LITHUANIAN UNIVERSITY OF HEALTH SCIENCES
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HAMLET COMPLEX AND FOLFOX CHEMOTHERAPY IMPACT ON COLORECTAL CANCER CELLS AND TISSUES WITH DIFFERENT *KRAS/BRAF* MUTATIONS

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HAMLET KOMPLEKSO IR FOLFOX CHEMOTERAPIJOS POVEIKIS STOROSIOS ŽARNOS VĖŽIO LĄSTELĖMS IR AUDINIAMS SU SKIRTINGOMIS *KRAS/BRAF* MUTACIJOMIS

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CONTENTS

ABBREVIATIONS

APC	–	Adenomatous Polyposis Coli
APR	–	Abdominoperineal resection
BRAF	–	Human gene that encodes a protein called B-Raf
CA19-9	–	Carbohydrate antigen 19-9
CEA	–	Carcinoembryonic antigen
CIMP	–	CpG Island Methylator Phenotype
CIN	–	Chromosomal Instability
CMS	–	Consensus Molecular Subtypes
CRC	–	Colorectal cancer
dATP	–	Deoxyadenosine triphosphate
DNA	–	Deoxyribonucleic acid
EGF	–	Epidermal growth factor
EGFR	–	Epidermal growth factor receptor
FAP	–	Familial adenomatous polyposis
HAMLET	–	Human Alpha-lactalbumin Made LEthal to Tumor
HNPCC	–	Hereditary Non-Polyposis Colorectal Cancer
IARC	–	International Agency for Research on Cancer
KRAS	–	Kirsten rat sarcoma viral oncogene homolog gene that encodes a protein K-ras.
MAPK	–	Mitogen-activated protein kinase
MGMT	–	O-6-methlyguanine DNA methyltransferase
mRNA	–	Messenger RNA
MSI	–	Microsatellite instability
MSI-H	–	High-level microsatellite instability
MSI-L	–	Low-level microsatellite instability
MSS	–	Microsatellite stability
MYC	–	A transcription factor that controls the expression of numerous genes and is involved in cell cycle progression, apoptosis, and cellular transformation.
PICK3CA	–	Phosphatodylinositol-4,5-bisphosphate 3-kinase catalytic subunit alpha
RNA	–	Ribonucleic acid
SMAD4	–	Small mother against decapentaplegic 4
TP53	–	Tumor protein 53
$\mathbf{V_{ADP\ (glu/mal/suc)}}$	–	Maximal-ADP-activated respiration rate
Wnt/β-catenin	–	A key effector in the WNT pathway, when stabilized, translocates to the nucleus and affects gene transcription.

INTRODUCTION

Worldwide, colorectal cancer (CRC) is a significant health burden, with nearly two million new diagnoses and more than 0.9 million deaths estimated in 2020 [1]. In the EU-27 countries, it ranks as the second most common cancer (13 %) and the second leading cause of cancer deaths (12,3 %) in 2022 [2]. The rising trend in CRC cases can be linked to aging populations, lifestyle modifications, and more effective screening [3–5]. All these factors and enhanced treatment options have raised the overall 5-year survival rate for colorectal cancer to 65 %. However, survival rates vary widely depending on the stage at diagnosis: localized cases have a high five-year survival rate of 91 %, while this rate drops to 14 % for distant disease [6–8]. Another significant issue is the recurrence of CRC after curative-intent surgery, which differs between 9–31 % according to stages I–III [9].

Personalized medicine has become increasingly important in treating CRC, focusing on the disease's heterogeneity and complex biology. Molecular alterations, particularly in the *KRAS* and *BRAF* genes, are central to understanding the diverse nature of CRC and guiding targeted therapy [10,11]. Recent advances have led to the development of innovative treatment strategies that use molecular insights to create more targeted and individualized treatments for CRC patients, tailoring therapies to the specific needs of each case [12].

The emergence of personalized medicine represents a pivotal moment in treating metastatic colorectal cancer (mCRC). Advances in this field have significantly improved patient outcomes, particularly with the introduction of targeted therapies. Key agents such as bevacizumab, which targets vascular endothelial growth factor, and cetuximab, which addresses the epidermal growth factor receptor (EGFR), have been crucial. The ongoing challenges in CRC are due to the heterogeneity of the disease and the variability in patients' molecular profiles, particularly with mutations in genes like RAS and *BRAF* leading to resistance against certain treatments [13].

Although new treatments have improved median survival rates in clinical trials, a disparity persists in the survival outcomes of the broader CRC patient population. Comparing clinical trial subjects with the broader mCRC population is complicated, given that trial participants tend to have fewer comorbidities and are younger and healthier [12,14,15].

In an effort to discover new therapeutic agents, researchers have been exploring natural products as an adjunct to CRC treatment [16,17]. One of the most promising new findings is the HAMLET complex (Human Alpha-lactalbumin Made LEthal to Tumor cells) [18]. HAMLET, derived from

components found in human milk, have a unique ability to target cancer cells while sparing healthy cells. Its efficacy in targeting cancer cells, for example, by acting on the EGFR pathway and potentially affecting mitochondrial function, positions it as a notable addition to cancer treatment options [19].

The dissertation hypothesis is that combining HAMLET with conventional chemotherapy regimens will enhance its efficacy against CRC. By analyzing cancer cell lines and patient-derived ex vivo biopsies, this study aims to elucidate the underlying mechanisms responsible for HAMLET's efficacy against CRC. Positioned within the field of precision medicine, this research provides valuable insights into personalized treatment strategies for CRC.

NOVELTY AND RELEVANCE OF THE STUDY

This research is of significant international importance as it pioneers the exploration of novel therapeutic avenues for treating CRC, focusing on the cytotoxic effects of HAMLET and its combination with the FOLFOX chemotherapy regimen. Little is known about the efficacy of HAMLET in CRC, particularly in the context of different *KRAS/BRAF* mutation status, and this study is the first to comprehensively analyze its impact on CRC cell viability, death pathways, and mitochondrial function. In addition, this study breaks new ground by investigating the synergistic effects of HAMLET and FOLFOX in CRC, particularly in the presence of the *BRAF* V600E mutation, which is associated with poor prognosis and resistance to therapy.

In addition, the study explores the underlying bioenergetic profiles of CRC cells, shedding light on the metabolic factors that influence cellular responses to HAMLET treatment. This research provides novel insights into CRC treatment efficacy by highlighting the role of mitochondrial respiration and bioenergetic parameters in modulating drug response. It paves the way for personalized therapeutic approaches tailored to individual mutational profiles.

In conclusion, this dissertation advances our understanding of personalized medicine in CRC treatment by exploring innovative therapeutic strategies and elucidating the complex interplay between genetic mutations, bioenergetics, and treatment response. By addressing critical gaps in CRC therapy, this research has the potential to change current treatment paradigms, offering new hope to patients with refractory CRC and contributing to improved treatment outcomes on a global scale.

1. AIM AND OBJECTIVES

Aim:

This study aimed to evaluate the effect of HAMLET complex and FOLFOX chemotherapy on colon cancer cells and tissues with different *KRAS*/*BRAF* mutations, *in vitro* and *ex vivo*.

Objectives:

1. To assess the impact of HAMLET on CRC cell lines with different *KRAS/BRAF* mutations *in vitro*, focusing on viability, cell death pathway, and colony formation.
2. To evaluate the effect of HAMLET on mitochondrial function and membrane permeability in colorectal cancer cells with different *KRAS/BRAF* mutations, *in vitro*.
3. To evaluate the response of CRC cell lines to FOLFOX and its potential synergistic effect with HAMLET in different *KRAS/BRAF* mutations, *in vitro*.
4. To evaluate the effect of HAMLET, FOLFOX chemotherapy, and their combination on viability of colorectal cancer explants with different *KRAS/BRAF* mutations*, ex vivo.*
5. To evaluate the effect of HAMLET on mitochondrial function of colon cancer explants with different *KRAS/BRAF* mutations, *ex vivo.*

2. REVIEW OF LITERATURE

2.1. Colorectal cancer

The World Health Organization (WHO) describes colorectal cancer as a malignancy that starts in the caecum, colon or rectum, parts of the large intestine. Common early symptoms of this condition can include rectal bleeding, changes in bowel habits, unexplained weight loss, abdominal discomfort, and fatigue [20]. This cancer typically develops from polyps, which are small, benign growths on the inner lining of these sections of the bowel that can become cancerous over time [21].

The development of colorectal cancer is influenced by a combination of genetic and epigenetic changes that can be inherited or result from environmental and lifestyle factors [6]. Identified risk factors include age, family history, inflammatory bowel disease, smoking, dietary habits, race, obesity, sedentary lifestyle, and alcohol consumption [5,20,22–24]. The transition from benign adenomas to cancer typically takes 10-15 years, highlighting the critical role of early detection and removal of adenomas to prevent progression to invasive cancer [21].

2.1.1. Epidemiology of colorectal cancer

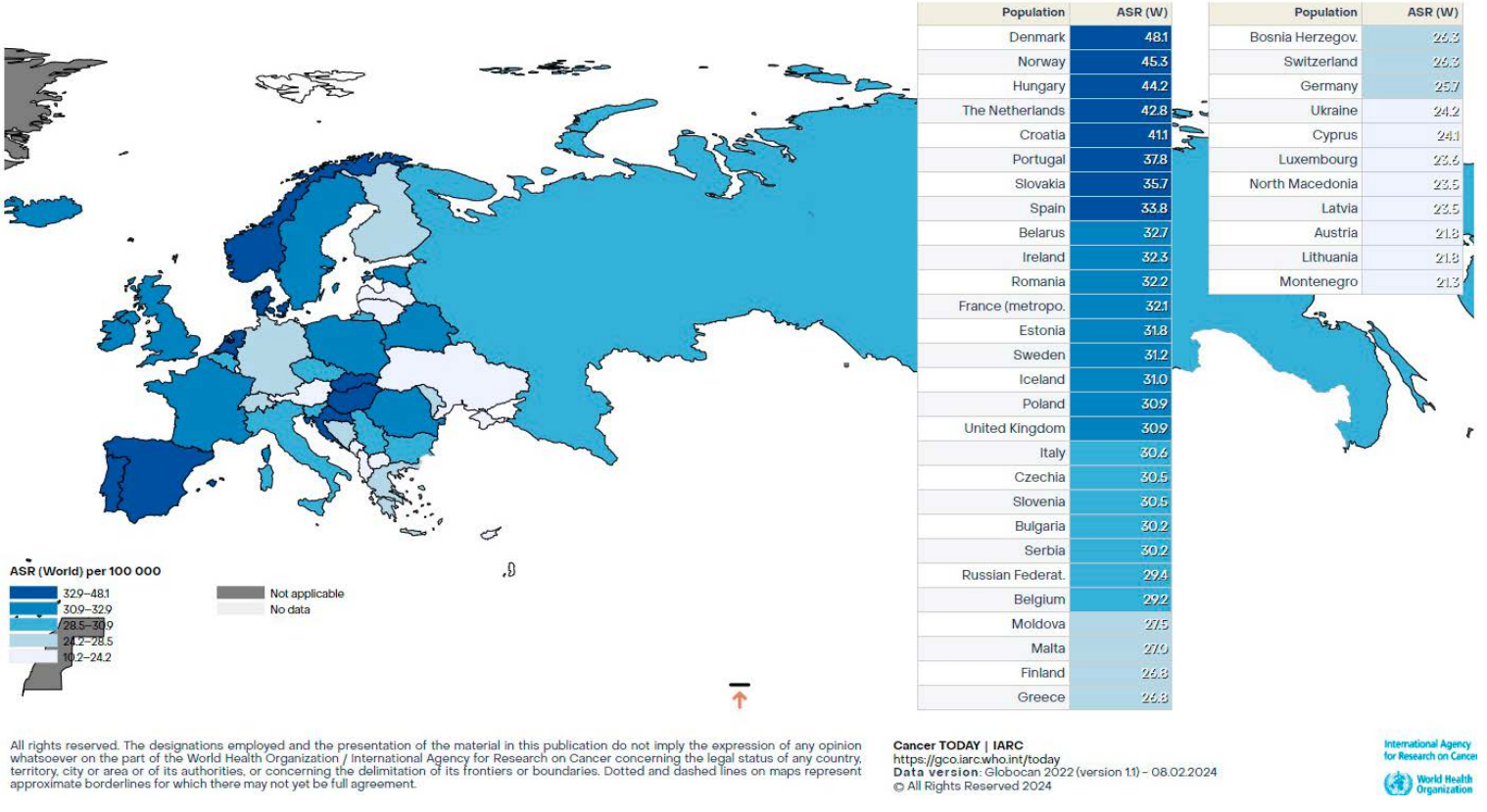
Worldwide, one-tenth of all cancers are found in the large intestine. GLOBOCAN estimates almost two million new cases (3rd in the world) and one million deaths (2nd in the world) of CRC each year [1]. In 2022, large bowel cancer was the second most diagnosed cancer and the second leading cause of cancer death in the EU, as summarized in Table 2.1.1.1 [25]. Hungary, Slovenia, Slovakia, the Netherlands, and Norway have the highest incidence rates in Europe. This is followed by Australia, New Zealand, North America, and East Asia [26]. For mortality, Hungary, Croatia, and Slovakia are among the highest, with three European countries in the top four in the world. While Lithuania reports one of the lowest incidence rates in Europe, surpassed only by Montenegro, its mortality rate positions it in the middle of the table [2]. Incidence and mortality among European countries in 2022 are presented in Figures 2.1.1.1 and 2.1.1.2.

Table 2.1.1.1. *The most diagnosed and most common cancer cause of death in Europe in 2022*

Most Diagnosed Cancers	Most Common Cancer Causes of Death
13.8 % Breast cancer*	19.5 % Lung cancer
13 % Colorectal cancer	**12.3 % Colorectal cancer**
12.1 %** Prostate cancer	7.5 %* Breast cancer
11.6 % Lung cancer	7.4 % Pancreatic cancer

(*) – "Breast cancer" 99 % of these cases affect women. (**) "Prostate cancer" cases affect men only.

Age is a significant risk factor for sporadic bowel cancer, which is rare in people under 40. However, the American Cancer Society reports that the incidence of colorectal cancer has been increasing by 1–2 % per year in people under 55 since the mid-1990s and has become the leading cause of cancer death in men under 50 and the second leading cause of cancer death in women [8,27,28].



Population	ASR (W)
Denmark	48.1
Norway	45.3
Hungary	44.2
The Netherlands	42.8
Croatia	41.1
Portugal	37.8
Slovakia	35.7
Spain	33.8
Belarus	32.7
Ireland	32.3
Romania	32.2
France (metropo.	32.1
Estonia	31.8
Sweden	31.2
Iceland	31.0
Poland	30.9
United Kingdom	30.9
Italy	30.6
Czechia	30.5
Slovenia	30.5
Bulgaria	30.2
Serbia	30.2
Russian Federat.	29.4
Belgium	29.2
Moldova	27.5
Malta	27.0
Finland	26.8
Greece	26.8

Population	ASR (W)
Bosnia Herzegov.	26.3
Switzerland	26.3
Germany	25.7
Ukraine	24.2
Cyprus	24.1
Luxembourg	23.6
North Macedonia	23.5
Latvia	23.5
Austria	21.8
Lithuania	21.8
Montenegro	21.3

Fig. 2.1.1.1. *CRC incidence among European countries in 2022. ASR (World) per 100,000 refers to the Age-Standardized Rate (World) for cancer incidence per 100,000 population.*

This figure is adapted GLOBOCAN [1].

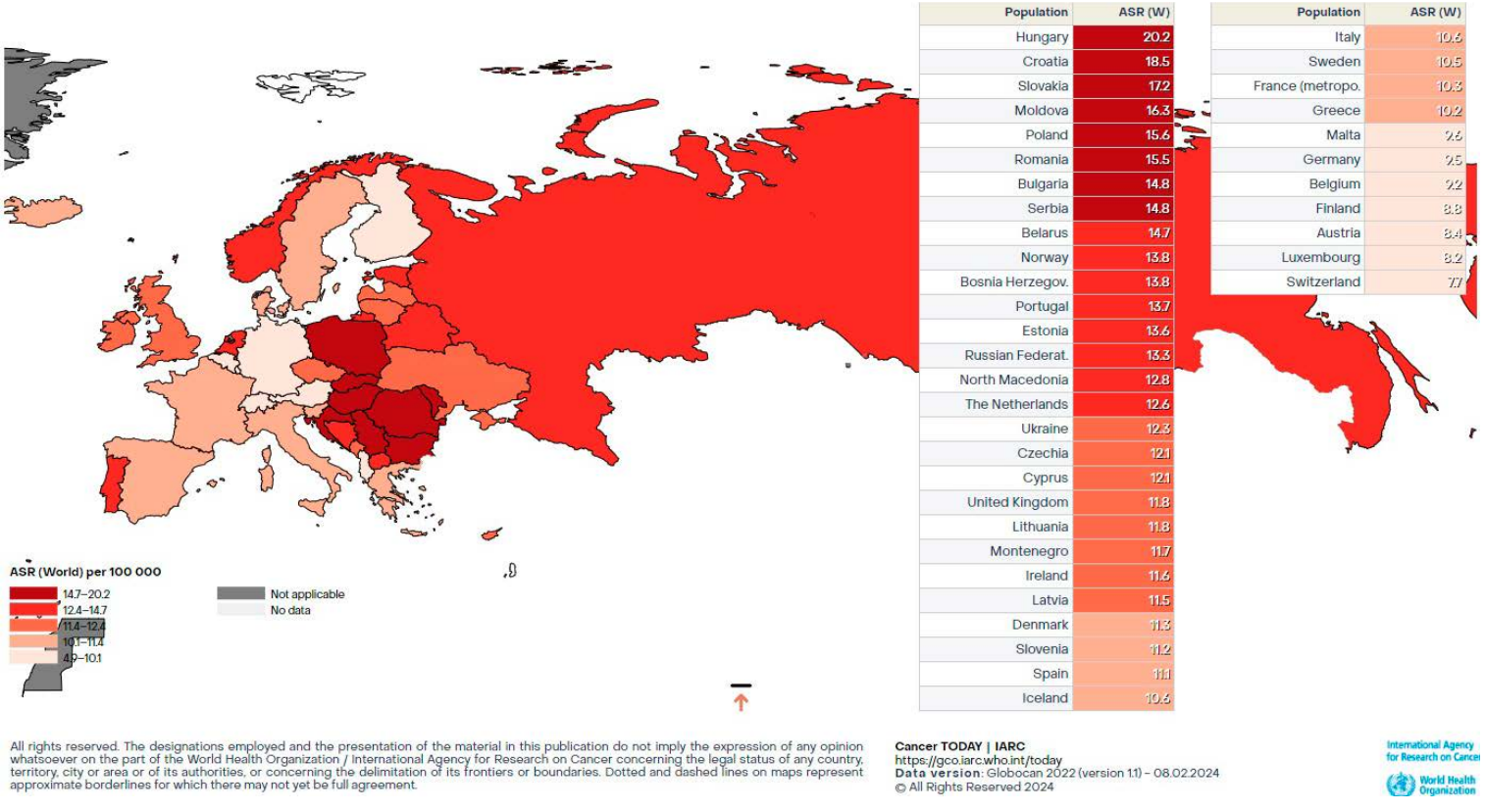


Fig. 2.1.1.2. *CRC mortality among European countries in 2022. ASR (0World) per 100,000 refers to the Age-Standardized Rate (World) for cancer mortality per 00,000 population.*

This figure is adapted GLOBOCAN [1].

2.1.2. Molecular pathogenesis of colorectal cancer

CRC develops from the normal colonic epithelium through molecular pathways, each distinguished by specific genetic and epigenetic changes. The adenomatous pathway, accounting for the majority of cases (70–90 %), follows a trajectory of chromosomal instability (CIN), beginning with APC gene mutations and progressing through a cascade of genetic changes, including *KRAS,* PI3KCA, SMAD4, and TP53. This sequence, which can progress from early to late adenomas, was summarized by Vogelstein in 1990 [24,29–31].

Simultaneously, the serrated pathway (10–20 %) involves hyperplastic polyps and sessile serrated lesions with mutations in *BRAF* or *KRAS* [32]. These lesions often feature mutations in the CpG island methylator phenotype (CIMP), which leads to gene silencing [33]. Some cases (2–7 %) also involve mutations in MLH1, part of the mismatch repair (MMR) system, which results in microsatellite instability (MSI) [24,34].

The interplay between these pathways can result in colorectal cancers with diverse molecular characteristics, including CIN, MSI, and CIMP [35]. The serrated pathway can lead to traditional serrated adenomas. In contrast, the adenomatous pathway may result in adenomatous polyps or serrated adenomas through the MMR mechanism, mainly when germline mutations in MMR genes are present [36]. The complexity of these overlapping pathways and their impact on CRC diversity is illustrated in Figure 2.1.2.1 [35].

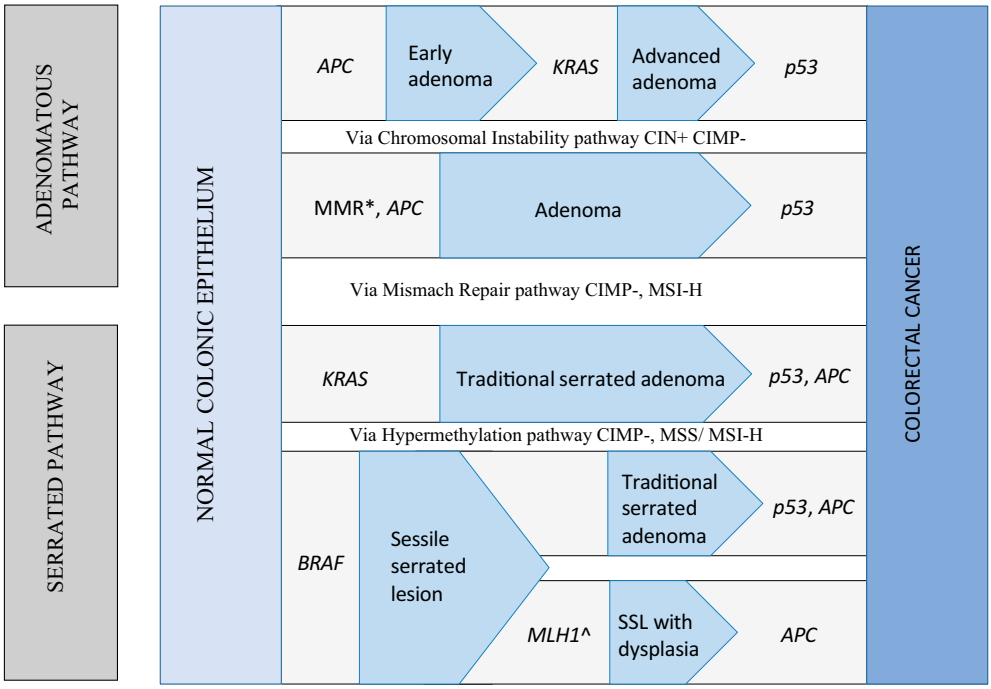


Fig. 2.1.2.1. *Molecular pathogenesis and pathways of colorectal cancer development.*

Adapted from Dekker E *et al*. [24], Kasi *et al*. reviews [35], and IARC Handbook of cancer prevention [20]. Genes such as APC, in the case of familial adenomatous polyposis, and mismatch repair genes [MMR*], like those involved in Lynch syndrome, can mutate before cancer develops. In sporadic colorectal cancer, these mutations may occur during carcinogenesis. Additionally, the MLH1 gene often becomes hypermethylated.

2.1.2.1. Colorectal cancer tumorigenesis molecular pathways

Colorectal cancer develops through three tumorigenesis pathways, each characterized by different types of genomic instability, including:

1. The CIN pathway, typically linked to familial adenomatous polyposis (FAP), features chromosomal alterations like deletions and insertions due to mutations in genes such as APC, *KRAS*, and others, affecting tumor suppressor genes and growth-promoting pathways [29].
2. The MMR pathway, often associated with Lynch syndrome, is characterized by germline mutations in genes like MLH1 or MSH2, causing MSI-H in tumors. Sporadic CRCs may also show MSI-H, frequently due to MLH1 promoter hypermethylation [37].

3. The CIMP pathway features hypermethylation that silences MMR genes, like MLH1. CRCs with CIMP positivity and MSI-H often have *BRAF* V600E mutations but not *KRAS* mutations, a pattern associated with a worse prognosis [35,36,38].

2.1.2.2. Genetic and epigenetic classifications of CRC subtypes

A total of five molecular subtypes of colorectal cancer, based on the presence of specific genetic mutations, chromosomal alterations, and methylation patterns, are presented in Table 2.1.2.2.1 below [21,39]:

1. CIN develops from adenomas, and it is predominantly microsatellite stable and CpG island methylator phenotype negative, following the APC pathway. It occurs both as a hereditary condition like FAP and sporadically.
2. Hereditary non-polyposis colorectal cancer (HNPCC) is associated with Lynch syndrome and is characterized by the absence of CIMP, MSI-H, the absence of chromosomal alterations, and *BRAF* mutations.
3. Serrated polyp-derived CRCs Type 1 represent 12 % of CRCs and are characterized by CIMP high (CIMP-H) status, *BRAF* mutations, chromosomal stability, and MSI-H. These tumors are likely to originate from sessile serrated adenomas in the right colon.
4. Serrated polyp-derived CRCs Type 2 represent 8 % of cases and are also CIMP-H. They are generally chromosomally stable, show MSI-L or MSS, and have MGMT methylation and *BRAF* mutations. These CRCs typically arise from traditional serrated adenomas and are more common on the left side of the colon.
5. Hybrid pathway CRCs constitute 20 % of CRCs and exhibit low CIMP (CIMP-L), chromosomal instability, MGMT methylation, *KRAS* mutations, and are MSI-L or MSS. They likely develop from a blend of the serrated and CIN pathways and can originate from either adenomatous polyps or serrated lesions with *KRAS* mutations.

Table 2.1.2.2.1. *Five molecular subtypes of colorectal cancer adapted from a review of Amy E [39]*

Heredity	**Chromosomal instability Pathway**	**Mismatch repair pathway**	**Serrated/CIMP pathway**		**Hybrid pathway**
	Hereditary and sporadic	**Hereditary**	**Hereditary and sporadic**		**Sporadic**
CIMP status	Negative	Negative	High	High	Low
MSI status	MSS	MSI-H	MSI-H	MSI-L	MSI-L or MSS
Chromosomal instability	Present	Absent	Absent	Absent	Present
KRAS mutation	+++	+/-	---	---	+++
BRAF mutation	---	---	+++	+++	---
MLH1 status	Normal	Mutation	Methylated	Partial methylation	Normal
MGMT methylation	---	---	+/-	+++	+++

+++: present; +/-: might or might not be present; ---: absent.

2.1.2.3. Transcriptomic and microenvironment *KRAS* mutation has been identified as a biomarker for resistance profiles in CRC classification

Consensus molecular subtypes (CMS) are based on RNA expression patterns reflecting different biological characteristics and the tumor microenvironment. Valuable for targeted therapies and predictive assessments. This system divides CRC into four biologically distinct subtypes [40,41]:

1. CMS1 (MSI Immune): characterized by microsatellite instability, a strong immune activation, and hypermutation, often with *BRAF* mutations. CMS1 has worse survival after relapse.
2. CMS2 (Canonical): marked by chromosomal instability, prevalent Wnt/β-catenin and MYC signaling activation, and typically MSS.
3. CMS3 (Metabolic): defined by metabolic dysregulation and intermediate levels of MSI and CIN, often with *KRAS* mutations.
4. CMS4 (Mesenchymal): distinguished by prominent transforming growth factor-beta activation, stromal invasion, and angiogenesis. CMS4 has worse relapse-free and overall survival.

2.1.3. Genetic alterations and MAPK/ERK pathway

2.1.3.1. *KRAS* oncogene

The *KRAS* gene, a proto-oncogene, produces a 21 kDa GTPase located on the short arm of chromosome 12. As a member of the Ras protein family, it plays a critical role in triggering cellular mechanisms by activating signaling pathways, particularly the MAPK and PI3K pathways. Together, these pathways regulate crucial cellular functions including proliferation, differentiation, motility, survival and intercellular trafficking. *KRAS* is a key component of the epidermal growth factor receptor (EGFR) signaling cascade, and mutations in the *KRAS* gene can lead to aberrant activation of this pathway [42].

In CRC, *KRAS* mutations are present in approximately 30-50 % of cases. These mutations most commonly occur as point mutations in codons 12 and 13, less commonly in codon 61, and rarely in codons 59, 146, 19, or 20 [43]. The presence of a *KRAS* mutation has been identified as a biomarker for resistance to anti-EGFR monoclonal antibody therapies used in the treatment of metastatic CRC. The prognostic significance of *KRAS* mutations in CRC remains controversial. While some studies suggest an association with poorer outcomes, others find no significant correlation with prognosis [42,43].

2.1.3.2. *BRAF* oncogene

The *BRAF* gene encodes a protein that is a key player in the MAPK/ERK signaling pathway, which regulates cell division, differentiation and secretion. Located on chromosome 7, *BRAF* is a member of the RAF kinase family, which is essential for cellular responses to growth signals. When activated, the *BRAF* protein facilitates a cascade of signaling events that can affect various cellular functions.

Mutations in the *BRAF* gene, in particular the V600E mutation, have been identified in a significant number of CRC cases, accounting for approximately 10-15 %. These mutations lead to continuous activation of the MAPK/ERK pathway, independent of external growth signals, driving uncontrolled cell proliferation and contributing to cancer development.

The presence of a *BRAF* mutation is also clinically important, as it is associated with a poorer prognosis and can influence response to certain therapies, particularly those targeting the EGFR pathway. *BRAF* mutations are recognized as a marker of resistance to anti-EGFR therapy in CRC, similar to *KRAS* mutations. Therefore, *BRAF* status is a consideration when developing treatment strategies for patients with metastatic CRC. The prognostic implications of *BRAF* mutations in CRC, together with their role

in resistance to therapy, highlight the importance of genetic profiling in the management of the disease [44,45].

2.1.3.3. MAPK/ERK signaling pathway

The MAPK/ERK signaling pathway plays a key role in CRC by regulating essential cellular functions such as growth, division, and survival. This pathway is particularly sensitive to mutations in oncogenes such as *KRAS* and *BRAF*, which facilitate tumorigenesis by enabling continuous cellular proliferation and differentiation, effectively bypassing the body's normal regulatory mechanisms. Such alterations in *KRAS* and *BRAF* drive the progression of CRC and affect the efficacy of therapeutic strategies. For example, these mutations are known to confer resistance to anti-EGFR therapies commonly used to treat metastatic CRC, thereby influencing treatment choices.

To further understand the function of this pathway, cancer cells use a network of signaling pathways to maintain growth signals and evade inhibitory or apoptotic influences. In contrast, these pathways are dormant in healthy cells and become active in cancer cells due to accumulated mutations in proto-oncogenes and inactivation of tumor suppressor genes. *KRAS* plays a critical role in the MAPK cascade, a key network segment. Activation begins when epidermal growth factor ligands bind to their respective tyrosine kinase receptors on the cell surface, triggering a series of biochemical events. These events involve the adaptor proteins GRB2 and SOS, which facilitate the exchange of GDP for GTP on *KRAS*, thereby activating it. Active *KRAS* then interacts with *BRAF*, leading to the formation and activation of the *BRAF* dimer. This activation sequence continues with the phosphorylation of MEK and then ERK, which translocates to the nucleus to activate transcription factors that promote cell proliferation and survival [46,47]. A detailed understanding of the MAPK/ERK pathway is presented in Figure 2.1.3.3.1 [44].

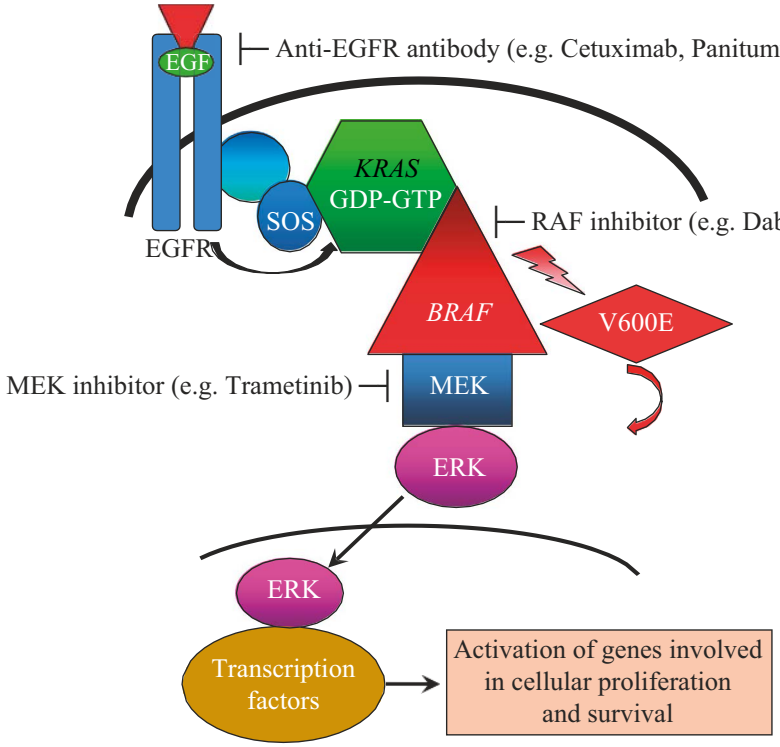


Fig. 2.1.3.3.1. *MAPK/ERK signaling pathway and potential targets. When the epidermal growth factor (EGF) attaches to its receptors (EGFR) on a cell's surface, it triggers EGFR phosphorylation, setting off a chain reaction. This reaction prompts the adaptor proteins GRB2 and SOS to catalyze the exchange of GDP for GTP on KRAS, activating it. Active KRAS enables BRAF to bind and phosphorylate it, advancing the signal downstream to MEK and then to MEK and ERK proteins. Once activated, ERK moves into the nucleus, which stimulates transcription factors that drive the survival and proliferation of cancer cells. Potential inhibitors have been identified to modulate this pathway: Anti-EGFR agents such as cetuximab and panitumumab target the initial steps by inhibiting EGFR, while trametinib acts as an MEK inhibitor and dabrafenib as an RAF inhibitor, each of which disrupts subsequent steps in the pathway.*

This figure is adapted from reviews by Bond *et al*. and CWK Wu *et al*., emphasizing both mechanism and therapeutic targets within the pathway. [11,44].

2.1.4. Personalized medicine in advanced CRC

Personalized medicine adapts medical treatment from the traditional "one-size-fits-all" strategy to individual patient characteristics. While surgery often remains, the initial treatment for localized, personalized medicine significantly improves therapeutic decisions after surgery, improving outcomes and minimizing side effects.

The evolution of personalized medicine in advanced CRC has been marked by significant milestones in both therapeutic approaches and research discoveries, as shown in the timeline below (Fig. 2.1.4.1). Since the 1950s, advances in CRC therapies have progressed from the introduction of 5-FU monotherapy to more complex regimens such as FOLFOX and FOLFIRI in the 1990s, and later to targeted therapies such as cetuximab and panitumumab that specifically inhibit EGFR (see Fig. 2.1.3.3.1). These developments of these therapies coincided with key discoveries in CRC research, such as the identification of the APC gene and the sequencing of the first CRC genome, which greatly improved our understanding of the molecular landscape of the disease [11].

