Tracing Chemical Alterations in Cancer Cells and Tissues

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Tracing chemical alterations in cancer cells and tissues

Tracing Chemical Alterations in Cancer Cells and Tissues

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LUND
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DOCTORAL DISSERTATION

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Abstract:

Cancer is a leading cause of death worldwide, with approximately ten million people dying from the disease each year. In most cases, cancer metastasizes to distant organs and develops resistance to therapy, highlighting the need for novel biomarkers for early detection. This thesis investigates the role of lipid metabolism changes, particularly involving cholesterol and related lipid species, as early biomarkers for cancer detection and therapeutic resistance. Using time-of-flight secondary ion mass spectrometry (ToF-SIMS), we explored tissue and cellular models to improve understanding of how spatial and molecular alterations in lipid profiles contribute to cancer progression and therapy adaptation.

At the tissue level, we examined the alteration of cholesterol fidelity in mammary glands of mice. We found a significant increase in the fractional abundance of cholesterol fragment peaks ($C_{27}H_{45}^+$) in cancerous tissues, suggesting dysregulated cholesterol metabolism within the tumor. In human glioblastoma multiforme (GBM), a highly aggressive brain cancer, we analyzed lipid distribution across different tumor regions and observed significant heterogeneity in cholesterol localization. These results indicate that cholesterol reprogramming is a key feature of tumor development, supporting uncontrolled growth, proliferation, and tumor heterogeneity.

At the cellular level, we investigated cisplatin-treated cancer cells, focusing on those that survived therapy. These cells exhibited a therapy-resistant phenotype, characterized by increased cell and nuclear size and the accumulation of lipid droplets. Multivariate analysis of ToF-SIMS data revealed distinct chemical profiles in lipid droplets compared to untreated cells, suggesting their role in chemotherapy resistance and cancer cell survival.

This thesis highlights the importance of lipid metabolism in cancer progression and its potential as a target for early detection and therapeutic intervention. By integrating high-resolution mass spectrometry, this research paves the way for developing diagnostic tools to detect cancer early and monitor treatment response more effectively.

Key words: cancer, cholesterol, lipid, time-of-flight secondary ion mass spectrometry, mammary glands, Glioblastoma multiforme, early detection

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Tracing Chemical Alterations in Cancer Cells and Tissues

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UNIVERSITY

Coverphoto by Worapat Sawatwong

A hare leaps fast through autumn's glow,
Where amber leaves scatter, soft winds flow.
The frost whispers winter's icy name,
Yet auroras paint skies in a fiery flame.

With each bounce, the seasons shift and change,
A timeless rhythm, both wild and strange.
Through dusk and dawn, the hare's heart beats,
Chasing the echoes of nature's feats.

Faculty of Medicine
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III. Chemical profiling of surviving cancer cells using ToF-SIMS and MCR analysis discriminates cell components
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Author's contribution

Paper I	Collaborated with co-authors in planning and designing the project. Conducted mouse experiments and performed ToF-SIMS analysis. Responsible for data analysis, interpretation, and figure preparation. Served as lead author for the manuscript and managed the journal submission and revision process.
Paper II	Contributed to data analysis and interpretation and co-wrote the first draft of the manuscript. Prepared figures for the paper. Managed the journal revision process.
Paper III	Planned and designed the project in collaboration with co-authors. Conducted cell culture experiments and performed ToF-SIMS analysis. Led data analysis and interpretation, prepared figures, and was the primary author of the manuscript. Managed the journal submission process.
Paper IV	Contributed to the planning and collection of data. Assisted in writing the first draft of the manuscript, particularly focusing on the method development to produce the surviving cells, and quantifying their cell numbers, size, and weight.

Abstract

Cancer is a leading cause of death worldwide, with approximately ten million people dying from the disease each year. In most cases, cancer metastasizes to distant organs and develops resistance to therapy, highlighting the need for novel biomarkers for early detection. This thesis investigates the role of lipid metabolism changes, particularly involving cholesterol and related lipid species, as early biomarkers for cancer detection and therapeutic resistance. Using time-of-flight secondary ion mass spectrometry (ToF-SIMS), we explored tissue and cellular models to improve understanding of how spatial and molecular alterations in lipid profiles contribute to cancer progression and therapy adaptation.

At the tissue level, we examined the alteration of cholesterol fidelity in mammary glands of mice, finding a significant increase in the fractional abundance of cholesterol fragment peaks ($C_{27}H_{45}^{+}$) in cancerous tissues. This suggests that dysregulated cholesterol metabolism within the tumor can be traced via the molecular structure of cholesterol. In human glioblastoma multiforme (GBM), a highly aggressive brain cancer, we analyzed lipid distribution across different tumor regions and observed significant heterogeneity in cholesterol localization. These results indicate that cholesterol reprogramming is a key feature of tumor development, supporting uncontrolled growth, proliferation, and tumor heterogeneity.

At the cellular level, we investigated cisplatin-treated cancer cells, focusing on those that survived therapy. These cells exhibited a therapy-resistant phenotype, characterized by increased cell and nuclear size and the accumulation of lipid droplets. Multivariate analysis of ToF-SIMS data revealed distinct chemical profiles in lipid droplets compared to untreated cells, suggesting their role in chemotherapy resistance and cancer cell survival.

This thesis highlights the importance of lipid metabolism in cancer progression and its potential as a target for early detection and therapeutic intervention. By integrating high-resolution mass spectrometry, these insights may pave the way for developing diagnostic tools to detect cancer early and monitor treatment response more effectively.

Popular science summary

Cancer remains one of the leading causes of death worldwide, with millions of people dying from the disease each year. Early detection and effective monitoring of treatment response are crucial in improving survival rates. However, current methods often fail to detect tumors at their earliest stages and struggle to predict therapy resistance. This thesis investigates how changes in lipid metabolism, particularly those involving cholesterol and related lipid species, could serve as early biomarkers for cancer detection and therapeutic resistance.

Lipids, including cholesterol, play a vital role in the structure and function of cells. Cancer cells often alter their lipid metabolism to support rapid growth, survival, and spread, making these changes potential indicators of cancer. In this research, we used a powerful technique called time-of-flight secondary ion mass spectrometry (ToF-SIMS) to explore how lipid profiles change in both cancer tissues and cells. By studying these changes, we aimed to uncover new ways to detect cancer early and track how it responds to treatment.

At the tissue level, we first examined how cholesterol metabolism is altered in the mammary glands of mice. In cancerous tissue, we observed a significant increase in the abundance of cholesterol-related fragments, indicating that the cancer cells had reprogrammed their cholesterol metabolism. This suggests that changes in lipid metabolism, especially cholesterol, are a key feature of tumor development. In human glioblastoma multiforme (GBM), an aggressive brain tumor, we also discovered significant variations in how cholesterol was distributed across different regions of the tumor. These findings highlight the complexity of cancer and its ability to adapt by reprogramming its metabolism, which may help tumors grow and become more heterogeneous.

At the cellular level, we studied how cancer cells treated with cisplatin, a chemotherapy drug, adapt to survive. We found that the surviving cells developed a therapy-resistant phenotype, characterized by larger cell and nuclear sizes, as well as the accumulation of lipid droplets. These lipid droplets, which store fats, were found to have unique chemical profiles compared to untreated cells. This suggests that lipid droplet formation could play an important role in chemotherapy resistance and help cancer cells survive in hostile environments.

This thesis emphasizes the crucial role of lipid metabolism in cancer progression and how these alterations could be traced from a molecular perspective. By combining high-resolution mass spectrometry with detailed chemical analysis, this research offers a new perspective on how we might detect cancer earlier, monitor treatment response, and improve therapeutic strategies. Understanding lipid alterations in cancer cells can pave the way for developing innovative diagnostic tools that can detect cancer at its earliest stages and monitor how well treatments are working.

Populärvetenskaplig sammanfattning

Cancer är en av de främsta dödsorsakerna globalt, med miljontals människor drabbade varje år. Tidig upptäckt är avgörande för att förbättra chanserna att överleva sjukdomen. Nuvarande metoder misslyckas dock ofta med att upptäcka tumörer i ett tidigt stadium. Vi har dessutom svårt att förstå och förutsäga terapiresistens. Denna avhandling undersöker hur förändringar i lipidmetabolismen, särskilt de som involverar kolesterol och relaterade lipider, kan fungera som tidiga biomarkörer för cancerupptäckt och terapeutisk resistens.

Lipider, inklusive kolesterol, spelar en viktig roll i cellers struktur och funktion. Cancerceller ändrar ofta sin lipidmetabolism vilket bidrar till snabb tillväxt, överlevnad och spridning. Ändrad lipidmetabolism kan därför vara potentiellt avslöja cancern. I denna studie använde vi tekniken som kallas time-of-flight sekundär jon-masspektrometri (ToF-SIMS) för att undersöka hur lipidprofiler, nivåerna av olika lipider, förändras i både cancervävnader och celler. Genom att studera dessa förändringar strävade vi efter att avslöja nya sätt att tidigt upptäcka cancer och följa hur den svarar på behandling.

På vävnadsnivå undersökte vi först hur kolesterolmetabolismen förändras i mössens bröstkörtlar. I tumörvävnad observerade vi en betydande ökning av förekomsten av kolesterolrelaterade fragment, vilket tyder på att cancercellerna hade omprogrammerat sin kolesterolmetabolism. Detta tyder på att förändringar i strukturen av kolesterol kan ändras till en nivå som i framtiden kan avslöja att tumörutveckling pågår i kroppen. Vid glioblastom multiforme (GBM), en aggressiv hjärntumör, upptäckte vi också betydande variationer i hur kolesterol fördelades över olika delar av tumören. Dessa resultat belyser cancercellernas förmåga att anpassa sig genom att omprogrammera sin metabolism, vilket kan leda till att tumörer växer och bli mer heterogena.

På cellulär nivå studerade vi hur cancerceller som behandlades med cisplatin, ett kemoterapeutiskt läkemedel, anpassade sig för att överleva. Vi fann att de överlevande cellerna utvecklade en terapiresistent fenotyp, kännetecknad av större cellstorlek, större kärna samt att fetter ansamlats i cellen. Dessa ansamlingar av fetter har unika kemiska profiler jämfört med obehandlade celler, vilket kan bidra till cancercellernas anpassning till ogynnsamma miljöer.

Sammanfattningsvis betonar denna avhandling den avgörande rollen för lipidmetabolism vid cancerprogression och att dessa förändringar kan bidra till nya sätt att upptäcka tumörtillväxt. Genom att kombinera högupplöst masspektrometri med detaljerad kemisk analys ger denna forskning ett nytt perspektiv på hur vi kan upptäcka cancer tidigare, övervaka behandlingens svar och förbättra terapeutiska strategier. Att förstå lipidförändringar i cancerceller kan bana vägen för att utveckla innovativa diagnostiska verktyg som kan upptäcka cancer i ett tidigt skede och följa hur väl behandlingarna fungerar.

