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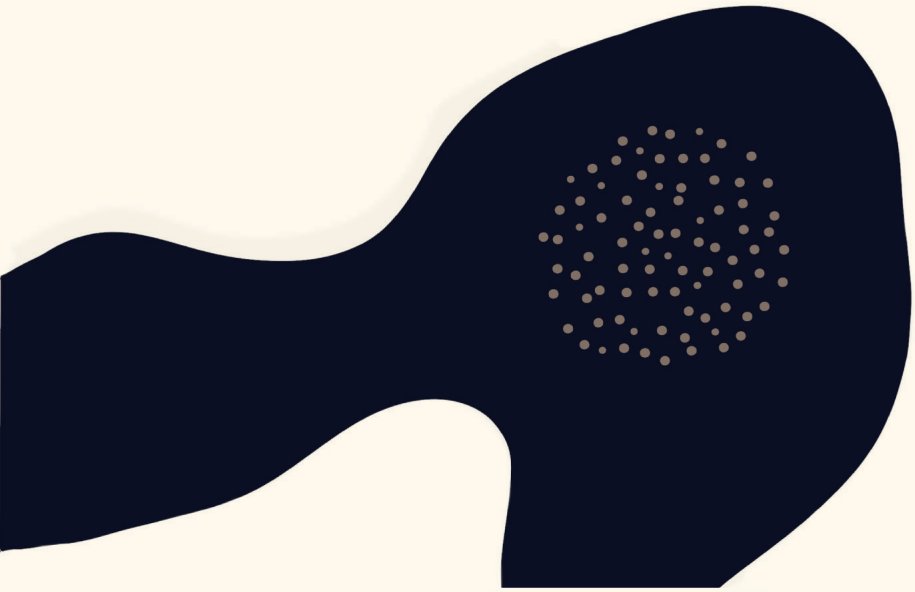

CANCER CELL METABOLISM AND COLORECTAL CANCER SURVIVAL: A ROLE FOR WARBURG-SUBTYPES?

Kelly Offermans

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Cancer cell metabolism and colorectal cancer survival: a role for Warburg-subtypes?

DISSERTATION

to obtain the degree of Doctor at Maastricht University,
on the authority of the Rector Magnificus, Prof. dr. Pamela Habibović
in accordance with the decision of the Board of Deans, to be defended in public on
Thursday 13 April, 2023 at 16.00 hours

by

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LIST OF ABBREVIATIONS

5-FU	5-Fluorouracil
AJCC	American Joint Committee on Cancer
AKT1	AKT Serine/Threonine Kinase 1
APC	Adenomatous polyposis coli
ATP	Adenosine triphosphate
BAX	Bcl-2-associated X
BMI	Body Mass Index
BRAF	V-raf murine sarcoma oncogene homolog B1
CAPOX	Capecitabine plus oxaliplatin
CHT	Chemotherapy
CI	Confidence Interval
CIMP	CpG island methylator phenotype
CIN	Chromosomal instability
CMS	Consensus Molecular Subtypes
COSMIC	Catalogue of Somatic Mutations in Cancer
CRC	Colorectal cancer
CRCSC	CRC Subtyping Consortium
cTNM	Clinical TNM stage
dMMR	MMR deficiency
DNA	Deoxyribonucleic acid
dsDNA	Double-stranded DNA
EBF	Energy balance-related factors
EGFR	Epidermal growth factor receptor
EMT	Epithelial-to-mesenchymal transition
ERK	Extracellular signal-regulated kinase
FAP	Familial adenomatous polyposis
FDG	Fluorodeoxyglucose
FFPE	Formalin-fixed paraffin-embedded
FOLFIRI	Intravenous 5-FU plus irinotecan
FOLFOX	Intravenous 5-FU plus oxaliplatin
FOLFOXIRI	Intravenous 5-FU plus oxaliplatin plus irinotecan
GEO	Gene Expression Omnibus
GLUT1	Glucose transporter 1
H&E	Hematoxylin & Eosin
HER2	Human epidermal growth factor receptor 2
HIF-1α	Hypoxia-inducible factor-1α
HK	Hexokinase
HR	Hazard Ratio

HRAS	Harvey rat sarcoma viral oncogene homolog
IHC	Immunohistochemistry
KRAS	Kirsten rat sarcoma virus
LDHA	Lactate dehydrogenase A
LOH	Loss of heterozygosity
M	Distant metastasis
MALDI-TOF	Matrix Assisted Laser Desorption Ionization Time of Flight
MAPK	Mitogen-activated protein kinase
MCT4	Monocarboxylate transporter 4
MEK	ERK kinase
MET	MNNG HOS transforming gene
METC	Medical Ethical Committee
miRNA	Micro RNA
MLH1	MutL Homolog 1
MMR	Mismatch repair
MPE	Molecular pathological epidemiology
mRNA	Messenger RNA
MSH2	MutS Homolog 2
MSH6	MutS Homolog 6
MSI	Microsatellite Instability
MSS	Microsatellite Stable
mTOR	Mammalian target of rapamycin
N	Lymph node involvement
NLCS	Netherlands Cohort Study
NRAS	Neuroblastoma RAS viral oncogene homolog
OXPHOS	Oxidative phosphorylation
PALGA	Dutch Pathology Registry
Pan-CK	Pan-cytokeratin
PDAC	Pancreatic ductal adenocarcinoma
PDK1	Pyruvate dehydrogenase kinase 1
PI3K	Phosphoinositide 3-kinase
PIK3CA	Phosphatidylinositol-4,5-bisphosphate 3-kinase catalytic subunit alpha
PKM2	Pyruvate kinase M2
pMMR	MMR proficient
PMS2	PKM1 Homolog 2
POLD1	DNA polymerase delta 1
POLE	DNA polymerase epsilon
PTEN	Phosphatase and tensin homolog
pTNM	Pathological TNM stage
RNA	Ribonucleic acid

RT	Radiotherapy
RTK	Receptor tyrosine kinase
SCNA	Somatic copy number alterations
T	Tumor extension
TCA	Tricarboxylic acid
TCGA	The Cancer Genome Atlas
TGFβRII	Transforming growth factor β receptor II
TMA	Tissue MicroArray
TNM	Tumor-node-metastasis
TP53	Tumor protein P53
UICC	Union for International Cancer

CHAPTER 1

General introduction

1. DESCRIPTIVE EPIDEMIOLOGY OF COLORECTAL CANCER

1.1. Incidence

Colorectal cancer (CRC) is the third most commonly diagnosed form of cancer globally (**Figure 1A**)[1]. In 2020, 1.93 million new CRC (including anal cancers) cases were diagnosed worldwide, representing 10% of all new cancer cases (**Figure 1A**)[1, 2]. According to estimates from GLOBOCAN 2020[1], 1.15 million new cases of colon cancer and 0.73 million new cases of rectal cancer were diagnosed in 2020. By 2040, these numbers are predicted to increase to 1.92 million and 1.16 million, respectively[3].

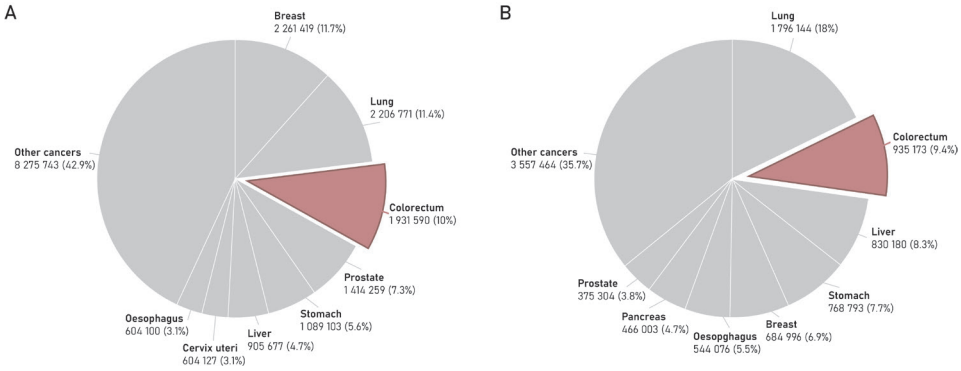

Figure 1 – Distribution of the estimated global numbers of (**A**) new cancer cases and (**B**) cancer-related deaths in 2020, both sexes combined, all ages (melanoma skin cancers excluded). Source: GLOBOCAN 2020, accessed September 2022[1].

With regard to the geographical distribution of CRC, most cases occur in more developed countries[1, 4]. At the country level, the highest age-standardized incidence rates (cases per 100,000 persons per year) in 2020 were observed for Hungary (45.3), Slovakia (43.9), Norway (41.9), the Netherlands (41.0) and Denmark (40.9)[1]. The lowest age-standardized incidence rates in 2020 were found for Guinea (3.3), the Republic of the Gambia (3.7), Bangladesh (3.8), Burkina Faso (3.8), and Bhutan (3.8)[1]. In the Netherlands, CRC was the most commonly diagnosed cancer in 2020 when men and women were combined, accounting for 12.9% of all new cancer cases[1]. In males and females separately, CRC was the second most commonly diagnosed cancer, after prostate cancer and breast cancer, respectively[1].

1.2. Mortality

CRC (including anal cancers) is the second leading cause of cancer-related mortality worldwide, accounting for more than 935,000 deaths in 2020 (**Figure 1B**)[1]. According

to estimates of GLOBOCAN 2020[1], this includes 576,858 deaths from colon cancer and 339,022 deaths from rectal cancer[1]. Mortality rates for CRC have been decreasing in most parts of the world, and are predicted to continue to decrease in the coming years, most likely due to early detection through screening and the availability of new treatments[5, 6].

As for incidence rates, mortality rates for CRC vary largely according to geographic location[1]. At country level, the highest age-standardized mortality rates (deaths per 100,000 persons per year) in 2020 were observed for Slovakia (21.0), Hungary (20.2), Croatia (19.6), Republic of Moldova (17.6), and Serbia (16.7)[1]. The lowest age-standardized mortality rates were found for Bangladesh (2.3), Nepal (2.5), Bhutan (2.5), Botswana (2.6), and India (2.8)[1]. In the Netherlands, CRC was the second most frequent cause of cancer-related death in 2020 when men and women were combined, accounting for 13.4% of all cancer-related deaths$_1$. CRC was the second leading cause of cancer-related death in men, after lung cancer$_1$. In women, CRC was ranked as the third leading cause of cancer-related death in 2020, after lung and breast cancer[1].

1.3. Staging and survival

CRC is staged according to the American Joint Committee on Cancer (AJCC) or Union for International Cancer Control (UICC) tumor-node-metastasis (TNM) classification and staging system[7, 8]. In this system, the stage is based on the characteristics of the primary tumor (T), the regional lymph node involvement (N) and distant metastasis (M)[9]. In CRC, T-values are used to describe the depth of invasion into the bowel wall and adjacent tissues, N-values are used to describe the number of regional lymph nodes with tumor, and M-values are used to describe the presence of distant metastases (**Figure 2**)[8, 9]. A combination of these values is used to define the overall stage of the tumor, with tumor stages ranging from I-IV[9]. The TNM stage may be defined clinically (c), based on preoperative clinical assessment, or pathologically (p), based on the histological evaluation of tissue specimens obtained during surgical resection[10, 11].

The first edition of the UICC staging system was published in 1968[12]. Every few years, the version of this classification system is updated according to new evidence. In 1987, the UICC and AJCC TNM classifications were unified[12]. Currently, the 8th edition of the AJCC/UICC TNM classification (2016) is used[12]. The main TNM stage groupings (I/II/III/IV) have remained essentially unchanged since the fourth edition (1987) [50, 57].

CRC survival rates are dependent on TNM stage at diagnosis[13]. In the Netherlands, the 5-year CRC-specific survival after diagnosis was 67% among all patients diagnosed with CRC between 2011-2020[14]. For patients diagnosed with TNM stage I CRC between 2010-2016 (7th edition of the AJCC staging system), the 5-year CRC-specific survival was 95%[14]. For TNM stage II, III and IV, 5-year CRC-specific survival rates were 86%, 72%, and 12%, respectively[14].

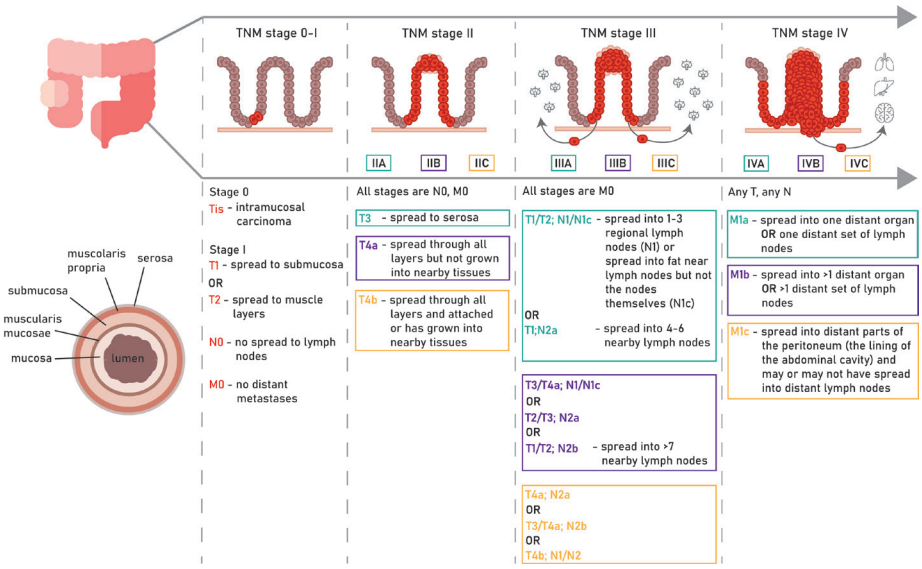

Figure 2 – Colorectal cancer TNM stages according to the 8th edition of the AJCC/UICC classification system[12]. The TNM stage is based on the characteristics of the primary tumor (T), the regional lymph node involvement (N) and distant metastasis (M)[12]. Adapted from Shek *et al.*[15] (CC-BY 4.0) Jauregui-Amezaga *et al.*[16] (CC-BY 4.0). Image colon: Flaticon.com. Abbreviations: TMM, tumor-node-metastasis; Tis, intramucosal carcinoma; T, primary tumor; N, lymph node involvement; M, distant metastasis.

2. ETIOLOGY AND RISK FACTORS

The large majority (>90%) of CRCs are adenocarcinomas originating from epithelial cells of the colorectal mucosa[17, 18]. Other rare types of CRCs include neuroendocrine, squamous cell, adenosquamous, spindle cell and undifferentiated carcinomas[17, 18].

Approximately 70% of all CRCs arise sporadically, meaning that these occur in individuals without a family history of CRC or any inherited genetic mutations that increase the risk of developing CRC[17, 19, 20]. Sporadic CRCs may occur through acquired somatic genetic and epigenetic aberrations which may for a large part be attributable to modifiable risk factors[17, 19]. Next to sporadic CRCs, ~30% of CRCs have a hereditary component[17, 19]. More specifically, ~25% can be attributed to a family history of CRC without any obvious genetic cancer syndrome[17, 19, 20], and ~5% can be attributed to hereditary cancer syndromes (e.g., Lynch syndrome or familial adenomatous polyposis (FAP)) caused by inherited germline mutations in rare, high-penetrance susceptibility genes (e.g., *MutL Homolog 1* (*MLH1)* and *adenomatous polyposis coli* (*APC)*, respectively)[17, 19, 20].

The lifetime risk of developing CRC is ~4.8% in the general Dutch population[1]. Modifiable risk factors that have been associated with an increased risk of developing CRC include

smoking, consuming red meat, consuming alcoholic drinks, and being overweight or obese[21]. On the contrary, being physically active, consuming wholegrains, dairy products, foods containing dietary fiber, and taking calcium supplements have been associated with a decreased risk of developing CRC[21]. Non-modifiable factors that have been associated with an increased risk of developing CRC include genetic factors, ethnicity, increased age, male sex, body height, and family history of CRC[20, 22].

3. MOLECULAR PATHOLOGY

The risk factors described above may potentially result in genetic and epigenetic changes in colorectal epithelial cells that, together with the inherited genetic makeup, have been related to the development of CRC[23]. In general, three major molecular pathways that are involved in the development of CRC are distinguished: (i) the chromosomal instability (CIN) pathway, (ii) the microsatellite instability (MSI) pathway, and (iii) the CpG island methylator phenotype (CIMP) pathway (**Figure 3**)[23-26]. It has been described that these molecular pathways are not mutually exclusive, and that each tumor harbors its own unique combination of genetic alterations thereby contributing to the heterogeneity of CRC[24, 26]. The heterogeneity of CRCs may be further attributed to interactions between these molecular pathways and other less described or undescribed pathways[24].

3.1. Chromosomal instability pathway

Aberrations in the CIN pathway are responsible for the majority (70-85%) of sporadic CRCs[23, 24]. The CIN pathway is characterized by alterations in the number and/or structure of chromosomes (aneuploidy)[26-28]. These chromosomal alterations are often accompanied by the accumulation of mutations in oncogenes and tumor suppressor genes[26, 27]. Inactivation of the *APC* gene is considered the earliest genetic event in the process of colorectal tumorigenesis, leading to activation of the Wnt-signaling pathway thereby stimulating growth and differentiation of epithelial cells, as well as resistance to apoptosis (**Figure 3A**) Activating mutations in *Kirsten rat sarcoma virus (KRAS)* often arise after mutations in *APC* (**Figure 3A**)[24, 28, 29]. Mutated *KRAS* is known to constitutively activate several growth factor signaling pathways, including the RAS/RAF/mitogen-activated protein kinase (MAPK)/ERK kinase (MEK)/extracellular signal-regulated kinase (ERK) and Phosphoinositide 3-kinase(PI3K)/AKT/mammalian target of rapamycin (mTOR) signaling pathways[24, 29]. Loss of heterozygosity (LOH; i.e., a form of allelic imbalance, caused by the loss of one of two alleles, causing a somatic cell to become homozygous[30]) for the long arm of chromosome 18 (18q) and subsequent inactivation of *tumor protein P53* (*TP53*) are often late events in the transition from adenoma to adenocarcinoma (**Figure 3A**)[24, 28]. These events provide additional growth advantages for these expanding cells, ultimately leading to cancer[24].

3.2. Microsatellite instability pathway

MSI is found in approximately 5-15% of all CRCs[31-33]. Microsatellites are stretches of deoxyribonucleic acid (DNA) in which a short motif of one to five nucleotides is repeated several times[34, 35]. These microsatellites are prone to DNA replication errors as a result of their repeated structure, leading to mismatched DNA strands[36, 37]. These errors are usually corrected through the mismatch repair (MMR) system, which consists of four main proteins: MLH1, MutS Homolog 2 (MSH2), MutS Homolog 6 (MSH6) and PKM1 Homolog 2 (PMS2)[37].

The MSI pathway is characterized by inactivation of any of these MMR genes[23, 38] (**Figure 3B**). The sporadic form of MSI CRC is almost always due to epigenetic inactivation of the *MLH1* gene, while the inherited form of MSI CRC (i.e. Lynch syndrome) is mainly due to inherited mutations in the *MLH1* and *MSH2* genes[39, 40]. Either way, loss of function of MMR results in the accumulation of mutations, including mutations in genes involved in proliferation, cellular differentiation (e.g. *transforming growth factor β receptor II* (*TGFβRII*)), and apoptosis (e.g. *Bcl-2-associated X (BAX)*), eventually leading to MSI cancers (**Figure 3B**)[24, 38].

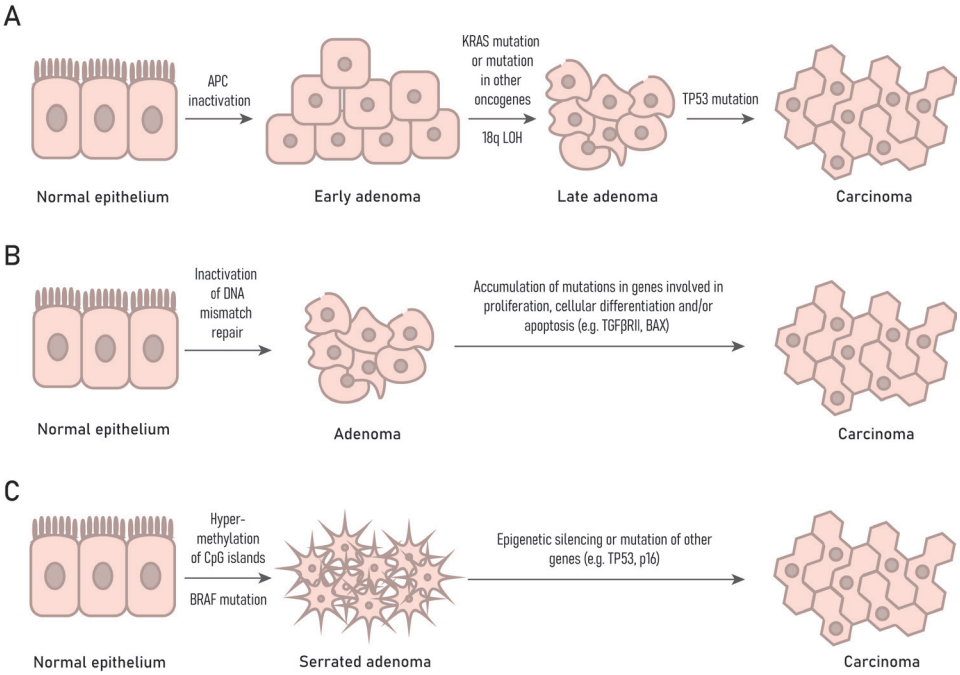

Figure 3 – Simplified illustration of the three major molecular pathways that are involved in the development of colorectal cancer: (**A**) the chromosomal instability pathway (CIN), (**B**) the microsatellite instability (MSI) pathway, and (**C**) the CpG island methylator phenotype pathway (CIMP). Adapted from Mundade et al.[24] (CC-BY 3.0). Abbreviations: APC, adenomatous polyposis coli; KRAS, Kirsten rat sarcoma viral oncogene homologue; LOH, loss of heterozygosity; TP53, tumor protein 53; DNA, deoxyribonucleic acid; TGFβRII, transforming growth factor β receptor II; BAX, Bcl-2-associated X protein.

3.3. CpG island methylator phenotype pathway

The CIMP pathway, also known as the serrated pathway in CRC, is responsible for approximately 15-20% of all CRCs2[4, 41]. It is characterized by significant hypermethylation of CpG islands of tumor suppressor genes and DNA repair genes (**Figure 3C**)[24, 42-44]. An early event in CIMP tumors is a mutation in the *BRAF* oncogene, leading to enhanced RAS/RAF/MAPK/MEK/ERK signaling and thereby inducing uncontrolled proliferation, immune evasion, angiogenesis, tissue invasion and metastasis, as well as resistance to apoptosis[24,44]. Subsequent epigenetic silencing or mutation of other genes (e.g. *TP53*, *p16*) eventually accelerates the progression of sporadic CRCs[24].

3.4. Molecular pathological classification of colorectal cancer

More recently, two molecular pathological classification systems for CRC have been proposed[45]. In 2012, The Cancer Genome Atlas (TCGA) research network conducted a genome-scale characterization of 276 CRC samples, analyzing exome sequences, DNA copy number, promotor methylation of messenger ribonucleic acid (mRNA) and micro RNA (miRNA) expression[46]. In addition, a subset of samples (n = 97) with matched normal tissue samples were subjected to whole-genome sequencing[46]. These results suggested that CRC could be split into three major subgroups: (i) hypermutated tumors (~13%; characterized by MSI/defective MMR, *BRAF V600E* (or similar) mutations, and CIMP), (ii) ultra-mutated tumors (~3%; characterized by mutations in DNA polymerase epsilon or delta 1 (*POLE* or *POLD1*) proofreading mutations), and (iii) CIN tumors (~84%; characterized by a high frequency of DNA somatic copy number alterations (SCNAs), low mutation rate, microsatellite stability (MSS), deregulation of the Wnt-pathway, and *APC* mutations)[45, 46].

In 2015, the Consensus Molecular Subtypes (CMS) Consortium compared and analyzed the data generated by six independent transcriptomics-based subtyping systems[47-53]. This resulted in a CMS classification consisting of four major subtypes that can be used to describe the heterogeneity of CRC at the gene-expression level: (i) CMS1-MSI immune subtype (~14%; characterized by hypermutation, MSI, CIMP, immune activation and *BRAF* mutation), (ii) CMS2-canonical subtype (~37%; characterized by epithelial features, activated Wnt and Myc signaling pathways, CIN, and MSS), (iii) CMS3-metabolic subtype (~13%; characterized by deregulation of metabolic pathways, *KRAS* mutations, and mixed CIN-MSI status), and (iv) the CMS4-mesenchymal subtype (~23%; characterized by upregulation of epithelial-to-mesenchymal transition (EMT), TGF-β activation, MSS, CIN, angiogenesis, and stromal invasion)[47].

4. CANCER CELL METABOLISM: THE WARBURG-EFFECT

In 2000, Hanahan and Weinberg[54] first described six biological capabilities acquired during the multistep development of human cancer. These so-called "Hallmarks of Cancer" included uncontrolled proliferative signaling, resistance to apoptosis, initiating angiogenesis, acquiring replicative immortality, activating invasion and metastasis, and evading growth suppressors[54]. These hallmarks were updated in 2011[55] and 2022[56], extending the list of cancer hallmarks to include 14 (emerging) hallmarks and enabling characteristics, including the hallmark "reprogramming cellular metabolism"[55, 56].

Otto Warburg was the first to describe the metabolic distinction between normal and tumor cells in the 1920s[57, 58]. In a normal cell, glucose is converted into pyruvate through glycolysis in the cytoplasm (**Figure 4A**)[58, 59]. Pyruvate then enters the mitochondria where it oxidized to CO_2 through the tricarboxylic acid (TCA) cycle and oxidative phosphorylation (OXPHOS) in the presence of oxygen[58, 59]. In normal conditions, pyruvate will only be metabolized into lactate when oxygen is limited[58, 59]. In a cancer cell, however, most glucose is converted into lactate, even in the presence of oxygen (**Figure 4A**)[58, 59]. This phenomenon of aerobic glycolysis, also known as the 'Warburg-effect', has since been observed in a variety of cancer types, including CRC[58].

Aerobic glycolysis is a very inefficient process compared to OXPHOS in terms of energy (i.e., adenosine triphosphate (ATP)) production, generating only 2 ATP/glucose instead of 38 ATP/glucose[60-62]. To compensate for this inefficiency, cancer cells increase their glycolytic flux by upregulating several glycolysis-related proteins (**Figure 4B**)[60, 63-65]. First, glucose uptake is stimulated by upregulation of glucose transporter 1 (GLUT1) expression[63, 64]. Second, glycolytic flux and subsequent lactate production are increased by upregulating the expression of hexokinase (HK), pyruvate kinase M2 (PKM2), pyruvate dehydrogenase kinase 1 (PDK1), and lactate dehydrogenase A (LDHA)[63, 65]. Third, lactate secretion is enhanced by increasing the expression of monocarboxylate transporter 4 (MCT4) to prevent cytoplasmic acidification[63, 64].

One of the most frequently activated molecular pathways in cancer cells is the PI3K/AKT/mTOR signaling pathway (**Figure 4B**)[66-68]. This pathway is known to be involved in a variety of cellular functions including proliferation, adhesion, migration, invasion, angiogenesis, and survival[66]. Most relevantly, however, the PI3K/AKT/mTOR signaling pathway is known to 'rewire' cancer metabolism from OXPHOS towards aerobic glycolysis through regulation of several transcription factors, including c-Myc and HIF-1α (**Figure 4B**)[63, 65, 66, 69-72].

