resistente ao CARB-PTX, mimetizando o cenário clínico. A técnica analítica escolhida foi a cromatografia gasosa acoplada à espectrometria de massa (GC-MS), que permitiu uma deteção pormenorizada dos perfis metabólicos intracelulares (endometaboloma) e extracelulares (exometaboloma) de ambas as linhas. Após a aquisição de dados, foi implementada uma série de etapas de processamento e posterior análise realizada através de ferramentas bioinformáticas, integrando métodos multivariados como a análise de componentes principais (PCA), juntamente com uma abordagem univariada como o Wilcoxon Test e o Kruskal Wallis Test. Foi ainda efetuada uma análise das vias metabólicas com o conjunto de metabolitos significativamente alterados, sugerindo as vias metabólicas associadas à resistência ao PTX nas células HGSC. Esta abordagem visou melhorar a compreensão da reprogramação metabólica associada à resistência ao PTX, abrindo caminho para estratégias terapêuticas mais eficazes.

A técnica de GC-MS permitiu a identificação de 47 metabolitos no endometaboloma e 36 no exometaboloma. No endometaboloma, foi possível observar que oito destes compostos (glicina, glicerol, taurina, glucose, mio-inositol, prolina, lactato e 5-oxoprolina) se encontravam alterados, sendo que dois deles (glucose e glicerol) se encontravam diminuídos nas células resistentes em relação às células parentais, e os restantes estavam aumentados. Isso pode ser explicado pelo facto de que a glucose serve como principal fonte de energia para sustentar a proliferação celular e o glicerol, por sua vez, atua como precursor na síntese de lipídios, contribuindo para a formação de membranas e servindo como uma fonte adicional de energia. O perfil de metabolitos no meio extracelular não apresentou variação estatisticamente significativa, indicando que as adaptações metabólicas relacionadas com a resistência ocorrem predominantemente no interior das células.

A análise estatística identificou um aumento significativo nos níveis intracelulares de taurina e mio-inositol nas células resistentes, sugerindo que a osmorregulação pode atuar como um mecanismo adaptativo crucial que contribui para a resistência ao PTX. A análise das vias metabólicas forneceu evidências adicionais de alterações no metabolismo da glutatona nas células resistentes, assim como alterações no metabolismo de aminoácidos, especialmente nos metabolismos da arginina e prolina.

Este estudo é o primeiro a utilizar uma abordagem baseada em metabolómica para investigar a reprogramação metabólica associada à resistência ao PTX em células de cancro de ovário, preenchendo uma lacuna significativa no conhecimento atual sobre os mecanismos específicos de resistência ao PTX.

Palavras-chaves: Cancro do ovário, quimiorresistência, metabolómica, cromatografia gasosa acoplada à espectrometria de massa, análise multivariada.

Abstract

High-grade serous carcinoma (HGSC) represents the predominant subtype among ovarian cancers, accounting for approximately 75% of all epithelial ovarian cancers. The elevated mortality rate observed in patients with HGSC can be attributed to the challenges of treatment resistance. Current therapeutic protocols involve cytoreductive surgery followed by a standard treatment regimen combining Paclitaxel (PTX) and Carboplatin (CARB). This platinum-taxane-based chemotherapy aims to disrupt various cellular mechanisms that orchestrate the cell cycle, ultimately leading to the demise of tumor cells. Despite these efforts, a significant number of HGSC patients experience disease relapse, resulting in an overall survival rate of approximately 12 months.

To unravel the chemoresistance in HGSC, this Master of Science (M.Sc.) dissertation is dedicated to an exploration of the metabolic mechanisms implicated in resistance to Paclitaxel (PTX) in an *in vitro* model. A metabolomics approach was used to perform a comprehensive analysis of a myriad of small molecules (metabolites) present in cells, such as amino acids, organic acids, carbohydrates, lipids, and others.

The research methodology involved the use of a cell line model with intrinsic resistance to Carboplatin (CARB), which was subsequently developed into a CARB-PTX-resistant variant, mimicking the clinical scenario. The chosen analytical technique was gas chromatography coupled with mass spectrometry (GC-MS), which enabled detailed detection of intracellular (endometabolome) and extracellular (exometabolome) metabolic profiles in both cell lines. Following data acquisition, a series of processing steps was implemented, and subsequent analysis was carried out using bioinformatic tools. This included multivariate methods such as principal component analysis (PCA), along with univariate approaches like the Wilcoxon test and the Kruskal–Wallis test. A metabolic pathway analysis was also conducted using the set of significantly altered metabolites, identifying the metabolic pathways potentially associated with PTX resistance in HGSC cells. This approach aimed to enhance the understanding of metabolic reprogramming linked to PTX resistance, paving the way for more effective therapeutic strategies.

The GC-MS technique enabled the identification of 47 metabolites in the endometabolome and 36 in the exometabolome. In the endometabolome, eight of these compounds (glycine, glycerol, taurine, glucose, myo-inositol, proline, lactate, and 5-oxoproline) were found to be altered. Among them, two (glucose and glycerol) were decreased in resistant cells compared to parental cells, while the remaining metabolites

were increased. This can be explained by the fact that glucose serves as a primary energy source to support cellular proliferation, and glycerol, in turn, acts as a precursor for lipid synthesis, contributing to membrane formation and serving as an additional energy source. The extracellular metabolite profile exhibited no variation, indicating that metabolic adaptations associated with drug resistance are primarily restricted to the intracellular environment.

Statistical analysis revealed a significant increase in intracellular levels of taurine and myo-inositol in resistant cells, suggesting that osmoregulation may function as a key adaptive mechanism contributing to PTX resistance. Pathway analysis provided further evidence of alterations in glutathione metabolism in resistant cells, as well as changes in amino acid metabolism, particularly in the arginine and proline pathways.

This is the first study to employ a metabolomics-based approach to explore the metabolic reprogramming associated with PTX resistance in ovarian cancer cells, addressing a significant gap in the current understanding of PTX-specific resistance mechanisms.

Keywords: Ovarian Cancer, Chemoresistance, Metabolomics, Gas chromatography-mass spectrometry, Multivariate analysis.

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List of Abbreviations

DMSO - Dimethyl Sulfoxide

ECACC - European Collection of Cell Cultures

ECM - Extracellular Matrix

ES – Effect Size

FBS - Fetal Bovine Serum

FDA - Food and Drug Administration

FDR – False Discovery Rate

GC-MS - Gas Chromatography-Mass Spectrometry

HDAC - Histone-Deacetylase

LC-MS - Liquid chromatography-mass spectrometry

LDA- Linear Discriminant Analysis

MS - Mass Spectrometry

NMR - Nuclear Magnetic Resonance Spectroscopy

OC – Ovarian Cancer

OS - Overall Survival

PCA - Principal Component Analysis

PLS- Partial Least Squares

PLS-DA - Partial Least Square Discriminant Analysis

QC - Quality Control

RI- Retention indices

R-Match- Reverse match

TA- Total area

VIP- Variable importance in the projection

1. Introduction

Over the past few decades, the use of omics technologies has become an invaluable tool in biomedical research, significantly enhancing our understanding of disease mechanisms within biological systems. Genomic studies were among the first to be widely applied, successfully identifying genetic variations associated with specific diseases and varying treatment responses. Building on the foundation of genomics, other omics disciplines—such as epigenomics, transcriptomics, proteomics, and metabolomics—have also demonstrated their importance in deepening our comprehension of biochemical processes (Hasin et al., 2017).

The field of metabolomics focuses on analyzing small molecules, or metabolites, within biological systems (Nicholson et al., 1999). These compounds play crucial roles in cellular growth and various physiological functions, and they are influenced by upstream biological changes. Analytical techniques such as nuclear magnetic resonance (NMR) spectroscopy, liquid chromatography-mass spectrometry (LC-MS), and gas chromatography-mass spectrometry (GC-MS) enable metabolomics to offer valuable insights into the biochemical mechanisms and pathways underlying diseases and to predict drug responses (Li et al., 2017; Stringer et al., 2017b). This omics approach is particularly useful in identifying biomarkers for prognostic, diagnostic, and screening purposes in conditions like diabetes, multiple sclerosis, and various cancers (Rispoli et al., 2021; Wishart, 2019). The search for disease-related metabolomic biomarkers is important, as they can improve diagnostic techniques by enabling non-invasive sample collection and potentially reducing costs compared to conventional methods. Moreover, metabolomic biomarkers can aid in selecting the most effective treatment strategies (Zhou & Zhong, 2022). The use of these biomarkers may also allow for earlier diagnosis, leading to better treatment outcomes (Res et al., 2018). As a result, metabolomic and multi-omics research is emerging as a critical tool for diagnostic, prognostic, and monitoring applications.

Cancer patients are a prime example of individuals who benefit from early diagnostic and staging approaches, as well as personalized treatment plans. Cancer remains one of the leading causes of death globally, with approximately 19 million new cases and 10 million deaths annually, with breast, lung, and prostate cancers being the most prevalent types. The incidence of cancer has been rising worldwide due to factors such as population growth, aging, and lifestyle choices (Lewandowska et al., 2019; Sung et al., 2021). The perception of cancer as a metabolic disease is supported by the notably higher energy demands of cancer cells, known as the "Warburg effect," the discovery of

oncometabolites, and the reliance of cancer cell growth on specific amino acids. Additionally, biomarkers that facilitate the early detection of cancer are crucial for improving survival rates and reducing potential complications (Di Meo et al., 2022).

Ovarian cancer (OC) is one of the most deadly gynecological cancers, with 21,410 cases and 13,770 deaths projected only in the United States in 2021 (Ortiz et al., 2022). According to GLOBOCAN, ovarian cancer occupies the eighteenth position of incidence (Sung et al., 2021). A woman's lifetime risk of developing ovarian cancer is 1 in 75, and her chance of dying from the disease is 1 in 100. It occurs in peri- and postmenopausal women, with 80% to 90% of cases occurring after the age of 40, with the peak incidence occurring at age 60 (Pokhriyal, 2019). Most patients with ovarian cancer are diagnosed at an advanced stage, resulting in a poor prognosis. The standard treatment for advanced ovarian cancer includes tumor debulking surgery followed by platinum-based chemotherapy (carboplatin or cisplatin) in combination with paclitaxel. It has remained the same for the past two decades. Despite improved clinical response and survival with this chemotherapy combination, approximately 80% of women relapse due to platinum resistance, highlighting the need to understand its molecular mechanisms to improve treatment efficacy and patient survival (Ortiz et al., 2022; Pokhriyal, 2019).

In recent years, the urgent need for reliable biomarkers has led researchers to consider metabolomics. This emerging omics science deals with analyzing the entire set of small molecules (≤ 1.5 kDa) present in biological systems (Saorin et al., 2020). Its continuous advancement has contributed to developing new medical approaches that better unravel the molecular changes underlying disease and inter-patient variability. Unlike the genomic profile, the metabolomic profile reflects the biochemical events that occur in the organism due to the complex interactions between age, sex, gene transcription, protein expression, physio-pathological conditions, including gut microbiome activity, and environmental exposures. Cancer can cause significant metabolic changes to sustain its growth, and metabolomics can highlight this, making it possible to detect cancer at an early stage of development. In the last decade, metabolomics has been widely used to identify different metabolic signatures to improve ovarian cancer diagnosis (Saorin et al., 2020).

1.1. Ovarian Cancer: staging and histologic subtypes

OC is characterized by the abnormal growth of cells in the ovaries, often presenting with subtle or non-specific symptoms in its early stages, making early detection challenging. As one of the deadliest gynecological malignancies, ovarian cancer is typically diagnosed at an advanced stage, which complicates treatment and reduces overall survival (Sambasivan, 2022). Several factors, including genetic predisposition, reproductive history, and hormonal factors, contribute to the development of ovarian cancer, highlighting the importance of comprehensive risk assessment and screening strategies (Sambasivan, 2022).

In 2020, OC was the eighth most commonly diagnosed malignancy worldwide, affecting approximately 314,000 women (Tavares et al., 2024). Worldwide, there are 313,959 new cases (1.6% of all cancers) and 207,252 deaths (2.1% of all cancer deaths) each year (Sambasivan, 2022; Sung et al., 2021).

Despite improvements in disease management, particularly in surgical techniques and maintenance therapy (treatment after first-line therapy to delay disease recurrence), OC patients still have a 5-year survival rate of less than 50% in most countries (Sambasivan, 2022). This is mainly due to the late diagnosis of the disease, its non-specific symptoms and the lack of appropriate screening methods, combined with the frequent development of chemoresistance leading to disease recurrence (Sambasivan, 2022). Several risk factors have been described as being associated with OC. We can divide these risk factors into demographic, reproductive, gynecological, hormonal, genetic, and lifestyle-related (Momenimovahed et al., 2019). It occurs mainly in postmenopausal women, with increasing age contributing to an increased risk. Nulliparity, endometriosis, obesity, perineal talc use, and smoking are the other described risk factors (Momenimovahed et al., 2019). Hereditary predisposition is a substantial risk factor, accounting for 20% of OC cases (Sung et al., 2021). Increased parity, higher age at birth, and use of oral contraceptives have been found to have a protective role in a few case-control studies (Sambasivan, 2022). Epidemiological evidence has also found regular use of non-steroidal anti-inflammatory drugs to be protective (Momenimovahed et al., 2019; Sambasivan, 2022). Mutations in BRCA1 and BRCA2 (genes that produce a protein that helps repair damaged DNA) are established risk factors for the development of HGSC of the ovary and are present in approximately 15–20% of women with ovarian cancer (Davis et al., 2014).

Most OC cases are sporadic, and only 5% to 10% are of familial origin. However, it is essential to note that one of the strongest risk factors for OC is a family history of the disease. Age has been described as a predisposing factor for the development of OC. An increased risk of OC has been observed in patients with a greater number of ovulatory cycles, such as those with a younger age at menarche and a later age at menopause. Increasing the number of ovulatory cycles increases cell division, which predisposes to the development of malignant neoplasms. Ovarian tumors are rare in young women, especially those under 30; however, older age increases the risk of having a more aggressive type of tumor (Momenimovahed et al., 2019; Tavares et al., 2024). Other factors, such as breastfeeding, protect against OC development (Gaona-Luviano et al., 2020). Breastfeeding for 3 months may be enough to reduce the risk. The association of protective factors has also been described in women who have used contraceptives and given birth (Gaona-Luviano et al., 2020).

Optimal surgery and platinum-based chemotherapy remain the standard of treatment. Treatment decision is based on the stage and performance status of the patient and should be taken after a multidisciplinary tumor board discussion. Primary treatment decides the chance of cure and depends on the stage and biology of the tumor. Early-stage disease has a 90% cure rate, but most cases are diagnosed in the advanced stage, limiting the chance of cure even with standard treatment (Tavares et al., 2024).

Treatment options for advanced-stage disease include either primary debulking surgery or interval cytoreduction after two to three cycles of neoadjuvant chemotherapy (NACT). Surgery for recurrence includes secondary or tertiary cytoreduction (De Oliveira et al., 2021). Patients undergoing primary cytoreduction for advanced-stage EOC should receive the standard six cycles of combination chemotherapy with paclitaxel and carboplatin. An alternate regimen with weekly paclitaxel increased overall survival in a Japanese trial, and many oncologists switched to weekly paclitaxel (Ortiz et al., 2022).

As with other tumors, the incidence and mortality of OC vary worldwide. While the disease is more common in European countries with a high Human Development Index, the lowest incidence rates are observed in African countries. In contrast, mortality rates tend to follow an inverse trend (Tavares et al., 2024). In 2022, there were 324,603 new cases reported and 206,956 deaths caused by OC (GLOBOCAN, 2022). According to ethnicity, the highest prevalence is among Caucasian women (12 per 100,000), followed by Hispanic women (10.3 per 100,000), African American women (0.4 per 100,000), and Asian women (9.2 per 100,000). However, the highest mortality from OC is in African

populations. This high mortality may be related to the social determinants of health. Factors such as poverty and poor access to health care may affect the outcome of OC, as with other diseases (Gaona-Luviano et al., 2020).

OC is classified into subtypes, the most common being epithelial OC, with only 10% being of non-epithelial origin. The predominant subtype has five major histological types that differ in origin, pathogenesis, molecular profile, risk factors for their development, and clinical prognosis. Among epithelial OC, 97% are non-mucinous and 3% are mucinous (Brierley et al., 2017). Mucinous tumors presented different histotypes such as serous (70%), endometrioid (10%), clear cell (10%), and unspecified (5%). Of the serous subtype, two further groups have been described: high grade and low grade (Brierley et al., 2017). The staging of OC was first defined by FIGO (International Federation of Gynecology and Obstetrics) in 1988 and updated in 2014 as described in Table 1 (Javadi et al., 2016).

Table 1 - International Federation of Gynecology and Obstetrics (FIGO) staging of ovarian cancer.

Stage	Description
Stage I	Tumor confined to ovaries
Stage IA	Tumor confined to one ovary (capsule intact); no tumor on ovarian tube surface; no malignant cells in the ascites;
Stage IB	Tumor confined to both ovaries (capsule intact); no tumor on ovarian tube surface; no malignant cells in the ascites;
Stage IC1	Tumor with surgical spillage in one or both ovaries;
Stage IC2	Tumor with preoperative capsule rupture or tumor on the ovarian surface
Stage IC3	Malignant cells in the ascites or peritoneal washings.
Stage II	Tumor involving one or both ovaries with pelvic extension or primary peritoneal carcinomatosis;
Stage IIA	Tumor has extension and/or implantation on the uterus and/or fallopian tubes;
Stage IIB	Tumor with extension to other pelvic intraperitoneal tissues;
Stage III	Tumor involving one or both ovaries with cytologically or histologically confirmed spread to the peritoneum outside the pelvis and/or metastasis to the retroperitoneal lymph nodes;
Stage IIIA	Tumor with positive retroperitoneal lymph nodes and/or microscopic metastasis outside the pelvis;
Stage IIIB	Tumor represents macroscopic peritoneal metastasis <2 cm outside the pelvis;

Stage IIIC	Tumor is defined as peritoneal metastasis >2 cm outside the pelvis and/or positive retroperitoneal lymph nodes;
Stage IV	Includes distant metastases outside the peritoneum;
Stage IVA	There is a malignant pleural effusion confirmed by cytology;
Stage IVB	When there are other types of distant metastases such as parenchymal liver or splenic metastases.

Adapted from International Federation of Gynecology and Obstetrics (FIGO) (Javadi et al., 2016).