Abbreviations

AFP	Alpha-fetoprotein
AI	Artificial intelligence
CA	Cancer antigen
CTCs	Circulating tumor cells
ctDNA	Circulating tumor deoxyribonucleic acid
DAG	Diacylglycerol
DBT	Digital breast tomosynthesis
DESI	Desorption electrospray ionization
DNA	Deoxyribonucleic acid
DPT	Day post treatment
EI	Electron ionization
ESI	Electrospray ionization
FA	Fatty acid
GBM	Glioblastoma multiforme
GC-MS	Gas chromatography mass spectrometry
GCIB	Gas cluster ion beam
HCC	Hepatocellular carcinoma
HPV	Human papillomavirus
HR-MS	High resolution mass spectrometry
ICP-MS	Inductively coupled plasma mass spectrometry
IHC	Immunohistochemistry
IRMS	Isotope ratio mass spectrometry
LC-MS	Liquid chromatography mass spectrometry
LMIG	Liquid metal ion gun
m/z	Mass to charge ratio
MAG	Monoacylglycerol
MALDI	Matrix-assisted laser desorption/ionization
MCR	Multivariate curve resolution

MMTV	Mouse mammary tumor virus
MRI	Magnetic resonance imaging
MRMS	Magnetic resonance mass spectrometry
MS	Mass spectrometry
MSI	Mass spectrometry imaging
MVA	Multivariate analysis
PBS	Phosphate-buffered saline
PC	Phosphatidylcholine
PCA	Principal component analysis
PE	Phosphatidylethanolamine
PET	Positron emission tomography
PI	Phosphatidylinositol
PLS-DA	Partial least squares discriminant analysis
PSA	Prostate-specific antigen
PyMT	Polyomavirus middle T antigen
RNA	Ribonucleic acid
SIMS	Secondary ion mass spectrometry
TAG	Triacylglycerol
ToF-SIMS	Time of flight secondary ion mass spectrometry
WT	Wild-type

Early detection of cancer

Cancer is a major global health issue, affecting millions of individuals and families every year. Globally, around 20 million people are diagnosed with cancer annually, with this number expected to rise as populations grow and age.[1] Cancer is also a leading cause of mortality, responsible for approximately 10 million deaths each year, making it one of the most lethal diseases worldwide.[1] The significant physical, emotional, and economic toll on patients, their families, and healthcare systems underscores the urgent need for effective cancer prevention, detection, and treatment strategies.

The need for early detection of cancer

Early detection of cancer is a cornerstone to improve patient outcomes, offering a greater chance for curative treatment and long-term survival. Identifying cancer at its earliest stages, when it is still localized, significantly broadens treatment options, including surgery, radiation, and targeted therapies, which are most effective when tumors are small and confined.[2] Early-stage cancers are typically less aggressive, more responsive to treatment, and less likely to have developed resistance to standard therapies.[3] For instance, the five-year survival rate for early-detected breast cancer is nearly 99%, compared to less than 30% for late-stage diagnoses.[2] Similar trends are seen in colorectal and cervical cancers, where timely intervention drastically improves survival rates and reduces the need for invasive treatments.[2]

Advances in screening technologies have improved early detection for several cancers, including breast, colorectal, lung, prostate, and cervical cancer. The United States Preventive Services Task Force (USPSTF) recommends routine screenings (Figure 1),[4] with typical sensitivities of 70% to 80% and specificities ranging from 60% to 70%.[5] However, these screening methods are not without limitations. Challenges such as lead-time bias, overdiagnosis, and false positives can lead to unnecessary interventions, causing anxiety and potential harm.[4] Compliance with screening programs also varies, with participation rates ranging from 69% to 80% for breast, cervical, and colorectal cancers, but dropping to just 5% for lung cancer.[6-9] Disparities in screening rates persist among underserved racial and ethnic groups, highlighting the need for equitable access to preventive care.[10]

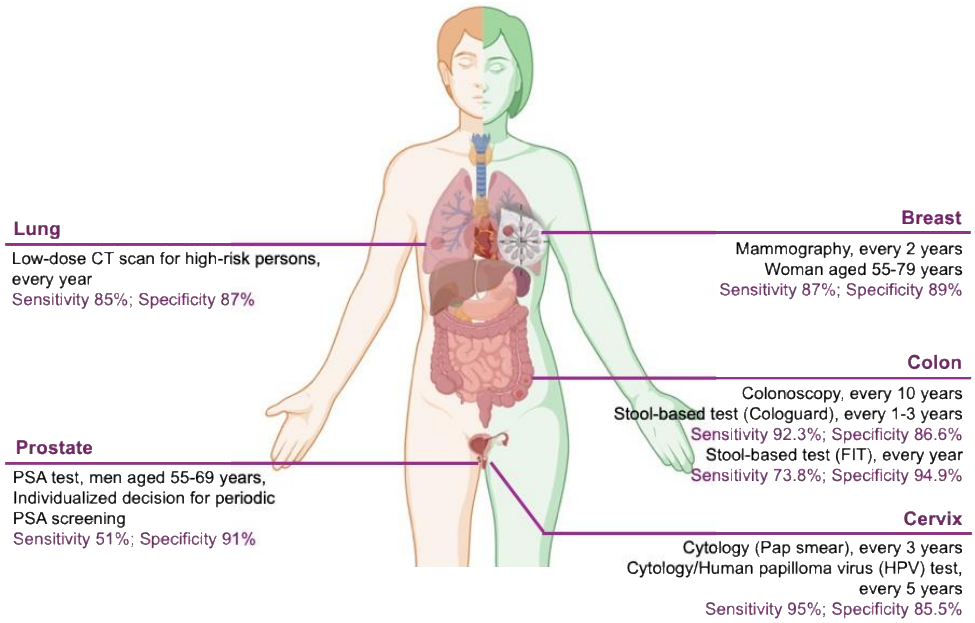

Figure 1. The United States Preventive Services Taskforce (USPSTF) recommended screening test for cancer (adapted from Kohaar et al.[4]).

Cancer's broader societal impact highlights the urgency of improving early detection. It is a leading cause of death worldwide, responsible for nearly 10 million deaths annually, and places a significant economic burden on healthcare systems.[2] As cancer progresses to advanced stages, it becomes more resistant to treatment, making therapy less effective, more complex, and increasingly expensive.[11] The adaptability of cancer cells, which enables them to evade the immune system and withstand various treatments, presents a major challenge in achieving a permanent cure.[12] Overcoming this challenge requires continued research into more precise early detection technologies, expanded access to screening programs, and increased public awareness of the importance of routine cancer screenings.[2]

Investing in early detection not only improves patient outcomes but also reduces the strain on healthcare systems, decreases treatment costs, and improves overall societal well-being.[2] By prioritizing early diagnosis, we can significantly reduce the global cancer burden, improve survival rates, and ultimately save millions of lives.

With advances in technology and a growing understanding of cancer biology, the focus is shifting toward developing innovative methods to identify cancer at its earliest stages. These approaches aim to detect subtle changes in the body that indicate the presence of a tumor long before clinical symptoms appear. Imaging-based, blood-based biomarkers, and cytology-based and molecular screening methods are at the forefront of early detection strategies. By detecting cancer when

it is most treatable and by tailoring treatment to the tumor's specific profile, these methods significantly improve patient outcomes, increase survival rates, and reduce the need for aggressive treatments.[4]

Imaging-based approaches for early detection of cancer

Imaging technologies remain fundamental tools for early cancer detection, allowing clinicians to visualize and identify tumors at their initial stages. These methods provide critical insights into tumor size, location, and morphology before symptoms become apparent.[5]

Mammography is one of the most widely used imaging tools for early breast cancer detection, specifically designed to identify abnormalities in breast tissue. It is particularly effective in detecting microcalcifications, tiny deposits of calcium that can be an early indicator of malignancy, as well as small masses that might not yet be palpable.[13] Modern mammography systems include digital mammography, which provides higher-resolution images for better analysis, and 3D mammography or digital breast tomosynthesis (DBT), which generates a three-dimensional reconstruction of the breast. DBT reduces overlapping tissue effects, increasing the sensitivity and accuracy of cancer detection, particularly in women with dense breast tissue.[14] The integration of artificial intelligence (AI) in mammographic analysis further increases its diagnostic power by aiding radiologists in identifying suspicious lesions and minimizing false positives or negatives.[15]

Similarly, low-dose CT scans are effective in detecting small lung nodules in high-risk individuals, such as smokers, improving the early diagnosis of lung cancer.[16] This method has been shown to reduce mortality in lung cancer screening programs by identifying tumors at stages where they are most treatable.[17]

Other imaging techniques complement mammography in cancer detection. Magnetic resonance imaging (MRI) is particularly valuable in detecting early-stage cancers in dense tissues like the brain, liver, and breasts, due to its superior resolution and ability to differentiate between soft tissue types.[18] Colonoscopy, though primarily an endoscopic procedure, incorporates real-time visualization of the colon and rectum to detect early polyps or lesions, making it the gold standard for early detection of colorectal cancer.[19]

Blood-based biomarkers for early detection of cancer

Blood-based biomarkers have revolutionized cancer screening by offering a minimally invasive, efficient, and repeatable means of identifying early

malignancies.[20-22] These biomarkers are molecules present in the blood that correlate with tumor development or progression, providing valuable diagnostic and prognostic information. By enabling the detection of tumors at an early stage, when treatments are most effective, they hold immense potential to improve cancer outcomes.

Alpha-fetoprotein (AFP) is one of the most well-established blood-based biomarkers, commonly used for detecting hepatocellular carcinoma (HCC).[23] Elevated levels of AFP in the blood can signal liver cancer or other liver-related conditions, allowing clinicians to identify tumors earlier, particularly in high-risk populations such as individuals with chronic hepatitis or cirrhosis. Regular monitoring of AFP levels, in conjunction with imaging techniques, has improved the early detection and management of HCC.

Another significant biomarker is prostate-specific antigen (PSA), widely used for early detection of prostate cancer. PSA testing is particularly valuable for screening asymptomatic individuals, helping to identify prostate cancer in its localized stages. Although PSA testing has limitations, such as false positives due to non-cancerous prostate conditions, it remains a cornerstone of prostate cancer screening.[24]

For ovarian cancer, cancer antigen 125 (CA-125) is an important biomarker. Elevated CA-125 levels are associated with ovarian malignancies, and its use is particularly effective in screening high-risk individuals or monitoring disease progression.[25] Combining CA-125 with other biomarkers and imaging tools improves its specificity and sensitivity, making it a more reliable tool for early detection.

While most blood-based biomarkers for early cancer detection are proteins, such as AFP, PSA, or CA-125, lipids are emerging as a promising class of biomarkers due to their significant roles in cancer biology. Lipids are fundamental components of cell membranes and serve as signaling molecules, energy storage units, and mediators of metabolic pathways, all of which can be altered in cancer.[26] For instance, altered levels of specific phospholipids, sphingolipids, and fatty acids have been associated with various cancers. Abnormal accumulation of lipid species, such as lysophosphatidylcholine (LPC)[26] and monoacylglycerols (MAGs),[27] has been linked to tumor growth and metabolic reprogramming. These lipid changes often reflect the increased lipid synthesis and turnover required to support the rapid proliferation of cancer cells.

In certain cancers, such as ovarian and colorectal cancers, changes in lipid metabolism can be detected in the blood. For example, elevated levels of cholesterol derivatives[28] have been observed in cancer patients, suggesting that these lipids may serve as early indicators of malignancy. The disruption of fatty acid oxidation pathways has been noted in various cancers, further supporting the potential of lipid biomarkers in early detection.[29]

Recent advances in biomarker research have introduced innovative tools for cancer screening, with liquid biopsies emerging as a transformative approach for early detection.[30] These minimally invasive techniques enable the detection of cancer-specific mutations and tumor dynamics through a simple blood draw, offering real-time insights into tumor behavior. Liquid biopsies are particularly promising for identifying cancers such as lung, breast, and colorectal cancer at early stages, improving the chances of effective intervention.[31] A key feature of liquid biopsies is their ability to analyze tumor-derived components in the bloodstream. Circulating tumor DNA (ctDNA) consists of small DNA fragments released by tumor cells, carrying genetic and epigenetic alterations that reflect the tumor's molecular profile.[31] Similarly, circulating tumor cells (CTCs) are intact cancer cells that detach from primary or metastatic tumors and enter the bloodstream, providing valuable insights into the biological properties of the tumor, including its metastatic potential.[31] By leveraging ctDNA and CTCs, liquid biopsies offer a dynamic and non-invasive approach to cancer detection, monitoring, and treatment planning. These advances represent a significant step forward in precision oncology, enabling earlier diagnosis and personalized care.

Together, blood-based biomarkers complement imaging-based strategies to improve the early detection of cancer. By integrating validated biomarkers with advanced technologies like liquid biopsy and AI-driven analysis, cancer care is shifting towards less invasive, more accurate, and globally accessible screening methods.[32] These advances offer hope for reducing cancer mortality and improving patient outcomes through earlier interventions.