The PI3K/AKT/mTOR signaling pathway is activated by mutations in tumor suppressor genes (e.g. *phosphatase and tensin homolog (PTEN)*, *TP53*), mutations in the components of the PI3K complex itself (e.g. *phosphatidylinositol-4,5-bisphosphate 3-kinase catalytic subunit alpha (PIK3CA)*), or by aberrant receptor tyrosine kinase (RTK) signaling (e.g. epidermal growth factor receptor (EGFR))[73, 74]. In addition, mutations in *RAS* (*KRAS, NRAS, HRAS*) or *BRAF,* which signal mainly through the RAS/RAF/MAPK/MEK/ERK signaling pathway can also activate the PI3K/AKT/mTOR pathway[75, 76].

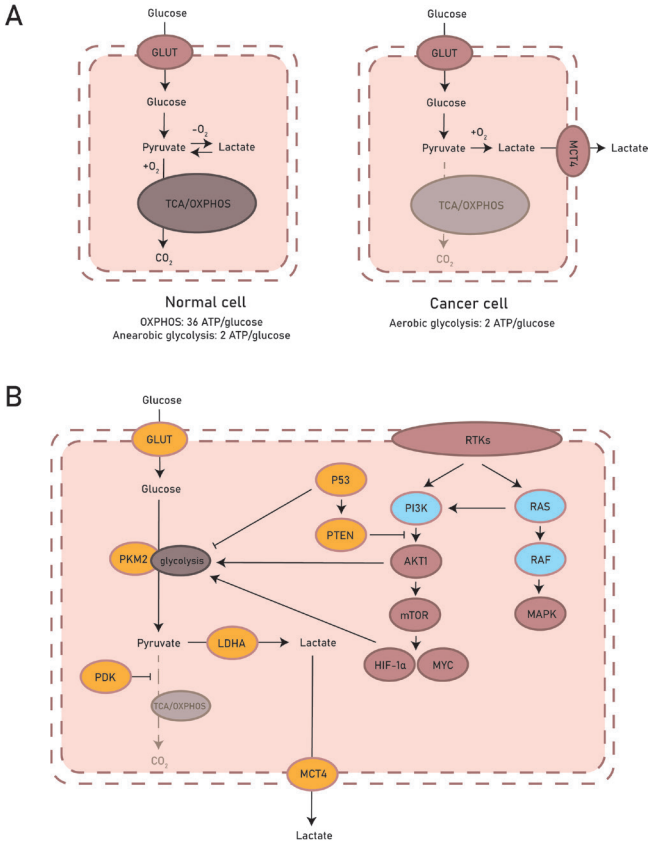

Figure 4 – Simplified schematic overview of the Warburg-effect. (**A**) Schematic overview of the metabolic differences between normal cells (complete glucose oxidation; left) and cancer cells (Warburg-effect; right). Adapted from Marie et al.[77] (CC-BY-NC 3.0) (**B**) More detailed overview of all proteins and drivers associated with the Warburg-effect (aerobic glycolysis), the PI3K/Akt/mTOR pathway, and RAS/RAF/MAPK pathway, and their relation. Sharp arrows (↑) indicate stimulation while blunt arrows (⊤) indicate inhibition. Note: a line does not always indicate a direct link as other factors may be left out for simplicity. Proteins and transcriptional regulators related to the Warburg-effect that were measured in this study (Chapter 3, 5, 6) are shown in yellow; mutations in drivers of the Warburg-effect that have been measured in this thesis (Chapter 4) are shown in blue. Figure based on Cairns et al. [74] and others[75, 78, 79]. Abbreviations: GLUT1, glucose transporter 1; PKM2, pyruvate kinase M2; PDK, pyruvate dehydrogenase; LDHA, lactate dehydrogenase A; MCT4, monocarboxylate transporter 4; P53, tumor protein P53; PTEN, phosphatase and tensin homolog; PI3K, phosphoinositide 3-kinase; AKT1, AKT serine/threonine kinase 1; mTOR, mammalian target of rapamycin; HIF-1α, hypoxia inducible factor-1α; MAPK, mitogen-activated protein kinase.

5. TREATMENT

Since its discovery in the 1960s, 5-fluorouracil (5-FU) has been the cornerstone in chemotherapy for advanced CRC[80]. Treatment options for CRC have been increasing in recent years, aimed at improving the survival of CRC patients[80]. By 2001, the oral precursor of 5-FU (capecitabine) as well as two new chemotherapeutic agents had been introduced (oxaliplatin and irinotecan)[81]. This resulted in the introduction of chemotherapeutic combination therapies, such as FOLFIRI (intravenous 5-FU plus irinotecan), FOLFOX (intravenous 5-FU plus oxaliplatin), CAPOX (capecitabine plus oxaliplatin), and FOLFOXIRI (intravenous 5-FU plus oxaliplatin plus irinotecan)[82]. More recently, targeted agents (i.e., anti-angiogenic agents, anti- EGFR agents and immunotherapeutic agents) have been integrated into chemotherapeutic combination therapies to further improve survival outcomes[80, 82]. Although these regimens have achieved good clinical benefits, 5-FU is still the most basic drug and the most important component in all these regimens[80].

5.1. Current treatment strategies

Treatment decisions in CRC patients are mainly guided by tumor location and TNM stage[83, 84]. Currently, patients with TNM stage I CRC and the majority of patients with TNM stage II CRC are treated with surgery alone and do not require adjuvant therapy[85, 86]. Patients diagnosed with high-risk TNM stage II (i.e., pT4 tumor, poor differentiation, bowel perforation or obstruction, lymphatic or venous or perineural invasion, or <12 lymph nodes found in the resection specimen[87]) or TNM stage III colon cancers are treated with surgery followed by adjuvant chemotherapy[85, 88]. Patients diagnosed with TNM stage II or III rectal cancers are treated with neoadjuvant chemoradiation therapy followed by surgery with or without adjuvant therapy[85]. Patients with metastatic (TNM stage IV) CRC are primarily treated with chemotherapy, although it has been described that a selected group of patients may be cured with metastasectomy[85]. In addition, anti-angiogenic agents (e.g., bevacizumab, ramucirumab), anti-EGFR agents (e.g., panitumumab, cetuximab), and immunotherapeutic agents (e.g., pembrolizumab, nivolumab) are sometimes used in combination with chemotherapy to treat patients with metastatic disease[88-90].

5.2. Changes in treatment strategies between 1986-2007

Treatment strategies for CRC in the Netherlands have changed over the years[91]. In this paragraph, we focus on the time period 1986-2007, as this is the time period during which the patients on which the results presented in this thesis were based were diagnosed with CRC. Between 1986-2007, the large majority (92-100%) of colon cancer patients in the Netherlands with TNM stage I-III disease were treated by resection of the primary tumor, regardless of the period of diagnosis and age[91]. Since the 1990s, the use of adjuvant chemotherapy for TNM stage III colon cancer increased drastically in all age groups, but to a lesser extent in the older age groups (from 1% in 1985-1989 to 79% in 2005-2007 in

patients aged 50-59; and from 0% in 1985-1989 to 49% in 2005-2007 in patients aged 70-79)[91]. The use of adjuvant chemotherapy for stage II colon cancer also slightly increased over the years (from 0% in 1985-1989 to 12% in 2005-2007 in patients aged 50-59; and from 0% in 1985-1989 to 3% in 2005-2007 in patients aged 70-79)[91]. Furthermore, the administration of chemotherapy in TNM stage IV colon cancer patients increased over time for all age groups, but for a lesser extent in older patients (from 11% in 1985-1989 to 75% in 2005-2007 in patients aged 50-59; and from 2% in 1985-1989 to 39% in 2005-2007 in patients aged 70-79)[91].

The large majority of patients with rectal cancer underwent surgery (88-99%)[91]. The use of postoperative radiotherapy in patients with TNM stage II and III rectal cancer decreased over time (from 49% in 1989-1993 to 4% in 2004-2006 in patients <75 years), while the use of preoperative radiotherapy increased drastically after its introduction in the mid-1990s (from 1% in 1989-1993 to 68% in 2004-2006 in patients <75 years)[91, 92]. The proportion of patients with TNM stage III or IV rectal cancer who received adjuvant chemotherapy increased over the years (from 9% in 1989-1993 to 29% in 2004-2006 in TNM stage III patients <75 years; and from 21% in 1989-1993 to 66% in 2004-2006 in TNM stage IV patients <75 years)[92]. Furthermore, neoadjuvant chemoradiation was introduced around 2004 for the treatment of stage II and III rectal cancer[92].

6. PROGNOSTIC AND PREDICTIVE BIOMARKERS

Biomarkers may be prognostic, predictive, or both[93]. Prognostic biomarkers are biomarkers that are used to identify the likelihood of a specific outcome (e.g. tumor progression, death, disease recurrence), but cannot be used to guide treatment decisions[93, 94]. Predictive biomarkers, on the other hand, are markers that are used to identify individuals who are more likely to benefit from a particular therapy, and therefore could be used to guide treatment decisions[93, 94]. Despite our growing understanding of the molecular pathways underlying CRC and the advances in the treatment of CRC, our understanding why patients do or do not respond to therapy remains poor[95].

Currently, TNM stage at the time of diagnosis remains the most important clinically used prognostic and predictive factor[83, 84, 94]. Over the last few years, extramural vascular, lymphatic or perineural invasion, poor histological differentiation, bowel obstruction or perforation, advanced tumor stage (pT4) at the time of surgery, and lymph node sampling lower than 12 were added as additional diagnostic factors to identify high-risk patients among early-stage CRCs[94, 96]. More recently, new guidelines for the testing of molecular markers have been established in order to assist in disease prognosis, surveillance, and treatment for CRC[94]. In CRC, this molecular testing has improved treatment selection and

clinical outcome, although this is so far limited to patients with metastatic disease[94].

Molecular biomarkers that have been implemented in current clinical practice include the mutational status of genes involved in CRC carcinogenesis (*KRAS, NRAS, BRAF*) or defects in the DNA MMR system[94]. MSI/MMR deficiency (dMMR) is observed in 5-15% of all CRCs[31-33]. It has been shown that patients with dMMR CRC have a better stage-adjusted survival compared to patients with MMR proficient (pMMR) CRC[94, 97]. Furthermore, dMMR tumors have a decreased risk of metastasizing, and are therefore associated with earlier-stage CRC[97]. Next to prognostic value, MMR status may also predict response to therapy[97]. Several clinical trials have shown that 5-FU-based adjuvant therapy may not provide survival benefit in stage II dMMR CRC[97]. Treatment with adjuvant FOLFOX is recommended in patients with TNM stage III CRCs, regardless of MMR status[97]. In the metastatic setting, it has been shown that patients with dMMR CRCs often show a good response to immunotherapy[97]. Testing for MMR status has therefore been included in the Dutch national CRC guidelines for all newly diagnosed CRC patients under the age of 70 years, and especially for patients who are eligible for adjuvant chemotherapy in stage II and III CRC or immunotherapy in stage IV CRC[90].

Mutations in *KRAS*, *NRAS,* and *BRAF* are observed in approximately 30-40%, 5-9%, and 4-20% of CRCs, respectively[31, 98]. Even though a poor prognosis has previously been reported in several studies, the prognostic significance of *RAS (KRAS* and *NRAS*) or *BRAF* mutations in CRC remains uncertain[31, 99]. Nevertheless, testing for *RAS* or *BRAF* mutations has predictive value in patients with metastatic CRCs, as only patients with wild-type *RAS* and *BRAF* genes were shown to benefit from anti-EGFR therapy[94, 100]. Testing for *RAS* and *BRAF* mutations has therefore been included in the Dutch national CRC guidelines for patients with metastatic CRCs who are eligible for systemic treatment[90].

Next to the markers described above, several promising biomarkers have been identified in recent years, including tumor budding[101], immunoscore®[102], tumor/stroma ratio[103], neutrophil/lymphocyte ratio[104], and CMS classification[47]. However, to date only tumor budding has been added to national and international guidelines[105, 106]. Hence, there remains an urgent clinical need to identify reliable prognostic and/or predictive biomarkers in CRC[95].

Potentially important markers that have been suggested to have prognostic and/or predictive potential in CRC include the expression of proteins related to the Warburg-effect[107-114], mutations related to the PI3K/AKT/mTOR pathway (e.g. *RAS, BRAF, PIK3CA, MET*)[115-119], and (long-term) energy balance-related factors[120] (i.e., pre-diagnostic BMI[121-125], weight change[125], non-occupational physical activity[126-130], adult-attained height[123], and energy-restriction[131, 132]). However, to date, the evidence is very limited and results are inconsistent.

7. MOLECULAR PATHOLOGIC EPIDEMIOLOGY

Traditional epidemiological research has investigated whether an exposure (e.g. lifestyle, environmental or genetic factor) is associated with the risk of developing cancer, patient prognosis, or response to treatment (**Figure 5A**)[133]. On the other hand, molecular pathology has traditionally been used to explore molecular characteristics of tumors that are related to patient prognosis or can be used to predict response to treatment (**Figure 5B**)[133]. These two approaches, epidemiology and molecular pathology, have recently been combined into a new field of epidemiology: molecular pathological epidemiology (MPE) (**Figure 5C**)[133].

One of the main aims of MPE is to investigate potential etiologic or survival factors across strata of molecular characteristics for the disease of interest[134]. With regard to etiology, it is hypothesized that diseases that share certain molecular alterations (e.g. Warburg-subtype) are more likely to share common risk factors (e.g. obesity) (**Figure 5C**)[134]. For prognostic studies, the underlying hypothesis is that some external or endogenous factors (e.g. energy balance-related factors) may influence the outcome of a certain disease (e.g. CRC) according to molecular alterations (e.g. Warburg-subtype), as those factors likely interact with the diseased cells in the tissue microenvironment (**Figure 5C**)[134].

One of the limitations inherent to MPE research is misclassification in tumor molecular subtyping[133]. Immunohistochemistry (IHC) assays are often used for molecular subtyping in MPE studies, and should be validated and monitored for their precision and accuracy[133]. To decrease variability in IHC assays, the use of tissue microarrays (TMAs) is recommended[133]. As manual IHC scoring of TMA sections can take a considerable amount of time if individual scores need to be provided for hundreds or thousands of tissue cores, IHC scoring is often done by non-pathologists[135-137]. However, studies on the validity and reproducibility of these IHC scoring results are currently very limited.

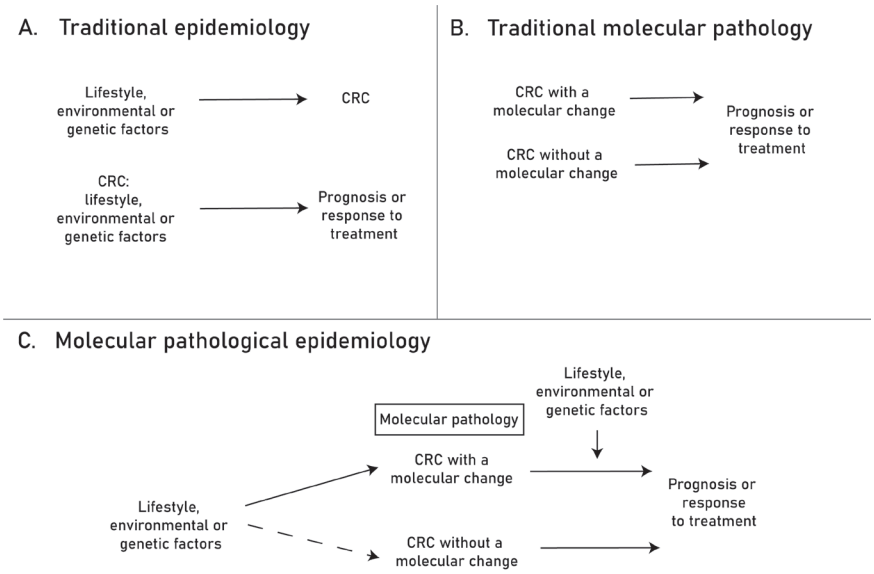

Figure 2 – Illustration showing (**A**) traditional epidemiology, (**B**) traditional molecular pathology, and (**C**) molecular pathological epidemiology. Molecular pathological epidemiology can be applied to investigate whether a certain exposure is associated with a specific molecular change in colorectal cancer (C, left side), as well as to investigate whether a specific molecular change can interact with a certain exposure factor to affect clinical outcome (C, right side). Reproduced from "Molecular pathological epidemiology of colorectal neoplasia: an emerging transdisciplinary and interdisciplinary field", Ogino, Shuji, Andrew T. Chan, Charles S. Fuchs, and Edward Giovannucci, Gut 60.3 (2011): 397-411[133] with permission from BMJ Publishing Group Ltd. Abbreviations: CRC, colorectal cancer

8. AIMS AND HYPOTHESES

In this thesis, we aimed to investigate whether Warburg-subtyping, based on the estimated presence of the Warburg-effect, has prognostic value and is able to predict survival benefit from adjuvant therapy in CRC patients, independent of known prognostic factors such as TNM stage. To this end, CRC patients were classified as having Warburg-low (i.e., low probability of the presence of the Warburg-effect), Warburg-moderate, or Warburg-high CRC using a pathway-based sum score based on the protein expression levels of six glycolytic proteins and transcriptional regulators indicative of the Warburg-effect (i.e., LDHA, GLUT1, MCT4, PKM2, p53, PTEN). First, we investigated whether non-pathologists can generate valid and reproducible IHC scoring results. Second, we studied the prognosis of CRC patients according to Warburg-subtype. Third, we investigated the prognosis of subgroups of patients based on mutations in oncogenes and/or tumor suppressor genes that have been related to the Warburg-effect (i.e., *RAS, BRAF, PIK3CA* and *MET*) and MMR status, as well as the additional prognostic value of Warburg-subtypes within these mutational subgroups. Fourth, we studied the prognostic value of long-term energy balance-related

factors (i.e., energy restriction during early-life, adult-attained height, weight change since age 20 years, adult BMI, and non-occupational physical activity) in the total series of CRC patients, as well as according to Warburg-subtype. Lastly, we explored whether Warburg-subtypes can be used to predict survival benefit from adjuvant therapy in CRC patients. We hypothesized that:

i. Non-pathologists can generate reproducible IHC scoring results, similar to those of an experienced pathologist;
ii. Patients with Warburg-high CRC have a poorer prognosis compared to patients with Warburg-low CRC;
iii. Mutational subgroups based on somatic mutations in *RAS, BRAF, PIK3CA* and *MET*, as well as patients' MMR status hold prognostic value in CRC, and Warburg-subtypes provide additional prognostic information within these mutational subgroups;
iv. Increased adult BMI, weight gain since age 20 years and adult-attained height are associated with a worse CRC-specific and overall survival, whereas energy restriction during childhood or adolescence and non-occupational physical activity are associated with a more favorable survival in CRC. The association between these long-term energy balance-related factors and CRC survival differs according to Warburg-subtype;
v. Patients with Warburg-high CRC will not derive a survival benefit from adjuvant chemo- or radiotherapy, whereas patients with Warburg-low CRC will derive survival benefit from adjuvant therapy.

9. STUDY DESIGN: THE NETHERLANDS COHORT STUDY

The results presented in this thesis are based on observational data from the Netherlands Cohort Study (NLCS) on diet and cancer[138]. This large, population-based prospective cohort study was initiated in 1986, and included 120,852 men and women aged 55-69 at baseline[138]. All participants completed a mailed, self-administered questionnaire on dietary habits, lifestyle, and other risk factors for cancer[138]. These factors included, but were not limited to, the following energy-balance related factors: adolescent (age 20 years) and adult (baseline) weight (kg), adult-attained height (cm), non-occupational physical activity (min/day), and energy-restriction proxy measures (place of residence during the Dutch Hunger Winter (1944-1945), place of residence during World War II (1940-1944), employment status of the father during the Dutch Economic Depression (1932-1940)) .

Follow-up for cancer incidence was established by annual record linkage with the Netherlands Cancer Registry and PALGA, the nationwide Dutch Pathology Registry, covering 20.3 years of follow-up since study initiation (September 17, 1986 until January 1, 2007)[139, 140]. After this follow-up period of 20.3 years, 4,597 incident CRC cases had occurred. Follow-up for vital status was carried out through linkage with the Central Bureau of Genealogy and the municipal population registries, until December 31, 2012. Cause of death was retrieved from Statistic Netherlands.

In 2012, the Rainbow-TMA project was initiated, aiming to enrich cohorts with TMAs and DNA[141]. Tumor and normal formalin-fixed paraffin-embedded (FFPE) tissue blocks from CRC patients were retrieved from pathology laboratories throughout the Netherlands. Pathologists reviewed Hematoxylin & Eosin (H&E)-stained sections and marked areas with the highest tumor density. From these areas, three 0.6 mm diameter tumor cores and three normal tissue cores were sampled and assembled in TMA blocks.

10. THESIS OUTLINE

The current chapter, **Chapter 1**, served as a general introduction to the topic of this thesis, the main concepts, aims and hypotheses, and the research design. **Chapter 2** describes the validity and reproducibility of IHC scoring by trained non-pathologists on TMAs. In **Chapter 3**, the survival of CRC patients according to our defined Warburg-subtypes is described. The next chapter, **Chapter 4**, describes the association between mutational subgroups, Warburg-subtypes, and CRC survival. In **Chapter 5**, the association between long-term energy balance-related factors and survival in CRC overall, and according to Warburg-subtype, is described. **Chapter 6** describes the association between adjuvant therapy and survival in CRC patients according to Warburg-subtype. Lastly, a general discussion of the main findings of this thesis is provided in **Chapter 7**.

REFERENCES

1. Ferlay J, Ervik M, Lam F, et al. Global Cancer Observatory: Cancer Today. Volume 2021: Lyon, France: International Agency for Research on Cancer, 2020.
2. Xi Y, Xu P. Global colorectal cancer burden in 2020 and projections to 2040. Translational Oncology 2021;14:101174.
3. Ferlay J, Laversanne M, Ervik M, et al. Global Cancer Observatory: Cancer Tomorrow. Volume 2022: Lyon, France: International Agency for Research on Cancer, 2020.
4. Gandomani HS, Aghajani M, Mohammadian-Hafshejani A, et al. Colorectal cancer in the world: incidence, mortality and risk factors. Biomedical Research and Therapy 2017;4:1656-1675.
5. Araghi M, Soerjomataram I, Jenkins M, et al. Global trends in colorectal cancer mortality: projections to the year 2035. International journal of cancer 2019;144:2992-3000.
6. Schreuders EH, Ruco A, Rabeneck L, et al. Colorectal cancer screening: a global overview of existing programmes. Gut 2015;64:1637-1649.
7. Weiser MR. AJCC 8th edition: colorectal cancer. Annals of surgical oncology 2018;25:1454-1455.
8. Edition S, Edge S, Byrd D. AJCC cancer staging manual. AJCC cancer staging manual 2017.
9. Rosen RD, Sapra A. TNM classification. StatPearls [Internet]: StatPearls Publishing, 2021.
10. Moawad EY. Clinical and pathological staging of the cancer at the nanoscale. Cancer nanotechnology 2012;3:37-46.
11. Koukis I, Gkiozos I, Ntanos I, et al. Clinical and surgical-pathological staging in early non-small cell lung cancer. Oncology Reviews 2013;7.
12. UfIC C. UICC and the TNM Classification of Malignant Tumours. 2021.
13. Haggar FA, Boushey RP. Colorectal cancer epidemiology: incidence, mortality, survival, and risk factors. Clin Colon Rectal Surg 2009;22:191-7.
14. (IKNL) IkN. NKR Cijfers. Volume 2022: IKNL, 2022.
15. Shek D, Akhuba L, Carlino MS, et al. Immune-checkpoint inhibitors for metastatic colorectal cancer: a systematic review of clinical outcomes. Cancers 2021;13:4345.
16. Jauregui-Amezaga A, Rimola J. Role of intestinal ultrasound in the management of patients with inflammatory bowel disease. Life 2021;11:603.
17. Keum N, Giovannucci E. Global burden of colorectal cancer: emerging trends, risk factors and prevention strategies. Nature reviews Gastroenterology & hepatology 2019;16:713-732.
18. Fleming M, Ravula S, Tatishchev SF, et al. Colorectal carcinoma: Pathologic aspects. Journal of gastrointestinal oncology 2012;3:153.
19. Mármol I, Sánchez-de-Diego C, Pradilla Dieste A, et al. Colorectal carcinoma: a general overview and future perspectives in colorectal cancer. International journal of molecular sciences 2017;18:197.
20. Wong MC, Ding H, Wang J, et al. Prevalence and risk factors of colorectal cancer in Asia. Intestinal research 2019;17:317.
21. Research WCRFAIfC. Continuous Update Project Expert Report 2018. Diet, nutrition, physical activity and colorectal cancer. Volume 2022.
22. Johnson CM, Wei C, Ensor JE, et al. Meta-analyses of colorectal cancer risk factors. Cancer causes & control

2013;24:1207-1222.

23. Mojarad EN, Kuppen PJ, Aghdaei HA, et al. The CpG island methylator phenotype (CIMP) in colorectal cancer. Gastroenterology and hepatology from bed to bench 2013;6:120.
24. Mundade R, Imperiale TF, Prabhu L, et al. Genetic pathways, prevention, and treatment of sporadic colorectal cancer. Oncoscience 2014;1:400.
25. Phipps AI, Limburg PJ, Baron JA, et al. Association between molecular subtypes of colorectal cancer and patient survival. Gastroenterology 2015;148:77-87. e2.
26. Al-Sohaily S, Biankin A, Leong R, et al. Molecular pathways in colorectal cancer. Journal of gastroenterology and hepatology 2012;27:1423-1431.
27. Bae JM, Kim JH, Kang GH. Molecular subtypes of colorectal cancer and their clinicopathologic features, with an emphasis on the serrated neoplasia pathway. Archives of pathology & laboratory medicine 2016;140:406-412.
28. Worthley DL, Leggett BA. Colorectal cancer: molecular features and clinical opportunities. The Clinical Biochemist Reviews 2010;31:31.
29. Nguyen LH, Goel A, Chung DC. Pathways of colorectal carcinogenesis. Gastroenterology 2020;158:291-302.
30. Maloy S, Hughes K. Brenner's encyclopedia of genetics: Academic Press, 2013.
31. Kadowaki S, Kakuta M, Takahashi S, et al. Prognostic value of KRAS and BRAF mutations in curatively resected colorectal cancer. World Journal of Gastroenterology: WJG 2015;21:1275.
32. Offermans K, Jenniskens JC, Simons CC, et al. Expression of proteins associated with the Warburg-effect and survival in colorectal cancer. The Journal of Pathology: Clinical Research 2021.
33. Offermans K, Jenniskens JC, Simons CC, et al. Association between mutational subgroups, Warburg-subtypes, and survival in patients with colorectal cancer. Cancer Medicine 2022.
34. de la Chapelle A. Microsatellite instability. New England Journal of Medicine 2003;349:209-210.
35. Singh MP, Rai S, Pandey A, et al. Molecular subtypes of colorectal cancer: An emerging therapeutic opportunity for personalized medicine. Genes & Diseases 2021;8:133-145.
36. Nojadeh JN, Sharif SB, Sakhinia E. Microsatellite instability in colorectal cancer. EXCLI journal 2018;17:159.
37. Baretti M, Le DT. DNA mismatch repair in cancer. Pharmacology & therapeutics 2018;189:45-62.
38. Carethers JM. Microsatellite instability pathway and EMAST in colorectal cancer. Current colorectal cancer reports 2017;13:73-80.
39. Sinicrope FA, Sargent DJ. Molecular pathways: microsatellite instability in colorectal cancer: prognostic, predictive, and therapeutic implications. Clinical cancer research 2012;18:1506-1512.
40. Arends MJ. Pathways of colorectal carcinogenesis. Applied Immunohistochemistry & Molecular Morphology 2013;21:97-102.
41. Fedorova MS, Krasnov GS, Lukyanova EN, et al. The CIMP-high phenotype is associated with energy metabolism alterations in colon adenocarcinoma. BMC Medical genetics 2019;20:15-27.
42. Advani SM, Advani P, DeSantis SM, et al. Clinical, pathological, and molecular characteristics of CpG island methylator phenotype in colorectal cancer: a systematic review and meta-analysis. Translational oncology 2018;11:1188-1201.
43. Rex DK, Ahnen DJ, Baron JA, et al. Serrated lesions of the colorectum: review and recommendations from an expert panel. The American journal of gastroenterology 2012;107:1315.
44. Hughes LA, Khalid-de Bakker CA, Smits KM, et al. The CpG island methylator phenotype in colorectal cancer:

progress and problems. Biochimica et Biophysica Acta (BBA)-Reviews on Cancer 2012;1825:77-85.