Histopathological evaluation is performed after surgical removal of an ovary, fallopian tube, or peritoneal biopsy. Treatment depends on the histology of the OC, the FIGO stage of the tumor, and the patient's circumstances (De Oliveira et al., 2021; Javadi et al., 2016)

The stage of OC varies according to the time of diagnosis. Serous carcinomas are usually diagnosed at an advanced stage, 51% at stage III and 29% at stage IV, being one of the most aggressive subtypes of OC. On the other hand, most cases are diagnosed at stage I (58% to 64%). As a result of diagnosis and stage, survival to 5 years is 43% in the serous subtype, in contrast to endometrioid (82%), mucinous (71%), and clear cell carcinoma (66%). As mentioned above, race/ethnicity also influences survival rates (Gaona-Luviano et al., 2020).

The typical clinical presentation is an adnexal mass on pelvic examination or imaging. An adnexal mass may cause symptoms such as pelvic pain or tenderness, or it may be found incidentally on routine pelvic examination or imaging for other clinical indications (Gaona-Luviano et al., 2020). Women with later stages may present with a pelvic mass that extends beyond the adnexa. Historically, OC has been considered a silent disease. Several studies have reported non-specific symptoms in many women, even in the early stages, such as abdominal distension, urinary symptoms such as urgency or increased frequency, some eating disorders, early satiety, and non-specific pelvic or abdominal pain (Sambasivan, 2022). Further evaluation of symptoms that raise suspicion of OC are those that are new onset, coexist with other symptoms, occur frequently, and increase in severity. Some women with OC present with postmenopausal bleeding, although women with postmenopausal bleeding should be evaluated for uterine pathology before proceeding with an evaluation for OC. Rarely, women with OC may present with a paraneoplastic syndrome or develop a syndrome during the disease. Paraneoplastic syndromes associated with epithelial ovarian cancer (EOC) include cerebellar degeneration, polyneuritis, dermatomyositis, hemolytic anemia, disseminated

intravascular coagulation, acanthosis, or nephrotic syndrome (Gaona-Luviano et al., 2020).

Given that most cases of ovarian cancer ultimately relapse and that resistance to drugs upon retreatment remains one of the main barriers to therapeutic success and patient survival, it is crucial to investigate the underlying metabolic mechanisms driving this phenomenon. Metabolomic approaches, by enabling the identification and characterization of resistance biomarkers and altered metabolic pathways, provide essential tools to understand, predict, and ultimately overcome therapy resistance, paving the way for more personalized and effective treatment strategies.

1.2. Metabolomics in Cancer Research

1.2.1. Definition and strategy

Metabolomics is the last in the cascade of omics sciences. This cascade also includes genomics, the study of genes; transcriptomics, the study of transcriptomes and their function; and proteomics, the study of proteins (Tokarz et al., 2020). Metabolomics is the comprehensive analysis of all metabolites involved in the metabolic network of living organisms and their response to pathophysiological or other stimuli (Nicholson et al., 1999). The metabolome includes all small molecules with a molecular mass of less than 1200 Da, such as lipids, sugars, and amino acids, in a biological sample (Tokarz et al., 2020). Different biospecimens can be used in the metabolomics workflow, ranging from immortalized or primary cultured cells to biological fluids (e.g., saliva, urine, blood plasma/serum) and tissues (Graziani et al., 2018).

The metabolomic approaches can be divided into two broad categories: untargeted (global) or targeted metabolomics. These approaches differ in several factors, mainly in the objective and purpose of the experiment, but also in the complexity of sample preparation, the number of metabolites detected, and the experimental accuracy and precision (Tokarz et al., 2020). Untargeted or global metabolomics, also known as metabolic profiling, aims to simultaneously determine all measurable analytes present in a sample (Tokarz et al., 2020; L. Wang et al., 2019). The main advantage of the untargeted metabolomics approach is the collection of large amounts of raw data without prior knowledge of the sample composition. In fact, this approach can potentially reveal altered metabolites and pathways that were previously unknown (Stringer et al., 2017b). The difficulty in identifying these unknown metabolites and the large amount of time required to process all the raw data obtained are some of the disadvantages associated with this approach (L. Wang et al., 2019). In addition, the untargeted approach does not

focus on the absolute quantification of metabolites, so it usually provides only semi-quantitative data (Tokarz et al., 2020). On the other hand, targeted metabolomics aims to determine the absolute concentration of a defined set of metabolites with similar chemical structure and/or related biological function to test a specific hypothesis (Turi et al., 2018). In this approach, a standard calibration curve can be generated for each metabolite for accurate quantification, as all metabolites in the study are pre-selected based on prior knowledge of the specific pathway involved in the hypothesis (Tokarz et al., 2020). Compared to the untargeted approach, targeted metabolomics is more accurate, sensitive, and reproducible, with fewer false positives. A disadvantage of this approach is that the compound of interest for the study must be known a priori and be available in its purified form. Furthermore, the limited set of simultaneously quantified metabolites may lead to an increased risk of missing interesting biological processes (Tokarz et al., 2020; Turi et al., 2018). An intermediate option, considering semi-targeted assays, can also be adopted, with the main aim of analysing large amounts of metabolites of known chemical structure and defining approximate metabolite concentrations (Broadhurst et al., 2018).

The workflow of a typical untargeted metabolomics analysis is illustrated in Figure 1. Samples are first collected from cancer-free controls and cancer patients at various stages to analyze their metabolic profiles. This approach helps identify the key metabolic alterations in patients (Amaro et al., 2022). Samples can be obtained directly as biofluids, cancer tissues, or through *in vitro* models. *In vitro* models are beneficial for preliminary studies on metabolic changes related to potential cancer therapies, as they are less complex and exhibit lower sample variability. However, translating findings from *in vitro* studies to *in vivo* systems can be challenging (Rodrigues et al., 2019). To achieve results that accurately reflect the real impact of metabolic changes on patients, human tissues or biofluids are preferred, with biofluids offering the additional advantage of being non-invasive. Several biofluids, including saliva, urine, and plasma, are suitable for metabolomics studies. Among these, urine and plasma/serum are the most commonly used in metabolomics research focused on cancer detection and staging (Iwamoto et al., 2022; Meihua et al., 2023).

Secondly, high-throughput analytical techniques are essential for detecting and characterizing the metabolites present in the samples. The primary platforms used for metabolic profiling are mass spectrometry (MS) and nuclear magnetic resonance (NMR) spectroscopy (Y. Chen et al., 2022).

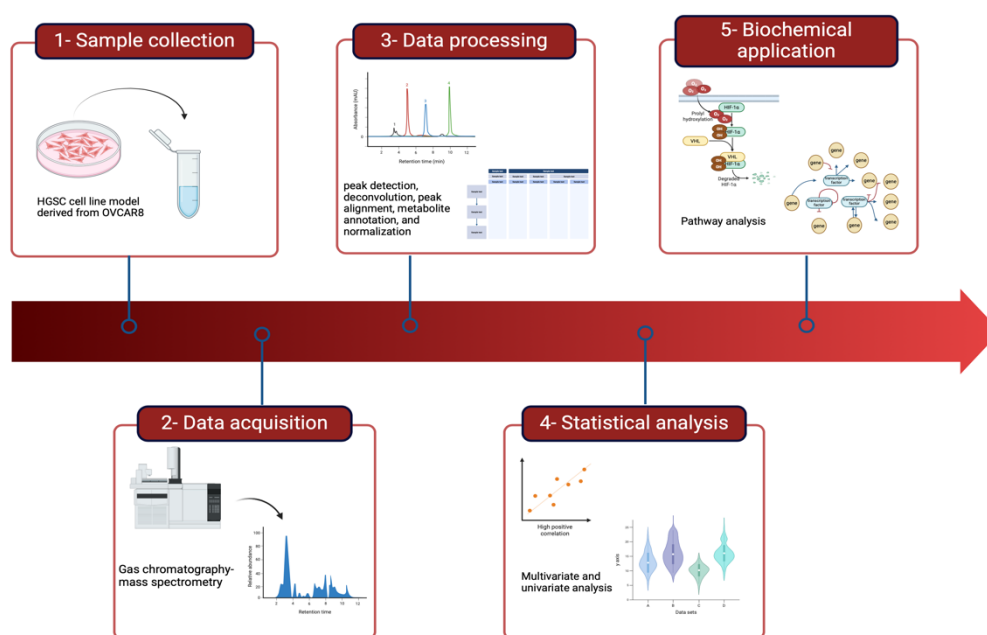


Figure 1 - Workflow of a typical untargeted metabolomics analysis using GC-MS as an analytical technique.

The next step in a metabolomics study is data processing. For GC-MS or LC-MS-based metabolomics data, this begins with the pre-processing of raw data, which includes tasks such as noise filtering, peak detection, retention time correction, chromatographic peak alignment, and deconvolution (Y. Chen et al., 2022). Following this, normalization is applied during data processing to minimize experimental bias, to correct different factors of dilution, and prevent the misidentification of compounds (Clish et al., 2020; Yi et al., 2016). Metabolites were identified by comparing what was obtained in the samples with the National Institute of Standards and Technology (NIST) standard reference database.

Once data processing is complete, statistical methods are employed to identify significantly expressed compounds. These methods are categorized into multivariate and univariate analyses. Multivariate analysis involves a large number of variables and can be further classified as unsupervised or supervised, depending on the approach used. Principal component analysis (unsupervised) and partial least squares-discriminant analysis (supervised) are common examples of these (Debik et al., 2022; Sung et al., 2021). PCA is widely used in metabolomic data analysis as a quality control step for metabolomics data to observe whether there are classification trends between groups and data outlier points. PCA can also be used to analyze whether quality control samples are clustered together or whether they are scattered or have some variability, which would indicate problems with the quality of the assay (Y. Chen et al., 2022). It has

been successfully applied in studies related to diseases such as diabetes and breast cancer to detect potential biomarkers (Gu et al., 2012).

In contrast, univariate analyses, such as the t-test, focus on examining one variable at a time (J. H. Wang et al., 2010). The statistical significance of the results is indicated by the *p-value*. Similarly, the Mann-Whitney test, a non-parametric counterpart to the t-test, assesses whether the central tendencies of the groups differ (Hart, 2015).

The last phase in a metabolomics study involves functional analysis, where the detected compounds are mapped to their biological pathways and functions. There are several biological databases that provide knowledge on a huge number of metabolic pathways. These databases are the Kyoto Encyclopedia of Genes and Genomes (KEGG), MetaCyc, Small Molecule Database (SMPDB), WikiPathways, and Plant Metabolic Network (PMN/ PlantCyc). Two primary approaches used in this analysis are enrichment analysis and pathway analysis (Y. Chen et al., 2022). Enrichment analysis aims to explore the profile of functionally relevant metabolites to determine the relationship between changes in metabolite expression and biological context. This allows a list of altered metabolites to be used to suggest biological pathways or disease states that would indicate the next steps in the study. Pathway analysis aims to identify pathways that have a significant impact on a particular biological process. Enrichment and pathway analyses are performed using specialized software tools that map metabolites to known biochemical pathways based on information in public databases such as KEGG (Y. Chen et al., 2022; Marco-Ramell et al., 2018). Pathway analysis, the one used in this study, seeks to identify significant pathways involved in specific biological processes. Tools like MetaboAnalyst utilize these methods to connect metabolites to particular biochemical pathways (Xia et al., 2009).

Metabolomics is increasingly gaining traction in biomedical research, particularly in oncology. Numerous studies have identified certain metabolites as potential biomarkers that can differentiate between tumor and non-tumor tissues (Gonzalez-Covarrubias et al., 2022). This approach holds promise for enhancing cancer diagnosis, monitoring, and treatment. In cancer research, metabolomics is clinically applied in several ways, including the identification of cancer risk factors, the discovery of biomarkers for cancer screening, diagnosis, and monitoring, and in fundamental research to understand cell-cell communication and the tumor microenvironment (Schmidt et al., 2021).

Metabolites are ideal biomarkers for cancer screening, diagnosis, and monitoring because they can be easily measured from non-invasive samples like blood or urine. This method enables the identification of potential biomarkers by comparing metabolite

levels between healthy and unhealthy individuals. In the past years, several comprehensive reviews have been published on metabolic biomarkers in various types of cancer (Goh et al., 2020; Stringer et al., 2017a).

Cancer biology and therapeutics represent additional clinical applications of metabolomics research that have garnered significant attention. Studying cancer biology is crucial for understanding the dynamics of metabolic pathways, which can help identify metabolites that are altered in specific cancers. Moreover, metabolomics can offer new insights for developing personalized treatments, potentially leading to more effective therapeutic outcomes than those achieved by standard therapies (L. Wang et al., 2019).

The following subchapters provide a detailed overview of the use of *in vitro* models in metabolomics applied to cancer research, as well as the main analytical techniques employed and the bioinformatic tools commonly used to interpret the complex datasets generated.

1.2.2. *In vitro* models for cancer metabolomic studies

As cancer is a widespread disease in the modern world, there is an innate necessity to study cancers adequately outside of *in vivo* systems to get a complete understanding of the disease (Katt et al., 2016). Through *in vitro* models, we can rapidly test the interaction of certain drugs with the cancer microenvironment and assess their therapeutic potential without the need to perform experiments on animals. We are also able to gain critical insights into the behavior of a tumor, namely in invasion potency, extracellular matrix remodeling, and extravasation. Models can vary in their constitution and complexity, ranging from primary cell lines to 3D models of the tumor microenvironment (Martinez-Pacheco & O'driscoll, 2021). The primary goal of the study of the cancer microenvironment ultimately dictates the complexity of the model. If the goal is to simply study the efflux of cancer drugs by multidrug resistance proteins of the cancerous cell line, a simple monoculture (2D model) will suffice. If the goal is to verify the metastatic potential and the invasion properties of the cells, a co-culture of the cancer cells with an extracellular matrix (ECM) or ECM-producing cells is performed, or a microenvironment with microvessels that allow blood flow (Katt et al., 2016). Having this in mind, the main component of any tumor model is the cancer cells. Commercially available cancer cell lines are widely used in research because they are easy to manipulate and grow, and are a staple of molecular biology throughout the research world (Martinez-Pacheco & O'driscoll, 2021).

Thus, immortalized cancer cell lines have been the most extensively used model in metabolomics studies (Prieto et al., 2020). It enables more controlled experimental designs and the removal of certain variables, such as age. The use of patient-derived cells enables a more accurate assessment of the specific tumor's gene profile and provides an *in vitro* model that closely reflects the behavior and microenvironment of the original tumor. However, the patient-derived sample typically contains a heterogeneity of cell types, resulting in cross-contamination of individual cultures. Despite the advantages, the simplicity of these models never allows for a correct understanding of the tumor microenvironment, such as their angiogenic potential, which in turn can lead to missed opportunities for novel therapeutic targets. In addition, cultured cells are usually placed in ideal nutrient and CO₂ levels, which do not occur in tumors, as both nutrient and O₂ deprivation (hypoxic core) are standard in many tumors (Katt et al., 2016; Martinez-Pacheco & O'driscoll, 2021).

1.2.3. Analytical techniques for metabolomic studies

Metabolomics research is typically conducted in complex matrices, such as cells, biofluids, or tissues, and various techniques are employed, each with its strengths and limitations. The primary aim of a metabolomic assay is to achieve comprehensive coverage of the metabolome, which often necessitates different techniques depending on the specific characteristics of the metabolites being analyzed (Munjal et al., 2022). Several techniques enable the quantification of small amounts of analytes with reliable and reproducible results. The most commonly used techniques include gas chromatography-mass spectrometry (GC-MS), liquid chromatography-mass spectrometry (LC-MS), and nuclear magnetic resonance (NMR) spectroscopy (J. H. Wang et al., 2010).

Mass spectrometry (MS) is a technique that involves the ionization of molecules, followed by mass analysis and ion detection, generating a spectrum that displays the abundance of detected ions as a function of their mass-to-charge (m/z) ratio. MS is particularly useful for analyzing metabolites at low concentrations, sometimes even reaching the nanomolar (nM) range (de Falco et al., 2022). Compared to NMR, MS offers higher resolution and is often paired with separation techniques like liquid chromatography (LC-MS) or gas chromatography (GC-MS). In GC-MS, a derivatization step may be necessary since this technique cannot analyze non-volatile or thermally labile compounds. In contrast, LC-MS does not face these issues and can analyze samples across a broad polarity range, including high molecular weight compounds (de Falco et al., 2022). While GC-MS is known for its high resolution, accuracy, and repeatability, and

its ability to distinguish between structurally similar molecules, LC-MS is highly sensitive but less reproducible. to ensure the reliability and reproducibility of analytical methods, quality control (QC) samples are used. QCs are a pool of small aliquots of every sample under study that are analyzed alongside real samples. Capillary electrophoresis can also be coupled with MS (CE-MS), but it is less commonly employed than the aforementioned techniques. MS requires a smaller sample volume, only a few microliters, compared to about 600 μ L in NMR. Although metabolomics experiments typically rely on a single technique, combining NMR and MS can enhance the identification of novel biomarkers (de Falco et al., 2022; J. H. Wang et al., 2010).

As outlined in Table 2, each technique has advantages and disadvantages that should be considered during study design.

Table 2 - Advantages and disadvantages of the analytical techniques used in metabolomics.

Techniques	Advantages	Disadvantages
GC-MS	The ability to provide both qualitative and quantitative information about a sample's composition and has a high resolution	Derivatization of non-volatile metabolites is needed before analysis; ineffective for thermolabile compounds
LC-MS	High sensitivity, good resolution, and is effective on thermolabile compounds	Lack of standard spectral libraries
NMR	Simple sample preparation, non-destructive and highly reproducible	Peak overlapping; lower sensitivity for metabolite detection

Adapted from (Y. Chen et al., 2022)

1.2.4. Data pre-processing and statistical analysis

Various techniques can be used to carry out the statistical analysis and interpretation of metabolomics data, including multivariate analysis, such as PCA analysis, and univariate analysis, such as the Wilcoxon test. Before statistical analysis, the data is centered and scaled. Centering converts all the concentrations to fluctuations around zero instead of around the mean of the metabolite concentrations, adjusting for differences in the offset between high and low-abundant metabolites. It is therefore used to focus on the fluctuating part of the data and leaves only the relevant variation for analysis (van den Berg et al., 2006). Scaling is used to make data more manageable and comparable,

especially when dealing with variables with different units or ranges. It is also helpful to understand and analyze data more effectively by putting all the numbers on a similar scale (van den Berg et al., 2006).

There are various possibilities within scaling, but the most widely used in metabolomics are autoscaling and Pareto scaling. Autoscaling, called unit or unit variance scaling, is commonly applied and uses the standard deviation as the scaling factor (van den Berg et al., 2006). After autoscaling, all metabolites have a standard deviation of one; therefore, the data is analyzed based on correlations instead of covariances. Pareto scaling is very similar to autoscaling. However, instead of the standard deviation, the square root of the standard deviation is used as the scaling factor (van den Berg et al., 2006).