Cytology and molecular screening for early detection of cancer

Cytology-based and molecular screening methods are essential tools in the early detection of cancers, particularly those with well-defined cellular or molecular precursors. These approaches analyze cells or DNA collected from the body to identify abnormalities that could signify early-stage cancer or precancerous conditions.[33] By enabling timely detection, these methods contribute significantly to cancer prevention and management. The pap smear is one of the most established cytology-based screening tests, primarily used to detect early changes in cervical cells that may lead to cervical cancer.[33] By examining exfoliated cells from the cervix under a microscope, clinicians can identify dysplasia or other abnormalities long before they develop into invasive cancer. This method has been instrumental in significantly reducing cervical cancer incidence and mortality worldwide.

Molecular screening for human papillomavirus (HPV), the primary cause of cervical cancer, has improved early detection strategies.[34] High-risk HPV DNA testing is

often performed alongside pap smears, allowing for a more comprehensive assessment of a patient's risk. This combined approach provides a reliable method for identifying women at increased risk of developing cervical cancer, facilitating earlier intervention.[34]

Cytology-based methods also extend to other cancers beyond cervical cancer. For instance, urine cytology is used to detect bladder cancer by analyzing exfoliated cells from urine. Similarly, sputum cytology can aid in the early detection of lung cancer, particularly in high-risk individuals.[35] These tests rely on the principle of identifying atypical or malignant cells shed into bodily fluids or accessible tissue samples.

Chemistry underpinning cancer biomarkers

Cancer is not only a biological phenomenon but also a complex chemical process.[36] The transformations that occur within cancer cells, such as their ability to switch metabolic pathways and consume higher levels of energy to sustain rapid division and growth, are deeply rooted in chemical changes.[37] By studying these chemical processes, researchers can gain a more detailed understanding of cancer biology, potentially uncovering new avenues for early detection.

One of the hallmarks of cancer cells is their altered metabolism, often described as the Warburg effect. In this phenomenon, cancer cells predominantly rely on glycolysis for energy production, even in the presence of sufficient oxygen, a process typically reserved for anaerobic conditions. This metabolic shift enables cancer cells to consume large amounts of glucose, producing ATP to meet the energy demands of their rapid and uncontrolled growth.[38,39] This shift is not limited to carbohydrate metabolism but also extends to lipid metabolism, which undergoes significant reprogramming in cancer cells.[37]

Lipid metabolism in cancer cells is crucial for supporting their aggressive growth and survival.[40,41] Cancer cells often exhibit elevated levels of lipogenesis, the process of synthesizing lipids from acetyl-CoA and malonyl-CoA.[42] This heightened lipogenesis provides essential components for building cellular membranes, signaling molecules, and energy storage, all of which are critical for maintaining rapid proliferation.[40,43] In parallel, cancer cells show increased uptake and utilization of exogenous lipids from their environment, further fueling their metabolic needs.[42,43]

Alterations in lipid metabolism are closely linked to the generation of bioactive lipid species, such as fatty acids, phospholipids, and sterols.[44] These molecules play key roles in modulating signal transduction, cell motility, and evasion of apoptosis. For instance, changes in the composition of membrane lipids can influence the fluidity and function of cellular membranes, impacting processes like nutrient transport and signaling pathways.[40] Abnormalities in fatty acid oxidation, a major pathway for energy production, are also frequently observed, enabling cancer cells to adapt to metabolic stress and nutrient scarcity.[44]

In recent years, clinics and research institutions have generated extensive datasets to uncover genetic and environmental factors that contribute to cancer risk, as well as to identify molecular targets for novel diagnostic and therapeutic strategies.[45] By analyzing these datasets, scientists have uncovered previously unknown patterns and markers, offering deeper insights into the mechanisms driving cancer development and progression.

A key aspect of this research involves understanding the chemical changes associated with cancer. These changes include both quantitative shifts, such as alterations in biomolecule concentrations, and qualitative modifications, including isotopic variations, distribution changes, and structural transformations. Examining these chemical dimensions reveals how cancer cells sustain their rapid growth, adapt to various environments, and evade conventional therapies. This section explores how cancer affects molecular chemistry, highlighting how these changes provide insights into cancer biology.

Studies of cancer-related biomolecules

Biomolecules such as lipids, proteins, carbohydrates, and nucleic acids are fundamental to cellular processes. In cancer, these biomolecules undergo significant changes in response to genetic mutations, metabolic demands, and microenvironmental stress.[46] These alterations can occur at different levels, including concentration, distribution, or molecular structure, improving the understanding of cancer biology and making them valuable tools for tracing cancer, monitoring its progression, and unraveling mechanisms of therapeutic resistance.

Cancer-specific alterations to the concentration of biomolecules

Quantifying the concentration of biomolecules, such as lipids, proteins, metabolites, and nucleic acids, is a powerful tool in cancer research.[12] These measurements often reveal critical insights into cancer-specific biochemical processes, making concentration analysis an essential step in identifying reliable biomarkers. Biomarkers, defined as specific molecules or combinations of molecules, offer early insights into cancer presence or progression by reflecting changes in their concentrations.[12,47]

Cancer cells exhibit unique biochemical behaviors, including reprogrammed metabolism and rapid cellular proliferation, which result in significant variations in biomolecule concentrations compared to normal cells.[37] For instance, increased levels of certain metabolites, lipids, or proteins may signal increased energy

production, biosynthesis, or signaling pathways that support tumor growth and survival.[48]

Advanced analytical techniques, such as mass spectrometry (MS), play a crucial role in these studies. Approaches like gas chromatography-MS (GC-MS) and liquid chromatography-MS (LC-MS) are widely used to measure biomolecule concentrations with high sensitivity and precision.[49,50] For example, LC-MS analyses of lipid profiles across various cancer types using various biological samples, such as serum,[51] plasma,[52] urine,[53] and tissue,[54] have uncovered critical mechanisms underlying cancer development and progression. These technologies enable researchers to distinguish between healthy and cancerous samples, facilitating early detection and better understanding of cancer biology.

Cancer-specific alterations to the distribution of biomolecules

In addition to changes in biomolecule concentration, cancer cells exhibit significant alterations in the spatial distribution of key biomolecules such as lipids, proteins, metabolites, and nucleic acids.[55] These distributional changes often reflect the unique biochemical and structural demands of cancerous cells and tissues, driven by processes like rapid cell proliferation, angiogenesis, altered extracellular matrix composition, and metabolic reprogramming.[37] Such spatial rearrangements play a crucial role in cancer progression, therapeutic resistance, and metastasis.

One of the hallmarks of cancer is its spatial heterogeneity, where different regions within a tumor display distinct molecular compositions.[37] For example, lipids required for membrane synthesis may accumulate in highly proliferative regions, while metabolites like lactate concentrate in hypoxic (low oxygen) zones.[56] This heterogeneity impacts tumor behavior and its response to treatment, making it essential to understand how biomolecules are distributed within cancerous tissues.

Advanced imaging techniques are used to examine these distributional changes. Mass spectrometry imaging (MSI) provides spatial maps of metabolites within cells and tissue sections. Matrix-assisted laser desorption/ionization (MALDI), desorption electrospray ionization (DESI), and secondary ion MS (SIMS) represent the primary ionization techniques employed in MSI.[55,57] Each method offers distinct advantages and specialized capabilities for various applications (Table 1). Lipid distribution analysis using MSI has been applied to tumor diagnosis, classification, and grading.[58-61] For example, a MALDI-MSI technique involving enzymatic lipid digestion was initially used to study sphingomyelins and ceramides in lung tissue and later adapted to clear cell renal cell carcinoma biopsies, successfully identifying biomarkers that distinguish between recurrent and non-recurrent disease.[62] Similarly, DESI-MSI demonstrated a localized enrichment of arachidonic acid within the tumor regions of basal cell carcinoma biopsies, underscoring the importance of lipid distribution in cancer pathology.[63] SIMS imaging of human

breast tissue biopsies revealed distinct variations in fatty acid composition between tumor and stromal regions, which corresponded to areas identified in consecutive H&E-stained sections, further emphasizing the heterogeneous nature of tumor microenvironments.[58] MALDI MSI is a highly effective technique for mapping protein distribution, with the ability to analyze biomolecules up to 100 kDa.[64] This broad mass range allows for the simultaneous detection and spatial visualization of hundreds of peptides and proteins directly from tissue sections, providing a detailed molecular profile of the sample.[65-68] Raman and infrared spectroscopy enable the visualization of biomolecules such as proteins and lipids based on vibrational properties,[69-72] such as the visualized signals of lipids and protein from CH_2 and CH_3 vibrations in fresh brain tissues. The multicolor coherent Raman images showed almost identical morphological information compared with the corresponding histopathological images.[73]

Table 1. Comparison of key parameters for MALDI, DESI, and SIMS mass spectrometry imaging (MSI) (adapted from Ma et al., 2022[55]).

	MALDI	DESI	SIMS
Required sample preparation (e.g., tissue embedding and sectioning)	Matrix deposition	None	Freeze fracturing and drying for subcellular imaging
Ionization conditions	Atmospheric/medium/high vacuum	Open atmosphere	Ultrahigh vacuum
Tissue conservation method	Fresh frozen/formalin-fixed paraffin embedding	Fresh frozen	Fresh frozen
Typical spatial resolution	5-200 μm	50-200 μm	0.05-100 μm (subcellular level imaging possible)
Compound coverage	Small molecule metabolites, lipids, peptides, and proteins (<50 kDa)	Small molecule metabolites, lipids, peptides, and proteins (<50 kDa)	Chemical elements, small molecule metabolites, and lipids, fragment ions (<1000 Da)
Destructive/non-destructive nature	Minimally destructive	Minimally destructive	Destructive

In this thesis, we focused on investigating the spatial distribution of lipids and cholesterol in cancer cells and tissues using mass spectrometry imaging (MSI). Lipids and cholesterol are essential components of cellular membranes and play critical roles in various biological processes, including energy storage, membrane fluidity, cell signaling, and intracellular transport.[74] Their altered distribution in

cancer is a hallmark of tumor progression, metastasis, and therapeutic resistance, making them key targets for cancer research.

Lipid metabolism and distribution are extensively reprogrammed in cancer cells to support rapid proliferation and ensure survival under hostile conditions.[40,43] Cancer cells often exhibit elevated lipid synthesis, accumulation of lipid droplets, and altered lipid transport mechanisms.[75,76] These changes are not merely a byproduct of cancer metabolism, but actively contribute to tumor growth by providing structural components for membrane biogenesis, signaling molecules for proliferation, and energy reserves to sustain high metabolic activity.[76]

Among the various lipids, cholesterol plays a particularly critical role in cancer development and progression.[77,78] Cholesterol is a fundamental component of lipid rafts in the plasma membrane that regulate signaling pathways involved in cell proliferation, migration, and survival.[79] Cancer cells often show dysregulated cholesterol homeostasis, leading to its accumulation in specific subcellular regions, further increasing their malignancy potential.[80]

Studying the spatial distribution of lipids and cholesterol in cancer provides valuable insights into tumor biology that cannot be obtained through bulk analysis alone. While bulk lipidomics can quantify the overall levels of lipids in a sample, it fails to capture their localization within different cellular compartments or tumor regions.[55,81] MSI addresses this limitation by providing high-resolution spatial maps of lipid and cholesterol distribution within intact tissue sections.[55]

Cancer-related alterations to the molecular structure of biomolecules

Life is fundamentally sustained by the complex interplay between atoms, metabolism, and genetics, all of which rely on the chemistry of the universe's most abundant elements: hydrogen, oxygen, nitrogen, sulfur, phosphorus, and carbon. These elements are the building blocks of living organisms, where the coordination of atomic, metabolic, and genetic processes enables the organization and reorganization of chemical information. This intricate equilibrium is essential for maintaining the structure and functionality of all living entities, including cancer cells.[82] However, cancer, as a pathological condition, disrupts this harmony through its uncontrolled proliferation and altered metabolic needs, resulting in profound changes to the molecular structure of biomolecules.[82]

Cancer cells exhibit extraordinarily high rates of biomolecule synthesis and turnover to support their rapid proliferation and growth.[37,44] These elevated metabolic demands lead to alterations in the chemical properties of biomolecules, including their bond characteristics and isotopic composition. The latter is explored in detail in the following section.