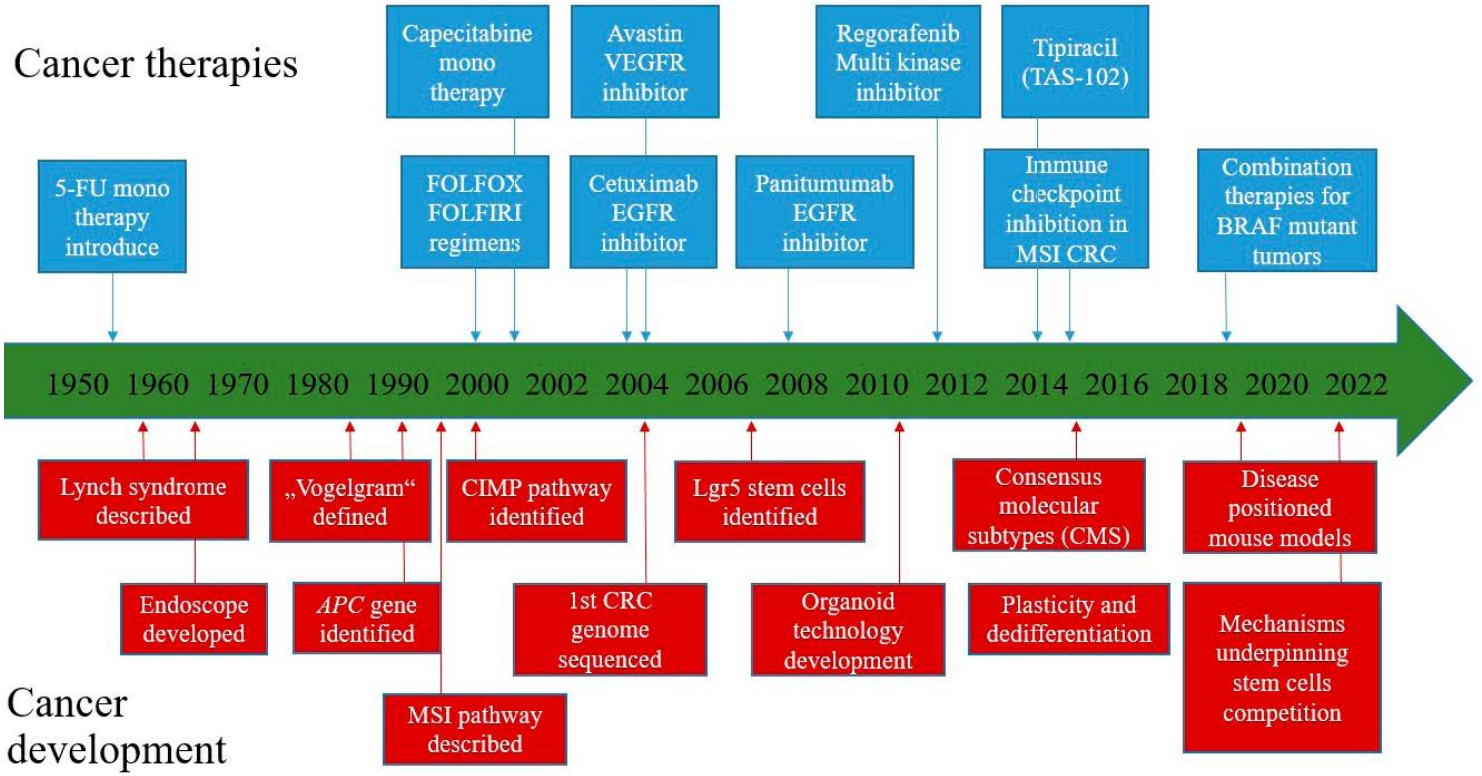


Fig. 2.1.4.1. *The evolution of personalized medicine in advanced colorectal cancer.*

This figure is adapted from a review by Bond CWK Wu *et al*. [11].

The recent introduction of multi-kinase and immune checkpoint inhibitors marks an era in which treatment is increasingly tailored to individual genetic profiles, mainly using MSI status to guide immunotherapy. The advent of organoid technology and the identification of consensus molecular subtypes (CMS) further reflect a shift towards precision medicine, allowing for more personalized treatment plans based on tumor characteristics and genetic variations. This personalized approach aims to optimize efficacy and minimize unnecessary toxicity, paving the way for more effective management of advanced CRC [40,48].

2.1.4.1. Initial systemic therapy for CRC

In the field of precision medicine for CRC, chemotherapy continues to play a critical role, now fine-tuned to align with individual genetic profiles. By the European Society for Medical Oncology (ESMO) guidelines [49], traditional chemotherapeutics such as 5-fluorouracil (5-FU), oxaliplatin, capecitabine, and irinotecan remain fundamental, frequently utilized in combinations such as FOLFOX and FOLFIRI to optimize efficacy. Molecular diagnostics has revolutionized the treatment landscape, enabling oncologists to tailor regimens to specific genetic markers, such as *KRAS* or *BRAF* mutations and microsatellite instability (MSI) status. This targeted approach enhances treatment precision, minimizes side effects, and optimizes outcomes for patients with mCRC [34,50].

Adjuvant chemotherapy is administered following surgery to eradicate residual cancer cells and reduce the likelihood of recurrence. Neoadjuvant chemotherapy is employed before surgery to reduce tumor size, thereby facilitating easier surgical removal. Additionally, radiation therapy is frequently combined with chemotherapy (chemoradiation), particularly in the case of rectal cancer, to shrink tumors before surgical intervention and destroy any residual cancer cells after that [51].

2.1.4.2 Targeted therapy for CRC

Following the use of traditional chemotherapies, targeted therapy has emerged as a key element in the management of CRC, particularly in its advanced stages. These therapies target specific molecular pathways essential for the survival and proliferation of cancer cells, offering a more tailored approach to treatment:

1. EGFR inhibitors (see Figure 2.1.3.3.1): These agents target the epidermal growth factor receptor (EGFR), a key mediator of tumor growth and survival.

a. Cetuximab is a monoclonal antibody that targets the epidermal growth factor receptor (EGFR), a key player in tumor growth and survival. It is utilized treating mCRC and is particularly effective in patients with wild type *KRAS* genes, where it inhibits the binding of growth factors to EGFR [52–54].
b. Panitumumab is another monoclonal antibody that is effective in treating mCRC. Similarly to cetuximab, this monoclonal antibody is employed under the same genetic conditions, thereby ensuring its efficacy by targeting the EGFR in *KRAS* wild type patients [54].

2. Vascular endothelial growth factor (VEGF) inhibitors: These agents target vascular endothelial growth factor (VEGF), a crucial factor in tumor angiogenesis, the process by which new blood vessels form to supply the growing tumor.
   a. Bevacizumab is a monoclonal antibody that inhibits angiogenesis, the process by which new blood vessels form to supply the growing tumor. This monoclonal antibody is employed with chemotherapy for mCRC to inhibit angiogenesis and, thus, tumor growth [55].
   b. Aflibercept is a recombinant humanized IgG1 kappa monoclonal antibody that binds to VEGF-A and inhibits its activity. As a decoy receptor for VEGF, it is used in combination with FOLFIRI in metastatic CRC following chemotherapy failure to hinder the vascular support needed by tumors [56].
3. *BRAF* Inhibitors: These agents specifically target and inhibit *BRAF* mutations, which are significant in various cancers, including CRC (see Figure 2.1.3.3.1).
   a. Vemurafenib, initially employed in the treatment of melanoma, has demonstrated efficacy in CRC with *BRAF* V600E mutations. It is typically administered in combination with agents such as irinotecan and cetuximab.
   b. Dabrafenib is another *BRAF* inhibitor that has been shown to be effective in the treatment of *BRAF* V600E-mutated CRC. In conjunction with trametinib, it is employed in the treatment of mCRC that exhibits *BRAF* V600E mutations, particularly in the event that previous treatments have proven ineffective [57].
4. Multi-kinase inhibitors are a class of drugs that inhibit multiple kinases, which are enzymes that regulate various cellular processes. These drugs target various kinases involved in the growth and angiogenesis of cancer.
   a. Regorafenib is effective in the treatment of mCRC, which has progressed following other treatments. This is employed for the treatment of mCRC that has progressed following other treatments.

It targets multiple pathways simultaneously, thereby halting cancer progression [58].

b. Sorafenib is a multi-kinase inhibitor that has been shown to be effective in the treatment of mCRC that has progressed following other treatments. Sorafenib is primarily utilized for the treatment of renal and liver cancers. However, it also has potential in the treatment of CRC, particularly in clinical trials involving patients with specific mutations [59].

2.1.4.3. Immunotherapy

Immunotherapy represents a transformative advancement in the treatment of CRC, particularly for tumors with specific genetic features such as microsatellite instability-high (MSI-H) or mismatch repair deficiency (dMMR). These tumors, which are characterized by a high mutation burden, are particularly susceptible to immune checkpoint inhibitors (Fig. 2.1.2.1 and paragraph 2.1.2.3):

1. Pembrolizumab and Nivolumab are two prominent checkpoint inhibitors that target the PD-1 pathway, a mechanism that tumors use to evade the immune system. These drugs have demonstrated significant efficacy in MSI-H or dMMR metastatic CRC by enhancing the immune system's ability to recognize and destroy cancer cells.
2. Ipilimumab - another checkpoint inhibitor that targets CTLA-4, is used in combination with nivolumab to further stimulate the immune response against cancer cells, particularly in CRC patients with specific genetic profiles [60].

2.1.4.4. CRC liver metastases - directed treatments

Radiofrequency ablation (RFA) and cryotherapy represent minimally invasive approaches to the targeted destruction of liver metastases from CRC. Radiofrequency ablation (RFA) employs high-energy radio waves, whereas cryotherapy utilizes extreme cold to eradicate cancer cells, rendering it a valuable adjunct to surgical removal when it is not feasible [61].

In the context of broader liver-directed treatments, Transarterial Chemoembolization (TACE) and Selective Internal Radiation Therapy (SIRT) are employed to address liver metastases. Transarterial chemoembolization (TACE) is a procedure that combines direct infusion of chemotherapy into the liver with the administration of an agent that blocks the hepatic artery. This approach has been shown to enhance drug retention and efficacy. SIRT involves the delivery of radioactive microspheres directly to liver tumors

through the same artery, thereby enabling the administration of radiation treatment with greater precision and efficacy [62,63].

2.1.4.5. Pharmacogenomics and drug metabolism

Pharmacogenomics greatly enhances the personalization of chemotherapy for CRC by determining how genetic variations influence drug response. Initially, drug testing involves the use of cancer cell lines with methods such as MTT assays, clonogenic assays, and flow cytometry to assess the efficacy and cytotoxicity of chemotherapeutic agents. These tests help identify the most promising drugs before moving on to genetic analysis.

This approach allows to adjust treatments based on genetic markers. For example, variations in the DPYD gene affect how a patient processes the chemotherapy drug 5-fluorouracil (5-FU). Identifying patients with these variants through genetic testing allows dose adjustments to minimize toxicity. Similarly, the detection of *KRAS* mutations can guide the selection of effective therapies, avoiding ineffective treatments such as EGFR inhibitors for those with specific mutations [64,65].

2.1.4.6. *Ex vivo* patient biopsies

Ex vivo testing plays a critical role in personalized cancer care, mainly through the use of living cancer tissue slices removed during surgery and cultured in growth media. This approach creates patient explant models that preserve the natural microenvironment of the tumor, allowing the evaluation of treatment efficacy prior to the initiation of systemic therapies. Various methods, such as 2D cultures of isolated tumor cells, 3D spheroid cultures, patient-derived xenograft (PDX) cultures, and organotypic tumor slice cultures, each provide unique insights into how cancer cells behave and respond to treatment. These techniques mimic actual tumor conditions, from simple cell interactions to complex tissue architectures, helping to tailor therapy for increased efficacy [66].

Studies by Sönnichsen *et al*. (2018) and Martin *et al*. (2019) underscore the utility of these ex vivo models, particularly patient-derived tissue slice cultures, in assessing treatment response in CRC. They allow real-time analysis of tumor responses to treatments, increasing the precision of therapeutic strategies tailored to individual patient needs. This targeted approach improves therapeutic outcomes and reduces the risk of ineffective treatments, marking a significant advance in the field of oncology and personalized medicine [53,67].

2.1.5. Cellular energetics and mitochondrial respiration

Mitochondrial respiration is increasingly being recognized as a key area of personalized medicine, particularly in CRC. Studies such as those by Rebane-Klemm *et al*. highlight how mitochondrial function varies among individuals and its significant impact on cancer cell dynamics and response to therapy. Their research shows that *KRAS*- and *BRAF*-mutant colorectal tumors and polyps have different mitochondrial respiration profiles, which may affect treatment strategies [68].

Further research by the same group extends this by demonstrating increased glycolytic activity in colorectal polyps, suggesting that metabolic pathways are prominently altered in early-stage colorectal lesions [69]. These findings underscore the potential for developing treatment approaches that target specific metabolic characteristics of cancer cells.

Incorporating metrics of mitochondrial respiration into the framework of personalized medicine not only deepens our understanding of tumor biology but also facilitates the development of targeted therapies that address the unique metabolic needs of individual tumors. This approach increases the precision and efficacy of CRC treatments by tailoring therapies to match better the metabolic profiles identified in different types of tumors and polyps.

2.1.6. Anticancer natural compounds as an adjunct treatment

Adjunct treatments have received considerable attention for their potential role in preventing and treating CRC. These compounds, often derived from plants, vegetables, fruits, and spices, are rich in antioxidants and possess anti-inflammatory properties that may inhibit cancer cell growth and induce apoptosis [16]. For example, curcumin [17], the active ingredient in turmeric, has shown promise in reducing inflammation and preventing cancer cell proliferation in CRC. Similarly, resveratrol, found in the skins of red grapes, exhibits anti-cancer properties by interfering with cancer cell DNA and promoting cell death [70,71]. Other notable compounds include sulforaphane from cruciferous vegetables such as broccoli, which has been studied for its ability to detoxify carcinogens and support normal cell function [72,73]. Incorporating adjunct treatment into the daily diet or as supplemental therapies has the potential not only to enhance the efficacy of conventional cancer treatments but also to offer a preventative approach by maintaining cellular health and reducing inflammation associated with cancer progression [74].

2.1.6.1. Human milk

Breast milk is the optimal and primary source of nutrition for newborns and infants. It begins to be produced around 16-22 weeks of pregnancy. Both WHO and UNICEF recommend that newborns should be breastfed within one hour of birth and that they should be exclusively breastfed for the first six months. The composition of breast milk varies widely between different feedings, over the course of lactation, and among women in different populations [75]. Breast milk contains essential nutrients such as fats, proteins, carbohydrates, and bioactive compounds. Proteins in breast milk are categorized into caseins (alpha-, beta-, and K-casein), whey proteins (alpha-lactalbumin, lactoferrin, lysozymes, and immunoglobulin A), and mucins from milk fat globule membranes [76]. It also contains significant amounts of palmitic and oleic acids, which are found in triglycerides [77]. The composition of breast milk differs significantly from that of cow's milk; it is more yellow and viscous, with higher levels of protein, sodium chloride, and magnesium, and lower levels of calcium, potassium, and metabolic by-products [78]. Colostrum, the first milk, is low in saturated fatty acids and linolenic acid, but high in immunoglobulin A, which protects newborns from gastrointestinal infections [79]. In addition, oleic acid and alpha-lactalbumin in human milk are important components of HAMLET, a compound with potential anti-tumor properties.

2.1.6.2. Human Alpha-lactalbumin

Alpha-lactalbumin, a whey protein found in breast milk, was first isolated by Arthur Wichmann in 1899 [80]. It has been extensively studied for its role in lactose synthesis and other biological functions. Initially estimated to have a molecular mass of 15,500 kDa in 1955 by W. G. Gordor and J. Ziegler [81], later research by Svensson, M., and Sabharwal, H., in 1999 revised this to only 14 kDa [82]. The structure of alpha-lactalbumin, well documented since 1966, includes both alpha-helix and beta-sheet components, contributing to its functional versatility in mammalian milk [83].

This protein is particularly prominent in human milk, which contains a much higher proportion of alpha-lactalbumin (28 % of total protein) compared to cow's milk (3 % of total protein), which significantly affects the amino acid profile and nutritional quality [84]. Its high tryptophan content is critical for neonatal development, affecting sleep and brain function. Studies have shown that consumption of alpha-lactalbumin increases plasma tryptophan levels, thereby improving sleep quality and cognitive function [85].

In addition, alpha-lactalbumin plays a critical role in neonatal immunity by preparing neutrophils for antigen encounters and has potential anti-cancer

properties when combined with oleic acid, selectively killing cancer cells [86]. Because of its wide range of applications, from improving the quality of infant formula to potential use in cancer therapy, alpha-lactalbumin is in high demand. This has led to the development of various extraction technologies that combine protein precipitation, membrane filtration, and chromatography to achieve high purity [87].

2.1.6.3. Oleic acid

Oleic acid is a monounsaturated fatty acid commonly found in both animal and plant sources. Its hydrocarbon chain contains 18 carbon atoms and a double bond, which defines its monounsaturated nature [88]. Oleic acid has a molecular weight of 282.5 g/mol, a melting point of 13–14 degrees Celsius, and a boiling point of 360 degrees Celsius.

This fatty acid is a major component of olive oil, making up 70–80 % of its content, and is associated with numerous health benefits, such as reduced blood pressure and improved overall well-being [89]. It is also abundant in other oils such as canola, palm, and corn oils, and in animal fats such as beef and pork tallow. Oleic acid plays a critical role in several physiological functions, including cancer prevention, reducing inflammation, and aiding wound healing [90]. In addition, it is beneficial in dieting by helping to reduce energy absorption from food [91].

2.1.7. HAMLET

HAMLET (human α-lactalbumin made lethal to tumor cells) is a complex formed by combining alpha-lactalbumin, a protein found in human milk, with oleic acid. This complex selectively induces apoptosis–like death in cancer cells without harming healthy, fully differentiated cells [92]. The initial discovery of HAMLET's effects occurred in 1995 at Lund University, Sweden, when researchers observed that a specific state of alpha-lactalbumin could lethally affect maternal, embryonic, and lymphoid cells but not mature epithelial cells [93].

Subsequent research showed that the formation of HAMLET requires not only alpha-lactalbumin but also oleic acid, with necessary conformational changes in both components [82]. Interestingly, this complex occurs naturally only in human milk, which is rich in both alpha-lactalbumin and oleic acid [94]. Despite the long recognition of HAMLET's potential and extensive research, no anticancer drugs based on the HAMLET complex have been approved to date.

2.1.7.1. HAMLET mechanism

The mechanism underlying the action of HAMLET on cancer cells is complex and not fully understood. Research indicates the existence of multiple possible pathways. HAMLET interacts with various tumor systems, resulting in alterations to cell morphology, metabolism, and viability. One of the key pathways involves the inhibition of the F-ATP synthetase enzyme, which is crucial for oxidative phosphorylation. Tumors rely predominantly on glycolysis for energy, and thus, blocking this pathway can result in the starvation of cancer cells, leading to their death [95]. In addition, HAMLET affects numerous targets across the cellular system, including over 8,000 proteins, with approximately 35 % being nucleotide-interacting proteins. This extensive interaction encompasses considerable effects on kinases, influencing numerous kinase families and modifying cell regulation [96].

Furthermore, HAMLET can alter chromatin structures by binding to histones within cancer cell nuclei, disrupting normal cell functions and leading to cell death (Düringer *et al*., 2003). Furthermore, it interacts with proteosomes, inhibiting their function and thus affecting protein turnover [97]. The efficacy of HAMLET also varies with the genetic makeup of the cancer cells, particularly with variations in the expression of the c-Myc oncogene, which affects cellular sensitivity to HAMLET [18].

The precise mechanism of cell death induced by HAMLET remains uncertain. Despite the presence of apoptotic markers, the typical pathways involving caspases, p53, or Bcl-2 proteins are not activated, suggesting that HAMLET may employ an alternative method to induce cell death, circumventing the usual anti-apoptotic defenses of cancer cells [98]. Further research is necessary to identify additional pathways through which HAMLET exerts its effects on cancer cells.

2.1.7.2. HAMLET therapeutic potential

Although further study is required to fully assess its therapeutic potential, HAMLET represents the first promising class of tumor-killing protein-fat complexes that could lead to safer cancer treatments. Researchers are actively investigating the potential of this agent against a range of cancers, including bladder, colon, glioblastoma, and skin papillomas. For example, studies have demonstrated that HAMLET can reduce the viability of bladder cancer cells, decrease tumor size, and slow its spread in mice, all without harming healthy surrounding.

Furthermore, human trials have demonstrated the potential of this approach, particularly in bladder cancer, where repeated intravesical administration has been shown to cause cancer cells to detach and be expelled in urine,

significantly reducing tumor size without affecting nearby cells [99]. In studies on colon cancer, oral administration of HAMLET in mice has been shown to slow tumor growth and improve survival, with the effects localized to cancerous tissues alone [18]. Furthermore, the topical application of HAMLET has been demonstrated to be an effective treatment for cutaneous papillomas, with a significant reduction in the number of lesions observed in patients who used HAMLET ointment compared to those who used a placebo [100]. Furthermore, research on glioblastoma in mice indicates that HAMLET is more effective than alpha-lactalbumin in delaying the onset of symptoms, with no adverse effects on healthy tissues [101]. These findings underscore the potential of HAMLET as a versatile and safe cancer treatment option.

Recent research has shown that alpha1-oleate, a compound similar to HAMLET, is effective in treating bladder cancer. A study by Tran Thi Hien *et al*. revealed that treating mice with alpha1-oleate alone or combined with low-dose chemotherapy such as Epirubicin or Mitomycin C halted tumor growth and provided lasting protection. Notably, repeated cycles of this treatment, especially when combined with Epirubicin, improved drug delivery to tumor cells, suggesting a synergistic effect that could prevent cell proliferation and initiate DNA fragmentation. These promising results support further exploration of alpha1-oleate as a potential long-term treatment for bladder cancer [102].

3. DESIGN OF A STUDY AND METHODS

3.1. Design of a study

This study consists of *in vitro* and *ex vivo* parts that will provide comprehensive insights into the efficacy of the HAMLET effect in colorectal cancer, using both cell line models and patient-derived tissue samples.

The *in vitro* study included five colorectal cancer cell lines with different mutation status:

1. Wild type: Caco-2 cell line.
2. *KRAS* mutation: LoVo and HCT-116 cell lines.
3. *BRAF* mutation: WiDr and HT-29 cell lines.

The *ex vivo* study included 32 patients who were operated on due to colorectal cancer at the Hospital of Lithuanian University of Health Sciences Kaunas Clinics between 2021 and 2022. Subjects were classified into subgroups according to mutation status: wild type, *KRAS* mutation and *BRAF* mutation (summarized in Fig. 3.1.1)

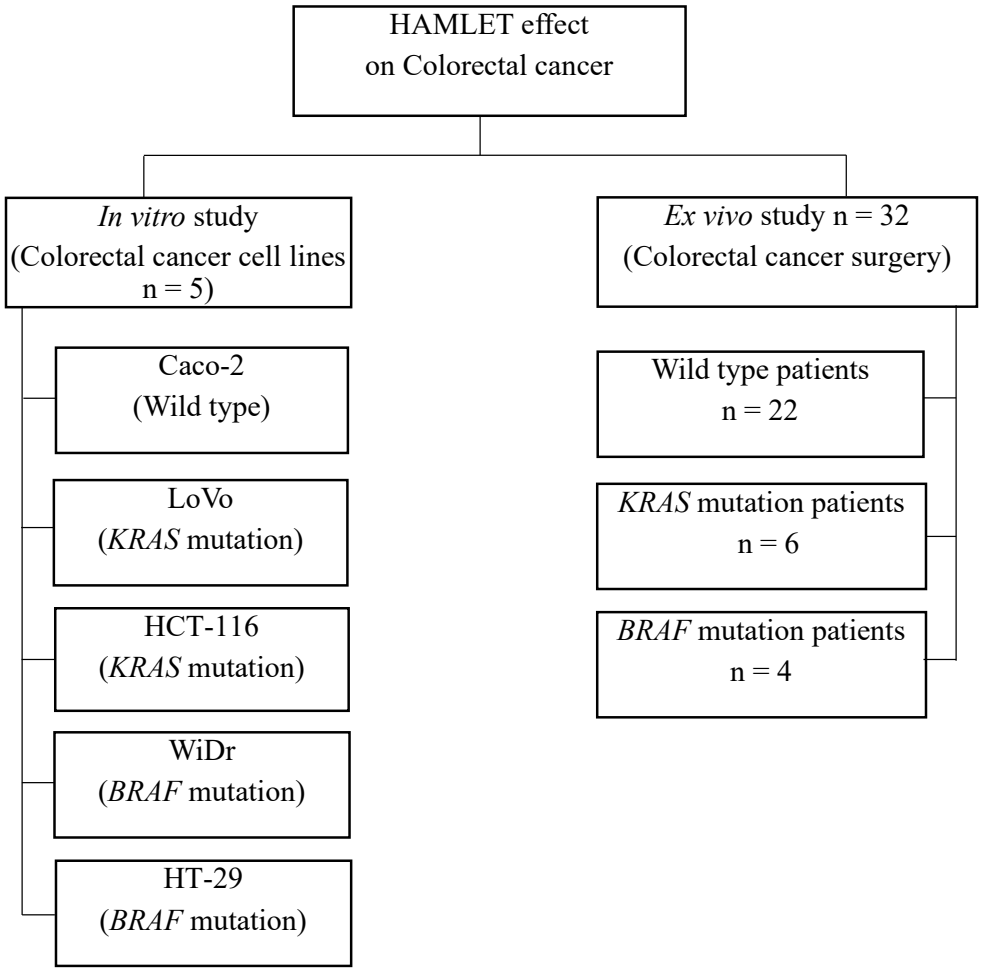


Fig. 3.1.1. *Overview of study for In vitro and ex vivo HAMLET Effect on CRC.*

3.1.1. The *ex vivo* study patients' inclusion and exclusion criteria

The detailed inclusion and exclusion criteria are outlined in the figure (Fig. 3.1.1.1).

Inclusion criteria:

1. Adult patients diagnosed with CRC and histologically confirmed.
2. Elective surgery.
3. Patients scheduled for surgeries on Mondays and Tuesdays due to the requirements of the *ex vivo* experiment process
4. Consented to and signed the informed consent form (ICF).

Exclusion criteria:

1. Patients under 18 years of age.
2. Patients who have not consented to or signed the ICF.
3. CRC cases after neoadjuvant treatment.
4. Cancer recurrence.
5. Colorectal surgery for diverticulosis or inflammatory bowel disease.
6. Intraoperative exclusion:
   a. tumor characteristics,
   b. change of operative tactics or complications,
   c. insufficient tissue sampling.

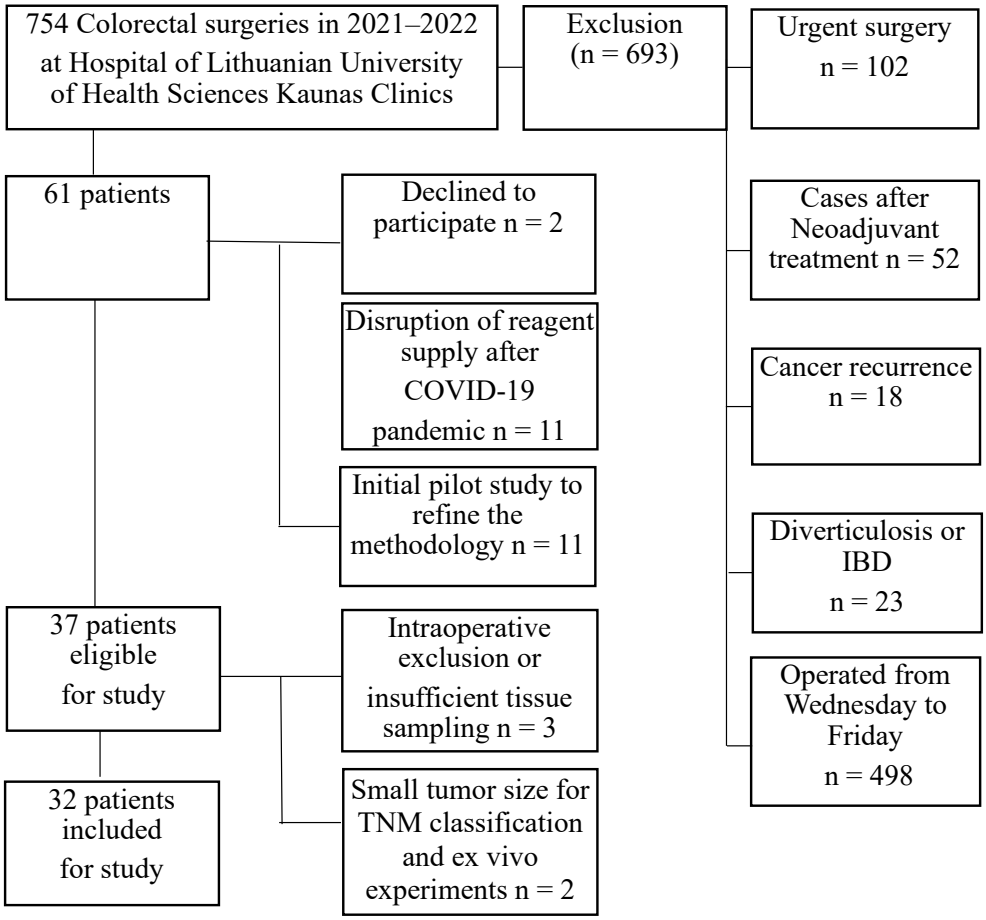


Fig. 3.1.1.1. *Explant study cohort flow chart (Inclusion/Exclusion criteria).*

3.2. Bioethics

This study received bioethics approval from Kaunas regional biomedical research ethics committee under approval number BE-2-64, study protocol No. 1 on August 1, 2019. The research was conducted in accordance with the principles of the Declaration of Helsinki and local laws and regulations. We affirm that all participants or their legal guardians provided informed consent after receiving comprehensive explanations regarding the study's objectives and procedures.

3.3. Methods of *in vitro* study

To achieve the first objective, we selected the most commonly used CRC cell lines, each representing a different mutation status: wild type, *KRAS* and *BRAF*. Mutation characteristics are detailed in Table 3.3.1. To assess the cytotoxic effects of HAMLET, we used the MTT assay to measure metabolic activity, the clonogenic assay to assess colony formation, and flow cytometry to quantify apoptotic and necrotic cells (see 2.1.4.5 paragraph).

Table 3.3.1. *The mutation status of cancer genes classifies colorectal cancer cell lines. Adapted from Ahmed D et al. [103]*

Colorectal Cell line	***KRAS* mutation**	***BRAF* mutation**
Caco-2	wild type	wild type
LoVo	G13D; A14V	wild type
HCT-116	G13D	wild type
WiDr	wild type	V600E
HT-29	wild type	V600E

3.3.1. Colorectal cancer cell lines

1. Caco-2 was purchased from the American Type Culture Collection (ATCC) in the United States.
2. LoVo was sourced from CLS cell lines service in Germany. Both Caco-2 and LoVo lines were cultured in Ham's F-12K (Kaighn's) medium (GIBCO) supplemented with 10 % fetal bovine serum (FBS, GIBCO) and 1 % penicillin-streptomycin (GIBCO).
3. HCT-116 was acquired from the American Type Culture Collection (ATCC) in the United States. It was cultured in McCoy's 5A medium (GIBCO) supplemented with 10 % FBS (GIBCO) and 1 % penicillin-streptomycin (GIBCO).

4. WiDr was obtained from CLS cell lines service in Germany. It was cultured in a 1:1 mixture of Ham's F-12K (Kaighn's) medium (GIBCO) and Dulbecco's modified eagle medium (DMEM, GIBCO), supplemented with 5 % FBS (GIBCO) and 1 % penicillin-streptomycin (GIBCO).
5. HT-29 was supplied from the American Type Culture Collection (ATCC) in the United States. This cell line was cultured similarly as HCT-116.

Cell lines were cultured at 37 °C in a 5 % CO_2 atmosphere.

3.3.2. HAMLET complex formation stages

The HAMLET complex was prepared from human α-lactalbumin (Sigma-Aldrich, Germany, Cat. No. L7269) and oleic acid (Sigma-Aldrich, Germany, Cat. No. O1383) according to the heat-treatment stages as illustrated in Figure 3.3.2.1 [86]:

1. Partial α-lactalbumin protein unfolding.
2. Oleic acid incorporation into protein structure.
3. Removal of excess oleic acid.
4. Storage.

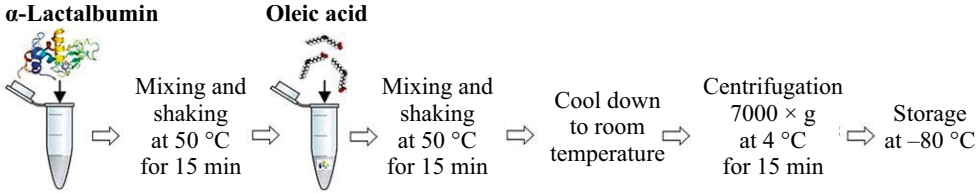


Fig. 3.3.2.1. *Detailed formation of HAMLET complex.*

Adapted from Kamijima, T *et al*.[86].

3.3.3. Cell viability assay stages

1. Cell seeding and hamlet treatment:
   a. Cells were seeded into a 96-well plate at a density ranging from 8×10^3 to 2×10^4 cells/well, depending on the specific cell line.
   b. After 24 hours of seeding, the HAMLET complex was added to the cell culture and incubated for 6 hours.
2. Medium change and MTT assay preparation:
   a. Following the 6-hour incubation with HAMLET, the growth medium was replaced with a fresh medium.
   b. Cells were incubated for an additional 18 hours.
   c. After the incubation period, the MTT reagent (Thermo Fisher Scientific, Cat. No. M6494) was added to each well.

3. MTT assay execution:
   a. The MTT reagent reacted with the cells for 3–4 hours at 37 °C.
   b. After the incubation, the growth medium was aspirated to remove excess MTT reagent.
   c. Formed formazan crystals were dissolved in 100 µL of DMSO (Carl Roth, Germany, Cat. No. A944.2).
   d. Absorption was measured at 570/620 nm using a spectrophotometer.
   e. Colorimetric absorption values were compared to those of the control group to assess cell growth inhibition in response to HAMLET treatment.

Cell viability and the following Clonogenic assays timeline illustrated in Figure 3.3.3.1.

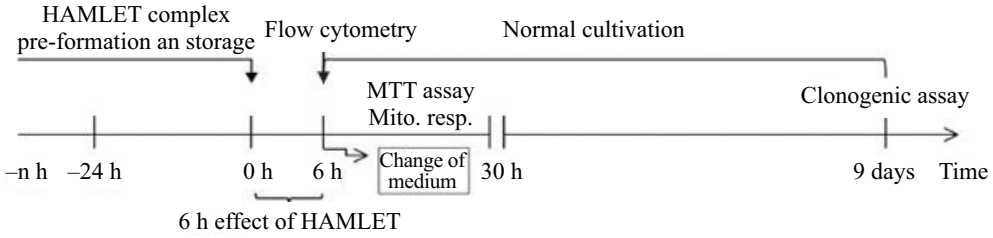


Fig. 3.3.3.1. *Schematic timeline of HAMLET formation, MTT, Clonogenic assay, and Mitochondrial respiration.*

Adapted from Kamijima, T *et al*. [86].

3.3.4. Clonogenic assay stages

1. Cell seeding:
   a. Cells were seeded into 24-well plates at a density ranging from 1×10^2 to 2×10^2 cells/well.
2. HAMLET treatment:
   a. After 24 hours of plating, the HAMLET complex was added to the cells.
   b. Cells were then incubated for 6 hours in the presence or absence of different concentrations of the HAMLET complex.
3. Medium change and incubation:
   a. Following the 6-hour incubation period, the culture medium was replaced with fresh medium devoid of HAMLET.
   b. Cells were subsequently incubated for eight days to allow colony formation.

4. Fixation and staining:
   a. After the eight-day incubation period, cells were fixed with ethanol.
   b. Subsequently, cells were stained with crystal violet.
5. Colony counting:
   a. The number of colonies containing more than 50 cells was counted using an inverted microscope.
6. Data analysis:
   a. All counted values were compared to those of the control group to assess the effects of HAMLET treatment on clonogenicity.

3.3.5. Stages of flow cytometry

1. Cell seeding:
   a. Cells were seeded into wells at a density ranging from 1×10^5 to 1.3×10^5 cells/well.
2. HAMLET treatment:
   a. After 24 hours of plating, the HAMLET complex was added to the cells.
   b. Cells were then incubated for 6 hours in the absence of different concentrations of the HAMLET complex.
3. Cell preparation for flow cytometry:
   a. After incubation, cells were detached using trypsin-EDTA, with floating cells retained.
   b. The culture medium was removed by centrifugation, and cells were resuspended in a binding buffer.
4. Staining:
   a. Cells were stained with annexin V-PE and 7-AAD dyes for subsequent flow cytometric analysis. EMD Millipore, United States, furnished the staining dyes Flow Cellect Mito Damage Kit (Cat. No. FCCH100106) and Annexin V-PE Apoptosis detection kit (Cat. No. CBA606).
5. Flow cytometry measurement:
   a. Stained cells were measured using the Guava Personal Cell Analysis Flow Cytometer (Merck; Millipore; Burlington; MA; United States).
   b. Data acquisition and analysis were performed using CytoSoft 2.1.4 software.

3.3.6. Stages of cancer cells mitochondrial respiration

To address the second objective, we used the same CRC cell lines with wild type, *KRAS* mutant and *BRAF* mutant profiles as previously described in Table 3.3.1.1. We assessed the effect of HAMLET on mitochondrial function

and membrane integrity using assays that measure mitochondrial respiration (see 2.1.5. paragraph).