45. Müller MF, Ibrahim AE, Arends MJ. Molecular pathological classification of colorectal cancer. Virchows Archiv 2016;469:125-134.
46. Cancer Genome Atlas Network. Comprehensive molecular characterization of human colon and rectal cancer. Nature 2012;487:330-337.
47. Guinney J, Dienstmann R, Wang X, et al. The consensus molecular subtypes of colorectal cancer. Nature medicine 2015;21:1350-1356.
48. Budinska E, Popovici V, Tejpar S, et al. Gene expression patterns unveil a new level of molecular heterogeneity in colorectal cancer. The Journal of pathology 2013;231:63-76.
49. De Sousa E Melo F, Wang X, Jansen M, et al. Poor-prognosis colon cancer is defined by a molecularly distinct subtype and develops from serrated precursor lesions. Nature medicine 2013;19:614-618.
50. Marisa L, de Reyniès A, Duval A, et al. Gene expression classification of colon cancer into molecular subtypes: characterization, validation, and prognostic value. PLoS medicine 2013;10:e1001453.
51. Roepman P, Schlicker A, Tabernero J, et al. Colorectal cancer intrinsic subtypes predict chemotherapy benefit, deficient mismatch repair and epithelial-to-mesenchymal transition. International journal of cancer 2014;134:552-562.
52. Sadanandam A, Lyssiotis CA, Homicsko K, et al. A colorectal cancer classification system that associates cellular phenotype and responses to therapy. Nature medicine 2013;19:619-625.
53. Schlicker A, Beran G, Chresta CM, et al. Subtypes of primary colorectal tumors correlate with response to targeted treatment in colorectal cell lines. BMC medical genomics 2012;5:1-15.
54. Hanahan D, Weinberg RA. The hallmarks of cancer. cell 2000;100:57-70.
55. Hanahan D, Weinberg RA. Hallmarks of cancer: the next generation. cell 2011;144:646-674.
56. Hanahan D. Hallmarks of cancer: new dimensions. Cancer discovery 2022;12:31-46.
57. Warburg O. The metabolism of carcinoma cells. The Journal of Cancer Research 1925;9:148-163.
58. Potter M, Newport E, Morten KJ. The Warburg effect: 80 years on. Biochemical Society Transactions 2016;44:1499-1505.
59. Jóźwiak P, Forma E, Bryś M, et al. O-GlcNAcylation and metabolic reprograming in cancer. Frontiers in endocrinology 2014;5:145.
60. Gravenmier CA, Siddique M, Gatenby RA. Adaptation to stochastic temporal variations in intratumoral blood flow: the Warburg effect as a bet hedging strategy. Bulletin of Mathematical Biology 2018;80:954-970.
61. Vander Heiden MG, Cantley LC, Thompson CB. Understanding the Warburg effect: the metabolic requirements of cell proliferation. science 2009;324:1029-1033.
62. Devic S. Warburg effect-a consequence or the cause of carcinogenesis? Journal of Cancer 2016;7:817.
63. Kato Y, Maeda T, Suzuki A, et al. Cancer metabolism: New insights into classic characteristics. Jpn Dent Sci Rev 2018;54:8-21.
64. Meijer TW, Schuurbiers OC, Kaanders JH, et al. Differences in metabolism between adeno- and squamous cell non-small cell lung carcinomas: spatial distribution and prognostic value of GLUT1 and MCT4. Lung Cancer 2012;76:316-23.
65. Bensinger SJ, Christofk HR. New aspects of the Warburg effect in cancer cell biology. Semin Cell Dev Biol 2012;23:352-61.

66. Karar J, Maity A. PI3K/AKT/mTOR pathway in angiogenesis. Frontiers in molecular neuroscience 2011;4:51.
67. Molinari F, Frattini M. Functions and Regulation of the PTEN Gene in Colorectal Cancer. Front Oncol 2013;3:326.
68. Yap TA, Bjerke L, Clarke PA, et al. Drugging PI3K in cancer: refining targets and therapeutic strategies. Curr Opin Pharmacol 2015;23:98-107.
69. Hussain A, Qazi AK, Mupparapu N, et al. Modulation of glycolysis and lipogenesis by novel PI3K selective molecule represses tumor angiogenesis and decreases colorectal cancer growth. Cancer Lett 2016;374:250-60.
70. Slattery ML, Mullany LE, Sakoda LC, et al. The PI3K/AKT signaling pathway: Associations of miRNAs with dysregulated gene expression in colorectal cancer. Mol Carcinog 2018;57:243-261.
71. Tian X, Liu M, Huang X, et al. Noscapine Induces Apoptosis in Human Colon Cancer Cells by Regulating Mitochondrial Damage and Warburg Effect via PTEN/PI3K/mTOR Signaling Pathway. Onco Targets Ther 2020;13:5419-5428.
72. Yeung SJ, Pan J, Lee MH. Roles of p53, MYC and HIF-1 in regulating glycolysis - the seventh hallmark of cancer. Cell Mol Life Sci 2008;65:3981-99.
73. Monsalves E, Juraschka K, Tateno T, et al. The PI3K/AKT/mTOR pathway in the pathophysiology and treatment of pituitary adenomas. Endocrine-Related Cancer 2014;21:R331-R344.
74. Cairns RA, Harris IS, Mak TW. Regulation of cancer cell metabolism. Nature Reviews Cancer 2011;11:85-95.
75. De Roock W, De Vriendt V, Normanno N, et al. KRAS, BRAF, PIK3CA, and PTEN mutations: implications for targeted therapies in metastatic colorectal cancer. The lancet oncology 2011;12:594-603.
76. Monzon JG, Dancey JE. Markers of Sensitivity and Resistance to EGFR Inhibitors in Colorectal Cancer. Handbook of Therapeutic Biomarkers in Cancer: Jenny Stanford Publishing, 2021:221-270.
77. Marie SKN, Shinjo SMO. Metabolism and brain cancer. Clinics 2011;66:33-43.
78. Yang J, Nie J, Ma X, et al. Targeting PI3K in cancer: mechanisms and advances in clinical trials. Molecular cancer 2019;18:1-28.
79. Aashiq M, Silverman DA, Na'ara S, et al. Radioiodine-refractory thyroid cancer: molecular basis of redifferentiation therapies, management, and novel therapies. Cancers 2019;11:1382.
80. Tong Z, Cheng M, Yu Y, et al. Correlation between pharmacokinetic parameters of 5-fluorouracil and related metabolites and adverse reactions in East-Asian patients with advanced colorectal cancer. Cancer Chemotherapy and Pharmacology 2022;89:323-330.
81. Andre N, Schmiegel W. Chemoradiotherapy for colorectal cancer. Gut 2005;54:1194-1202.
82. Aparicio J, Esposito F, Serrano S, et al. Metastatic colorectal cancer. First line therapy for unresectable disease. Journal of Clinical Medicine 2020;9:3889.
83. Roelands J, Kuppen PJ, Vermeulen L, et al. Immunogenomic classification of colorectal cancer and therapeutic implications. International journal of molecular sciences 2017;18:2229.
84. Kawakami H, Zaanan A, Sinicrope FA. Microsatellite instability testing and its role in the management of colorectal cancer. Current treatment options in oncology 2015;16:1-15.
85. Ahmed S, Johnson K, Ahmed O, et al. Advances in the management of colorectal cancer: from biology to treatment. International journal of colorectal disease 2014;29:1031-1042.
86. Reinert T, Henriksen TV, Christensen E, et al. Analysis of plasma cell-free DNA by ultradeep sequencing in patients with stages I to III colorectal cancer. JAMA oncology 2019;5:1124-1131.
87. Labianca R, Nordlinger B, Beretta G, et al. Early colon cancer: ESMO Clinical Practice Guidelines for diagnosis,

treatment and follow-up. Annals of oncology 2013;24:vi64-vi72.

88. Vodenkova S, Buchler T, Cervena K, et al. 5-fluorouracil and other fluoropyrimidines in colorectal cancer: Past, present and future. Pharmacology & therapeutics 2020;206:107447.
89. Ganesh K, Stadler ZK, Cercek A, et al. Immunotherapy in colorectal cancer: rationale, challenges and potential. Nature reviews Gastroenterology & hepatology 2019;16:361-375.
90. Specialisten FM. Richtlijnendatabase. URL: https://richtlijnendatabase.nl/richtlijn/colorectaal_carcinoom_crc/gemetastaseerd_colorectaalcarcinoom_crc.html 2020.
91. Lemmens V, Steenbergen Lv, Janssen-Heijnen M, et al. Trends in colorectal cancer in the south of the Netherlands 1975–2007: rectal cancer survival levels with colon cancer survival. Acta oncologica 2010;49:784-796.
92. Elferink M, Van Steenbergen L, Krijnen P, et al. Marked improvements in survival of patients with rectal cancer in the Netherlands following changes in therapy, 1989–2006. European journal of cancer 2010;46:1421-1429.
93. Group F-NBW. BEST (Biomarkers, endpoints, and other tools) resource [Internet]. 2016.
94. Koncina E, Haan S, Rauh S, et al. Prognostic and predictive molecular biomarkers for colorectal cancer: updates and challenges. Cancers 2020;12:319.
95. Lee JK, Chan AT. Molecular prognostic and predictive markers in colorectal cancer: current status. Current colorectal cancer reports 2011;7:136-144.
96. Eriksen AC, Sørensen FB, Lindebjerg J, et al. The prognostic value of tumour stroma ratio and tumour budding in stage II colon cancer. A nationwide population-based study. International journal of colorectal disease 2018;33:1115-1124.
97. Marks K, West N, Morris E, et al. Clinicopathological, genomic and immunological factors in colorectal cancer prognosis. Journal of British Surgery 2018;105:e99-e109.
98. Kuhn N, Klinger B, Uhlitz F, et al. Mutation-specific effects of NRAS oncogenes in colorectal cancer cells. Advances in Biological Regulation 2021;79:100778.
99. Koulouridi A, Karagianni M, Messaritakis I, et al. Prognostic value of kras mutations in colorectal cancer patients. Cancers 2022;14:3320.
100. Saeed O, Lopez-Beltran A, Fisher KW, et al. RAS genes in colorectal carcinoma: pathogenesis, testing guidelines and treatment implications. Journal of clinical pathology 2019;72:135-139.
101. Lugli A, Kirsch R, Ajioka Y, et al. Recommendations for reporting tumor budding in colorectal cancer based on the International Tumor Budding Consensus Conference (ITBCC) 2016. Modern pathology 2017;30:1299-1311.
102. Fridman WH, Sautès-Fridman C, Galon J. The immune contexture in human tumours: impact on clinical outcome. Nature Reviews Cancer 2012;12:298-306.
103. Mesker WE, Junggeburt J, Szuhai K, et al. The carcinoma–stromal ratio of colon carcinoma is an independent factor for survival compared to lymph node status and tumor stage. Analytical Cellular Pathology 2007;29:387-398.
104. Li MX, Liu XM, Zhang XF, et al. Prognostic role of neutrophil-to-lymphocyte ratio in colorectal cancer: A systematic review and meta-analysis. International journal of cancer 2014;134:2403-2413.
105. Lugli A, Zlobec I, Berger MD, et al. Tumour budding in solid cancers. Nature Reviews Clinical Oncology 2021;18:101-115.
106. Heelkunde NVv. Update Richtlijn Colorectaal Carcinoom Juni 2022. URL: https://pathology.nl/wp-content/uploads/2022/06/Update-Richtlijn-Colorectaal-Carcinoom-11-nieuwe-conceptmodules-Juni-2022.pdf 2022.

107. Yu M, Chen S, Hong W, et al. Prognostic role of glycolysis for cancer outcome: evidence from 86 studies. J Cancer Res Clin Oncol 2019;145:967-999.
108. Dong S, Liang S, Cheng Z, et al. ROS/PI3K/Akt and Wnt/β-catenin signalings activate HIF-1α-induced metabolic reprogramming to impart 5-fluorouracil resistance in colorectal cancer. Journal of Experimental & Clinical Cancer Research 2022;41:1-27.
109. Liu C, Jin Y, Fan Z. The Mechanism of Warburg Effect-Induced Chemoresistance in Cancer. Frontiers in oncology 2021:3408.
110. Zaal EA, Berkers CR. The influence of metabolism on drug response in cancer. Frontiers in oncology 2018;8:500.
111. Desbats MA, Giacomini I, Prayer-Galetti T, et al. Metabolic plasticity in chemotherapy resistance. Frontiers in oncology 2020;10:281.
112. Zhong J-T, Zhou S-H. Warburg effect, hexokinase-II, and radioresistance of laryngeal carcinoma. Oncotarget 2017;8:14133.
113. Morandi A, Indraccolo S. Linking metabolic reprogramming to therapy resistance in cancer. Biochimica et Biophysica Acta (BBA)-Reviews on Cancer 2017;1868:1-6.
114. Kitazawa M, Hatta T, Sasaki Y, et al. Promotion of the Warburg effect is associated with poor benefit from adjuvant chemotherapy in colorectal cancer. Cancer science 2020;111:658.
115. Papa S, Choy PM, Bubici C. The ERK and JNK pathways in the regulation of metabolic reprogramming. Oncogene 2019;38:2223-2240.
116. Kang S, Bader AG, Vogt PK. Phosphatidylinositol 3-kinase mutations identified in human cancer are oncogenic. Proceedings of the National Academy of Sciences 2005;102:802-807.
117. Feron O. Pyruvate into lactate and back: from the Warburg effect to symbiotic energy fuel exchange in cancer cells. Radiotherapy and oncology 2009;92:329-333.
118. Chandel V, Raj S, Choudhari R, et al. Role of c-Met/HGF Axis in Altered Cancer Metabolism. Cancer Cell Metabolism: A Potential Target for Cancer Therapy: Springer, 2020:89-102.
119. Vasaikar S, Huang C, Wang X, et al. Proteogenomic analysis of human colon cancer reveals new therapeutic opportunities. Cell 2019;177:1035-1049. e19.
120. Weijenberg MP, Hughes LA, Bours MJ, et al. The mTOR Pathway and the Role of Energy Balance Throughout Life in Colorectal Cancer Etiology and Prognosis: Unravelling Mechanisms Through a Multidimensional Molecular Epidemiologic Approach. Curr Nutr Rep 2013;2:19-26.
121. Wang N, Khankari NK, Cai H, et al. Prediagnosis body mass index and waist–hip circumference ratio in association with colorectal cancer survival. International journal of cancer 2017;140:292-301.
122. Laake I, Larsen IK, Selmer R, et al. Pre-diagnostic body mass index and weight change in relation to colorectal cancer survival among incident cases from a population-based cohort study. BMC cancer 2016;16:1-10.
123. Fedirko V, Romieu I, Aleksandrova K, et al. Pre-diagnostic anthropometry and survival after colorectal cancer diagnosis in Western European populations. International journal of cancer 2014;135:1949-1960.
124. Taghizadeh N, Boezen HM, Schouten JP, et al. BMI and lifetime changes in BMI and cancer mortality risk. PloS one 2015;10:e0125261.
125. Campbell PT, Newton CC, Newcomb PA, et al. Association between body mass index and mortality for colorectal cancer survivors: overall and by tumor molecular phenotype. Cancer Epidemiology and Prevention Biomarkers 2015;24:1229-1238.

126. Hardikar S, Newcomb PA, Campbell PT, et al. Prediagnostic Physical Activity and Colorectal Cancer Survival: Overall and Stratified by Tumor CharacteristicsPhysical Activity and Colorectal Cancer Survival. Cancer Epidemiology, Biomarkers & Prevention 2015;24:1130-1137.
127. Jayasekara H, English DR, Haydon A, et al. Associations of alcohol intake, smoking, physical activity and obesity with survival following colorectal cancer diagnosis by stage, anatomic site and tumor molecular subtype. International journal of cancer 2018;142:238-250.
128. Walter V, Jansen L, Knebel P, et al. Physical activity and survival of colorectal cancer patients: Population-based study from Germany. International journal of cancer 2017;140:1985-1997.
129. Arem H, Pfeiffer RM, Engels EA, et al. Pre-and postdiagnosis physical activity, television viewing, and mortality among patients with colorectal cancer in the National Institutes of Health–AARP diet and health study. Journal of Clinical Oncology 2015;33:180.
130. Boyle T, Fritschi L, Platell C, et al. Lifestyle factors associated with survival after colorectal cancer diagnosis. British journal of cancer 2013;109:814-822.
131. Castejón M, Plaza A, Martinez-Romero J, et al. Energy restriction and colorectal cancer: a call for additional research. Nutrients 2020;12:114.
132. Lv M, Zhu X, Wang H, et al. Roles of caloric restriction, ketogenic diet and intermittent fasting during initiation, progression and metastasis of cancer in animal models: a systematic review and meta-analysis. PloS one 2014;9:e115147.
133. Ogino S, Chan AT, Fuchs CS, et al. Molecular pathological epidemiology of colorectal neoplasia: an emerging transdisciplinary and interdisciplinary field. Gut 2011;60:397-411.
134. Campbell PT, Ambrosone CB, Nishihara R, et al. Proceedings of the fourth international molecular pathological epidemiology (MPE) meeting. Cancer Causes & Control 2019;30:799-811.
135. Nielsen JS, Jakobsen E, Hølund B, et al. Prognostic significance of p53, Her-2, and EGFR overexpression in borderline and epithelial ovarian cancer. International Journal of Gynecologic Cancer 2004;14.
136. Dijkema I, Struikmans H, Dullens H, et al. Influence of p53 and bcl-2 on proliferative activity and treatment outcome in head and neck cancer patients. Oral oncology 2000;36:54-60.
137. Jenniskens JC, Offermans K, Samarska I, et al. Validity and reproducibility of immunohistochemical scoring by trained non-pathologists on Tissue MicroArrays. Cancer Epidemiology and Prevention Biomarkers 2021.
138. van den Brandt PA, Goldbohm RA, van 't Veer P, et al. A large-scale prospective cohort study on diet and cancer in The Netherlands. J Clin Epidemiol 1990;43:285-95.
139. Casparie M, Tiebosch AT, Burger G, et al. Pathology databanking and biobanking in The Netherlands, a central role for PALGA, the nationwide histopathology and cytopathology data network and archive. Cell Oncol 2007;29:19-24.
140. van den Brandt PA, Schouten LJ, Goldbohm RA, et al. Development of a record linkage protocol for use in the Dutch Cancer Registry for Epidemiological Research. Int J Epidemiol 1990;19:553-8.
141. van den Brandt PA. Molecular pathological epidemiology of lifestyle factors and colorectal and renal cell cancer risk. Maastricht Pathology 2018. 11th Joint Meeting of the British Division of the International Academy of Pathology and the Pathological Society of Great Britain & Ireland, 19-22 June 2018. . J Pathol 2018;246 Suppl 1: S9.

CHAPTER 2

Validity and reproducibility of immunohistochemical scoring by trained non-pathologists on tissue microarrays

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ABSTRACT

Background

Scoring of immunohistochemistry (IHC) staining is often done by non-pathologists, especially in large-scale tissue microarray (TMA)-based studies. Studies on the validity and reproducibility of scoring results from non-pathologists are limited. Therefore, our main aim was to assess inter-observer agreement between trained non-pathologists and an experienced histopathologist for three IHC markers with different subcellular localization (nucleus/membrane/cytoplasm).

Methods

Three non-pathologists were trained in recognizing adenocarcinoma and IHC scoring by a senior histopathologist. Kappa statistics were used to analyze inter-observer and intra-observer agreement for 6,249 TMA cores from a colorectal cancer series.

Results

Inter-observer agreement between non-pathologists (independently scored) and the histopathologist was "substantial" for nuclear and membranous IHC markers (κ_{range} = 0.67–0.75 and κ_{range} = 0.61–0.69, respectively), and "moderate" for the cytoplasmic IHC marker (κ_{range} = 0.43–0.57). Scores of the three non-pathologists were also combined into a "combination score" (if at least two non-pathologists independently assigned the same score to a core, this was the combination score). This increased agreement with the pathologist ($\kappa_{nuclear}$ = 0.74; $\kappa_{membranous}$ = 0.73; $\kappa_{cytoplasmic}$ = 0.57). Inter-observer agreement between non-pathologists was "substantial" ($\kappa_{nuclear}$ = 0.78; $\kappa_{membranous}$ = 0.72; $\kappa_{cytoplasmic}$ = 0.61). Intra-observer agreement of non-pathologists was "substantial" to "almost perfect" ($\kappa_{nuclear,range}$ = 0.83–0.87; $\kappa_{membranous,range}$ = 0.75–0.82; $\kappa_{cytoplasmic}$ = 0.69). Overall, agreement was lowest for the cytoplasmic IHC marker.

Conclusions

This study shows that adequately trained non-pathologists are able to generate reproducible IHC scoring results, that are similar to those of an experienced histopathologist. A combination score of at least two non-pathologists yielded optimal results.

Impact

Non-pathologists can generate reproducible IHC results after appropriate training, making analyses of large-scale molecular pathological epidemiology studies feasible within an acceptable time frame.

INTRODUCTION

The introduction of the tissue microarray (TMA) technology by Kononen and colleagues[1] in 1998 has enabled large-scale studies using archival formalin-fixed paraffin-embedded (FFPE) tissue blocks[2, 3]. The TMA technology has the advantage that sampling of cores leaves the donor block relatively intact, allowing it to be sampled multiple times[3,4]. Furthermore, immunohistochemistry (IHC) on TMAs is cost effective and less time consuming than performing IHC on full tissue sections[2-6]. In addition, a higher level of assay standardization can be achieved, improving reproducibility of results[3, 4, 6–8].

Several studies have shown a high degree of concordance between IHC results obtained from TMA sections and full sections when three 0.6 mm cores per case were used[9–13]. Interestingly, a study by Gavrielides and colleagues[14] found slightly higher inter-observer agreement for human epidermal growth factor receptor 2 (HER2) scoring on TMAs compared with full sections, suggesting a potential benefit of the restricted field of view. Manual scoring of TMA sections can take a considerable amount of time if individual scores need to be provided for hundreds or thousands of cores[7, 15]. Although scoring by automated image analysis has been proposed as a potential alternative to manual scoring, IHC markers present in tumor cells and other cell populations at the same time are challenging to assess automatically[16].

Scoring of IHC stained sections is often done by non-pathologists[17, 18]. However, studies on the validity of results from non-pathologists are limited. Jaraj and colleagues[19] suggested that after adequate training, non-pathologists are able to produce valid and reproducible IHC results for a cytoplasmic marker. However, it has been suggested that apart from the expert histopathologist knowledge, the agreement of IHC results between observers might also be affected by the subcellular localization of the marker of interest (nucleus/membrane/cytoplasm)[20]. There is a limited number of studies investigating scoring agreement of markers with different subcellular localizations. One of these studies reported similar overall kappa values for scoring of staining in different subcellular compartments[21, 22], whereas another study reported considerably lower agreement for scoring of cytoplasmic immunostaining[23].

We hypothesized that there is good inter-observer agreement between trained non-pathologists and pathologists for IHC scoring on TMAs, and that the inter-observer agreement does not depend on the subcellular localization of the staining. Therefore, the aims of the current study were to (i) assess inter-observer agreement between trained non-pathologists and an experienced pathologist, and (ii) assess agreement of three IHC markers with different subcellular localization (nucleus, membrane, cytoplasm)

METHODS

Study population, tissue collection, and tissue microarray construction

For TMA construction, tissue blocks from colorectal cancer resections of cases from the Netherlands Cohort Study (NLCS) were collected retrospectively from Dutch hospitals[24–26]. Hematoxylin & eosin (H&E)-stained sections were reviewed and the area with the highest tumor density was identified. From this area, three 0.6-mm-diameter cores with tumor and three cores with normal epithelium were sampled per case for TMA construction (TMA-Grandmaster, 3DHISTEC). In total, 78 TMA blocks were constructed containing 7,963 tumor cores. Ethical approval was obtained from Medical Ethical Committee MUMC, number METC 2019-1085.

Immunohistochemistry

Five mm thick serial sections were cut from all 78 TMA blocks and subjected to IHC using an automated immunostainer (DAKO Autostainer Link 48, Glostrup). p53, GLUT1, and PTEN were chosen as markers to assess inter-observer and intra-observer agreement in scoring nuclear, membranous, and cytoplasmic immunoreactivity, respectively, as these are established IHC markers routinely used in clinical setting. Details of primary antibodies and staining protocols are shown in **Table 1**. Staining protocols for all markers were optimized to eliminate background and nonspecific staining. Sections were counterstained with Mayer's Hematoxylin (VWR International B.V.), dehydrated, and mounted with a glass coverslip and xylene-based mounting medium (DPX, Sigma-Aldrich). All TMA sections were scanned using the Aperio scanner (Leica Microsystems) at 40x magnification at the University of Leeds (Leeds, UK) Scanning Facility.

Quality control

Presence of adenocarcinoma was confirmed for every individual core by reviewing the H&E-stained TMA sections. In case of tumor identification difficulties because of poor tumor differentiation or a large number of inflammatory cells, pan-cytokeratin staining was used to identify tumor cells.

Table 1 - Overview staining protocols, all performed using the DAKO Autostainer Link 48.

Antibody	Clone	Supplier (catalogue number)	Antigen retrieval	Dilution	Incubation time
Pan-CK	AE1/AE3	DAKO (GA05361-2)	PT high[a]	RTU[b]	10 min
p53	DO-7	DAKO (M700101-2)	PT high[a]	RTU[b]	20 min
GLUT1	-	Thermo Scientific (RB-9052-P)	PT low[c]	1:200	20 min
PTEN	6H2.1	DAKO (M362729-2)	PT high[a]	1:100	20 min

Note: visualization system, Envision FLEX Visualization Kit (K8008, DAKO); chromogen, 3,30-diaminobenzidine (DAB).

[a]High pH retrieval (K8004) for 20 minutes on the Dako PT link (Agilent Technologies)

[b]RTU: ready-to-use

[c]Low pH retrieval (K8005) for 20 minutes on the Dako PT link (Agilent Technologies)

Immunohistochemical scoring

Three non-pathologists (G.E. Fazzi: histology technician; K. Offermans: PhD student; J.C.A. Jenniskens: PhD student) were trained by a senior histopathologist (H.I. Grabsch) in (i) recognizing adenocarcinoma on H&E-stained TMA sections; (ii) recognizing immunoreactivity and distinguishing between immunoreactivity in the nucleus, membrane, and cytoplasm; and (iii) scoring of two TMA sections (200 cores) for every immunostaining to ensure that the same criteria were used by all assessors.

After training, the three non-pathologists scored all tumor cores for p53, GLUT1, and PTEN immunostainings. The scores from the three non-pathologists were combined into a "combination score". If at least two non-pathologists independently assigned the same score to a core, this score became the combination score. If all non-pathologists assigned different scores, the core was categorized as "no agreement". Because not all cores were scored by three non-pathologists for GLUT1 (**Table 2**), the remaining scores of the combination score were based on two non-pathologists. When comparing scores from pairs of trained non-pathologists to the score of the pathologist, non-pathologists' scores were combined as described for the combination score of three non-pathologists.