A significant contributor to these changes is the isotope effect, which is a chemical phenomenon where the substitution of one isotope for another affects reaction rates and pathways.[83,84] Lighter isotopes, with their weaker bond strength, generally enable faster reaction rates compared to heavier isotopes.[85] This difference in reaction dynamics influences the synthesis and stability of biomolecules in cancer cells, causing shifts in their structural and energetic properties.[86] Isotopic substitution not only alters the formation and dissociation of bonds but also impacts the energy stability of molecules, often leading to altered fragmentation patterns during the ionization process. For instance, such changes in molecular stability can lead to the production of distinct molecular fragments, which can be effectively identified and analyzed using techniques like mass spectrometry.[87-89]

In this thesis, we investigated the alteration of cholesterol fidelity in cancer tissues and its impact on the molecule's fragmentation patterns during mass spectrometry analysis. In this context, fidelity refers to the structural accuracy, integrity, and stability of the cholesterol molecule, particularly its resistance to fragmentation under ionization conditions.

Cholesterol consists of three key structural components: the hydroxyl group, the steroid nucleus, and the hydrocarbon side chain (Figure 2). The hydroxyl group is attached to C-3 of the steroid nucleus, making cholesterol an alcohol. This functional group contributes to the amphipathic nature of cholesterol, allowing it to interact with both hydrophilic and hydrophobic environments. The steroid nucleus is a rigid, planar structure composed of four fused hydrocarbon rings, including three six-membered rings and one five-membered ring, which form the core of all steroid molecules. The hydrocarbon side chain is attached to C-17 of the steroid nucleus and is a hydrophobic aliphatic chain that enhances the lipid-like nature of cholesterol.[90] These structural features make cholesterol a critical component of cell membranes, where it plays a key role in modulating membrane fluidity and acting as a precursor for the biosynthesis of steroid hormones, bile acids, and vitamin D.[91]

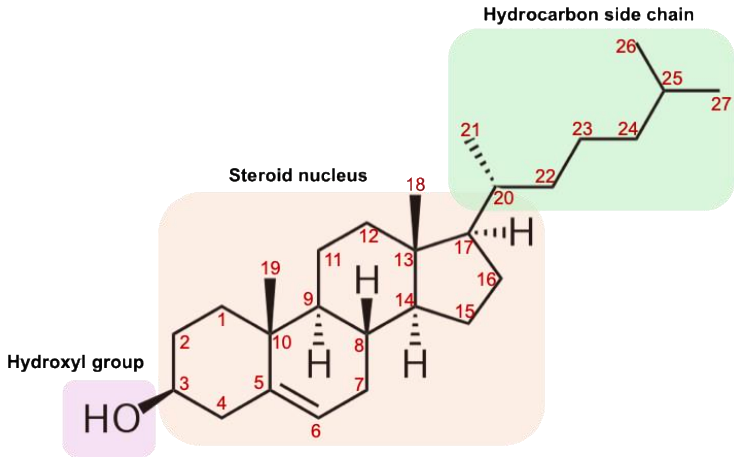

Figure 2. Chemical structure of cholesterol.

Cholesterol is essential for maintaining membrane integrity, facilitating cell signaling, and supporting structural stability across various cell types, including cancer cells.[92] In cancer, the uncontrolled proliferation of cells is accompanied by an upregulation of both cholesterol uptake from extracellular sources and de novo synthesis.[93-95] This metabolic reprogramming is necessary to meet the increased bioenergetic and biosynthetic demands of rapidly dividing cancer cells.[77,79] Several studies have demonstrated a positive correlation between elevated cholesterol synthesis and cancer cell proliferation. Increased cholesterol levels are associated with enhanced membrane dynamics, activation of oncogenic signaling pathways, and accelerated tumor progression.[77,79,93-95] However, this elevated synthesis may compromise the structural fidelity of cholesterol, making it more susceptible to fragmentation during ionization in mass spectrometry.[96]

Studying the alteration of cholesterol fidelity in cancer tissue provides important insights into how metabolic dysregulation in cancer affects cholesterol stability and fragmentation behavior. By understanding these changes, we can gain a deeper understanding of the role of cholesterol in cancer progression and identify potential biomarkers for early detection of cancer.

Cancer-related changes to stable isotopes

What is a stable isotope?

An isotope is a variation of an element that maintains the same number of protons (and therefore has the same atomic number) but differs in the number of neutrons within its nucleus, resulting in variations in atomic mass.[97,98] For instance, carbon exists in two common isotopic forms: carbon-12 (^{12}C) and carbon-13 (^{13}C). Both isotopes have six protons, but while ^{12}C has six neutrons, ^{13}C contains seven, giving it a slightly higher atomic mass.

Stable isotopes are a subset of isotopes that do not undergo radioactive decay, meaning they remain chemically stable over time.[97,98] These naturally occurring isotopes are indispensable in various scientific fields due to their ability to integrate into natural and biological processes. Examples of stable isotopes include carbon-13 (^{13}C), nitrogen-15 (^{15}N), oxygen-18 (^{18}O), and hydrogen-2 (commonly known as deuterium, ^{2}H), widely used in biological and metabolic research to trace pathways and monitor cellular activity.[99-104] These isotopes provide a window into the intricacies of molecular interactions and environmental processes, making them powerful tools for research and discovery.[98]

Why isotopic effects may trace changes in cancer

Cancer cells exhibit significantly higher rates of biomolecule utilization and synthesis to support their uncontrolled growth and proliferation.[37,44] This heightened metabolic activity can alter natural isotope fractionation processes, where lighter isotopes are typically favored in biochemical reactions due to their lower energy requirements.[105] However, under such accelerated metabolic conditions, cancer cells may increasingly consume heavier isotopes as lighter isotopes are depleted. This results in a distinct isotopic composition of biomolecules in cancer cells compared to normal cells.

The energy demands of biochemical processes are influenced by the mass differences between isotopes, which affect reaction rates. In rapidly growing tumors, these energy requirements drive the preferential utilization of specific isotopes, enabling cancer cells to meet their heightened bioenergetic and biosynthetic needs.[37,106] For instance, the metabolic adaptations in cancer cells are particularly geared toward maximizing the utilization of nitrogen and carbon sources for anabolic processes and the biosynthesis of macromolecules essential for cell proliferation and tumor growth. Notably, cancer cells exhibit significantly increased rates of glucose and glutamine consumption, which contribute to lactate production and increased nitrogen excretion (Figure 3). Deregulation of the urea cycle (UC), a key pathway in nitrogen metabolism, further exacerbates these

changes, leading to detectable variations in ^{13}C and ^{15}N isotopic signatures.[105,107] These shifts reflect the metabolic reprogramming inherent to cancer cells, and underscore the potential of isotopic analysis to trace the biochemical and metabolic alterations associated with tumor progression.

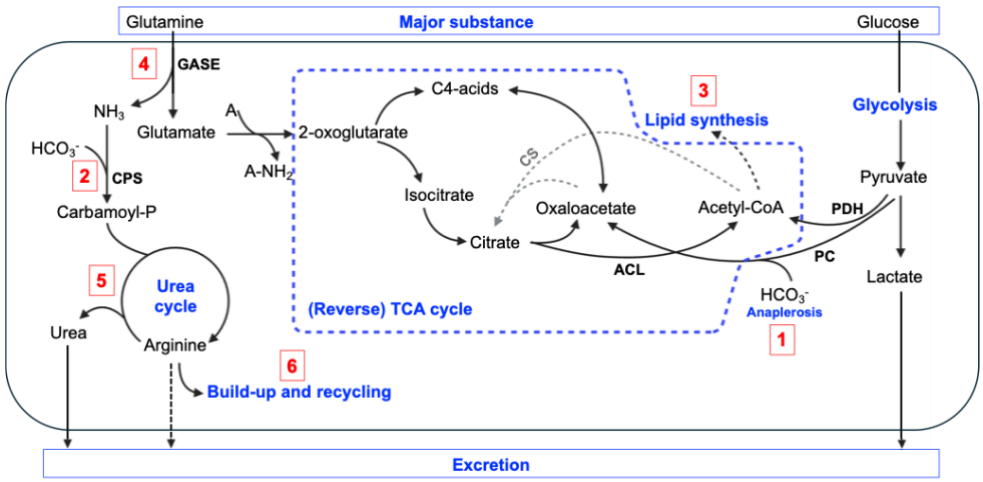

Figure 3. Major metabolic pathways explaining the natural ^{13}C and ^{15}N isotope abundance in cancerous cells (adapted from Tea et al.[107]).

The ^{13}C-enrichment mostly comes from the anaplerotic fixation of bicarbonate by pyruvatecarboxylase (PC, 1) and carbamoyl-phosphate synthase (CPS, 2) to feed the urea cycle, as well as a lower accumulation and ^{13}C value of non-structural lipids (3); the ^{15}N-depletion comes from the consumption of glutamine via glutaminase (GASE, 4), isotope effects in the urea cycle (5) and a decreased excretion of ^{15}N-depleted arginine (6). Abbreviations: A, generic amino group acceptor; CS, citrate synthase; PDH, pyruvate dehydrogenase complex.

These isotopic variations are influenced by a chemical phenomenon known as the isotope effect, where the substitution of one isotope for another alters reaction rates and pathways. Lighter isotopes, due to their weaker bond strength, typically facilitate faster reaction rates compared to their heavier counterparts. This difference in reaction dynamics affects the synthesis and stability of biomolecules in cancer cells, leading to shifts in their structural and energetic properties. Isotopic substitution can not only modify bond formation and dissociation but also impact the energy stability of molecules. In some cases, this instability may result in molecular fragmentation, which can be precisely detected and analyzed using mass spectrometry, providing valuable insights into these alterations.

Studies of isotopic signatures in cancer cells, tissues, and blood

Isotopic analysis usually examines the relative abundance of different isotopes of elements within biological molecules using inductively coupled plasma mass spectrometry (ICP-MS) and isotope ratio mass spectrometry (IRMS). Studies have explored isotopes of macro-elements such as carbon, nitrogen, and sulfur, along with trace metals, in biological samples obtained from cancer patients.[105,108] Cancer cells often exhibit unique metabolic pathways, leading to altered isotopic ratios in certain metabolites due to changes in biosynthetic processes.[105] These alterations highlight shifts in isotopic balances observed in cancer cell lines, tissues, blood, and serum, driven by the increased biosynthesis and nutrient uptake characteristic of cancer cells. For example, isotopic analysis of tissue samples from patients and cultured breast cancer cell lines reveals that cancerous cells, particularly those with invasive potential, are typically depleted in ^{15}N and enriched in ^{13}C compared to healthy tissues and cells.[107] Comparable isotopic patterns are observed in oral tissues, where patients with oral squamous cell carcinoma exhibit decreased ^{15}N and increased ^{13}C, consistent with findings in breast cancer.[109] Furthermore, the serum of hepatocellular carcinoma patients shows increased enrichment of ^{32}S and ^{63}Cu compared to ^{34}S and ^{65}Cu isotopes.[110] This is consistent with the lower ^{65}Cu isotopic ratio in the blood from ovarian, thyroid, breast, and colorectal cancer patients.[111-113] In breast cancer tumor tissues, the proportion of the ^{64}Zn isotope is higher compared to that of the ^{66}Zn isotope,[114] further supporting these metabolic alterations in cancer. The study of isotopic signatures in cancer cells, tissues, blood, and serum has provided valuable insights into the metabolic alterations associated with cancer.[105,108]

Biofluid and tissue for chemical alteration studies

The study of biomolecule alterations in cancer research often involves analyzing a variety of sample types, including blood, urine, and tumor tissues.[115] Each sample type offers distinct advantages and insights into the molecular alterations associated with cancer.