1. Mitochondrial respiration measurement setup:
   a. Mitochondrial respiration (oxygen consumption) rate was recorded using the high-resolution respirometry system Oxygraph-2k from OROBOROS Instruments, located in Innsbruck, Austria.
   b. Measurements were conducted at 37 °C in a medium comprising 0.5 mM EGTA, 3 mM $MgCl_2$, 60 mM K-lactobionate, 20 mM Taurine, 10 mM KH_2PO_4, 20 mM HEPES, and 110 mM sucrose (pH 7.1 at 37 °C).
2. Investigation of mitochondrial functions:
   a. Mitochondrial functions were assessed using a multiple substrate–inhibitor titration method.
   b. Digitonin (16 µg/mL) was added to permeabilize the cell membrane, facilitating substrates and inhibitors' access to mitochondria.
3. Measurement of respiration rates:
   a. Mitochondrial non-phosphorylating State 2 (V_0) respiration rate was recorded in the medium supplemented with cells and mitochondrial Complex I substrate (5 mM glutamate + 2 mM malate).
   b. The state 3 respiration rate (V_{ADP}) was determined after the addition of 1 mM ADP.
   c. To achieve maximal mitochondrial respiration ($V_{ADP\ (glu/mal/suc)}$), a Complex II substrate, succinate (12 mM), was introduced.
   d. The effect of cytochrome c on the respiration rate, indicative of mitochondrial outer membrane permeability, was assessed by adding 32 µM cytochrome c.
   e. To evaluate permeability of inner mitochondrial membrane CAT (carboxyatractylozide, 0.75 µmol) was added and mitochondrial respiration rate V_{CAT} was measured.
4. Calculation of respiratory control index (RCI):
   a. RCI for glutamate/malate was calculated as the ratio between V_{ADP} and V_0 respiration rate.
5. Data acquisition and analysis:
   a. Real-time data acquisition and analysis were performed using Datlab 5 software (Oroboros Instruments)
   b. Oxygen consumption rates were normalized to cell number (pmol/s/1 mln cells) for comparative analysis.

The summary scheme of mitochondrial respiration measurement is provided in Figure 3.3.6.1.

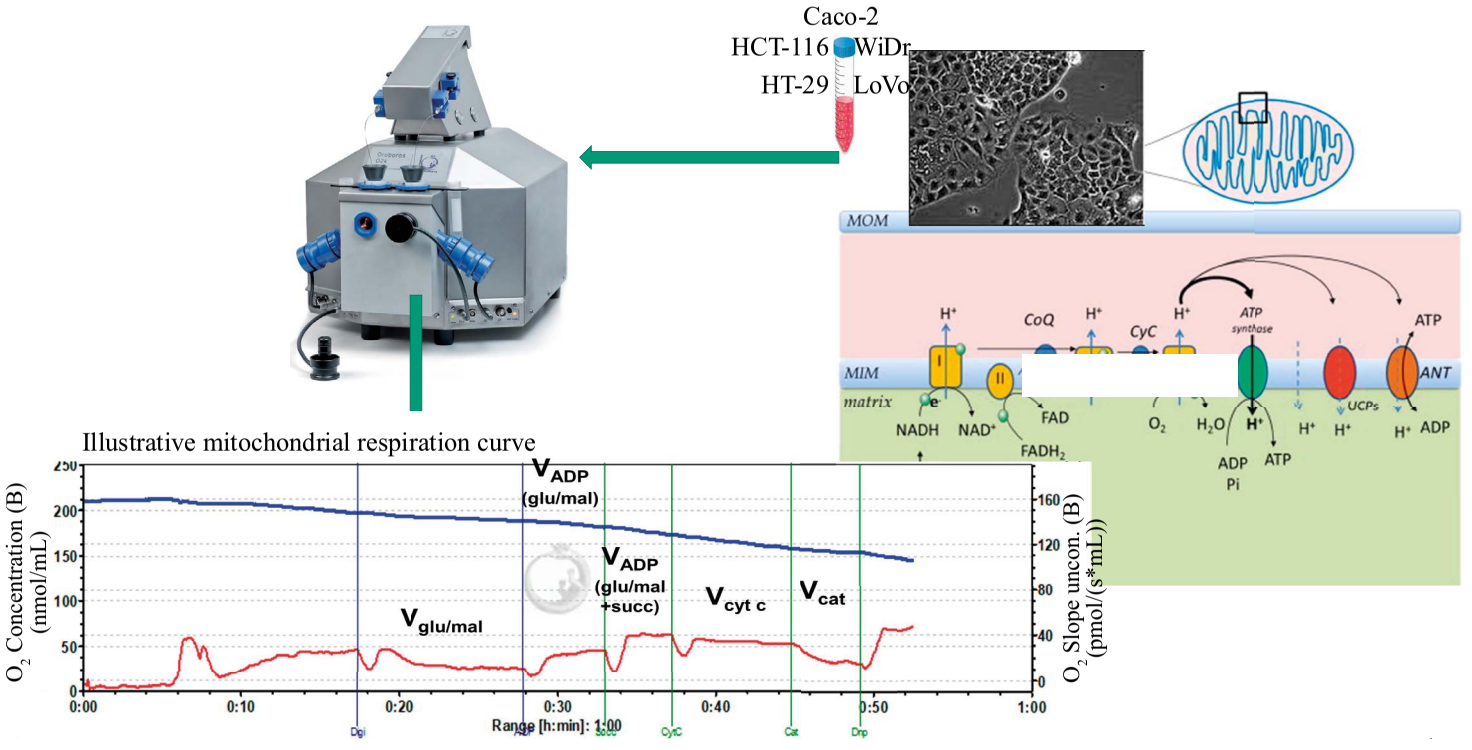


Fig. 3.3.6.1. *Schematic picture of mitochondrial function measurement in cancer cells.*

Adapted from Sukovas, A *et al.* [104].

3.3.7. Drug combination effect calculation

To address the third objective, we evaluated the efficacy of HAMLET and FOLFOX, individually and in combination, on colorectal cancer cell lines to determine mutation-specific resistance and synergistic effects. We used the MTT assay to measure the combination effects and used Combenefit software for calculations, applying the Bliss independence model [105,106].

1. Drug combination effect measurement:
   a. The combination effect of the HAMLET complex and FOLFOX (5-fluorouracil (5-FU) + oxaliplatin) was assessed using the MTT assay.
2. Calculation method:
   a. Combenefit (v2.021) software was employed for the calculation of the combination effect.
3. Theory selection:
   a. Bliss theory was chosen as the calculation theory. This theory operates under the assumption that both drugs work independently but can synergistically increase each other's cytotoxic effect.
4. Dose variation:
   a. Varying doses of HAMLET (1, 3, 5 μM) and FOLFOX (with concentrations of 5-FU and oxaliplatin ranging from 3,125 + 0.078 to 800 + 20 μM) were used in the experiments to determine the combination effect.

3.4. Methods of *ex vivo* study

To address the fourth objective, we prepared ex vivo patient cancer model and mutation analysis. Then we assessed the impact of HAMLET and FOLFOX on viability in colorectal cancer explants with different mutations. We treated the explants with HAMLET, followed by metabolic activity measurement using resazurin.

3.4.1. Stages to prepare *ex vivo* patient cancer model

1. Collection and preparation of tissue:
   a. After colorectal cancer resection surgery, a piece of tumor is collected during the examination.
2. Sterile washing procedure:
   a. The collected tissue pieces are washed three times under sterile conditions to remove microorganisms and blood cells as thoroughly as possible.

3. Precision cutting:
   a. Using biopsy needles, the tissue pieces are delicately cut into uniform 2 mm segments, minimizing tissue shredding.
4. Formation of explants:
   a. The precision cutting yields uniform 2 mm^3 explants from the colon cancer tissue.
5. Placement in Culture Plates:
   a. Each individual explant is carefully placed into a separate well of a 96-well plate containing nutrient medium.
6. Incubation conditions:
   a. The culture plates are then incubated in a controlled environment at 37 °C, with 95–98 % humidity, and a 5 % CO_2 atmosphere to facilitate cell growth and maintenance.

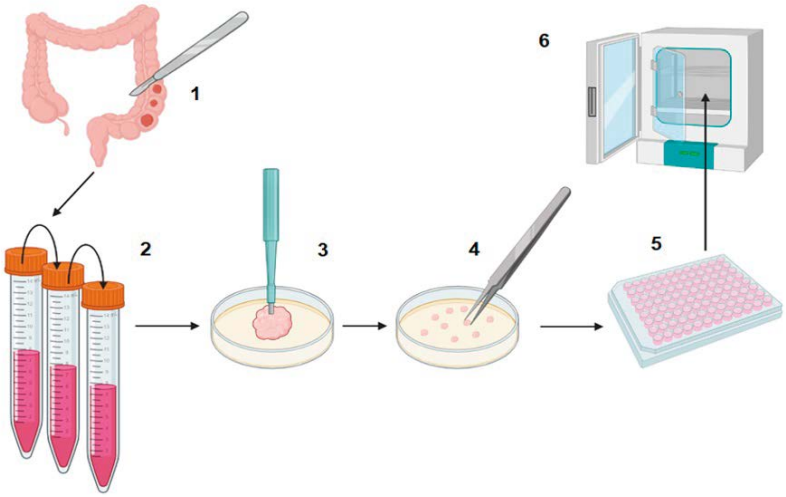


Fig. 3.4.1.1. *Schematic establishment of ex vivo cancer model. 1. Collection and Preparation of Tissue. 2. Sterile Washing Procedure. 3. Precision Cutting. 4. Formation of Explants. 5. Placement in Culture Plates. 6. Incubation*

Adapted from Žilinskas, J *et al*. [107].

3.4.2. *Ex vivo* patient biopsies mutation analysis

In our study on *ex vivo* CRC patient biopsies samples, genomic DNA was isolated using the DNeasy Blood and Tissue kit by (Qiagen, Hilden, Germany). Following isolation, DNA samples were quantified, and their purity was

assessed through UV spectrophotometry. For the detection of mutations, we employed TaqMan assays designed for specific mutations:

1. *BRAF* V600E (reference rs113488022).
2. *KRAS* G12V (reference rs121913529).
3. *KRAS* G12C (reference rs121913530).
4. *KRAS* G12D (reference rs112445441).

We used the TaqMan Universal PCR Master Mix, No UNG, supplied by Life Technologies in Carlsbad, CA, USA. A DNA sample of approximately 20 ng per well was used for amplification in the ABI 7500 fast Real-Time PCR system. Genotype assignments were accurately determined through manual inspection using the ABI 2.3 software, which is compatible with the TaqMan® system. To ensure the integrity of our results, 25 % of the samples from each category were re-analyzed, demonstrating a 100 % concordance rate in the findings. This methodical approach yielded a detailed analysis of mutations within the CRC samples, providing their genetic landscape.

3.4.3. Explant treatment with HAMLET

1. HAMLET treatment:
   a. After 24 hours of incubation, 60 μM HAMLET was added to the appropriate wells of the plate.
   b. The plate was then incubated for another 24 hours.
2. Dose selection:
   a. The chosen dose of 60 μM HAMLET for 24 hours was based on previous experiments with explants which showed a statistically significant effect under these conditions.
3. Medium change and resazurin supplementation:
   a. After 24 hours of HAMLET treatment, the medium in the wells was changed to fresh medium supplemented with 10 % resazurin.
4. Measurement of metabolism:
   a. Explants metabolize the purple compound, resazurin, to a pink compound, resorufin.
   b. Resorufin production was measured using a spectrometer equipped with 570 nm and 620 nm filters.
5. Time points for measurement:
   a. Since resazurin and resorufin are non-toxic, measurements were taken 24 hours after treatment and repeated by removing old medium and adding fresh medium with resazurin 48 hours after HAMLET treatment to monitor metabolic activity over time.

3.4.4. Explant mitochondrial respiration investigation

To address the fifth objective, we prepared ex vivo patient cancer model and mutation analysis. Then we assessed the impact of HAMLET on mitochondrial respiration in colorectal cancer explants with different mutations. We evaluated mitochondrial respiration using the Oxygraph-2k system, adapting methods from prior studies on CRC cell lines to suit explant tissues (see 2.1.5. paragraph).

We used a method similar to that previously described for CRC cell lines (3.3.6. Stages of cancer cell mitochondrial respiration) but adapted for *ex vivo* tissue samples with the Oxygraph-2k high-resolution respirometry system. We assessed mitochondrial non-phosphorylating (V_0) respiration rate and mitochondrial state 3 (V_{ADP}) respiration rate.

3.5. Statistical analysis

Statistical analyses were performed using GraphPad Prism 6 and SigmaPlot software. Nonparametric data sets were evaluated using the Mann-Whitney U test. Correlations between qualitative measures in comparison cohorts were examined using the chi-squared test (χ^2). In addition, Student's t-test was used to evaluate interval and categorical data. A significance level of $p < 0.05$ was used for all analyses.

3.6. Funding

The study was funded by the Research Council of Lithuania and the Lithuanian University of Health Sciences (LUHS) in 2019–2023:

- Research Council of Lithuania (2019 June–August): project No 09.3.3.-LMT-K-712-15-0288. "Comparison of human α-lactalbumin (HAMLET) cytotoxicity between *KRAS*/*BRAF* mutant and non-mutant colon cancer cells, in vitro".
- LUHS and KTU collaboration (2019 April–December): project "Formation and study of milk protein complexes in preclinical models of colon cancer" (FAMPOPAS).
- Research Council of Lithuania (2019 October–2020 June): project No 09.3.3-LMT-K-712-16-0007 "Comparison of the cytotoxicity of human a-lactalbumin (HAMLET) between cancerous and non-cancerous colon cells, in vitro"
- Research Council of Lithuania (October 2020–April 2021): project No 09.3.3.-LMT-K-712-22-0052 "Establishment of a human colon cancer explant model and the effect of human α-lactalbumin (HAMLET), ex vivo.
- Order of the Rector of LUHS 28 December 2020 No V-786. LUHS Science Foundation's PhD research grant for 2021.

4. RESULTS

4.1. The HAMLET complex's impact on cell viability, cell death and apoptosis/necrosis

The results for the 1st objective:

4.1.1. Wild type

In the investigation of HAMLET's effect on cell viability among wild type CRC cells, the Caco-2 cell line, which is *KRAS* and *BRAF* wild type, exhibited a unique response to HAMLET treatment. At the lowest concentration of 2 μM, Caco-2 cells showed a slight increase in viability, suggesting minimal cytotoxicity of HAMLET at lower doses. However, as the concentration of HAMLET was increased to 5 μM and beyond, a significant reduction in cell viability was observed, with viability decreasing to 64 % at 10 μM. This dose-dependent decrease in viability indicates that while Caco-2 cells may initially resist HAMLET-induced cytotoxicity at lower concentrations, they are susceptible to higher concentrations, underscoring the complex interaction between HAMLET and CRC cell lines with wild type *KRAS/BRAF* status.

4.1.2. *KRAS* mutant

For *KRAS* mutant CRC cell lines, LoVo and HCT-116, which are both *KRAS* mutant and *BRAF* wild type, the response to HAMLET treatment varied across different concentrations. Initially, at 2 μM HAMLET, both cell lines demonstrated minimal changes in viability, akin to the trend observed in wild type cells. With an increase in HAMLET concentration to 5 μM and above, a notable decline in viability was evident, reaching 60 % in LoVo and 43 % in HCT-116 at 10 μM. This pattern suggests that *KRAS* mutations do not confer significant resistance to HAMLET's cytotoxic effects, especially at higher concentrations. The pronounced suppression of viability in HCT-116 cells compared to LoVo might reflect differences in mutation-specific vulnerability to HAMLET.

4.1.3. *BRAF* mutant

Among *BRAF* mutant CRC cell lines, WiDr and HT-29, both with *KRAS* wild type and *BRAF* mutant status, the impact of HAMLET on cell viability was particularly significant. While at the lowest concentration of 2 μM, HT-29 cells already showed a decrease in viability to 90 %, indicating a sensitivity to HAMLET, WiDr cells did not exhibit significant changes at

this concentration. However, at higher concentrations (5 μM and 10 μM), both cell lines experienced a considerable reduction in viability, with HT-29 cells showing a more significant suppression to 40 % viability at 10 μM compared to WiDr's 61 %. At 20 μM, a general decline in viability was noted across all cell lines, but WiDr cells demonstrated a somewhat greater resistance to HAMLET, marking a unique aspect of *BRAF* mutant cells' response to HAMLET treatment. This resistance in WiDr cells, in contrast to the vulnerability observed in HT-29, suggests variability in the response to HAMLET among *BRAF* mutant CRC cell lines. Data summarized in Figure 4.1.3.1.

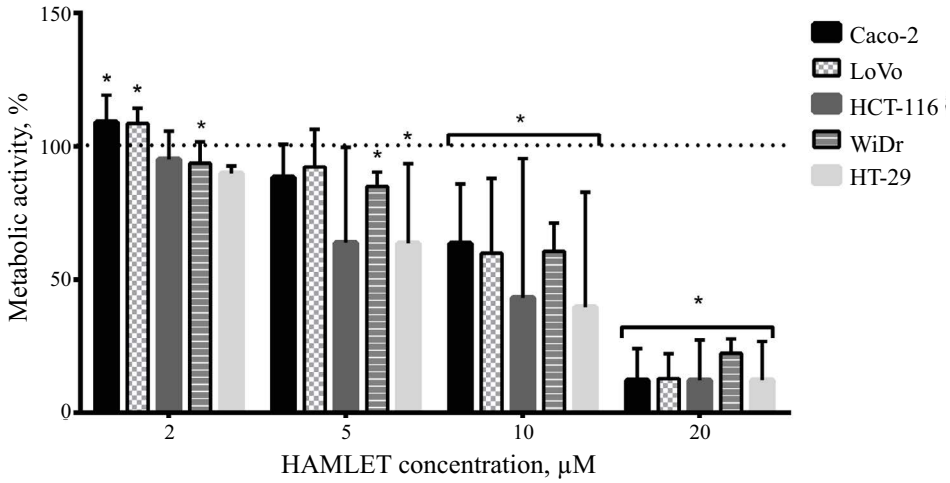


Fig. 4.1.3.1. *The effect of HAMLET on colorectal cancer cell viability.*

Dose-dependent response and enhanced resistance of WiDr cell line to HAMLET complex
*$p < 0.05$, compared to control group data (100 %) – dotted line.

The results of MTT assay were seconded by flow cytometry assay (Figure 4.1.3.2, A), which showed similar tendencies where HT-29 and HCT-116 cell lines were more sensitive to HAMLET, and WiDr cell line was more resistant. The numbers of cells undergoing necrosis or apoptosis after treatment with the 20 μM HAMLET complex were evaluated by flow cytometry. The assay showed a meager increase or even a decrease in apoptotic cell population when comparing untreated samples with samples treated with 20 μM HAMLET complex (Figure 4.1.3.2, A). Apoptotic cell population increased from 1.6 % to 3.1 % in Caco-2 cells; decreased from 2.8 % to 1.8 % in LoVo cells; increased from 1.87 % to 1.9 % in WiDr cells; increased 0.3 % to 2.8 % in HT-29 cells and from 0.4 % to 5 % in HCT-116 cells line. The increase in

apoptotic cell population was statistically significant in only HT-29 and HCT-116 cell lines.

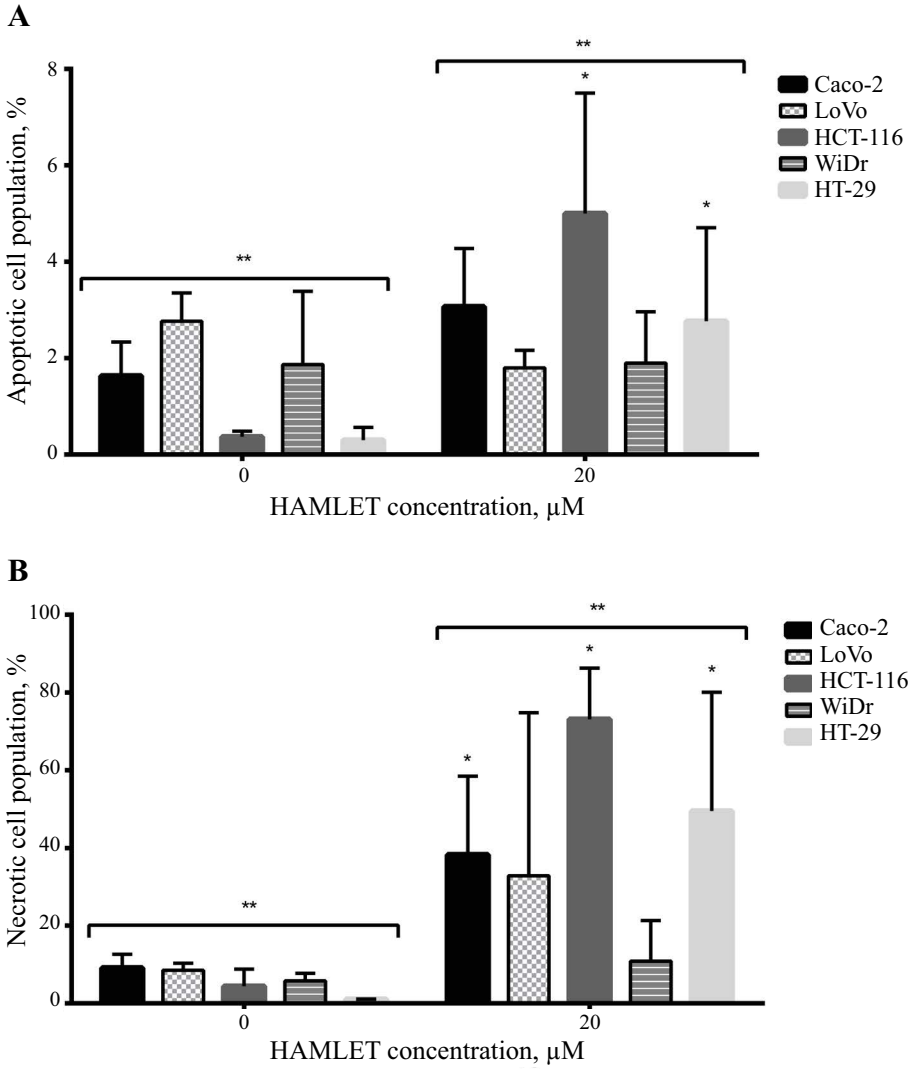


Figure 4.1.3.2. *Flow cytometric analysis of the effect of HAMLET complex on five colorectal cancer cell lines. A: Illustrates a modest increase in apoptotic cell population with subtle variations among the cell lines tested. B: Depicts a significant rise in necrotic cell population across all cell lines, except for WiDr, where the increase is notably lower.*

*$p \leq 0.05$ indicates statistical significance compared to untreated samples (0 µM); **$p < 0.05$ denotes significance when comparing apoptosis and necrosis within the same sample.

However, the increase in the necrotic cell population (Figure 4.1.3.2, B) was much more prominent than the apoptotic population. Cell population undergoing necrosis increased from 9 % to 38 % in Caco-2 cells; from 9 % to 33 % in LoVo cells; from 6 % to 11 % in WiDr cells; from 1 % to 50 % in HT-29 cells and from 4 % to 73 % in HCT-116 cell line. The increase in necrotic cell population was statistically significant in Caco-2, HT-29 and HCT-116 cell lines. Likewise, the WiDr cell line had the lowest increase in a necrotic cell population, while HT-29 and HCT-116 had the most noticeable increase in a necrotic cell population. The results indicate that the HAMLET complex mainly causes necrotic death in CRC cell lines without showing tendencies between cells with different *KRAS/BRAF* mutations.

4.2. Evaluation of HAMLET complex effect on clonogenic assay

In addition to suppressing cell viability, the HAMLET complex also significantly impacted colony formation (Figure 4.2.1). The pattern of results obtained by clonogenic assay was similar to those obtained by MTT (Figure 4.1.3.1) and flow cytometry (Figure 4.1.3.2). Similarly, the *KRAS/BRAF* mutation did not seem to impact the HAMLET complex response significantly, and the WiDr cell line was the most resistant to the 20 μM HAMLET complex. HT-29 and HCT-116 cell lines were more sensitive to the HAMLET complex since they did not form any colonies after the effect of HAMLET.

To summarize the results presented in the figures (Fig. 4.1.3.1–4.1.3.2.), there was no correlation between the HAMLET-induced cell death level and *KRAS/BRAF* mutations. However, the WiDr cell line differs from the other lines as being more resistant in terms of cell metabolism, increase in necrotic cell population, and colony formation, while cells with similar genetic profiles (HT-29) were the most sensitive. One of the possible explanations for this could be the differences between the energetic metabolism phenotypes of the cells.

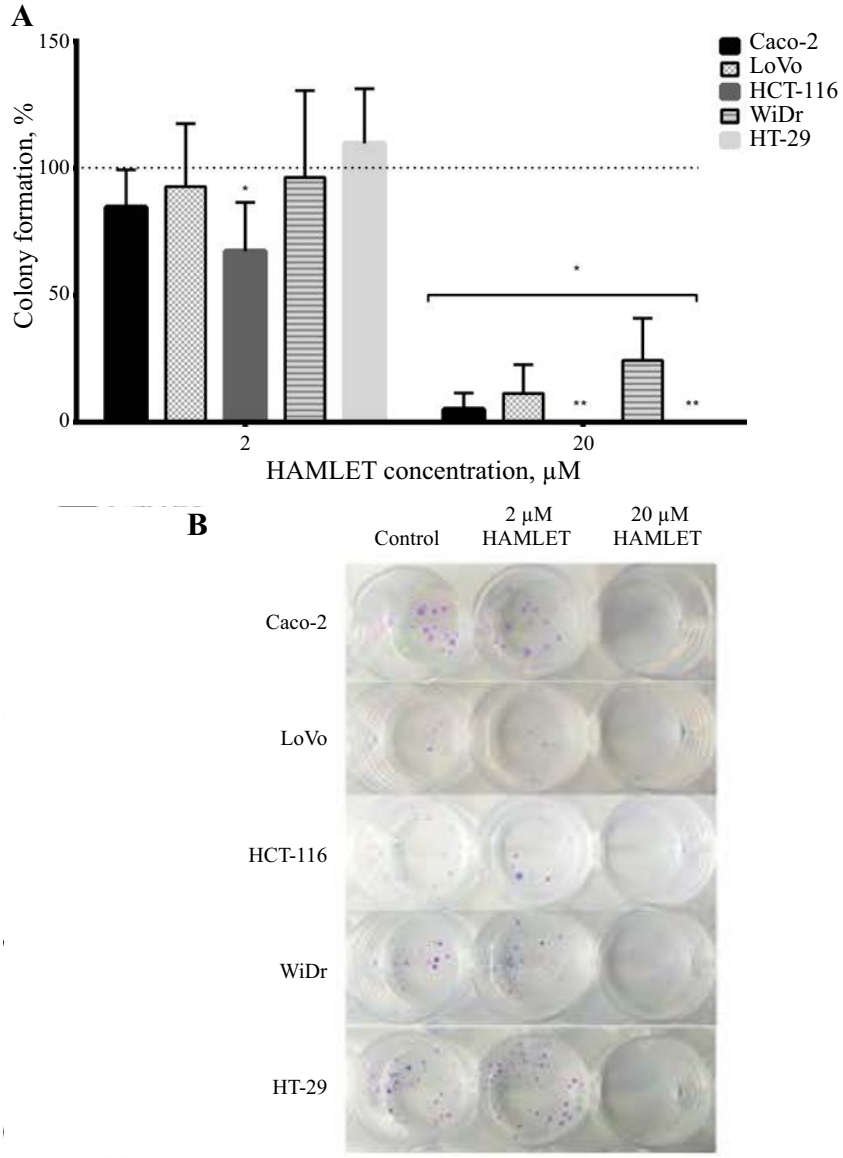


Fig. 4.2.1. *The Impact of HAMLET on colony formation in five colorectal cancer cell lines. A: Results of the clonogenic assay conducted eight days post-incubation with the HAMLET complex, revealing a complete colony formation loss in two lines, while the WiDr cell line was the most resistant to 20 µM HAMLET; B: Representative images depicting the colony formation assay.*

*Significant at $p < 0.05$ compared to control group data (100 %) represented by the dotted line; ** Denotes absence of colony formation.

4.3. HAMLET treatment effects on mitochondrial respiration and membrane permeability in colorectal cancer cells

The results for the 2nd objective:

4.3.1. Wild type – Caco-2 CRC Cells

In wild type Caco-2 CRC cells (Figure 4.3.1.1), treatment with 5 μM HAMLET resulted in an 18 % reduction in non-phosphorylating respiration rates compared to untreated cells. This finding indicates a significant impairment in mitochondrial function specific to this cell line. Furthermore, HAMLET was observed to inhibit mitochondrial complex I and complex I+II-dependent ADP-stimulated respiration rates by 19–45 %. These results suggest that HAMLET's mechanism of action includes the disruption of critical mitochondrial respiratory processes in Caco-2 cells, highlighting its potential as a therapeutic agent targeting mitochondrial function in CRC.

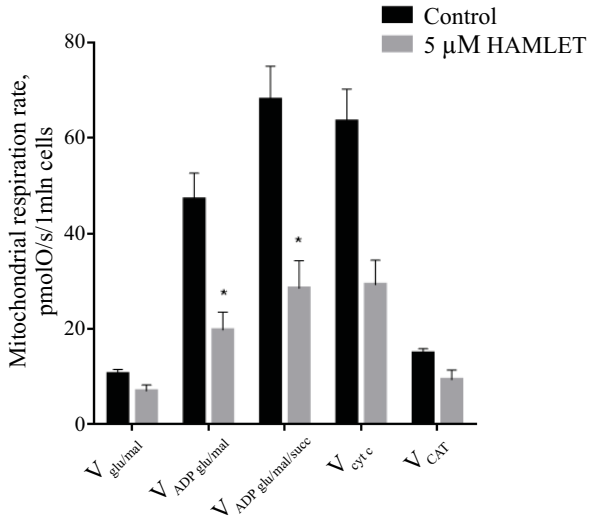


Fig. 4.3.1.1 *HAMLET effect on mitochondrial respiration and membrane permeability in colorectal cancer cells. Wild type Caco-2 cell line.*

$V_{glutamate/malate}$ – respiration of mitochondria without ADP; $V_{ADP\ (glu/mal)}$ – respiration related to ATP synthesis with mitochondrial complex I substrates; $V_{ADP\ (glu/mal/suc)}$ – maximal mitochondrial respiration related to ATP synthesis with complex I and II substrates; $V_{cyt\ c}$ – respiration with added external cytochrome C; V_{CAT} – respiration rate with added carboxyatracylocide, ATP/ADP transporter inhibitor. Respiration rate shows permeability of inner mitochondrial membrane.

4.3.2. *KRAS* mutant – LoVo and HCT-116 CRC Cells

In *KRAS* mutant CRC cell lines, LoVo and HCT-116 (Fig. 4.3.2.1), HAMLET treatment led to decreases in non-phosphorylating respiration rates by 6 % and 17 %, respectively. Moreover, HAMLET significantly inhibited mitochondrial complex I and complex I+II-dependent ADP-stimulated respiration rates across these cell lines. This effect underlines the sensitivity of *KRAS* mutant cells to HAMLET, pointing to a differential impact based on genetic mutations within CRC cells. Notably, LoVo cells also experienced a 30 % increase in the permeability of the mitochondrial inner membrane, suggesting a mutation-specific vulnerability to HAMLET's action.

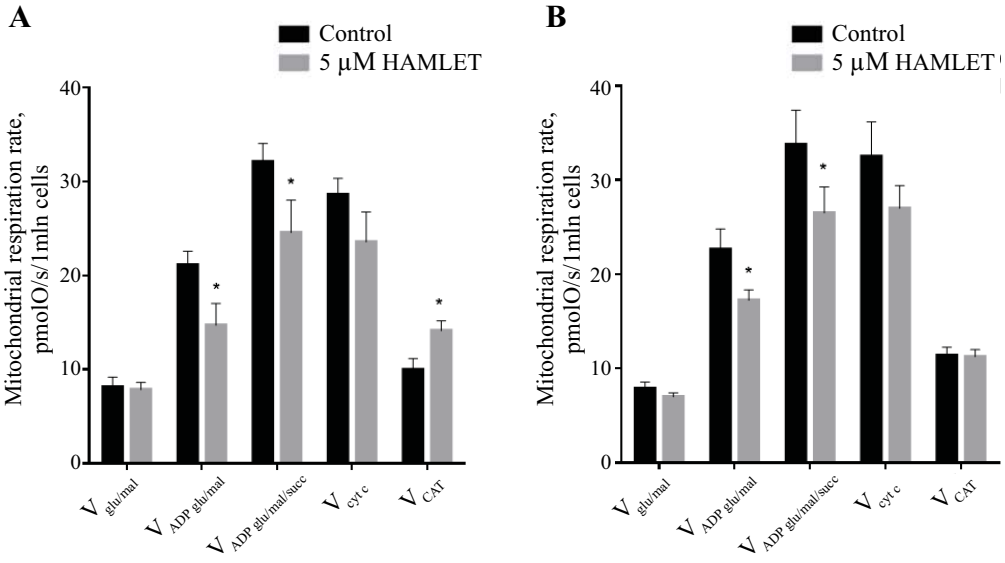


Fig. 4.3.2.1. *HAMLET effect on mitochondrial respiration and membrane permeability in colorectal cancer cells. (A) LoVo and (B) HCT-116 V glutamate/malate - respiration of mitochondria without ADP.*

$V_{ADP\ (glu/mal)}$ – respiration related to ATP synthesis with mitochondrial complex I substrates; $V_{ADP\ (glu/mal/suc)}$ – maximal mitochondrial respiration related to ATP synthesis with complex I and II substrates; $V_{cyt\ c}$ – respiration with added external cytochrome C; V_{CAT} – respiration rate with added carboxyatracylocide, ATP/ADP transporter inhibitor. Respiration rate shows permeability of inner mitochondrial membrane.

4.3.3. *BRAF* mutant – WiDr and HT-29 CRC Cells

In contrast, *BRAF* mutant WiDr cells (Figure 4.3.3.1) showed no clear impact on non-phosphorylating respiration rates following HAMLET treatment, and only a slight decrease in complex I-dependent ADP-stimulated respiration rates by 12 %. However, HT-29 cells also harboring the *BRAF* mutation, experienced a significant decrease in non-phosphorylating respiration rates by 26 %. These findings indicate a heterogeneous response among *BRAF* mutant CRC cells to HAMLET, with HT-29 cells being notably more susceptible to mitochondrial function impairment than WiDr cells. The differential responses of *BRAF* mutant cells underscore the complexity of HAMLET's effects and suggest the possibility of specific vulnerability markers within this subgroup.

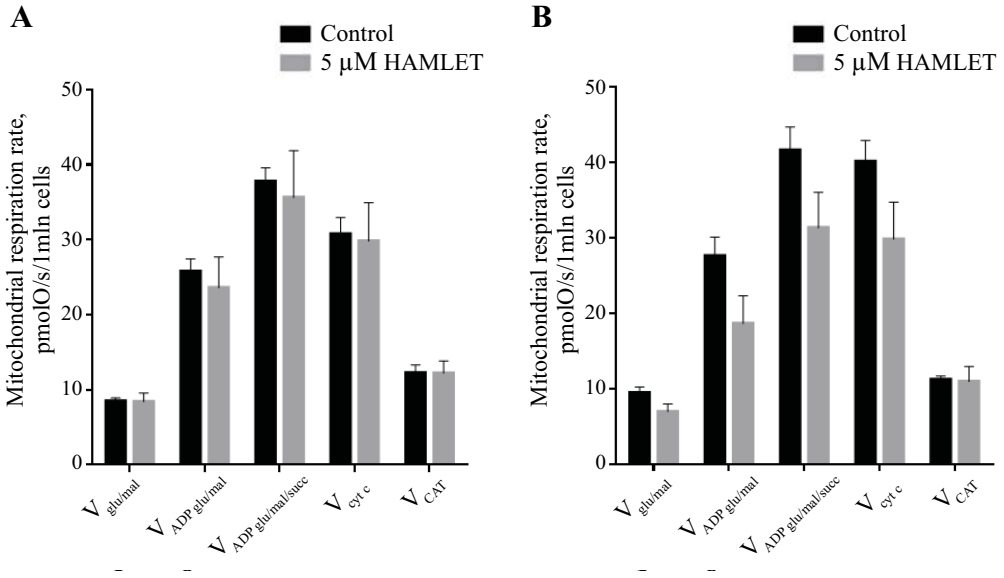


Fig. 4.3.3.1. *HAMLET effect on mitochondrial respiration and membrane permeability in colorectal cancer cells. (A) WiDr and (B) HT-29 BRAF mutant cell lines.*

$V_{glutamate/malate}$ – respiration of mitochondria without ADP; $V_{ADP\ (glu/mal)}$ – respiration related to ATP synthesis with mitochondrial complex I substrates; $V_{ADP\ (glu/mal/suc)}$ – maximal mitochondrial respiration related to ATP synthesis with complex I and II substrates; $V_{cyt\ c}$ – respiration with added external cytochrome C; V_{CAT} – respiration rate with added carboxyatracylocide, ATP/ADP transporter inhibitor. Respiration rate shows permeability of inner mitochondrial membrane.