Regarding the multivariate analysis, PCA uses sophisticated underlying mathematical principles to transform several possibly correlated variables into a smaller number of variables called principal components (Jolliffe & Cadima, 2016). PCA has been described as one of the most important tools of applied linear algebra, and perhaps its most common use is to analyze large data sets. Some of the other common applications include: signal denoising, blind source separation, and data compression (Jolliffe & Cadima, 2016). In general, PCA uses a vector space transformation to reduce the dimensionality of large data sets. Using mathematical projection, the original data set, which may have contained many variables, can often be interpreted as just a few variables (i.e., the principal components) (Mishra, 2001). The central idea of PCA is to reduce the dimensionality of a data set in which there are a large number of interrelated variables, while preserving as much of the variation present in the data set as possible. This reduction is achieved by transforming into a new set of variables, the principal components, which are uncorrelated and ordered so that the first few retain most of the variation in all the original variables (Jolliffe & Cadima, 2016; Mishra, 2001). Computing the principal components reduces to solving an eigenvalue-eigenvector problem for a positive semidefinite symmetric matrix.

In univariate analysis, a 'variable' typically refers to a condition or category into which the data is divided. Parametric tests are applicable only when the assumption of normal distribution is met and the data follow a specific distribution with known mathematical properties (e.g., normal, binomial), except for certain unknown parameters (such as the mean or variance) that are estimated from the data (Angelini, 2019). Examples include the t-test for comparing means, the binomial test for proportions, and the F-test used in ANOVA models. The t-test is a parametric statistical method used to compare the means

of two groups in order to determine whether the observed differences are statistically significant. It relies on certain assumptions, including that the data are approximately normally distributed, that the variable being analyzed is continuous, and that the observations are independent. In some cases, it also assumes equal variances between the groups being compared (Al-Kassab & Majeed, 2022; Angelini, 2019). When this assumption is violated, a variant known as Welch's t-test can be used to adjust for unequal variances. The test calculates a t-statistic based on the difference between means, the variability of the data, and the sample size. This statistic is then used to obtain a p-value, which indicates the probability of observing such a difference by chance if the null hypothesis is true. A p-value below a chosen significance threshold (commonly 0.05) suggests that the difference is statistically significant (Al-Kassab & Majeed, 2022).

Non-parametric methods involve the evaluation of a single variable without assuming any specific underlying probability distribution, such as normality. These methods are particularly suitable when the data do not meet the assumptions required for parametric tests, including the assumption of normally distributed variables or homogeneity of variances. Unlike parametric tests, which rely on the actual data values, non-parametric approaches use ranks or frequencies, making them more robust in the presence of deviations from distributional assumptions (Janssen & Veraverbeke, 2024).

Non-parametric tests are especially advantageous in situations involving small sample sizes, where parametric tests may lack power or reliability. They are also preferable when outliers are present and cannot be excluded, as their reliance on ranks minimizes the influence of extreme values (Janssen & Veraverbeke, 2024). Furthermore, when the hypothesis concerns the median of a distribution rather than the mean, non-parametric methods provide a more appropriate analytical framework. In cases where the data significantly deviates from a normal distribution, the use of non-parametric methods ensures more valid and reliable results (Janssen & Veraverbeke, 2024).

Typical examples of univariate non-parametric tests include the Mann–Whitney U test, also known as the Wilcoxon rank-sum test, and the Kruskal–Wallis test. This is a non-parametric statistical test used to compare two samples or groups (Kim, 2014). It assesses whether two sampled groups are likely to come from the same population, essentially asking: do these two populations have the same shape in their data? In other words, we want to know whether the groups are drawn from populations with different levels of a variable of interest. The hypotheses in a Mann-Whitney U test are: the null hypothesis (H_0) is that the two populations are equal. The alternative hypothesis (H_1) is that the two populations are unequal (McClenaghan, 2024). Some researchers interpret

this as comparing the medians between the two populations (in contrast, parametric tests compare the means between two independent groups). In certain situations where the data are similarly shaped, this is valid. Still, it should be noted that the medians are not involved in calculating the Mann-Whitney U test statistic. According to the Mann-Whitney U test, two groups could have the same median and still be significantly different (McClenaghan, 2024). The Kruskal-Wallis test serves as the nonparametric counterpart to the one-way ANOVA (a statistical method used to determine whether there are significant differences among the means of three or more populations), used to determine whether multiple samples come from the same distribution. It extends the Wilcoxon-Mann-Whitney test to situations involving more than two independent groups (Ostertagová et al., 2014).

Based on existing knowledge of biological pathways, molecular entities such as genes, proteins, and metabolites can be mapped onto curated pathway sets that aim to represent how these entities collectively function and interact in a biological context (Wieder et al., 2021). Originally developed for the interpretation of transcriptomic data, pathway analysis has become a popular method for the analysis of metabolomic data (Tsouka & Masoodi, 2023; Wieder et al., 2021). However, there are several inherent differences between transcriptomic and untargeted metabolomic data that need to be considered when performing the analysis on metabolites. First, metabolomics datasets tend to cover a much smaller fraction of the total metabolome than transcriptomic datasets do of the genome. Therefore, metabolomics datasets tend to contain far fewer metabolites than the transcripts found in transcriptomic datasets. Second, the mapping of compounds to pathways is not as straightforward as the equivalent mapping with genes and proteins, and there is often a significant degree of uncertainty in metabolite identification, both in terms of structures and database identifiers in any metabolomics dataset (Wieder et al., 2021).

1.3. Drug resistance in OC

A wide range of drugs have been studied over the years to find better treatments for OC and to improve patients' quality of life. Carboplatin and Paclitaxel (PTX) are two drugs extensively studied in this field that have demonstrated efficacy against the disease, being used as first-line drugs for OC since 1999 (Du Bois et al., 1999).

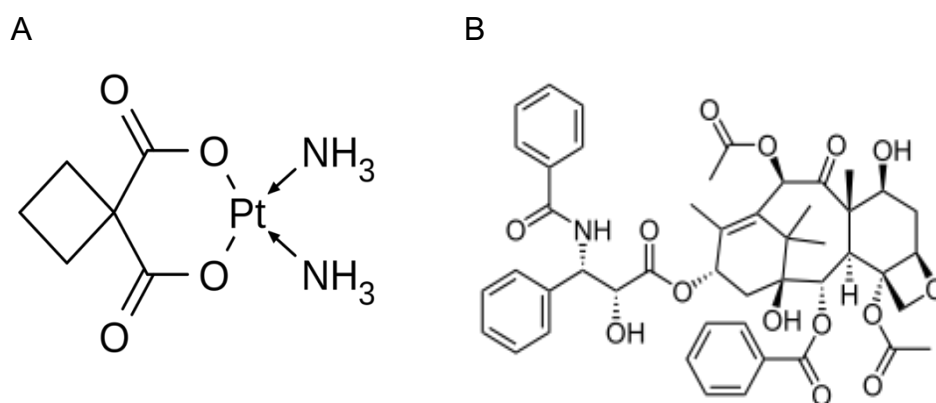


Figure 2 - Molecular structures of Carboplatin (A) and Paclitaxel (B).

Carboplatin (Figure 2A) is a 'second-generation' platinum compound that has been advocated for use in patients with OC and has also shown promise in small cell lung cancer, squamous cell carcinoma of the head and neck, and seminoma (Pokhriyal, 2019). Overall, it appears to have a similar qualitative spectrum of activity to cisplatin. There have been few comparative trials with carboplatin, to try to improve its efficiency and avoid resistance, either alone or in combination with other chemotherapeutic agents such as paclitaxel, but the limited data suggest comparable efficacy to cisplatin in OC (Pokhriyal, 2019). Like cisplatin, carboplatin reacts with nucleophilic sites on DNA, causing intrastrand, interstrand, and DNA-protein crosslinks. However, these crosslinks are formed later with carboplatin than with cisplatin (de Sousa et al., 2014; Pokhriyal, 2019).

PTX (Figure 2B), also known as Taxol, is a secondary metabolite produced by *Taxus sp.* and, to a lesser extent, *Coniferales Cephalotaxus*, *Podocarpus gracilior*, or *Corylus avellana* (Gallego-Jara et al., 2020). The first identification of PTX was made by Dr. Jonathan L. Hartwell (National Cancer Institute, NCI) in the 1960s, when a screening program for antitumor agents in the plant kingdom was carried out by the National Cancer Institute and the US Department of Agriculture. PTX from *Taxus brevifolia* (T. brevifolia) was identified as a potent anticancer agent (Gallego-Jara et al., 2020). A chemical synthesis route for PTX was developed, and worldwide sales of Taxol® have been in the millions since it was first launched (Gallego-Jara et al., 2020). The Food and Drug Administration (FDA) and the European Medicines Agency (EMA) have approved Taxol® for the treatment of breast, ovarian, and lung cancers, and Kaposi's sarcoma (Sharifi-rad et al., 2021). Although the response rate depends on a number of factors, including the type of cancer, Taxol® has been shown to have an approximate response rate of 30% in OC (Gallego-Jara et al., 2020).

As an anti-microtubule agent, PTX promotes microtubule assembly, increases the activity of tubulin dimers, and stabilizes existing microtubules while inhibiting their disassembly (Gallego-Jara et al., 2020). The increased stability of microtubules in the presence of PTX results in cell cycle arrest in late G2, leading to inhibition of cell replication. PTX can also distort mitotic spindles and lead to chromosome fragmentation (Sharifi-rad et al., 2021). One of the disadvantages of this compound is its limited solubility, recrystallisation on dilution, and co-solvent induced toxicity.

It should be emphasized that most platinum-sensitive patients eventually become platinum-resistant at an advanced stage. Acquired chemotherapeutic resistance can be related to different molecular mechanisms such as drug efflux, altered cell cycle checkpoints, drug uptake, and epigenetic changes (Marchetti et al., 2021). First, in platinum-based chemotherapy, both cisplatin and carboplatin induce cell apoptosis and cross-link with the purine bases on deoxyribonucleic acid (DNA), causing DNA damage. Each step in this complex mechanism, from drug release into the cell cytoplasm to DNA binding or drug-induced signaling pathways, may be impaired in platinum-resistant cells. In addition, the susceptibility of a cancer cell to DNA alkylating agents is determined by its ability to detect and repair DNA damage. Therefore, cells that can repair DNA from the outset or restore initially impaired DNA repair systems may be able to resist chemotherapy. Once this goal is achieved, immune checkpoint genes are activated to induce apoptosis. Alterations in this complex mechanism appear to correlate with resistance to platinum-based chemotherapy (Marchetti et al., 2021).

The cytotoxic action of cisplatin (and its analogs) involves active uptake into cells and subsequent binding to DNA, forming adducts which then lead to the formation of intra- and inter-strand cross-links and, in turn, single- or double-strand DNA breaks. This DNA damage can lead to activation of the apoptosis cascade and cell death if the cell is unable to repair the DNA. Platinum can also cause mitochondrial damage, which is associated with decreased ATPase activity and cell death (Davis et al., 2014). Factors associated with platinum resistance include those that limit the formation of cytotoxic platinum-DNA adducts and those that prevent cell death following platinum adduct formation. The former may result from either reduced uptake of cisplatin into cells or increased efflux via alterations in transport proteins or inactivation of intracellular cisplatin by conversion to cisplatin-thiol conjugates. The latter form of resistance can occur through increased DNA repair following adduct formation (Davis et al., 2014). Alterations in various proteins associated with these repair mechanisms have been implicated in platinum resistance, including high levels of excision repair cross-complementation group 1 (ERCC1) protein

and secondary mutations in BRCA1 or 2 that can cause reversion to the BRCA genotype and restoration of BRCA function. These different factors may be present at diagnosis or acquired over time (Davis et al., 2014; Marchetti et al., 2021).

Chemoresistance results are complex and have not been fully understood, with mechanisms that involve multiple concurrent intra- and extracellular factors. This complexity can significantly hamper the management of drug-resistant disease. In platinum-resistant OC, medical treatment is challenging, and its failure is ultimately responsible for nearly all deaths from this cancer (Marchetti et al., 2021).

2. Aims of this dissertation

The work carried out throughout this dissertation aimed at performing an *in vitro* study, based on GC-MS-based metabolomics, to unravel the metabolic events associated with chemoresistance to PTX in an OC cell line. A deeper knowledge of the mechanism of resistance to this drug at the molecular level may contribute to improving the treatment guidelines for HGSC. The specific aims of this thesis were:

- To perform comprehensive metabolic profiling of intracellular (endometabolome) and extracellular (exometabolome) compartments in wild-type (OVCAR8) and PTX-resistant OC cells using GC-MS;
- To identify differentially regulated intracellular and extracellular metabolites between PTX-resistant and wild-type OVCAR8 cells;
- To conduct pathway analysis using significantly altered metabolites to elucidate the metabolic events associated with PTX resistance.

3. Experimental section

3.1. Chemicals

Valine ($\geq 98\%$), succinic acid ($\geq 99\%$), serine ($\geq 99\%$), malic acid ($\geq 99\%$), cholesterol ($\geq 99\%$), tyrosine ($\geq 98\%$), glutamine ($\geq 99\%$), alanine ($\geq 98\%$), isoleucine ($\geq 98\%$), threonine ($\geq 98\%$), phenylalanine ($\geq 98\%$), citric acid ($\geq 99.5\%$), glycine ($\geq 99\%$), Lactate ($\geq 99\%$), alanine ($\geq 99\%$), urea ($\geq 99\%$), glycerol ($\geq 99\%$), butanedioic acid ($\geq 99\%$), nonanoic acid ($\geq 99\%$), aspartic acid ($\geq 99\%$), oxoproline ($\geq 99\%$), asparagine ($\geq 99\%$), taurine ($\geq 99\%$), myristic acid ($\geq 99\%$), oxalic acid ($\geq 99\%$), proline ($\geq 99\%$), glucose ($\geq 99.5\%$), fructose ($\geq 99.5\%$), mannose ($\geq 99.5\%$), threose ($\geq 99.5\%$), monopalmitin ($\geq 99.5\%$), glycerol monostearate ($\geq 99.5\%$), ornithine ($\geq 98.5\%$), tryptophan ($\geq 98\%$), leucine ($\geq 98\%$), methionine ($\geq 98\%$), xylitol ($\geq 99\%$), cysteine ($\geq 98.5\%$), were purchased from Sigma-Aldrich (Madrid, Spain) for standard mixtures preparation. Sodium chloride was purchased from VWR (Leuven, Belgium). N,O-Bis(trimethylsilyl)trifluoroacetamide with 1% trimethylchlorosilane (BSTFA-TMCS), chloroform ($\geq 99.8\%$), desmosterol ($\geq 84\%$), and methoxyamine hydrochloride (MOX) ($\geq 98\%$) were purchased from Sigma-Aldrich (Madrid, Spain). Pyridine ($\geq 99\%$) was supplied by Carlo Erba (Val-de-Reuil, France). PTX was purchased from Selleckchem (Houston, Texas, USA) and dimethylsulfoxide (DMSO) from AppliChem (Barcelona, Spain).

3.2. Cell lines and culture conditions

Protocols already optimized in previous work were used to prepare the cell lines (M. Nunes et al., 2020, 2021). The wild-type OVCAR8 cell line was kindly provided by Dr Francis Jacob, Gynecological Cancer Centre and Ovarian Cancer Research, Department of Biomedicine, University Hospital Basel and University of Basel, Basel, Switzerland. Cells were grown in complete media, specifically RPMI-1640 medium (ThermoFisher Scientific, Massachusetts, USA) supplemented with 10% (v/v) inactivated and filtered fetal bovine serum (FBS; Biowest, Nuaille, France) and 1% (v/v) penicillin/streptomycin (ThermoFisher Scientific) and maintained at 37°C in 5% CO₂. OVCAR8 cells were authenticated by short tandem repeat profiling and regularly tested for the absence of mycoplasma. PTX was dissolved in DMSO and stored at -80°C according to the manufacturer's instructions (M. Nunes et al., 2021). Immediately before use, an aliquot was diluted to the required concentrations.

An OVCAR8 PTX-resistant variant was established from wild-type OVCAR8 by exposure to a stepwise increasing PTX concentration (2 to 74.9 nM) for 3 months. The starting concentration used for PTX-resistance induction corresponded to the inhibitory concentration of 10% (IC₁₀) value obtained from dose-response curves after exposure of wild-type OVCAR8 to PTX (1.56 to 200 nM) for 48 h. Then, it was tested whether the PTX-resistant phenotype was stable in drug-free culture. Therefore, after 2 months of freezing cycles, cells were cultured in the absence of PTX supplementation (M. Nunes et al., 2021).

3.3. Metabolomics Assay

3.3.1. Cell lines and culture conditions

Sample collection was divided into two separate days, one for cell culture under ideal conditions to perform the assay and the other for cell collection and preparation for GC-MS analysis. The first day started with pre-warming (37°C, 30 min) of the complete medium, PBS 1x and trypsin, and labelling of Eppendorfs 1.5 mL (2; 1 for each cell line), Falcons 50 mL (4; 2 for each cell line), and 6-well plates. Falcons were then prepared, and 19 mL of complete medium was added to each Falcon. The medium was removed from the previously prepared cell culture flasks, and the cells were washed with PBS 1x (~3-4 mL), and the supernatant was discarded into the biological container. The cells were then trypsinized (~1.5 mL trypsin, 37°C for 3-5 min), and complete medium (5 mL) was added, resuspended, and the suspended cells transferred to a 15 mL tube. The cells were then centrifuged at 1200 rpm for 5 min, the supernatant was removed to a biological container, and the cells were resuspended in complete medium (~3 mL). The cells were then counted (in an Eppendorf, 10 µL of trypan blue and 10 µL of cell suspension were added), and a cell counter was used. We then seeded OVCAR-8 and OVCAR8 PTX R C cells into 19 wells at a density of 6×10^5 cells/mL. After doing the calculations, we came to the conclusion that we needed 803 µL cells and 18.2µL complete medium for the OVCAR-8 and 2007 µL cells and 17.0µL complete medium for the OVCAR8 PTX R C. We then resuspended the cell suspension (in falcons), removed the calculated volume from each and added the corresponding cell volume to the falcon. We plated 1000 µL/P6 of each cell line and incubated the plates at 37°C and 5% CO₂, and allowed them to adhere overnight.