For evaluation of intra-observer agreement, two non-pathologists (assessor 2 and 3) evaluated 10% randomly selected TMA sections (range: 538–681 cores) per marker for a second time after a period of at least 5 months. These scores were only used to assess intra-observer agreement. To assess inter-observer agreement between pathologist and non-pathologists, an experienced pathologist (I. Samarska) evaluated the same 10% randomly selected TMA sections for every marker. The contribution of each assessor to the IHC scoring of the different markers is shown in **Table 2**.

p53 positivity was defined as unequivocal strong nuclear staining and scored semi-quantitatively as published previously[13, 27], with minor adaptations, as: (i) no positive tumor nuclei; (ii) ≤10% positive tumor nuclei; (iii) 11% to 50% positive tumor nuclei; (iv) 51% to 90% positive tumor nuclei; and (v) 91% to 100% positive tumor nuclei (**Figure 1A**).

Table 2 - Percentage of slides evaluated per assessor for all immunohistochemical markers.

Assessor	Experience	Nuclear (p53)	Membranous (GLUT1)	Cytoplasmic (PTEN)	Intra-observer[b]
1	NP	100%	25%[a]	100%	X
2	NP	100%	100%	100%	10%
3	NP	100%	100%	100%	10%
4	P	10%	10%	10%	X

NP: Non-pathologist; P: Pathologist.
[a]Assessor 1 left the project early because of an unforeseen work relocation.
[b]Percentage of slides re-scored per protein.

GLUT1 positivity was defined as any membranous (complete or incomplete) immunostaining of tumor cells, and scored as published previously[28,29]: (i) no tumor cells with membranous immunostaining; (ii) ≤10% tumor cells with membranous immunostaining; (iii) 11% to 50% tumor cells with membranous immunostaining; (iv) >50% tumor cells with membranous immunostaining (**Figure 1B**).

PTEN scoring was performed as described previously[30], comparing cytoplasmic immunostaining intensity of the tumor cells with that of adjacent stromal cells. PTEN immunostaining was classified as: (i) negative (no PTEN staining in the tumor cells); (ii) weak (staining intensity in the tumor cells weaker than in the stromal cells); (iii) moderate (similar staining intensity in tumor and stromal cells); or (iv) strong (staining intensity in the tumor cells stronger than in the stromal cells), see **Figure 1C**. In case of heterogeneous immunostaining, the region with the highest staining intensity prevailed.

Uninterpretable (e.g., folded cores) or missing cores were categorized as "uninterpretable" and excluded from analyses for all markers.

Statistical analysis

Inter-observer and intra-observer agreement was assessed using all cores that passed quality control. Cohen's kappa was used for assessing inter-observer agreement between assessor pairs and for assessing intra-observer agreement within one assessor[31]. Fleiss' kappa was used for assessing inter-observer agreement between more than two assessors[32]. All kappa values were weighted[33], taking into account the magnitude of the disagreement (e.g., ≤10% vs. >50% is worse than ≤10% vs. 11%–50%). A weight of 0.5 was chosen for scoring an adjacent category and a weight of zero for non-adjacent categories. Non-weighted Fleiss' kappa was used for assessing the variation in inter-observer agreement between scoring categories. To calculate kappa confidence intervals, the bootstrap method was used with 1,000 repetitions[34–36]. The interpretation of kappa values is shown in **Supplementary Table S1**. Agreement between each pair of assessors was determined, as well as agreement between the combination score of two or three non-pathologists and the pathologist's score (for the latter, cores for which no agreement was reached were excluded from analyses). Data were analyzed using Stata (version 15.1, Statacorp).

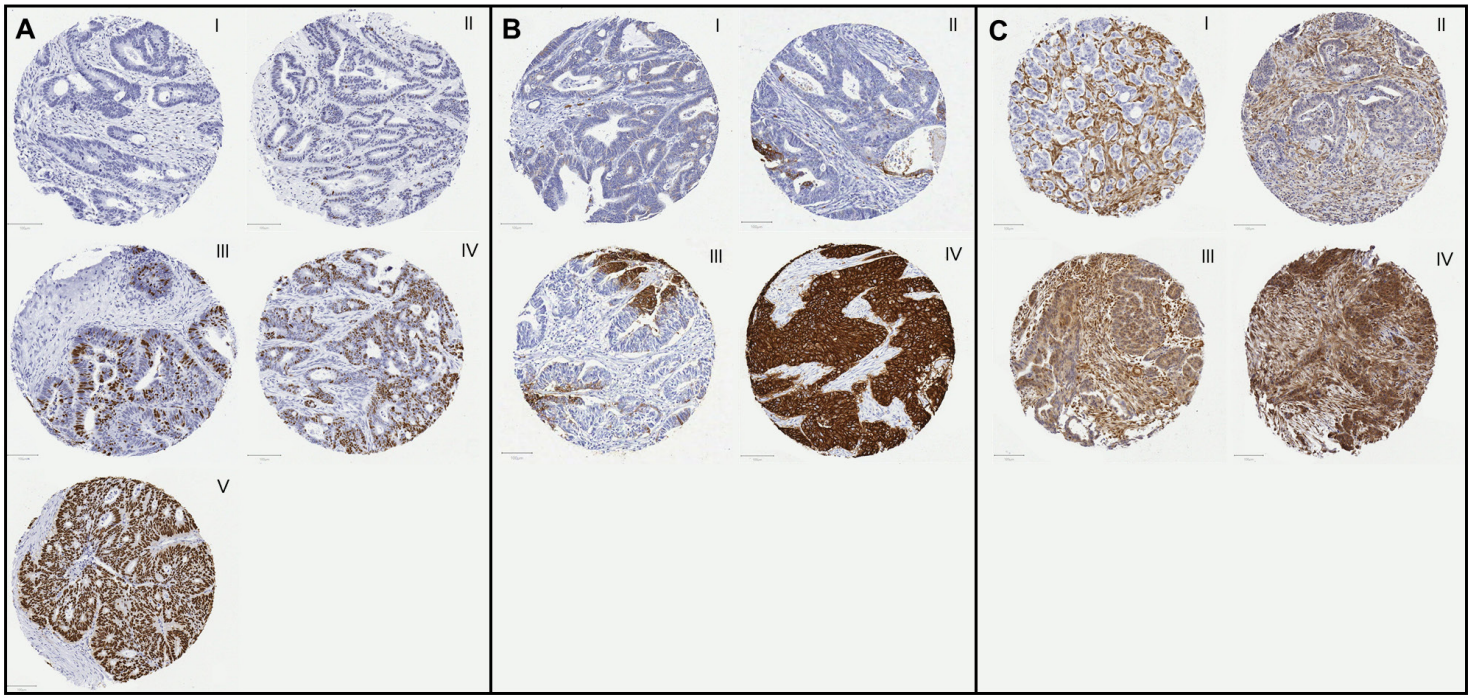

Figure 1 – IHC scoring of nuclear (p53), membranous (GLUT1), and cytoplasmic (PTEN) protein expression on TMAs. The localization of the antigen–antibody complex is visualized in brown (3,3′-diaminobenzidine) and counterstained in blue (hematoxylin). (**A**) Nuclear positivity was defined as unequivocal strong nuclear staining and scored as negative nuclear immunoreactivity (I); ≤10% nuclear immunoreactivity (II); 11%–50% nuclear immunoreactivity (III); 51%–90% nuclear immunoreactivity (IV); 90%–100% nuclear immunoreactivity (V). (**B**) Membranous positivity was defined as any membranous (complete or incomplete) immunostaining of tumor cells and scored as negative membranous immunoreactivity (I); ≤10% membranous immunoreactivity (II); 11%–50% membranous immunoreactivity (III); >50% membranous immunoreactivity (IV). (**C**) Cytoplasmic protein expression was evaluated by comparing the cytoplasmic immunostaining intensity of the tumor cells to that of adjacent stromal cells, and scored as negative cytoplasmic immunoreactivity (I); weak positive cytoplasmic immunoreactivity (II); moderate positive cytoplasmic immunoreactivity (III); strong positive cytoplasmic immunoreactivity (IV). In case of heterogeneous immunostaining, the region with the highest staining intensity prevailed.

RESULTS

In total, 78 TMA blocks containing 7,963 tumor cores were available. After quality control, 1,714 (21.5%) cores were excluded (464 missing cores; 1,135 cores lacking tumor tissue; 115 uninterpretable tissue cores), leaving 6,249 tumor cores for analyses. All cores were evaluated by at least two assessors (**Table 2**). Frequency distributions of scores assigned by all assessors for nuclear (p53), membranous (GLUT1), and cytoplasmic (PTEN) immunoreactivity are shown in **Supplementary Tables S2–S4**.

Inter-observer agreement

Non-pathologist versus pathologist

Weighted kappa values of inter-observer agreement between non-pathologists and pathologist are shown in **Table 3** (non-weighted kappa values in **Supplementary Table S5**). Kappa values of each individual non-pathologist with the pathologist showed "substantial" agreement for nuclear (κ_{range} = 0.67–0.75) and membranous immunostainings (κ_{range} = 0.61–0.69), and "moderate" for cytoplasmic immunostaining (κ_{range} = 0.43–0.57). The combination score of the three non-pathologists showed "substantial" agreement with the pathologist's score for nuclear (κ = 0.74) and membranous immunoreactivity (κ = 0.73), and "moderate" agreement for cytoplasmic immunoreactivity (κ = 0.57). The combination score of two non-pathologists showed similar agreement with the pathologist's score as the combination score of three non-pathologists ($\kappa_{nuclear,range}$ = 0.75–0.81; $\kappa_{membranous,range}$ = 0.75–0.79; $\kappa_{cytoplasmic,range}$ = 0.54–0.65). For the majority of scores (range, 90.3%–98.6%), equal or adjacent scoring categories were assigned (**Table 4**) by pathologist and non-pathologists.

In **Supplementary Table S6**, the agreement per scoring category is shown by non-weighted kappa values. The lowest and highest scoring categories show higher agreement among non-pathologist assessors ($\kappa_{nuclear}$ 0.83 and 0.79; $\kappa_{membranous}$ 0.68 and 0.82; $\kappa_{cytoplasmic}$ 0.61 and 0.51, respectively) than the scoring categories in between ($\kappa_{nuclear,range}$ = 0.35–0.56; $\kappa_{membranous,range}$ = 0.45–0.53; $\kappa_{cytoplasmic,range}$ = 0.49–0.53). Adding the pathologist assessor, this again led to highest agreement in the most extreme categories for nuclear and membranous stainings ($\kappa_{nuclear}$ 0.86 and 0.67; $\kappa_{membranous}$ 0.74 and 0.76, respectively). For cytoplasmic stainings the agreement was highest for the lowest scoring category, and decreased with increasing scoring categories ($\kappa_{category0}$ = 0.60; $\kappa_{category1}$ = 0.53; $\kappa_{category2}$ = 0.37; $\kappa_{category3}$ = 0.32).

Non-pathologist versus non-pathologist

Inter-observer agreement among non-pathologists is shown in **Table 3** (non-weighted kappa values in **Supplementary Table S5**). Overall kappa values between all three non-pathologists were similar to those comparing the combination score and the pathologist's score ($\kappa_{nuclear}$ 0.78 vs. 0.74; $\kappa_{membranous}$ 0.72 vs. 0.73; $\kappa_{cytoplasmic}$ 0.61 vs. 0.56, respectively). Scores for nuclear and membranous immunoreactivity showed the highest kappa

values among non-pathologists, with an overall weighted kappa of 0.78 (κ_{range} = 0.74–0.80) and 0.72 (κ_{range} = 0.66–0.81), respectively. Agreement was lowest for cytoplasmic immunoreactivity, with an overall kappa of 0.61 (κ_{range} = 0.55– 0.65). In the majority of non-pathologists' scores (range, 96.2%– 99.8%), equal or adjacent scoring categories were assigned (**Supplementary Table S6**).

Intra-observer agreement of non-pathologists

Weighted intra-observer kappa values of two non-pathologists are shown in **Table 5** (non-weighted kappa values in **Supplementary Table S7**). The intra-observer agreement was highest for scoring nuclear and membranous immunoreactivity, showing "almost perfect" agreement ($\kappa_{observer2}$ = 0.83; $\kappa_{observer3}$ = 0.87), and "substantial" to "almost perfect" agreement ($\kappa_{observer2}$ = 0.82; $\kappa_{observer3}$ = 0.75), respectively. Scoring of cytoplasmic immunoreactivity showed "substantial" agreement ($\kappa_{observer2}$ = 0.69; $\kappa_{observer3}$ = 0.69). In the majority of scores (range, 98.9%–100%), equal or adjacent categories were assigned at the first and second timepoint (**Supplementary Table S6**).

Table 3 – Inter-observer agreement (weighted) between non-pathologists and pathologist.

	Nuclear κ (95%-CI)	**Membranous κ (95%-CI)**	**Cytoplasmic κ (95%-CI)**
NP vs P[a]			
1 vs 4	0.75 (0.72-0.79)	0.61 (0.55-0.67)[f]	0.57 (0.53-0.61)
2 vs 4	0.67 (0.63-0.71)	0.69 (0.65-0.73)	0.43 (0.38-0.48)
3 vs 4	0.70 (0.67-0.74)	0.69 (0.66-0.73)	0.56 (0.52-0.60)
1+2 vs 4[b,c]	0.80 (0.77-0.84)	0.77 (0.70-0.83)[f]	0.57 (0.51-0.62)
1+3 vs 4[b,c]	0.81 (0.77-0.84)	0.79 (0.73-0.85)[f]	0.65 (0.60-0.70)
2+3 vs 4[b,c]	0.75 (0.72-0.79)	0.75 (0.72-0.79)	0.54 (0.50-0.60)
Combination score[c,d] vs 4	0.74 (0.71-0.78)	0.73 (0.69-0.77)	0.56 (0.52-0.61)
NP vs NP[e]			
1 vs 2	0.74 (0.73-0.75)	0.69 (0.67-0.72)[f]	0.55 (0.54-0.57)
1 vs 3	0.79 (0.79-0.80)	0.66 (0.64-0.69)[f]	0.64 (0.62-0.65)
2 vs 3	0.80 (0.79-0.81)	0.81 (0.80-0.82)	0.65 (0.64-0.67)
1 vs 2 vs 3[g]	0.78	0.72[f]	0.61

NP: non-pathologist; P: pathologist. Nuclear: p53; membranous: GLUT1; cytoplasmic: PTEN.
[a]Based on a random 10% of TMA sections (range 538-681 cores).
[b]Comparison of a combination of two non-pathologists with the pathologist: if the two non-pathologists independently assigned the same score to a core, this was the combined score. If the non-pathologists assigned a different score, the core was categorized as no agreement.
[c]Cores where no agreement was reached between non-pathologists (combination score = no agreement) were excluded for analyses.
[d]The combination score is based on all three non-pathologist's scores: if at least two assessors independently assigned the same score to a core, this was the combination score. If none of the assessors assigned the same score, the core was categorized as no agreement.
[e]Based on all cores (N_{cores} = 6249).
[f]Assessor 1 left the project early because of an unforeseen work relocation, 1457 cores were evaluated.
[g]Confidence interval for weighted kappa of multiple assessors (>2) could not be calculated using Stata.

Table 4 – Percentages of discrepancies between assessors.

	Nuclear Difference in categories[b]				Membranous[a] Difference in categories[b]				Cytoplasmic Difference in categories[b]			
	0	1	2	3/4	0	1	2	3	0	1	2	3
Inter-observer												
NP vs P[c]												
1 vs 4	57.9	32.4	8.0	1.8	62.5	29.4	6.0	2.2	54.1	42.1	3.9	0.0
2 vs 4	51.4	41.0	6.7	0.9	70.5	24.5	4.0	1.0	63.0	33.9	2.7	0.5
3 vs 4	61.7	32.0	5.1	1.2	70.6	25.2	3.1	1.0	62.1	36.5	1.4	0.0
Combination vs 4	69.8	27.1	2.9	0.2	73.7	22.7	3.0	0.7	64.6	34.8	0.7	0.0
NP vs NP[d]												
1 vs 2	72.0	24.2	3.5	0.3	68.3	29.2	2.1	0.4	65.4	34.0	0.6	0.0
1 vs 3	76.3	22.2	1.4	0.1	65.5	30.9	3.2	0.4	73.2	26.5	0.3	0.0
2 vs 3	76.3	22.4	1.2	0.1	81.4	17.2	1.3	0.1	76.0	23.8	0.2	0.0
Intra-observer												
NP vs NP[c]												
2 vs 2	82.6	16.3	1.0	0.0	82.4	16.6	1.0	0.0	78.8	21.2	0.0	0.0
3 vs 3	84.1	15.3	0.6	0.0	74.2	24.8	0.8	0.3	80.0	20.0	0.0	0.0

NP: non-pathologist; P: pathologist. Nuclear: p53; membranous: GLUT1; cytoplasmic: PTEN.
Uninterpretable cores were excluded.
[a]Assessor 1 left the project early because of an unforeseen work relocation.
[b]Difference in categories assigned by the two assessors: 0=same category assigned (no discrepancy); 1=adjacent categories were assigned (e.g., <10% positive and 11-50% positive); 2=difference between assigned categories was 2 (e.g., <10% positive and >50% positive); 3(/4)=difference between assigned categories was 3 or 4 (e.g., negative and >50%).
[c]Based on a random 10% of TMA sections.
[d]Based on all TMA sections.

Intra-observer agreement of non-pathologists

Weighted intra-observer kappa values of two non-pathologists are shown in **Table 5** (non-weighted kappa values in **Supplementary Table S7**). The intra-observer agreement was highest for scoring nuclear and membranous immunoreactivity, showing "almost perfect" agreement ($\kappa_{observer2}$ = 0.83; $\kappa_{observer3}$ = 0.87), and "substantial" to "almost perfect" agreement ($\kappa_{observer2}$ = 0.82; $\kappa_{observer3}$ = 0.75), respectively. Scoring of cytoplasmic immunoreactivity showed "substantial" agreement ($\kappa_{observer2}$ = 0.69; $\kappa_{observer3}$ = 0.69). In the majority of scores (range, 98.9%–100%), equal or adjacent categories were assigned at the first and second timepoint (**Supplementary Table S6**).

Table 5 – Intra-observer agreement (weighted) of two non-pathologists, based on 10% randomly selected TMA sections.

	Assessor 2 κ (95%-CI)	Assessor 3 κ (95%-CI)
Nuclear	0.83 (0.80-0.86)	0.87 (0.84-0.90)
Membranous	0.82 (0.79-0.85)	0.75 (0.72-0.78)
Cytoplasmic	0.69 (0.64-0.74)	0.69 (0.64-0.74)

Nuclear: p53; membranous: GLUT1; cytoplasmic: PTEN.

DISCUSSION

TMAs are increasingly used to analyze protein expression by IHC in large-scale studies[2, 3, 5, 37]. Scoring is often done by non-pathologists[17, 18]; however, only few studies reported validity and reproducibility of scoring results[38, 39]. To the best of our knowledge, our study is one of the first to investigate agreement of TMA-based scoring of immunoreactivity in different subcellular localizations by non-pathologists. Our study showed that inter-observer agreement between an experienced histopathologist and trained non-pathologists was "moderate" to "substantial." Agreement with the pathologist's score did not further increase when a combination score from three instead of two trained non-pathologists was used.

Inter-observer agreement non-pathologists versus pathologist

Our study demonstrates that non-pathologists can generate reproducible results. These results are in line with a previous study by Jaraj and colleagues[19], reporting comparable kappa values for inter-observer agreement between pathologists and non-pathologists. Even though it was not their main objective, two other studies reported comparable inter-observer agreement between pathologists and non-pathologists[22, 40]. However, some of the studies reported weighted kappa values[19, 22], but did not state what weights were assigned to adjacent scoring categories, making a direct comparison of kappa values with our study impossible.

Considering the subjectivity of immunoreactivity scoring, several studies recommended that scoring should be done by multiple assessors to improve inter-observer agreement[39, 41, 42]. Our study confirmed that combining scores from multiple non-pathologists into a combination score increased inter-observer agreement with the pathologist's score. Combining scores of three non-pathologists instead of two did not change inter-observer agreement with the pathologist, indicating that IHC scoring by two non-pathologists seems to be sufficient to yield reliable IHC results.

Immunoreactivity scoring in different subcellular localizations

A limited number of studies investigated scoring agreement of immunoreactivity in different subcellular localizations, showing inconsistent results[21–23]. We showed that scoring of nuclear and membranous immunoreactivity generally leads to higher inter-observer agreement compared with cytoplasmic immunoreactivity, consistent with results of Bolton and colleagues[23]. However, this is in contrast to two other studies which did not find a difference in the intra-observer and inter-observer agreement when scoring nuclear, membranous and cytoplasmic immunoreactivity[21, 22]. These discrepant results might be explained by the use of different IHC scoring methods between studies.

The IHC markers selected for the current study were chosen to provide a range of subcellular localizations (nucleus/membrane/cytoplasm) for scoring purposes. These markers are generalizable to other IHC stainings considering the subcellular localization.

Inter-observer agreement among non-pathologists

Hitherto, few studies reported inter-observer agreement of IHC results among non-pathologists. In the current study, we found "substantial" to "almost perfect" agreement among trained non-pathologists, which is in line with previously published results on TMAs and whole tissue sections[17–19].

Intra-observer agreement of non-pathologists

IHC studies often report intra-observer kappa values as a measure of reproducibility. Our study shows that non-pathologists are able to generate reproducible IHC scores after appropriate training, which is in line with previous studies[17–19, 40]. Interestingly, intra-observer kappa values of non-pathologists in the current study were similar to those previously reported for pathologists[23, 43]. In general, across all three markers, disagreements were limited to one-category discordances (e.g., <10% vs. 11%–50%) for all comparisons.

Limitations

Our study has some limitations. We have no information on intra-observer and inter-observer agreement of pathologists, as this was beyond the scope of this article. Furthermore, the current study used TMA cores to assess inter-observer and intra-observer agreement. It has been described in the literature that inter-observer agreement increases when using TMA cores compared with whole tissue sections[14]. Thus, it remains to be clarified whether the agreement among non-pathologists and between non-pathologists and pathologists is similar in full tissue sections. However, the aim of this study was specifically to investigate IHC scoring on TMAs, because non-pathologists will mainly be involved in IHC scoring in large-scale studies using TMAs. Also, we did not directly compare the scoring performance between trained non-pathologists and untrained non-pathologists; thus, we are not able to draw direct conclusions on the necessity of training, and in particular whether similar results would have been obtained without training.

Recommendations

We propose some recommendations which could improve comparability of IHC studies. First, it is important to report what weights were used for analyses of weighted kappa values. In addition, we think it would be of value to report both weighted and non-weighted kappa values. Second, it should be mentioned clearly in the methods what the IHC scoring experience of assessors was. If done by non-pathologists, it is important to report their training. Third, our results showed that disagreements were mostly limited to one-category discordances, suggesting that less refined scoring protocols may potentially improve

agreement. This is in line with previous studies[44, 45], in which the authors showed that agreement improved when using scoring protocols with less categories. However, we acknowledge that the number of categories of the scoring protocol depends on the novelty and clinical relevance of the biomarker being studied. Scoring protocols for potential new biomarkers might comprise more categories compared with well-known biomarkers. Finally, we suggest that IHC scoring should be performed by at least two non-pathologists to be able to assess inter-observer agreement among assessors. Ideally, these non-pathologists are trained by an expert pathologist and a certain percentage of samples (e.g., 10%) are double-scored by the pathologist to ensure quality of scoring.

CONCLUSION

In this large study investigating inter-observer and intra-observer agreement of TMA-based immunoreactivity scores between pathologists and non-pathologists, we have shown that non- pathologists can generate reproducible IHC scoring results that are similar to those of an experienced pathologist. A combination score of at least two non-pathologists yielded optimal results. Future studies are required to validate our findings and to examine the practical implications and impact of potential misclassification, by comparing effect estimates for established stain-outcome associations when using the pathologist's score versus the non-pathologists' combination score.

REFERENCES

1. Kononen J, Bubendorf L, Kallioniemi A, Bärlund M, Schraml P, Leighton S, et al. Tissue microarrays for high-throughput molecular profiling of tumor specimens. Nat Med 1998;4:844–7.
2. Tawfik El-Mansi M, Williams AR. Validation of tissue microarray technology using cervical adenocarcinoma and its precursors as a model system. Int J Gynecol Cancer 2006;16:1225–33.
3. Ilyas M, Grabsch H, Ellis IO, Womack C, Brown R, Berney D, et al. Guidelines and considerations for conducting experiments using tissue microarrays. Histopathology 2013;62:827–39.
4. Camp RL, Neumeister V, Rimm DL. A decade of tissue microarrays: progress in the discovery and validation of cancer biomarkers. J Clin Oncol 2008;26: 5630–7.
5. Domeny-Duarte P, Niero L, Domingues MAC. Tissue microarrays of bone marrow aspirate clot allow assessment of multiple samples. Pathology, research and practice. 2020;216:152721.
6. Shergill IS, Shergill NK, Arya M, Patel HRH. Tissue microarrays: a current medical research tool. Curr Med Res Opin 2004;20:707–12.
7. Bubendorf L, Nocito A, Moch H, Sauter G. Tissue microarray (TMA) technology: miniaturized pathology archives for high-throughput in situ studies. J Pathol 2001;195:72–9.
8. Al Kuraya K, Simon R, Sauter G. Tissue microarrays for high-throughput molecular pathology. Ann Saudi Med 2004;24:169–74.
9. Boone J, van Hillegersberg R, van Diest PJ, Offerhaus GJA, Borel Rinkes IHM, Ten Kate FJW. Validation of tissue microarray technology in squamous cell carcinoma of the esophagus. Virchows Arch 2008;452:507–14.
10. Zhang D, Salto-Tellez M, Putti TC, Do E, Koay ESC. Reliability of tissue microarrays in detecting protein expression and gene amplification in breast cancer. Mod Pathol 2003;16:79–84.
11. Hassan S, Ferrario C, Mamo A, Basik M. Tissue microarrays: emerging standard for biomarker validation. Curr Opin Biotechnol 2008;19:19–25.
12. Hoos A, Urist MJ, Stojadinovic A, Mastorides S, Dudas ME, Leung DH, et al. Validation of tissue microarrays for immunohistochemical profiling of cancer specimens using the example of human fibroblastic tumors. Am J Pathol 2001; 158:1245–51.
13. Jourdan F, Sebbagh N, Comperat E, Mourra N, Flahault A, Olschwang S, et al. Tissue microarray technology: validation in colorectal carcinoma and analysis of p53, hMLH1, and hMSH2 immunohistochemical expression. Virchows Arch 2003;443:115–21.
14. Gavrielides MA, Conway C, O'Flaherty N, Gallas BD, Hewitt SM. Observer performance in the use of digital and optical microscopy for the interpretation of tissue-based biomarkers. Anal Cell Pathol 2014;2014:157308.
15. Jawhar NM. Tissue microarray: a rapidly evolving diagnostic and research tool. Ann Saudi Med 2009;29:123–7.
16. O'Hurley G, Sjostedt E, Rahman A, Li B, Kampf C, Ponten F, et al. Garbage in, garbage out: a critical evaluation of strategies used for validation of immunohistochemical biomarkers. Mol Oncol 2014;8:783–98.
17. Nielsen JS, Jakobsen E, Holund B, Bertelsen K, Jakobsen A. Prognostic significance of p53, Her-2, and EGFR overexpression in borderline and epithelial ovarian cancer. Int J Gynecol Cancer 2004;14:1086–96.
18. Dijkema IM, Struikmans H, Dullens HF, Kal HB, van der Tweel I, Battermann JJ. Influence of p53 and bcl-2 on proliferative activity and treatment outcome in head and neck cancer patients. Oral Oncol 2000;36:54–60.