4.4. Isobolograms: Bliss synergy model of HAMLET and FOLFOX treatment on CRC cell lines

The results for the 3rd objective:

4.4.1. CRC cell lines response to FOLFOX

The study of five CRC cell lines exposed to the chemotherapeutic drug combination FOLFOX (5-FU and oxaliplatin) has provided valuable information on drug resistance and the potential for drug-drug interactions. HCT-116 cells were significantly less sensitive to FOLFOX, requiring a higher IC50 dose of 73.1 μM to achieve the same level of cell death as the other cell lines tested, which had IC50 values between 6 and 15.1 μM. All values illustrated in Figure 4.4.1.1. These findings suggest that HCT-116 has enhanced defense mechanisms that enable it to counteract the toxic effects of FOLFOX more effectively than the other cell lines.

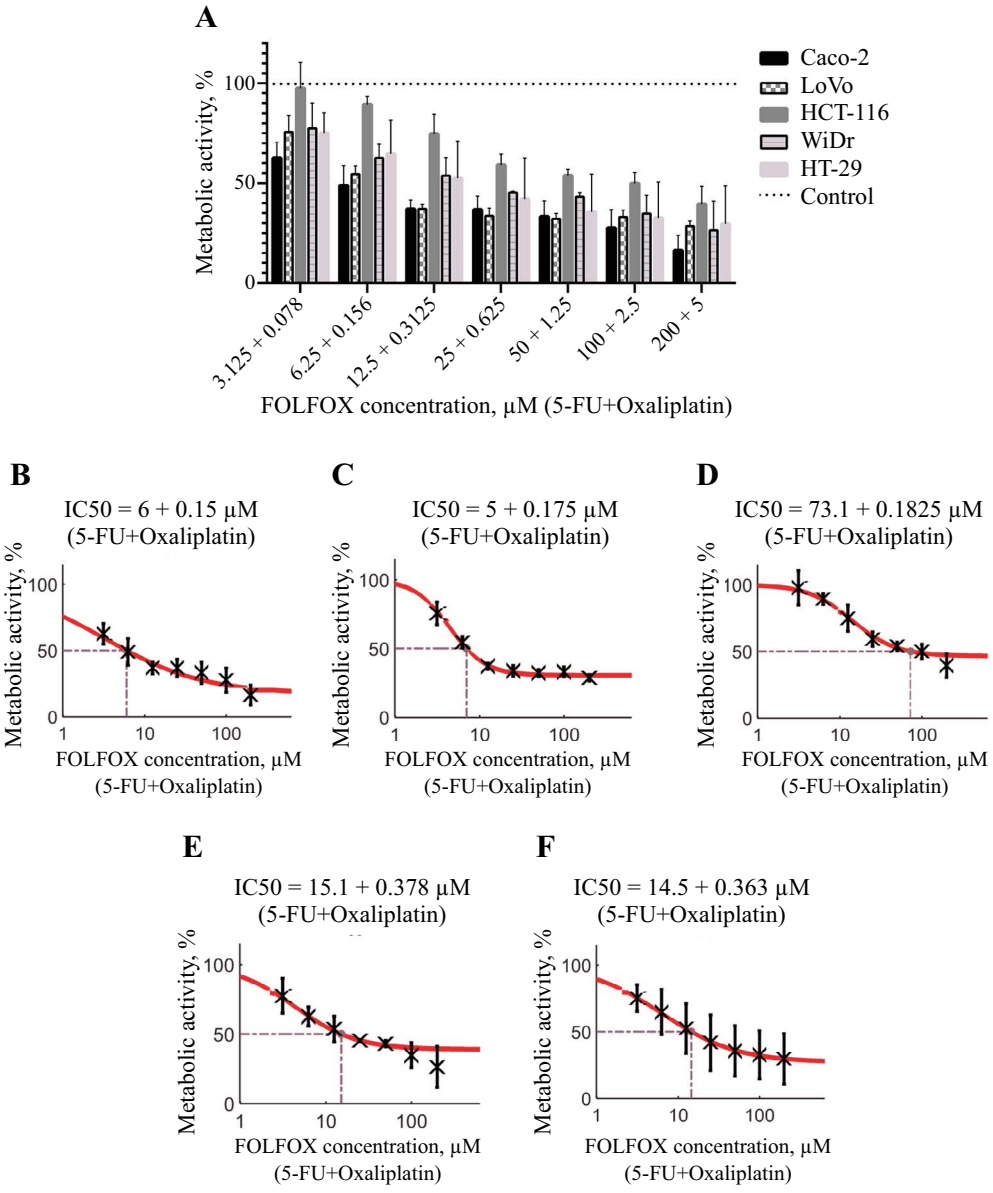


Fig. 4.4.1.1. *CRC cell line response to FOLFOX. (A) Dose-response graph of CRC cell lines (Caco-2, LoVo, HCT-29, WiDr and HT-29) response to different doses of FOLFOX. (B) FOLFOX IC50 dose calculation of Caco-2 cell line. (C) FOLFOX IC50 dose calculation of LoVo cell line. (D) FOLFOX IC50 dose calculation of HCT-116 cell line. (E) FOLFOX IC50 dose calculation of WiDr cell line. (F) FOLFOX IC50 dose calculation of HT-29 cell line.*

N = 3, MEAN ± SD.

4.4.2. The synergy between FOLFOX and HAMLET was evaluated using the Bliss independence model

1. The Caco-2 heatmap reveals a general trend of decreasing viability with increasing doses of FOLFOX and HAMLET. The table does not present any significant synergy or antagonism as most of the values are around zero, suggesting mostly additive effects.

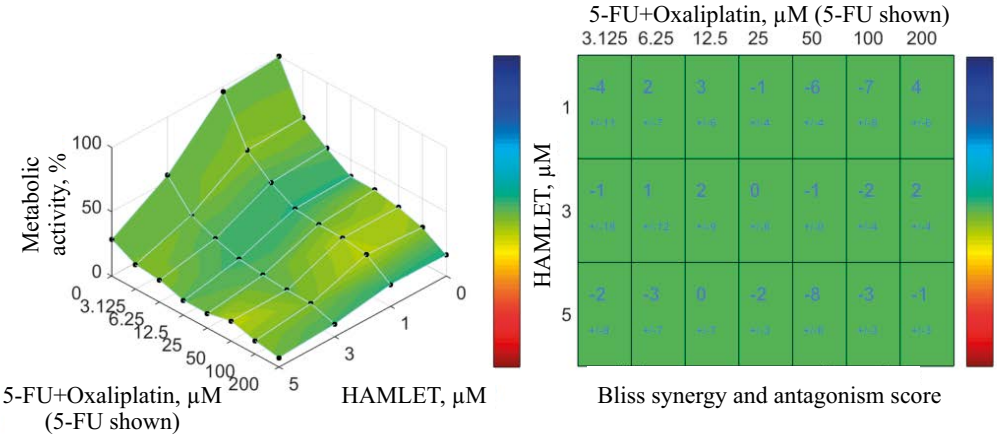


Fig. 4.4.2.1. *Caco-2 cell line heatmaps and tables illustrate Bliss synergy and antagonism calculations. Displays a decrease in viability upon treatment with different doses of FOLFOX and/or HAMLET compared to the control (100 % viability). Tables indicate synergy or antagonism index, with higher positive numbers highlighted in blue indicating significant synergy, while negative numbers highlighted in red denote significant antagonism (not present).*

* – $p < 0.05$.

2. LoVo is similar to Caco-2, there is a tendency for decreased viability at higher doses, but the Bliss scores again remain around zero, suggesting an additive effect rather than synergy or antagonism.

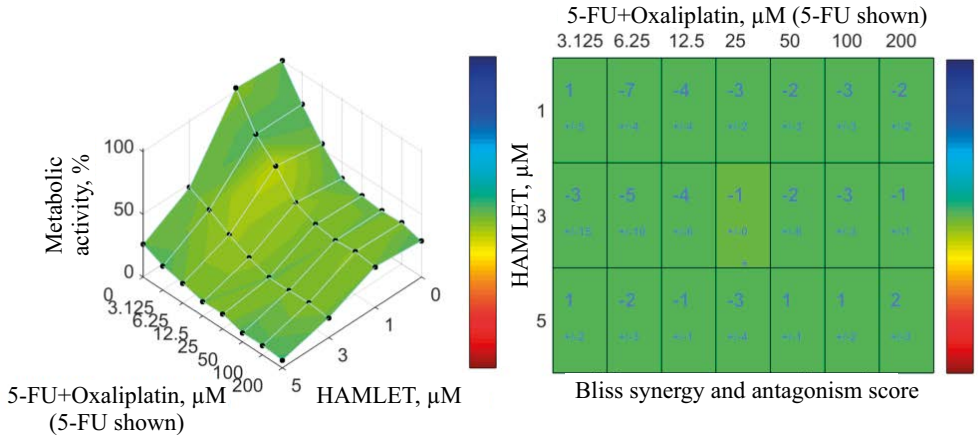


Fig. 4.4.2.2. *LoVo cell line heatmaps and tables illustrate Bliss synergy and antagonism calculations.*

3. HCT-116 heatmap illustrates a loss of viability with increasing drug concentrations, while the table shows occasional positive values suggesting some synergism at certain dose combinations, although these instances are not dominant.

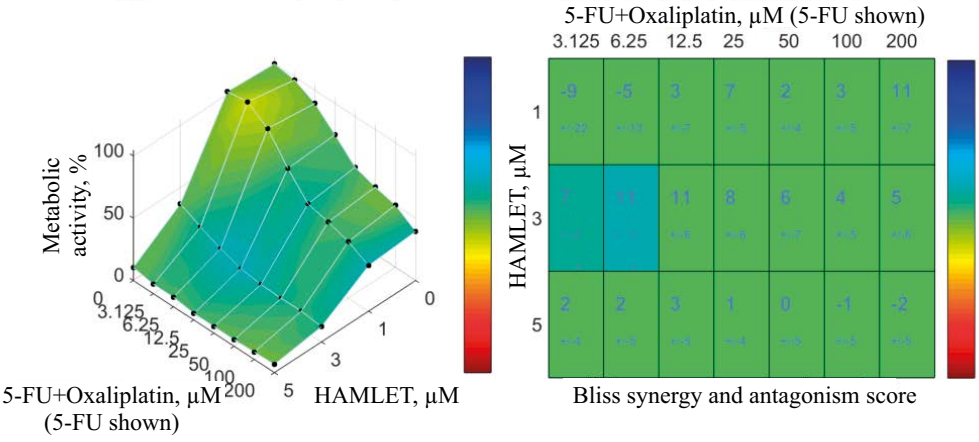


Fig. 4.4.2.3. *HCT-116 cell line heatmaps and tables illustrate Bliss synergy and antagonism calculations.*

4. WiDr cell line shows a significant decrease in cell viability, especially at a combination of 3 µM HAMLET and 1.25 µM FOLFOX. The table demonstrates consistently positive results, especially at the mid-range doses, indicating significant synergy.

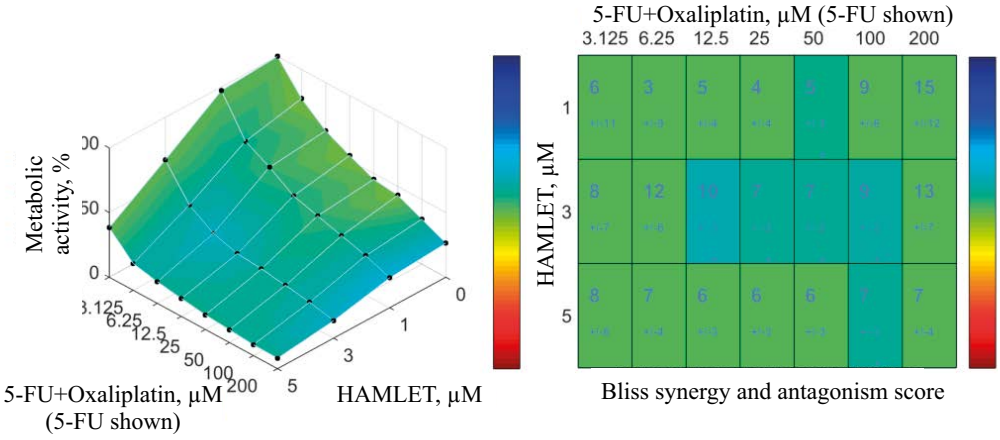


Fig. 4.4.2.4. *WiDR cell line heatmaps and tables illustrate Bliss synergy and antagonism calculations.*

5. The decrease in viability is clear in the HT-29 heatmap and mirrors Caco-2 and LoVo results.

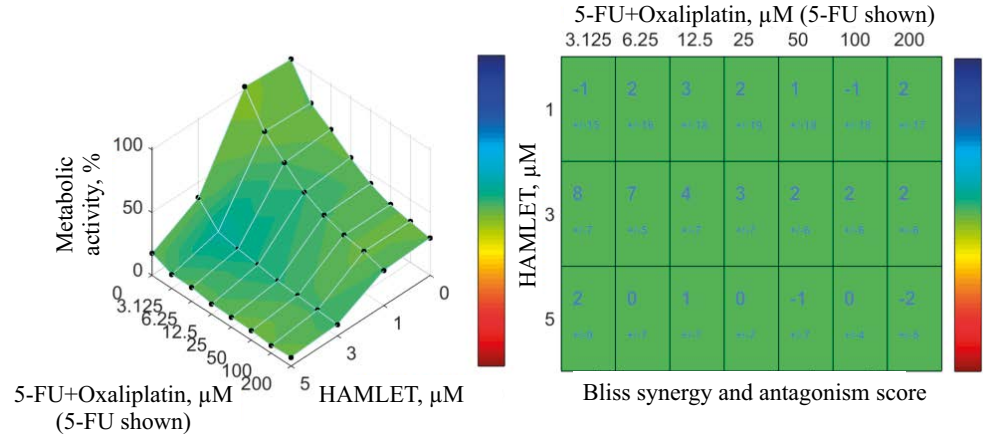


Fig. 4.4.2.5. *HT-29 cell line heatmaps and tables illustrate Bliss synergy and antagonism calculations.*

In summary, it is reasonable to hypothesize that the synergism observed, particularly in the WiDr cell line, might be related to the intrinsic resistance of the cells to FOLFOX. The synergistic interaction in WiDr and, to some extent, in HCT-116 suggests that HAMLET may play a role in mitigating resistance to FOLFOX. What is more, the lack of significant synergism or antagonism in the Caco-2, LoVo, and HT-29 cell lines when treated with FOLFOX and HAMLET in combination suggests that the effect of HAMLET

on the efficacy of FOLFOX is inconsistent across different CRC cell lines and may not be directly correlated with mutation status.

4.5. Explant Results

The results for 4th objective:

4.5.1. Colorectal cancer patients' explant characteristics

In this research, we included a cohort of 32 patients diagnosed with colorectal cancer, noting an average patient age of 68 years. Females constituted two-thirds of the study group, indicating a gender predominance. Genetic evaluations revealed *KRAS* mutations in six patients (18.8 % of the cohort) and *BRAF* mutations in four individuals (12.5 % of the cohort). Serum marker assessments showed elevated CEA levels in 34.4 % of cases and CA 19-9 levels in 12.5 %. The staging analysis indicated that a substantial majority, more than two-thirds, were diagnosed with stage II and III cancer, with rectal cancer being the prevalent type in half of the cases. A significant observation was that 84.4 % of the cancers were moderately differentiated and classified as G2. Notably, there was one patient death attributed to complicated postoperative pneumonia and comorbidities. Table 4.5.1.1 presents comprehensive data on TNM staging, postoperative complications according to the Clavien-Dindo classification, and the follow-up details of the patients, offering valuable insights into the treatment outcomes and patient progress.

Table 4.5.1.1. *Ex vivo patient cohort clinicopathological characteristics*

Patient demographics	Characteristics	N = 32 (%)
Age at diagnosis	<65 year ≥65 year	11 (34.4 %) 21 (65.6 %)
Gender	Female Male	21 (65.6 %) 11 (34.4 %)
Mutation status	Wild type *KRAS* mutant *BRAF* mutant	22 (68.75 %) 6 (18.75 %) 4 (12.5 %)
CEA	normal <5.8 g/L elavated ≥5.8 g/L	21 (65.6 %) 11 (34.4 %)
Ca 19-9	normal <37 g/L elavated ≥37 g/L	28 (87.5 %) 4 (12.5 %)
Localization	Right colon Left colon Rectum	8 (25.0 %) 8 (25.0 %) 16 (50.0 %)

Table 4.5.1.1. cont.

Patient demographics	Characteristics	N = 32 (%)
Surgery type	Right Hemicolectomy Left hemicolectomy Sigmoid resection Rectal resection APR	9 (28.1 %) 2 (6.2 %) 6 (18.8 %) 11 (34.4 %) 4 (12.5 %)
Tumor Differentiation	Well differentiated,G1 Mod. Differentiated, G2 Poorly differentiated, G3	3 (9.4 %) 27 (84.4 %) 2 (6.2 %)
TNM Stage	Stage I Stage II Stage III Stage IV	7 (21.9 %) 15 (46.9 %) 7 (21.9 %) 3 (9.4 %)
pT Stage	pT1 pT2 pT3 pT4	1 (3.1 %) 9 (28.2 %) 21 (65.6 %) 1 (3.1 %)
pN Stage	pN0 pN1 pN2	20 (62.5 %) 10 (31.3 %) 2 (6.2 %)
V Vascular invasion	No invasion invasion	24 (75.0 %) 8 (25.0 %)
L invasion into lymphatic vessels	No invasion invasion	25 (78.1 %) 7 (21. 9 %)
Clavien-Dindo postoperative complications 30 days follow-up	Grade 0 (no complications) Grade I Grade II Grade IIIA Grade IIIB Grade IV Grade V	23 (71.9 %) 1 (3.1 %) 3 (9.4 %) None 4 (12.5 %) None 1 (3.1 %)
Postoperative hospital stay	days	8.47 d ± 5.81 (range: 3–27)
Follow-up months	months	21.97 ± 10.90 (range: 1–36)

4.5.2. CRC Explant patient survival analysis

In an ex vivo cohort analysis over 36 months, patients with *KRAS* mutations had the longest survival with a median survival of 27.84 ± 10.19 months (range: 17 to 36). Wild type patients had a median survival of 21.03 ± 10.61 months (range: 1 to 36), and patients with *BRAF* mutations had the lowest median survival of 21.97 ± 7.59 months (range: 2 to 25). Although survival varied between groups, the differences were not statistically significant ($p = 0.414$). The survival graph (Figure 4.5.2.1) suggests that while *KRAS*

mutations may be associated with a better prognosis, the true survival benefit requires longer follow-up to draw more definitive conclusions. Particularly when all patients in the *KRAS*-mutated group were censored and three deaths occurred in other groups.

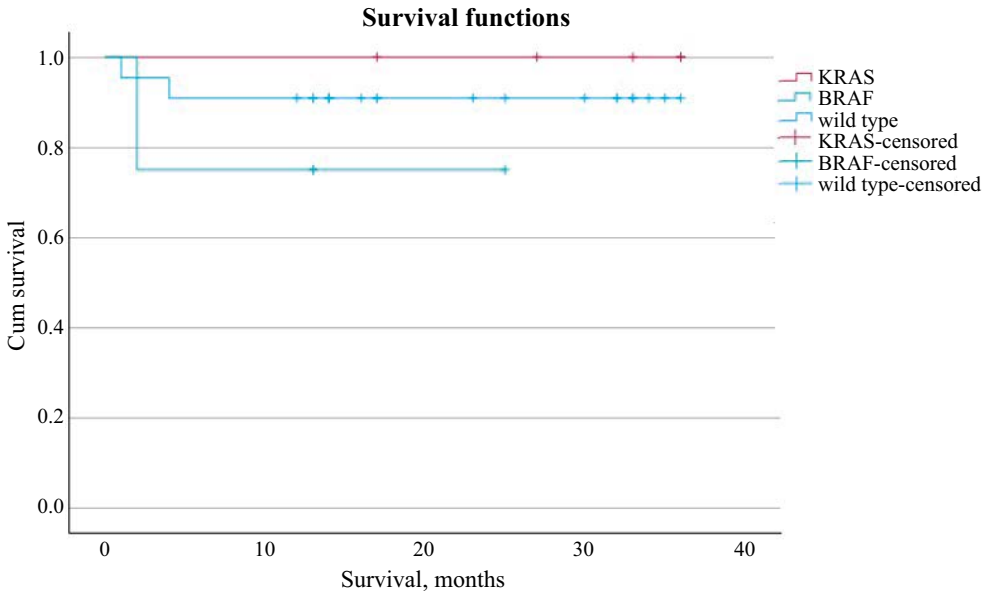


Fig. 4.5.2.1. *Kaplan-Meier survival curve illustrating the survival probabilities of colorectal cancer patients over time, stratified by genetic mutation status: KRAS, BRAF, and wild type. Each line represents the cumulative survival probability for patients within each subgroup.*

4.5.3. Colorectal cancer explant viability

4.5.3.1. *KRAS* mutant

The viability of *KRAS* mutant explants was impacted differently by the two treatments without reaching statistical significance. HAMLET treatment reduced viability to 78.32 % at 24 hours and further to 74.33 % at 48 hours. In contrast, FOLFOX had a slightly different effect, showing an initial viability of 88.89 % at 24 hours but then paradoxically increasing viability to 96.90 % at 48 hours, indicating an unexpected rise. This deviation from the expected cytotoxic effect suggests that FOLFOX may have a delayed interaction with *KRAS* mutant cells that do not immediately result in reduced viability. On the other hand, the combined treatment of HAMLET and FOLFOX resulted in viability close to those of HAMLET alone, with 78.09 % at 24 hours and 73.25 % at 48 hours.

Table 4.5.3.1.1. *Mean and SD of KRAS mutant explant viability*

KRAS	**HAMLET**	**FOLFOX**	**HAMLET + FOLFOX**
24 hours	78.32 ± 31.37	88.89 ± 18.59	78.09 ± 29.86
48 hours	74.33 ± 39.87	96.90 ± 37.87	73.25 ± 41.55

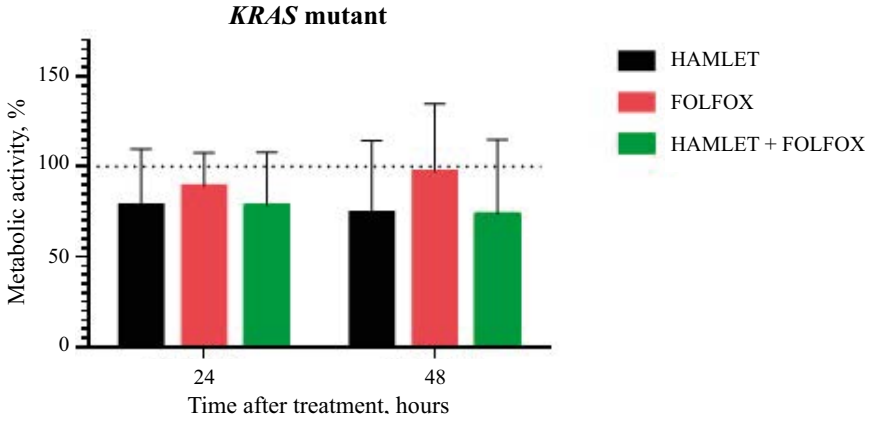


Fig. 4.5.3.1.1. *KRAS mutant explant viability after 24 hours and 48 hours.*

4.5.3.2. *BRAF* mutant explant viability

BRAF-mutated explants had different viability than *KRAS*-mutated ones after treatment. HAMLET treatment resulted in a statistically significant reduction in viability to 86.75 % at 24 hours and further reduced viability to 84.31 % at 48 hours. Similarly, FOLFOX treatment achieved statistically significant effects in only 48 hours, where viability was reduced to 77.73 %. The combination of the two regimens did not demonstrate a statistically significant reduction in viability.

Table 4.5.3.2.1. *Mean and SD of BRAF mutant explant viability*

BRAF	**HAMLET**	**FOLFOX**	**HAMLET + FOLFOX**
24 hours	86,75 ± 5,07	92,12 ± 36,49	99,99 ± 32,19
48 hours	84,31 ± 12,77	77,73 ± 22,18	79,64 ± 38,39

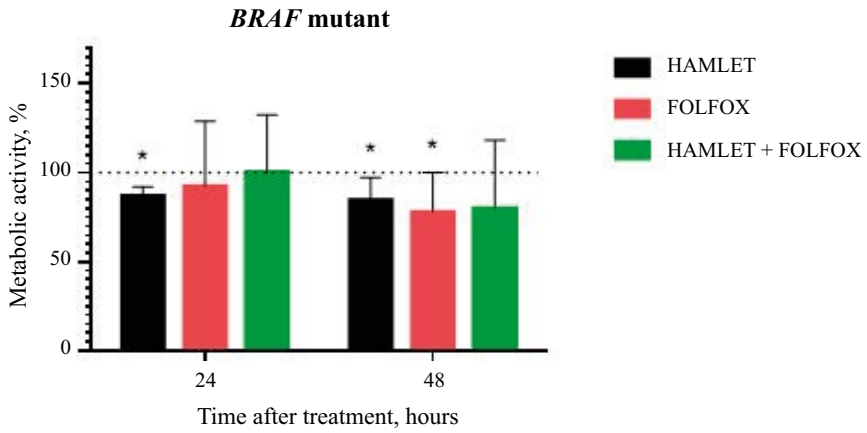


Fig. 4.5.3.2.1. *BRAF mutant explant viability after 24 hours and 48 hours.*

4.5.3.3. *KRAS/BRAF* wild type explant viability

The results of the HAMLET regimen were not statistically significant in wild type explants. In contrast, FOLFOX treatment alone had a statistically significant effect on viability, reducing it to 80.88 % at 24 hours and further to 73.13 % at 48 hours. This highlights the strong cytotoxic capacity of FOLFOX against WT explants over the period observed. Furthermore, the combined treatment of HAMLET and FOLFOX demonstrated the most pronounced and statistically significant reduction in viability among the treatments tested, dropping to 78.56 % at 24 hours and further to 67.71 % at 48 hours. These results highlight the therapeutic potential of combining HAMLET and FOLFOX to target wild type explants.

Table 4.5.3.3.1. *Mean and SD of KRAS/BRAF wild type explant viability*

Wild type	HAMLET	FOLFOX	HAMLET + FOLFOX
24 hours	97.64 ± 28.32	80.88 ± 11.72	78.56 ± 18.40
48 hours	93.65 ± 25.14	73.13 ± 14.96	67.71 ± 15.92

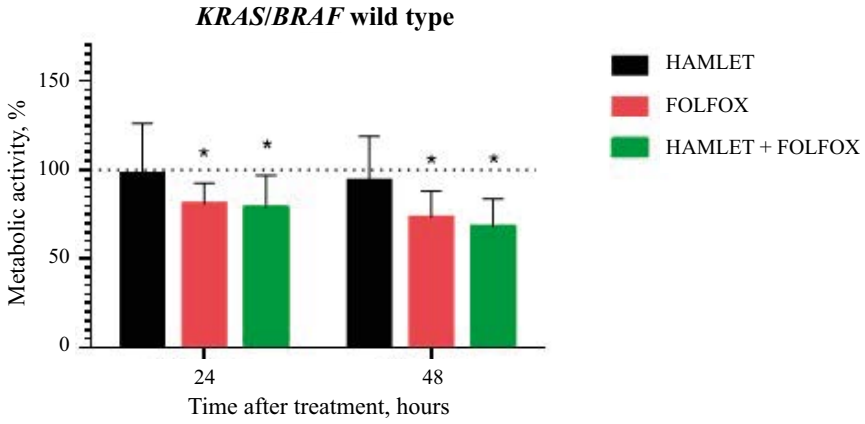


Fig. 4.5.3.3.1. *KRAS/BRAF wild type explant viability after 24 hours and 48 hours.*

4.5.4. CRC explant mitochondrial respiration

The results for 5th objective:

In our investigation of mitochondrial respiration in human CRC tissue explants, we examined the effects of HAMLET (60 μM) on mitochondrial function. We assessed mitochondrial respiration rate at 37 °C using glutamate/malate (Complex I) and succinate (Complex II) as substrates. Our results indicate that HAMLET has a tendency to inhibit mitochondrial respiration in both *BRAF* mutant and wild type CRC tissue samples, although this inhibition did not reach statistical significance ($p > 0.05$). Specifically, in CRC samples without the *BRAF* mutation, treatment with HAMLET led to a 39 % decrease in the non-phosphorylating (V_0) respiration rate (from 12 ± 2.646 to 7.3 ± 3.215 pmolO/s/mg dry weight) and a 33.3 % reduction in the $V_{ADP\ (glu/mal/suc)}$ respiration rate (from 13 ± 1 to 8.67 ± 3.786 pmolO/s/mg dry weight). Conversely, tissue samples with the *BRAF* mutation exhibited a more pronounced effect, with a 71 % decrease in the V_0 respiration rate (from 12 ± 4.243 to 3.5 ± 0.7 pmolO/s/mg dry weight) and a 60 % reduction in the $V_{ADP\ (glu/mal/suc)}$ respiration rate (from 12.5 ± 3.54 to 5 ± 1.41 pmolO/s/mg dry weight). These results suggest a differential response to mitochondrial respiration inhibition by HAMLET between *BRAF* mutant and wild type CRC samples, with a more significant inhibition observed in the *BRAF* mutant samples (see Table 4.5.4.1).

Table 4.5.4.1. *Comparison of the HAMLET impact and control on mitochondrial functions between CRC wild type and BRAF mutant.*

	Control		**HAMLET**	
	$\mathbf{V_0}$	$\mathbf{V_{ADP\ (glu/mal/suc)}}$	$\mathbf{V_0}$	$\mathbf{V_{ADP\ (glu/mal/suc)}}$
CRC wild type	12.0 ± 1.52	13.0 ± 0.58	12.0 ± 3.0	12.5 ± 2.5
***BRAF* mutant**	7.3 ± 1.85	8.7 ± 2.18	3.5 ± 0.5	5.0 + 1.0

5. DISCUSSION

5.1. The HAMLET complex's impact on cell viability, cell death and apoptosis/necrosis principal findings

Our research focused on evaluating the cytotoxic effects of HAMLET on various colorectal cancer (CRC) cell lines characterized by different *KRAS*/*BRAF* mutations, focusing on their viability, the occurrence of apoptotic and necrotic death, and their ability to form colonies. HAMLET has been highlighted as a promising candidate for targeted cancer therapy due to its selective accumulation in tumor tissue and significant reduction in tumor burden, as demonstrated in previous research using the Apc(Min)(/+) mouse model [18]. Recent clinical evidence has further supported the safety and efficacy of HAMLET, increasing its potential for broader therapeutic applications [108].

The principal findings of present study showed a dose-dependent decrease in cell viability in all cell lines, with higher concentrations of HAMLET significantly reducing viability. Wild type Caco-2 cells showed resistance at lower doses but were susceptible at higher concentrations. *KRAS* mutant lines (LoVo and HCT-116) also showed decreased viability at higher HAMLET concentrations, with HCT-116 being more sensitive. *BRAF* mutant lines (WiDr and HT-29) showed significant variability; WiDr cells were more resistant, while HT-29 cells were highly sensitive to HAMLET.

Cell death analysis revealed that HAMLET primarily induced necrosis rather than apoptosis. The necrotic cell population increased significantly in all cell lines, especially in HT-29 and HCT-116. Apoptosis was less pronounced, with only slight increases observed, mainly in HT-29 and HCT-116. Colony formation assays supported these findings, showing a marked reduction in colony formation, particularly in the sensitive HT-29 and HCT-116 lines. These results suggest that the cytotoxic effects of HAMLET are largely independent of *KRAS*/*BRAF* mutation status, with necrosis being the dominant form of cell death. The differential responses observed, particularly the resistance in WiDr cells compared to the sensitivity in HT-29 cells, suggest that other factors, such as mitochondrial respiration, may influence the efficacy of HAMLET.

5.1.1. Comparison with adjunct treatment effect on wild type CRC cell lines

Multiple adjunct treatment can influence the viability and apoptosis of Caco-2 cells through a number of different mechanisms. For instance,

cannabidiol (CBD) has been demonstrated to exhibit a chemopreventive effect by decreasing cell proliferation [109]. This effect is achieved through the activation of cannabinoid receptor 1 (CB1), transient receptor potential vanilloid 1 (TRPV1), and peroxisome proliferator-activated receptor gamma (PPARγ), a mechanism that is similar to that of HAMLET in its protective role against oxidative damage. However, the pathways involved in these processes are distinct [110]. Furthermore, natural agent quercetin sensitize these cells to tumor necrosis factor-related apoptosis-inducing ligand (TRAIL), primarily leading to apoptosis, while HAMLET's action appears to focus more on inducing necrosis at higher concentrations [111].

Furthermore, fucoxanthin induces apoptosis in Caco-2 cells by downregulating Bcl-2 protein expression, in contrast to the primary necrotic cell death caused by HAMLET [112]. Geraniol, an essential oil component, has also been demonstrated to increase apoptosis in Caco-2 cells when combined with 5-FU [113].

5.1.2. Comparison with adjunct treatment effect on *KRAS* CRC cell lines

A comparison of the effects of HAMLET on *KRAS* mutant CRC cell lines with those of other adjunct treatment reveals several parallels and contrasts. Curcumin enhances the effects of conventional chemotherapeutics like FOLFOX in HCT-116 cells by suppressing invasion through AMPK-induced inhibition of NF-kB, which is consistent with the ability of HAMLET to reduce cell viability [114,115]. Resveratrol sensitizes HCT-116 cells to 5-FU and induces apoptosis, a process that is analogous to the reduction of cell viability observed with HAMLET. However, resveratrol primarily induces apoptosis rather than necrosis [116].

CBD displays antiproliferative effects in HCT-116 cells by reducing proliferation through CB1, TRPV1, and PPARγ pathways, analogous to the induction of cell death observed with HAMLET [109]. Conversely, compounds such as isoliquiritigenin and gossypol primarily promote apoptosis in HCT-116 cells through caspase activation and downregulation of anti-apoptotic proteins, which differs from HAMLET's necrosis induction [117,118]. Furthermore, Sulforaphane induces cell death in HCT-116 cells through G2/M phase arrest and apoptosis, offering potential synergy with HAMLET's necrotic effects. Similarly, artesunate and panaxadiol induce cell cycle arrest and apoptosis in HCT-116 cells, which may complement HAMLET's necrotic pathways. [119–121].

Taken together, while HAMLET predominantly induces necrosis in *KRAS*-mutant CRC cells, adjunct treatment such as curcumin, resveratrol, and

cannabidiol demonstrate synergistic cytotoxic effects via diverse mechanisms, including apoptosis and cell cycle arrest [115,116,119].

5.1.3. Comparison with adjunct treatment effect on *BRAF* CRC cell lines

Adjunct treatment have shown similar efficacy to HAMLET in inhibiting the proliferation of WiDr cells. Fucoxanthin, a carotenoid found in seaweed, and its metabolite fucoxanthinol have shown significant anti-cancer activity in several CRC cell lines, including WiDr. At 20 μM, fucoxanthin and fucoxanthinol significantly reduced cell viability in WiDr cells, with fucoxanthinol reducing viability to 12.0 % [122].

In addition, quercetin exhibits significant anti-proliferative effects by inhibiting RASA1 and preventing RAS activation in WiDr cells. Studies have shown that 10 μM quercetin reduces p21-ras protein levels by approximately 50 % within 24 hours in a time- and concentration-dependent manner. This reduction affects K-, H- and N-ras proteins and is specific rather than a general suppression of protein synthesis [123].

In HT-29 cells, adjunct treatment have been observed to exhibit mechanisms that may complement the effects of conventional chemotherapeutics similarly to HAMLET. Quercetin induces apoptosis through a number of mechanisms, including caspase-3 activation, increased cytosolic cytochrome c, and COX-2-dependent reactive oxygen species (ROS) generation. This differs from the effects of HAMLET, which primarily induce necrosis. However, both compounds effectively reduce cell viability and could potentially complement each other to enhance overall cytotoxicity against CRC cells [124]. Curcumin has been demonstrated to enhance the efficacy of conventional chemotherapeutics such as FOLFOX, potentiating pro-apoptotic pathways and reducing cell viability in a manner analogous to that observed with HAMLET. This was associated with decreased expression and activation of EGFR [114].

Resveratrol sensitizes HT-29 cells to 5-FU-induced oxidative stress and primarily induces apoptosis, differing from HAMLET's necrotic induction but suggesting potential for synergistic effects [125]. Genistein and apigenin have been observed to increase the expression of pro-apoptotic proteins such as Bax and p21, while simultaneously inhibiting cell growth, thereby enhancing apoptosis in HT-29 cells [126–128].

Other compounds, such as kaempferol, which induces cell cycle arrest and apoptosis, and allyl isothiocyanate, which inhibits metastasis, also demonstrate promising activity in HT-29 cells. The combination of these

compounds with HAMLET may result in the targeting of multiple pathways, thereby enhancing the overall cytotoxic effects [129–131].