3.3.2. Metabolite extraction

Successful metabolite extraction is a critical step in metabolite profiling. By optimizing metabolite extraction, the scope and quantitative capacity of metabolomics studies can be improved (Gonzalez-Covarrubias et al., 2022). On the second day, we divided the procedure into extracellular and intracellular metabolite extraction. For the extracellular, we collected 500 μL of the culture medium from both cell lines and blank controls (medium without cells) into a centrifuge tube and centrifuged at 2000 g for 5 min at 4 °C (to eliminate cell debris). We then collected 100 μL of the supernatant into a 2 mL Eppendorf tube, added 900 μL of ice-cold methanol, and vortexed for 5 minutes. We then centrifuged again at 10,000 g for 10 min at 4 °C and collected 200 μL of supernatant in a glass vial for GC-MS analysis and 200 μL of supernatant in another glass vial for QCs (pooling of all samples in a 4 mL glass, later divided into several aliquots of 200 μL). All tubes were dried under a nitrogen stream and stored at -80 °C until preparation for GC-MS analysis.

For intracellular fraction collection, adherent cells were gently washed twice with 0.9% NaCl at room temperature (to remove interferences). Then, we added 700 μL of ice-cold methanol:water solution (80:20 v/v) (to quench metabolism and extract metabolites) and scraped cells from each well and transferred both from the same condition to an Eppendorf (2 mL capacity). We then sonicated on ice (3 x 30 s at 23 kHz and 10 μm amplitude using an exponential probe, with a 30 s interval between sonications). We centrifuged at 3000 g for 10 min at 4 °C and collected 800 μL of supernatant in one glass vial for GC-MS analysis and 300 μL of supernatant in another glass vial for QCs. All glass vials were stored at -80 °C and lyophilized.

Quality control (QC) samples for both intracellular and extracellular analyses were prepared by pooling small aliquots from all respective samples. These QC samples were analyzed alongside study samples to assess analytical reproducibility. All samples and QCs were lyophilized and stored at -80 °C until further processing.

3.3.3. Sample preparation for GC-MS analysis

Samples were prepared using the recommended derivatization protocol for GC-MS (Want et al., 2010). Prior to GC-MS analysis, 10 μL of desmosterol (1 mg/mL in chloroform) was introduced into each sample and quality control (QC) vial to serve as an internal standard. The solvent was then evaporated using a gentle nitrogen stream. For intracellular metabolites, the dried extracts were treated with 80 μL of methoxyamine hydrochloride solution (20 mg/mL in pyridine) and incubated at 37 °C for 90 minutes. This

was followed by the addition of 100 μ L of BSTFA + TMCS, with a further incubation at the same temperature for 30 minutes. For extracellular metabolites, derivatization began with 50 μ L of the same methoxyamine solution, incubated at 60 °C for 1 hour. Afterwards, 100 μ L of BSTFA + TMCS was added, and the reaction continued for an additional 60 minutes at 60 °C. In both workflows, 100 μ L of the final derivatized mixture was transferred into GC-compatible vials, and 2 μ L aliquots were injected into the GC-MS using a 1:20 split ratio. Injections were randomized to minimize batch effects, and QC samples were run after every six experimental samples to assess system stability and ensure data reliability.

3.3.4. GC-MS acquisition parameters

Derivatized intra- and extracellular metabolites were analyzed using a standardized gas chromatography-mass spectrometry (GC-MS) workflow. The instrumentation comprised a Bruker 436-GC system coupled to a SCION single quadrupole mass spectrometer (Bruker Daltonics), equipped with a Combi-PAL autosampler (Varian), and controlled through Bruker MS Workstation software (version 8.2.1; Bruker Daltonics, Bremen, Germany). Separation of compounds was achieved on an Rxi-5Sil MS capillary column (30 m $\times$ 0.25 mm i.d., 0.25 μ m film thickness; Restek, Bellefonte, PA, USA). High-purity helium (C-60, Gasin, Porto, Portugal) was used as the carrier gas, delivered at a constant flow rate of 1 mL/min.

The oven temperature was programmed as follows: initial hold at 70 °C for 2 minutes, followed by a ramp to 250 °C at 15 °C/min with a 2-minute hold, then further increased to 300 °C at 10 °C/min and held for 5 minutes at the final temperature. The injector was maintained at 250 °C, while the transfer line, ion source, and manifold were set to 250 °C, 260 °C, and 39 °C, respectively. Electron ionization was performed at 70 eV in full-scan acquisition mode, covering a mass-to-charge (m/z) range of 50 to 1000.

3.3.5. Metabolite annotation

Metabolites detected in both intracellular and extracellular fractions were identified by comparing the mass spectrum of the metabolite present in the sample with the mass spectra contained in the National Institute of Standards and Technology (NIST14) database and authentic reference standards (where commercially available). In addition, Rmatch, a similarity factor that evaluates the similarity between the peaks in our sample and those in the database, was also considered for metabolite annotation (acceptance threshold Rmatch $\geq$ 700).

3.3.6. Data pre-processing for statistical analysis

The GC-MS data obtained from the samples and QCs were converted to netCDF format to further perform statistical analysis. Metabolite annotation and peak area integration were carried out using the PARADISE software (PARAFAC2-based Deconvolution and Identification System) (Quintanilla-Casas et al., 2023). Automatic data alignment was selected, and a maximum of 4 components were defined for each ROI to avoid overlapping peaks and co-diluted compounds. The MS spectra obtained in each ROI were compared with the NIST database, and each component was assigned to the best NIST match. The PARADISE report was then exported in .xls format and provided the list of each identified compound with their respective peak areas, retention times, match quality, and m/z ions. The list of compounds in the PARADISE report was compared with the manual metabolite annotation using the QCs' chromatographic data to ensure that the compounds were appropriately selected. The MS spectra were compared against the NIST database, with each component assigned to the best-matching NIST entry. When possible, identification was further confirmed by analysis of commercial standards under identical conditions. This methodology allowed the establishment of confidence levels for metabolite identification following recommendations for metabolomic studies (Viant et al., 2017).

The contaminating peaks caused by the column were eliminated, and only those with an RSD (relative standard deviation) calculated for the QCs of less than 40 percent were considered. The total of metabolites identified had their peak areas normalized by total area (TA), which consists of dividing the area of each metabolite by the total sum of the metabolites in the sample to mitigate uncertainties related to sample concentration and injection order.

3.3.7. Statistical analysis

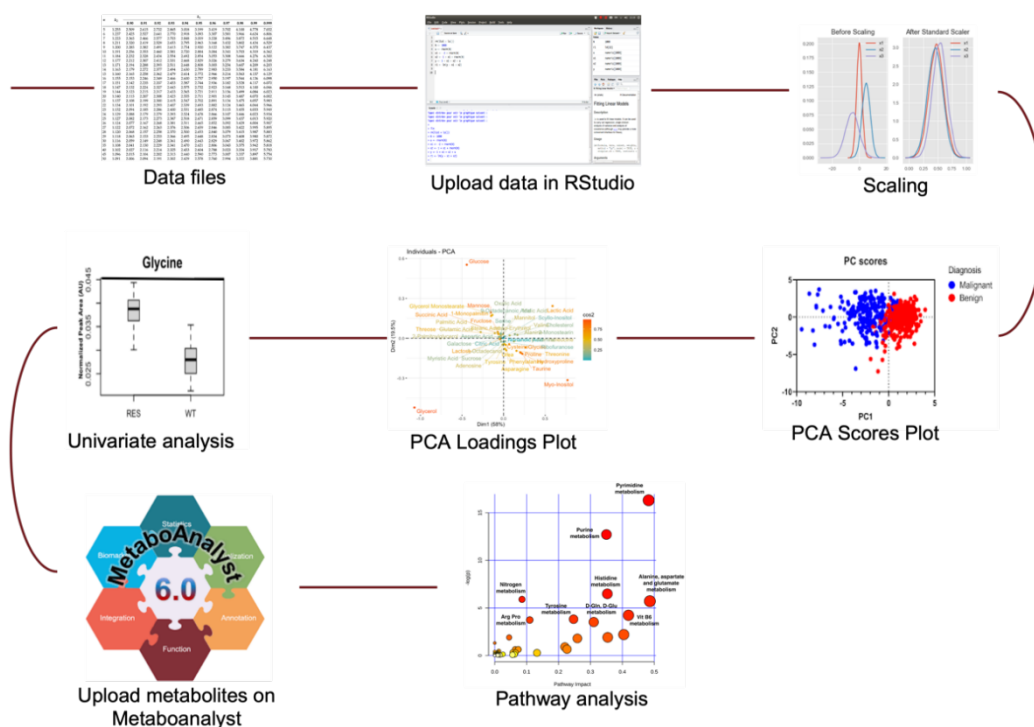


Figure 3 - Workflow of the conducted statistical analysis and pathway analysis.

RStudio is an integrated development environment that allows easy and productive computational analysis using the R language (Hair et al., 2021). It is an open-source project that aims to combine the various components of R (source editor, console, help, history, graphics) into a productive workbench. It is designed both to ease the learning curve for new R users and to provide highly productive tools for more advanced users. After pre-processing the data, statistical analysis was conducted at this software, an integrated development environment (Hair et al., 2021). Comparisons were made between resistant and wild-type cells.

The pre-processed data was first uploaded to RStudio in an Excel. file format. The data was subjected to a scaling procedure, namely Pareto scaling, which involved 4 steps to mean-centering the data and dividing by the square root of the standard deviation of each variable. The code used is shown in the Appendix - Section 1.

PCA was performed to generate score plots for each comparison, displaying 95% confidence regions (Keita, 2023). The code used is shown in the Appendix - Section 2. To analyze the PCA, score plots were made of all the samples against the QCs to test the reproducibility of the analytical method, score plots of the PTX-resistant samples

against the wild-type samples, and a loadings plot to identify which metabolites were differently expressed. These graphs were made for both the endometabolome and the exometabolome. In the exometabolome, a further comparison graph was made between the blanks (culture medium without cells), the PTX-resistant group (RES), and the wild-type group (WT).

To carry out the univariate analysis, the metabolites that were most relevant in the PCA loadings plot were selected, and to generate the respective boxplots, a code adapted from a script provided by Gonçalo Graça was used (Graca et al., 2010). The code used to carry out these analyses can be found in Appendix 1, Section 3. Multiple comparisons were corrected using the False Discovery Rate (FDR) method implemented via the `p.adjust()` function in R (version 4.3.2) (Team, 2006). The effect size of significantly altered metabolites was calculated as the standardized mean difference between groups, using the pooled standard deviation (Berben et al., 2012).

Pathway Analysis was conducted using MetaboAnalyst 6.0 to explore the potentially disturbed metabolic pathways (Pang et al., 2024). Significantly altered metabolites between PTX-resistant compared to wild-type cells were entered into a column and matched with their corresponding IDs from the Human Metabolome Database (HMDB) (Wishart et al., 2007). The enrichment method was the hypergeometric test, and the topology measure was defined as relative-betweenness centrality. The analysis utilized the Homo sapiens Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway library (Kanehisa & Goto, 1999).

4. Results

4.1. Metabolic profiles of intracellular and extracellular domains of OVCAR-8

A typical GC-MS chromatogram of the endometabolome (intracellular aqueous extract) of OVCAR-8 cells is shown in Figure 4. Overall, 47 metabolites were identified using the NIST library, including 16 amino acids, 11 organic acids and derivatives, 8 fatty acids, and 12 carbohydrates. These metabolites were identified using the NIST library. Their characteristic ions, retention time (RT), RMatch, retention index (RI) from NIST, and RSD are shown in Table 3.

Figure 4 - Representative GC-MS chromatogram of the endometabolome (intracellular aqueous extract) of wild-type OVCAR-8 cells.

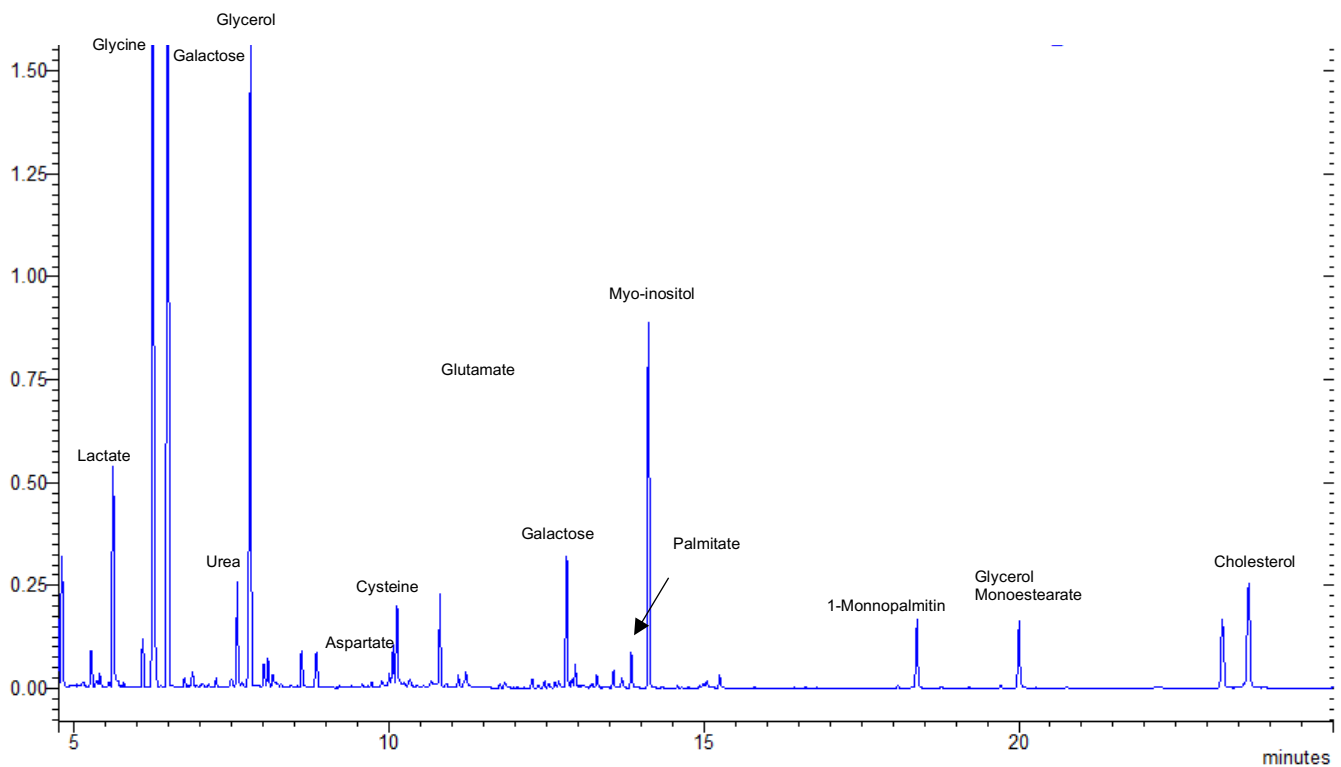


Table 3 - Metabolites identified in the GC-MS analysis of the intracellular aqueous extracts (endometabolome) of PTX-resistant and wild-type OVCAR-8 cells.

Compound	RT (min)	Ions (m/z)	Rmatch	RI NIST	RSD QCs (%)
Lactate, 2TMS derivative	5.58	73, 117, 147, 191	941	1064	27.0
Alanine, 2TMS derivative	6.07	73, 116, 147	907	1113	14.7
Glycine, 2TMS derivative	6.26	73, 102, 147	867	1119	22.7
Oxalic Acid	6.36	73, 147	920	1139	24.9
L-Valine, 2TMS derivative	7.25	73, 144, 218	876	1222	17.8
Urea, 2TMS derivative	7.49	73, 147, 189	763	1260	35.3
Serine, 2TMS derivative	7.68	73, 116	748	1244	12.9
Glycerol, 3TMS derivative	7.79	73, 147, 205	955	1278	33.4
Isoleucine, 2TMS derivative	8.01	73, 158	902	1316	16.8
Proline, 2TMS derivative	8.08	73, 142	894	1311	13.5
Butanedioic acid, 2TMS derivative	8.20	147, 247	639	1321	18.0
Nonanoic Acid, TMS derivative	8.61	73, 204, 218	909	1581	17.0
L-Threonine, 3TMS derivative	8.65	117, 215	788	1409	14.5
L-Aspartate, 2TMS derivative	8.84	73, 117, 218	882	1413	21.6
β-Alanine, 3TMS derivative	9.26	73, 174, 248	791	1446	53.4
Malate, 3TMS derivative	9.72	73, 147	858	1498	16.9
meso-Erythritol, 4TMS derivative	9.87	73, 147, 217	822	1506	12.0
5-Oxoproline, 3TMS derivative	10.06	73, 156	838	1500	16.9
Cysteine, 3TMS derivative	10.31	73, 220	833	1547	22.5
L-Glutamate, 3TMS derivative	10.80	73, 128, 246	925	1625	3.8
Phenylalanine, 2TMS derivative	10.91	73, 192, 218	777	1633	28.5
Asparagine, 3TMS derivative	11.19	73, 231	767	1668	28.9
Taurine, 3TMS derivative	11.23	73, 326	854	1691	43.4
Citrate, 4TMS derivative	12.27	73, 147, 273	834	1835	35.9

Compound	RT (min)	Ions (m/z)	Rmatch	RI NIST	RSD QCs (%)
Myristate, TMS derivative	12.53	73, 117, 285	846	1846	37.6
D-Fructose, 1,3,4,5,6-pentakis-O-(trimethylsilyl)-, O-methyloxime	12.64	73, 147, 217	849	2182	20.7
d-Glucose, 2,3,4,5,6-pentakis-O-(trimethylsilyl)-, o-methyloxime, (1Z)-	12.82	73, 147, 319	937	1877	18.4
L-(+)-Threose, tris(trimethylsilyl) ether, ethyloxime (isomer 1)	12.90	73, 147, 205	756	1556	30.3
d-Mannose, 2,3,4,5,6-pentakis-O-(trimethylsilyl)-, o-methyloxime, (1E)-	12.97	73, 147, 319	790	1821	10.6
D-Mannitol, 6TMS derivative	13.08	73, 147, 319	806	1979	18.7
L-Tyrosine, 3TMS derivative	13.13	73, 218	627	1994	49.5
D-Galactose, 5TMS derivative	13.33	73, 217	597	1877	26.4
Scyllo-Inositol, 6TMS derivative	13.70	73, 217	820	2009	12.2
Palmitic Acid, TMS derivative	13.84	73, 117, 129, 313	895	2045	21.0
Myo-Inositol, 6TMS derivative	14.11	73, 147, 217, 305	914	2009	16.2
1-Octadecanol, TMS derivative	14.58	75, 327	869	2150	20.4
9-Octadecenoic acid, (E)-, TMS derivative	15.05	73, 117, 339	882	2218	21.3
Stearic acid, TMS derivative	15.25	73, 117, 341	858	2242	12.8
2-Palmitoylglycerol, 2TMS derivative	18.07	73, 129, 218	790	5488	5.2
1-Monopalmitin, 2TMS derivative	18.37	73, 147, 371	896	2546	11.7
Sucrose, 8TMS derivative	18.77	73, 361	746	2813	11.9
D-Lactose, octakis(trimethylsilyl) ether, methyloxime (isomer 2)	19.21	73, 204, 361	701	2890	7.8
2-Monostearin, 2TMS derivative	19.71	73, 129, 218	824	3005	3.7
Glycerol monostearate, 2TMS derivative	20.00	73, 147, 399	898	3007	7.6

Compound	RT (min)	Ions (m/z)	Rmatch	RI NIST	RSD QCs (%)
D-Ribofuranose, 1,2,3-tris-O-(trimethylsilyl)-, bis(trimethylsilyl) phosphate	22.19	73, 315	652	3086	39.0
Adenosine, N-(trimethylsilyl)-2',3'-bis-O-(trimethylsilyl)-, 5'-[bis(trimethylsilyl) phosphate]	22.25	73, 169, 315	669	3222	49.2
Cholesterol, TMS derivative	23.23	73, 129, 329	915	3386	3.4

RT – Retention time; RI – retention index; R-match – Reverse match; RSD – Relative Standard Deviation

A typical GC-MS chromatogram of the OVCAR-8 exometabolome, the set of metabolites present in the medium in which the cells were cultured, is shown in Figure 5. Overall, 36 metabolites were identified, including 17 amino acids, 8 organic acids and derivatives, 3 fatty acids, and 8 carbohydrates. Their characteristic ions, retention time, RMatch, and RI from NIST are shown in Table 4. Between the exometabolome and the endometabolome, 26 compounds were found in common, namely lactic acid, alanine, glycine, oxalic acid, valine, urea, isoleucine, proline, threonine, mesa-erythritol, aspartic acid, hydroxyproline, glutamic acid, phenylalanine, asparagine, citric acid, fructose, mannose, galactose, glucose, mannitol, tyrosine, palmitic acid, myo-inositol, 1-monopalmitin and glycerol monostearate.