Biofluid analysis, which examines biomolecules in easily accessible fluids such as blood, urine, or saliva, provides a non-invasive method to track cancer-related changes over time.[115] This approach is particularly valuable for early detection, monitoring disease progression, and assessing treatment responses. For example, changes in metabolite levels or protein biomarkers in blood can reflect systemic metabolic disruptions caused by cancer,[116] while urinary biomarkers may indicate altered excretion patterns linked to tumor activity.[117] Technologies like mass spectrometry and liquid chromatography have made biofluid analysis more efficient, enabling more accurate and timely biomarker discovery.

Tissue analysis focuses on the direct examination of tumor or surrounding tissues to provide localized insights into cancer-specific molecular changes.[55] This approach allows for a detailed understanding of the tumor microenvironment, including metabolic reprogramming, signaling pathway alterations, and biomolecular heterogeneity within the tumor. Tissue samples often reveal spatial variations in biomolecule concentrations, shedding light on how cancer progresses and interacts with its environment.[58]

Together, biofluid and tissue analyses complement each other, offering a comprehensive understanding of cancer biology. Biofluid analysis enables convenient and repeated sampling, while tissue analysis provides depth and specificity, making both approaches critical tools for cancer research and diagnostics.

Time-of-flight secondary ion mass spectrometry

This thesis focuses on investigating molecular structure, or chemical fidelity, and the spatial distribution of chemical signatures in biological samples. These aspects are crucial for cancer detection, as they offer detailed structural and spatial insights, allowing for higher specificity in identifying cancerous cells and tissues. By uncovering unique chemical signatures associated with cancer, this research aims to contribute to early detection of cancer.

To achieve these objectives, we employed time-of-flight secondary ion mass spectrometry (ToF-SIMS), a powerful mass spectrometry technique that combines a time-of-flight analyzer with the ionization capabilities of secondary ion mass spectrometry. ToF-SIMS is a highly surface-sensitive technique that analyzes the outermost molecular layer of a sample, providing both chemical specificity and high spatial resolution. By using a micro-focused energetic ion beam, ToF-SIMS ionizes molecules from the sample surface, enabling the spectra and the detailed imaging of chemical distributions of biological samples through mass spectrometry imaging (MSI). This approach not only supports early-stage cancer detection but also deepens our understanding of cancer biology at the molecular level by mapping spatially localized molecular changes in the tumor microenvironment.

Mass spectrometry

Mass spectrometry (MS) is a powerful analytical technique used to identify and quantify chemical compounds based on their mass-to-charge (*m/z*) ratios.[118-121] MS has a wide range of applications, enabling the identification of unknown compounds in a sample, the relative quantification of specific compounds, and the elucidation of the structures and chemical properties of various molecules.[121-123] The fundamental principle of mass spectrometry involves three main stages: ionization, mass analysis, and detection (Figure 4).

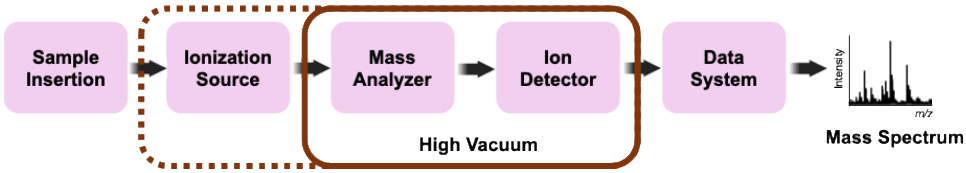

Figure 4. Basic components of a mass spectrometer.

Ionization: The process begins with ionization of the sample, where neutral molecules are converted into charged ions. Whether a vacuum is required depends on the ionization technique. Methods like electron ionization (EI) and matrix-assisted laser desorption/ionization (MALDI) typically operate under high vacuum, while electrospray ionization (ESI) and desorption electrospray ionization (DESI) operate at atmospheric pressure. Ionization techniques are broadly categorized into soft and hard ionization methods. Soft ionization methods, such as ESI[124,125] and MALDI,[125,126] produce ions with minimal fragmentation, preserving the molecular structure and making them suitable for analyzing large, intact biomolecules.[127,128] In contrast, hard ionization techniques like EI, commonly used in gas chromatography-mass spectrometry (GC-MS),[129,130] induce extensive fragmentation, providing detailed structural information about the analyte. This fragmentation is advantageous for structural elucidation but is less suitable for intact biomolecules.[131]

Mass analysis: Once the ions are generated, they are introduced into the mass analyzer. The mass analyzer separates the ions based on their *m/z* ratios, allowing for the determination of their masses.[132] Various types of mass analyzers exist, including quadrupole, time-of-flight (ToF), and ion trap, each with distinct operational principles and capabilities.[133]

Detection: After separation, the ions are directed toward a detector that measures their abundance. The detector generates a mass spectrum, a graphical representation that plots *m/z* values on the x-axis against ion intensity on the y-axis. This spectrum provides valuable information about the composition and quantity of the analytes present in the sample.[134]

A significant advantage of mass spectrometry is its label-free nature, enabling the identification and quantification of compounds without prior knowledge of their identity. This versatility makes MS an invaluable tool across various fields, including chemistry, biology, and environmental science.[135,136]

One specific application of mass spectrometry is ToF-SIMS. ToF-SIMS uses a focused ion beam to sputter and ionize molecules directly from the surface of a sample, generating secondary ions that are then analyzed based on their *m/z* ratios. This technique allows for high-resolution imaging and detailed chemical analysis of surfaces, making it particularly useful for studying biological samples and complex materials.[137-139] By understanding the general principles of mass spectrometry, the

unique capabilities and applications of ToF-SIMS in molecular and surface analysis become evident.

Secondary ion mass spectrometry

In secondary ion mass spectrometry (SIMS), the primary ion beam plays a critical role in analyzing the surface composition of a sample. The analysis process begins when a focused primary ion beam is directed onto the sample's surface. This beam, consisting of high-energy ions, bombards the sample surface, transferring energy to the atoms and molecules within, leading to a process known as sputtering[140] (Figure 5). During sputtering, particles are ejected from the sample surface, and a fraction of these particles become ionized, producing what are known as secondary ions. These secondary ions are then collected and analyzed to determine the sample's composition and structure.[141]

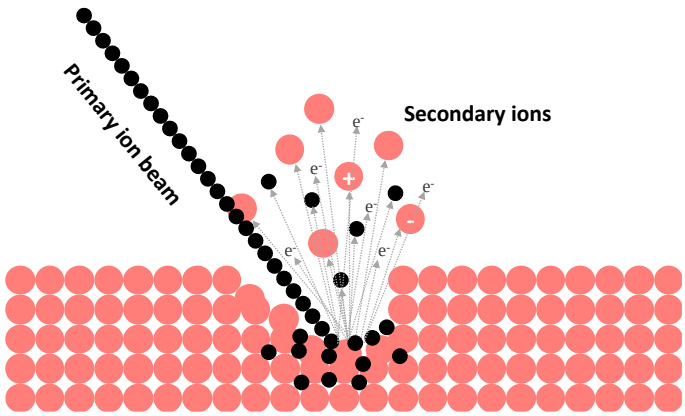

Figure 5. Schematic description of the sputtering process in ToF-SIMS.

Different types of primary ion beams can be used in SIMS, each suited to specific analytical needs (Table 2). Monoatomic ion beams, such as gallium (Ga^+), cesium (Cs^+), and oxygen (O_2^+), are commonly employed for elemental analysis and depth profiling in materials with robust atomic bonds.[142] Their high sputter rates make them ideal for such applications, although they may cause damage to organic materials due to their high energy. Cluster ion beams, such as bismuth (Bi_3^+), gold (Au_3^+), and fullerene (C_{60}^+), offer a gentler sputtering process, preserving molecular information and thereby proving useful for analyzing organic molecules and biological samples.[142,143] This makes cluster ion beams particularly beneficial for applications requiring molecular imaging, as they allow for minimal fragmentation. Gas cluster ion beams (GCIB), typically composed of large argon clusters (e.g.,

Ar_{1000}^{+}), are the softest of the ion beams used in SIMS. GCIBs are well-suited for analyzing fragile materials, enabling ultra-soft sputtering that preserves the integrity of delicate samples, making them ideal for depth profiling of polymers, biological tissues, and other soft materials.[144,145]

Table 2. Summary of SIMS ion beam and application

Ion Beam Type	Characteristics	Typical Applications
Monoatomic	High-energy, single ions (e.g., Ga^{+}, Cs^{+})	Elemental analysis, depth profiling of hard materials
Cluster	Gentle, molecular-friendly clusters (e.g., Bi_3^{+}, C_{60}^{+})	Organic and biomolecular analysis, molecular imaging
Gas Cluster Ion Beam (GCIB)	Ultra-gentle, large clusters (e.g., Ar_{1000}^{+})	Depth profiling in soft materials, polymers, biological samples

The primary ion beam in SIMS serves multiple functions, including sputtering the sample surface to generate secondary ions, enabling depth profiling by progressively removing layers of material and facilitating high spatial resolution.[146] By scanning (or rastering) the ion beam across the surface, SIMS can create spatially resolved chemical maps, providing detailed insights into the sample's composition and distribution of elements and molecules. Once the secondary ions are generated, they are analyzed by a mass spectrometer, typically a ToF analyzer in ToF-SIMS, which separates ions based on their mass-to-charge ratios (*m/z*). This produces a mass spectrum that provides both qualitative and quantitative information about the elements, isotopes, and molecular species present on the sample's surface.[147,148]

By selecting an appropriate primary ion beam type, SIMS can be tailored to meet the specific requirements of various applications. Monoatomic ion beams are often preferred for elemental analysis in hard materials, while cluster ion beams are advantageous for organic and biological sample analysis, preserving molecular structures. Gas cluster ion beams are particularly useful in ultra-soft sputtering for analyzing soft materials where molecular integrity is paramount. This flexibility makes SIMS a powerful and adaptable tool in fields such as materials science, semiconductor analysis, and biological research, allowing for detailed surface composition and chemical distribution analysis across a wide range of sample types.

Time-of-flight mass analyzer

The time-of-flight (ToF) mass analyzer is a critical component in mass spectrometry, used to distinguish ions based on their *m/z* ratios by measuring their travel time over a set distance. The ToF analyzer operates on the principle that ions of different masses will travel at distinct velocities when subjected to the same initial kinetic energy.[149]

In the ToF process, ions are first accelerated by an electric field, imparting each ion with the same amount of kinetic energy. The kinetic energy (KE) of a particle of a certain mass is given by the equation,

$$KE = \frac{1}{2}mv^2 \qquad (1)$$

where m is the mass and v is the velocity of the particle.

The lighter ions travel faster while heavier ions move slower (Figure 6). After acceleration, ions enter a field-free drift region, where they continue toward the detector at speeds dependent on their masses.[150] The time each ion takes to reach the detector, its time-of-flight, is recorded and directly related to the ion's *m/z* ratio, allowing the analyzer to differentiate between ions of various masses. Knowing the exact length of the drift tube and the electric field strength, the analyzer calculates the *m/z* ratio for each ion based on its flight time.

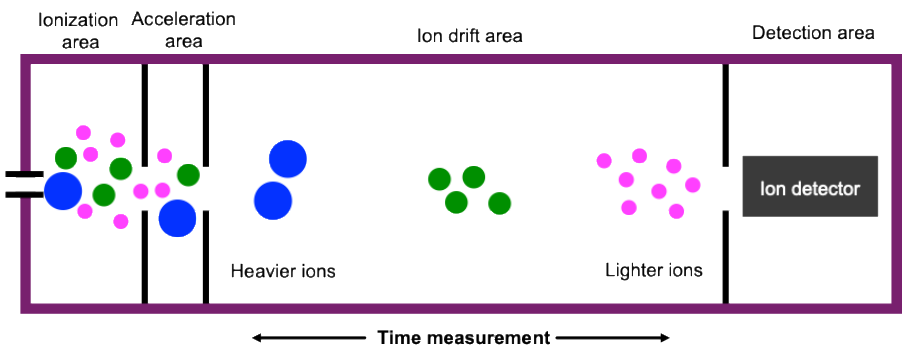

Figure 6. Time of flight mass spectrometer schematic diagram.