5.1.4. Cell death mechanism

Our study's observation that HAMLET predominantly induces necrosis, as opposed to the apoptosis-like cell death reported in other studies, highlights a key area of interest in understanding the mechanisms behind HAMLET's cytotoxic effects [132]. The literature from Lund University indicates that HAMLET typically triggers apoptosis through the mitochondrial pathway, releasing cytochrome C and activating caspases-2, -3, and -9 [92,96]. However, our findings indicate a significant increase in necrotic cell populations, particularly in *BRAF* mutant cell lines, suggesting that alternative cell death pathways may be involved.

The discrepancy between our results and those of previous studies may be attributed to differences in experimental conditions, such as variations in cell line characteristics, HAMLET concentrations, or treatment durations. Furthermore, our analysis of apoptosis-related markers, including APAF-1, BIRC3, and XIAP, demonstrated that HAMLET treatment did not significantly alter the expression of genes associated with apoptosis in *BRAF* mutant cells. This lack of activation of the apoptotic pathway genes suggests that necrosis may be the primary mode of cell death in these contexts. It is also possible that the mutational status of the cell lines plays a crucial role in determining the cell death mechanism. In our study, *BRAF* mutant WiDr cells exhibited a lower number of necrotic cells compared to other lines, and there was no significant overexpression of apoptosis-related genes [133]. This resistance to apoptosis may be a result of the activation of anti-apoptotic mechanisms or the engagement of alternative survival pathways in these cells. These pathways may prevent the apoptotic cascade from proceeding despite the initial mitochondrial disruption [97,98].

Furthermore, our clonogenic assays demonstrated that *BRAF* mutant WiDr cells exhibited resistance to HAMLET, indicating that these cells utilize distinct survival mechanisms. These findings indicate that the *KRAS*/*BRAF* mutational status may influence the effectiveness of HAMLET, potentially through varied impacts on mitochondrial function and cell death pathways. A more detailed understanding of these mechanisms will support our hypothesis that combining HAMLET with conventional chemotherapy regimens could enhance its efficacy against CRC.

5.2. HAMLET treatment effects on mitochondrial respiration and membrane permeability in colorectal cancer cells

5.2.1. Principal findings

The objective of this study was to investigate the effects of HAMLET on mitochondrial respiration and membrane permeability in colorectal cancer (CRC) cells with wild type, *KRAS* mutant, and *BRAF* mutant genetic profiles. Mitochondrial function is of great importance for the survival and apoptosis of cells, and alterations in mitochondrial respiration can significantly impact the metabolism and viability of cancer cells.

The findings of this study revealed that HAMLET selectively impairs mitochondrial respiration and membrane permeability in colon cancer cells. The most notable dysfunction was observed in wild type and *KRAS* mutant lines. Specifically, HAMLET significantly impaired mitochondrial complex I and complex I+II-dependent respiration rates across various CRC cell lines. This was particularly evident in wild type Caco-2 cells, where non-phosphorylating respiration was reduced by 18 %, and in *KRAS* mutant HCT-116 cells, where it was reduced by 17 %. Moreover, in the LoVo cell line, which harbors a *KRAS* mutation, HAMLET increased mitochondrial inner membrane permeability by 30 %. These findings indicate that the mitochondrial targeting of HAMLET may be influenced by genetic profile, suggesting its potential as a selective therapeutic agent.

5.2.2. Mitochondrial respiration and membrane permeability

The HAMLET treatment resulted in significant impairment of mitochondrial respiration and increased membrane permeability in CRC cell lines, with effects varying based on the status of the *KRAS*/*BRAF* mutations. In wild type Caco-2 cells, HAMLET disrupted mitochondrial complex I and complex I+II-dependent respiration, aligning with Hallgren *et al.* study showing HAMLET's ability to impair mitochondrial function [98]. Similarly, a study by Trumbeckaite *et al.* showed that cisplatin treatment impaired mitochondrial functions in Caco-2 cells by increasing the proton permeability of the inner mitochondrial membrane and decreasing the efficiency of oxidative phosphorylation. However, hyperthermia did not enhance the effects of cisplatin in Caco-2 cells, highlighting the complexity of mitochondrial responses to different treatments [134].

KRAS mutant cell lines (LoVo and HCT-116) exhibited significant inhibition of mitochondrial respiration, consistent with previous research indicating mitochondrial dysfunction as a key mechanism of action for HAMLET [97]. These findings are further supported by Rebane-Klemm

et al., who demonstrated that *KRAS* mutations are associated with altered mitochondrial respiration, suggesting a link between mutation status and metabolic phenotype in CRC cells [68].

In contrast, *BRAF* mutant cells exhibited a heterogeneous response. HT-29 cells demonstrated significant sensitivity to HAMLET, with a marked impairment in mitochondrial function, while WiDr cells exhibited minimal changes. This variability is consistent with findings from other research indicating that *BRAF* mutations often lead to altered mitochondrial dynamics and glycolytic shifts in cancer cells [68,69]. Furthermore, Fang *et al.* demonstrated that the α-lactalbumin-oleic acid complex, analogous to HAMLET, enhanced mitochondrial functions and substrate utilization, suggesting a complex interplay between mitochondrial function and cellular energy metabolism [135].

5.2.3. Potential for combination therapies

The disruption of mitochondrial respiration and increased membrane permeability by HAMLET indicate its potential for combination with other treatments targeting mitochondrial function. For example, diosmetin, a natural flavonoid, has been shown to synergize with 5-fluorouracil (5-FU) and enhance its anticancer activity by inducing apoptosis and disrupting mitosis in colon cancer cells [136]. Curcumin enhances the efficacy of chemotherapeutics like FOLFOX by promoting apoptosis and reducing cell viability, mechanisms that could be synergistic with HAMLET's effects [17]. Resveratrol, which induces oxidative stress and apoptosis, may also complement HAMLET by leveraging multiple pathways to induce cancer cell death more effectively [125]. The combination of HAMLET with these compounds could overcome resistance mechanisms and improve therapeutic outcomes.

5.3. Isobolograms: Bliss synergy model of HAMLET and FOLFOX treatment on CRC cell lines

The objective of this study was to evaluate the efficacy of HAMLET and FOLFOX as single agents and in combination on colorectal cancer (CRC) cell lines, with a particular focus on mutation-specific resistance or synergistic effects. The synergy between HAMLET and FOLFOX was evaluated using the Bliss independence model.

5.3.1. Principal findings

The Caco-2 heatmap shows a general trend of decreasing viability with increasing doses of FOLFOX and HAMLET, suggesting mostly additive

effects with no significant synergy or antagonism. Similarly, LoVo cells showed decreased viability at higher doses, with Bliss scores around zero, indicating an additive effect. In contrast, the HCT-116 heatmap shows occasional positive Bliss scores, suggesting some synergism at certain dose combinations, although these instances were not dominant. Most notably, the WiDr cell line showed a significant decrease in cell viability, particularly with the combination of 3 μM HAMLET and 1.25 μM FOLFOX, indicating significant synergy at mid-range doses. HT-29 cells mirrored the Caco-2 and LoVo results, showing a significant decrease in viability but no significant synergism or antagonism.

5.3.2. Comparison with other adjunct treatment

The observed synergy in WiDr and partial synergy in HCT-116 cells are consistent with the results of other studies investigating the combination of adjunct treatment with chemotherapeutic agents. For example, Fernández *et al.* demonstrated that apigenin and luteolin, flavonoids with anticancer properties, exhibited synergistic effects with 5-fluorouracil (5-FU) in HCT-116 cells by increasing apoptosis and inhibiting cell proliferation [16]. Kamran *et al.* highlighted that diosmetin, another natural flavonoid, significantly enhanced the efficacy of 5-FU in CRC cells by inducing apoptosis and disrupting mitosis [136].

Shaheer *et al.* investigated the combination of thymoquinone, a component of black seed, with 5-FU and found significant anti-proliferative activity and enhanced effects in CRC cells, including HCT-116 and HT-29 [137]. In addition, studies showed that curcumin, in combination with FOLFOX, significantly improved apoptosis and reduced cell viability in CRC cells. A randomized Phase IIa study further demonstrated the safety and tolerability of this combination in patients with metastatic colorectal cancer, supporting our findings of potential synergistic effects in specific CRC cell lines [114,138].

5.3.3. Comparison with altering treatment conditions

An examination of alternative strategies for enhancing the effectiveness of chemotherapy reveals promising approaches. For example, studies of hyperthermia in combination with chemotherapy have shown promising results. Čėsna *et al.* analyzed the combined effects of hyperthermia and cisplatin on Caco-2 cells using isobologram analysis, demonstrating a range of interactions from synergistic to antagonistic depending on the specific conditions [139]. Another study showed that modulated electro-hyperthermia combined with curcumin and resveratrol enhanced antitumor efficacy in CRC cells by inducing apoptosis and improving immune responses [140]. These

results suggest that combining HAMLET with hyperthermia or modulating treatment conditions could potentially enhance therapeutic responses.

Other options for modifying treatment conditions include adjusting the pH of the microenvironment, the use of radiation therapy for rectal cancer, or the incorporation of photodynamic therapy [141,142].

5.4. *Ex vivo* study principal findings

5.4.1. Explant viability

Our study investigated the effect of HAMLET and FOLFOX on the viability of ex vivo CRC explants with different genetic profiles, focusing on *KRAS* and *BRAF* mutations.

The viability of CRC explants varied with mutation status and treatment. *KRAS* mutant explants showed a significant decrease in viability with HAMLET, while FOLFOX paradoxically increased viability at 48 hours. Combined treatment mirrored HAMLET alone. *BRAF* mutant explants experienced significant viability reductions with both treatments, with no increase with the combination. Wild type explants showed the greatest reduction in viability with combined HAMLET and FOLFOX treatment.

These findings are consistent with those of Novo *et al.*, who showed that the MEK1/2 inhibitor selumetinib had differential efficacy on colorectal tumor biopsies depending on the genetic profile [143]. Similarly, the study by Khan *et al.* (2022) highlighted the differential response of adenoma and CRC explants to different treatments, supporting the need for mutation-specific approaches [144]. Ji *et al.* (2017) used MTT assays to assess chemosensitivity in CRC patients, highlighting the variability in response due to genetic differences, which is consistent with our observations [145]. In addition, Yoon *et al.* (2017) demonstrated that the integrative tumor response assay (ITRA) can predict therapeutic efficacy and select appropriate anticancer regimens, further highlighting the value of ex vivo models to assess treatment response based on genetic profiles [146].

5.4.2. Explant mitochondrial respiration

Our investigation of mitochondrial respiration revealed that HAMLET tended to inhibit mitochondrial function, particularly in *BRAF* mutant samples, although these findings did not reach statistical significance. In *BRAF* mutant tissues, HAMLET caused a marked decrease in both non-phosphorylated and ADP-stimulated respiration rates, suggesting a significant impairment of mitochondrial activity. A less pronounced inhibition was observed in wild type tissues.

These observations are consistent with studies demonstrating the sensitivity of *BRAF*-mutant CRC cells to mitochondrial inhibitors. The differential mitochondrial response observed in our study is supported by the dissertation of Rebane-Klemm, who demonstrated significant variation in mitochondrial respiration based on *KRAS* and *BRAF* mutation status, with *BRAF* mutations often correlating with a more glycolytic phenotype [147]. Our findings are consistent with the potential of targeting mitochondrial function as a therapeutic strategy in CRC and highlight the utility of ex vivo models to evaluate these effects.

CONCLUSIONS

1. HAMLET induces a dose-dependent cytotoxic effect across CRC cell lines regardless of *KRAS/BRAF* mutation status, with a notable preference for necrotic over apoptotic cell death and variable effects on colony formation.
2. HAMLET selectively impairs mitochondrial respiration and outer and inner membrane permeability in colon cancer cells, with the most notable dysfunction observed in wild type and *KRAS* mutant lines.
3. The combination of HAMLET and FOLFOX showed variable responses in different colorectal cancer cell lines, with synergy in WiDr cells, potentially overcoming FOLFOX resistance, while other lines showed additive effects, underscoring the complexity of their interaction beyond mutation-specific influences.
4. HAMLET and FOLFOX have different effects on colorectal cancer explants, with combined treatments showing efficacy.
5. *BRAF* mutants show a sensitivity to HAMLET-induced mitochondrial respiration inhibition.

PRACTICAL RECOMMENDATIONS

1. The combination of HAMLET with conventional chemotherapeutic agents such as FOLFOX is recommended as our study showed a significant reduction in cell viability, especially in wild type and *KRAS* mutant cells. This combination could improve therapeutic outcomes by exploiting the different mechanisms of both treatments.
2. Given HAMLET's impairment of mitochondrial respiration, particularly in *BRAF*-mutant cells, combination with mitochondrial inhibitors or metabolic modulators may enhance its effects. This approach could enhance the efficacy of HAMLET by disrupting the metabolic adaptations of cancer cells.
3. Matching treatments to the genetic profiles of CRC patients is critical. Our study showed different responses to HAMLET in *KRAS* and *BRAF* mutant cell lines, highlighting the need for personalized treatment plans. For example, *BRAF*-mutant cells were more sensitive to mitochondrial inhibition by HAMLET. In addition, it is important to consider other mutations and microsatellite stability as these factors also influence treatment response and outcome.

LIMITATIONS OF THE STUDY

While our study showed promising results with HAMLET in various CRC cell lines and ex vivo explants, the results are limited to in vitro and ex vivo models. These environments do not fully replicate the complexity of living organisms, and therefore the therapeutic effects and potential side effects of HAMLET in the clinical setting remain uncertain.

The study focused primarily on the genetic profiles of *KRAS* and *BRAF* mutations in CRC cells. However, colorectal cancer is highly heterogeneous and other mutations and factors such as microsatellite instability (MSI) and chromosomal instability (CIN) may also significantly influence treatment response. Excluding these factors limits the comprehensiveness of our findings.

Our analysis of mitochondrial function and membrane permeability focused on specific respiratory parameters. A broader investigation, including additional mitochondrial functions such as reactive oxygen species (ROS) production and mitochondrial dynamics, would provide a more complete picture of HAMLET's effects on cellular metabolism.

The study explored the combination of HAMLET with FOLFOX but did not extensively evaluate the optimal dosing, timing, and sequence of these combinations. Detailed pharmacokinetic and pharmacodynamic studies are necessary to determine the most effective combination strategies.

The study would benefit from the inclusion of histopathologic and immunohistochemical analyses to provide deeper insights into the cellular and molecular changes induced by HAMLET treatment. These methods could help to elucidate the mechanisms of cell death and validate the findings related to cell viability and mitochondrial function.

SANTRAUKA

ĮVADAS

Pasaulyje storosios žarnos vėžys (SŽV) yra didelė našta sveikatos priežiūros sistemai. 2020 m. buvo diagnozuota beveik du milijonai naujų SŽV atvejų ir daugiau kaip 0,9 milijono mirčių [1]. 2022 m. ES-27 šalių grupėje tarp visų vėžio lokalizacijų SŽV užėmė antrą vietą pagal sergamumą (13 proc.) ir buvo antra pagrindinė mirties nuo vėžio priežastis (12,3 proc.) [2]. SŽV atvejų didėjimą galima sieti su gyventojų senėjimu, gyvenimo būdo pokyčiais ir veiksmingesne atrankine patikra [3–5]. Tiesa, išgyvenamumo rodikliai labai skiriasi priklausomai nuo ligos stadijos: ankstyvos stadijos atveju penkerių metų išgyvenamumo rodiklis yra aukštas – 91 proc., o ligų su tolimuoju išplitimu šis rodiklis sumažėja iki 14 proc. [6–8]. Kita opi problema – SŽV atkryčių dažnis po operacijos, kuris pagal I-III stadijas siekia nuo 9 iki 31 proc. [9].

Individualizuota medicina tampa vis svarbesnė gydant SŽV, sutelkiant dėmesį į ligos heterogeniškumą ir jos sudėtingą naviko biologiją. Molekuliniai pokyčiai, ypač *KRAS* ir *BRAF* genuose, yra svarbiausi siekiant suprasti SŽV įvairovę ir nukreipti taikinių terapiją [10,11]. Naujausi pasiekimai leido sukurti novatoriškas gydymo schemas, kuriose molekulinės informacijos pagrindu kuriamas taiklesnis ir individualizuotas SŽV pacientų gydymas, pritaikant terapiją pagal konkrečius kiekvieno atvejo ypatumus [12].

Individualizuotos medicinos atsiradimas yra esminis metastazavusio storosios žarnos vėžio gydymo postūmis. Pažanga šioje srityje gerokai pagerino pacientų gydymo rezultatus, ypač pradėjus taikyti taikinių terapiją. Labai reikšmingas vėžio gydymui pagrindinių preparatų atsiradimas, tokių kaip bevacizumabas, veikiantis kraujagyslių endotelio augimo faktorių (VEGF (angl. *Vascular endotelial growth factor*)), bei cetuksimabas, veikiantis epidermio augimo faktoriaus receptorių (EGFR (angl. *Epidermal growth factor receptor*)). SŽV srityje nuolat kyla sunkumų dėl ligos heterogeniškumo ir pacientų molekulinių profilių įvairovės, ypač dėl tokių genų kaip *RAS* ir *BRAF* mutacijų, kurios lemia atsparumą tam tikriems preparatams ar gydymo schemoms [13].

Nors klinikinių tyrimų metu buvo nustatyta, kad naujų gydymo metodų taikymas pagerino išgyvenamumą, tačiau, palyginus su platesne SŽV sergančių pacientų populiacija, išgyvenamumo rezultatai nėra tokie džiuginantys. Klinikinių tyrimų dalyviai dažniausiai turi mažiau gretutinių ligų, yra jaunesni ir sveikesni nei visa populiacija [12,14,15].

Mokslininkai siekia atrasti naujų gydymo būdų, tyrinėja natūralius produktus, kuriais galėtų papildyti įprastą SŽV sisteminį gydymą. [16,17]. Vie-

nas iš daugiausiai vilčių teikiančių naujųjų preparatų yra HAMLET kompleksas (angl. *Human Alpha-lactalbumin Made LEthal to Tumor cells*) [18]. Dėl savo veiksmingumo, pavyzdžiui, veikiant vėžines ląsteles EGFR keliu bei veikiant mitochondrijų funkciją, jis gali tapti svarbiu papildymu prie esamų vėžio gydymo pasirinkimų. [19].

Disertacijoje keliama hipotezė, jog HAMLET derinimas su įprastiniais chemoterapijos preparatais padidins SŽV gydymo veiksmingumą. Analizuojant vėžio ląstelių linijas ir pacientų *ex vivo* biopsijas, šiuo tyrimu siekiama išsiaiškinti pagrindinius mechanizmus, lemiančius HAMLET veiksmingumą prieš SŽV. Šis tyrimas suteiks vertingų duomenų apie individualizuotas SŽV gydymo strategijas.

TIKSLAS

Įvertinti HAMLET komplekso ir FOLFOX chemoterapijos poveikį storosios žarnos vėžio ląstelėms ir audiniams su skirtingu *KRAS* ir *BRAF* mutacijomis *in vitro* ir *ex vivo*.

UŽDAVINIAI

1. Įvertinti HAMLET poveikį SŽV ląstelių metaboliniam aktyvumui, kolonijų formavimui ir žūties keliui linijose su skirtingomis *KRAS/BRAF* mutacijomis, *in vitro*.
2. Įvertinti HAMLET poveikį mitochondrijų funkcijai ir išorinės bei vidinės membranų laidumui SŽV ląstelių linijose su skirtingomis *KRAS/BRAF* mutacijomis, *in vitro*.
3. Įvertinti SŽV ląstelių linijų atsaką į FOLFOX ir jo kombinacinį poveikį su HAMLET, esant skirtingoms *KRAS/BRAF mutacijoms*, *in vitro*.
4. Įvertinti HAMLET, FOLFOX chemoterapijos ir jų derinio poveikį storosios žarnos vėžio eksplantų gyvybingumui su skirtingomis *KRAS/BRAF* mutacijomis, *ex vivo.*
5. Įvertinti HAMLET poveikį storosios žarnos vėžio eksplantų mitochondrijų funkcijoms su skirtingomis *KRAS/BRAF* mutacijomis, *ex vivo.*

METODIKA

Tyrimą sudaro *in vitro* ir *ex vivo* dalys, kuriose vertinamas HAMLET komplekso poveikio veiksmingumas storosios žarnos vėžio atveju, naudojant vėžinių ląstelių linijų ir pacientų audinių *ex vivo* mėginius – eksplanto modelius.

Tyrimo *in vitro* dalyje naudotos penkios SŽV ląstelių linijos, kurios turėjo skirtingą mutacijos statusą: Caco-2 (laukinio tipo), LoVo ir HCT-116 (*KRAS* mutacijos), WiDr ir HT-29 (*BRAF* mutacijos). Šios ląstelių linijos buvo kulti-

vuojamos, remiantis standartizuotais ląstelių banko (ATCC) protokolais ir laboratorijos protokolais, specialiose terpėse siekiant palaikyti optimalias augimo sąlygas: 37 °C temperatūra, 5 proc. CO_2 atmosfera, 95–98 proc. drėgnumas. Remiantis literatūroje aprašytu metodu [86], HAMLET kompleksas buvo paruoštas naudojant žmogaus α-laktalbuminą ir oleino rūgštį. Gaminant junginį atliktas dalinis α-laktalbumino baltymo denatūravimas karščio metodu (50 °C, 1500 apsisukimų per minutę termomikseryje, 15 min). Antrame etape buvo įvesta oleino rūgšties į komplekso struktūrą: maišymas (50 °C, 1500 apsisukimų per minutę termomikseryje, 10 min)), sudaryto junginio atvėsinimas iki kambario temperatūros, centrifugavimas (7000 G greitis, 4 °C, 15 min) ir komplekso saugojimas (–-80 °C) iki panaudojimo tyrimams.

SŽV ląstelių linijų metabolinis aktyvumas po poveikio HAMLET (ar FOLFOX) buvo matuojamas naudojant MTT kolorimetrinį testą. Ląstelės sėjamos į 96 šulinėlių plokšteles priklausomai nuo ląstelių linijos ir (8×10^3)–(2×10^4) ląstelių/šulinėlio tankio. Praėjus 24 valandoms buvo pridėtas HAMLET kompleksas 6 valandų poveikiui skirtingomis koncentracijomis. Po to terpė atnaujinta ir ląstelės inkubuotos dar 18 valandų prieš pridedant MTT reagento. Po 3–4 valandų 37 °C temperatūroje terpė pašalinta, o formazano kristalai ištirpinti 100 µl DMSO terpėje. Absorbcija išmatuota esant 570/620 nm bangos ilgiui ir palyginta su kontrolinėmis vertėmis.

Ląstelių kolonijų formavimo testas, kitaip vadinamas klonogeniniu testu, skirtas įvertinti ilgalaikius ląstelių dauginimosi skirtumus tarp HAMLET paveiktų ląstelių ir kontrolės. Tyrimas atliekamas ląsteles sėjant (1×10^2)–(2×10^2) koncentracija į 24 šulinėlių plokšteles. Po 24 val. sėjimo buvo pridėta HAMLET komplekso ir ląstelės inkubuojamos 6 val. esant skirtingoms HAMLET komplekso koncentracijoms. Po 6 val. mitybinė terpė pakeista į šviežią mitybinę terpę be HAMLET ir ląstelės inkubuojamos 9 dienas. Tada ląstelės buvo fiksuojamos etanoliu ir dažomos kristalinio violeto tirpalu. Kolonijos (tik > 50 ląstelių) buvo suskaičiuotos naudojant mikroskopą. Visos vertės buvo lyginamos su kontroline grupe.

Tėkmės citometrijos analizė atlikta naudojant „Guava Personal Cell Analysis“ tėkmės citometrą („Merck“; „Millipore“; Burlingtonas; MA; JAV) ir „CytoSoft 2.1.4“ programinę įrangą. Tyrimas atliktas pasėjus (1×10^5)–($1{,}3 \times 10^5$) ląstelių į šulinėlį. Po 24 val. buvo pridėta HAMLET komplekso ir ląstelės inkubuojamos 6 val. su skirtingomis HAMLET koncentracijomis. Po 6 val. ląstelės atskirtos naudojant tripsiną-EDTA. Ląstelių terpė buvo pašalinta centrifuguojant, o ląstelės suspenduotos jungiamajame buferiniame tirpale. Ląstelės nudažytos aneksino V-PE ir 7-AAD dažais ir analizuotos tekmės citometrijos metodu.

Mitochondrijų kvėpavimas buvo registruojamas oksigrafu (Oroboros Oxygraph-2k) 37 °C temperatūroje, mitochondrijų kvėpavimo terpėje pagal

gamintojo protokolą. Buvo vertinamas HAMLET poveikis mitochondrijų kvėpavimui ex vivo audinių mėginiuose ir storosios žarnos vėžio ląstelių linijose. Digitoninas (16 μg/ml) padidino ląstelių membranos pralaidumą, kad substratai ir metabolitai iš terpės patektų į ląsteles. Registravome kvėpavimo greitį V_0 (kvėpavimo greitis laidumo būsenoje) su I ir/arba II mitochondrijų kvėpavimo grandinės kompleksų substratais. Pridėjus 1 mM ADP, išmatuotas kvėpavimo greitis fosforilinimo būsenoje (V_{ADP}). Kad būtų pasiektas maksimalus mitochondrijų kvėpavimas ($V_{ADP\ (glu/mal/suc)}$), įvestas II komplekso substratas – sukcinatas (12 mM). Vcyt c – kvėpavimas, pridėjus išorinio citochromo C, įvertinama išorinės mitochondrijų membranos pažaida. V_{CAT} – išmatuojamas kvėpavimo greitis pridėjus karboksiatraktilozido, ADP/ATP nešiklio slopiklio, atspindi mitochondrijų vidinės membranos pažaidą.

HAMLET komplekso ir FOLFOX (5-fluorouracilas (5-FU) + oksaliplatina) derinio poveikis buvo išmatuotas MTT testu ir apskaičiuotas naudojant „Combenefit“ (v2.021) programinę įrangą. Skaičiavimams pasirinkta Bliss teorija, pagrįsta prielaida, kad abu vaistai veikia nepriklausomai, tačiau gali padidinti vienas kito citotoksinį poveikį [105,106].

Į antrąją *ex vivo* tyrimo dalį buvo įtraukti pacientai, kuriems Lietuvos sveikatos mokslų universiteto ligoninėje Kauno klinikose atlikta storosios žarnos vėžio operacija. Audinių mėginiai buvo paimti operacijos metu. Iš šios biopsijos pagal protokolą paruoštas *ex vivo* eksplanto modelis: audinio preparavimas, sterilus plovimas, tikslus pjovimas, eksplanto formavimas ir auginimas terpėje. *Ex vivo* mėginių mutacijų analizei išskirta genominė DNR ir naudojant TaqMan testus bei realaus laiko PGR nustatytos konkrečios mutacijos ir suskirstyta į pogrupius pagal požymius: laukinio tipas, *KRAS* ir *BRAF* mutacijos. *Ex vivo* eksplantų gyvybingumo vertinimas buvo atliekamas po poveikio HAMLET ir/arba FOLFOX naudojant resazurino redukcijos testą. Mitochondrijų kvėpavimas eksplantuose įvertintas naudojant anksčiau paminėtą oksigrafą, remiantis standartizuotu protokolu SŽV audinių tyrimams.

Statistinė analizė atlikta naudojant „GraphPad Prism“ ir „SigmaPlot“ programinę įrangą. Neparametriniams duomenų rinkiniams analizuoti taikėme Mann-Whitney U testą. Koreliacinius ryšius tarp kokybinių rodiklių lyginamosiose kohortose nagrinėjome naudodami chi kvadrato (χ^2) testą, o intervaliniams ir kategoriniams duomenims vertinti taikytas Student’s t-testas. Nustatytas statistinis reikšmingumo lygmuo $p < 0,05$.

BIOETIKA

Šiam tyrimui 2019 m. rugpjūčio 1 d. buvo gautas Kauno regioninio biomedicininių tyrimų etikos komiteto bioetikos leidimas Nr. BE-2-64, tyrimo

protokolo Nr. 1. Tyrimas atliktas laikantis Helsinkio deklaracijos principų ir vietinių įstatymų bei teisės aktų. Patvirtiname, kad visi dalyviai arba jų teisėti globėjai davė informuoto asmens sutikimą, gavę išsamius paaiškinimus apie tyrimo tikslus ir procedūras.

REZULTATAI

1. Uždavinys: HAMLET poveikis SŽV ląstelių metaboliniam aktyvumui, jų gyvybingumui kolonijų formavimimo testu, ląstelių žūties būdui

Iš pradžių įvertinome HAMLET komplekso poveikį SŽV ląstelių linijų, turinčių skirtingą mutacijos statusą, metaboliniam aktyvumui. Ištyrėme ląstelių metabolizmo reakciją į 2 μM, 5 μM, 10 μM ir 20 μM HAMLET komplekso koncentracijas. Visos HAMLET komplekso koncentracijos veikė visas ląstelių linijas pagal dozės-atsako priklausomybę.

Mažiausia tirta HAMLET koncentracija (2 μM) turėjo minimalų poveikį ląstelių metaboliniam aktyvumui. Caco-2 (*KRAS/BRAF* laukinio tipo) ir LoVo (*KRAS* mutacija) ląstelių linijų metabolinis aktyvumas net padidėjo iki 109 proc., o HT-29 (*BRAF* mutacija) ląstelių linijos labiausiai sumažėjo – iki 90 proc. WiDr (*BRAF* mutacija) ir HCT-116 (*KRAS* mutacija) ląstelių linijų metabolinis aktyvumas po poveikio žymiau nepasikeitė.

Padidinus HAMLET komplekso koncentraciją iki 5 μM, visų ląstelių linijų metabolinis aktyvumas sumažėjo stipriau. Tačiau statistiškai reikšmingai sumažėjimas stebėtas tik WiDr (85 proc.) ir HT-29 (64 proc.) ląstelių linijose ($p < 0,05$).

Ląstelių, paveiktų 10 μM HAMLET komplekso, metabolinis aktyvumas reikšmingai sumažėjo lyginant su kontrole ($p < 0,05$). Su 10 μM HAMLET koncentracija Caco-2 metabolinis aktyvumas statistiškai reikšmingai sumažėjo iki 64 proc., LoVo – 60 proc., WiDr – 61 proc., HT-29 – 40 proc., HCT-116 – 43 proc. Ląstelių linijų, paveiktų 5 ir 10 μM HAMLET kompleksu, tendencijos buvo panašios.

Po poveikio 20 μM HAMLET koncentracija, metabolinis aktyvumas statistiškai reikšmingai sumažėjo visose ląstelių linijose ($p < 0,05$), tačiau WiDr ląstelės pasižymėjo šiek tiek didesniu atsparumu. Tai rodo unikalų *BRAF* mutavusių ląstelių atsako į gydymą aspektą. WiDr ląstelių atsparumas, skirtingai nei HT-29 ląstelių pažeidžiamumas, rodo, kad *BRAF* mutavusių SŽV ląstelių linijų atsakas į HAMLET yra nevienodas, nors WiDr ir HT-29 ląstelių linijos turi tą pačią mutaciją. Todėl galima daryti prielaidą, kad *BRAF* mutacija nėra tiesiogiai susijusi su didesniu WiDr ląstelių atsparumu. Be to, panašus ląstelių

linijų su skirtingomis mutacijomis atsakas rodo, kad *KRAS/BRAF* mutacija neturi įtakos atsakui į HAMLET kompleksą.

MTT tyrimo rezultatus patvirtino tėkmės citometrijos tyrimas, kuris parodė panašias tendencijas: HT-29 ir HCT-116 ląstelių linijos buvo jautresnės HAMLET, o WiDr ląstelių linija buvo atsparesnė po HAMLET poveikio su 20 μM koncentracija. Apoptotinių ląstelių populiacija padidėjo nuo 1,6 iki 3,1 proc. Caco-2 ląstelėse; sumažėjo nuo 2,8 iki 1,8 proc. LoVo ląstelėse; padidėjo nuo 1,87 iki 1,9 proc. WiDr ląstelėse; padidėjo nuo 0,3 iki 2,8 proc. HT-29 ląstelėse ir nuo 0,4 iki 5 proc. HCT-116 ląstelių linijoje. Apoptotinių ląstelių populiacijos padidėjimas buvo statistiškai reikšmingas tik HT-29 ir HCT-116 ląstelių linijose ($p < 0,05$).

Tačiau nekrotinių ląstelių populiacijos padidėjimas daug ryškesnis nei apoptotinių. Ląstelių populiacija, patirianti nekrozę, padidėjo nuo 9 iki 38 proc. Caco-2 ląstelėse, nuo 9 iki 33 proc. LoVo ląstelėse, nuo 6 iki 11 proc. WiDr ląstelėse, nuo 1 iki 50 proc. HT-29 ląstelėse ir nuo 4 iki 73 proc. HCT-116 ląstelių linijoje. Nekrotinių ląstelių populiacijos padidėjimas buvo statistiškai reikšmingas Caco-2, HT-29 ir HCT-116 ląstelių linijose ($p < 0,05$). WiDr ląstelių linijoje nekrotinių ląstelių populiacija padidėjo mažiausiai, o HT-29 ir HCT-116 linijose nekrotinių ląstelių populiacija padidėjo labiausiai. Rezultatai rodo, kad HAMLET kompleksas beveik nesukelia apoptozės ir daugiausia SŽV ląstelių linijas žudo nekrozės būdu bei nepriklauso nuo *KRAS/BRAF* mutacijos statuso.

HAMLET kompleksas ne tik slopino ląstelių metabolinį poveikį, bet ir darė didelę įtaką kolonijų formavimuisi. Rezultatai, gauti atlikus kolonijų formavimo testą, buvo panašūs į MTT ir tėkmės citometrijos rezultatus. *KRAS/BRAF* mutacija neturėjo didelės įtakos HAMLET komplekso reakcijai, o WiDr ląstelių linija buvo atspariausia 20 μM HAMLET kompleksui. HT-29 ir HCT-116 ląstelių linijos buvo jautriausios HAMLET kompleksui, nes po HAMLET poveikio nebesusiformavo nei viena kolonija.

Apibendrinant pirmojo uždavinio rezultatus galima teigti, kad tarp HAMLET sukelto ląstelių žūties būdo ir *KRAS*/*BRAF* mutacijų koreliacijos nebuvo. Tačiau WiDr ląstelių linija skiriasi nuo kitų linijų, nes buvo atsparesnė ląstelių metabolizmo, nekrotinių ląstelių populiacijos padidėjimo ir kolonijų formavimosi požiūriu, o panašaus genetinio profilio ląstelės (HT-29) buvo jautriausios. Vienas iš galimų to paaiškinimų galėtų būti ląstelių mitochondrijų skirtumai.

2. Uždavinys: HAMLET poveikis mitochondrijų funkcijai ir mitochondrijų išorinės bei vidinės membranų laidumui SŽV ląstelėse

Laukinio tipo Caco-2 SŽV ląstelėse, po poveikio HAMLET (5 mM) slopino kvėpavimo greitį laidumo būsenoje 18 proc. lyginant su HAMLET nepaveiktomis ląstelėmis po HAMLET poveikio su 5 μM. Be to, HAMLET 19–45 proc. statistiškai reikšmingai slopina mitochondrijų kvėpavimą fosforilinimo būsenoje (esant ADP priedui) su mitochondrijų kvėpavimo grandinės I ir I+II kompleksų substratais ($p < 0,05$). Šie rezultatai rodo, kad Caco-2 ląstelėse HAMLET slopina mitochondrijų oksidacinio fosforilinimo sistemą, sutrikdydamas deguonies panaudojimą ir ATP sintezę mitochondrijose.

KRAS mutavusių SŽV ląstelių linijose LoVo ir HCT-116 HAMLET slopino mitochondrijų kvėpavimo greitį laidumo būsenoje, atitinkamai 6 ir 17 proc. Be to, HAMLET šiose ląstelių linijose statistiškai reikšmingai slopino nuo mitochondrijų I komplekso ir I+II komplekso substratų priklausomą ADP aktyvinamą kvėpavimo greitį ($p < 0,05$). Šis poveikis pabrėžia *KRAS* mutavusių ląstelių jautrumą HAMLET, o tai rodo, kad SŽV ląstelėse genetinės mutacijos daro skirtingą poveikį. Pažymėtina, kad LoVo ląstelėse taip pat 30 proc. padidėjo mitochondrijų vidinės membranos pralaidumas, o tai rodo, jog HAMLET poveikis mitochondrijų funkcijoms priklauso nuo mutacijos.

Priešingai, *BRAF* mutavusios WiDr ląstelėse, HAMLET neturėjo poveikio mitochondrijų kvėpavimo greičiui laidumo būsenoje, o nuo I komplekso substratų priklausomas ADP stimuliuojamas kvėpavimo greitis sumažėjo tik nežymiai, t. y. 12 proc. Tačiau HT-29 ląstelėse, taip pat turinčiose *BRAF* mutaciją, mitochondrijų kvėpavimo greitis laidumo būsenoje sumažėjo 26 proc. Šie rezultatai rodo, kad *BRAF* mutavusių SŽV ląstelių atsakas į HAMLET yra nevienodas, o HT-29 ląstelės yra žymiai jautresnės HAMLET poveikiui ir mitochondrijų funkcijos slopinamos stipriau nei WiDr ląstelėse.