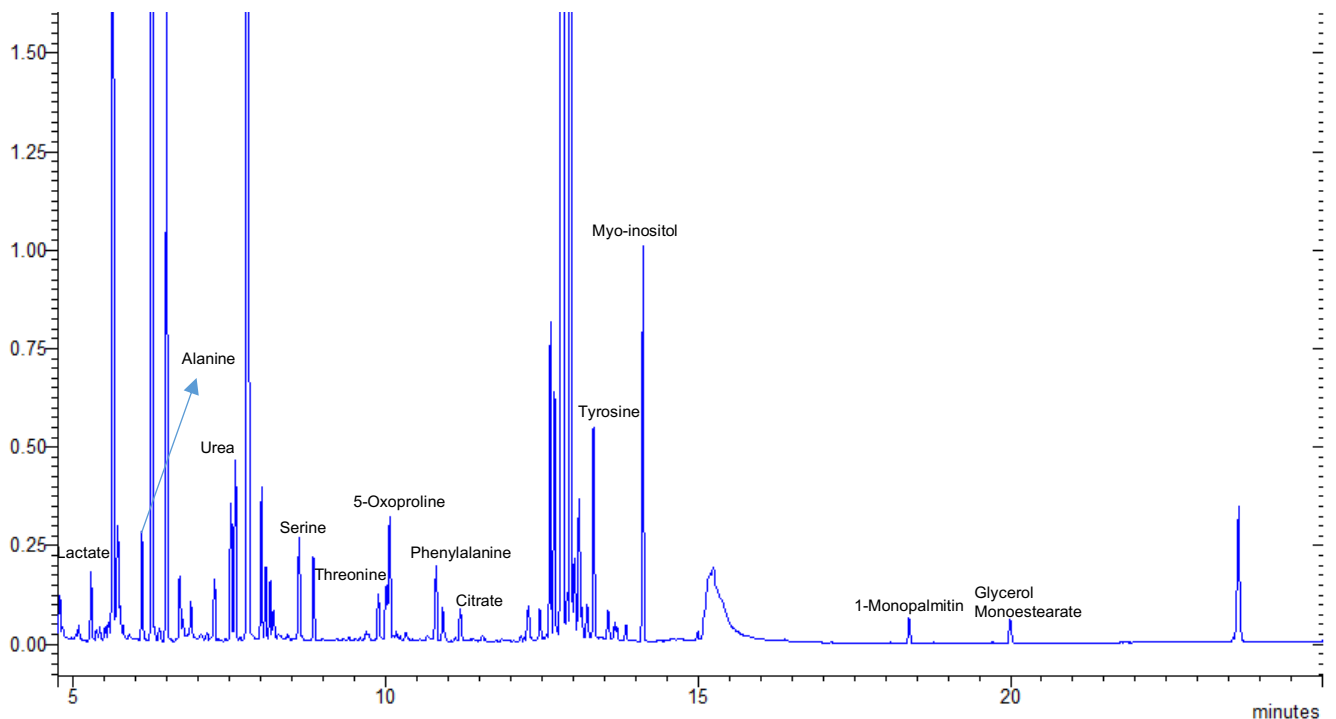


Figure 5 - GC-MS chromatogram of the exometabolome of wild-type OVCAR-8 cells.

Table 4 - Metabolites identified in the GC-MS analysis of the culture media (exometabolome) of PTX-resistant and wild-type OVCAR-8 cells.

Compound	RT (min)	Ions (m/z)	Rmatch	RI	RSD QCs (%)
Propanoic acid, 2-(methoxyimino)-, trimethylsilyl ester	5.49	59, 73, 89, 174	841	1063	10.2
L-Alanine, 2TMS derivative	6.11	73, 116, 147	895	1113	37.9
Glycine, 2TMS derivative	6.29	73, 102, 147, 204	899	1119	39.1
Oxalate, 2TMS derivative	6.38	73, 147, 190	950	1140	62.7
L-Valine, 2TMS derivative	7.26	73, 100, 144, 218	904	1222	56.6
Urea, 2TMS derivative	7.51	73, 147	902	1260	56.8
L-Leucine, 2TMS derivative	7.81	73, 158, 232	909	1291	38.9
L-Isoleucine, 2TMS derivative	8.01	73, 100, 158, 218	906	1317	32.2
L-Proline, 2TMS derivative	8.07	73, 142, 216	881	1311	48.3
Butanedioic acid, 2TMS derivative	8.19	73, 147, 247	922	1321	41.6
Serine, 3TMS derivative	8.61	73, 147, 204	923	1410	43.4
L-Threonine, 3TMS derivative	8.85	73, 117, 218	911	1410	44.7
meso-Erythritol, 4TMS derivative	9.87	73, 147, 217	914	1507	44.4
L-Aspartate, 3TMS derivative	10.01	73, 100, 232	859	1549	42.2
L-5-Oxoproline, 2TMS derivative	10.06	73, 156, 258	856	1509	51.8
L-Hydroxyproline, (E)-, 3TMS derivative	10.07	73, 140, 230	90	1501	50.8
L-Threonic acid, tris(trimethylsilyl) ether, trimethylsilyl ester	10.17	73, 147, 292	873	1591	50.0
L-Glutamate, 3TMS derivative	10.80	73, 128, 147, 246	937	1626	40.9
Phenylalanine, 2TMS derivative	10.90	73, 147, 218	920	1634	47.7
Asparagine, 3TMS derivative	11.20	73, 116, 231	933	1669	49.8
D-(+)-Arabitol, 5TMS derivative	11.55	73, 147, 217, 307	848	1735	42.3
DL-Ornithine, 3TMS derivative	11.86	73, 174, 244, 348	843	1742	24.4

Compound	RT (min)	Ions (m/z)	Rmatch	RI	RSD QCs (%)
D-(-)-Fructofuranose, pentakis(trimethylsilyl) ether (isomer 1)	12.17	73, 147, 217	834	1748	61.9
Citrate, 4TMS derivative	12.27	73, 147, 273	848	1835	32.5
D-(-)-Fructopyranose, 5TMS derivative (isomer 2)	12.28	73, 147, 204	795	2049	47.4
D-Fructose, 1,3,4,5,6-pentakis-O- (trimethylsilyl)-, O-methyloxime	12.70	73, 103, 217, 307	922	2182	33.2
d-Mannose, 2,3,4,5,6-pentakis-O- (trimethylsilyl)-, o-methyloxyme, (1Z)-	12.74	73, 147, 205, 319	854	2173	43.0
d-Galactose, 2,3,4,5,6-pentakis-O- (trimethylsilyl)-, o-methyloxyme, (1E)-	12.83	73, 147, 205, 319	916	1738	15.8
d-Glucose, 2,3,4,5,6-pentakis-O- (trimethylsilyl)-, o-methyloxyme, (1E)-	12.96	73, 147, 205, 319	907	1877	26.3
L-Lysine, 4TMS derivative	13.01	73, 317	839	1908	17.8
D-Mannitol, 6TMS derivative	13.09	73, 147, 319	924	1979	25.4
L-Tyrosine, 3TMS derivative	13.13	73, 147, 218	902	1994	33.0
Palmitic Acid, TMS derivative	13.84	73, 147, 313	898	2045	37.8
Myo-Inositol, 6TMS derivative	14.11	73, 147, 217, 305	933	2009	27.1
1-Monopalmitin, 2TMS derivative	18.38	73, 147, 371	887	2546	24.3
Glycerol monostearate, 2TMS derivative	20.00	73, 147, 399	917	3007	19.2

RT – Retention time; RI – retention index; R-match – Reverse match; RSD – Relative Standard Deviation

4.2. Metabolic events associated with PTX-resistance in OC cells

4.2.1. Metabolic alterations in the endometabolome

Regarding statistical analyses, PCA operates by making principal components, which are new variables created as linear combinations of the original variables. These combinations are designed to ensure that the new variables (principal components) are uncorrelated, with the majority of the information from the initial variables being concentrated in the first few components. Organizing information into principal components allows for the reduction of dimensionality while preserving most of the information. This is achieved by discarding components with minimal information and retaining the remaining components as your new variables.

PCA was initially conducted using the QC samples along with all samples under investigation to assess the analytical reproducibility of the GC-MS. The resulting score plot, shown in Figure 6, reveals that the QC samples clustered closely together, indicating that the experimental data were consistent and not impacted by technical variation, thereby supporting the reliability of the analytical method.

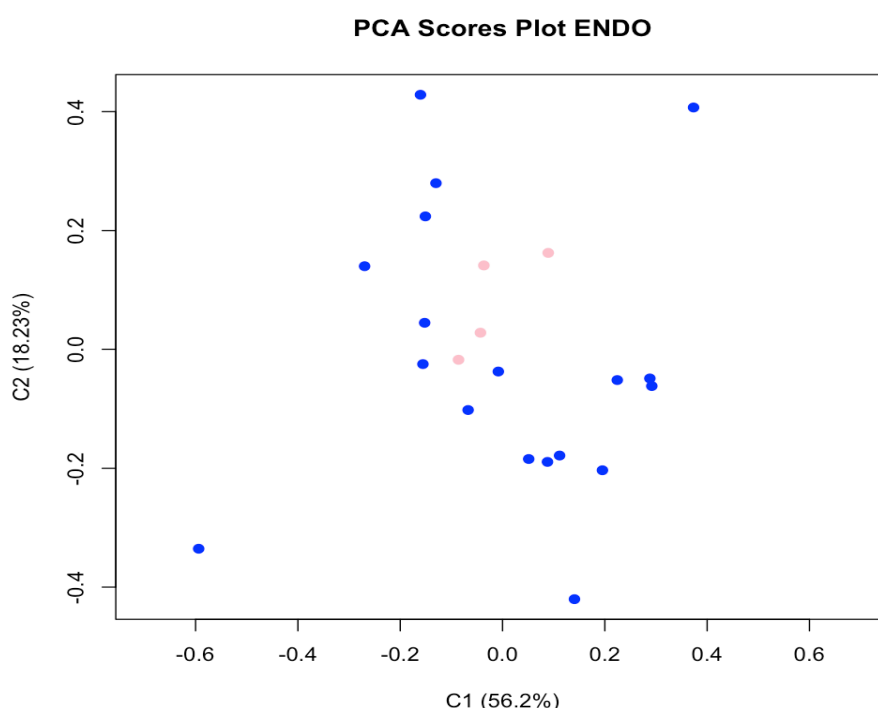


Figure 6 - PCA score plot of the endometabolome of all the samples and corresponding QCs. Pink dots – QC's, Blue dots – samples.

PCA was also employed to investigate differences between the metabolic profiles of PTX-resistant and parental cells.

The first approach to presenting the data was a scree plot, shown in Figure 7. This was used to visualize the importance of each principal component. This plot shows the eigenvalues in a downward curve, from highest to lowest, and confirms what was presented in the PCA summary, where the first two components represent the data.

Each component explains a percentage of the total variance in the data set, and in the cumulative proportions section, the first principal component explains 58% of the total variance. This means that nearly two-thirds of the data in the set of 18 variables can be represented by the first principal component alone. The second explains 19.5% of the total variance. The cumulative proportion of Comp.1 and Comp.2 explains almost 78% of the total variance. This means that the first two principal components can accurately describe the data.

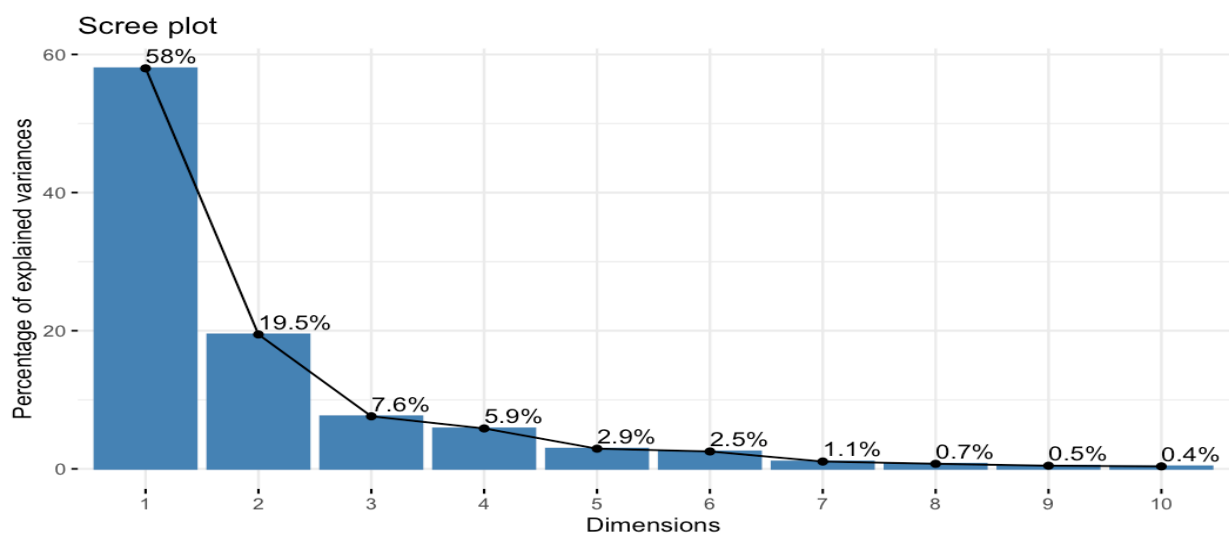


Figure 7 - Scree plot of the importance of each principal component.

Figure 8A shows that the PCA model efficiently distinguished the PTX-resistant cells (RES) from the wild-type cells. Figure 8B represents the loadings plot of the metabolites found on the endometabolome. According to the scale of colors, the ones that are colored with red/orange are the ones that are altered the most (glucose, glycerol, taurine, myo-inositol, taurine, glycine, proline, 5-oxoproline, and lactate).

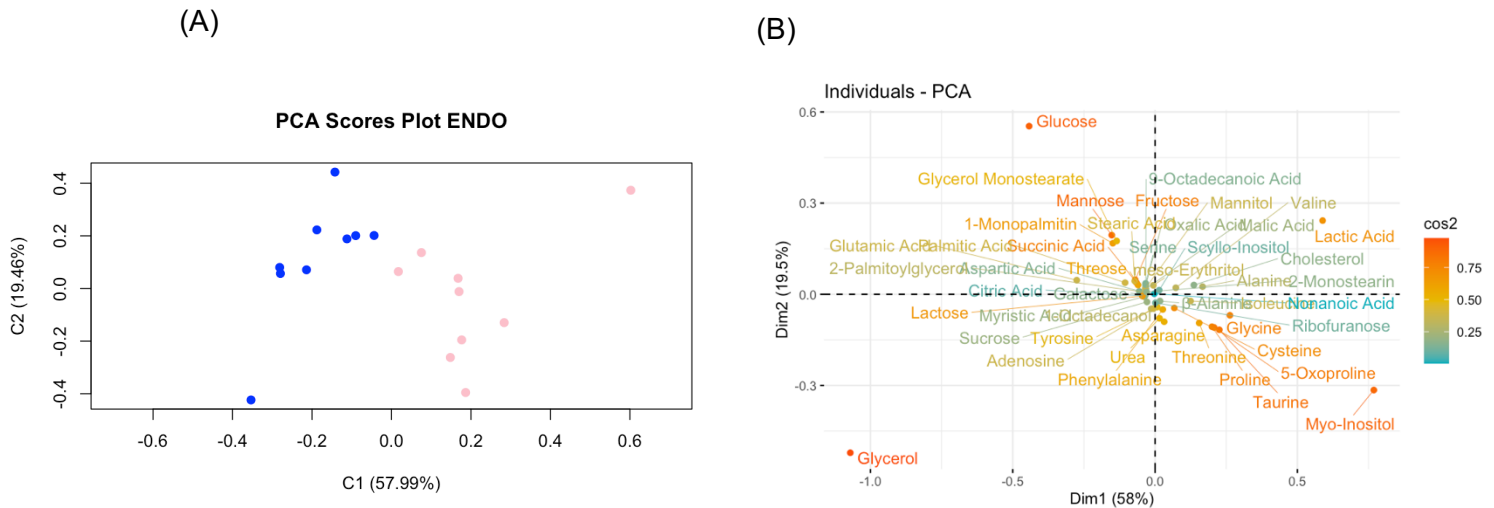


Figure 8 - (A) PCA score plot of the endometabolome of RES cells vs. WT cells. Pink dots – Resistant cells; Blue dots – wild-type cells; (B) Loadings plot with scale of color of the most relevant metabolites (Orange – most significant; Blue – least significant).

From the loadings plot, we can visualize which metabolites are most relevant to separate both groups in the endometabolome (glucose, glycerol, taurine, myo-inositol, glycine, proline, 5-oxoproline, and lactate). Therefore, these were the ones selected for the univariate analysis.