19. Jaraj SJ, Camparo P, Boyle H, Germain F, Nilsson B, Petersson F, et al. Intra-and interobserver reproducibility of interpretation of immunohistochemical stains of prostate cancer. Virchows Arch 2009;455:375–81.
20. Lejeune M, Jaen J, Pons L, Lopez C, Salvado MT, Bosch R, et al. Quantification of diverse subcellular immunohistochemical markers with clinicobiological relevancies: validation of a new computer-assisted image analysis procedure. J Anat 2008;212:868–78.
21. Zlobec I, Terracciano L, Jass JR, Lugli A. Value of staining intensity in the interpretation of immunohistochemistry for tumor markers in colorectal cancer. Virchows Arch 2007;451:763–9.
22. Kirkegaard T, Edwards J, Tovey S, McGlynn LM, Krishna SN, Mukherjee R, et al. Observer variation in immunohistochemical analysis of protein expression, time for a change? Histopathology 2006;48:787–94.
23. Bolton KL, Garcia-Closas M, Pfeiffer RM, Duggan MA, Howat WJ, Hewitt SM, et al. Assessment of automated image analysis of breast cancer tissue microarrays for epidemiologic studies. Cancer Epidemiol Biomarkers Prev 2010;19:992–9.
24. van den Brandt PA, Goldbohm RA, van't Veer P, Volovics A, Hermus RJ, Sturmans F. A large-scale prospective cohort study on diet and cancer in The Netherlands. J Clin Epidemiol 1990;43:285–95.
25. van den Brandt PA, Schouten LJ, Goldbohm RA, Dorant E, Hunen PM. Development of a record linkage protocol for use in the Dutch Cancer Registry for Epidemiological Research. Int J Epidemiol 1990;19:553–8.
26. van den Brandt PA. Molecular pathological epidemiology of lifestyle factors and colorectal and renal cell cancer risk. Maastricht Pathology 2018. 11th Joint Meeting of the British Division of the International Academy of Pathology and the Pathological Society of Great Britain & Ireland, 19–22 June 2018. J Pathol 2018;246:S1–S46.
27. Resnick MB, Routhier J, Konkin T, Sabo E, Pricolo VE. Epidermal growth factor receptor, c-MET, b-catenin, and p53 expression as prognostic indicators in stage II colon cancer: a tissue microarray study. Clin Cancer Res 2004;10:3069–75.
28. Cooper R, Sarioglu S, Sökmen S, Füzün M, Küpelioglu A, Valentine H, et al. Glucose transporter-1 (GLUT-1): a potential marker of prognosis in rectal carcinoma? Br J Cancer 2003;89:870–6.
29. Sakashita M, Aoyama N, Minami R, Maekawa S, Kuroda K, Shirasaka D, et al. Glut1 expression in T1 and T2 stage colorectal carcinomas: its relationship to clinicopathological features. Eur J Cancer 2001;37:204–9.
30. Richman SD, Adams R, Quirke P, Butler R, Hemmings G, Chambers P, et al. Pretrial inter-laboratory analytical validation of the FOCUS4 personalised therapy trial. J Clin Pathol 2016;69:35–41.
31. Cohen J. A coefficient of agreement for nominal scales. Educ Psychol Meas 1960; 20:37–46.
32. Fleiss JL. Measuring nominal scale agreement among many raters. Psychol Bull 1971;76:378–82.
33. Cohen J. Weighted kappa: nominal scale agreement provision for scaled disagreement or partial credit. Psychol Bull 1968;70:213–20.
34. Efron B, Tibshirani RJ. An introduction to the bootstrap Boca Raton, FL: Chapman & Hall; 1993.
35. Lee J, Fung KP. Confidence interval of the kappa coefficient by bootstrap resampling. Psychiatry Res 1993;49:97–8.
36. Reichenheim ME. Confidence intervals for the kappa statistic. Stata J 2004;4: 421–8.
37. Shiraishi T, Shinto E, Nearchou IP, Tsuda H, Kajiwara Y, Einama T, et al. Prognostic significance of mesothelin expression in colorectal cancer disclosed by area-specific four-point tissue microarrays. Virchows Arch 2020;477:409–20.

38. Cross SS. Observer accuracy in estimating proportions in images: implications for the semiquantitative assessment of staining reactions and a proposal for a new system. J Clin Pathol 2001;54:385–90
39. Adams EJ, Green JA, Clark AH, Youngson JH. Comparison of different scoring systems for immunohistochemical staining. J Clin Pathol 1999;52:75–7.
40. Gavrielides MA, Gallas BD, Lenz P, Badano A, Hewitt SM. Observer variability in the interpretation of HER2/neu immunohistochemical expression with unaided and computer-aided digital microscopy. Arch Pathol Lab Med 2011;135:233–42.
41. Masmoudi H, Hewitt SM, Petrick N, Myers KJ, Gavrielides MA. Automated quantitative assessment of HER-2/neu immunohistochemical expression in breast cancer. IEEE Trans Med Imaging 2009;28:916–25.
42. Obuchowski NA. How many observers are needed in clinical studies of medical imaging? AJR Am J Roentgenol 2004;182:867–9.
43. Ali A, Bell S, Bilsland A, Slavin J, Lynch V, Elgoweini M, et al. Investigating various thresholds as immunohistochemistry cutoffs for observer agreement. Appl Immunohistochem Mol Morphol 2017;25:599–608.
44. de Jong D, Rosenwald A, Chhanabhai M, Gaulard P, Klapper W, Lee A, et al. Immunohistochemical prognostic markers in diffuse large B-cell lymphoma: validation of tissue microarray as a prerequisite for broad clinical applications–a study from the Lunenburg Lymphoma Biomarker Consortium. J Clin Oncol 2007;25:805–12.
45. Rüschoff J, Dietel M, Baretton G, Arbogast S, Walch A, Monges G, et al. HER2 diagnostics in gastric cancer—guideline validation and development of standardized immunohistochemical testing. Virchows Arch 2010;457:299–307.

SUPPLEMENTARY MATERIAL

Supplementary Table S1 - Interpretation of Kappa values

Kappa coefficient	Agreement
0-0.20	Slight
0.21-0.40	Fair
0.41-0.60	Moderate
0.61-0.80	Substantial
0.81-1.00	Almost perfect

Supplementary Table S2 - Scoring percentages of nuclear (p53) immunoreactivity in tumor cells between non-pathologists, the combination score, and the pathologist score. In total, 6,249 tumor cores that passed quality control were evaluated.

Scoring categories	All cores N_{cores} = 6,249				Random 10% of cores N_{cores} = 538	
	NP1	NP2	NP3	Combination NP[c]	Combination NP[c]	P4
Negative	39.5	31.6	30.7	32.7	29.6	31.0
1-10%	12.1	14.0	18.7	15.4	15.2	11.5
11-50%	10.8	10.6	10.8	9.7	11.9	19.1
51-90%	9.9	7.9	14.4	10.1	10.4	22.1
91-100%	26.0	34.3	23.8	27.2	27.9	15.4
Uninterpretable[a]	1.7	1.6	1.6	1.3	1.3	0.7
No agreement[b]	-	-	-	3.5	3.7	-

NP: non-pathologist; P: pathologist

[a]Excluded from IHC analyses because of missing core, no tumor tissue in core, no immunostaining, or folded core.

[b]If none of the assessors assigned the same score, the core was categorized as no agreement for the combination score.

[c]The combination score is based on the non-pathologist's scores: if at least two assessors independently assigned the same score to a core, this was the combination score.

Supplementary Table S3 - Scoring percentages of membranous (GLUT1) immunoreactivity in tumor cells between non-pathologists, the combination score, and the pathologist score. In total, 6249 tumor cores that passed quality control were evaluated.

Scoring categories	All cores N_{cores} = 6,249				Random 10% of cores N_{cores} = 681	
	NP1[c]	NP2	NP3	Combination NP[d]	Combination NP[d]	P4
Negative	36.2	24.3	23.8	21.6	24.1	25.4
1-10%	23.5	17.6	15.9	12.4	12.8	18.2
11-50%	17.8	31.2	33.8	27.1	26.0	25.8
51-100%	20.3	25.0	24.0	22.0	26.6	30.1
Uninterpretable[a]	2.1	2.0	2.5	1.7	0.9	0.4
No agreement[b]	-	-	-	15.2	9.7	-

NP: non-pathologist; P: pathologist
[a]Excluded from IHC analyses because of missing core, no tumor tissue in core, no immunostaining, or folded core.
[b]If none of the assessors assigned the same score, the core was categorized as no agreement for the combination score.
[c]Assessor 1 left the project early because of an unforeseen work relocation, 1457 cores were evaluated.
[d]The combination score is based on the non-pathologist's scores: if at least two assessors independently assigned the same score to a core, this was the combination score.

Supplementary Table S4 - Scoring percentages of cytoplasmic (PTEN) immunoreactivity in tumor cells between non-pathologists, the combination score, and the pathologist score. In total, 6,249 tumor cores that passed quality control were evaluated.

Scoring categories	All cores N_{cores} = 6,249				Random 10% of cores N_{cores} = 645	
	NP1	NP2	NP3	Combination NP[c]	Combination NP[c]	P4
Negative	14.5	7.0	7.5	7.8	8.5	12.9
Weak positive	49.7	51.8	52.3	53.0	51.6	45.7
Moderate positive	22.5	32.6	31.2	28.7	30.2	20.2
Strong positive	8.6	5.6	5.4	5.8	4.7	17.8
Uninterpretable[a]	4.8	3.1	3.5	3.3	3.3	3.4
No agreement[b]	-	-	-	1.4	1.7	-

NP: non-pathologist; P: pathologist
[a]Excluded from IHC analyses because of missing core, no tumor tissue in core, no immunostaining, or folded core.
[b]If none of the assessors assigned the same score, the core was categorized as no agreement for the combination score.
[c]The combination score is based on the non-pathologist's scores: if at least two assessors independently assigned the same score to a core, this was the combination score.

Supplementary Table S5 – Inter-observer agreement (non-weighted) between the non-pathologists and the pathologist.

	Nuclear κ (95%-CI)	Membranous κ (95%-CI)	Cytoplasmic κ (95%-CI)
NP vs P[a]			
1 vs 4	0.63 (0.59-0.68)	0.50 (0.43-0.57)[f]	0.47 (0.42-0.52)
2 vs 4	0.51 (0.47-0.56)	0.60 (0.56-0.64)	0.31 (0.26-0.37)
3 vs 4	0.57 (0.52-0.62)	0.60 (0.56-0.64)	0.45 (0.39-0.50)
1+2 vs 4[b,c]	0.69 (0.64-0.74)	0.69 (0.61-0.77)[f]	0.47 (0.41-0.53)
1+3 vs 4[b,c]	0.70 (0.65-0.75)	0.72 (0.64-0.80)[f]	0.55 (0.49-0.61)
2+3 vs 4[b,c]	0.62 (0.57-0.67)	0.67 (0.62-0.72)	0.43 (0.37-0.49)
Combination score[c,d] vs 4	0.61 (0.57-0.66)	0.64 (0.59-0.69)	0.45 (0.40-0.50)
NP vs NP[e]			
1 vs 2	0.62 (0.60–0.63)	0.58 (0.55-0.61)[f]	0.45 (0.44-0.47)
1 vs 3	0.68 (0.67-0.70)	0.54 (0.51-0.58)[f]	0.56 (0.55-0.58)
2 vs 3	0.69 (0.67-0.70)	0.74 (0.73-0.76)	0.59 (0.58-0.61)
1 vs 2 vs 3	0.66 (0.65-0.67)	0.62 (0.59-0.64)[f]	0.54 (0.52-0.55)

NP: non-pathologist; P: pathologist. Nuclear: p53; membranous: GLUT1; cytoplasmic: PTEN.
[a]Based on a random 10% of TMA sections (range 538-681 cores).
[b]Comparison of a combination of two non-pathologists with the pathologist: if the two non-pathologists independently assigned the same score to a core, this was the combined score. If the non-pathologists assigned a different score, the core was categorized as no agreement.
[c]Cores where no agreement was reached between non-pathologists (combination score = no agreement) were excluded for analyses.
[d]The combination score is based on all three non-pathologist's scores: if at least two assessors independently assigned the same score to a core, this was the combination score. If none of the assessors assigned the same score, the core was categorized as no agreement.
[e]Based on all cores (N_{cores} = 6249).
[f]Assessor 1 left the project early because of an unforeseen work relocation, 1457 cores were evaluated.

Supplementary Table S6 – Inter-observer agreement (non-weighted) per scoring category of non-pathologist assessors (NP1-3) and all (NP1-3 & P4) assessors.

	Agreement non-pathologist assessors (NP1-3) N_{cores} = 6,249			Agreement all assessors (NP1-3 & P4) N_{cores} = 538 - 681		
Scoring categories	**Nuclear[a] κ**	**Membranous[b,c] κ**	**Cytoplasmic[d] κ**	**Nuclear[a] κ**	**Membranous[b,c] κ**	**Cytoplasmic[d] κ**
0	0.83	0.68	0.61	0.86	0.74	0.60
1	0.56	0.45	0.53	0.57	0.44	0.53
2	0.45	0.53	0.49	0.46	0.56	0.37
3	0.35	0.82	0.51	0.32	0.76	0.32
4	0.79	-	-	0.67	-	-
Not interpretable	0.65	0.71	0.62	0.64	0.59	0.54
Combined	0.66	0.62	0.53	0.62	0.64	0.47

Confidence intervals for kappa values per category could not be calculated using Stata.
[a]Nuclear (p53): 0 = negative; 1 = <10% positive nuclei; 2 = 11-50% positive nuclei; 3 = 51-90% positive nuclei; 4 = 91-100% positive nuclei.
[b]Membranous (GLUT1): 0 = negative; 1 = <10% positive membranes; 2 = 11-50% positive membranes; 3 = 51-100% positive membranes.
[c]Assessor 1 left the project early because of an unforeseen work relocation.
[d]Cytoplasmic (PTEN): 0 = negative; 1 = weak positive cytoplasm; 2 = moderate positive cytoplasm; 3 = strong positive.

Supplementary Table S7 – Intra-observer agreement (non-weighted) of two non-pathologists, based on 10% randomly selected TMA sections (N_{cores} = 538-681).

	Assessor 2 κ (95%-CI)	**Assessor 3 κ (95%-CI)**
Nuclear	0.73 (0.69-0.77)	0.80 (0.76-0.84)
Membranous	0.75 (0.72-0.79)	0.65 (0.60-0.69)
Cytoplasmic	0.63 (0.58-0.69)	0.65 (0.60-0.70)

Nuclear: p53; membranous: GLUT1; cytoplasmic: PTEN.

CHAPTER 3

Expression of proteins associated with the Warburg-effect and survival in colorectal cancer

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ABSTRACT

Previous research has suggested that the expression of proteins related to the Warburg-effect may have prognostic value in colorectal cancer (CRC), but results remain inconsistent. Our objective was to investigate the relationship between Warburg-subtypes and patient survival in a large population-based series of CRC patients. In the present study, we investigated the expression of six proteins related to the Warburg-effect (LDHA, GLUT1, MCT4, PKM2, p53, PTEN) by immunohistochemistry on tissue microarrays (TMAs) from 2,399 incident CRC patients from the prospective Netherlands Cohort Study. Expression levels of the six proteins were combined into a pathway-based sum-score and patients were categorized into three Warburg-subtypes (low/moderate/high). The associations between Warburg-subtypes and CRC-specific and overall survival were investigated using Kaplan–Meier curves and Cox regression models. CRC patients were classified as Warburg-low (*n* = 695, 29.0%), Warburg-moderate (*n* = 858, 35.8%) or Warburg-high (*n* = 841, 35.1%). Patients with Warburg-high CRC had the poorest CRC-specific (hazard ratio (HR) 1.17; 95% CI 1.00–1.38) and overall survival (HR 1.19; 95% CI 1.05–1.35), independent of known prognostic factors. In stratified analyses, this was particularly true for patients with tumor-node-metastasis (TNM) stage III CRC ($HR_{CRC\text{-}specific}$ 1.45; 95% CI 1.10–1.92 and $HR_{overall}$ 1.47; 95% CI 1.15–1.87), and cancers located in the rectum ($HR_{overall}$ 1.56; 95% CI 1.15–2.13). To our knowledge, this is the first study to identify the prognostic value of immunohistochemistry-based Warburg-subtypes in CRC. Our data suggest that Warburg-subtypes are related to potentially important differences in CRC survival. Further research is required to validate our findings and to investigate the potential clinical utility of these Warburg-subtypes in CRC.

INTRODUCTION

Colorectal cancer (CRC) is the third most common cancer worldwide[1, 2]. Despite advances in early detection and treatment of CRC patients, it remains the second most deadly cancer worldwide, accounting for more than 900,000 deaths every year[1, 2]. Currently, the tumor-node-metastasis (TNM) staging system remains the most important clinically used factor to predict patient prognosis[3, 4]. However, even within the same TNM stage, the prognosis of patients may differ significantly, most likely due to heterogeneity in patient and CRC characteristics[3-6]. It has been suggested that CRCs represent a heterogeneous group of tumors that develop via several distinct molecular pathways involving different genetic and epigenetic alterations[7-10]. One of the most frequently activated molecular pathways is the PI3K/AKT/mTOR signaling pathway, which is regulated by the tumor suppressor PTEN[11, 12]. It has been proposed that the PI3K/AKT/mTOR signaling pathway is involved in 'rewiring' cancer metabolism from oxidative phosphorylation towards aerobic glycolysis through the regulation of three transcription factors: HIF-1α, MYC, and p53[13-18].

Aerobic glycolysis, also known as the 'Warburg-effect', is characterized by increased glucose uptake and lactate secretion, even in the presence of oxygen[16, 17, 19]. First, glucose uptake by tumor cells is stimulated by upregulation of the expression of glucose transporter 1 (GLUT1)[17, 20]. Then, glycolytic flux and lactate production are increased by upregulation of the expression of pyruvate kinase M2 (PKM2), pyruvate dehydrogenase kinase 1 (PDK1) and lactate dehydrogenase A (LDHA)[16, 17]. Finally, the expression of monocarboxylate transporter 4 (MCT4) is increased to promote lactate secretion and prevent cytoplasmic acidification[17, 20].

The Warburg-effect is thought to increase the malignant potential of tumor cells[17] and may contribute to therapy resistance[21]. Glycolysis-related proteins are therefore considered to have potential prognostic value[22]. Numerous studies have investigated the prognostic potential of key glycolytic enzymes and transcriptional regulators in various types of cancer, including CRC, but results remain inconsistent (see Yu *et al.*[22] for meta-analysis). However, most previous studies focused on investigating a single protein involved in the Warburg-effect, while this pathway is much more complicated. Therefore, there may not be one single protein driving the Warburg effect, but rather a combination of proteins.

In the present study, we therefore attempted to capture the Warburg-effect by ensuring that the different steps of the pathway were represented by at least one protein. These steps include: (1) upstream regulation of the Warburg effect (PTEN, p53), (2) more glucose entering the pathway (GLUT1), (3) enhanced glycolysis (PKM2), (4) increased lactate production (LDHA), and (4) enhanced lactate secretion (MCT4). The expression levels of these six proteins (PTEN, p53, GLUT1, PKM2, LDHA, MCT4) were combined into a sum

score. Based on the sum score, patients were divided into three subgroups representing low, moderate or high likelihood of the presence of the Warburg-effect, hereafter referred to as the "Warburg-subtypes" (Warburg-low, -moderate, and -high, respectively). We then investigated the relationship between these Warburg-subtypes and patient survival in a large population-based series of CRC patients. We hypothesized that patients with Warburg-high CRC have a worse prognosis compared to patients with Warburg-low CRC.

METHODS

Design and study population

The population-based series of CRC patients was derived from the prospective Netherlands Cohort Study (NLCS), which has been described in detail previously[23]. In short, the NLCS was initiated in September 1986 (baseline) when 120,852 men and women, aged 55–69 years, completed a mailed, self-administered questionnaire on diet and other cancer risk factors[23]. The NLCS was approved by the institutional review boards of the TNO Quality of Life Research Institute (Zeist, the Netherlands) and Maastricht University (Maastricht, the Netherlands) (METC number 85-012). All cohort members consented to participation by completing the questionnaire. Follow-up for cancer incidence was established by annual record linkage with the Netherlands Cancer Registry and PALGA, the nationwide Dutch Pathology Registry[24, 25], covering 20.3 years of follow-up since study initiation (17 September 1986 until 1 January 2007). The estimated completeness of cancer incidence follow-up was >96%[26]. After excluding patients who reported a history of cancer (excluding non-melanoma skin cancer) at baseline, 4,597 incident CRC patients were available (**Figure 1**).

Tissue collection and TMA construction

Formalin-fixed paraffin-embedded (FFPE) tissue blocks from CRC patients were collected as part of the Rainbow-Tissue MicroArray (TMA) project during 2012–2017[27] (**Supplementary Table S1**). Tumor and normal tissue FFPE blocks were requested for 3,872 incident CRC patients, selected based on available linkage to PALGA record (which provides access to pathology laboratories) and surgical or endoscopic resection specimen with pathology report, excluding those who received neoadjuvant therapy. FFPE blocks from 3,021 CRC patients were retrieved from 43 pathology laboratories throughout the Netherlands (78% retrieval rate), after excluding patients without approval of donor pathology labs, without pathology report or FFPE blocks. For TMA construction, H&E-stained sections were reviewed by pathologists and areas with the highest tumor density were marked. Three 0.6 mm diameter cores with tumor and three cores with normal epithelium were sampled per patient (TMA-Grandmaster, 3D-Histech, Hungary). After excluding patients with unusable FFPE blocks, tumor cores from 2,694 CRC patients were successfully assembled in 78 TMA blocks (**Figure 1**).

Immunohistochemistry

Five micrometer thick serial sections were cut from all 78 TMA blocks and subjected to immunohistochemistry (IHC). Details of the primary antibodies and staining protocols are shown in **Supplementary Table S2**. After IHC, TMA sections were scanned using the Aperio scanner (Leica Microsystems, Milton Keynes, UK) at 40× magnification at the University of Leeds (UK) Scanning Facility.

Three non-pathologist observers (GEF: histology technician; KO: PhD student; JCAJ: PhD student) were trained by a senior histopathologist (HIG) in recognizing adenocarcinoma and IHC scoring. Presence of adenocarcinoma was confirmed for every individual core by reviewing the H&E-stained TMA sections in combination with the pan-cytokeratin-stained sections if necessary. Requiring at least one tumor core per patient, tumor cores of 2,497 CRC patients passed quality control (**Figure 1**).

After quality control, all tumor cores were scored by at least two observers, independently and blinded for patient characteristics (see **Supplementary Table S3** for percentage of slides evaluated per observer). Scoring protocols for all proteins and kappa values for inter- and intra-observer agreement are shown in **Supplementary Table S4**. In brief, the expression of p53 in the tumor cells was scored as negative; 1–10% positive nuclei; 11–50% positive nuclei; 51–90% positive nuclei; and 91–100% positive nuclei. PTEN expression was scored as negative (no staining in tumor cytoplasm); weak (staining of tumor cytoplasm weaker than adjacent stroma); moderate (similar staining intensity in tumor cytoplasm and adjacent stroma); or strong (staining of tumor cytoplasm stronger than adjacent stroma), according to the protocol of Richman *et al.*[28]. GLUT1 and MCT4 expression were scored as negative; 1–10% tumor cells with membranous staining; 11–50% positive tumor cells; and >50% positive tumor cells. LDHA was evaluated according to the protocol of Koukourakis *et al.*[29], with minor adaptations. LDHA expression was scored in the tumor cells as negative/weak cytoplasmic staining; 1–50% tumor cells strong cytoplasmic staining; >50% tumor cells strong cytoplasmic staining. PKM2 expression was scored in the tumor cells as negative/weak cytoplasmic staining; moderate cytoplasmic staining; 1–50% tumor cells strong cytoplasmic staining; and >50% tumor cells strong cytoplasmic staining.

Supplementary Figure S1 shows a flow diagram of the process of combining multiple core-level scores into patient-level Warburg-subtypes. Scores from individual observers were combined into a 'combination score' if the same score was given by at least two observers. If the score was discrepant between observers, cores were either reviewed jointly by the two initial observers to agree on a final score, or an experienced pathologist (IS) with special interest in gastrointestinal pathology determined the final score. To obtain patient-level data for every protein, the scores of all available tumor cores (1–3

tumor cores per patient) were averaged and the value was rounded to the nearest scoring category. The average score was categorized to achieve three approximately equal-sized groups, representing low, moderate, and high protein expression. Cut-offs for PTEN and p53 were based on published literature[28, 30], cut-offs for other proteins were determined based on the distribution of patients (**Supplementary Table S4** shows cut-offs per protein).

Establishing Warburg-subtypes

Warburg-subtypes were created using a sum score, where high protein expression for p53, GLUT1, LDHA, MCT4 or PKM2 added a score of 2 per protein, moderate expression a score of 1 per protein, and low expression a score of 0 per protein. Since PTEN is inversely associated with the Warburg-effect[31], its score was reversed, i.e., 2 = low expression, 1 = moderate expression, 0 = high expression. The resulting sum score ranged from 0 to 12, where a higher sum score indicated a higher probability of the presence of the Warburg effect. Patients with missing data for one or more of the proteins were excluded from further analyses, resulting in 2,399 CRC patients for which a Warburg-subtype could be determined (**Figure 1**). Based on the sum score, CRC patients were categorized into the 'Warburg-low' subtype (sum score 0–3, *n* = 698), 'Warburg-moderate' subtype (sum score 4–5, *n* = 859) or 'Warburg-high' subtype (sum score 6–12, *n* = 842) (**Supplementary Figure S1**).

DNA mismatch repair status

DNA mismatch repair (MMR) status was assessed by IHC for MLH1 and MSH2 proteins (see **Supplementary Table S2** for primary antibodies and staining protocols), and immunostaining was evaluated according to the protocol of Richman *et al.*[28]. Nuclear immunostaining of stromal cells or lymphocytes adjacent to the tumor served as internal positive controls. Tumors with loss of either MLH1 or MSH2 expression, in the presence of internal positive controls, were considered MMR deficient (dMMR). Tumors that expressed both MLH1 and MSH2 were considered MMR proficient (pMMR).

Clinical characteristics and follow-up

Information on patient and tumor characteristics, such as incidence date, TNM stage, tumor location, tumor differentiation grade, and initial treatment information was retrieved from the cancer registry or PALGA histopathology reports. Patients who were diagnosed at autopsy were excluded (*n* = 5), leaving 2,394 CRC patients for analyses (**Figure 1**). Follow-up for vital status of the CRC patients was carried out through linkage with the Central Bureau of Genealogy and the municipal population registries until 31 December 2012. Cause of death was retrieved from Statistics Netherlands. CRC-specific deaths included those with an underlying cause attributed to malignant neoplasms of the colon, rectosigmoid junction, and rectum (ICD-10 codes C18–C20). Vital status was available for 2,393 patients, and information regarding CRC-specific death was available for 2,356 patients.