3. Uždavinys: HAMLET ir FOLFOX derinio poveikis SŽV ląstelių linijoms

Ištyrus penkias SŽV ląstelių linijas, veikiamas FOLFOX (5-FU ir oksaliplatina), gauta vertingos informacijos apie atsparumą vaistams ir galimą vaistų sąveiką. HCT-116 ląstelės buvo gerokai mažiau jautrios FOLFOX, joms reikėjo didesnės IC50 dozės – 73,1 μM, kad būtų pasiektas toks pat ląstelių žūties lygis, kaip ir kitoms tirtoms ląstelių linijoms, kurių IC50 vertės buvo nuo 6 iki 15,1 μM. Šie rezultatai rodo, kad HCT-116 veiksmingiau nei kitoms ląstelių linijos neutralizuoja toksinį FOLFOX poveikį.

Remiantis FOLFOX ir HAMLET poveikio SŽV linijoms MTT metodu, galimas sinergizmas ir antagonizmas buvo įvertintas taikant Bliss nepriklau-

somybės modelį. Caco-2 šilumos žemėlapis atskleidžia bendrą tendenciją, kad gyvybingumas mažėja didėjant FOLFOX ir HAMLET dozėms, todėl reikšmingo sinergizmo ar antagonizmo nenustatyta. Dauguma reikšmių yra apie nulį, o tai rodo daugiausia adityvų poveikį. Panašiai ir LoVo ląstelių linijos gyvybingumas mažėja vartojant didesnes dozes, tačiau Bliss rodikliai vėl išlieka apie nulį (adityvus poveikis). Priešingai, HCT-116 šilumos žemėlapyje matyti, kad gyvybingumas mažėja didėjant vaisto koncentracijai, o gautos teigiamos reikšmės, rodančios sinergiją su tam tikrais dozių deriniais (HAMLET 3 μM ir didėjant FOLFOX koncentracijai iki 6,25 μM).

WiDr ląstelių linija rodo, kad ląstelių metabolinis aktyvumas labai sumažėja, ypač esant 3 μM HAMLET ir 1,25 μM FOLFOX deriniui, o gauti teigiami rezultatai, ypač esant vidutinėms dozėms, rodo sinergizmą. Gyvybingumo sumažėjimas taip pat aiškiai matomas HT-29 šilumos žemėlapyje ir atspindi Caco-2 ir LoVo ląstelių rezultatus. Apibendrinant galima teigti, jog stebėtas sinergizmas, ypač WiDr ląstelių linijoje, gali būti susijęs su ląstelių linijų atsparumu FOLFOX. Sinergetinė sąveika WiDr ir iš dalies HCT-116 ląstelėse leidžia manyti, kad HAMLET gali būti svarbus mažinant atsparumą FOLFOX. Be to, reikšmingo sinergizmo ar antagonizmo nebuvimas Caco-2, LoVo ir HT-29 ląstelių linijose, kai jos buvo veikiamos kartu su FOLFOX ir HAMLET, rodo, kad HAMLET poveikis FOLFOX veiksmingumui nėra nuoseklus skirtingose SŽV ląstelių linijose ir gali būti tiesiogiai nesusijęs su mutacijos statusu.

4. Uždavinys HAMLET ir FOLFOX chemoterapijos schemos derinio gyvybingumą *ex vivo* storosios žarnos vėžio eksplantui

Ex vivo eksplantų tyrime dalyvavo 32 pacientai, kuriems diagnozuotas storosios žarnos vėžys, o vidutinis pacientų amžius buvo 68 metai. Moterys sudarė du trečdalius tiriamosios grupės. Atlikus genetinį vertinimą, *KRAS* mutacijos nustatytos šešiems pacientams (18,8 proc. grupės), o *BRAF* mutacijos - keturiems asmenims (12,5 proc. grupės). Įvertinus serumo žymenis, 34,4 proc. atvejų nustatytas padidėjęs CEA kiekis, o 12,5 proc. – CA 19-9 kiekis. Daugiau nei dviem trečdaliams diagnozuotas II ir III stadijos vėžys, o tiesiosios žarnos vėžys buvo vyraujanti lokalizacija pusėje atvejų.

Atlikus 36 mėnesių *ex vivo* kohortos analizę, ilgiausiai išgyveno *KRAS* mutacijas turintys pacientai, kurių išgyvenamumo mediana buvo 27,84 ± 10,19 mėnesio (nuo 17 iki 36 mėnesių). Laukinio tipo pacientų išgyvenamumo mediana buvo 21,03 ± 10,61 mėnesio (intervalas nuo 1 iki 36), o *BRAF* mutacijų turinčių pacientų išgyvenamumo mediana mažiausia – 21,97 ± 7,59 mėnesio (intervalas nuo 2 iki 25). Nors išgyvenamumas skirtingose grupėse skyrėsi, skirtumai nebuvo statistiškai reikšmingi (p=0,414).

KRAS mutavusių eksplantų metaboliniam aktyvumui HAMLET ir FOLFOX turėjo skirtingą poveikį, tačiau statistinis reikšmingumas nebuvo pasiektas. HAMLET poveikis 24 val. sumažino eksplanto gyvybingumą iki 78,32 proc., o 48 val.– iki 74,33 proc. Tuo tarpu FOLFOX poveikis buvo šiek tiek kitoks: 24 val. gyvybingumas iš pradžių buvo 88,89 proc., tačiau 48 val. gyvybingumas paradoksaliai padidėjo iki 96,90 proc., o tai rodo netikėtą gyvybingumo padidėjimą. Šis nukrypimas nuo tikėtino citotoksinio poveikio rodo, kad FOLFOX gali turėti uždelstą sąveiką su *KRAS* mutavusiomis ląstelėmis, dėl kurios gyvybingumas iš karto nesumažėja. Kita vertus, kartu gydant HAMLET ir FOLFOX, gyvybingumas buvo panašus į vien tik HAMLET, t. y. 78,09 proc. 24 val. ir 73,25 proc. 48 val.

BRAF mutavusių eksplantų gyvybingumas po gydymo skyrėsi nuo *KRAS* grupės. Po HAMLET poveikio, gyvybingumas statistiškai reikšmingai sumažėjo iki 86,75 proc. po 24 valandų, o po 48 valandų gyvybingumas dar labiau sumažėjo iki 84,31 proc. Panašiai, gydant FOLFOX, statistiškai reikšmingas poveikis buvo pasiektas tik per 48 valandas, kai gyvybingumas sumažėjo iki 77,73 proc. Dviejų preparatų derinys statistiškai reikšmingai nesumažino metabolinio aktyvumo.

HAMLET poveikio rezultatai nebuvo statistiškai reikšmingi laukinio tipo eksplantams. Priešingai, vien gydymas FOLFOX turėjo statistiškai reikšmingą poveikį gyvybingumui – 24 val. jis sumažėjo iki 80,88 proc., o 48 val. – iki 73,13 proc. Tai rodo stiprų FOLFOX citotoksinį poveikį WT eksplantams. Be to, kombinuotas HAMLET ir FOLFOX poveikis parodė ryškiausią ir statistiškai reikšmingiausią gyvybingumo sumažėjimą iš visų poveikio būdų: 24 val. gyvybingumas sumažėjo iki 78,56 proc., o 48 val. – iki 67,71 proc. Šie rezultatai rodo, kad HAMLET ir FOLFOX derinimas turi terapinį potencialą, siekiant paveikti laukinio tipo eksplantus.

5. HAMLET gydymo poveikį storosios žarnos vėžio *ex vivo* eksplantų mitochondrijų kvėpavimui

Tirdami mitochondrijų kvėpavimą žmogaus SŽV audinių eksplantuose, ištyrėme HAMLET (60 μM) poveikį mitochondrijų funkcijai. Įvertinome mitochondrijų kvėpavimo greitį 37 °C temperatūroje, kaip substratus naudodami glutamatą/malatą (I kompleksas) ir sukcinatą (II kompleksas).

Mūsų rezultatai rodo, kad HAMLET turi tendenciją slopinti mitochondrijų kvėpavimą tiek *BRAF* mutavusio, tiek laukinio tipo SŽV audinių mėginiuose, nors šis slopinimas nebuvo statistiškai reikšmingas ($p > 0,05$). Tiksliau, SŽV mėginiuose be *BRAF* mutacijos, veikiant HAMLET, 39 proc. sumažėjo mitochondrijų kvėpavimo greitis laidumo (V_0) būsenoje (nuo 12 ± 2,646 iki 7,3 ± 3,215 pmolO/s/mg sausos medžiagos svorio) ir 33,3 proc.

sumažėjo mitochondrijų kvėpavimo greitis ($V_{ADP\ (glu/mal/suc)}$ fosforilinimo būsenoje (nuo 13 ± 1 iki 8,67 ± 3,786 pmolO/s/mg sausos medžiagos svorio). Priešingai, *BRAF* mutaciją turinčių audinių mėginiai pasižymėjo ryškesniu poveikiu: V_0 kvėpavimo greitis sumažėjo 71 proc. (nuo 12 ± 4,243 iki 3,5 ± 0,7 pmolO/s/mg sausos masės), o $V_{ADP\ (glu/mal/suc)}$ kvėpavimo greitis – 60 proc. (nuo 12,5 ± 3,54 iki 5 ± 1,41 pmolO/s/mg sausos masės). Šie rezultatai rodo, kad *BRAF* mutavusio ir laukinio tipo SŽV eksplantai skirtingai reaguoja į mitochondrijų kvėpavimo slopinimą HAMLET, o *BRAF* mutavusio SŽV eksplantai slopinami labiau.

IŠVADOS

1. HAMLET sukelia nuo dozės priklausomą citotoksinį poveikį visoms SŽV ląstelių linijoms, nepriklausomai nuo *KRAS*/*BRAF* mutacijos statuso, su pastebimu polinkiu nekrotinei, o ne apoptotinei ląstelių žūčiai ir skirtingu poveikiu kolonijų formavimuisi.
2. HAMLET selektyviai sutrikdo mitochondrijų kvėpavimą ir mitochondrijų išorinės bei vidinės membranų laidumą storosios žarnos vėžio ląstelėse, ryškiausias poveikis stebimas laukinio tipo ir *KRAS* mutantų linijose.
3. HAMLET ir FOLFOX derinys skirtingose storosios žarnos vėžio ląstelių linijose pasižymėjo skirtingu atsaku: WiDr ląstelėse pasireiškė sinergija, galinti įveikti atsparumą FOLFOX, o kitose linijose pasireiškė papildomas poveikis, pabrėžiantis jų sąveikos sudėtingumą, neapsiribojant tik mutacijai būdinga įtaka.
4. HAMLET ir FOLFOX skirtingai veikia storosios žarnos vėžio eksplantus, o kombinuotas gydymas pasižymi veiksmingumu.
5. HAMLET slopino mitochondrijų kvėpavimą *BRAF* mutaciją turinčiuose eksplantuose.

PRAKTINĖS REKOMENDACIJOS

Rekomenduojama HAMLET derinti su įprastiniais chemoterapiniais preparatais, tokiais kaip FOLFOX. Mūsų tyrimo duomenimis ląstelių metabolinis aktyvumas, ypač laukinio tipo ir *KRAS* mutavusių ląstelių, labai sumažėjo. Šis derinys galėtų pagerinti gydymo rezultatus, nes būtų išnaudoti skirtingi abiejų gydymo būdų mechanizmai.

Atsižvelgiant į tai, kad HAMLET sutrikdo mitochondrijų kvėpavimą, ypač *BRAF* mutavusiose ląstelėse, derinys su mitochondrijų inhibitoriais arba medžiagų apykaitos moduliatoriais gali sustiprinti jo poveikį.

Labai svarbu gydymą pritaikyti prie SŽV sergančių pacientų genetinių profilių. Mūsų tyrimas parodė, kad *KRAS* ir *BRAF* mutavusių ląstelių linijų atsakas į HAMLET skiriasi, todėl būtina pritaikyti individualizuotus gydymo planus. Pavyzdžiui, *BRAF* mutavusios ląstelės buvo jautresnės mitochondrijų slopinimui HAMLET. Be to, svarbu atsižvelgti į kitas mutacijas ir mikrosatelitų stabilumą, nes šie veiksniai taip pat turi įtakos atsakui į gydymą ir rezultatams.

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LIST OF PUBLICATIONS

Publications related to the results of dissertation

1. **Žilinskas J**, Stukas D, Jasukaitienė A, Žievytė I, Balion Z, Šapauskienė J, *et al.* Assessing the Therapeutic Impacts of HAMLET and FOLFOX on *BRAF*-Mutated Colorectal Cancer: A Study of Cancer Cell Survival and Mitochondrial Dynamics In Vitro and Ex Vivo. Med. 2024;60(1). doi:10.1007/s00432-023-04777-0.
2. **Žilinskas J**, Stukas D, Jasukaitienė A, Šapauskienė J, Banienė R, Trumbeckaitė S, *et al.* HAMLET effect on cell death and mitochondrial respiration in colorectal cancer cell lines with *KRAS*/*BRAF* mutations. J Cancer Res Clin Oncol. 2023;149(11). doi: 10.3390/medicina60010142.

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1. Pikūnienė I, Strakšytė V, Basevičius A, **Žilinskas J**, Ambrazienė R, Jančiauskienė R, *et al.* Prognostic Value of Tumor Volume, Tumor Volume Reduction Rate and Magnetic Resonance Tumor Regression Grade in Rectal Cancer. Med. 2023;59(12). doi:10.3390/medicina59122194.
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LIST OF SCIENTIFIC CONFERENCES

The results of dissertation were presented in the scientific conferences:

1. **Žilinskas, Justas**; Šapauskienė, Jurgita; Kazakevičiūtė, Gintarė; Stukas, Darius; Gulbinas, Antanas; Tamelis, Algimantas; Banienė, Rasa. The effect of human α-lactalbumin and oleic acid complex (HAMLET) on mitochondrial function in colon cancer cells // XVIth International Conference of the Lithuanian Biochemical Society "Biochemistry Targeting Diseases": Taujėnai, Lithuania, June 28-30, 2023: programme and abstract book / Lietuvos Biochemikų Draugija., 2023, p. 34 - 34, ISBN 9786099603032. Prieiga per internetą: https://hdl.handle.net/20.500.12512/237924
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RESEARCH

HAMLET effect on cell death and mitochondrial respiration in colorectal cancer cell lines with KRAS/BRAF mutations

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Abstract

Purpose Treatment of advanced colorectal cancer (CRC) depends on the correct selection of personalized strategies. HAMLET (Human Alpha-lactalbumin Made LEthal to Tumor cells) is a natural proteolipid milk compound that might serve as a novel cancer prevention and therapy candidate. Our purpose was to investigate HAMLET effect on viability, death pathway and mitochondrial bioenergetics of CRC cells with different KRAS/BRAF mutational status in vitro.

Methods We treated three cell lines (Caco-2, LoVo, WiDr) with HAMLET to evaluate cell metabolic activity and viability, flow cytometry of apoptotic and necrotic cells, pro- and anti-apoptotic genes, and protein expressions. Mitochondrial respiration (oxygen consumption) rate was recorded by high-resolution respirometry system Oxygraph-2 k.

Results The HAMLET complex was cytotoxic to all investigated CRC cell lines and this effect is irreversible. Flow cytometry revealed that HAMLET induces necrotic cell death with a slight increase in an apoptotic cell population. WiDr cell metabolism, clonogenicity, necrosis/apoptosis level, and mitochondrial respiration were affected significantly less than other cells.

Conclusion HAMLET exhibits irreversible cytotoxicity on human CRC cells in a dose-dependent manner, leading to necrotic cell death and inhibiting the extrinsic apoptosis pathway. BRAF-mutant cell line is more resistant than other type lines. HAMLET decreased mitochondrial respiration and ATP synthesis in CaCo-2 and LoVo cell lines but did not affect WiDr cells' respiration. Pretreatment of cancer cells with HAMLET has no impact on mitochondrial outer and inner membrane permeability.

Keywords Colorectal cancer · Bioactive milk components · KRAS and BRAF mutation · EGFR · Mitochondrial respiration

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Introduction

Approximately, 2 million new colorectal cancer (CRC) cases and 935,000 deaths were estimated to occur in 2020, accounting for about 10% of all diagnosed malignancies and cancer-related deaths worldwide. Therefore, significant bowel cancer rates are third in incidence and second in mortality (Sung et al. 2021). However, a quarter of these patients have advanced disease at the time of diagnosis, while 20% of patients will be diagnosed with metastatic at a later time (Aasebø et al. 2020).

Personalized medicine development of new active agents as an adjunct to chemotherapy has enhanced metastatic CRC (mCRC) outcomes in the 21st century. For instance, monoclonal antibodies such as bevacizumab target vascular endothelial growth factor, while cetuximab acts directly against epidermal growth factor receptor (EGFR) (Douillard et al. 2013; Cohen

et al. 2021; Tougeron et al. 2013). Second, the scientific progress of patient molecular profile and heterogeneous tumor microenvironment demands complex oncological treatment decisions. For example, activating mutations in RAS, BRAF V600E induce resistance to EGFR inhibitors (Molinari et al. 2018; Oikonomou et al. 2014). Other essential agents include human EGFR 2, programmed death receptor 1 and tropomyosin receptor kinase inhibitors (Fujii et al. 2020; André et al. 2020; Cooper et al. 2020).

Regardless of therapeutic advancement, the median overall survival of selected mCRC patients improved to the extent of 20–30 months in clinical trials. In comparison, the prognosis for an unselected population from the Scandinavian cancer registry remains significantly shorter, with a median overall survival of 10–15 months. In addition, subjects from clinical trials usually have better performance status, younger age, and less comorbidity, making them incomparable to the general mCRC population (Aasebø et al. 2020; Hamers et al. 2019). Opposite survival findings from trials and registries challenge the need to develop novel therapeutic agents.

Traditionally, natural products have been the prim3

02}>M+ry origin of bioactive syntheses used in the pharmaceutical industry and traditional healthcare systems (Genovese et al. 2020). A novel promising candidate is human alpha-lactalbumin made lethal to tumor cells (HAMLET), a new type of cancer-killing molecule developed by the Lund University research group. It is a complex of two of the most abundant units in human milk: Protein alpha-lactalbumin and lipid oleic acid. Together they form a compound with a broad tumoricidal effect against cancer cells without harming mature, healthy cells (Ho et al. 2017, 2016; Arcila et al. 2011).

HAMLET independently hits multiple cell targets, including the EGFR signaling pathway. Two of the key signaling molecules of the pathway are RAS and RAF, encoded by the *KRAS* and *BRAF* genes, respectively (Kim and Bodmer 2022). Typically, these mutations occur in CRC, and their presence links to EGFR inhibitor resistance (Ho et al. 2017) revealing that HAMLET inhibits oncogenic Ras and Braf activity (Ho et al. 2016). In addition, HAMLET is known to activate mitochondria-dependent apoptosis and might interfere with mitochondrial function (Boekema et al. 2015). However, the cell death mechanism is undefined. Thus, we hypothesized that HAMLET anticancer effectiveness might be affected by different *KRAS*/*BRAF* mutational status and mitochondrial activity of CRC cell lines.

Materials and methods

Cell cultures and reagents

Human CRC cell lines WiDr (colorectal adenocarcinoma) and LoVo (colorectal adenocarcinoma from metastatic site) were obtained from CLS cell lines service, Germany. Caco-2 (colorectal adenocarcinoma) cell line was obtained from American Type Culture Collection (ATCC, United States). The mutation status of CRC cell lines is summarized in Table 1 (Ahmed et al. 2013). Caco-2 and LoVo cell lines were cultured in Ham's F-12K (Kaighn's) medium (GIBCO) supplemented with 10% fetal bovine serum (GIBCO) and 1% penicillin-streptomycin (GIBCO). WiDr cell line was cultured in 1:1 Ham's F-12K (Kaighn's) medium (GIBCO) and Dulbecco's modified eagle medium (GIBCO) supplemented with 5% fetal bovine serum (GIBCO) and 1% penicillin-streptomycin (GIBCO). The cell lines were incubated at 37 °C in a 5% CO_2 atmosphere.

Human alpha-lactalbumin (Cat. No. L7269) and oleic acid (Cat. No. O1383) were purchased from Sigma-Aldrich, Germany. 3-(4,5-Dimethylthiazol-2yl)-2,5-diphenyltetrazolium bromide (MTT) (Cat. No. M6494) was purchased from Thermo Fisher Scientific. Dimethyl sulfoxide (Cat. No. A944.2) was purchased from Carl Roth, Germany. The staining dyes, Flow Cellect Mito Damage Kit (Cat. No. FCCH100106) and Annexin V-PE Apoptosis detection kit (Cat. No. CBA606), were purchased from EMD Millipore, United States.

Formation of the HAMLET complex

The HAMLET complex was formed from human alpha-lactalbumin and oleic acid using the heat-treatment method as described (Kamijima et al. 2008). Human alpha-lactalbumin was dissolved in phosphate-buffered saline and incubated at 50 °C for 15 min, shaking. After 15 min of incubation and shaking, oleic acid was added. The solution was repeatedly incubated at 50 °C for 10 min, shaking. The solution was then cooled to room temperature, and excess oleic acid was removed via centrifugation. After production, the HAMLET complex was stored at − 80 °C.

Cell viability assay

Inhibition of cell growth in response to HAMLET was measured by MTT colorimetric assay. During the assay, HAMLET cytotoxicity was measured for 48 h by seeding cells into a 96 well plate at a density of 8 × 103 to 2 × 104 cells/well

Table 1 Colon cancer cell lines are classified by the mutation status of cancer genes

Colorectal Cell line	KRAS mutation	BRAF mutation
Caco-2	Wild type	Wild type
LoVo	G13D; A14V	Wild type
WiDr	wild type	V600E

Adapted from Ahmed et al. (2013)

(exact concentration was cell line-dependent). The HAMLET complex was added to the cell culture 24 h after plating, and cells were further incubated for 6 h. Subsequently, the growth medium was changed, and cells were incubated further for 18 h, followed by the addition of MTT reagent. The chemical reaction with MTT took place for 3–4 h at 37 °C, and the growth medium was then removed by aspiration. Formed formazan crystals were dissolved in 100 µL dimethyl sulfoxide, and the absorption was measured at 570/620 nm. Colorimetric absorption values were compared to the control group.

Clonogenic assay

Clonogenic assays were performed by seeding 1 × 102 to 2 × 102 cells/well in 24-well plates. After 24 h of plating, the HAMLET complex was added, and cells were incubated for 6 h in the presence or absence of different HAMLET complex concentrations. After 6 h, the culture medium was changed into a fresh culture medium without HAMLET, and the cells were incubated for 8 days. Cells were then fixed with ethanol and stained with crystal violet. The number of colonies (> 50 cells) was counted using an inverted microscope. All values were compared to the control group.

Flow cytometric analysis

Flow cytometric analysis was performed using Guava Personal Cell Analysis Flow Cytometer (Merck, Millipore, Burlington, MA, United States) and CytoSoft 2.1.4 software. The assay was performed by seeding 1 × 105 to 1.3 × 105 cells/well. After 24 h of plating, the HAMLET complex was added, and cells were incubated for 6 h in the absence of different HAMLET complex concentrations. After 6 h, cells were detached using trypsin-EDTA without discarding the floating cells. The culture medium was removed by centrifugation, and cells were suspended in a binding buffer. The cells were stained with annexin V-PE and 7-AAD dyes and measured by flow cytometry.

RNA extraction and real-time polymerase chain reaction (RT-PCR)

Total RNA extraction was performed from cultured cells using the RNA extraction kit (Abbexa) according to the manufacturer's protocol. Purified RNA was quantified and assessed for purity by UV spectrophotometry (NanoDrop). cDNA was generated from 2 µg of RNA with High-Capacity cDNA Reverse Transcription Kit, (Applied Biosystems). The amplification of specific RNA was performed in a 20 µl reaction mixture containing 2 µl of cDNA template, 1X PCR master mix, and the primers. The PCR primers used for the detection of BIRC2 (Hs01112284_m1), BIRC3 (Hs00985031_g1), BIRC5 (Hs00153353_m1), XIAP (Hs00745222_s1), APAF-1 (Hs00559441_m1) and housekeeping gene GAPDH (Hs02758991_g1) were from Applied Biosystems.

Western blot analysis

Lysates from cells were prepared using radioimmunoprecipitation lysis buffer (Abcam, Cambridge, UK) containing protease inhibitors (Roche Diagnostics, Basel, Switzerland). A bicinchoninic acid protein assay kit (Thermo Fisher Scientific, Inc.) was used to determine the protein concentration according to the manufacturer's protocols. Following heating at 97 °C for 5 min, protein samples (50 µg) were subjected to 4–12% SDS-PAGE and transferred to polyvinylidene fluoride membranes at 30 V for 50 min. Membranes were blocked with a blocking buffer (20% diluent A, 30% diluent B; Western Breeze Blocker/Diluent; Invitrogen; Thermo Fisher Scientific, Inc.) at room temperature for 30 min and incubated with the primary antibodies rabbit anti-cas9 (dilution 1:1000; cat. no., PA5-19904; Invitrogen; Thermo Fisher Scientific, Inc) and mouse anti-GAPDH (dilution, 1:3000; cat. no., AM4300; Ambion; Thermo Fisher Scientific, Inc.) at 4 °C overnight. The following day, the blots were incubated with ready-to-use secondary antibodies against rabbit (cat. no. WP20007; Invitrogen, Thermo Fisher Scientific, Inc.) or mouse immunoglobulin G (cat. no. WP20006; Invitrogen; Thermo Fisher Scientific, Inc.) for 30 min at room temperature. Chemiluminescence substrate (CDP-Star; Invitrogen; Thermo Fisher Scientific, Inc.) was added, and the ChemiDoc imaging system (Bio-Rad Laboratories, Inc.) was used for visualization. ImageJ software (version 1.48; National Institutes of Health, Bethesda, MD, USA) was used for quantification of western blots.

Measurement of mitochondrial function in cancer cells

Mitochondrial respiration (oxygen consumption) rate was recorded by high-resolution respirometry system Oxygraph-2k (OROBOROS Instruments, Innsbruck, Austria) at 37 °C in the medium containing 0.5 mM EGTA, 3 mM MgCl2, 60 mM K-lactobionate, 20 mM Taurine, 10 mM KH2PO4, 20 mM HEPES, 110 mM sucrose (pH 7.1 at 37 °C). WE investigated mitochondrial functions according to a multiple substrate–inhibitor titration (Fig. 1). Digitonin (16 µg/ml) was added in order to permeabilize the cell membrane. Mitochondrial non-phosphorylating state State 2 (V_0) respiration rate was recorded in the medium supplemented with cells and mitochondrial Complex I substrate (5 mM glutamate +2 mM malate). The state 3 respiration rate (V_{ADP}) was determined after adding 1 mM ADP. Complex II substrate succinate (12 mM) was used to achieve maximal mitochondrial

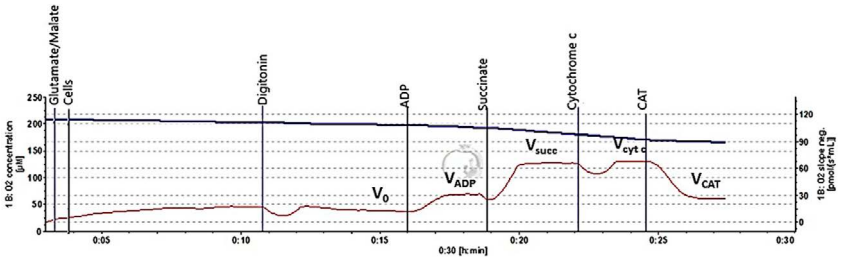

Fig. 1 Typical trace of colon cancer cells mitochondrial respiration. Mitochondrial non-phosphorylating state 2 (V_0) respiration rate was recorded in the medium supplemented with colorectal cancer cells (1 mln cells/2 ml) and mitochondrial Complex I substrate (5 mM glutamate + 2 mM malate). Digitonin (16 µg/ml) was added in order to permeabilize cell membrane. The state 3 respiration rate (V_{ADP}) was determined following the addition of 1 mM ADP. Complex II substrate succinate (12 mM) was used to achieve maximal mitochondrial respiration (Vsucc). The effect of cytochrome c on respiration rate (indicating mitochondrial outer membrane permeability) was determined by adding 32 µM cytochrome c. Carboxyatractyloside (1 µM), an inhibitor of ADP/ATP translocator (V_{CAT}), was added to evaluate the permeability of mitochondrial inner membrane

respiration (V_{succ}). The effect of cytochrome c on respiration rate (indicating mitochondrial outer membrane permeability) was determined by adding 32 µM cytochrome c. The respiratory control index (RCI) for glutamate/malate was calculated as the ratio between V_{ADP}/V_0 respiration rate. Datlab 5 software (Oroboros Instruments) was used for real-time data acquisition and data analysis. Oxygen consumption was related to cell number (pmol/s/1 mln cells).

Statistical analysis

GraphPad Prism 6 and SigmaPlot software were used for statistical analysis. The Mann–Whitney test was used for non-parametric data. Association between qualitative values in comparative groups was assessed by the $\chi 2$ test and interval and categorical by the Student's *t* test. The level of significance was set at 0.05.

Results

Cell viability suppression caused by the HAMLET complex

Initially, we evaluated the effect of the HAMLET complex on the viability of CRC cell lines with different mutational statuses. We examined cell metabolism response to 2 µM, 5 µM, 10 µM and 20 µM concentrations of the HAMLET complex. All concentrations of the HAMLET complex affected all cell lines in a dose–response relationship (Fig. 2). The lowest tested concentration of HAMLET (2 µM) had a minimal effect on cell viability. Caco-2 [KRAS/BRAF wild-type (wt)] and LoVo [KRAS mutant (mt), BRAF wt] viability increased significantly to 109% and WiDr (KRAS wt, BRAF mt) had no significant change in viability. When the HAMLET complex concentration was increased to 5 µM, the viability of all cell lines decreased. However, the suppression of viability was significant only in WiDr (85%) cell line. Cells treated with 10 µM of HAMLET complex significantly decreased cell viability. When comparing the effect of 10 µM HAMLET to control (100%), Caco-2 viability was reduced to 64%, LoVo to 60%, WiDr to 61%. At the highest concentration (20 µM) of the HAMLET complex a difference between BRAF mutant and KRAS mt or WT cells was observed—WiDr cell line was more resistant to the effects of 20 µM HAMLET complex (12-12 % viability for Caco-2 and LoVo compared to 22 % viability for WiDr cell line).

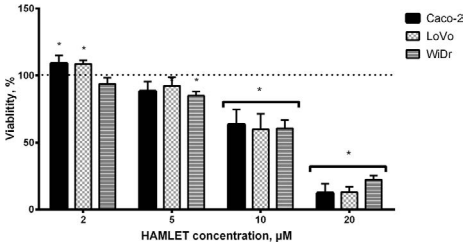

Fig. 2 The MTT assay performed 24 h after a 6 h incubation revealed a dose-dependent response and higher WiDr cell line resistance to the 20 µM human alpha-lactalbumin made lethal to tumor cells complex. *$p < 0.05$: Compared to control group data (100%, dotted line). HAMLET: Human alpha-lactalbumin made lethal to tumor cells

HAMLET complex effect on prolonged cell survival by clonogenic assay

In addition to suppressing cell viability, the HAMLET complex also significantly affected colony formation (Fig. 3). The pattern of results obtained by the clonogenic assay was comparable to those obtained by MTT (Fig. 2). Similarly, BRAF mutation seemed to impact the HAMLET complex response since the WiDr cell line was the most resistant to the 20 µM HAMLET complex.

HAMLET-induced apoptosis/necrosis signal analysis by flow cytometry

The results of the MTT assay and colony formation test were confirmed by flow cytometry (Fig. 4), which showed similar tendencies of rejecting our hypothesis with HAMLET and mutational status relationship. For clarity, we present the flow cytometry outcomes of only three cell lines corresponding to a different type of mutation: Caco-2 [KRAS/BRAF wild-type (wt)], LoVo [KRAS mutant (mt), BRAF wt] and WiDr [KRAS wt, BRAF mt].

The numbers of cells undergoing necrosis or apoptosis after treatment with the 10 and 20 µM HAMLET complex were evaluated by flow cytometry. The assay showed a meager increase or even a decrease in apoptotic cell population when comparing untreated samples with samples treated with 10 or 20 µM HAMLET complex (Fig. 3A). After treatment with 10 µM HAMLET complex Caco-2 cell population increased 1.15 times, LoVo decreased 0.84 times and WiDr—0.91 when compared to control samples. Treating with 20 µM, Caco-2 apoptotic cell population increased 1.9 times, LoVo decreased 0.66 times and WiDr increased 1.25 times when compared to control. However, after the exposure of 20 µM, the increase in the necrotic cell population (Fig. 3B) was much more prominent than the apoptotic

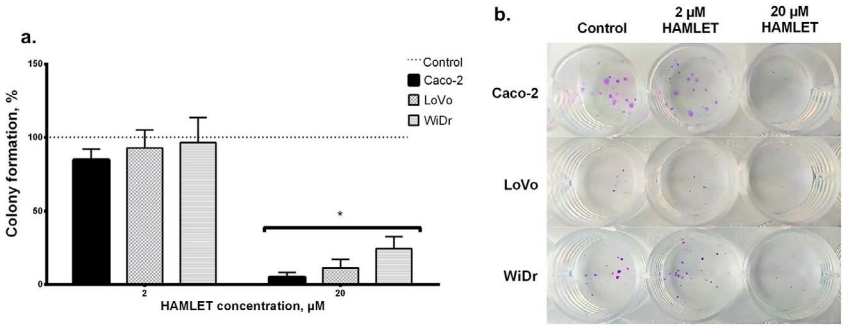

Fig. 3 The effect of HAMLET on colony formation in different colorectal cancer cell lines. **A** Clonogenic assay performed 8 days after incubation with the HAMLET complex revealed that WiDr cell line was the most resistant to 20 µM HAMLET; **B** Representative pictures of the colony formation assay. *< 0.05: compared to control group data (100%, dotted line)

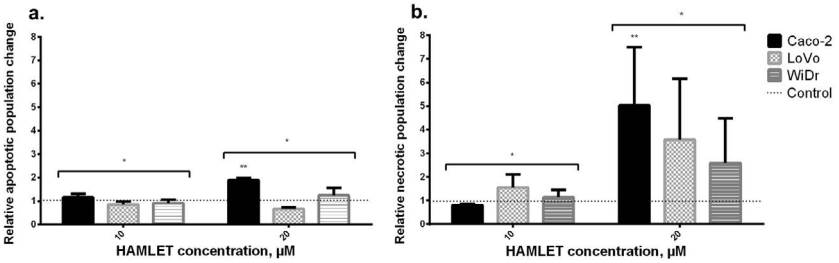

Fig. 4 Flow cytometric analysis of HAMLET effect to different cell lines. **A** A low increase in apoptotic cell population and slight differences between cell lines; **B** a high increase in necrotic cell population in all cell lines. *$p \leq 0.05$ when comparing apoptosis and necrosis of the same sample. **$p < 0.05$ when comparing between 10 µM and 20 µM. Control group data = 1—dotted line

population. After treatment with 10 µM HAMLET complex Caco-2 necrotic cell population decreased 0.8 times, LoVo increased 1.55 times and WiDr—1.13 times when compared to control samples. Treating with 20 µM, Caco-2 apoptotic cell population increased 5 times, LoVo—3.59 times and WiDr—2.69 times when compared to control. Yet again, WiDr cell line was the most resistant and had the lowest increase in a necrotic cell population. The results indicate that the HAMLET complex mainly causes necrotic death in colorectal cancer cell lines.

To summarize the results in the figures (Figs. 2, 3, 4), there was no correlation between the HAMLET-induced cell death level and KRAS/BRAF mutations. However, the WiDr cell line differs from the other lines as being more resistant in terms of cell metabolism, increased necrotic cell population and colony formation.