Figure 9 represents the boxplots of each metabolite, where we can see that glycine, taurine, myo-inositol, proline, 5-oxoproline, and lactate are significantly increased in the PTX-resistant cells (RES) compared to the wild-type ones. On the other hand, glycerol and glucose levels are decreased in PTX-resistant (RES) compared to wild-type cells.

These findings suggest that resistant cells consume more glucose and glycerol than wild-type cells.

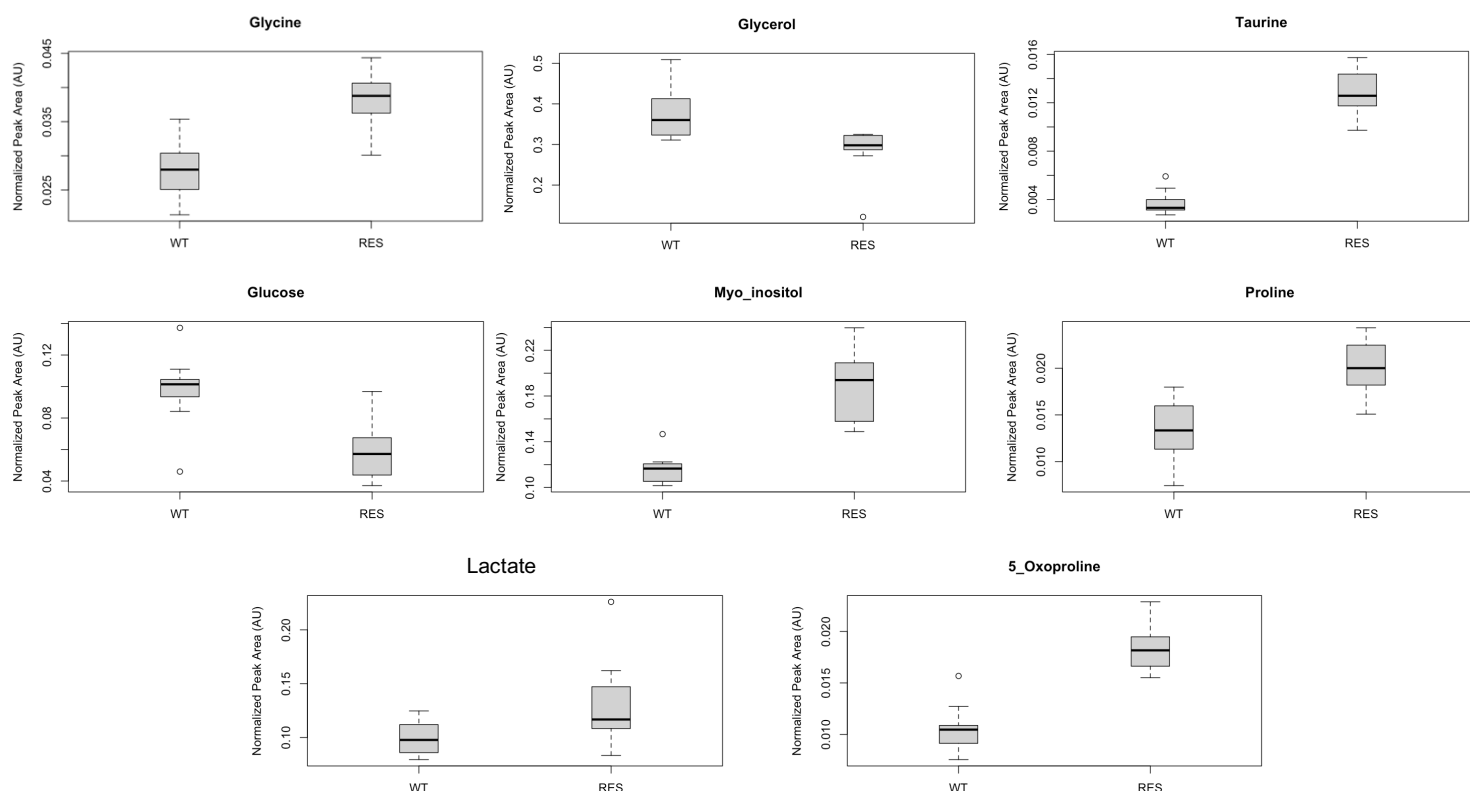


Figure 9 - Box plots of altered metabolites in the endometabolome of PTX-resistant cells vs wild-type cells.

Table 5 presents the corresponding effect sizes, p-values, and FDR-corrected p-values. Regarding effect size, both glycerol and glucose show negative values, which translates to them being diminished in the resistant cells compared to the wild-type ones. Glycine, taurine, myo-inositol, proline, lactate, and 5-oxoproline all present positive values of effect size, being elevated in the resistant cells. From all of these, myo-inositol is the one that is most elevated when compared with wild-type ones. All metabolites present a p-value lower than 0.05, except lactate. Glycine, taurine, proline, and 5-oxoproline showed an FDR-adjusted p-value below 0.05.

Table 5 - Metabolites altered in the endometabolome of PTX-resistant cells compared with WT OVCAR8 cells and their respective *p*-value.

Metabolites	ES $\pm$ 95% CI	<i>p</i> -value	FDR adjusted <i>p</i> -value
Glycine	2.3 $\pm$ 1.2	0.0004	0.044
Glycerol	-1.3 $\pm$ 1.0	0.0056	0.121
Taurine	5.1 $\pm$ 1.9	<0.0001	0.014
Glucose	-1.5 $\pm$ 1.0	0,0040	0.059
Myo-inositol	2.8 $\pm$ 1.3	<0.0001	0.224
Proline	1.9 $\pm$ 1.1	0.0005	0.024
Lactate	1.0 $\pm$ 0.9	0.0503	0.226
5-Oxoproline	3.1 $\pm$ 1.3	<0.0001	0.019

ES – effect size; CI – confidence interval; FDR- False Discovery Rate; * - *p*-value $\leq$ 0.05, ** - *p*-value $\leq$ 0.01, ***- *p*-value $\leq$ 0.001.

4.2.2. Metabolic alterations in the exometabolome

To assess the analytical reproducibility of the GC-MS for analysis of the exometabolome, PCA was initially conducted using the QC samples along with all samples under investigation. The resulting score plot is shown in Figure 10. The QCs are scattered across the plot, not clustered as would be expected for the analysis to be reproducible. This may be explained by the high RSDs of the metabolites identified in the exometabolome, especially when compared to those in the endometabolome, as most metabolites have RSD values above 40%.

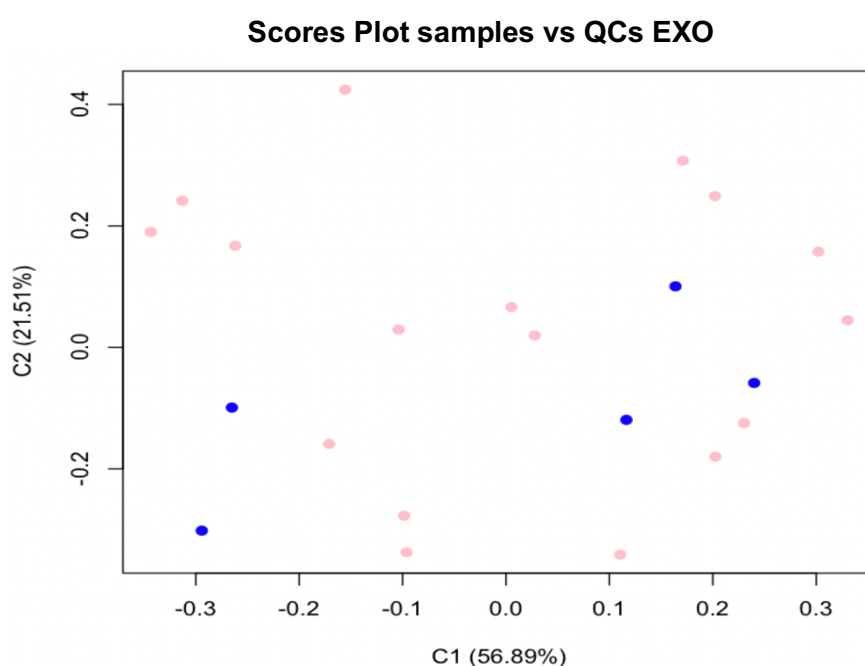


Figure 10 - PCA score plot of the exometabolome of all the samples and the corresponding QCs. Blue dots – QC's, Pink dots - Samples.

For the exometabolome, Eleven principal components were generated, which matches the number of samples in the dataset. Each component accounts for a certain percentage of the total variance in the dataset. The cumulative proportions section reveals that the first principal component alone explains nearly 49% of the total variance, indicating that almost half of the data from the 11 variables can be represented by this component. The second principal component explains 31% of the total variance. The first and second principal components account for nearly 80% of the total variance, suggesting that these two components provide a comprehensive representation of the data.

The first approach to presenting the data was a scree plot, shown in Figure 11. This plot visualizes the importance of each principal component by displaying eigenvalues in a descending curve, confirming that the first two components are particularly representative of the data, as highlighted in the PCA summary.

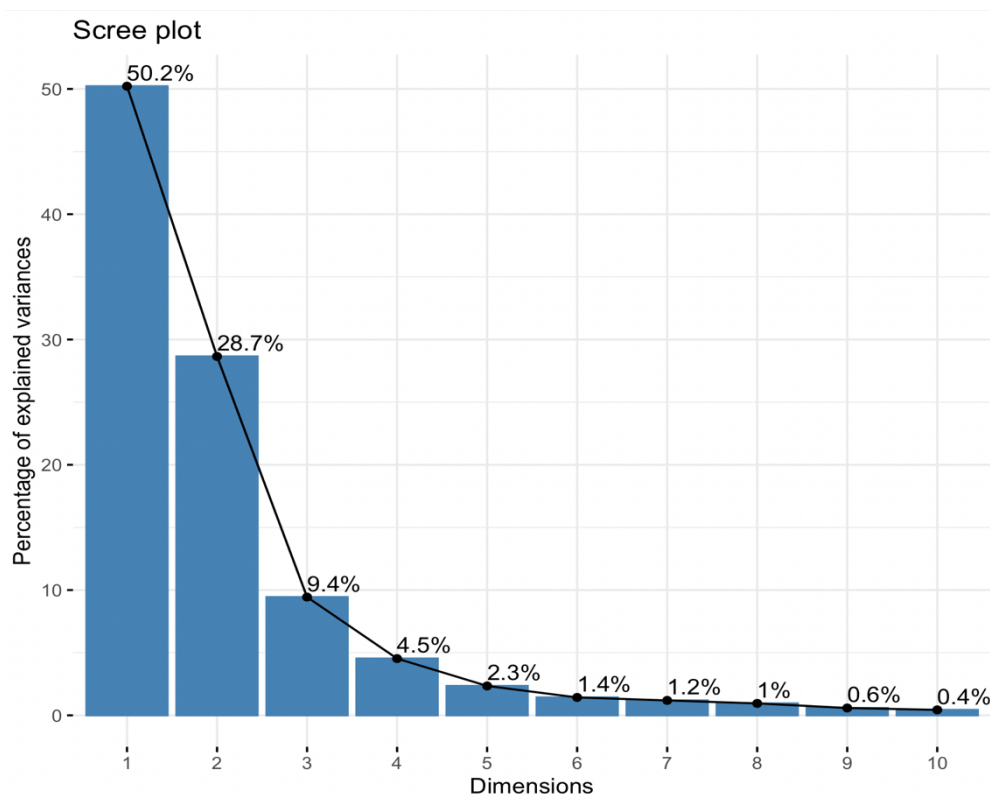


Figure 11 - Scree plot of the importance of each principal component.

PCA was also utilized to explore differences in extracellular metabolic profiles between resistant and wild-type OC cells. However, the PCA model did not effectively distinguish between these groups. Figure 12A represents the scores plot of the exometabolome of PTX-resistant cells against WT cells and the blanks (culture media without cells), where we can see a separation of the PTX-resistant cells vs blanks in the second principal component only. This is consistent with the graphics shown in Figure 13, which explains the variations in the consumption/excretion rate of metabolites, as it is higher in the extracellular medium of the PTX-resistant cells than in the blanks. On the other hand, Figure 12B shows the scores plot of RES against WT ones, where we can't see any separation.

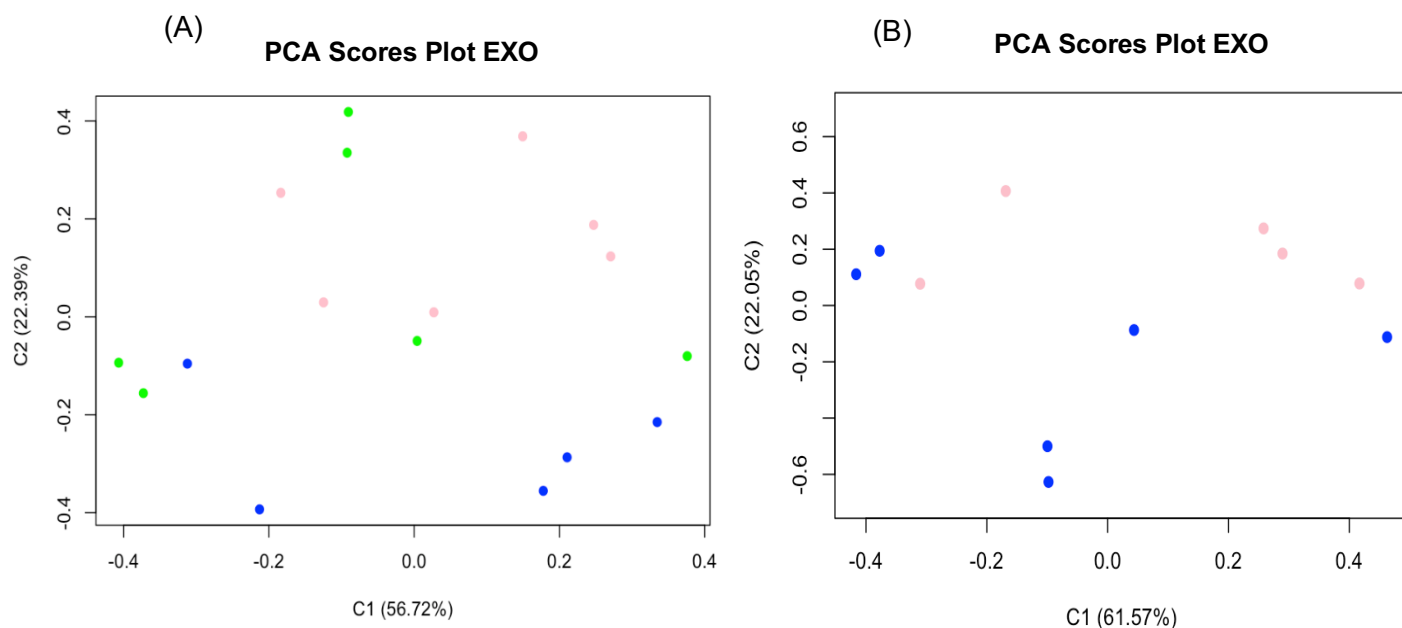


Figure 12 - (A) PCA score plot of the exometabolome of resistant cells vs. wild-type cells vs. blanks. Pink dots – Blanks, Blue dots – Resistant (RES), Green dots – wild-type (WT); (B) PCA score plot of the exometabolome of resistant cells vs. wild-type cells. Pink dots – Resistant cells; Blue dots- wild-type cells.

In the case of the exometabolome with blanks, seventeen principal components were generated, corresponding to the total number of samples analyzed. Each of these components captures a portion of the dataset's overall variance. Notably, the first principal component alone captures approximately 57% of the variance, meaning it effectively summarizes half of the information from the 17 variables. The second principal component contributes an additional 22%. Together, the first two components explain close to 80% of the total variance, highlighting their strong ability to represent the underlying structure of the data.

In Figure 13, a scree plot is presented comparing the resistant cells, wild-type, and blanks, where the eigenvalues of each principal component are displayed in descending order, allowing for the visualization of their relative importance. The steep decline in the curve confirms that the first two principal components capture the most significant portion of the data's variability, reinforcing the findings from the PCA summary.

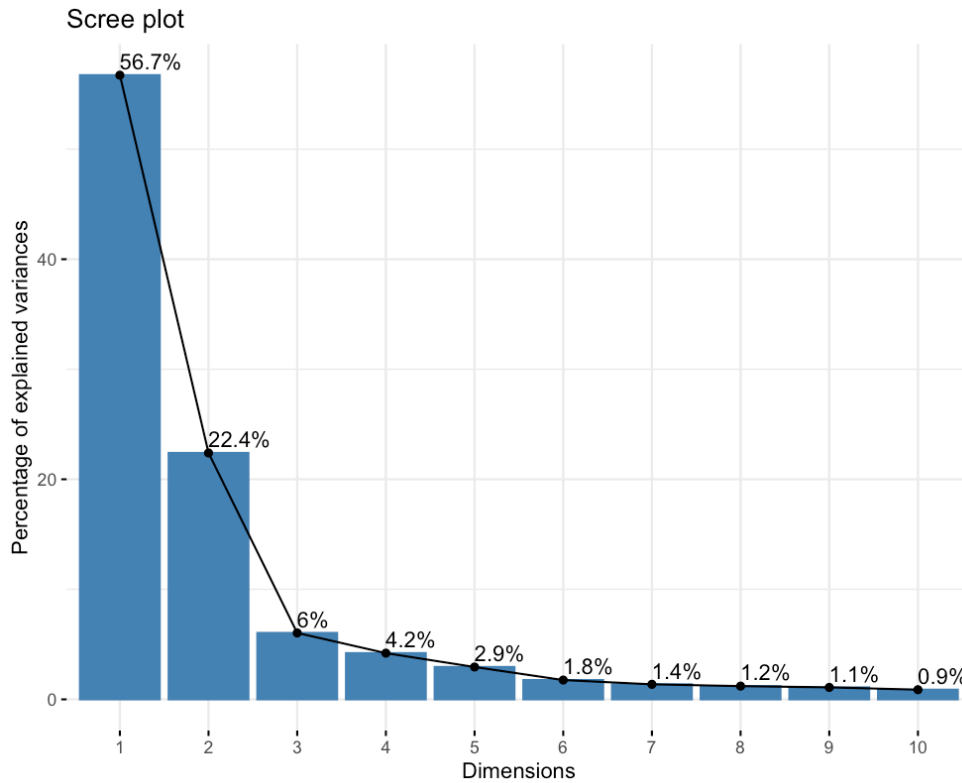


Figure 13 - Scree plot of the exometabolome of resistant cells vs. wild-type vs. blanks

Figure 14 shows the boxplots of the metabolites found in the exometabolome that are in common with the ones significantly altered in the endometabolome. Those are glycine, glucose, myo-inositol, proline, and 5-oxoproline. This analysis was conducted to gain a deeper understanding of the dynamics of consumption and excretion between resistant and wild-type cells and the surrounding medium.