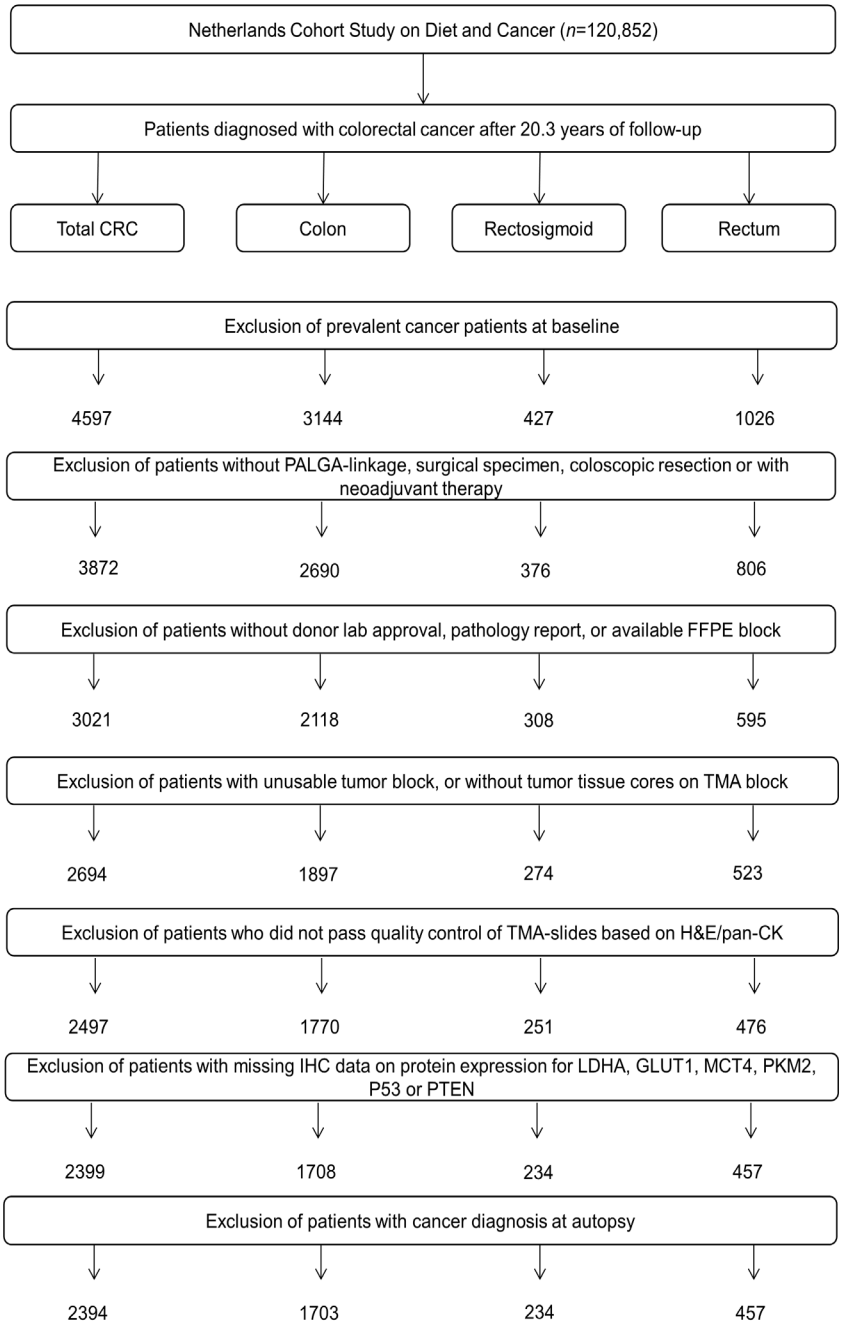


Figure 1 – Flow diagram of the number of CRC patients available for analyses in the Netherlands Cohort Study (NLCS), 1986-2006. CRC, colorectal cancer; PALGA, Netherlands pathology database; TMA, tissue microarray.

Statistical analyses

Descriptive statistics and frequency distributions were calculated for clinical characteristics. Differences between Warburg-subtypes were evaluated using Chi-square for categorical variables and Kruskal–Wallis tests for continuous variables. The primary endpoints of the current study were CRC-specific survival, defined as the time from CRC diagnosis to CRC-related death or end of follow-up, and overall survival, defined as the time from CRC diagnosis to death from any cause or end of follow-up. Because of the limited number of events in the later period with follow-up of more than 10 years (CRC-specific deaths: *n* = 33, 3.2%; overall deaths: *n* = 275, 15.1%), survival analyses were restricted to 10 years of follow-up. Analyses were stratified for TNM stage and tumor location. The relationship between Warburg-subtypes and CRC-specific or overall survival was estimated using Kaplan–Meier curves and Wilcoxon tests. Hazard ratios (HRs) and 95% CIs were estimated using Cox proportional hazards regression.

The proportional hazards assumption was tested using the scaled Schoenfeld residuals[32], by evaluating −log−log transformed survival curves and by introducing time-covariate interactions into the models. HRs were adjusted for a set of a priori selected prognostic factors: age at diagnosis, sex, tumor location, TNM stage, differentiation grade, MMR status and adjuvant therapy. A separate category ('unknown') was used for patients with unknown clinical information regarding TNM stage, differentiation grade, adjuvant therapy, or MMR status to enable inclusion of these patients in the Cox proportional hazards models.

Cancer stage was based on the pathological TNM classification, according to the edition that was valid at the time of cancer diagnosis (**Supplementary Table S5**). Hence, five different TNM versions have been used during the follow-up period (TNM versions 3–6). However, the main TNM stage groupings (I/II/III/IV) have remained essentially unchanged[33]. Year of diagnosis (1986–2006) and TNM version were considered as potential confounders to account for potential differences in clinical practice over the years. Both variables were not included in our final models because they did not introduce a ≥10% change in HRs.

In sensitivity analyses, we repeated analyses after excluding CRC patients who died within 30 days after diagnosis (*n* = 93). Furthermore, analyses were repeated after excluding CRC patients with unknown clinical information regarding TNM stage, differentiation grade, adjuvant therapy or MMR status (*n* = 265).

All analyses were conducted in Stata Statistical Software: Release 16 (StataCorp., College Station, TX, USA). P values <0.05 were considered significant.

RESULTS

In total, 2,394 CRC patients were available for analyses and classified as Warburg-low (*n* = 695, 29.0%), Warburg-moderate (*n* = 858, 35.8%) or Warburg-high (*n* = 841, 35.1%), based on the combined protein expression levels of LDHA, GLUT1, MCT4, PKM2, p53, and PTEN (**Supplementary Table S6**).

Clinical characteristics

Clinical characteristics of the 2,394 included CRC patients are shown in **Table 1**. Warburg-subtypes differed significantly with respect to tumor location, TNM stage, tumor extension (pT), lymph node involvement (pN), differentiation grade, and MMR status, but did not differ with respect to age at diagnosis, sex, and adjuvant therapy status. The Warburg-high subtype was more often observed in tumors located in the colon, whereas the Warburg-low and -moderate subtypes were more often observed in tumors located in the rectum or rectosigmoid ($p = 0.027$). Furthermore, the Warburg-high subtype was more common in TNM stage IV tumors, whereas Warburg-low and -moderate subtypes were more common in TNM stage I tumors ($p = 0.001$). The Warburg-high subtype was more frequently observed in tumors with a higher primary tumor extension (pT, $p = 0.007$) and tumors with lymph node involvement (pN, $p = 0.006$). Lastly, Warburg-high tumors were more often poorly differentiated ($p < 0.001$) and MMR deficient ($p < 0.001$) compared to Warburg-low and -moderate tumors.

Survival

The median (range) follow-up time since diagnosis was 4.86 years (0.0027–25.99 years). Survival analyses were restricted to 10 years of follow-up. During these first 10 years of follow-up, 1,551 (64.8%) deaths were observed, of which 986 (63.6%) were CRC-related deaths.

Univariable Kaplan–Meier curves showed differences between Warburg-subtypes for CRC-specific survival ($p = 0.0037$) and overall survival ($p = 0.0004$) (**Figure 2**). Patients with Warburg-high tumors had a significantly worse CRC-specific survival (HR 1.30; 95% CI 1.11–1.52) and overall survival (HR 1.26; 95% CI 1.12–1.43) compared to patients with Warburg-low tumors in univariable analyses (**Table 2**). The Warburg-high subtype remained a significant predictor of prognosis in multivariable-adjusted analyses (HR 1.17; 95% CI 1.00–1.38, and HR 1.19; 95% 1.05–1.35 respectively) (**Table 2**). No significant difference in survival was observed for CRC patients with Warburg-moderate compared to Warburg-low tumors.

Table 1 – Clinical characteristics of the colorectal cancer patients within the Netherlands Cohort Study (NLCS, 1986-2006, total *n* = 2,394) according to Warburg-subtypes.

Clinical characteristics	Total CRC (*n* = 2,394)	Warburg-subtypes			P-value[a]
		Warburg-low (*n* = 695)	Warburg-moderate (*n* = 858)	Warburg-high (*n* = 841)	
Year of diagnosis, n (%)					
1986-1988	109 (4.6)	38 (5.5)	35 (4.1)	36 (4.3)	
1989-1991	206 (8.6)	60 (8.6)	81 (9.4)	65 (7.7)	
1992-1994	306 (12.8)	80 (11.5)	107 (12.5)	119 (14.2)	
1995-1997	426 (17.8)	128 (18.4)	161 (18.8)	137 (16.3)	0.211
1998-2000	444 (18.6)	146 (21.0)	152 (17.7)	146 (17.4)	
2001-2003	442 (18.5)	109 (15.7)	159 (18.5)	174 (20.7)	
2004-2006	461 (19.3)	134 (19.3)	163 (19.0)	164 (19.5)	
Age at diagnosis in years, median (range)	74.0 (55.0-89.0)	74.0 (55.0-89.0)	74.0 (56.0-88.0)	74.0 (56.0-89.0)	0.645[b]
Sex, *n* (%)					
Men	1333 (55.7)	406 (58.4)	485 (56.5)	442 (52.6)	0.058
Women	1061 (44.3)	289 (41.6)	373 (43.5)	399 (47.4)	
Tumor location, *n* (%)					
Colon	1703 (71.1)	467 (67.2)	608 (70.9)	628 (74.7)	
Rectosigmoid	234 (9.8)	81 (11.7)	81 (9.4)	72 (8.6)	0.027
Rectum	457 (19.1)	147 (21.2)	169 (19.7)	141 (16.8)	
pTNM stage, n (%)					
I	468 (19.6)	170 (24.5)	172 (20.1)	126 (15.0)	
II	909 (38.0)	260 (37.4)	309 (36.0)	340 (40.4)	
III	625 (26.1)	163 (23.5)	233 (27.2)	229 (27.2)	0.001
IV	335 (14.0)	82 (11.8)	123 (14.3)	130 (15.5)	
Unknown	57 (2.4)	20 (2.9)	21 (2.5)	16 (1.9)	
Tumor extension (pT), n (%)					
T1	101 (4.2)	39 (5.6)	35 (4.1)	27 (3.2)	
T2	454 (19.0)	152 (21.9)	174 (20.3)	128 (15.2)	
T3	1535 (64.1)	421 (60.6)	542 (63.2)	572 (68.0)	0.007
T4	239 (10.0)	62 (8.9)	84 (9.8)	93 (11.1)	
Unknown	65 (2.7)	21 (3.0)	23 (2.7)	21 (2.5)	
Lymph node metastasis (pN), n (%)					
N0	1247 (52.1)	377 (54.2)	450 (52.5)	420 (49.9)	
N+	870 (36.3)	220 (31.7)	314 (36.6)	336 (40.0)	0.006
Unknown	277 (11.6)	98 (14.1)	94 (11.0)	85 (10.1)	
Differentiation grade, n (%)					
Well	205 (8.6)	80 (11.5)	76 (8.9)	49 (5.8)	
Moderate	1571 (65.6)	463 (66.6)	565 (65.9)	543 (64.6)	<0.001
Poor/undifferentiated	415 (17.3)	89 (12.8)	139 (16.2)	187 (22.2)	
Unknown	203 (8.5)	63 (9.1)	78 (9.1)	62 (7.4)	
Adjuvant therapy, n (%)					
No	1874 (78.3)	547 (78.7)	668 (77.9)	659 (78.4)	
Yes	499 (20.8)	137 (19.7)	185 (21.6)	177 (21.1)	0.181
Unknown	21 (0.9)	11 (1.6)	5 (0.6)	5 (0.6)	
dMMR, n (%)					
No	2116 (88.4)	628 (90.4)	775 (90.3)	713 (84.8)	
Yes	254 (10.6)	58 (8.4)	79 (9.2)	117 (13.9)	0.001
Unknown	24 (1.0)	9 (1.3)	4 (0.5)	11 (1.3)	

[a]P-value for the χ^2 test, unless otherwise specified

[b]P-value for the Kruskall-Wallis test

CRC, colorectal cancer; TNM, tumor node metastasis; dMMR, mismatch repair deficient.

Univariable and multivariable-adjusted HRs for other relevant prognostic factors included in the model are shown in **Supplementary Table S7**. In multivariable-adjusted analyses, age at diagnosis (per year), TNM stage, and tumor differentiation grade were associated with a significantly worse CRC-specific and overall survival, while adjuvant therapy and MMR deficiency were associated with better survival. Moreover, women had improved overall survival, but not CRC-specific survival. No significant associations were found between tumor location and CRC-specific or overall survival.

Next, we stratified CRC patients by TNM stage to assess the disease stage-dependent prognostic value of Warburg-subtypes. Univariable Kaplan–Meier curves showed that CRC-specific survival differed between Warburg-subtypes in TNM stage III (p = 0.0011), but not in the other TNM stages (**Supplementary Figure S2**). Univariable Cox regression analyses revealed that patients with Warburg-high tumors had a significantly poorer CRC-specific (HR 1.40; 95% CI 1.06–1.85) and overall survival (HR 1.42; 95% CI 1.12–1.80) compared to patients with Warburg-low tumors in TNM stage III (**Table 3**). After multivariable adjustment, the Warburg-high subtype remained a significant predictor of CRC-specific (HR 1.45; 95% CI 1.10–1.92) and overall mortality (HR 1.47; 95% CI 1.15–1.87) in TNM stage III (**Table 3**).

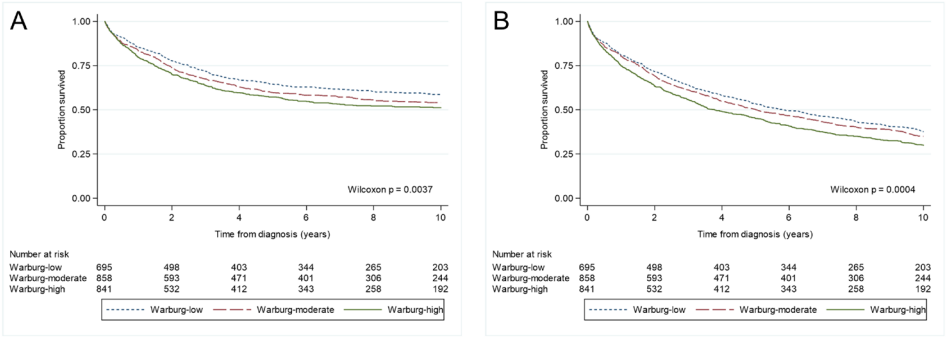


Figure 2 – Kaplan-Meier curves according to metabolic subtypes (i.e., "Warburg-low", "Warburg-moderate", "Warburg-high") in colorectal cancer patients within the Netherlands Cohort Study (NLCS, 1986-2006, total *n* = 2,394), showing (**A**) CRC-specific survival and (**B**) overall survival.

Table 2 – Univariable and multivariable-adjusted hazard ratios (HRs) for associations between Warburg-subtypes and survival within the Netherlands Cohort Study (NLCS, 1986-2006).

	N	CRC-specific survival			Overall survival		
		CRC deaths (%)	HR (95% CI)		Deaths (%)	HR (95% CI)	
			Univariable	Multivariable-adjusted[a]		Univariable	Multivariable-adjusted[a]
Warburg subtypes							
Warburg-low	695	258 (37.1)	1.00 (ref)	1.00 (ref)	424 (61.0)	1.00 (ref)	1.00 (ref)
Warburg-moderate	858	360 (42.0)	1.16 (0.99-1.37)	1.05 (0.89-1.23)	548 (63.9)	1.08 (0.95-1.23)	1.02 (0.90-1.16)
Warburg-high	841	368 (43.8)	1.30 (1.11-1.52)	1.17 (1.00-1.38)	579 (68.8)	1.26 (1.12-1.43)	1.19 (1.05-1.35)

[a]Adjusted for age at diagnosis, sex (men/women), tumor location (colon/rectosigmoid/rectum), TNM stage (I/II/III/IV/unknown), differentiation grade (well/moderate/poor/unknown), adjuvant therapy (yes/no/unknown) and MMR deficiency (yes/no/unknown)

CRC, colorectal cancer; HR, hazard ratio; CI, confidence interval.

Table 3 – TNM stage-specific univariable and multivariable-adjusted hazard ratios (HRs) for associations between Warburg-subtypes and survival within the Netherlands Cohort Study (NLCS, 1986-2006).

	N	CRC-specific survival			Overall survival		
		CRC deaths (%)	HR (95% CI)		Deaths (%)	HR (95% CI)	
			Univariable	Multivariable-adjusted[a]		Univariable	Multivariable-adjusted[a]
TNM stage I							
Warburg-low	170	27 (15.9)	1.00 (ref)	1.00 (ref)	80 (47.1)	1.00 (ref)	1.00 (ref)
Warburg-moderate	172	31 (18.0)	1.14 (0.68-1.91)	1.12 (0.67-1.89)	74 (43.0)	0.92 (0.67-1.27)	0.89 (0.65-1.23)
Warburg-high	126	21 (16.7)	1.09 (0.62-1.92)	1.19 (0.66-2.14)	62 (49.2)	1.09 (0.78-1.52)	1.01 (0.71-1.43)
TNM stage II							
Warburg-low	260	67 (25.8)	1.00 (ref)	1.00 (ref)	141 (54.2)	1.00 (ref)	1.00 (ref)
Warburg-moderate	309	89 (28.8)	1.15 (0.84-1.58)	1.15 (0.84-1.59)	173 (56.0)	1.06 (0.85-1.33)	1.03 (0.83-1.29)
Warburg-high	340	90 (26.5)	1.04 (0.76-1.43)	1.05 (0.76-1.45)	197 (57.9)	1.10 (0.89-1.37)	1.10 (0.88-1.36)
TNM stage III							
Warburg-low	163	81 (49.7)	1.00 (ref)	1.00 (ref)	109 (66.9)	1.00 (ref)	1.00 (ref)
Warburg-moderate	233	114 (48.9)	0.94 (0.71-1.25)	0.98 (0.73-1.30)	165 (70.8)	1.01 (0.79-1.29)	1.07 (0.84-1.37)
Warburg-high	229	133 (58.1)	1.40 (1.06-1.85)	1.45 (1.10-1.92)	179 (78.2)	1.42 (1.12-1.80)	1.47 (1.15-1.87)
TNM stage IV							
Warburg-low	82	74 (90.2)	1.00 (ref)	1.00 (ref)	81 (98.8)	1.00 (ref)	1.00 (ref)
Warburg-moderate	123	114 (92.7)	1.07 (0.80-1.43)	1.00 (0.74-1.34)	122 (99.2)	1.03 (0.78-1.37)	0.95 (0.72-1.27)
Warburg-high	130	118 (90.8)	1.28 (0.96-1.72)	1.06 (0.78-1.44)	130 (100.0)	1.30 (0.99-1.72)	1.08 (0.81-1.45)

[a]Adjusted for age at diagnosis, sex (men/women), tumor location (colon/rectosigmoid/rectum), differentiation grade (well/moderate/poor/unknown), adjuvant therapy (yes/no/unknown) and MMR deficiency (yes/no/unknown)
CRC, colorectal cancer; HR, hazard ratio; CI, confidence interval; TNM, tumor node metastasis.

In addition, CRC-specific survival differed between Warburg-subtypes in patients with tumors located in the rectosigmoid (p = 0.0003) (**Supplementary Figure S3**). Patients with Warburg-high tumors located in the rectosigmoid or rectum had a significantly worse CRC-specific and overall survival compared to patients with Warburg-low tumors (rectosigmoid: HR 2.37; 95% CI 1.40–4.01 and HR 1.73; 95% CI 1.17–2.54; rectum: HR 1.55; 95% CI 1.07–2.24 and HR 1.77; 95% CI 1.31–2.39) in univariable analyses (**Table 4**). In multivariable-adjusted analyses, the Warburg-high subtype remained a significant predictor of overall survival in patients with tumors in the rectum (HR 1.56; 95% CI 1.15–2.13) (**Table 4**).

In sensitivity analyses, excluding CRC patients who died within 30 days after diagnosis (*n* = 93) did not lead to essential changes (*data not shown*). Furthermore, excluding CRC patients with unknown clinical information (*n* = 265) (i.e., unknown TNM stage, differentiation grade, adjuvant therapy, or MMR status) yielded similar results, except for a statistically significant positive association between the Warburg-high subtype and CRC-specific survival (HR 1.51; 95% CI 1.01–2.26 *versus* HR 1.29; 95% CI 0.88–1.88) in patients with tumors in the rectum after multivariable-adjustment (*data not shown*).

Table 4 – Tumor location-specific univariable and multivariable-adjusted hazard ratios (HRs) for associations between Warburg-subtypes and survival within the Netherlands Cohort Study (NLCS, 1986-2006),

	N	CRC-specific survival			Overall survival		
		CRC deaths (%)	HR (95% CI)		Deaths (%)	HR (95% CI)	
			Univariable	Multivariable-adjusted[a]		Univariable	Multivariable-adjusted[a]
Colon							
Warburg-low	467	183 (39.2)	1.00 (ref)	1.00 (ref)	299 (64.0)	1.00 (ref)	1.00 (ref)
Warburg-moderate	608	263 (43.3)	1.14 (0.95-1.38)	1.05 (0.87-1.27)	398 (65.5)	1.06 (0.91-1.23)	1.01 (0.87-1.17)
Warburg-high	628	268 (42.7)	1.14 (0.95-1.38)	1.14 (0.94-1.38)	424 (67.5)	1.11 (0.96-1.29)	1.12 (0.96-1.30)
Rectosigmoid							
Warburg-low	81	23 (28.4)	1.00 (ref)	1.00 (ref)	50 (61.7)	1.00 (ref)	1.00 (ref)
Warburg-moderate	81	27 (33.3)	1.20 (0.69-2.09)	0.97 (0.55-1.71)	50 (61.7)	1.03 (0.69-1.52)	0.96 (0.64-1.44)
Warburg-high	72	36 (50.0)	2.37 (1.40-4.01)	1.48 (0.84-2.63)	54 (75.0)	1.73 (1.17-2.54)	1.40 (0.92-2.11)
Rectum							
Warburg-low	147	52 (35.4)	1.00 (ref)	1.00 (ref)	75 (51.0)	1.00 (ref)	1.00 (ref)
Warburg-moderate	169	70 (41.4)	1.16 (0.81-1.67)	0.96 (0.66-1.39)	100 (59.2)	1.18 (0.87-1.59)	1.04 (0.76-1.41)
Warburg-high	141	64 (45.4)	1.55 (1.07-2.24)	1.29 (0.88-1.88)	101 (71.6)	1.77 (1.31-2.39)	1.56 (1.15-2.13)

[a]Adjusted for age at diagnosis, sex (men/women), TNM stage (I/II/III/IV/unknown), differentiation grade (well/moderate/poor/unknown), adjuvant therapy (yes/no/unknown) and MMR deficiency (yes/no/unknown)
CRC, colorectal cancer; HR, hazard ratio; CI, confidence interval.

DISCUSSION

To the best of our knowledge, this is the first study to investigate the prognostic value of immunohistochemistry (IHC)-based Warburg-subtypes in a large population-based series of in colorectal cancer (CRC) patients. Warburg-subtypes were characterized using a pathway-based sum score of the IHC expression levels of six glycolytic proteins and transcriptional regulators indicative of the Warburg-effect (LDHA, GLUT1, MCT4, PKM2, p53, PTEN). Based on this sum score, CRC patients were classified as Warburg-low (low probability of the presence of the Warburg effect), Warburg-moderate or Warburg-high (high probability of the presence of the Warburg effect). Our results indicate that CRC patients with Warburg-high tumors had a worse CRC-specific and overall survival, independent of known prognostic factors such as TNM stage. Stratified analyses indicated that the Warburg-high subtype was particularly associated with a poor prognosis in patients with TNM stage III CRC, and tumors located in the rectum.

There have been some studies investigating the existence and prognostic value of metabolic subtypes in other cancer types. Karasinska *et al.*[34] identified four metabolic subtypes (quiescent, glycolytic, cholesterogenic and mixed) in pancreatic ductal adenocarcinoma (PDAC), based on RNA-sequencing data of glycolytic and cholesterogenic genes. Their results indicated that patients with glycolytic PDACs had a poorer overall survival[34]. Choi *et al.*[35] stratified breast cancer patients into four metabolic subtypes, based on IHC data on the expression of GLUT1 and CAIX: (1) the Warburg type (glycolytic tumor, non-glycolytic stroma); (2) the null type (non-glycolytic tumor, non-glycolytic stroma); (3) the mixed type; and (4) the reverse Warburg type (non-glycolytic tumor, glycolytic stroma). The Warburg-subtype was associated with a poor survival in breast cancer. Although these studies were performed in different cancer types using different subtyping methodology, our results are consistent with those previously reported, supporting the potential prognostic value of Warburg/glycolytic-subtypes in CRC.

Furthermore, our results support the findings reported in the meta-analysis by Yu *et al.*[22], in which the results of 86 observational studies, including four studies in CRC (n = 648), were pooled to investigate the association between glycolysis-related markers and cancer prognosis. The authors reported that glycolysis-related proteins were associated with a poor overall survival in various cancers, including CRC[22]. Moreover, Zhu *et al.*[36] constructed a glycolysis-related risk score model for CRC patients based on mRNA sequencing data from TCGA and GEO databases and showed that a glycolysis-related risk score was associated with a poor prognosis in CRC and could be used to predict CRC patient's outcomes[36]. However, their study was based on a limited number of CRC patients (n = 379) because of incomplete follow-up data. In addition, Zhu *et al.*[36] reported that the five genes used to establish the risk score were not reported to be key genes in the glycolysis

pathway. Nevertheless, the findings in the current study are consistent with their findings and suggest that the Warburg-effect is associated with a poor prognosis in CRC.

The biological explanation for the differences in survival we observed for the Warburg-subtypes, and especially within the different TNM stages and tumor locations, remains to be investigated in future studies. A potential mechanism through which the Warburg-effect is thought to contribute to a poor prognosis in cancer patients is the acidification of the tumor environment[37], which is caused by the increased secretion of lactate by cancer cells[38]. It has previously been suggested that extracellular acidification contributes to tumor aggressiveness by allowing cancer cells to invade normal surrounding tissues and by causing cancer cells to detach from the extracellular matrix and metastasize[37]. In addition, acidification of the tumor environment has been associated with therapy resistance and immunosuppression[21, 39].

Targeting the Warburg-effect is a major area of focus in the development of novel anti-cancer drugs[40]. Inhibition of the Warburg effect may reduce tumor cell proliferation and metastasis[41]. Several inhibitors of glycolytic enzymes and transporters (e.g., GLUT, PKM2, LDHA, MCT1) are currently in (pre)clinical development; however, to date there has been little clinical success[42, 43]. Vanhove *et al*.[43] described that a major pitfall in the trials to test drugs targeting metabolism is the limited knowledge about the metabolic pathways involved, as no metabolic profiling is performed before initiation of therapy. Indeed, although research has shown that the Warburg effect is frequently observed in cancer, it is not a universal trait of all tumor cells[43, 44]. Therefore, Warburg-subtyping may aid the design of Warburg-targeted therapies and improve therapeutic outcomes.

Strengths of this study include its use of a large population-based series of CRC patients, the nearly complete follow-up, and the fact that patients were mainly treated with surgery. Our study has some potential limitations. First, we decided to categorize CRC patients into Warburg-subtypes by using a pathway-based sum score of six proteins involved in the Warburg effect. With such an approach, the Warburg-low group includes CRC patients with moderate or high protein expression for some of the proteins, whereas the Warburg-high group includes CRC patients with low or moderate expression for some of the proteins. Second, the six proteins used to identify Warburg-subtypes represent a selection of proteins involved in the pathway. However, we believe that using a multi-marker approach which incorporated six proteins involved in different levels of the pathway (i.e., transcriptional regulation, glucose transport, glycolysis, lactate secretion), provided a relatively comprehensive insight into the Warburg-effect. A third potential limitation is related to the use of TMAs, which may not fully represent the whole of the tumour[45]. However, it has been shown previously that triplicate 0.6-mm cores are a reliable alternative for high-throughput molecular profiling using IHC compared to whole-tissue sections[46]. Lastly, our study did

not have a validation cohort available to confirm the observed associations.