RT-PCR and WB analysis

To clarify the flow cytometry results, RT-PCR and WB analysis of apoptosis-related markers were performed. HAMLET complex did not affect BIRC2 or BIRC5 expression levels (data not demonstrated). There was also no noticeable increase in investigated gene expressions after treating cells with 2 µM HAMLET complex. When treating cells with 10 µM HAMLET complex Caco-2 cell line and LoVo cell line had a slight, although statistically insignificant, increase of pro-apoptotic APAF-1 gene expression (Caco-2—1,37 times; LoVo—1,16 times) and a high, statistically significant increase in anti-apoptotic BIRC3 (Caco-2—2,71 times; LoVo 4,86 times) and XIAP (Caco-2—2,43 times (statistically insignificant); LoVo—1,11 times (Statistically significant)) genes suggesting that apoptosis was being suppressed after HAMLET treatment (Fig. 5A). None of the apoptosis-related genes had any expression change in the WiDr cell line after treatment with HAMLET. To elucidate the functional response of cells to 10 µM HAMLET treatment, WB analysis of caspase 9 (cas9) protein was performed (Fig. 5B, C). Both Caco-2 and LoVo cell lines had decreased cas9 levels (Caco-2 0.8 times, LoVo—0.65 times). However, there was no change of cas9 in WiDr cell line. The results of WB conform with RT-PCR data demonstrating that increased levels of XIAP inhibited cas9 protein synthesis and in turn inhibited apoptosis of Caco-2 and LoVo cells.

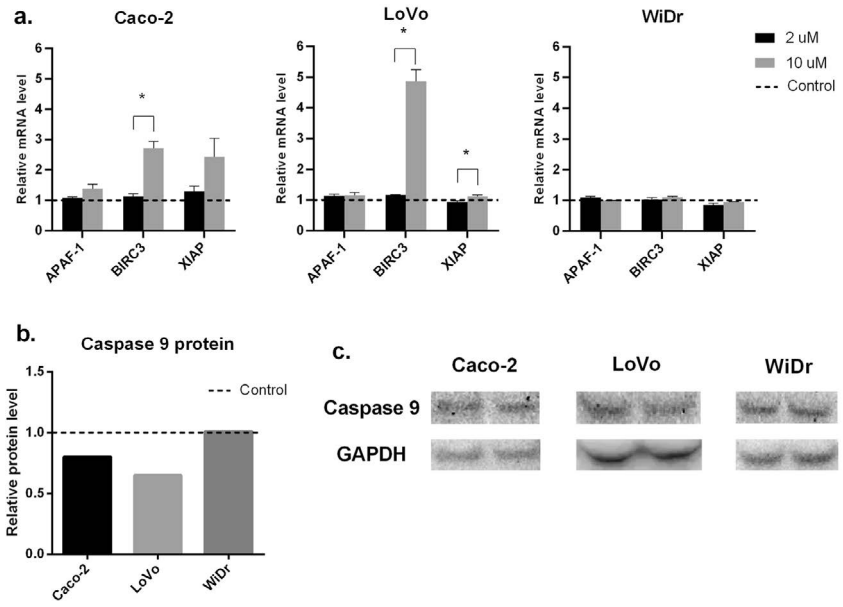

Fig. 5 RT-PCR analysis of APAF-1, BIRC3, XIAP gene and Caspase 9 protein expression after HAMLET treatment of different cell lines. **A** RT-PCR analysis. **B** Western blot analysis of caspase 9 protein expression. **C** Western blot membrane band photo. *$p < 0.05$ when comparing between 2 µM and 10 µM. Control group data = 1—dotted line

The effect of HAMLET on mitochondrial functions in colon cancer cells

We assessed the effect of HAMLET (5 µM) on mitochondrial functions in cancer cells (37 °C) by measuring mitochondrial respiration rate with glutamate/malate and succinate as substrates in three different cell lines (CaCo-2, LoVo and WiDr). HAMLET caused a statistically significant decrease in non-phosphorylating (V_0) respiration rate (substrate glutamate/malate) by 38% in CaCo-2 cell lines but had no effect on non-phosphorylating (V_0) respiration rate in LoVo and WiDr cell lines (Fig. 6A). Furthermore, mitochondrial State 3 (V_{ADP}) respiration rate was reduced by 62%, 46% in CaCo and LoVo cell lines, respectively ($p<0.05$). HAMLET tended to decrease State 3 (V_{ADP}) mitochondrial respiration rate in WiDr cell lines by 47% ($p=0.057$) (Fig. 6B). Moreover, pretreatment with HAMLET caused the decrease in maximal mitochondrial respiration (V_{succ}) with complex II dependent substrate succinate by 62 % and 36 %, respectively, $p<0.05$, in CaCo-2 and LoVo cell lines, (Fig. 6C), and by 47% ($p=0.054$) in WiDr cell lines as compared to untreated cells ($p<0.05$). The addition of cytochrome c to mitochondria (Fig. 6D) showed that pretreatment of cancer cells with HAMLET has no effect on mitochondrial respiration rate V_{cyt}. Therefore, these results suggest that HAMLET did not affect the mitochondrial outer membrane permeability. The respiratory control index (RCI, Fig. 6E) after pretreatment cells with HAMLET decreased by 33 and 19%, respectively, in Caco-2 and LoVo cell lines, as compared with untreated cells ($p<0.05$). However, there

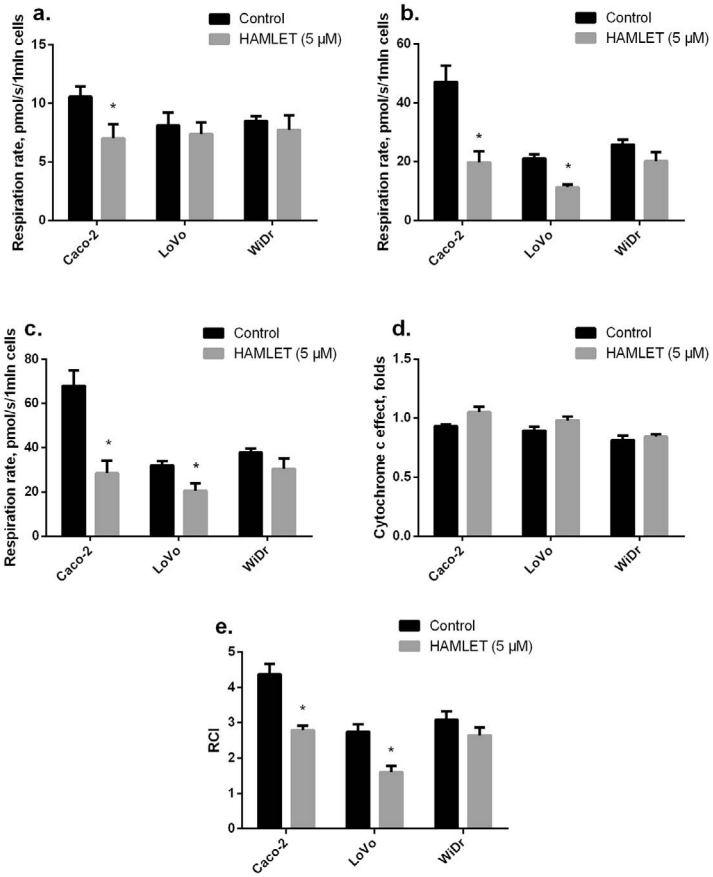

Fig. 6 Effect of HAMLET on mitochondrial respiration and respiratory control index (RCI). Mitochondrial respiration rate was measured as described in "Methods". **a** Mitochondrial non-phosphorylating (V_0) respiration rate in the presence of 1 mln/mL of cells and glutamate (5 mM) plus malate (2 mM); **b** state 3 respiration rate in the presence of ADP (1 mM, VADP); **c** mitochondrial maximal respiration rate in the presence of succinate (12 mM, Vsucc); **d** mitochondrial respiration rate in the presence of cytochrome c (32 µM, Vcyt c); **e** mitochondrial respiratory control index (RCI) (VADP/V0)). *$p<0.05$ as compared to the control group

was no effect on RCI in WiDr cells. Pretreatment of cancer cells with HAMLET did not induce changes in carboxyatractyloside-dependent (V_{CAT}) respiration rate (data not shown). Thus, HAMLET does not affect mitochondrial inner membrane permeability.

Discussion

Treatment of advanced colorectal carcinoma is a clinical challenge for precision oncology due to the variation of molecular profiles, tumor microenvironment, and response to cytotoxic drugs and targeted agents.[6] Despite positive outcomes published from selected trial patients, the effect on survival exceeding the specified study treatment remains uncertain (Modest et al. 2019). The concern is that the present first-line combination of chemotherapy and targeted treatment has little benefit and poor prognosis when applied to BRAF- and KRAS-mutation-bearing patients with mCRC (Li et al. 2020).

The relationship between mutations and susceptibility to treatment helps elucidate personalization trends. Before the study, we reviewed advanced CRC systemic treatment survival compared to the chemotherapy and biological therapy group. First, our hospital results revealed that the median survival of KRAS wild-type patients was statistically significantly longer by 2.5 months than KRAS mutation patients (33.0 months vs. 30.5 months) (Ilekis et al. 2017). These findings coincide with other published studies where median survival varies from 21 to 33 months (Stintzing et al. 2017; Modest et al. 2016). Second, compared to cohort groups, survival was not significantly different between the patients receiving and not receiving monoclonal antibodies. According to the trials, cetuximab significantly improves median overall survival by 3.5 months (Cutsem et al. 2011) and bevacizumab by 2 months (Hurwitz et al. 2013). Regrettably, we did not find any randomized trials with panitumumab. Nonetheless, this compound has a better effect than bevacizumab and is similar to cetuximab (McGregor et al. 2018). Furthermore, researchers from the University of Texas (Loree et al. 2018) suggested monoclonal antibodies as an adjunct to chemotherapy only for mCRC KRAS/BRAF double wild-type left-sided primary malignancies.

The advantage of biologic agents is relatively less profitable than expected, particularly when we consider possible side effects and select a suitable patient for therapy (Mármol et al. 2017). Currently, scientists concentrate on developing new personalized treatment options that are less aggressive and more effective than conventional ones. A novel anticancer drug, HAMLET, offers significant therapeutic potential with low toxicity (Ho et al. 2021). As demonstrated in this study, this complex efficiently suppressed three human colon cancer cells: double wild type; BRAF mutant; and KRAS mutant. Although this study did not confirm the initial hypothesis that KRAS and BRAF genes were associated with HAMLET, we found BRAF-mutant cell line resistance.

Compared to other all-natural mixtures found in food that can act as antitumor drugs, Genovese et al. (2020) reported gercumin. An active blend of curcumins inhibited two human colon cancer cells. At the same time, Fernández et al. (2021) tested five plant flavonoids for their potential as antitumor drugs against the same human CRC cell lines plus T84 (epithelial morphology, adenocarcinoma, metastasis in lung, KRAS mutant, BRAF wild type). Xanthohumol displayed the most significant antiproliferative activity of all flavonoids, even higher than the clinically used chemotherapy drug 5-fluorouracil.

Our flow cytometric analysis reported that HAMLET induced predominantly necrotic cell death. However, this disagrees with the literature data. A group of scientists from Lund University published that HAMLET causes mostly apoptosis-like death in tumor cells (Svanborg C et al. 2003). Presently, the suggested promising strategies for targeting CRC apoptotic pathways are direct activation of the extrinsic pathway by pro-apoptotic receptors, inactivation of BCL-2 proteins, caspase modification, and apoptosis protein inhibition (Abraha et al. 2016). HAMLET was shown to induce apoptosis via the mitochondrial pathway; according to the authors, apoptosis was initiated by releasing cytochrome C, activating caspase-2, -3, -9 and phosphatidylserine exposure (Ho et al. 2017). However, other studies indicated that caspase inhibitors, BCL-2 protein or p53 mutation did not prevent apoptosis, and apoptotic caspase cascade was not the leading cause of cell death (Hallgren et al. 2006; Mossberg et al. 2010; Gustafsson et al. 2009). The research question remains of cell death mechanism.

We investigated some apoptosis-related markers, such as the apoptosis-initiating gene APAF-1 and the apoptosis-inhibiting genes BIRC3 (IAP2), and XIAP, presented in the mechanistic scheme (Fig. 7). Our reverse transcription polymerase chain reaction gene expression analysis data revealed that HAMLET was not associated with the BIRC2 and BIRC5. The presence of non-mutant BRAF cells after HAMLET treatment allows the activation of more anti-apoptotic mechanisms through pathways such as increased BIRC3 and XIAP gene expression, which inhibits apoptosis through extrinsic or intrinsic activation. While pro-apoptotic gene APAF-1 expression has been slightly activated only in wild-type cells. BRAF-mutant WiDr cells do not have overexpression of any of these genes. In addition, we noticed that after HAMLET treatment, the BRAF-mutant WiDr cell line had a smaller number of necrotic cells than other investigated cells.

RT-PCR and flow cytometry findings indicate that there is no apoptotic death pathway, especially regarding WiDr cells. It is possible that the BRAF mutant could have necrotic or

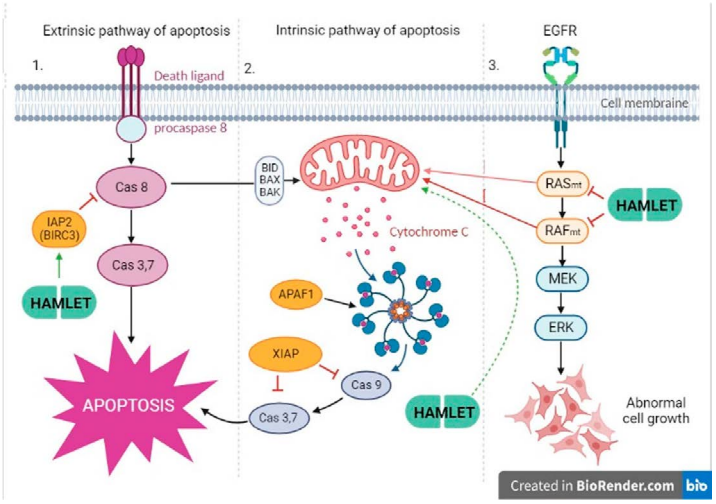

Fig. 7 Mechanism of this study illustrating suppression of apoptosis and HAMLET targets. EGFR and its related proteins are involved in cell signaling pathways that control cell division and survival. EGFR-related RAF and RAS gene mutations cause proteins to produce higher than normal amounts in CRC. HAMLET inhibits oncogenic Ras and Braf activity which causes tumor death (Ho et al. 2017, 2016). KRAS/BRAF mutational status is also implicated in mitochondrial activity of CRC cancer (Rebane-Klemm et al. 2020). The relationship between HAMLET, RAS/RAF gene mutations and mitochondrial phenotype suggests that HAMLET affects cells through mitochondria depending on their activity. HAMLET is also known to activate apoptosis-like death mechanisms via intrinsic pathways (Boekema et al. 2015). However, APAF-1, an apoptotic protease activating factor that activates apoptosis, does not change after HAMLET treatment, and an increase of XIAP, one of the apoptosis-inhibiting proteins, shows that HAMLET does not cause canonical intrinsic pathway apoptosis. Up-regulation of BIRC3 (IAP2) also suggests that HAMLET treatment inhibits apoptosis in the extrinsic pathway as well, again showing that HAMLET causes cell death in a complex, non-apoptotic way which cannot be directly associated with KRAS or BRAF mutation

another death mechanism after HAMLET exposure. We hypothesized that different KRAS/BRAF mutational statuses of colorectal cancer cell lines affect the effectiveness of HAMLET anticancer and mitochondrial activity. Our clonogenicity analysis illustrates that the BRAF-mutant WiDr cell line was resistant to treatment with HAMLET.

Rebane-Klemm et al. (2020) revealed that mitochondrial activity varied between tumors of a similar genetic profile, and this is characteristic of KRAS and BRAF mutated and wild-type tumors. KRAS/BRAF mutational status is also implicated in mitochondrial activity of CRC with KRAS mutants having lower ADP-activated respiration rate than KRAS/BRAF wt and unchanged outer membrane permeability, suggesting an oxidative phenotype. BRAF mutant has an even lower respiration rate and altered outer membrane permeability suggesting glycolytic phenotype. Consequently, different metabolic resources can foresee a response to remedy, which helps with precision therapy.

Therefore, in the future, it would be appropriate to evaluate mitochondrial activity in a couple more BRAF normal/mutant cells after exposure to HAMLET. It could answer more mechanistic questions about the potential resistant tendency of BRAF mutation. In addition, it would be appropriate to analyze other markers related to necrosis or ferroptosis, which may occur due to lipid peroxidation.

A limitation of the study was that we investigated only three CRC cell lines. However, there was no clear link between the HAMLET cytotoxicity level and the bioenergetic profile provided. A more comprehensive analysis is required to detail further the effect of HAMLET on mitochondria and the glycolysis process. First, more CRC cell lines must be screened for the sensitivity of mitochondrial and glycolytic function to HAMLET treatment. Next, if the effects of HAMLET on mitochondria are observed, then the mechanism of action should be uncovered. A study on isolated rat liver mitochondria showed that HAMLET induced mitochondrial permeability transition, potentially leading to mitochondrial dysfunction and apoptosis (Köhler et al. 2001). This suggests possible testing if HAMLET affects permeability transition and related events, such as cytochrome c release. Another suggestion is that HAMLET might target mitochondrial ATP synthase (Boekema et al.

2015); thus, the sensitivity of this enzyme and other key enzyme complexes should also be assessed after HAMLET treatment. In addition to mitochondrial efficiency, cell glycolytic pathway sensitivity to HAMLET is crucial and might define the death/survival decision. HAMLET inhibits the glycolytic enzymes fructose bisphosphate aldolase and glyceraldehyde-3-phosphate dehydrogenase in bacteria (Roche-Hakansson et al. 2019); thus, similar glycolysis suppressing activity could also take place in eukaryotic cells. The precise definition of HAMLET-sensitive and not-sensitive members of mitochondrial and glycolytic energetic pathways will allow the creation of a strategy for patient stratification and identification of additional treatment targets.

To the best of our knowledge, this is the first study evaluating how KRAS/BRAF mutation status affects HAMLET anticancer activity. One of the recently described HAMLET efficiency regulating mechanisms is related to alpha-helical- or beta-sheet domains of alpha-lactalbumin in heat shock proteins, resulting in an immediate death response or a delay due to transient accumulation of the HAMLET complex in lysosomes (Nadeem A et al. 2019). However, this finding provides no direct clues to the relationship between KRAS/BRAF pathway and energetic metabolism. Nevertheless, the complex is actively exploited because of its prominent selective toxicity to cancer cells. A group of scientists from Lund University has recently published the first HAMLET data on a single-center, placebo-controlled, double-blinded randomized phase I/II interventional clinical trial of non-muscle invasive bladder cancer. Researchers concluded that intravesical inoculation of alpha1-oleate was safe and effective in patients with bladder cancer (Brisuda et al. 2021). After this successful trial, the Lund University group shared other trial ideas of having this proteolipid compound in drinking water as prevention. Targeting early, locally growing tumors is essential to reduce tumor progression and metastatic disease risk (Smith 2013). A further research direction would be testing the efficiency of HAMLET on fresh surgically resected human colorectal tumor biopsies ex vivo (Novo et al. 2017) to identify patient responses together with analysis of tumor bioenergetic profiles for a patient stratification strategy.

Conclusions

HAMLET exhibits irreversible cytotoxicity on human CRC cells in a dose-dependent manner, leading to necrotic cell death and inhibiting the extrinsic apoptosis pathway. BRAF-mutant cell line is more resistant than other type lines. HAMLET decreased mitochondrial respiration and ATP synthesis in CaCo-2 and LoVo cell lines but did not affect WiDr cells' respiration. Pretreatment of cancer cells with HAMLET has no impact on mitochondrial outer and inner membrane permeability.

Author contributions Conceptualization: AG, ŽD and AT; methodology: DS, JŽ and JŠ; software: DS, JŠ; validation: RB, ŽD, MC and AJ; formal analysis: DS, JŠ, JŽ; investigation: DS, JŠ, JŽ, ST; resources: SŠ; data curation: DS, JŠ and JŽ; writing—original draft preparation: JŽ, DS, AJ; writing—review and editing: AG, AJ, ŽD and AT; visualization: AJ, JŽ; supervision: RB, AG, MC; project administration: SŠ; funding acquisition: AT, AG. All authors read and approved the final version of the manuscript.

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Data availability The data sets analyzed during the current study are available from the corresponding author on reasonable request.

Declarations

Conflict of interest The authors declare that they have no conflict of interest.

Ethical approval This article does not contain any studies with human participants or animals performed by any of the authors.

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Assessing the Therapeutic Impacts of HAMLET and FOLFOX on BRAF-Mutated Colorectal Cancer: A Study of Cancer Cell Survival and Mitochondrial Dynamics In Vitro and Ex Vivo

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Abstract: *Background and Objectives*: Colorectal cancer (CRC) is a major global health challenge. The BRAF V600E mutation, found in 8–12% of CRC patients, exacerbates this by conferring poor prognosis and resistance to therapy. Our study focuses on the efficacy of the HAMLET complex, a molecular substance derived from human breast milk, on CRC cell lines and ex vivo biopsies harboring this mutation, given its previously observed selective toxicity to cancer cells. *Materials and Methods:* we explored the effects of combining HAMLET with the FOLFOX chemotherapy regimen on CRC cell lines and ex vivo models. Key assessments included cell viability, apoptosis/necrosis induction, and mitochondrial function, aiming to understand the mutation-specific resistance or other cellular response mechanisms. *Results:* HAMLET and FOLFOX alone decreased viability in CRC explants, irrespective of the BRAF mutation status. Notably, their combination yielded a marked decrease in viability, particularly in the BRAF wild-type samples, suggesting a synergistic effect. While HAMLET showed a modest inhibitory effect on mitochondrial respiration across both mutant and wild-type samples, the response varied depending on the mutation status. Significant differences emerged in the responses of the HT-29 and WiDr cell lines to HAMLET, with WiDr cells showing greater resistance, pointing to factors beyond genetic mutations influencing drug responses. A slight synergy between HAMLET and FOLFOX was observed in WiDr cells, independent of the BRAF mutation. The bioenergetic analysis highlighted differences in mitochondrial respiration between HT-29 and WiDr cells, suggesting that bioenergetic profiles could be key in determining cellular responses to HAMLET. *Conclusions:* We highlight the potential of HAMLET and FOLFOX as a combined therapeutic approach in BRAF wild-type CRC, significantly reducing cancer cell viability. The varied responses in CRC cell lines, especially regarding bioenergetic and mitochondrial factors, emphasize the need for a comprehensive approach considering both genetic and metabolic aspects in CRC treatment strategies.

Keywords: colorectal cancer; BRAF mutation; HAMLET; bioactive milk compound; mitochondrial function; ex vivo treatment; precision medicine

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1. Introduction

Colorectal cancer (CRC) stands as the third most prevalent neoplasm, with over 1.9 million cases annually, and is the second most lethal, accounting for nearly a million deaths each year [1,2]. Despite advances in diagnostics, screening, lifestyle awareness, and combined therapeutic regimens that have elevated the 5-year overall survival rate to 65% [3], the 5-year survival rate drastically drops to 13% for patients who either present with distant disease at diagnosis or develop it later [4,5].

A deeper understanding of the biology and heterogeneity of CRC is the next challenge for precision medicine. Researchers intend to categorize CRC based on genetic and molecular markers [6].

A notable molecular variation, the BRAF V600E mutation (mt), prevalent in 8–12% of CRC patients, is linked to a poor prognosis and diminished treatment responsiveness. This mutation, often associated with alterations in the MAPK pathway and the tumor environment, frequently coexists with other molecular markers, thus complicating therapeutic approaches [7,8].

Numerous studies consistently suggest that patients whose tumors carry the BRAF V600E mutation show reduced response rates to EGFR inhibitors, regardless of whether these drugs are administered alone or alongside chemotherapy. This diminished efficacy remains evident even in tumors with a RAS wild-type (wt) status, underlining the therapeutic challenges specific to the BRAF V600E mutation. For those with BRAF V600E mutant mCRC, FOLFOX- and bevacizumab-based chemotherapy is still the preferred initial treatment strategy [9–12].

In the ongoing search for innovative therapeutic agents, particularly for challenging cases of CRC, the HAMLET complex (human alpha-lactalbumin made lethal to tumor cells) has emerged as a potential game-changer. Derived from components of human breast milk, HAMLET has a unique ability to selectively target cancer cells while leaving healthy cells unharmed [13,14]. This is critical in BRAF mutant CRC, which often resists standard therapies [15]. Our previous findings with HAMLET have demonstrated a pronounced different effect on the BRAF mutant cell line than others, revealing its dose-dependent cytotoxic effects on CRC cells, predominantly leading to necrotic death, and altered mitochondrial functions in specific cell lines [16].

A breakthrough approach in CRC research involves ex vivo patient biopsies, where tumor tissues are cultivated outside the body to assess treatment responses. This method offers a window into individual tumor drug sensitivity and emphasizes the heterogeneity intrinsic to CRC tumors [17]. However, maintaining intrinsic tumor characteristics in an ex vivo environment presents significant challenges. With the increasing emphasis on precision medicine, it is imperative to comprehend the advantages and disadvantages of ex vivo patient biopsies in treating CRC [18,19].

In light of our initial observations, we aimed to evaluate the effects of combining HAMLET with the standard chemotherapy FOLFOX on BRAF mutant CRC cell lines and ex vivo explant models to assess whether the response to HAMLET is driven by the BRAF mutation or by mutation-independent mechanisms such as mitochondrial function.

2. Materials and Methods

2.1. Patient Cohort and Explant Formation of Human Colorectal Cancer Biopsy

We included adult patients diagnosed with colorectal cancer who underwent surgery at the Hospital of the Lithuanian University of Health Sciences Kaunas Clinics between 2021 and 2022. Each patient provided informed consent, and this research received full ethical approval from the Kaunas Regional Biomedical Research Ethics Committee (approval number BE-2-64, dated 1 August 2019). All procedures performed in this study complied with the relevant regulations following the Declaration of Helsinki.

During the specified time period, 754 colorectal surgeries were performed at our institution, of which a selected group of 32 cases met the specific inclusion criteria of this study. The strict parameters of our selection process required us to exclude surgeries performed

from Wednesday to Friday to coincide with the timing of ex vivo experimental evaluations, as well as emergency surgeries, cases of recurrent cancer, and cases involving neo-adjuvant therapy, diverticulosis, or inflammatory bowel disease (IBD). Our methodology was further refined by omitting cases from the initial pilot study, accounting for patients who declined to participate, and adjusting for a pause in this study due to the impact of the COVID-19 pandemic on reagent availability. The study design is detailed in Chart 1.

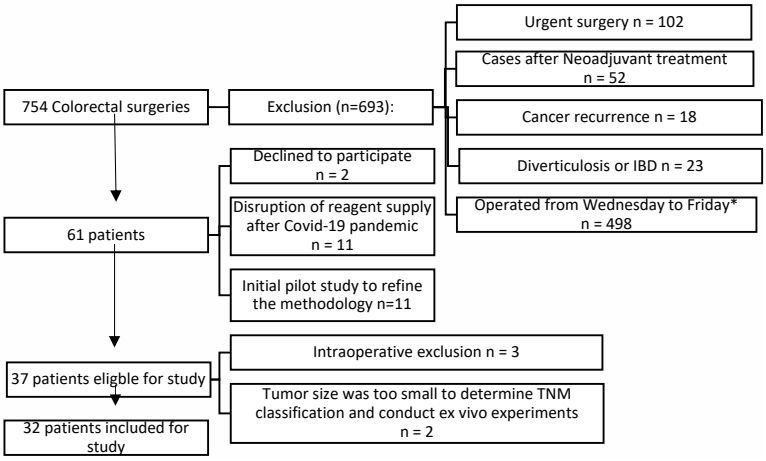

Chart 1. Overall flow chart design of colorectal surgeries in 2021 and 2022 and inclusion for study. IBD—inflammatory bowel disease. * We only included patients operated on Monday or Tuesday due to methodology.

After colorectal cancer resection surgery, pathologists evaluated the excised section of the large bowel. After assessing the tissue and determining the localization of the tumor, a piece of the tumor was cut and placed in a cold cell culture medium (minimal essential medium (MEM)) with 10% fetal bovine serum (FBS) and antibiotics/with a mixture of antimycotics. A piece of tissue was brought to a vertical flow laminar, and, furthermore, all manipulations were performed under sterile conditions with sterile tools and reagents.

Since the large intestine contains various intestinal bacteria and the excised part of the intestine was evaluated under non-sterile conditions, the tissue inevitably became infected with various bacteria and fungi. To stop the growth of bacteria or fungi but not harm the cells, the nutrient medium was supplemented with commonly used and non-toxic doses of antibiotics: penicillin/streptomycin and amphotericin B solution, metronidazole, cefuroxime, and gentamicin, as well as 10% FBS.

The piece of tissue was washed three times with a nutrient medium to remove as many microorganisms and blood cells as possible. After washing, the tissue was cut with 2 mm diameter biopsy needles, trying to avoid tearing the tissue. Colon cancer pieces of a uniform size of approximately 2 mm^3 were obtained. Each piece was individually placed in a well of a 96-well plate with a nutrient medium and incubated for 24 h. The pieces were incubated in an incubator that maintained a temperature of 37 °C, 95–98% humidity, and a 5% CO_2-saturated environment (Figure 1).

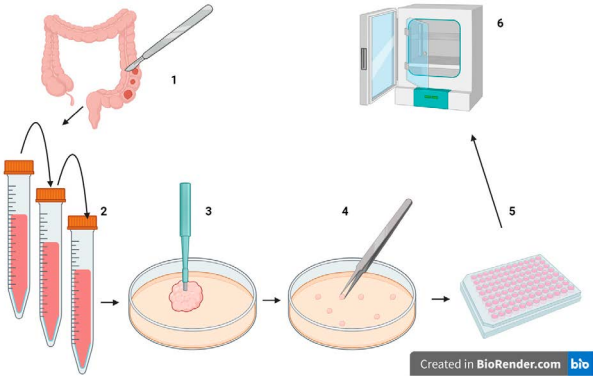

Figure 1. Establishing the explant model: 1—a piece of tumor and healthy tissue is collected after operation and examination; 2—the pieces are washed three times under sterile conditions; 3—the pieces are cut into 2 mm pieces with a biopsy needle, avoiding shredding as much as possible; 4—we are left with 2 mm^3 explants; 5—every single explant is placed into a well of 96-well plate; and 6—incubation at 37 °C, 95—98% humidity, and 5% CO_2 atmosphere.

2.2. Mutation Analysis—RT PCR

Genomic DNA was extracted from CRC tissue using a DNeasy Blood and Tissue kit (Qiagen, Hilden, Germany). Purified DNA was quantified and assessed for purity using UV spectrophotometry. For BRAF V600E mutation detection, we used predesigned TaqMan assays (Life Technologies, Carlsbad, CA, USA) (rs113488022) with a TaqMan Universal PCR Master Mix, No UNG (Life Technologies, Carlsbad, CA, USA) and an approximately 20 ng per well DNA sample. Amplification was performed using the ABI 7500 fast Real-Time PCR system. Genotype assignments were manually confirmed via visual inspection with the ABI 2.3 software compatible with the TaqMan® system. After initial genotyping, 25% of all samples in each group were included in a repetitive analysis, which showed a 100% concordance rate.

2.3. Cell Cultures

We purchased the WiDr (colorectal adenocarcinoma) cell line from CLS Cell Lines Service in Germany and the HT-29 (colorectal adenocarcinoma) cell line from the American Type Culture Collection (ATCC) in the United States. The HT-29 cell line was cultured in McCoy's 5A medium, supplemented with 10% fetal bovine serum and 1% penicillin–streptomycin, all obtained from GIBCO. The WiDr cell line was maintained in a 1:1 mixture of Ham's F-12K (Kaighn's) medium and Dulbecco's Modified Eagle Medium, enriched with 5% fetal bovine serum and 1% penicillin–streptomycin. Both cell lines were incubated at 37 °C in an atmosphere containing 5% CO_2.

2.4. Formation of the HAMLET Complex

We prepared the HAMLET complex by combining human alpha-lactalbumin with oleic acid (obtained from Sigma-Aldrich in Steinheim, Germany), following the heat treatment method outlined in the literature [20]. We first dissolved human alpha-lactalbumin in phosphate-buffered saline and then shook the solution at 50 °C for 15 min. Subsequently, we added oleic acid to the mixture and continued the incubation with shaking for another 10 min at the same temperature. We allowed the solution to cool down to room temperature before centrifuging to remove any excess oleic acid. Finally, we stored the HAMLET complex at −80 °C for future use.

2.5. Explant Treatment with HAMLET

After 24 h of incubation, 60 μM of HAMLET was added into the appropriate wells, and the plate was incubated for 24 h. The dose was chosen according to previous experiments with explants, which showed a statistically significant effect only when treated with 60 μM of HAMLET for 24 h. Subsequently, the medium was changed to a medium supplemented with 10% resazurin. The explant metabolizes the purple-colored compound, resazurin, into a pink-colored compound, resorufin, which can be measured with a spectrometer using 570 nm and 620 nm filters. Since resazurin is a non-toxic compound, the measurements were made 24 and 48 h after HAMLET treatment.

Alternatively, after a 24 h treatment with HAMLET, the explant samples were collected, and the oxygen consumption and extracellular acidification rates were measured.

2.6. Mitochondrial Respiration

We studied the mitochondrial respiration, specifically, oxygen consumption, of ex vivo tissue samples and two colon cancer cell lines harboring BRAF mutations (HT-29 and WiDr) using the high-resolution respirometry system Oxygraph-2k (OROBOROS Instruments, Innsbruck, Austria) at 37 °C. The medium for the measurements contained 0.5 mM of EGTA, 3 mM of $MgCl_2$, 60 mM of K-lactobionate, 20 mM of taurine, 10 mM of KH_2PO_4, 20 mM of HEPES, and 110 mM of sucrose, adjusted to pH 7.1 at 37 °C. To permeabilize the cell membranes, 16 μg/mL of digitonin was added. We documented the non-phosphorylating state 2 (V0) respiration rate in the medium containing the tissue samples, mitochondrial complex I substrates (5 mM of glutamate + 2 mM of malate), and the complex II substrate succinate (12 mM). After adding 1 mM of ADP, we assessed the state 3 respiration rate (VADP). All respiration rates were normalized to the dry weight of the tissue in milligrams.

2.7. Cell Viability Flow Cytometric Analysis

We used the MTT colorimetric assay (obtained from Thermo Fisher Scientific and Carl Roth in Steinheim, Germany and Karlsruhe, Germany) to assess cell viability and the cytotoxic effects of HAMLET over 48 h. We initially seeded cells into 96-well plates at densities appropriate for each cell line, ranging from 8000 to 20,000 cells per well. After 24 h, we treated the cultures with the HAMLET complex and incubated them for another 6 h. We then replaced the growth medium and allowed the cells to incubate for another 18 h before adding the MTT reagent. After incubation for 3–4 h at 37 °C, we dissolved the formazan crystals in dimethyl sulfoxide, measured the absorbance at 570/620 nm, and compared it with a control group.

2.8. Clonogenic Assay

For the clonogenic assay, we plated cells at a density of 100 to 200 cells per well in 24-well plates. After allowing 24 h for attachment, we administered various concentrations of the HAMLET complex for 6 h. Then, we changed the medium and incubated the cells for 8 days. After incubation, we fixed and stained the colonies with crystal violet and counted those with more than 50 cells using an inverted microscope and compared the results with controls.

2.9. Flow Cytometric Analysis

We used the Flow Cellect Mito Damage Kit and Annexin V-PE Apoptosis Detection Kit obtained from EMD Millipore in the United States. To perform the flow cytometric analysis and understand how cells responded to HAMLET, we seeded 100,000 to 130,000 cells per well. After one day, we treated these cells with different concentrations of HAMLET for 6 h. We then detached the cells, stained them with Annexin V-PE and 7-AAD, and analyzed them using the Guava Personal Cell Analysis Flow Cytometer and CytoSoft software (version 2.1.4; Guava; EMD Millipore, Burlington, MA, USA).

2.10. Drug Combination Effect Calculation

The combination effect of the HAMLET complex and FOLFOX (5-fluorouracil (5-FU) + oxaliplatin) was measured via MTT assay and calculated using the Combenefit (v2.021) software [21]. Bliss theory was chosen for the calculation theory based on the assumption that both drugs work independently but can increase each other's cytotoxic effects [22]. Varying doses of HAMLET (1, 3, and 5 μM) and FOLFOX (5-FU (μM) + oxaliplatin (μM): 3.125 + 0.078; 6.25 + 0.156; 12.5 + 0.3125; 25 + 0.625; 50 + 1.25; 100 + 2.5; 200 + 5; 400 + 10; and 800 + 20) were used.

2.11. Glycolytic and Mitochondrial Activity Determination

The mitochondrial and glycolytic activity of WiDr and HT-29 cells was measured with a Seahorse XFp Analyzer (Agilent Technologies, Santa Clara, CA, USA) using the Seahorse XFp Cell Mito Stress Test Kit (Agilent Technologies) according to the manufacturer's instructions. All the assay and data interpretation details are available in the User Guide [23]. Briefly, the cells were seeded into Agilent Seahorse XFp miniplates at a density of 1.5×10^3 to 3×10^3 cells/well and kept in the cell culture medium indicated above. The cells were incubated for 4 d until 50–80% confluency was reached. One hour before the measurement, the medium was replaced with Seahorse XF Assay Medium supplemented with 2 mmol/L of L-glutamine, 1 mmol/L of sodium pyruvate, and 10 mmol/L of glucose, and the cells were placed in a non-CO_2 incubator. Just before the measurement, the medium was changed again to fresh Assay Medium with the same supplements. The final inhibitor concentrations in the wells were 1.5 μM oligomycin, 1 μM carbonyl cyanide-4-phenylhydrazone, 0.5 μM antimycin A, and 0.5 μM rotenone. The oxygen consumption rate (OCR) and extracellular acidification rate were normalized to the total cellular protein content determined directly in the plate using the Bradford assay. The data were analyzed using Wave software 2.6.1 (Agilent Technologies), and graphical images were created employing SigmaPlot vs. 13 (Systat Software, Slough, UK).