ToF analyzers are particularly valued in secondary ion mass spectrometry (SIMS) for their high mass resolution and ability to detect a wide range of masses simultaneously.[151] This makes them especially useful in applications requiring rapid and precise mass determination, such as surface analysis, where detailed mass spectra reveal insights into a sample's composition and structure. The mass spectrum enables identification and quantification of elements and compounds,

while advanced data techniques support depth profiling and imaging, mapping the spatial distribution of analytes within the sample. SIMS is especially valuable for its nanometer-to-micrometer spatial resolution and minimal sample preparation needs, establishing it as a crucial tool in fields like materials science, semiconductor analysis, and biological research, where precise surface composition and chemical distribution information are essential.

Mass spectrometry imaging

Mass spectrometry imaging (MSI) is a powerful analytical technique that enables the spatial mapping of chemical compounds directly within biological samples.[152] Unlike traditional mass spectrometry, which provides information on the overall composition of a sample, MSI offers both chemical and spatial information, allowing researchers to visualize the distribution of specific molecules, such as lipids, proteins, and metabolites, within tissues or cells.[57,153] This spatially resolved chemical information is crucial for understanding molecular organization and biological processes within complex samples.[154]

Several ionization methods are employed in MSI, the most common including MALDI and ToF-SIMS. MALDI is typically favored for analyzing large biomolecules, such as proteins, peptides, and large lipids, due to its ability to gently ionize these compounds with minimal fragmentation. However, MALDI generally provides a spatial resolution of around 10-20 microns which, while suitable for tissue-level imaging, may lack the finer resolution needed for subcellular studies of smaller molecules.[155,156]

In this thesis, we chose ToF-SIMS for MSI because of its superior spatial resolution and its effectiveness in analyzing small molecules, such as lipids and cholesterol.[157] ToF-SIMS uses a focused ion beam to bombard the sample surface, causing the ejection of secondary ions, which are then analyzed based on their *m/z* ratios. This technique achieves submicron spatial resolution (often below 1 micron), making it particularly well-suited for high-resolution imaging of small molecules.[157] This high spatial precision allows us to map the distribution of lipids and cholesterol with fine detail, enabling an in-depth investigation of these compounds within cellular structures and tissue regions.[158] Additionally, ToF-SIMS is a "soft" ionization technique, which helps to preserve the molecular integrity of lipids and other small molecules, providing clear and accurate molecular images.

MSI, particularly ToF-SIMS, has numerous applications in the biomedical and life sciences. In this research, ToF-SIMS was employed to investigate the distribution of lipids and cholesterol within biological samples, offering insights into the molecular composition of cellular membranes and tissue structures. These biomolecules are crucial to cell function and structure, and their spatial distribution

can provide information about cellular organization, disease progression, and potential therapeutic targets. MSI is widely used in cancer research, where it can reveal lipid and metabolite variations between tumor and normal tissue regions.[159-162] In neuroscience, MSI enables the visualization of neurotransmitter and lipid distributions in brain tissue, offering new insights into neurological processes and diseases.[163-165] MSI is also instrumental in pharmacology, allowing for precise tracking of drug localization and metabolism within tissues.[166-168]

In summary, MSI is a powerful tool for mapping the molecular landscape of biological samples, and ToF-SIMS, with its high spatial resolution, is particularly suited for imaging small molecules like lipids and cholesterol. By using ToF-SIMS in this thesis, we aimed to capture the detailed chemical architecture of cellular regions, enabling a deeper understanding of the molecular environment at a level of precision that would be challenging to achieve with other MSI techniques like MALDI.

Multivariate analysis

During ToF-SIMS imaging, an extensive amount of data is generated. Each pixel point in the image contains a full mass spectrum, with numerous mass channels representing both true peaks and background noise. This vast data set can be challenging to interpret, particularly for complex biological samples with a mixture of diverse compounds.[169,170] Multivariate analysis (MVA) has proven valuable in simplifying this data, extracting key chemical information from the sample.[171-173] Two commonly used MVA techniques for analyzing ToF-SIMS images are principal component analysis (PCA) and multivariate curve resolution (MCR), both of which help highlight relevant chemical patterns within the complex data.

PCA is primarily used for data compression and dimensionality reduction.[169] It identifies patterns within the data by finding directions, or "principal components" along which variance is maximized. This technique provides both positive and negative loadings, which help to highlight distinct chemical variations within the sample.[170] PCA is particularly useful for revealing major trends and reducing the complexity of ToF-SIMS datasets while preserving the most informative features.[171]

MCR, on the other hand, is focused on separating and identifying individual chemical components within a mixture.[174] Unlike PCA, MCR only generates positive loadings, making it better suited for isolating pure chemical profiles in samples where specific components are present at varying concentrations.[171] This approach is especially useful for distinguishing the unique contributions of each chemical species in complex biological samples, allowing for a clearer interpretation of individual compound distributions.

Together, PCA and MCR provide complementary insights into ToF-SIMS data: PCA simplifies and highlights overall trends, while MCR separates and identifies the unique chemical components within a sample.[175]

Sample preparation for ToF-SIMS analysis

Sample preparation is one of the most challenging and crucial steps in obtaining reliable data in ToF-SIMS analysis. To achieve high-quality and meaningful results, particular care is taken to preserve the native state of biological samples, while avoiding contamination that could skew the findings. This section outlines the specific protocols and considerations applied when preparing tissue and cellular samples for ToF-SIMS analysis, focusing on avoiding embedding media contamination, ensuring vacuum compatibility through freeze-drying, and minimizing salt residue from cell culture media.

Avoiding embedding media contamination

To prevent contamination from foreign materials that could interfere with ToF-SIMS analysis, tissue samples are prepared without the use of embedding media such as optimal cutting temperature (OCT) compound or gelatin.[168,176] These materials, commonly used to stabilize tissue for cryo-sectioning, can introduce additional ions or artifacts that may obscure the natural composition of the tissue samples. By foregoing embedding media, we ensure that the ToF-SIMS results reflect only the native chemical composition of the tissue.

To preserve biomolecular structures in their native state, we employ snap-freezing, a technique that involves rapidly freezing the samples to maintain the integrity of most biomolecules. In snap-freezing, tissue samples are quickly frozen to ultra-low temperatures, typically using liquid nitrogen. This rapid cooling process halts molecular motion almost instantly, preventing enzymatic degradation and minimizing chemical alterations in the sample. Snap-freezing is highly effective at preserving cellular structures and the spatial distribution of biomolecules, which is essential for achieving accurate chemical mapping in ToF-SIMS.

However, snap-frozen tissues are inherently more brittle, making them challenging to section for imaging. During the cryo-sectioning process, tissues are carefully cut at cryogenic temperatures to avoid structural damage, enabling detailed ToF-SIMS analysis. This approach ensures that the tissues retain their molecular composition and structure, allowing us to examine the native biomolecular distributions within the sample without interference from embedding compounds or thawing-related changes.

Freeze-drying for vacuum compatibility

One critical requirement of ToF-SIMS is that samples must be completely water-free to prevent issues within the high-vacuum environment of the instrument.[177] Any residual water can lead to sample distortion or artifacts due to evaporation under vacuum conditions, which could affect the accuracy of the ToF-SIMS measurements. To address this, we employ freeze-drying (lyophilization),[162,178] which is a dehydration process conducted at low temperatures to avoid chemical changes in sensitive biomolecules. By sublimating water directly from the frozen state, freeze-drying preserves the structural and molecular composition of the sample,[177] ensuring it remains compatible with the ToF-SIMS vacuum without introducing artificial alterations.

Minimizing salt residue from cell culture media

Excess salts, commonly present in cell culture media, present a significant challenge for ToF-SIMS analysis. High salt content can cause ion suppression effects, generating background noise and interfering with the detection of biologically relevant ions.[179] To address this issue, cell samples are carefully rinsed with volatile buffers, such as 0.15 M ammonium formate or ammonium acetate at pH 7.4, to reduce salt concentration while preserving essential cellular components.[177,179-181] This rinsing step is particularly crucial for samples derived from cell cultures, where residual salts can obscure or complicate the interpretation of chemical distributions within cells. By selectively reducing salt residues, we improve the clarity and accuracy of the ToF-SIMS data, enabling more precise insights into the sample's chemical composition.

Model systems

In this thesis, we used two model systems, tissue-scale and cellular-scale, to study chemical alterations using ToF-SIMS. The tissue-scale model involved mammary glands from a murine model to investigate cholesterol fidelity, along with glioblastoma multiforme (GBM) samples from patients to analyze lipid distribution differences. The cellular-scale model used breast cancer cell lines (HCC-1806), including both untreated cells and cells that survived post-cisplatin treatment, to distinguish chemical profiles of cellular components.

Tissue scale

Mammary glands from murine model

In Paper I of this thesis, we investigated alterations in cholesterol fidelity using mammary glands from a murine model. We employed FVB/N female mice, with two mouse phenotypes: wild-type (WT) mice as the healthy controls, and MMTV-PyMT (mouse mammary tumor virus - polyomavirus middle T antigen) mice as the cancer model (Figure 7).

The FVB mouse strain is commonly used in biomedical research due to its genetic uniformity, large litter sizes, and high reproductive performance, making it ideal for breeding and genetic studies.[182] FVB mice are particularly suitable for transgenic research, as their visible pronuclei facilitate the introduction of foreign genes. Their genetic stability also allows researchers to create cancer models, such as the MMTV-PyMT model, which closely mimics the development and progression of human breast cancer.[183] In this study, FVB/N mice provided a controllable system to study cholesterol fidelity in both healthy and cancerous mammary tissue.

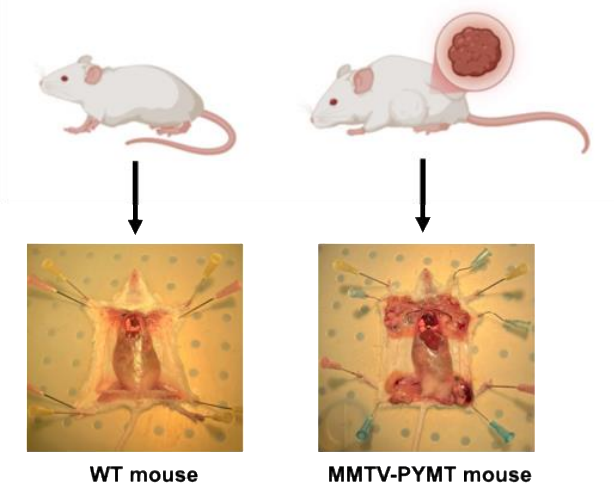

Figure 7. Experimental mouse models.
Wild-type (WT) mouse as the control model and MMTV-PyMT (mouse mammary tumor virus-polyomavirus middle T antigen) mouse as the cancer model.

Glioblastoma multiforme

In Paper II of this thesis, we used human-derived glioblastoma multiforme (GBM) (Figure 8) as a model system to explore lipid distribution across the different tumor regions.

GBM is an aggressive and fast-growing type of brain tumor that originates from glial cells, which are supportive cells in the central nervous system.[184,185] Classified as a Grade IV glioma, the most severe grade according to the World Health Organization, GBM is one of the most common and deadly primary brain tumors in adults.[186] It typically occurs in the cerebral hemispheres but can also develop in other parts of the brain and spinal cord.