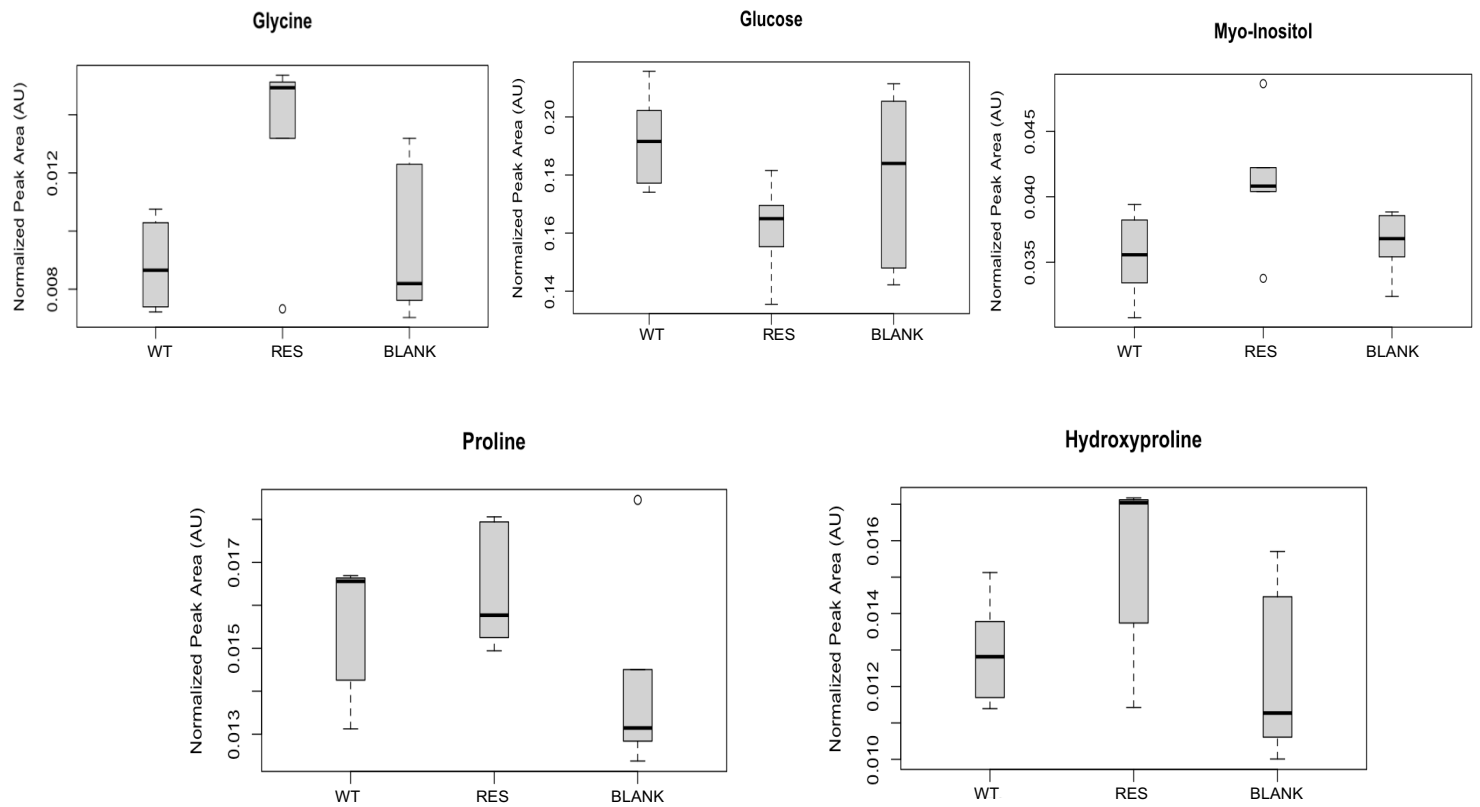


Figure 14 - Boxplots for extracellular metabolites found in common with the statistically significant metabolites identified in the endometabolome.

Table 6 presents the *p*-values of the extracellular metabolites found to be common with the significant ones on the endometabolome. These metabolites can't be considered significant, once they have a *p*-value higher than 0.05.

Table 6 - Extracellular metabolites and their respective *p*-values when comparing PTX-resistant with wild type cells.

Metabolites	<i>p</i> -value
Glycine	0.13
Glucose	0.10
Myo-Inositol	0.08
Proline	0.14
5-Oxoproline	0.12

Glycine tends to be increased in the PTX-resistant cells compared to the blank, indicating that it accumulates intracellularly and is therefore excreted into the exometabolome. In wild-type cells, its levels are similar to those in the culture medium, suggesting a balanced rate of consumption and excretion.

As for glucose, it tends to be decreased in the exometabolome of PTX-resistant cells compared to the culture medium, indicating that the cells require glucose and are actively consuming it from the medium. This result is consistent with the findings from the endometabolome, where glucose was one of the intracellular metabolites found to be decreased in drug-resistant cancer cells, since these cells use glucose as a source of energy to replicate themselves. The consumption and excretion rates in WT cells appear balanced, reflecting normal cellular metabolism.

Myo-inositol tends to be elevated in the exometabolome of resistant cells, suggesting they are excreting it, whereas in WT cells, the consumption/excretion rate appears to be normal.

In the case of proline and 5-oxoproline, both WT and resistant cells tend to have higher levels than the blank, indicating that both cell types excrete them.

4.3. Biological interpretation of metabolic alterations in PTX-resistant OC cells

A pathway analysis was conducted to better understand the resistance in OC cells. When focusing on the significantly altered metabolites found in the endometabolome (10 metabolites), various metabolic pathways are putatively involved, as shown in Figure 20. The most relevant metabolic pathways are galactose metabolism (including glycerol, glucose, myo-inositol), starch and sucrose metabolism (involving glucose), taurine and hypotaurine metabolism (taurine), arginine and proline metabolism (proline), glycerolipid metabolism (glycerol), and glutathione metabolism (5-oxoproline).

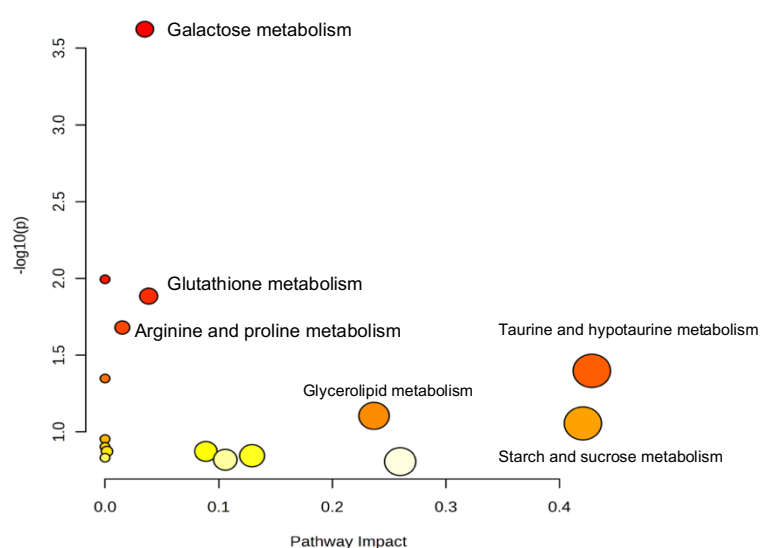


Figure 15 - Putatively altered metabolic pathways in PTX-resistant OC cells, considering only the metabolites altered in the endometabolome.

Table 7 - Putatively altered metabolic pathways in PTX-resistant OC cells considering the 10 significantly altered intracellular metabolites.

Metabolites	Type of variation	Metabolic Pathways
Glycine	Increased	Glycine, serine and threonine metabolism
Glycerol	Decreased	Galactose metabolism and glycerolipid metabolism
Taurine	Increased	Taurine and hypotaurine metabolism
Glucose	Decreased	Glycolysis / Gluconeogenesis
Myo-inositol	Increased	Inositol phosphate metabolism
Proline	Increased	Arginine and proline metabolism
Lactate	Increased	Glycolysis / Gluconeogenesis and pyruvate metabolism
5-Oxoproline	Increased	Glutathione metabolism

5. Discussion

Platinum-based drugs combined with PTX are widely recognized as the first-line therapy for OC, owing to their synergistic effects and the absence of cross-resistance (González-Martín et al., 2023). Nevertheless, the intrinsic heterogeneity of tumor cells promotes the development of chemoresistance, driven by alterations in signaling pathways such as tumor suppressor gene inhibition, metabolic reprogramming, and oncogene activation (Pokhriyal, 2019). Therapeutic resistance and subsequent disease recurrence are often driven by adaptive cellular mechanisms.

Despite the continued reliance on paclitaxel (PTX) as a fundamental component of ovarian cancer treatment, the precise biological factors underlying treatment failure and relapse in certain patients remain elusive. Metabolomics provides a robust platform for exploring the complex and evolving biochemical shifts linked to drug resistance, especially given the central role of metabolic reprogramming in resistant cancer cells. Yet, the lack of in-depth, untargeted metabolomic analyses focused specifically on PTX resistance in ovarian cancer has hindered efforts to uncover exploitable metabolic targets.

To bridge this gap, the present study employed an untargeted metabolomics strategy using GC-MS to systematically investigate the metabolic shifts associated with PTX resistance. By comparing PTX-resistant OC cells (RES) with their wild-type sensitive line (WT), an in-depth analysis of both intracellular (endometabolome) and extracellular (exometabolome) metabolites was performed. This approach enabled the capture of the biochemical dynamics underpinning cellular adaptation and survival in the context of drug resistance.

A total of 47 compounds were identified on the endometabolome and 36 on the exometabolome. Notably, some compounds, such as glucose, phenylalanine, and aspartate, have been associated with OC, showing higher levels in resistant cells to paclitaxel compared to the wild-type ones (Fong et al., 2011).

Regarding the endometabolome profile, most of the metabolites identified were amino acids and organic acids. Notably, myo-inositol, taurine, glycine, proline, and 5-oxoproline levels were significantly elevated in the resistant cells of the endometabolome, while glucose and glycerol were significantly decreased. These metabolic alterations may be associated with dysregulations in several metabolic pathways, such as galactose

metabolism, arginine and proline metabolism, glutathione metabolism, as well as taurine and hypotaurine metabolism. Interestingly, the extracellular metabolic profile showed minimal variation, suggesting that the metabolic adaptations linked to drug resistance are primarily confined to the intracellular space. This compartmentalized metabolic reprogramming may indicate an internal cellular strategy to support survival under chemotherapy; however, further investigation is necessary to better understand the underlying mechanisms of this phenomenon.

Interestingly, this study found a significant accumulation of myo-inositol in PTX-resistant cell lines. This compound has previously been explored as a potential therapeutic agent in other malignancies, including colorectal and pulmonary cancers, primarily due to its function in maintaining osmotic balance (Bizzarri et al., 2016; Q. Chen et al., 2023; Kassie et al., 2022). Myo-inositol acts as an osmolyte, regulating cell volume and potentially providing a protective effect by mitigating apoptotic signaling. Supporting this observation, Ghini et al. also reported increased intracellular myo-inositol concentrations in cell lines resistant to platinum-based therapies during a metabolomic profiling study (Ghini et al., 2022). The concurrent elevation of both myo-inositol and taurine in resistant cells suggests that osmoregulation might be a critical adaptive mechanism for PTX resistance. This raises important questions about the role of osmolyte dynamics in chemotherapy resistance and underscores the need for further research into how these molecules influence cellular survival under treatment.

This metabolomic analysis identified a marked increase in intracellular taurine levels in resistant cells, with taurine emerging as the metabolite exhibiting the highest effect size (Table 5). Taurine is known to influence cellular antioxidant defense mechanisms (Ma N. et al., 2022) and has been reported to enhance the anticancer efficacy of various chemotherapeutic agents in several malignancies (W. Chen et al., 2022; Liang et al., 2024; Okano et al., 2023). It participates in various metabolic processes, including osmoregulation, membrane stabilization, modulation of intracellular calcium levels, and detoxification. However, its role in OC appears to be complex and sometimes contradictory. For instance, while taurine has been shown to inhibit the proliferation of OC cells exposed to cisplatin, patient-derived organoid models have demonstrated a protective effect of taurine against cisplatin-induced DNA damage (Centeno et al., 2024). On the other hand, elevated intracellular taurine levels have also been linked to reduced apoptotic cell shrinkage in Pt-resistant cell lines, thereby impairing the initiation of programmed cell death (Ghini et al., 2022; Sørensen et al., 2014). In addition, a study (Bishnu et al., 2019) revealed that in resistant cells, taurine levels were found to increase

in response to metformin exposure. Based on this observation, the study manipulated taurine concentrations to investigate whether modulating its levels could help reduce cancer stem cell populations and overcome chemoresistance, suggesting a novel role for taurine in promoting differentiation of OC stem cells (Bishnu et al., 2019). Although additional research is necessary to confirm these observations, these findings highlight the potential relevance of taurine accumulation in PTX resistance. Given this complexity, further functional assays are needed to delineate whether taurine contributes to chemoresistance or could be leveraged as a therapeutic enhancer in OC.

Glutathione's involvement in chemotherapy resistance, particularly in OC, has been extensively documented (S. C. Nunes & Serpa, 2018; Simic et al., 2022). As a major antioxidant and detoxifying molecule, glutathione protects cancer cells from reactive oxygen species (ROS) and aids in the elimination of drugs, thereby enhancing cell survival (Kennedy et al., 2020). Moreover, glutathione can form conjugates with PTX, which are subsequently transported out of the cell via P-glycoprotein (P-gp), further contributing to reduced intracellular drug levels and diminished therapeutic response (S. C. Nunes & Serpa, 2018). Therapeutic strategies aimed at inhibiting glutathione synthesis may offer potential avenues for overcoming PTX resistance and re-sensitizing cancer cells to treatment.

The intracellular metabolomic profiling of PTX-resistant cells revealed potential shifts in energy-related metabolic pathways, particularly involving galactose metabolism. Notably, glucose levels were lower in resistant cells compared to their wild-type counterparts. This observation is especially relevant given that tumor cells, including those from OC, often rely on the Warburg effect—a metabolic adaptation marked by elevated glucose uptake and lactate production, even under normal levels of oxygen (Ali et al., 2024; Tondo-Steele & McLean, 2022). While excess glucose availability suppresses oxidative phosphorylation (OXPHOS), the observed decline in glucose concentration in the resistant cells may indicate a metabolic shift towards greater reliance on OXPHOS for ATP generation (Nayak et al., 2018). Prior research supports this notion, suggesting that OC cells can depend on oxidative metabolism, and that cisplatin-resistant OC cells exhibit the capacity to switch between glycolytic and oxidative energy pathways (Dar et al., 2017; Nayak et al., 2018).

Glycerol is a metabolic intermediate that can be converted into sugars and non-essential amino acids, which may fuel cell proliferation and survival in the resistant phenotype (Yan et al., 2023). Altogether, these changes underscore the adaptive metabolic

strategies adopted by PTX-resistant cells, highlighting their capacity to rewire energy metabolism to support continued growth and therapy evasion.

Alterations in amino acid metabolism, particularly involving arginine and proline pathways, were identified in PTX-resistant cells. Amino acids play a central role in aiding cancer cell survival under therapeutic pressure by helping maintain redox balance, fueling biosynthetic processes, and supplying intermediates necessary for energy metabolism (Yoo & Han, 2022). Arginine metabolism is especially significant in oncogenesis, as it contributes to both tumour cell viability and immune system modulation (D. Wang et al., 2024). For example, T-cell activation depends on arginine availability; once activated, T-cells transition from glycolysis to OXPHOS to meet their energy demands. Moreover, arginine supports the formation of memory T cells with enhanced longevity and function (Geiger et al., 2016). Given this dual functionality, targeting arginine metabolism has become an emerging therapeutic avenue, with strategies such as arginase inhibition being investigated for their potential in OC treatment (Niu et al., 2022; D. Wang et al., 2024). Preclinical studies using murine models of OC have shown that inhibiting arginase activity reduces immune suppression, thereby curbing tumour growth (Bak et al., 2008; Czystowska-Kuzmicz et al., 2019). Nonetheless, translating these findings into clinical practice remains challenging and necessitates further research.

Proline metabolism also plays a key role in cancer, influencing cellular signaling and energy dynamics critical for tumour development (Yoo & Han, 2022). In particular, previous work identified significant disruptions in proline metabolism as one of the major metabolic distinctions between OVCAR-3 cells and OC stem cells (Vermeersch et al., 2015). To fully understand the therapeutic potential of targeting arginine and proline pathways, future investigations should focus on combining metabolic interventions with current chemotherapeutic regimens and validating outcomes in more representative models, such as patient-derived organoids and *in vivo* systems.

This study represents the first metabolomics-driven investigation aimed at unraveling the metabolic shifts underlying paclitaxel (PTX) resistance in ovarian cancer (OC) cells, thereby filling a critical gap in the current knowledge of PTX-specific resistance pathways. In contrast to the well-characterized mechanisms of resistance to platinum-based therapies, the metabolic basis of PTX resistance remains poorly understood. While the use of the OVCAR8 cell line *in vitro* may not fully reflect the clinical and molecular heterogeneity of OC, it provides a consistent and controllable experimental

model. Such precision in experimental design strengthens the reliability of the metabolomic data, allowing for the detection of distinct metabolic changes associated with the development of drug resistance.

To build upon these insights, future studies should incorporate integrative omics approaches to clarify further the role of these metabolic changes in PTX resistance development. This cellular model can also be used to experimentally target these altered metabolic pathways, facilitating the investigation of potential strategies for overcoming resistance.

6. Conclusion

This study employed a metabolomics approach to identify low molecular weight compounds to better understand what causes the resistance to treatment in OC. Metabolites were detected in the endometabolome and exometabolome of PTX-resistant and wild-type OC cells by GC-MS analysis. The non-volatile compounds, such as organic acids, amino acids, fatty acids, and sugars, were analyzed using a derivatization procedure. Comparing PTX-resistant with the wild-type cells, seven significantly altered metabolites were identified in the endometabolome, namely myo-inositol, glycerol, glucose, taurine, glycine, 5-oxoproline, and proline. These results indicate that the endometabolome can aid in understanding PTX resistance in OC. The resistant phenotype is marked by enhanced antioxidant capacity—evidenced by increased levels of glutathione precursors—alongside mechanisms that support cellular adaptation to osmotic stress, as shown by the accumulation of metabolites such as taurine and myo-inositol. Additionally, alterations in energy metabolism pathways indicate a shift towards greater metabolic plasticity. Notably, disruptions in arginine and proline metabolism point to their possible involvement in fulfilling the increased biosynthetic requirements associated with drug resistance. These results highlight key metabolic pathways—namely glutathione, arginine/proline, and taurine/hypotaurine metabolism—as potential intervention points to sensitize cells to PTX. Integrating metabolomics with other omics approaches and bioinformatic tools may provide deeper insights into OC metabolism and chemotherapy resistance, and should be prioritized in future research.