In conclusion, in the present study, we have investigated the prognostic value of immunohistochemistry (IHC)-based Warburg-subtypes in colorectal cancer (CRC). The Warburg-high subtype was associated with the poorest prognosis in CRC patients, especially in TNM stage III CRC, and cancers located in the rectum. Metabolic subtyping, based on the presence of the Warburg effect, resulted in potentially important differences in CRC survival and may be used in the future for risk stratification, the design of Warburg-targeted therapies, and to improve therapeutic outcomes. However, further research is required to validate our findings and to investigate the potential clinical utility of these Warburg-subtypes in CRC.

REFERENCES

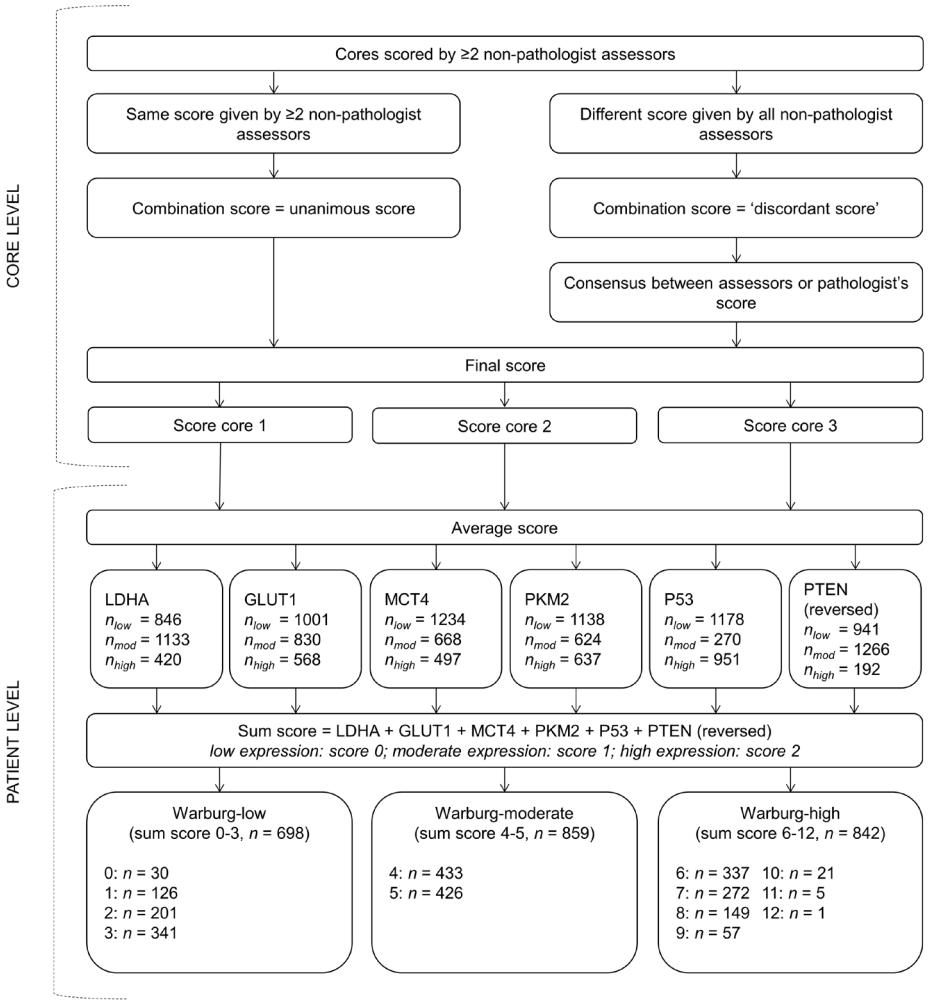
1. Rawla P, Sunkara T, Barsouk A. Epidemiology of colorectal cancer: incidence, mortality, survival, and risk factors. *Prz Gastroenterol* 2019; 14: 89-103.
2. Ferlay J, Ervik M, Lam F, *et al.* Global Cancer Observatory: Cancer Today Lyon, France: International Agency for Research on Cancer. [Accessed 15 February 2021]: Available from: https://gco.iarc.fr/today
3. Wei XL, Wang DS, Xi SY, *et al.* Clinicopathologic and prognostic relevance of ARID1A protein loss in colorectal cancer. *World J Gastroenterol* 2014; 20: 18404-18412.
4. Mo S, Zhou Z, Li Y, *et al.* Establishment and validation of a novel nomogram incorporating clinicopathological parameters into the TNM staging system to predict prognosis for stage II colorectal cancer. *Cancer Cell Int* 2020; 20: 285.
5. Dienstmann R, Mason MJ, Sinicrope FA, *et al.* Prediction of overall survival in stage II and III colon cancer beyond TNM system: a retrospective, pooled biomarker study. *Ann Oncol* 2017; 28: 1023-1031.
6. Marks KM, West NP, Morris E, *et al.* Clinicopathological, genomic and immunological factors in colorectal cancer prognosis. *Br J Surg* 2018; 105: e99-e109.
7. Roseweir AK, McMillan DC, Horgan PG, *et al.* Colorectal cancer subtypes: Translation to routine clinical pathology. *Cancer Treat Rev* 2017; 57: 1-7.
8. Ogino S, Chan AT, Fuchs CS, *et al.* Molecular pathological epidemiology of colorectal neoplasia: an emerging transdisciplinary and interdisciplinary field. *Gut* 2011; 60: 397-411.
9. Phipps AI, Limburg PJ, Baron JA, *et al.* Association between molecular subtypes of colorectal cancer and patient survival. *Gastroenterology* 2015; 148: 77-87 e72.
10. Long Z, Zhou J, Xie K, *et al.* Metabolomic Markers of Colorectal Tumor With Different Clinicopathological Features. *Front Oncol* 2020; 10: 981.
11. Molinari F, Frattini M. Functions and Regulation of the PTEN Gene in Colorectal Cancer. *Front Oncol* 2013; 3: 326.
12. Yap TA, Bjerke L, Clarke PA, *et al.* Drugging PI3K in cancer: refining targets and therapeutic strategies. *Curr Opin Pharmacol* 2015; 23: 98-107.
13. Hussain A, Qazi AK, Mupparapu N, *et al.* Modulation of glycolysis and lipogenesis by novel PI3K selective molecule represses tumor angiogenesis and decreases colorectal cancer growth. *Cancer Lett* 2016; 374: 250-260.
14. Slattery ML, Mullany LE, Sakoda LC, *et al.* The PI3K/AKT signaling pathway: Associations of miRNAs with dysregulated gene expression in colorectal cancer. *Mol Carcinog* 2018; 57: 243-261.
15. Tian X, Liu M, Huang X, *et al.* Noscapine Induces Apoptosis in Human Colon Cancer Cells by Regulating Mitochondrial Damage and Warburg Effect via PTEN/PI3K/mTOR Signaling Pathway. *Onco Targets Ther* 2020; 13: 5419-5428.
16. Bensinger SJ, Christofk HR. New aspects of the Warburg effect in cancer cell biology. *Semin Cell Dev Biol* 2012; 23: 352-361.
17. Kato Y, Maeda T, Suzuki A, *et al.* Cancer metabolism: New insights into classic characteristics. *Jpn Dent Sci Rev* 2018; 54: 8-21.
18. Yeung SJ, Pan J, Lee MH. Roles of p53, MYC and HIF-1 in regulating glycolysis - the seventh hallmark of cancer.

Cell Mol Life Sci 2008; 65: 3981-3999.

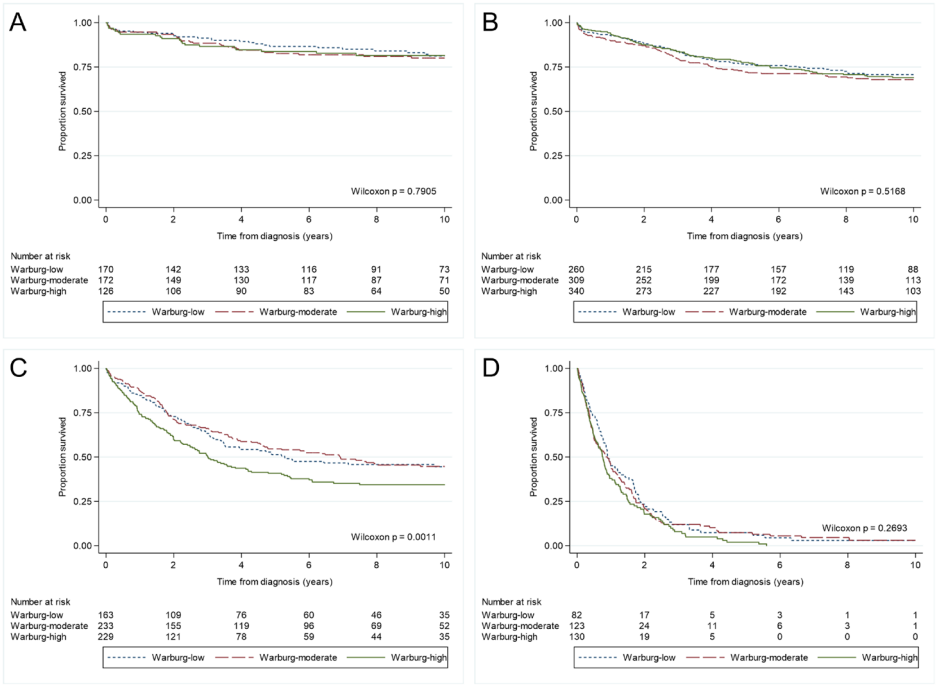
19. Wolpaw AJ, Dang CV. Exploiting Metabolic Vulnerabilities of Cancer with Precision and Accuracy. *Trends Cell Biol* 2018; 28: 201-212.
20. Meijer TW, Schuurbiers OC, Kaanders JH*, et al.* Differences in metabolism between adeno- and squamous cell non-small cell lung carcinomas: spatial distribution and prognostic value of GLUT1 and MCT4. *Lung Cancer* 2012; 76: 316-323.
21. Schuurbiers OC, Kaanders JH, van der Heijden HF*, et al.* The PI3-K/AKT-pathway and radiation resistance mechanisms in non-small cell lung cancer. *J Thorac Oncol* 2009; 4: 761-767.
22. Yu M, Chen S, Hong W*, et al.* Prognostic role of glycolysis for cancer outcome: evidence from 86 studies. *J Cancer Res Clin Oncol* 2019; 145: 967-999.
23. van den Brandt PA, Goldbohm RA, van 't Veer P*, et al.* A large-scale prospective cohort study on diet and cancer in The Netherlands. *J Clin Epidemiol* 1990; 43: 285-295.
24. Casparie M, Tiebosch AT, Burger G*, et al.* Pathology databanking and biobanking in The Netherlands, a central role for PALGA, the nationwide histopathology and cytopathology data network and archive. *Cell Oncol* 2007; 29: 19-24.
25. van den Brandt PA, Schouten LJ, Goldbohm RA*, et al.* Development of a record linkage protocol for use in the Dutch Cancer Registry for Epidemiological Research. *Int J Epidemiol* 1990; 19: 553-558.
26. Goldbohm RA, van den Brandt PA, Dorant E. Estimation of the coverage of municipalities by cancer registries and PALGA using hospital discharge data. *Tijdschr Soc Gezondheidsz* 1994; 72: 80-84.
27. van den Brandt PA. Molecular pathological epidemiology of lifestyle factors and colorectal and renal cell cancer risk. Maastricht Pathology 2018. 11th Joint Meeting of the British Division of the International Academy of Pathology and the Pathological Society of Great Britain & Ireland, 19-22 June 2018. *J Pathol* 2018; 246 (Suppl 1): S9.
28. Richman SD, Adams R, Quirke P*, et al.* Pre-trial inter-laboratory analytical validation of the FOCUS4 personalised therapy trial. *J Clin Pathol* 2016; 69: 35-41.
29. Koukourakis MI, Giatromanolaki A, Sivridis E*, et al.* Lactate dehydrogenase 5 expression in operable colorectal cancer: strong association with survival and activated vascular endothelial growth factor pathway—a report of the Tumour Angiogenesis Research Group. *J Clin Oncol* 2006; 24: 4301-4308.
30. Resnick MB, Routhier J, Konkin T*, et al.* Epidermal growth factor receptor, c-MET, β-catenin, and p53 expression as prognostic indicators in stage II colon cancer: a tissue microarray study. *Clin Cancer Res* 2004; 10: 3069-3075.
31. Ortega-Molina A, Serrano M. PTEN in cancer, metabolism, and aging. *Trends Endocrinol Metab* 2013; 24: 184-189.
32. Schoenfeld D. Partial residuals for the proportional hazards regression model. *Biometrika* 1982; 69: 239-241.
33. Sobin LH, Compton CA, Gospodarowicz M*, et al.* 'Evidence-based medicine: the time has come to set standards for staging'. Is a radical overhaul really needed? *J Pathol* 2010; 221: 361-362.
34. Karasinska JM, Topham JT, Kalloger SE*, et al.* Altered gene expression along the glycolysis–cholesterol synthesis axis is associated with outcome in pancreatic cancer. *Clin Cancer Res* 2020; 26: 135-146.
35. Choi J, Jung WH, Koo JS. Metabolic interaction between cancer cells and stromal cells according to breast cancer molecular subtype. *Breast Cancer Res* 2013; 15: 1-20.

36. Zhu J, Wang S, Bai H, *et al.* Identification of Five Glycolysis-Related Gene Signature and Risk Score Model for Colorectal Cancer. *Front Oncol* 2021; 11: 211.
37. Spencer NY, Stanton RC. The Warburg effect, lactate, and nearly a century of trying to cure cancer. *Semin Nephrol* 2019; 39: 380-393.
38. Jiang B. Aerobic glycolysis and high level of lactate in cancer metabolism and microenvironment. *Genes & diseases* 2017; 4: 25-27.
39. Peppicelli S, Bianchini F, Calorini L. Extracellular acidity, a "reappreciated" trait of tumor environment driving malignancy: perspectives in diagnosis and therapy. *Cancer Metastasis Rev* 2014; 33: 823-832.
40. Li Y, Wang Y, Liu Z, *et al.* Atractylenolide I Induces Apoptosis and Suppresses Glycolysis by Blocking the JAK2/STAT3 Signaling Pathway in Colorectal Cancer Cells. *Front Pharmacol* 2020; 11: 273.
41. Nenkov M, Ma Y, Gassler N, *et al.* Metabolic Reprogramming of Colorectal Cancer Cells and the Microenvironment: Implication for Therapy. *Int J Mol Sci* 2021; 22: 6262.
42. Le A (Ed). The Heterogeneity of Cancer Metabolism. Springer Nature, 2021.
43. Vanhove K, Graulus G-J, Mesotten L, *et al.* The metabolic landscape of lung cancer: new insights in a disturbed glucose metabolism. *Front Oncol* 2019; 9: 1215.
44. Correa PN, Correa AN. A unitarian biochemical and bioenergetic theory of adaptive oncogenesis: from hypoxia and energy starvation (aerobic and ambipolar) to the roles of HIF-1, IGF-I, and vitamins C and D. *J Biophys Hematol Oncol* 2010; 1: 1-93.
45. Wang H, Wang H, Zhang W, *et al.* Tissue microarrays: applications in neuropathology research, diagnosis, and education. *Brain Pathol* 2002; 12: 95-107.
46. Hoos A, Urist MJ, Stojadinovic A, *et al.* Validation of tissue microarrays for immunohistochemical profiling of cancer specimens using the example of human fibroblastic tumors. *Am J Pathol* 2001; 158: 1245-1251.

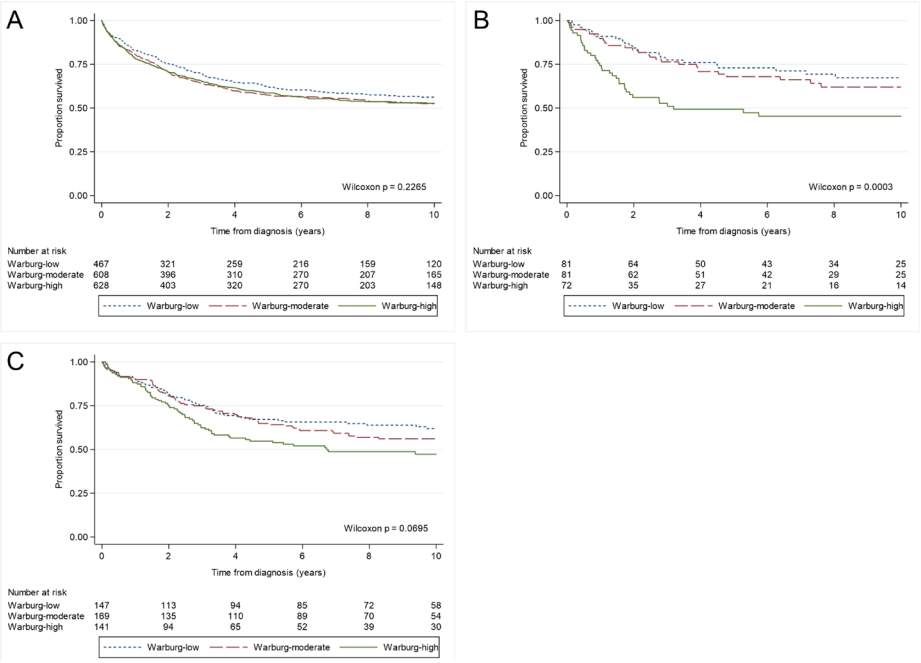
SUPPLEMENTARY MATERIAL



Supplementary Figure S1 – Flow diagram of the process of combining core-level scores into patient-level Warburg-subtypes. N_{low}, number of CRC patients with low protein expression; n_{mod}, number of CRC patients with moderate protein expression; n_{high}, number of CRC patients with high protein expression.



Supplementary Figure S2 – Kaplan-Meier curves showing CRC-specific survival of Warburg subtypes (i.e., Warburg-low, Warburg-moderate and Warburg-high), within (**A**) TNM stage I (*n* = 468); (**B**) TNM stage II (*n* = 909); (**C**) TNM stage III (*n* = 625); (**D**) TNM stage IV (*n* = 335). Netherlands Cohort Study (NLCS), 1986-2006.



Supplementary Figure S3 – Kaplan-Meier curves showing CRC-specific survival of Warburg subtypes (i.e., Warburg-low, Warburg-moderate and Warburg-high), within (**A**) colon (*n* = 1703); (**B**) rectosigmoid (*n* = 234); and (**C**) rectum (*n* = 457). Netherlands Cohort Study (NLCS), 1986-2006.

Supplementary Table S1 – Acknowledgements for the Rainbow-TMA project.

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Supplementary Table S2 – Details of primary antibodies and staining protocols.

Antibody	Clone	Source	Dilution	Staining procedure	Antigen retrieval	Incubation time	Visualization system	Chromogen
Pan-CK	AE1/AE3	DAKO (GA053)	RTU	DAKO Autostainer[a]	PT high[b]	10 minutes	EnVision FLEX[f]	DAB
MLH1	ES05	DAKO (M3640)	RTU	DAKO Autostainer[a]	PT high[b]	40 minutes	EnVision FLEX[f]	DAB
MSH2	FE11	DAKO (M3639)	RTU	DAKO Autostainer[a]	PT high[b]	40 minutes	EnVision FLEX[f]	DAB
p53	DO-7	DAKO (M7001)	RTU	DAKO Autostainer[a]	PT high[b]	20 minutes	EnVision FLEX[f]	DAB
PTEN	6H2.1	DAKO (M3627)	1:100	DAKO Autostainer[a]	PT high[b]	20 minutes	EnVision FLEX[f]	DAB
GLUT1	-	TFS (RB-9052-P1)	1:200	DAKO Autostainer[a]	PT low[c]	20 minutes	EnVision FLEX[f]	DAB
LDHA	E-9	SCBT (sc-137243)	1:800	Manual	HIER high[d]	Overnight, 4°C	REAL EnVision[g]	DAB
MCT4	D-1	SCBT (sc-376140)	1:100	Manual	HIER high[d]	Overnight, 4°C	REAL EnVision[g]	DAB
PKM2	C-11	SCBT (sc-365684)	1:100	Manual	HIER low[e]	1hr, 37°C	LSAB2 Kit/HRP[h]	DAB

[a]DAKO Autostainer Link 48
[b]High pH retrieval (K8004) for 20 minutes on the Dako PT link (Agilent Technologies)
[c]Low pH retrieval (K8005) for 20 minutes on the Dako PT link (Agilent Technologies)
[d]Heat-induced antigen retrieval using a solution of Tris/EDTA (pH 9.0)
[e]Heat-induced antigen retrieval using a solution of sodium citrate (pH 6.0)
[f]EnVision FLEX Visualization Kit (K8008, DAKO)
[g]REAL EnVision Detection System (K5007, DAKO)
[h]Universal LSAB2 kit/HRP (K0675, Agilent)
TFS, Thermo Fisher Scientific; SBCT, Santa Cruz Biotechnology; RTU, ready-to-use; DAB, 3,3'-diaminobenzide.

Supplementary Table S3 – Percentage of slides evaluated per observer for the six immunohistochemical markers of proteins incorporated in the Warburg-subtypes.

	Observer 1 Non-pathologist	Observer 2 Non-pathologist		Observer 3 Non-pathologist		Observer 4 Pathologist[b]
		Time point 1	Time point 2[a]	Time point 1	Time point 2[a]	
p53	100%	100%	10%	100%	10%	10%
PTEN	100%	100%	10%	100%	10%	10%
GLUT1	25%[c]	100%	10%	100%	10%	10%
LDHA	-	100%	10%	100%	10%	10%
MCT4	-	100%	10%	100%	10%	10%
PKM2	-	100%	10%	100%	10%	10%

[a]10% randomly selected TMA sections per marker were scored for a second time after a period of at least five months to assess intra-observer reproducibility.
[b]10% randomly selected TMA sections per marker were scored by an experienced pathologist to assess inter-observer agreement between pathologist and non-pathologists
[c]Observer 1 left the project early because of an unforeseen work relocation.

Supplementary Table S4 – Scoring protocols and kappa values with 95% confidence intervals for inter- and intra-observer agreement of the six proteins incorporated in the Warburg-subtypes.

	p53	PTEN	GLUT1	LDHA	MCT4	PKM2
Localization[a]	**Nucleus**	**Cytoplasm**[b]	**Membrane**	**Cytoplasm**	**Membrane**	**Cytoplasm**
Scoring protocol						
Low						
Category 1	(1) negative	(1) negative	(1) negative	(1) negative/weak	(1) negative	(1) negative/weak
Category 2	(2) 1-10% positive		(2) 1-10% positive		(2) 1-10% positive	
Moderate						
Category 2		(2) weak		(2) 1-50% strong positive		(2) moderate positive
Category 3	(3) 11-50% positive	(3) moderate	(3) 11-50% positive		(3) 11-50% positive	(3) 1-50% strong positive
High						
Category 3				(3) >50% strong positive		
Category 4	(4) 51-90% positive	(4) strong	(4) >50% positive		(4) >50% positive	(4) >50% strong positive
Category 5	(5) >90% positive					
Scoring agreement						
Inter-observer agreement[c]	κ (95%-CI)	κ (95%-CI)	κ (95%-CI)	κ (95%-CI)	κ (95%-CI)	κ (95%-CI)
Final score[d] vs pathologist						
Weighted kappa[e]	0.75 (0.72-0.79)	0.58 (0.53-0.62)	0.71 (0.67-0.74)	0.65 (0.60-0.69)	0.74 (0.71-0.77)	0.65 (0.61-0.69)
Non-weighted kappa	0.63 (0.58-0.68)	0.47 (0.41-0.52)	0.61 (0.57-0.66)	0.59 (0.54-0.64)	0.63 (0.59-0.68)	0.56 (0.51-0.60)
Intra-observer agreement[c, f]						
Non-pathologist assessor 1						
Weighted kappa[e]	0.83 (0.80-0.86)	0.69 (0.65-0.74)	0.82 (0.79-0.85)	0.78 (0.74-0.82)	0.86 (0.83-0.88)	0.70 (0.67-0.74)
Non-weighted kappa	0.73 (0.69-0.77)	0.63 (0.58-0.69)	0.75 (0.72-0.79)	0.76 (0.71-0.80)	0.79 (0.75-0.82)	0.58 (0.53-0.62)
Non-pathologist assessor 2						
Weighted kappa[e]	0.87 (0.84-0.90)	0.69 (0.64-0.74)	0.75 (0.72-0.78)	0.77 (0.73-0.81)	0.83 (0.81-0.86)	0.72 (0.68-0.75)
Non-weighted kappa	0.80 (0.76-0.84)	0.65 (0.60-0.70)	0.65 (0.60-0.69)	0.73 (0.69-0.78)	0.75 (0.71-0.79)	0.62 (0.58-0.67)

[a]Only immunoreactivity in reported cellular localization was considered positive staining.
[b]For PTEN scoring, staining intensity of tumor cells was compared with that of stromal cells: (1) no PTEN staining in the tumor cells; (2) staining intensity in the tumor cells weaker than in the stromal cells; (3) similar staining intensity in tumor and stromal cells; (4) staining intensity in the tumor cells stronger than in the stromal cells.
[c]Based on a random 10% of TMA sections
[d]The final score is based on at least two non-pathologists, with discrepancies replaced by a consensus score or pathologist's score.
[e]Weight of 0.5 for adjacent categories and 0 for non-adjacent categories.
[f]10% of TMA sections were scored for a second time after at least 2 months.

Supplementary Table S5 – TNM classification of colorectal cancer, according to incidence year.

Topography	**153.0-154.1 or C18-C20**		
Histology	**Epithelial cancers (M8010-8580)**		
Incidence years	1988-2002		
TNM version(s)	4.1-5		
Stage	T	N	M
I	1-2	0/X	0/X
II	3-4	0/X	0/X
III	Any T	1-3	0/X
IV	Any T	Any N	1
X	X	0/X	0/X
Incidence years	2003-2009		
TNM version(s)	6		
Stage	T	N	M
I	1-2	0/X	0/X
IIA	3	0/X	0/X
IIB	4	0/X	0/X
III	X	1	0/X
IIIA	1-2	1	0/X
IIIB	3-4	1	0/X
IIIC	Any T	2	0/X
IV	Any T	Any N	1
X	X	0/X	0/X

Supplementary Table S6 – Individual protein expression data of colorectal cancer patients from the Netherlands Cohort Study (NLCS, 1986-2006, total n = 2,394), according to tumor location and Warburg-subtypes.