GBM is characterized by rapid growth and high invasiveness, spreading quickly within the brain and infiltrating nearby tissue. This makes complete surgical removal challenging.[184] It is also highly resistant to conventional treatments, such as chemotherapy and radiation therapy, and often recurs even after aggressive treatment.[187] Another defining feature of GBM is its heterogeneous cell population, comprising a mixture of different cell types within a single tumor. This heterogeneity contributes to its resilience, treatment resistance, and the difficulty of targeting it effectively.[187]

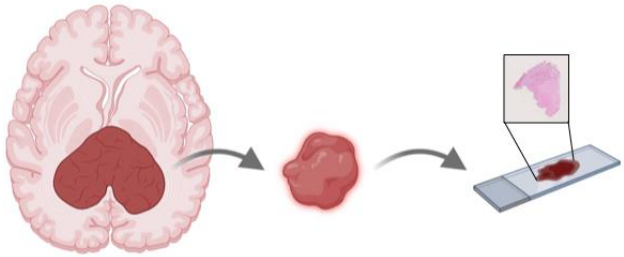

Figure 8. Illustration of glioblastoma multiforme (GBM).
The inset shows an H&E image of a GBM.

Cellular scale

In Papers III and IV, we used the epithelial breast cancer cell line HCC-1806 to examine both untreated cells and cells that survived cisplatin treatment (Figure 9). Paper III focused on analyzing the chemical profiles of cellular components using mass spectrometry imaging (MSI), while Paper IV explored the biological characterization of both untreated and surviving cells.

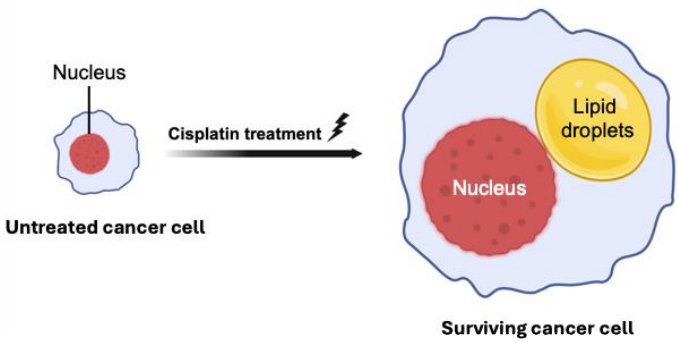

Figure 9. Illustration of untreated cancer cells and cancer cells surviving cisplatin treatment.

Untreated cancer cells

HCC-1806 is an epithelial cell line isolated from the mammary gland of a 60-year-old black female patient with acantholytic squamous cell carcinoma (ASCC). This cell line is triple-negative, lacking estrogen receptors (ER), progesterone receptors (PR), and human epidermal growth factor receptor 2 (HER2), which makes it a

suitable model for studying triple-negative breast cancer (TNBC),[188] a subtype known for its aggressiveness and resistance to treatment. Due to its stability, reproducibility, and aggressive characteristics, HCC-1806 is widely used for investigating tumor biology and testing novel therapeutic approaches for TNBC.[189]

Surviving cancer cells

To investigate drug-resistant cancer cells, HCC-1806 cells were treated with the chemotherapy drug cisplatin at LD50 for 72 hours, resulting in the death of approximately 90% of cells. The remaining 10%, which adapted to the cisplatin treatment, were designated "surviving cancer cells" in this thesis. These cells exhibited increased nuclear and cell size. Previous studies have also highlighted an increased abundance of lipid droplets in surviving cancer cells, which is associated with greater survival against cancer treatments compared to untreated cells. These characteristics pose challenges in treatment, as they may contribute to tumor recurrence and metastasis. Ongoing research seeks to address cisplatin resistance through combination therapies, alternative chemotherapy agents, and targeted approaches to eliminate these resilient cancer cells.

The present investigation

In this thesis, we aim to explore whether cancer leaves distinct chemical signatures that can be used as markers for detection and characterization. We used ToF-SIMS to investigate chemical alterations in both cancer cells and tissues. Our focus was on three key aspects: spatial mapping of chemical distributions, analysis of molecular structure, and examination of isotopic composition, all of which contribute to understanding the biochemical changes associated with cancer and treatment response.

The aims of the four papers were as follows:

Paper I: To investigate chemical alterations in cholesterol profiles within cancerous tissues, using ToF-SIMS to access the cholesterol fidelity.

Paper II: To investigate the spatial distribution of lipids, particularly cholesterol, within glioblastoma multiforme (GBM) tissue from homogeneous and heterogeneous regions using ToF-SIMS.

Paper III: To map and compare the chemical distribution and molecular profiles of untreated and cisplatin-surviving cancer cells using ToF-SIMS.

Paper IV: To investigate how polyploidization in cancer cells contributes to drug resistance.

Paper I: Alterations of the chemical profile of cholesterol in cancer tissue as traced with ToF-SIMS

In Paper I, we investigated the chemical alterations in cholesterol profiles within cancerous tissues using ToF-SIMS. Cholesterol is a critical component of cellular membranes and plays a vital role in regulating cell signaling, membrane fluidity, and various metabolic pathways.[79] Its involvement in cancer progression is well-documented, particularly in how cancer cells reprogram cholesterol metabolism by increasing the synthesis rate to sustain rapid proliferation and increase survival under therapeutic stress.[77,93] Complementary findings in Paper II further reinforced this notion by demonstrating elevated cholesterol levels in homogeneous tumor regions of human glioblastoma multiforme (GBM). Despite its critical role, analyzing cholesterol in biological tissues remains challenging due to its structural complexity and the difficulties associated with ionizing it using conventional mass spectrometry techniques. The focus of this study was to assess the fidelity of cholesterol in cancer tissues, which refers to the structural integrity and stability of the cholesterol molecule, specifically its resistance to fragmentation during ionization processes.

The rationale for evaluating cholesterol fidelity stems from the hypothesis that changes in cholesterol-related molecular fragments could serve as potential biomarkers for cancer detection. By investigating these alterations, we aimed to identify distinctive chemical signatures that could provide insights into metabolic reprogramming in cancer. Understanding how cholesterol metabolism is altered in cancer could open new avenues for early cancer detection and improved diagnostic tools.

ToF-SIMS was selected for this study due to its unique ability to address the limitations of other analytical methods, which often struggle to effectively ionize cholesterol and other lipids. Unlike traditional mass spectrometry techniques, which typically require matrix-assisted ionization or other complex preparations, ToF-SIMS enables high spatial resolution and direct detection of small molecular fragments within tissue samples, preserving their native chemical environment.[190] This approach allowed for more precise localization and characterization of cholesterol-related changes in cancerous tissues without the need for matrix addition. By retaining the integrity of the native tissue environment, ToF-SIMS provided a more accurate and detailed view of how cholesterol is altered in cancer, thereby offering a significant advantage over conventional mass spectrometry techniques.

In Paper I, we found significant alterations in cholesterol fragmentation patterns in cancerous tissues compared to normal tissues. Specifically, histological analysis of mammary gland tissues from MMTV-PyMT mice (a mouse model for breast

cancer) revealed a higher density of malignantly transformed cells with irregular morphology and increased nuclear-to-cytoplasmic ratios. These transformed cells exhibited changes consistent with cancerous progression, such as loss of normal tissue architecture. ToF-SIMS analysis also showed a marked increase in fragmented cholesterol ($C_{27}H_{45}^+$, *m/z* 369) in cancerous tissues, while the intact cholesterol peak ($C_{27}H_{43}O^+$, *m/z* 383) was significantly reduced. Interestingly, the intact cholesterol at *m/z* 385 ($C_{27}H_{45}O^+$) showed no significant differences between normal and transformed tissues, suggesting that this specific cholesterol species may not be affected by cancerous transformation. These findings indicate that the structural integrity of cholesterol is compromised during neoplastic transformation, leading to a shift in cholesterol metabolism and fragmentation.

Further isotopic analysis revealed altered incorporation of heavier carbon isotopes in cholesterol molecules from transformed tissues. Cholesterol molecules containing one ^{13}C isotope (*m/z* 368: $^{13}CC_{26}H_{43}^+$ and 370: $^{13}CC_{26}H_{45}^+$) were found to be overrepresented in cancerous tissues, while molecules with two ^{13}C isotopes (*m/z* 371: $^{13}C_2C_{25}H_{45}^+$ and 387: $^{13}C_2C_{25}H_{45}O^+$) were more prevalent in normal tissues. This shift in isotopic patterns suggests changes in cholesterol biosynthesis and metabolism in cancer. Specifically, cancer cells may alter their cholesterol synthesis pathways to support rapid cell division and growth. These findings highlight the potential for using isotopic shifts in cholesterol as an additional marker for cancer diagnosis and metabolic changes within tumors.

Machine learning models, including principal component analysis (PCA) and partial least squares discriminant analysis (PLS-DA), were also applied to the data to determine whether cholesterol fragmentation patterns could effectively distinguish cancerous tissues from normal ones. The models successfully identified key *m/z* ratios (368, 371, and 383) as significant contributors to sample classification, achieving complete separation between cancerous and normal tissues. This demonstrates the power of ToF-SIMS-derived fragmentation patterns in providing molecular-level information that can differentiate between cancerous and non-cancerous tissue samples.

The combination of structural analysis, isotopic profiling, and machine learning approaches in this study led to the identification of cholesterol fragmentation patterns as potential biomarkers for cancer detection. These findings suggest that cholesterol metabolism, including alterations in cholesterol fragmentation and synthesis, plays a crucial role in cancer progression and heterogeneity. By providing a detailed molecular map of cholesterol-related changes in cancer, this research offers new insights into the metabolic vulnerabilities of cancer cells and their potential exploitation for early cancer detection and the development of novel diagnostic tools.

Discussion and limitations

The observed increase in cholesterol fragmentation in transformed mammary tissues suggests that cancer alters the structural stability of cholesterol, possibly due to deregulated lipid synthesis pathways. The accumulation of intermediates such as squalene and lanosterol, known to be disrupted in cancer, may lead to the formation of structurally unstable cholesterol molecules that are more susceptible to fragmentation under ionization. The higher incorporation of one ^{13}C isotope in cancer tissues may reflect a shift in metabolic processes, indicating that transformed tissues prioritize different biochemical pathways for lipid synthesis. However, the unexpected underrepresentation of molecules with two ^{13}C isotopes in cancer tissues raises questions about potential limitations in isotope discrimination during analysis.

The differences observed between ToF-SIMS and DESI-MS emphasize the importance of validating results across multiple analytical techniques to account for ionization-specific variability. Moreover, the resolution limitations of ToF-SIMS may have hindered accurate differentiation between isotopic effects and protonation, which could influence fragmentation patterns. Higher-resolution methods such as GC-HR-MS or MRMS could provide more precise insights into these processes. The influence of isotopic substitutions, such as deuterium or oxygen-18, on cholesterol fragmentation was not fully explored and may require further investigation. The absence of direct analysis of cholesterol intermediates limits our understanding of their role in the observed fragmentation patterns, warranting future studies to comprehensively explore lipid metabolism in cancerous tissues.

Paper II: Distinct cholesterol localization in glioblastoma multiforme revealed by mass spectrometry imaging

Paper II aimed to investigate the spatial distribution of lipids, particularly cholesterol, within glioblastoma multiforme (GBM) tissue, focusing on two regions with distinct histopathology: homogeneous and heterogeneous areas. GBM is an aggressive brain cancer known for its significant metabolic dysregulation, including enhanced lipid synthesis and altered cholesterol metabolism, both of which support key hallmarks of the disease such as uncontrolled proliferation, angiogenesis, and immune evasion.[191,192] Cholesterol, a crucial component of cellular membranes and signaling pathways, is particularly important for GBM survival, and its metabolism in the tumor has been found to differ from that of healthy brain tissue.[193] Previous research has demonstrated the tumor's reliance on cholesterol for growth and resilience.[77] Given this, the aim of Paper II was to use ToF-SIMS to examine the distribution of cholesterol and other lipids across different regions of GBM tumors. The goal was to explore the metabolic irregularities in GBM and identify potential therapeutic targets by analyzing lipid variations in the tumor's heterogeneous landscape.