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Appendix

Section 1- Normalization and scaling

Normalization of the data:

```
class(data)

str(data)

soma <- colSums(Matrizn_normalizada[,-1])

class(soma)


matriz_norm=Matrizn_normalizada

for(i in 2:24){

  matriz_norm[,i]=Matrizn_normalizada[,i]/soma[i-1]

} #fim do for
```

Transpose the matrix and set the metabolite names as the header:

```
matriz_norm_t <- t(matriz_norm)

colnames(matriz_norm_t) <- as.character(matriz_norm_t[1,])

matriz_normalizada <- matriz_norm_t[2:24,]
```

Performing Pareto scaling:

```
#Calcular a média de cada coluna

media <- colMeans(Matriz_normalizada_R[,-1])

class(media)


#Centering

centering=Matriz_normalizada_R
```

```
for (i in 2:48){
  centering[,i]=Matriz_normalizada_R[,i]-media[i-1]
}
```

#Calculate the standard deviation of each column.

```
library(matrixStats)
desvio_padrao <- colSds(as.matrix(Matriz_normalizada_R[,2:48]), na.rm=TRUE)
```

```
#Perform scaling
pareto <- centering
for(i in 2:48){
  pareto[,i]=centering[,i]/(sqrt(desvio_padrao[i-1]))
}
```

```
pareto_scaling <- pareto[, 2:48]
pareto_t <- as.data.frame(t(pareto[,2:48]))
colnames(pareto_t) <- t(pareto[,1])
```

```
#Create a table without QC's samples
scaling_sem_QCs <- pareto_t[,-(1:5)]
```

Sample of the table:

	RES_R1_ENDO	RES_R2_ENDO	RES_R3_ENDO	RES_R4_ENDO	RES_R5_ENDO	RES_R6_ENDO	RES_R7_ENDO	RES_R8_ENDO	RES_R9_ENDO	WT_R1_ENDO	WT_R2_ENDO	WT_R3_ENDO	WT_R4_ENDO	WT_R5_ENDO
Lactic Acid	0.595535728	0.075410423	-0.051176453	-0.0449041756	0.0009478496	-0.0151983644	2.476864e-01	-0.1811368675	0.1664877447	-0.0248897517	0.0441477368	-0.013753071	-0.1025041173	-0.1927685697
Alanine	0.0938463437	-0.0958446644	-0.025789485	0.036065294	0.0263094143	0.0422319349	3.740287e-02	-0.0024196492	0.2187458667	-0.0774062849	-0.0544528534	0.056387912	-0.0602735789	-0.0570464354
Glycine	0.1435146260	-0.031018219	0.117838666	0.0523414523	0.0752014996	0.0442839783	2.103017e-02	0.0839307387	0.0978900741	-0.0925411986	-0.0216802057	-0.0275084482	-0.1107455896	-0.0438146694
Oxalic Acid	0.0112509094	-0.0163177652	-0.026877000	-0.0016483240	-0.0263288312	0.0278290013	-2.022117e-02	0.0109703037	0.0141199319	0.0035149968	0.0384891410	0.009551389	-0.0093097138	-0.0037735198
Valine	0.0715558783	-0.0253640946	-0.033805415	0.0441717209	-0.0155314723	0.0570483183	2.921398e-02	0.0141798235	0.0744811649	-0.0262974760	-0.0153141084	0.008981494	-0.0370480978	-0.0203042715
Urea	0.01252213775	0.0001835367	0.024879721	0.0137845359	-0.0038226929	-0.0268840066	2.032126e-02	0.0636393372	0.0208427523	-0.0427040690	-0.0450007115	0.024049361	-0.0196059594	-0.0150302715
Serine	0.0145455412	0.0007495337	-0.010748197	0.0114913792	0.0013448589	0.0145991131	-2.864518e-02	-0.0078010585	-0.0121472855	0.0205923708	0.1207028275	-0.024952571	-0.0022045055	-0.0062991399
Glycerol	-0.7754935329	-0.0709117362	-0.060508481	-0.0511265366	-0.1857456270	-0.1469483428	-1.582449e-01	-0.0551965248	-0.2389685902	0.3294392005	-0.0571377578	0.0002617803	0.0748969306	0.1478212018
Isoleucine	0.0704840753	-0.0639229760	0.054388090	0.02711542059	0.0042491781	0.0780122866	6.134748e-02	0.0240070515	0.0639460231	-0.080955022	-0.0542745254	0.022994157	-0.1053643184	-0.0241685710
Proline	0.1013871338	0.0322150111	0.084648023	-0.0254240407	0.0059164980	0.0479751211	2.098990e-02	0.0530094712	0.1122967132	-0.1395169864	-0.0122216763	0.017748412	-0.0542945472	-0.0812679590
Succinic Acid	0.0065988452	-0.0045795684	-0.077941226	0.0022802827	-0.0135191174	0.0130678338	-1.747674e-02	-0.0319771125	-0.0264807186	0.0195149167	0.0306069454	0.023806498	0.0156713905	0.0077067745
Nonanoic Acid	0.0576372218	0.0006244111	-0.020821320	-0.0155992099	-0.0151104091	-0.0235439996	-4.420060e-03	-0.0001386028	-0.0081651578	-0.0166988423	0.0211000634	0.013777824	0.0005831059	-0.0014477491
Threonine	0.0379420925	0.0160553599	0.055772686	0.0092065815	-0.0009153850	0.0547988414	8.142730e-02	0.0309430306	0.1227236618	-0.0708062585	-0.0683172993	0.013093811	-0.0654501138	-0.0693061843
Aspartic Acid	-0.0176361893	-0.0304154992	0.002650232	-0.0251046459	-0.0369614815	-0.0248017340	-3.965706e-03	-0.0036697339	0.0112959500	-0.0413422122	0.0074110372	0.044022899	-0.0030254625	0.0083347007
β-Alanine	0.0365016030	-0.0163578952	0.012247537	0.0335491602	0.0180842108	0.0009349995	-9.637452e-03	0.0158114775	0.0168613071	-0.0043042110	-0.0039013104	-0.021221202	-0.0198021810	-0.0081411702
Malic Acid	0.0147044662	-0.0276062341	-0.005285926	-0.0163624857	-0.0191413438	0.0097385655	-5.448740e-03	-0.0060815574	0.0045214130	-0.0150504966	0.0398883000	0.039051087	-0.0052642519	0.0167257875
meso-Erythritol	0.0104941637	0.0059428454	-0.010926278	-0.0022277822	-0.0108337236	0.0246654455	-5.606139e-03	-0.0152944261	-0.0072016411	-0.0131637891	0.0264317290	0.017239844	-0.0040647374	-0.0064727506
Hydroxyproline	0.0774486579	0.0163505868	0.092485031	0.0335067692	0.0192959449	0.0456209598	5.722079e-02	0.0597361700	0.1298047560	-0.1063670107	-0.0819623790	-0.026766813	-0.0276736697	-0.0614797404
Cysteine	0.0568359995	0.0039760555	0.037143842	0.0302090745	0.0082192520	0.0067013679	2.297598e-02	0.0231514306	0.0301912078	-0.0309052329	-0.0129320592	-0.010090979	-0.0275104777	-0.0279149018
Glutamic Acid	-0.0624012820	-0.1512514870	0.019822159	-0.0970063410	-0.1041230308	-0.1682293856	-6.523834e-02	-0.0610305178	-0.0186549614	-0.0009328118	0.0397945235	0.152350180	-0.0219407799	0.1218887528
Phenylalanine	0.0179664799	0.0019607054	0.034291056	0.0165134365	0.0128648725	-0.0031886211	2.767352e-02	0.0129744625	0.0238682785	-0.0241311999	-0.0346734054	0.004079003	-0.0138548438	-0.0198698281
Asparagine	-0.0057079272	-0.0186806844	0.073898757	0.0189860857	0.0193782606	-0.0218095811	2.422523e-02	0.0199506693	0.0552686243	-0.0282676578	-0.0435746045	-0.004372947	-0.0237751335	-0.0138369481
Taurine	0.0939112297	0.0274777598	0.104724444	0.0604315152	0.0609585473	0.0245671290	6.713956e-02	0.0546341439	0.1142108801	-0.0738599989	-0.0798663836	-0.046817191	-0.0711860062	-0.0718564360
Citric Acid	-0.0333551408	-0.0400029859	-0.038106874	-0.0225799111	-0.0206539351	0.0468823245	3.834632e-05	0.0161489865	0.0561042162	-0.0473928014	-0.0130078385	-0.018179982	-0.0153731014	0.0102910275
Myristic Acid	-0.0394396835	0.0338039334	0.007069038	0.0001232985	0.0104837066	0.0110548526	8.073243e-03	-0.0089783229	0.0049080188	-0.0001225079	0.0326646370	0.013712692	-0.0068876800	-0.0008959166
Fructose	0.0071295471	-0.0043359592	-0.023678261	0.0037295280	-0.0124911631	0.0110928824	-1.911481e-02	-0.0293500459	-0.0328519311	0.0202679455	0.0304615137	0.010300253	0.01970221173	0.0040736142
Glucose	-0.0973850595	-0.1881823747	-0.138558206	0.1069825120	-0.0455512252	0.1313883819	-1.474760e-01	-0.2287264595	-0.2248815307	0.1757238894	0.1111698578	0.17700138	0.216995901	0.0552227655
Threonine	0.0075422061	0.003748314	-0.022253769	0.0118893215	-0.0232303780	0.0003100025	-1.374309e-02	-0.0308397664	-0.0272692041	0.0133378370	-0.0001305169	0.018331660	0.0129304846	-0.0085544603
Mannose	0.0171710604	-0.0501728812	-0.060047276	0.0014733545	-0.0284996583	0.0228080050	-5.979708e-02	-0.0758031746	-0.0918876766	0.0705005569	0.0659339252	0.060054949	0.0612951051	0.0123818922
Mannitol	0.0021299830	-0.0078088583	-0.004275715	0.0163025268	0.0001357496	0.0241501763	-6.394750e-03	0.0007581298	-0.0230007007	0.0170911427	0.0216651352	0.015047879	0.0046986057	0.0027287348
Tyrosine	0.0111767854	-0.0049572320	0.026514789	0.024560203	0.0154371835	-0.0068214167	2.924868e-02	0.0099653675	0.0125285844	-0.0132807849	-0.0176986991	-0.002942717	-0.0079245948	-0.0094502251
Galactose	-0.0028195886	-0.0023495458	-0.014882726	0.0082949157	-0.0022178979	0.0616143092	-8.351239e-03	0.0095618699	-0.0081047580	0.0033552600	0.0002988103	-0.011393936	0.0403667378	0.0039357310
Seylo-Inositol	0.0154906289	0.0190085593	0.010780500	-0.0138230602	0.0122571412	0.0160312899	2.288122e-04	-0.0101666549	0.0052318156	-0.0320785836	0.0477271161	0.005505115	-0.0051869734	-0.018915132
Palmitic Acid	-0.0644379067	0.0376339675	-0.061952565	-0.0071225238	-0.0114651189	0.0530141237	1.782078e-02	-0.0196294226	-0.0018799987	-0.0521465458	0.0590693368	-0.014106810	0.0504130883	0.0322504998
Myo-Inositol	0.3558114340	0.2818293628	0.206682424	-0.0118477294	0.2123904590	0.0271094327	-1.752302e-02	0.4339038862	0.1523582164	-0.2459249121	-0.0282401091	-0.185582238	-0.1492999349	-0.1635242020
1-Octadecanol	-0.0259357446	0.0469580428	0.007616358	0.0047231092	0.0098476791	-0.0011296004	5.0061092e-03	0.0453014366	-0.0001424214	-0.0022962022	0.0208015105	-0.005105389	-0.0148508670	-0.0046049809
9-Octadecanoic Acid	0.0004237174	0.0423160511	-0.004106328	-0.0107170835	0.0222200319	0.0169526730	1.219031e-02	-0.0200129581	-0.0194439596	-0.0041928374	0.0692331241	-0.010643525	0.0178961109	0.0254828360
Stearic Acid	0.0030066023	-0.0054470656	-0.028975538	-0.0047813036	0.0058674610	-0.0014285605	-4.865268e-03	-0.0228814984	-0.0028578784	-0.0316887475	0.0372923827	-0.0058565534	0.0622955185	0.0750569808
2-Palmitoylglycerol	0.0084940136	-0.0027411056	-0.024394946	-0.0018109616	0.0128327756	0.0004914425	1.235957e-03	-0.0075692189	-0.0107347195	-0.0277718499	0.0107998368	-0.015998147	0.0353186896	0.0294573892
1-Monopalmitin	0.0028673104	-0.0761613866	-0.115974763	0.0160197806	0.0394419607	0.0073208854	-1.227605e-02	-0.0661955705	-0.0668524787	-0.0342817625	0.0183652097	0.010707266	0.1094559652	0.1397950654
Sucrose	0.0063101021	0.0601402499	-0.007546071	-0.0112849883	-0.0047769995	-0.0091055744	-5.447772e-03	-0.0012463585	-0.0135444440	-0.0067691169	0.0178221619	0.004089812	0.0040446662	0.0085084430
Lactose	0.0009497224	0.0071890489	-0.003277632	-0.0065891232	-0.0019184025	-0.0070348985	-3.514008e-03	-0.0054325899	-0.0090105917	-0.0014943522	0.0202423775	0.005172471	0.0109788026	0.0043375635

Showing 1 to 41 of 47 entries, 18 total columns

Console



Section 2 - Multivariate analysis:

PCA:

```
pca <- princomp(scaling_sem_QCs)
```

```
#View(pca)
```

```
summary(pca)
```

```
tip0_am <- factor(substr(colnames(scaling_sem_QCs), 1, 3))
```

```
cores <- c("green", "red")
```

```
#Scores Plot
```

```
library(ggfortify)
```

```
autoplot(pca)
```

```
# Explained variance
```

```
v <- pca$sdev**2
```

```
#Loadings Plot
```

```
plot(
```

```
  pca$loadings,
```

```
  main = "Sample loadings",
```

```
  xlab = paste0("C1 (", round(v[1]/sum(v)*100, 2), "%)"),
```

```
  ylab = paste0("C2 (", round(v[2]/sum(v)*100, 2), "%)"),
```

```
  col = cores[tipo_am],
```

```
  pch = 19
```

```
)
```

```
d <- as.data.frame(pca$loadings[,1:2])
```

```
d$tipo_am <- tipo_am
```

```
plot(d)
```

```
# create a scatter plot with points colored by group
```

```
library(ggforce)
```

```
ggplot(d, aes(x = Comp.1,
```

```
              y = Comp.2))+
```

```
  geom_point(aes(color = tipo_am))+
```

```
  geom_mark_ellipse(aes(fill = tipo_am), expand = unit(0.5,"mm"))+
```

```
  theme(legend.position = "right")
```

#Plot of the projection of variables

```
fviz_pca_ind(pca,
             col.ind = "cos2", #cor pela qualidade da representação
             gradient.cols = c("#00AFBB", "#E7B800", "#FC4E07"),
             repel = TRUE    #Evitar a sobreposição de texto
)
```

Section 3 - Univariate analysis:

Wilcox Test for the endometabolome:

```
m<Endo_Univariada
```

```
ma<-m[,2:length(m[1,])]
```

```
ma<-t(ma)
```

#matriz n que contem os compostos por linhas e amostras por colunas

```
n<-m[,1]
```

```
n1<-as.matrix(n)
```

Create a vector with groups 0 and 1 for control and case. Then update the number of samples in each group for 0 and 1 accordingly.

```
grp<-c(rep(0,9),rep(1,9))
```

loop that generates boxplots and saves them as JPG files

```
for (i in 1:length(ma[1,]))
```

```
{
```

```
boxplot(ma[,i]~grp, boxwex=0.2,names=c("RES","WT"),ylab="Normalized Peak area
(AU)", main=n1[i,1])
```

```
savePlot(filename=paste(n1[i,1]),type=c("png"),device=dev.cur())  
}
```

Create a dummy matrix with the desired number of rows and columns to store the data.

```
d<-matrix(rep(1,length(n1[,1])*3), ncol=3)
```

Loop to calculate the p-value and the means of the 'control' and 'case' groups.

Update the sample indices in each group.

```
for (i in 1:length(ma[1,]))  
{  
  w<-wilcox.test(ma[,i]~grp, ma)
```

```
  d[i,1]<-w$p.value
```

```
  d[i,2]<-mean(ma[1:9,i])
```

```
  d[i,3]<-mean(ma[9:18,i])
```

```
}
```

```
t<-cbind(n1,d)
```

```
write.table(t,file="result_wilcox test.txt",sep="\t")
```

Kruskal Wallis for the exometabolome:

```
r<-read.table("Exounivariada.txt", header=TRUE)
```

#matriz m tem que ter amostras por colunas e compostos por linhas

```
ra <- t(r)
```

#matriz n que cont m numa coluna o nome dos ppm ou nome do composto

```

x <- row.names(r)

x1<-as.matrix(x)

# Create a vector with groups 0, 1 and 2. Then update the number of samples in each
group for 0 and 1 accordingly.

grp<-c(rep(0,6),rep(1,5),rep(2,6))

# loop that generates boxplots and saves them as JPG files
for (i in 1:length(ra[1,]))
{
  boxplot(ra[,i]~grp, boxwex=0.2,names=c("RES","WT","Blank"),ylab="Normalized Peak
Area (AU)",main=x1[i,1])

  #savePlot(filename=paste(n1[i,1]),type=c("png"),device=dev.cur())
}

# Create a dummy matrix with the desired number of rows and columns to store the
data

d<-matrix(rep(1,length(x1[,1])), ncol=1)

# Loop to calculate the p-value and the means of the 'control' and 'case' groups.
Update the sample indices in each group.

for (i in 1:length(ra[1,]))
{
  w<-kruskal.test(ra[,i]~grp, ma)

  d[i,1]<-w$p.value
}

```

```
t<-cbind(x1,d)
```

```
write.table(t,file="result_Kruskal test.txt",sep="\t")
```

Calculate FDR adjusted p-value:

```
c<-RSDendo
```

```
d<- c[,2]
```

```
d_adjusted <- p.adjust(d, method = "fdr", n = length(d))
```

```
write.table(d_adjusted,file="pvalues fdr correction.txt",sep="\t")
```