2.12. Statistical Analysis

We carried out our statistical analyses using GraphPad Prism 6 with SigmaPlot. To analyze non-parametric datasets, we applied the Mann–Whitney *U* test. We examined the correlations between qualitative measures in comparative cohorts using the chi-square (χ^2) test, while Student's *t*-test was employed for interval and categorical data assessments. The significance level was set at $p < 0.05$.

3. Results

3.1. Colorectal Cancer Patients' Explant Characteristics

We analyzed 32 patients with colorectal carcinoma (mostly moderately differentiated, G2 (84.4%)). The mean age of CRC patients was 68.06 ± 11.95, with a gender distribution of 21 females and 11 males. BRAF mutations were identified in four patients, accounting for 12.5% of the cohort. The serum markers CEA and CA 19-9 were predominantly below normal limits in 65.6% and 87.5% of patients, respectively. The primary site of cancer was predominantly the rectum, with six patients undergoing neoadjuvant treatment. A detailed analysis of the TNM staging, postoperative Clavien–Dindo complications, and patient follow-up data are outlined in Table 1.

In our Kaplan–Meier survival analysis (Figure 2), we observed that patients with BRAF wt mutations had a longer median survival of 32.68 ± 1.58 months (95% CI: 29.58–35.78) compared with 18.5 ± 4.76 months (95% CI: 9.16–27.84) in patients with BRAF mutant types. However, the difference in the survival curves was not statistically significant ($p = 0.247$). Notably, follow-up was longer in the BRAF wt group (22.29 ± 10.53 months; range: 1–35) than in the BRAF mt group (14.42 ± 7 months; range: 2–24).

Table 1. Colorectal cancer patient clinicopathological characteristics. CEA—carcinoembryonic antigen; CA19-9—carbohydrate antigen 19-9, and APR—abdominoperineal resection.

Patient Demographics	**N (%)**
Gender	Female, 21 (65.6%) Male, 11 (34.4%)
Age at diagnosis	<60 years, 5 (15.6%) ≥60 years, 27 (84.4%)
BRAF mutation status	Wild type, 28 (87.5%) BRAF mutant, 4 (12.5%)
CEA	Normal <5.8, 21 (65.6%) Elevated ≥5.8, 11 (34.4%)
Ca 19-9	Normal <37, 28 (87.5%) Elevated ≥37, 4 (12.5%)
Localization	Right colon, 8 (25.0%) Left colon, 8 (25.0%) Rectum, 16 (50.0%)
Surgery type	Right hemicolectomy, 9 (28.1%) Left hemicolectomy, 2 (6.2%) Sigmoid resection, 6 (18.8%) Rectal resection, 11 (34.4%) APR, 4 (12.5%)
Tumor differentiation	Well-differentiated G1, 3 (9.4%) Mod. differentiated G2, 27 (84.4%) Poorly differentiated G3, 2 (6.2%)
TNM stage	Stage I, 7 (21.9%) Stage II, 15 (46.9%) Stage III, 7 (21.9%) Stage IV, 3 (9.4%)
pT stage	pT1, 1 (3.1%) pT2, 9 (28.2%) pT3, 21 (65.6%) pT4, 1 (3.1%)
pN Stage	pN0, 20 (62.5%) pN1, 10 (31.3%) pN2, 2 (6.2%)
V—vascular invasion	(−) 24 (75.0%) (+) 8 (25.0%)
L—invasion into lymphatic vessels	(−) 25 (78.1%) (+) 7 (21.9%)
Clavien–Dindo postoperative complications at 30-day follow-up	Grade 0 (no complications), 23 (71.9%) Grade I, 1 (3.1%) Grade II, 3 (9.4%) Grade IIIA, none Grade IIIB, 4 (12.5%) Grade IV, none Grade V, 1 (3.1%)
Median postoperative hospital stay	7 days (range: 3–27 days)
Follow-up months	21.06 mo ± 10.73 (min. 1–max. 35)

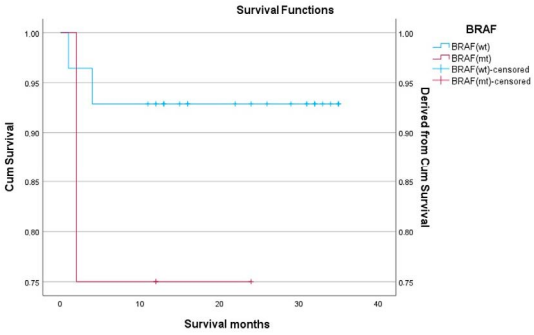

Figure 2. Kaplan–Meier analysis by month, stratified by mutation status.

3.2. Colorectal Cancer Explant Viability

In our study, we observed that both HAMLET and FOLFOX, when used alone, exerted similar cytotoxic effects on CRC explants, regardless of the BRAF mutation status. Specifically, HAMLET reduced the viability of both wild-type and mutant BRAF explants to approximately 87% at 24 h and 86% wt and 84% mt at 48 h. Similarly, FOLFOX treatment reduced the viability of the BRAF wt explants to 85% at 24 h and 79% at 48 h, while the mt explants showed a comparable reduction to 78% at 48 h.

However, the combination of HAMLET and FOLFOX showed a more pronounced effect on the BRAF wt group. This combined treatment reduced the viability of BRAF wt explants to 80% at 24 h and to 69% at 48 h, indicating a synergistic effect. In contrast, the same combination did not significantly alter the viability of BRAF mt explants (Figure 3).

3.3. Explant Mitochondrial Respiration

In our investigation of mitochondrial respiration in human CRC tissue explants, we evaluated the impact of HAMLET (60 µM) on mitochondrial function. Our assays focused on measuring the mitochondrial respiration rate at 37 °C, utilizing glutamate/malate (Complex I) and succinate (Complex II) as substrates. We observed that HAMLET tended to inhibit mitochondrial respiration in both BRAF mutant and wild-type CRC tissue samples, though this inhibition was not statistically significant ($p > 0.05$).

Specifically, in CRC samples without the BRAF mutation, HAMLET treatment led to a 39% reduction in the non-phosphorylating (V0) respiration rate (from 12 ± 2.646 to 7.3 ± 3.215 pmolO/s/mg dry weight) and a 33.3% decrease in the state 3 (VADP) respiration rate (from 13 ± 1 to 8.67 ± 3.786 pmolO/s/mg dry weight). In contrast, tissue samples with the BRAF mutation exhibited a more substantial impact, with a 71% reduction in the V0 respiration rate (from 12 ± 4.243 to 3.5 ± 0.7 pmolO/s/mg dry weight) and a 60% decrease in the VADP respiration rate (from 12.5 ± 3.54 to 5 ± 1.41 pmolO/s/mg dry weight). These findings suggest a differential response in mitochondrial respiration inhibition by HAMLET between BRAF mt and wt CRC samples, with a greater extent of inhibition observed in the BRAF mt samples (Figure 4b).

3.4. Comprehensive Analysis of HAMLET Complex Effects on CRC BRAF Mutant Cells: Viability, Clonogenic Survival, and Induced Apoptosis/Necrosis

We then investigated why the BRAF mutant has distinctive features in viability and respiration studies. Therefore, we chose two genetically identical colon cancer cell lines with the BRAF mutation.

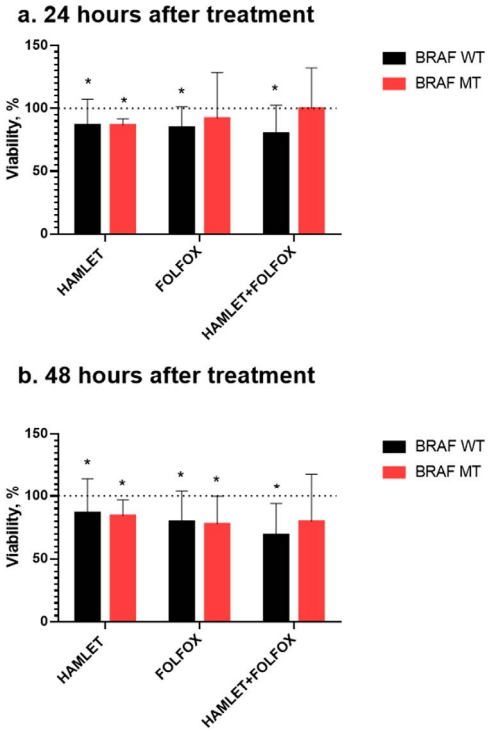

Figure 3. The effect of 60 μM of HAMLET and/or FOLFOX (3 mM of 5-FU + 75 μM of oxaliplatin) on CRC explant viability. (**a**) Explant viability 24 h after treatment; (**b**) explant viability 48 h after treatment. Comparing BRAF wild-type and mutant explants. Means ± SD. N ≥ 3. Dotted line—control group data (100%). *—$p < 0.05$ comparing with control group data.

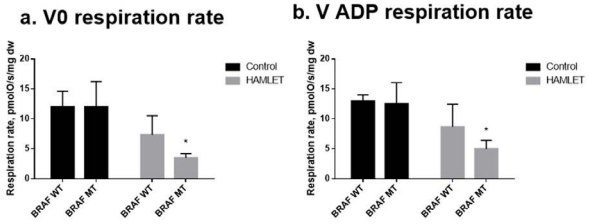

Figure 4. Comparison of the impacts of HAMLET and control on mitochondrial functions between BRAF wild type and BRAF mutant: (**a**) mitochondrial non-phosphorylating (V0) respiration rate and (**b**) mitochondrial state 3 (VADP) respiration rate are means ± SD. *—$p < 0.05$ comparing non-treated control and HAMLET-treated samples.

In our evaluation of the effects of the HAMLET complex on different CRC cell lines (HT-29 and WiDr (a derivative of HT-29)) with V600E BRAF mutation status, we found consistent dose-dependent effects on viability at 2 μM, 5 μM, 10 μM, and 20 μM concentrations. At 2 μM, the viability of the WiDr cell line remained largely unaffected, while the HT-29 line experienced an 11% reduction in viability to 89%. As the concentration increased, the differences in resistance between the two lines became more pronounced: At 5 μM, WiDr retained 85% viability compared with 43% for HT-29. At higher concentrations of 10 and 20 μM, WiDr's viability dropped to 61% and 22%, respectively, while HT-29's viability dropped to 8% and 2% (Figure 5a).

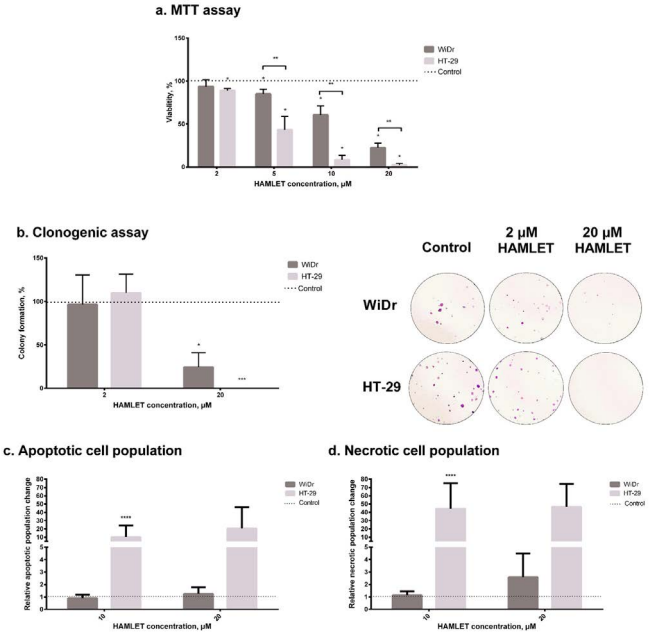

Figure 5. Dose-dependent responses of CRC cell lines to HAMLET complex. (**a**) The MTT assay performed 24 h after a 6 h incubation revealed a dose-dependent response and higher WiDr cell line resistance to HAMLET than HT-29. (**b**) The effect of HAMLET on colony formation in different BRAF mutant CRC cell lines. Graphic representation and representative photos. (**c**) Apoptotic cell line population after the effect of HAMLET. (**d**) Necrotic cell line population after the effect of HAMLET. Control group data—dotted line. * $p < 0.05$—compared with control group data. ** $p < 0.05$—comparing HAMLET's effects on WiDr and HT-29. ***—no colony formation. **** $p \leq 0.05$ when comparing apoptosis and necrosis of the same sample. Means ± SD. N ≥ 3.

Further analysis, focusing on clonogenic potential, showed that while 2 μM of the HAMLET complex showed no discernible reduction in colony formation for either cell line, a drastic effect was observed at 20 μM. Herein, the colony formation of the WiDr line dropped to 24%, while the HT-29 line showed a complete loss of its ability to form colonies (Figure 5b).

Flow cytometry confirmed these findings (Figure 5c,d), with a marked shift toward apoptosis and necrosis after HAMLET treatments. The apoptotic cell population of WiDr decreased by 10% at 10 μM and increased 1.25-fold at 20 μM, whereas HT-29 showed a dramatic increase: 10-fold and 20-fold at 10 μM and 20 μM, respectively. The shift toward necrosis was even more pronounced, especially for HT-29, which showed staggering 44-fold and 47-fold increases at these concentrations.

However, in all these assays, WiDr consistently showed greater resistance. Taking these observations together (Figure 5a–d), it is clear that WiDr's resistance to HAMLET far exceeds that of the HT-29 line despite having identical mutations. Such results indicate that the response to the HAMLET complex may be controlled by factors beyond mere genomic or mutational characteristics, prompting us to investigate the different energetics of these cell lines.

3.5. Bliss Synergy Model Calculation

Opposite to their responses to HAMLET, both cell lines showed similar tendencies when treated with FOLFOX. They responded to FOLFOX in a dose-dependent manner (Figure 6a). When calculating the IC50 doses of both cell lines, there was a minimal difference between them, with WiDr's IC50 dose being 15.1 + 0.378 μM and HT-29's being 14.5 + 0.363 μM (5-FU + oxaliplatin) (Figure 6a,b).

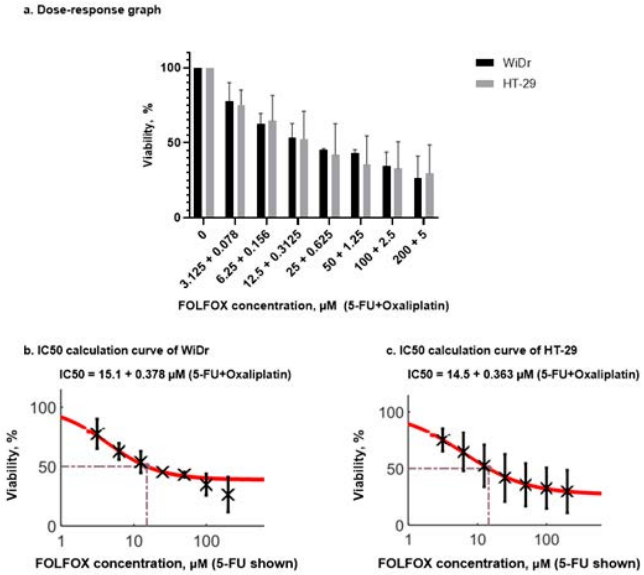

Figure 6. Cell responses to FOLFOX. (**a**) Dose–response graph of WiDr's and HT-29's responses to different doses of FOLFOX. (**b**) FOLFOX IC50 dose calculation for WiDr cell line. (**c**) FOLFOX IC50 dose calculation for HT-29 cell line. N = 3; means ± SD. Purple dot marks 50% viability (IC50).

The results of the commonly used chemotherapy drug combination of FOLFOX and the HAMLET complex showed a slight synergy between FOLFOX and HAMLET but only in the WiDr cell line. The highest significant synergy score obtained was when treating cells with 3 μM of HAMLET + 1.25 of FOLFOX (50 μM of 5-FU + 1.25 μM of oxaliplatin).

However, most of the combinations of 3 µM of HAMLET and FOLFOX showed a synergistic tendency, while most of the combinations with 1 or 5 µM of HAMLET + FOLFOX showed an additive tendency (Figure 7). The results suggest that these two drugs do not interfere with each other and, in some cases, could even exert a synergistic cytotoxic effect on the cells, which does not depend on the BRAF mutation.

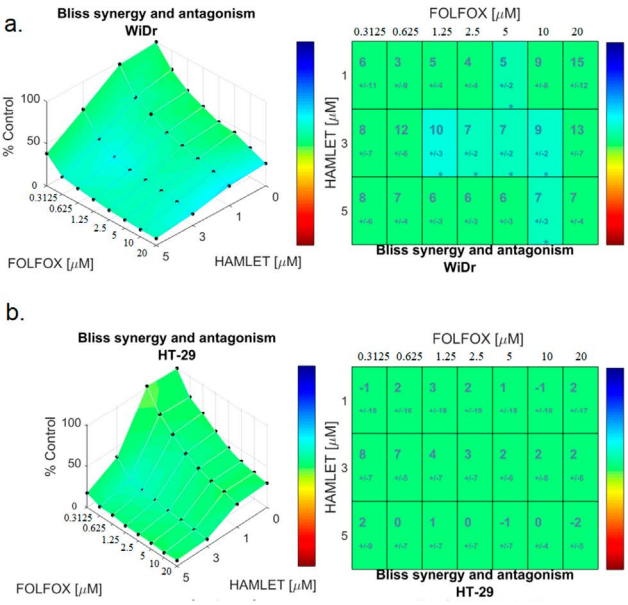

Figure 7. Heatmaps and tables of Bliss synergy and antagonism calculation. (**a**) WiDr cell line; (**b**) HT-29 cell line. Heatmaps show decrease in viability when treating with varying doses of FOLFOX and/or HAMLET compared with control (100% viability). Tables show synergy or antagonism index. Higher positive number with blue color shows significant synergy, and negative number with red color shows significant antagonism (not present). *—$p < 0.05$.

3.6. Glycolytic and Mitochondrial Activity Determination

The observations in Figure 5 show a remarkable divergence in cell lines despite sharing a similar BRAF mutation profile. This prompted an investigation into whether variations in energetic metabolism could account for these differences. Therefore, we compared the cell lines with BRAF mutations for their mitochondrial and glycolytic activity to determine if this was the case. To test the hypothesis, we selected the WiDr and HT-29 cell lines with the same mutational status but different sensitivity to the HAMLET complex and compared their mitochondrial and glycolytic activity. The data of the bioenergetic analysis are presented in Figure 8. The basal mitochondrial respiration of HT-29 cells was significantly higher than that of WiDr cells (the first three points in the oxygen consumption curves in Figure 8a). However, after the addition of the ATP synthase inhibitor oligomycin (to reveal the proton-leak-stimulated OCR), carbonyl cyanide-4-phenylhydrazone (to discover the maximal respiratory capacity), and the respiratory chain complexes I and III inhibitors rotenone and antimycin A (to assess the non-mitochondrial oxygen consumption), there were no significant differences between the respiration rates of WiDr and HT-29 cells. To

summarize the results, the WiDr cell line showed a higher resistance to HAMLET than the HT-29 cell line. Moreover, the cellular response to HAMLET was influenced by factors independent of the genomic characteristics of the cells or their mutational status, leading us to characterize the two cell lines by their bioenergetic properties for further insights.

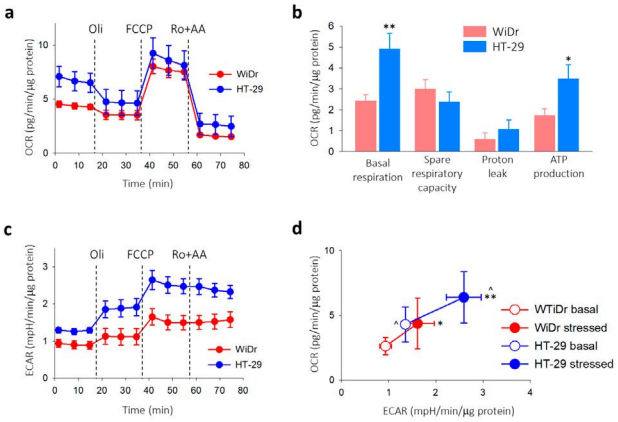

Figure 8. Comparison of the mitochondrial and glycolytic activity of WiDr and HT-29 cells. In (**a**), mitochondrial oxygen consumption curves are presented as averages ± standard deviations of each measurement time point (n = 3 of 3 technical replicates). In (**b**), summarized mitochondrial capacity data are calculated from the curves in (**a**). * and **—statistically significant differences compared with WiDr; $p < 0.05$ and $p < 0.01$, respectively. In (**c**), glycolytic activity is monitored as pH changes simultaneously with the oxygen consumption rate. In (**d**), energy phenotype plots represent mitochondrial and glycolytic energy capacity distributions under normal (basal) and stressed conditions. * and **—statistically significant differences compared with basal conditions for the same cell type; $p < 0.05$ and $p < 0.01$, respectively. ^—statistically significant difference compared with the same parameter of WiDr; $p < 0.05$. OCR—oxygen consumption rate, ECAR—extracellular acidification rate, Oli—oligomycin, FCCP—carbonyl cyanide 4-(trifluoromethoxy) phenylhydrazone, Ro—rotenone, and AA—antimycin A.

Further analysis of mitochondrial function revealed a significant increase in the basal mitochondrial OCR of HT-29 cells compared with the WiDr line (Figure 8b). The average OCR in HT-29 was about two-fold higher than in WiDr. Similarly, the oligomycin-sensitive or mitochondrial-ATP-production-coupled OCR was two-fold lower in WiDr cells. However, the spare respiratory capacity detected after permeabilizing the inner membrane for H^+ with carbonyl cyanide-4-phenylhydrazone and proton-leak-driven respiration was not significantly different in WiDr and HT-29 cells.

The glycolysis activity of the cells was assessed as the extracellular acidification rate simultaneously with the OCRs in the identical probes. The basal glycolytic activity in HT-29 cells was significantly higher than that in WiDr, and the difference became even more prominent after each addition of mitochondrial inhibitors (Figure 8c). In addition, cell energy phenotype analysis indicated that unstressed HT-29 cells had the same glycolytic activity as WiDr under maximal mitochondrial stress conditions (Figure 8d). Thus, the more intensive glycolytic response to mitochondrial failure indicated that HT-29 cells had a higher capacity to maintain energetic balance. On the other hand, the sensitive regulation of bioenergetic metabolism might point to the elevated energetic demands of the HT-29 cells, possibly explaining their higher sensitivity to HAMLET treatment.

3.7. Mitochondrial Respiration

We conducted experiments to assess HAMLET's impact on mitochondrial respiration in HT-29 and WiDr cancer cells, displaying similar BRAF mutant genetic characteristics. Using glutamate/malate for complex I and succinate for complex II as substrates, we measured the mitochondrial respiration rates at 37 °C. We found that HAMLET, at doses of 2 and 5 μM, did not modify the non-phosphorylating (V0) respiration rate in either cell type, as indicated in Figure 9a. However, HAMLET significantly diminished the state 3 (VADP) respiration rate in HT-29 cells by 17% at 2 μM and by 23% at 5 μM, while it had no such effect on WiDr cells, as depicted in Figure 9b when compared with control cells ($p < 0.05$). In HT-29 cells, pretreatment with HAMLET at 5 μM led to a 17% reduction in the maximal mitochondrial respiration (Vmax) with complex I and II substrates, whereas 2 μM of HAMLET had no significant effect, as shown in Figure 9c against untreated cells ($p < 0.05$). Neither 2 μM nor 5 μM of HAMLET influenced the maximum mitochondrial respiration rate in WiDr cells. Adding cytochrome c to the mitochondrial mix, as seen in Figure 9d, confirmed that HAMLET pretreatment at both concentrations did not impact the mitochondrial respiration rate (Vcyt. c) in either cell line, suggesting no change in mitochondrial outer membrane permeability. Lastly, the respiratory control index (RCI), observed in Figure 9e, remained stable in cells pre-treated with HAMLET at both concentrations compared with the control cells in both the HT-29 and WiDr cell lines.

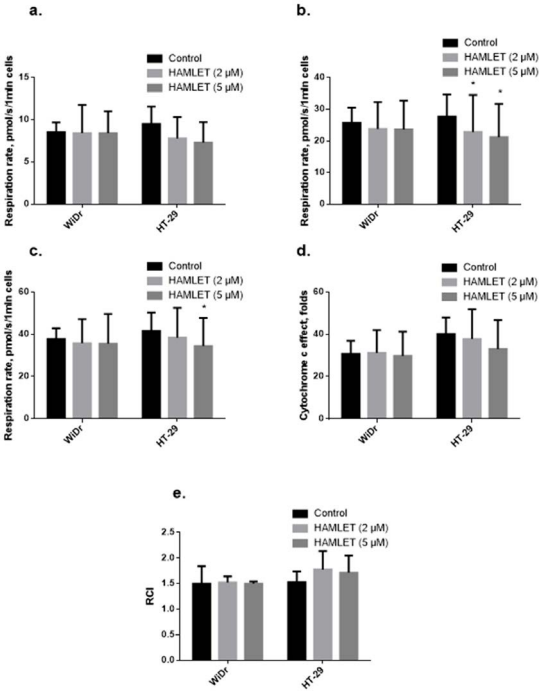

Figure 9. Effect of HAMLET on mitochondrial respiration and respiratory control index (RCI). Mitochondrial respiration rate was measured as described in "Methods". (**a**) Mitochondrial non-phosphorylating

(V_0) respiration rate in the presence of 1 mln/mL of cells and glutamate (5 mM) plus malate (2 mM); (**b**) state 3 respiration rate in the presence of ADP (1 mM; VADP); (**c**) mitochondrial maximal respiration rate in the presence of succinate (12 mM; Vsucc); (**d**) mitochondrial respiration rate in the presence of cytochrome c (32 μM; Vcyt c); and (**e**) mitochondrial respiratory control index (RCI) (VADP/V0)). * $p < 0.05$ compared with the control group.

4. Discussion

In a breakthrough approach, researchers at Lund University have pioneered the use of HAMLET as a novel molecular agent for the treatment and prevention of CRC. HAMLET's ability to selectively accumulate in tumor tissue and its demonstrated efficacy in reducing tumor burden and mortality in Apc(Min)(/+) mice heralds a new era in targeted therapies for CRC [13,24].

In our previous study, we found resistance of BRAF mutant cancer cells to HAMLET [16]. One of the objectives of the current research was to determine whether this mutation is related to the effects of this complex. According to the data from this study, in cell lines with an identical BRAF mutation, the resistance of WiDr to HAMLET is significantly higher than that of the HT-29 line. To date, there has been limited investigation of the interaction between HAMLET and CRC. This gap is further bridged only by the parallel investigation of BAMLET, a similar compound derived from bovine alpha-lactalbumin. Behrouj and colleagues have been investigating the effect of BAMLET on cell survival mechanisms in RAS-mutant HCT116 cells. BAMLET reduces CK1α expression, interferes with key signaling pathways, and inhibits cellular recycling processes, leading to increased cell death, particularly when combined with specific kinase inhibitors [25]. Compared with the BAMLET study cell line, intriguingly, unpublished observations from our laboratory suggest that HCT116 cells, despite having a different mutational profile than HT-29 cells, respond positively to HAMLET, mirroring the response in HT-29. Such findings hint that HAMLET may be broadly applicable across different CRC mutations. However, factors other than genomic or mutational specificities may control the response to the HAMLET complex.

One of the potential factors are mitochondrial functions and bioenergetic analysis. We found that HAMLET's inhibitory effect on mitochondrial respiration is cell-type-specific within the context of BRAF mutant colorectal cancer cells. The different sensitivities to HAMLET in BRAF mutant cells might be due to differences in their energy metabolism. Specifically, HT-29 cells exhibit significantly higher basal mitochondrial respiration and glycolytic activity compared with WiDr cells. Despite their mutation status, HT-29 cells are more sensitive to HAMLET, which could be linked to their higher energetic demands, as indicated by their more intensive glycolytic response to mitochondrial stress. This suggests that the ability of HT-29 cells to maintain energy balance is greater than that of WiDr cells, which might contribute to their differing responses to HAMLET treatment. The results imply that factors beyond genomic characteristics, such as cellular energy metabolism, play a role in the efficacy of HAMLET against colorectal cancer cells. Only one study has investigated the interaction between HAMLET and mitochondrial respiration. The authors posited that HAMLET induces cell death, highlighting its potential to target and kill tumor cells via a direct effect on their mitochondrial function [26].

Our study confirms the critical role of metabolic profiles in the response of cancer cells to treatments, as previously noted by Lin et al. [27]. Specifically, we found that HT-29 cells, which have higher glycolytic rates than WiDr cells, are more susceptible to HAMLET, a therapeutic protein complex. This susceptibility is based not only on genetic markers but also on distinct metabolic behaviors, a notion supported by Rebane-Klemm et al., who found metabolic phenotype variations in colorectal tumors with KRAS and BRAF mutations [28]. We found that HAMLET selectively impairs mitochondrial respiration in HT-29 cells, even though both HT-29 and WiDr cells share a BRAF mutation, suggesting that the metabolic phenotype dictates treatment sensitivity. This is consistent with the findings

of Cha et al. that genetic alterations such as APC loss can drive metabolic changes in cancer cells [29]. Our results differ from the effects of vitamin C on KRAS-mutant colon cancer reported by Cenigaonandia-Campillo et al., suggesting that different treatments may exploit unique metabolic vulnerabilities [30]. In addition, the studies by Spier et al. and Monterisi et al. highlight the role of mitochondrial function in cell fate and survival, emphasizing the interplay between metabolic and genetic factors in cancer therapeutics [31,32]. Kealey et al. also highlight the complexity of cancer cell bioenergetics, as TP53 deficiency and KRAS signaling can alter cellular responses to various substrates, indicating that metabolic responses are multifaceted and context-dependent [33]. Collectively, our findings highlight the importance of an integrative approach that considers both genetic mutations and metabolic features in the development of targeted cancer therapies.

We included a scheme that illustrates the mechanism (Figure 10) where a combination of HAMLET and the components of FOLFOX (oxaliplatin and 5-fluorouracil) induces cancer cell death. The diagram highlights the effect of these agents on mitochondrial function, a critical aspect of this study's findings. It shows that while HAMLET alone reduces ATP production and cell respiration, its combination with oxaliplatin further disrupts these processes, as demonstrated by our institution's previous studies with platinum-based drugs in various cancer cell lines or Wei Sun et al.'s study [34,35]. This combined effect impairs mitochondrial function and increases mitochondrial membrane permeability, leading to a synergistic escalation of cell death. The following visual representation clarifies the underlying biochemical interactions and supports this study's hypothesis of a combined therapeutic effect against CRC cells.

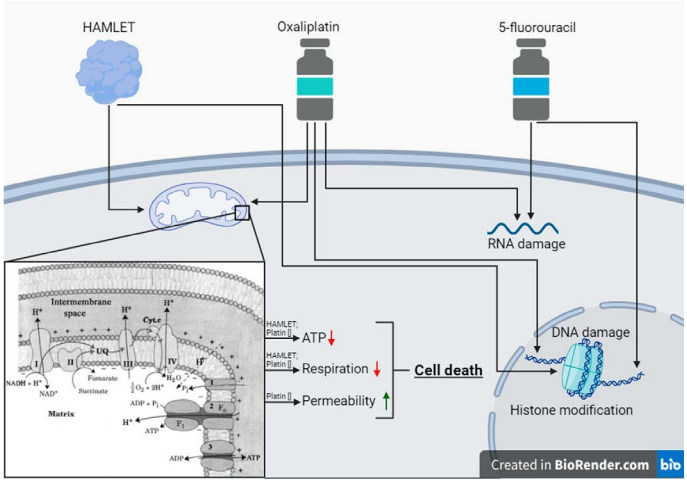

Figure 10. Schematic representation of the possible combined effect of HAMLET and FOLFOX components (oxaliplatin and 5-fluorouracil). The combined effect of FOLFOX component oxaliplatin and HAMLET is most likely exerted via an effect on mitochondria. HAMLET decreases cell ATP production and cell respiration, and our previous studies show that platin-based drugs reduce ATP production, respiration, and mitochondria membrane permeability (Caco-2, AGS, and T3M4 with cisplatin). The combined effect of HAMLET and platin-based drugs on mitochondria can, in some cases, have synergistic effects, leading to increased cell death via mitochondrial damage. ↑ increaced ↓ decreased.

The results of our study on the efficacy of HAMLET in combination with FOLFOX in CRC are consistent with the work of James M.I. et al., who reported that curcumin safely enhances the effects of FOLFOX in a clinical setting [36]. We extend this by showing that the HAMLET–FOLFOX combination selectively reduces viability in BRAF wild-type explants, highlighting the potential for tailored therapies based on genetic profiles and laying the foundation for future clinical applications. For example, a similar bioactive agent to HAMLET is resveratrol in combination with 5-fluorouracil [37], which could be used as an adjunct to conventional chemotherapy.

Our goal is to translate our promising in vitro and ex vivo findings into clinical trials to evaluate the efficacy and safety of this combination therapy in CRC patients. This approach has the potential to improve outcomes, particularly in cases resistant to conventional treatments.

In addition, the diverse responses of CRC cell lines to treatment, which are influenced by mitochondrial dynamics, underscore the need for personalized medicine in CRC therapies [18,19]. Our research suggests that incorporating genetic and metabolic profiling into treatment design could lead to more targeted and effective strategies, with the ultimate goal of improving patient prognoses and treatment responses.

The drawbacks of our research include a limited sample size, which may not reflect the diversity of the broader CRC population, affecting the generalizability of our findings. In addition, we focused on the BRAF mutation and may have overlooked the impact of other mutations on the efficacy of HAMLET [38]. Moreover, keeping the biopsy tissue alive and maintaining its native characteristics outside the body is challenging. Finally, while informative, our in vitro and ex vivo models cannot fully replicate the complex biology of human CRC, including the tumor microenvironment and metabolic effects, which are key to clinical translation. For instance, the examination of intra-tumoral heterogeneity addresses the challenge of differential responses within different cancer cell subpopulations within a single tumor [39]. Further investigations with more comprehensive models are required to validate our findings.

To summarize, our research supports the concept that personalized medicine, tailored to individual genetic and metabolic profiles, may improve outcomes in CRC. Despite this study's limitations, HAMLET, a combination therapy component for CRC, paves the way for future research that will hopefully translate these findings into clinical practice, offering new hope for targeted, effective treatments for patients with this challenging disease.

5. Conclusions

Our study demonstrates that HAMLET and FOLFOX together significantly lower the viability of BRAF wild-type CRC explants via a synergistic effect. HAMLET also moderately inhibits mitochondrial respiration in these cancer tissues. We observed varied responses to HAMLET in CRC BRAF mt cell lines, with WiDr cells being more resistant than HT-29 cells, highlighting the influence of bioenergetic and mitochondrial factors on drug responses. The synergy between HAMLET and FOLFOX in WiDr cells underscores the potential of combined therapies, emphasizing the need to consider both genetic and metabolic aspects in CRC treatment.

Author Contributions: Conceptualization, A.G., Ž.D., R.B. and A.T.; methodology, D.S., J.Ž., V.Č. and A.J.; software, D.S., Z.B. and J.Š.; validation, R.B., Ž.D., J.Ž. and A.J.; formal analysis, D.S., Z.B. and J.Ž.; investigation, D.S., Z.B., J.Ž., A.J., I.Ž. and J.Š.; resources, H.P. and I.Ž.; data curation, D.S., Z.B. and J.Ž.; writing—original draft preparation, J.Ž., D.S. and Z.B.; writing—review and editing, A.G., A.J., Ž.D., R.B. and A.T.; visualization, A.J. and J.Ž.; supervision, V.Č., H.P. and P.L.; project administration, V.Č., H.P. and P.L.; funding acquisition, A.T. and A.G. All authors have read and agreed to the published version of the manuscript.

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Institutional Review Board Statement: This study was approved by The Kaunas Regional Biomedical Research Ethics Committee (no. BE-2-64; study protocol no. 1 on 1 August 2019) and conducted

under the principles of the Declaration of Helsinki and local laws and regulations. We confirm that informed consent was obtained from all subjects and/or their legal guardian(s).

Informed Consent Statement: Informed consent was obtained from all subjects involved in this study.

Data Availability Statement: The data presented in this study are available on request from the corresponding author.

Conflicts of Interest: The authors declare no conflicts of interest.

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