The main findings of the study revealed significant differences in lipid distribution between the homogeneous and heterogeneous regions of GBM. Cholesterol was found to be more abundant in the homogeneous region, suggesting its critical role in supporting tumor cell membrane stability and signaling, which are essential for tumor survival. This observation reinforces the importance of cholesterol in maintaining the integrity and function of cancer cells within the homogeneous region. In contrast, the heterogeneous region showed a higher prevalence of phosphatidylcholine (PC) fragments at *m/z* 184.1, indicating that lipid metabolism differs between the two areas. Other lipids, such as phosphatidylethanolamine (PE) and phosphatidylinositol (PI), displayed distinct regional variations. Some phospholipids like PC (34:1), PE (32:1), and PI (38:4) were more uniformly distributed across the tumor, while others like PC (36:1) and PE (38:1) were enriched in the homogeneous region.

Fatty acids (FAs) and diacylglycerols (DAGs) were found to accumulate at the interface between the two regions, suggesting they may play a role in lipid turnover and tumor invasion. The co-localization of DAGs and FAs hints at the involvement of triacylglycerol (TAG) metabolism in GBM proliferation and metastasis. These findings indicate that GBM tumors exhibit complex lipid metabolic processes that are spatially organized, with different tumor regions engaging in distinct metabolic pathways. This spatial heterogeneity may contribute to the tumor's ability to evade treatment and metastasize, offering valuable insights into potential therapeutic approaches for targeting lipid metabolism in GBM. By highlighting these metabolic differences, the study lays the groundwork for future research focused on

developing targeted therapies aimed at disrupting lipid metabolism in GBM, potentially improving treatment outcomes for this aggressive cancer.

Discussion and limitations

The findings suggest that GBM tumors exhibit regional variations in lipid composition, with cholesterol playing a key role in the homogeneous regions, potentially supporting tumor cell metabolism. The increased accumulation of lipids, including DAGs and fatty acids, at the tumor interface indicates possible lipid-driven mechanisms for tumor invasion and growth. These results align with existing literature on lipid metabolism alterations in GBM, particularly the increased lipid uptake and storage in transformed tissues. The co-localization of fatty acids and DAGs at the interface may also suggest the involvement of lipid droplet accumulation in promoting tumor proliferation.

The study's reliance on ToF-SIMS for lipid mapping, while offering high spatial resolution, is limited by ion fragmentation, which can reduce the detection of intact molecules. The findings on lipid distribution do not fully elucidate the molecular mechanisms behind the observed changes in lipid composition. The absence of functional studies exploring the impact of these lipid changes on GBM progression further limits the ability to draw definitive conclusions regarding the role of lipids in tumor behavior. Future studies incorporating more complementary techniques and functional analyses are necessary to confirm the implications of lipid metabolism in GBM.

Paper III: Chemical profiling of surviving cancer cells using ToF-SIMS and MCR analysis discriminates cell components

The aim of this study was to investigate the chemical distribution and molecular differences between untreated and chemotherapy-surviving cancer cells using ToF-SIMS. The rationale behind this study stems from the growing interest in cancer's chemical signatures beyond genetic mutations. Cancer cells exhibit significant alterations in the chemical composition of elements, isotopes, and lipids, all of which contribute to cancer progression, metastasis, and therapeutic resistance.[107,194] Previous studies have identified chemical changes in elements and altered lipid profiles that could serve as valuable biomarkers for cancer detection.[12] Mapping these chemical alterations at the cellular level offers valuable insights into cancer biology and therapeutic mechanisms, including the development of resistance to treatments such as cisplatin, as explored in Paper IV.

Mass spectrometry, particularly ToF-SIMS, offers the high spatial resolution necessary to explore the molecular composition of individual cancer cells.[180] ToF-SIMS provides submicron to nanometer-scale resolution, making it particularly suited for capturing the chemical signatures within cells, including both untreated and surviving cancer cells. While traditional mass spectrometry techniques like MALDI-MS lack the required resolution for cellular-scale mapping,[156] ToF-SIMS stands out by offering detailed molecular profiles at the subcellular level. This capability is crucial for understanding how surviving cancer cells, which often exhibit larger sizes and altered structures, adapt to chemotherapy and contribute to therapeutic resistance.[195]

In this study, we aimed to differentiate between untreated and surviving cancer cells by analyzing their chemical profiles, specifically focusing on lipid metabolism, phospholipid modifications, and other cellular components that might contribute to survival mechanisms. Using both positive and negative ion modes of ToF-SIMS, we analyzed the molecular distribution within HCC-1806 breast cancer cells treated with cisplatin, and compared them to untreated controls. The results revealed distinct chemical differences between the two cell populations. Surviving cancer cells exhibited prominent peaks associated with lipid metabolites such as monoacylglycerol (MAG) and diacylglycerol (DAG) lipids, which were absent in untreated cells. In the negative ion mode, surviving cancer cells showed unique phosphatidylcholine (PC) species, including epoxidized PCs, indicative of oxidative stress and altered lipid metabolism. These alterations suggest that surviving cells undergo significant biochemical changes that help them endure the therapeutic stress induced by cisplatin.

Principal component analysis (PCA) and multivariate curve resolution (MCR) were employed to evaluate the chemical data from ToF-SIMS. PCA did not reveal a clear distinction between untreated and surviving cancer cells, nor did it distinguish the major intracellular components. However, MCR, a more advanced multivariate analysis method, successfully separated the chemical profiles of untreated and surviving cells, as well as individual components such as the nucleus, lipid droplets, and cytoplasm. This analysis highlighted the importance of lipid droplets, which were notably more abundant in the surviving cells.[196] The lipid droplets, often linked to increased resistance to oxidative stress, could contribute to the cells' ability to survive chemotherapy.

Discussion and limitations

This study demonstrates the power of ToF-SIMS combined with multivariate analysis techniques like MCR for unraveling the chemical signatures of untreated and chemotherapy-surviving cancer cells. The main findings reveal significant differences in the lipid profiles, specifically the accumulation of monoacylglycerols and diacylglycerols, and the presence of altered phosphatidylcholine species in the surviving cells. These findings suggest that lipid metabolism plays a crucial role in the adaptation of cancer cells to chemotherapy. The increased number of lipid droplets in surviving cells aligns with previous studies linking these organelles to therapeutic resistance, possibly by protecting cells from oxidative damage.

While the results offer valuable insights, there are limitations to the study. While ToF-SIMS provides high spatial resolution, it does not offer comprehensive information on all chemical species, especially proteins and other large biomolecules that may also play a significant role in therapeutic resistance. MCR outperformed PCA in distinguishing chemical profiles, but the complexity of biological systems may still limit the interpretability of the data, as overlapping chemical signals or experimental noise could influence the results. Despite these limitations, the study demonstrates the potential of ToF-SIMS as a tool for investigating cellular chemistry in cancer and offers a framework for further research into the molecular mechanisms underlying cancer survival and resistance.

Paper IV: Drug-resilient cancer cell phenotype is acquired via polyploidization associated with early stress response coupled to HIF2α transcriptional regulation

The aim of Paper IV was to investigate how polyploidization in cancer cells contributes to drug resistance, with a specific focus on its association with early stress responses and HIF2α transcriptional regulation. Cancer cells that survive chemotherapy often develop resistance through adaptive mechanisms, and polyploidization, the acquisition of multiple sets of chromosomes, is one such strategy that may improve their ability to endure treatment-induced stress. This study explored the link between polyploidy and the activation of early stress response pathways, particularly those regulated by HIF2α, a transcription factor crucial for cellular adaptation to hypoxia and other stress conditions. Understanding the interplay between polyploidization and HIF2α may provide new therapeutic insights to target drug-resilient cancer phenotypes. My contribution to Paper IV focused on examining how cancer cells increase in size following exposure to cytotoxic drugs.

In this study, cisplatin treatment of various cancer cell lines, including breast (HCC1806), colon (HCT116), lung (U1690), and kidney (786-0) carcinomas, induced the development of therapy-resilient phenotypes characterized by significant increases in both cell and nuclear size. Across all tested cell lines, cisplatin-treated cells displayed a 3- to 5-fold increase in size by 5 days post-treatment (DPT), which escalated to a 9- to 11-fold increase by 10 DPT. This size expansion was consistent across six additional cancer cell lines. Quantitative analysis using FlowCam confirmed a progressive increase in cellular size, from 1.4 times at 0 DPT to 2.3 times by 10 DPT, highlighting the sustained growth of therapy-resilient cancer cells. These findings provided crucial data on the phenotypic changes associated with drug resistance, which subsequently led to the investigation of chemical alterations at the cellular level in Paper III.

Conclusions and future perspectives

This thesis investigates the chemical alterations in cancer cells and tissues, with a particular emphasis on the molecular implications of altered lipid metabolism, specifically cholesterol, and its role in driving cancer progression and therapeutic resistance. Our investigations explored biochemical changes associated with cancer at both tissue scale and cellular scale.

At the tissue level, we investigated cholesterol alterations in two cancer models: mouse mammary glands and human glioblastoma multiforme (GBM). In mammary glands, dysregulated metabolism linked to tumor growth appears to be reflected in increased cholesterol fragments. Future research could seek this indication in other liquid or tissue biopsies to trace early tumor growth. Hair, as a readily accessible biological matrix, provides a unique reflection of the chemical conditions within our living environment.[197] The analysis of cholesterol in hair serves as a valuable diagnostic tool for identifying age-related changes in disorders such as Werner's syndrome,[198] as well as metabolic conditions like Smith-Lemli-Opitz syndrome.[199] Therefore, hair can be a matrix used in the future for early cancer detection and comprehensive metabolic health assessment.

GBM analysis revealed heterogeneous cholesterol distribution across tumor regions. Using ToF-SIMS, we mapped these changes in situ, demonstrating that cholesterol reprogramming supports cancer progression, thereby highlighting the technique’s potential for studying cancer metabolism in intact tissues. Future studies could investigate the molecular pathways underlying altered cholesterol localization, linking lipid dysregulation to tumor progression and resistance, and explore therapeutic strategies such as cholesterol-modulating drugs (e.g., statins) or targeting cholesterol-rich regions for localized drug delivery to enhance treatment efficacy.

Limitations to the ToF-SIMS method concern how its offered resolution fails to distinguish isotopic effects from protonation during fragmentation of complex molecules like cholesterol. Future studies employing higher-resolution techniques, such as gas chromatography-high resolution mass spectrometry (GC-HR-MS) or magnetic resonance mass spectrometry (MRMS), could provide deeper insights into these isotopic effects and their impact on fragmentation patterns.

At the cellular level, our research demonstrated the power of ToF-SIMS combined with multivariate analysis techniques like MCR to distinguish the chemical profiles

of untreated and chemotherapy-surviving cancer cells. We identified distinct chemical signatures in different cellular components, such as the nucleus, cytoplasm, and lipid droplets. Notably, surviving cancer cells exhibited significant lipid droplet accumulation and altered chemical profiles following cisplatin treatment. These findings suggest that lipid droplet formation may play a critical role in the adaptive response of cancer cells to chemotherapy, contributing to their survival and therapeutic resistance.

Future work could focus on unraveling the biochemical pathways that regulate lipid droplet formation and their functional roles in cancer cell survival. A deeper understanding of how these structures influence specific stages of the cell cycle and contribute to cancer cell aggressiveness may identify novel therapeutic targets. Investigating the crosstalk between lipid metabolism and other stress response pathways, such as the HIF2α pathway discussed in Paper IV, could shed light on the complex mechanisms driving drug resistance and reveal additional strategies for intervention.

In conclusion, this thesis highlights the importance of chemical alterations in cancer biology, emphasizing the need for advanced analytical techniques to study metabolic reprogramming and adaptive responses in cancer cells. By bridging tissue-level observations with cellular-level insights, we can develop a more comprehensive understanding of cancer metabolism, paving the way for novel diagnostic and therapeutic strategies aimed at overcoming therapeutic resistance and improving patient outcomes.

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In wonder's glow, we dream and fly."

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