	Total CRC (*n* = 2,394)	Colon (n = 1,703)				Rectosigmoid (*n* = 234)				Rectum (*n* = 457)			
		Total	Warburg-low (*n* = 467)	Warburg-moderate (*n* = 608)	Warburg-high (*n* = 628)	Total	Warburg-low (*n* = 81)	Warburg-moderate (*n* = 81)	Warburg-high (*n* = 72)	Total	Warburg-low (*n* = 147)	Warburg-moderate (*n* = 169)	Warburg-high (*n* = 141)
p53 (%)													
Low	49.1	50.7	78.8	50.0	30.6	48.7	75.3	48.2	19.4	43.3	76.9	37.9	14.9
Moderate	11.2	12.3	9.4	13.3	13.5	6.8	2.5	7.4	11.1	9.4	6.1	12.4	9.2
High	39.6	36.9	11.8	36.7	55.9	44.4	22.2	44.4	69.4	47.3	17.0	49.7	75.9
PTEN (%)													
Low	8.0	9.3	3.4	6.3	16.7	5.1	2.5	2.5	11.1	4.6	2.7	4.1	7.1
Moderate	52.8	51.6	51.2	52.1	51.3	56.4	42.0	61.7	66.7	55.4	46.9	54.4	65.3
High	39.2	39.1	45.4	41.6	32.0	38.5	55.6	35.8	22.2	40.0	50.3	41.4	27.7
GLUT1 (%)													
Low	41.7	42.5	69.8	44.4	20.4	38.9	65.4	34.6	13.9	40.3	67.4	39.6	12.8
Moderate	34.6	34.1	24.6	37.5	37.7	36.3	27.2	42.0	40.3	35.7	28.6	42.0	35.5
High	23.7	23.4	5.6	18.1	41.9	24.8	7.4	23.5	45.8	24.1	4.1	18.3	51.8
LDHA (%)													
Low	35.1	34.2	61.5	32.4	15.6	38.0	59.3	32.1	20.8	37.2	58.5	33.1	19.9
Moderate	47.3	46.4	34.5	51.3	50.5	49.6	39.5	53.1	56.9	49.7	38.8	55.0	54.6
High	17.5	19.4	4.1	16.3	33.9	12.4	1.2	14.8	22.2	13.1	2.7	11.8	25.5
MCT4 (%)													
Low	51.4	49.1	80.7	50.7	24.0	56.4	72.8	60.5	33.3	57.6	79.6	56.8	35.5
Moderate	27.9	27.4	15.4	29.1	34.6	29.1	24.7	25.9	37.5	29.1	18.4	30.2	39.0
High	20.7	23.6	3.9	20.2	41.4	14.5	2.5	13.6	29.2	13.4	2.0	13.0	25.5
PKM2 (%)													
Low	47.4	44.7	76.5	47.9	18.0	53.9	74.1	59.3	25.0	54.1	81.0	59.8	19.2
Moderate	26.0	26.7	19.3	29.9	29.1	24.4	22.2	24.7	26.4	24.3	13.6	27.8	31.2
High	26.6	28.6	4.3	22.2	52.9	21.8	3.7	16.1	48.6	21.7	5.4	12.4	49.7

Supplementary Table S7 – Univariable and multivariable-adjusted analyses of the association between known prognostic factors that were included in the final Cox regression models and survival within the Netherlands Cohort Study (NLCS, 1986-2006)

	N	CRC-specific survival			Overall survival		
		CRC-deaths (%)	HR (95% CI)		Deaths (%)	HR (95% CI)	
			Univariable	Multivariable-adjusteda		Univariable	Multivariable-adjusteda
Age at diagnosis, per year	2394	986 (41.2)	1.02 (1.01-1.03)	1.02 (1.01-1.03)	1551 (64.8)	1.04 (1.03-1.05)	1.05 (1.04-1.06)
Sex							
Men	1333	551 (41.3)	1.00 (ref)	1.00 (ref)	904 (67.8)	1.00 (ref)	1.00 (ref)
Women	1061	435 (41.0)	0.97 (0.86-1.10)	0.94 (0.82-1.07)	647 (61.0)	0.87 (0.79-0.97)	0.81 (0.73-0.89)
Tumor location							
Colon	1703	714 (41.9)	1.00 (ref)	1.00 (ref)	1121 (65.8)	1.00 (ref)	1.00 (ref)
Rectosigmoid	234	86 (36.8)	0.84 (0.67-1.05)	0.92 (0.74-1.16)	154 (65.8)	0.96 (0.81-1.14)	1.08 (0.91-1.28)
Rectum	457	186 (40.7)	0.88 (0.75-1.03)	1.12 (0.94-1.33)	276 (60.4)	0.82 (0.72-0.94)	1.03 (0.89-1.19)
TNM stage							
I	468	79 (16.9)	1.00 (ref)	1.00 (ref)	216 (46.2)	1.00 (ref)	1.00 (ref)
II	909	246 (27.1)	1.72 (1.33-2.21)	1.72 (1.33-2.22)	511 (56.2)	1.33 (1.14-1.56)	1.27 (1.08-1.50)
III	625	328 (52.5)	4.29 (3.35-5.49)	4.29 (3.32-5.54)	453 (72.5)	2.32 (1.97-2.73)	2.29 (1.93-2.72)
IV	335	306 (91.3)	17.70 (13.72-22.84)	18.11 (13.89-23.61)	333 (99.4)	8.54 (7.13-10.23)	8.70 (7.20-10.52)
Unknown	57	27 (47.4)	3.24 (2.10-5.02)	3.36 (2.09-5.43)	38 (66.7)	1.72 (1.22-2.42)	2.07 (1.42-3.00)
Differentiation grade							
Well	205	67 (32.7)	1.00 (ref)	1.00 (ref)	113 (55.1)	1.00 (ref)	1.00 (ref)
Moderate	1571	607 (38.6)	1.27 (0.98-1.63)	1.03 (0.80-1.33)	991 (63.1)	1.26 (1.03-1.52)	1.09 (0.89-1.32)
Poor/undifferentiated	415	232 (55.9)	2.39 (1.82-3.14)	1.60 (1.21-2.13)	318 (76.6)	2.05 (1.65-2.54)	1.52 (1.22-1.91)
Unknown	203	80 (39.4)	1.32 (0.96-1.83)	1.00 (0.72-1.39)	129 (63.5)	1.30 (1.01-1.68)	1.09 (0.84-1.41)
Adjuvant therapy							
No	1874	714 (38.1)	1.00 (ref)	1.00 (ref)	1191 (63.6)	1.00 (ref)	1.00 (ref)
Yes	499	262 (52.5)	1.36 (1.18-1.57)	0.75 (0.65-0.88)	346 (69.3)	1.12 (0.99-1.26)	0.76 (0.66-0.86)
Unknown	21	10 (47.6)	1.23 (0.66-2.29)	1.33 (0.67-2.62)	14 (66.7)	1.04 (0.61-1.76)	1.35 (0.77-2.39)
dMMR							
No	2116	908 (42.9)	1.00 (ref)	1.00 (ref)	1389 (65.6)	1.00 (ref)	1.00 (ref)
Yes	254	68 (26.8)	0.56 (0.44-0.72)	0.53 (0.41-0.69)	147 (57.9)	0.79 (0.66-0.93)	0.71 (0.59-0.85)
Unknown	24	10 (41.7)	1.01 (0.54-1.89)	1.13 (0.61-2.11)	15 (62.5)	0.98 (0.59-1.63)	1.08 (0.65-1.81)

[a]The model included age at diagnosis, Warburg-subtypes (low/moderate/high), sex (men/women), tumor location (colon/rectosigmoid/rectum), TNM stage (I/II/III/IV/unknown), differentiation grade (well/moderate/poor/unknown), adjuvant therapy (yes/no/unknown) and dMMR (yes/no/unknown)
CRC, colorectal cancer; HR, hazard ratio; CI, confidence interval; TNM, tumor node metastasis; dMMR, mismatch repair deficiency.

CHAPTER 4

Association between mutational subgroups, Warburg-subtypes, and survival in patients with colorectal cancer

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ABSTRACT

Background

Previous research suggests that Warburg-subtypes are related to potentially important survival differences in colorectal cancer (CRC) patients. In the present study, we investigated whether mutational subgroups based on somatic mutations in *RAS*, *BRAF*, *PIK3CA*, and *MET*, which are known to promote the Warburg-effect, as well as mismatch repair (MMR) status, hold prognostic value in CRC. In addition, we investigated whether Warburg-subtypes provide additional prognostic information, independent of known prognostic factors like TNM stage.

Methods

CRC patients (n = 2,344) from the prospective Netherlands Cohort Study (NLCS) were classified into eight mutually exclusive mutational subgroups, based on observed mutations in *RAS, BRAF, PIK3CA*, and *MET*, and MMR status: all-wild-type + $MMR_{proficient}$, $KRAS_{mut}$ + $MMR_{proficient}$, $KRAS_{mut}$ + $PIK3CA_{mut}$ + $MMR_{proficient}$, $PIK3CA_{mut}$ + $MMR_{proficient}$, $BRAF_{mut}$ + $MMR_{proficient}$, $BRAF_{mut}$ + $MMR_{deficient}$, other + $MMR_{proficient}$, and other + $MMR_{deficient}$. Kaplan–Meier curves and Cox regression models were used to investigate associations between mutational subgroups and survival, as well as associations between our previously defined Warburg-subtypes and survival within these mutational subgroups.

Results

Compared to patients with all-wild-type + $MMR_{proficient}$ CRC, patients with $KRAS_{mut}$ + $MMR_{proficient}$, $KRAS_{mut}$ + $PIK3CA_{mut}$ + $MMR_{proficient}$, $BRAF_{mut}$ + $MMR_{proficient}$, or other + $MMR_{proficient}$ CRC had a statistically significant worse survival ($HR_{CRC\text{-}specific}$ ranged from 1.29 to 1.88). In contrast, patients with other + $MMR_{deficient}$ CRC had the most favorable survival ($HR_{CRC\text{-}specific}$ 0.48). No statistically significant survival differences were observed for the Warburg-subtypes within mutational subgroups.

Conclusion

Our results highlight the prognostic potential of mutational subgroups in CRC. Warburg-subtypes did not provide additional significant prognostic information within these mutational subgroups. Future larger-scale prospective studies are necessary to validate our findings and to examine the potential clinical utility of CRC subtyping based on mutational subgroups.

INTRODUCTION

Colorectal cancer (CRC) is the third most prevalent cancer and the second leading cause of cancer-related mortality worldwide, accounting for more than 900,000 deaths every year.[1] Despite all efforts to identify molecular prognostic biomarkers in CRC, the tumor-node-metastasis (TNM) staging system remains the only clinically used prognostic factor.[2] However, patients with the same TNM stage can have large differences in survival.[2] Cancer cells are known to reprogram their metabolism from oxidative phosphorylation towards aerobic glycolysis, a phenomenon commonly referred to as the "Warburg-effect".[3, 4] The Warburg-effect is characterized by increased glucose uptake and lactate secretion in the presence of oxygen.[3, 4] Since its discovery by Otto Warburg in the 1920s,[5] the presence of the Warburg-effect has been described in a number of different cancer types, including CRC,[6] and has recently been proposed as one of the emerging hallmarks of cancer.[7]

Metabolic reprogramming towards the Warburg-effect is influenced by two major oncogenic pathways: the *PI3K/AKT/mTOR* and *RAS/RAF/MEK/ERK* pathways.[8-11] Key genes involved in these pathways including *RAS* (*KRAS, NRAS, HRAS*), *BRAF*, *PIK3CA*, and *MET* are often mutated in human cancers,[12-14] and these mutations have been suggested to promote the Warburg-effect.[12-15] In CRC, it has previously been shown that especially *KRAS*, *BRAF*, and *PIK3CA* are frequently mutated.[10, 16, 17] In addition, mutations in more than one of the genes (e.g., presence of *PI3KCA* mutations in combination with *RAS* or *BRAF* mutations) have been described previously.[18, 19]

Recently, it has become clear that *BRAF* mutations can be present in microsatellite instable (MSI) as well as in microsatellite stable (MSS) CRC.[20] Several studies have shown that MSS *BRAF*-mutated CRC have an aggressive phenotype (i.e., occurring at younger age, diagnosed at more advanced TNM stage, often poorly differentiated) and are associated with a poorer prognosis compared to MSI *BRAF*-mutated CRC.[20, 21] It has been described that presence of MSI 'overrides' the negative prognostic potential of *BRAF* mutations.[22]

Previously, we identified Warburg-subtypes using a pathway-based sum score after measuring the expression levels of six glycolytic proteins and transcriptional regulators indicative of the Warburg-effect (LDHA, GLUT1, MCT4, PKM2, p53, PTEN) using immunohistochemistry (IHC).[23] Based on this sum score, we classified CRC patients as having Warburg-low (i.e., low probability of the presence of the Warburg-effect), Warburg-moderate, or Warburg-high cancers. Our previous study suggested that Warburg-subtypes are related to differences in survival in CRC patients, independent of known prognostic factors like TNM stage.[23] We hypothesized that (1) mutational subgroups based on somatic mutations in *RAS*, *BRAF*, *PIK3CA*, and *MET*, which are known to promote the Warburg-

effect,[12-15] as well as patients' mismatch repair (MMR) status, may hold prognostic value in CRC, and (2) Warburg-subtypes may provide additional prognostic information within these mutational subgroups, independent of known prognostic factors like TNM stage.

In this large population-based series of CRC patients, we therefore aimed to (1) study the association between mutational subgroups based on the presence of somatic mutations in *RAS* (*KRAS*, *NRAS*, *HRAS*), *BRAF*, *PIK3CA*, and *MET*, as well as MMR status, and survival, and (2) to study the relationship between previously identified Warburg-subtypes and survival within these mutational subgroups to examine whether Warburg-subtypes provide additional prognostic information.

MATERIALS AND METHODS

Design and study population

This population-based series of colorectal cancer (CRC) patients was derived from the prospective Netherlands Cohort Study (NLCS), which has been described in detail previously.[24] Briefly, the NLCS was initiated in September 1986 (baseline) and included 120,852 men and women, aged 55–69 years. At baseline, all participants completed a mailed, self-administered questionnaire on diet and other cancer risk factors.[24] By completing and returning the questionnaire, participants agreed to participate in the study. The NLCS was approved by the institutional review boards of the TNO Quality of Life Research Institute (Zeist, the Netherlands) and Maastricht University (Maastricht, the Netherlands). Ethical approval was obtained from the Medical Ethical Committee (METC) of Maastricht University Medical Center+.

Follow-up for cancer incidence was established by annual record linkage with the Netherlands Cancer Registry and PALGA, the nationwide Dutch Pathology Registry,[25, 26] covering 20.3 years of follow-up (September 17, 1986 until January 1, 2007). The completeness of cancer incidence follow-up was estimated to be >96%.[27] After excluding patients who reported a history of cancer (excluding non-melanoma skin cancer) at baseline, 4597 incident CRC patients were available (**Figure 1**).

Tissue collection and TMA construction

Formalin-fixed paraffin-embedded (FFPE) tissue blocks from CRC patients were collected as part of the Rainbow-Tissue MicroArray (TMA) project during 2012–2017.[28] Details of TMA construction have been described previously.[23] In short, FFPE blocks with primary tumor and matched normal tissue of 3021 CRC patients were retrieved (78% retrieval rate) from 43 pathology laboratories throughout the Netherlands. Hematoxylin&Eosin (H&E)-stained sections were reviewed by pathologists and areas with the highest tumor density

were marked for TMA construction (TMA-Grandmaster, 3D-Histech, Hungary). In total, 78 TMA blocks were constructed containing three 0.6 mm cores from tumor and three from normal epithelium of 2,694 CRC patients (**Figure 1**). In addition, two 20 µm tissue sections were cut from the tumor FFPE blocks for DNA extraction.

Immunohistochemistry

Five µm thick serial sections were cut from all 78 TMA blocks and subjected to either H&E staining according to a standard protocol, or subjected to immunohistochemistry (IHC) for LDHA, GLUT1, MCT4, PKM2, p53, and PTEN using an automated immunostainer (DAKO Autostainer Link 48, Glostrup, Denmark) or manual scoring protocol. Details of the primary antibodies and staining protocols have been described previously,[23] see also **Supplementary Table S1**. After IHC, TMA sections were scanned using the Aperio scanner (Leica Microsystems, Milton Keynes, UK) at 40x magnification at the University of Leeds (UK) Scanning Facility.

First, the presence of adenocarcinoma was confirmed for every individual core by reviewing the H&E-stained TMA sections in combination with pan-cytokeratin-stained sections if necessary. Requiring at least one tumor core per patient, 2,497 CRC patients passed quality control (**Figure 1**). Then, scoring of IHC was performed by three non-pathologists (G.E. Fazzi: histology technician; K. Offermans: PhD-student; J.C.A. Jenniskens: PhD-student), after appropriate training.[23, 29] IHC scoring protocols for all proteins, including kappa values for inter- and intra-observer agreement, are shown in **Supplementary Table S2** and have been described in detail previously.[23]

DNA mismatch repair status

DNA mismatch repair (MMR) status, as a proxy for MSI status,[30] was assessed by IHC for MLH1 and MSH2 as described previously.[23] Briefly, cancers with loss of either MLH1 or MSH2 expression, in the presence of internal positive controls, were considered MMR deficient (dMMR). Cancers that expressed both MLH1 and MSH2 were considered MMR proficient (pMMR). Information regarding MMR status was available for 2,455 CRC patients (**Figure 1**).

DNA isolation and mutational status

Two 20 µm thick FFPE tissue sections were deparaffinized manually using the Buffer ATL (Cat. No. 939011, Qiagen, Hilden, Germany), Proteinase K (Cat. No. 19131, Qiagen), and the Deparaffinization Solution (Cat. No. 19093, Qiagen), using an adapted version of the manufacturer's protocol. DNA isolation was performed using the DSP DNA Mini Kit (Cat. No. 937236, Qiagen) and the QIAsymphony® (Qiagen) instrument, following the manufacturer's protocol (Tissue_HC_200 protocol). Double-stranded DNA (dsDNA) concentrations were quantified using the Quantus™ Fluorometer (Promega, Madison, WI,

USA) with a QuantiFluor® dsDNA system (Promega).

Mutations were analyzed at the Institute for Immunology and Genetics (Kaiserslautern, Germany) using Matrix Assisted Laser Desorption Ionization Time of Flight (MALDI-TOF) mass spectrometry and the ColoCarta Panel (Agena Bioscience, Hamburg), which screens for 32 mutations in six genes known to be commonly mutated in CRC (*KRAS*, *NRAS*, *HRAS*, *BRAF*, *PIK3CA*, *MET*; **Supplementary Table S3**). Data analysis was performed at the Institute for Immunology and Genetics (Kaiserslautern, Germany) using MassArray Typer Analyzer software 4.0.4.20 (Sequenom) and the following cut-offs: mutation frequency cut-off ≥0.075; Z-score ≥4.00; spectrum quality ≥0.750; typer peak probability ≥0.850; primer extension rate cut-off ≥0.200.

Patients testing positive for any mutation-specific assay were classified as mutant for the respective gene; patients with no detectable mutations were classified as wild-type; and patients for whom testing failed or for whom equivocal results were obtained (i.e., one or more assay(s) failed and for other assays no detectable mutations were identified) were classified as having an unknown mutation status. After excluding patients with unknown mutation status for *KRAS*, *BRAF*, *PIK3CA*, *NRAS*, or *MET*, 2,344 CRC patients were available for mutational subgrouping (**Figure 1**).

Mutational subgroups

In total, 2,344 CRC patients were classified into eight mutually exclusive mutational subgroups based on observed frequencies of tumor markers or combinations of tumor markers, requiring at least 100 patients per subgroup: (1) all-wild-type + pMMR (*n* = 851, 36.3%), (2) $KRAS_{mut}$ + pMMR (*n* = 580, 24.7%), (3) $KRAS_{mut}$ + $PIK3CA_{mut}$ + pMMR (*n* = 173, 7.4%), (4) $PIK3CA_{mut}$ + pMMR (*n* = 124, 5.3%), (5) $BRAF_{mut}$ + pMMR (*n* = 147, 6.3%), (6) $BRAF_{mut}$ + dMMR (*n* = 134, 5.7%), (7) other + pMMR (*n* = 218, 9.3%), and (8) other + dMMR (*n* = 117, 5.0%) (see **Supplementary Table S4** for details on mutational subgroups).

Note, the other + pMMR group comprises all CRC patients with other (combinations of) markers and proficient MMR status (see **Supplementary Table S4** and **Figure 2** for details). The other + dMMR subgroup includes patients with all-wild-type + dMMR tumors, as well as other (combinations of) markers and deficient MMR status.

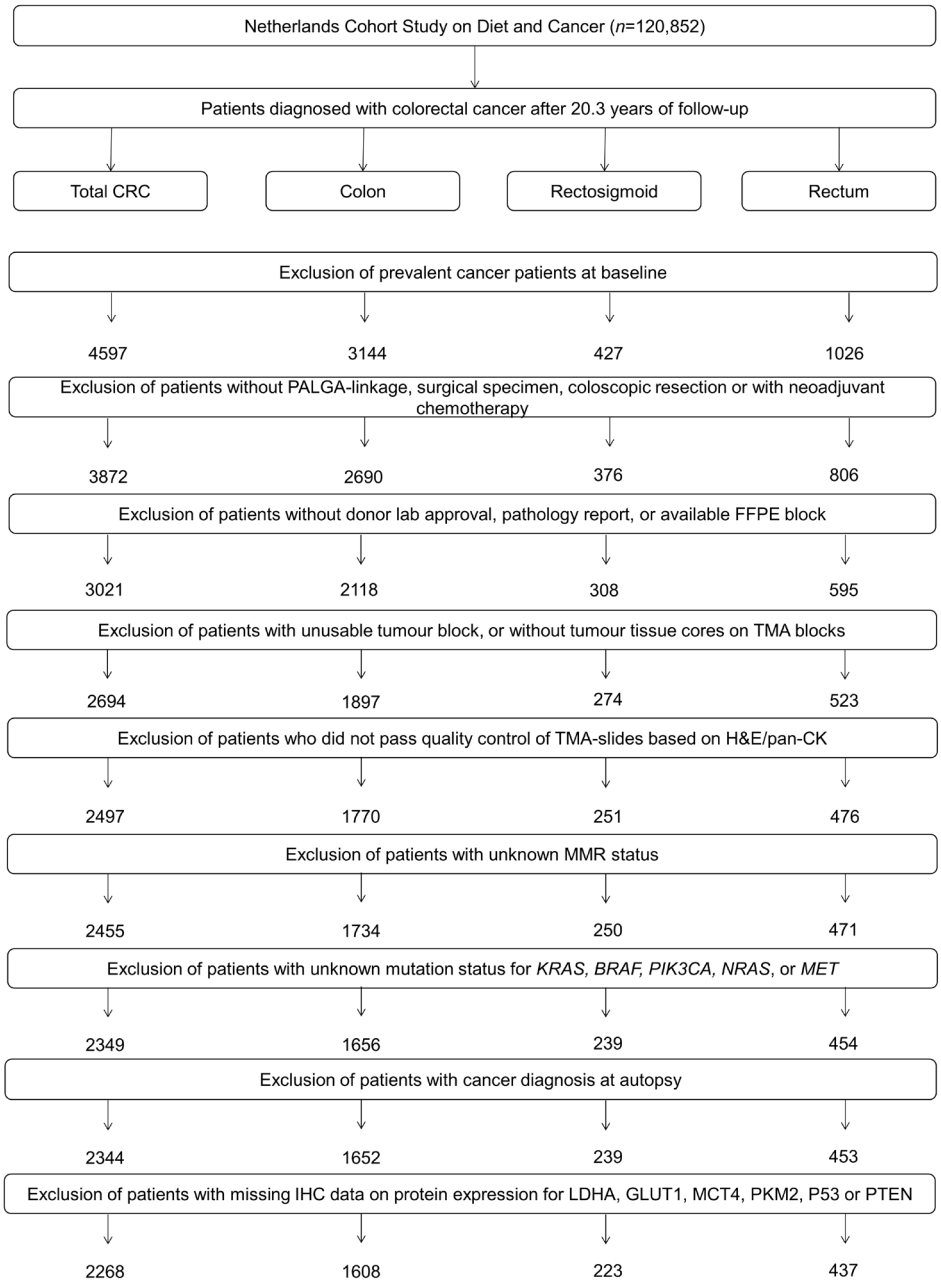


Figure 1 – Flow diagram of the number of CRC patients available for analyses in the Netherlands Cohort Study (NLCS), 1986–2006. CRC, colorectal cancer; PALGA, Netherlands pathology database; TMA, tissue microarray.

Clinical characteristics and follow-up

Information on patient and tumor characteristics, such as age at diagnosis, pathological (p) TNM stage, tumor location, and tumor differentiation grade was retrieved from the cancer registry or PALGA histopathology reports. Follow-up for vital status of the CRC patients was carried out through linkage to the Central Bureau of Genealogy and the municipal population registries until December 31, 2012. Patients who were found to have CRC at autopsy were excluded (*n* = 5) (**Figure 1**). The cause of death was retrieved from Statistics Netherlands. CRC-specific deaths included those with an underlying cause attributed to malignant neoplasms of the colon, rectosigmoid junction, or rectum. Vital status was available for 2,343 patients, and information regarding CRC-specific death was available for 2,305 patients.

Warburg-subtypes

The process of combining multiple core-level scores of proteins involved in the Warburg-effect (LDHA, GLUT1, MCT4, PKM2, p53, or PTEN) into patient-level Warburg-subtypes has been described previously.[23] Briefly: (1) scores from individual observers were combined into a "combination score" if the same score was given by at least two observers; (2) remaining discrepancies were either resolved by consensus agreement or an experienced pathologist determined the final score; (3) the final scores of all available tumor cores were averaged and the value was rounded to the nearest scoring category to obtain a patient-level score; (4) the average scores per patient were categorized as low, moderate, or high protein expression; (5) the expression levels of all six proteins were combined into a pathway-based sum score (range 0–12); (6) based on the sum score, 2,268 CRC patients were categorized into the "Warburg-low" (sum score 0–3, *n* = 646, 28.5%), "Warburg-moderate" (sum score 4–5, *n* = 820, 36.2%) or "Warburg-high" subtype (sum score 6–12, *n* = 802, 35.4%) (**Figure 1**).

Statistical analyses

Descriptive statistics and frequency distributions were calculated for clinical characteristics. Differences between mutational subgroups were evaluated using Chi-square for categorical variables and Kruskal–Wallis tests for continuous variables. The primary endpoints of the current study were CRC-specific survival, defined as the time from CRC diagnosis to CRC-related death or end of follow-up, and overall survival, defined as the time from CRC diagnosis to death from any cause or end of follow-up. Because of the limited number of events in the later period with follow-up of more than 10 years (CRC-specific deaths: *n* = 33, 3.3%; overall deaths: *n* = 266, 14.9%), survival analyses were restricted to 10 years of follow-up. The relationship between mutational subgroups and CRC-specific or overall survival was estimated using Kaplan–Meier curves and Wilcoxon tests. Hazard ratios (HRs) and 95% confidence intervals (CIs) were estimated using Cox proportional hazards regression. In addition, analyses were performed stratifying CRC

patients by pTNM stage or tumor location. Furthermore, the relationship between Warburg-subtypes and CRC-specific or overall survival within mutational subgroups was examined. The proportional hazards assumption was tested using the scaled Schoenfeld residuals,[31] by evaluating-log transformed survival curves or by introducing time-covariate interactions into the models. HRs were adjusted for a set of a priori selected prognostic factors: age at diagnosis, sex, tumor location, pTNM stage, differentiation grade, and adjuvant therapy. A separate category ('unknown') was used for patients with unknown clinical information regarding pTNM stage, differentiation grade, or adjuvant therapy to enable inclusion of these patients in the Cox proportional hazards models.

Disease stage was based on the pTNM classification according to the edition valid at the time of cancer diagnosis (**Supplementary Table S5**) resulting in the use of five different TNM editions (UICC TNM edition 3–6). However, the main TNM stage groupings (I/II/III/IV) have remained essentially unchanged.[32] Year of diagnosis and pTNM version were considered as potential confounders. Both variables were not included in the final models because they did not introduce a ≥ 10% change in HRs.

In sensitivity analyses, we repeated analyses after excluding CRC patients with unknown clinical information regarding pTNM stage, differentiation grade or adjuvant therapy (n = 247).

All analyses were conducted in Stata Statistical Software: Release 16 (StataCorp., College Station, TX). p-values <0.05 were considered significant.

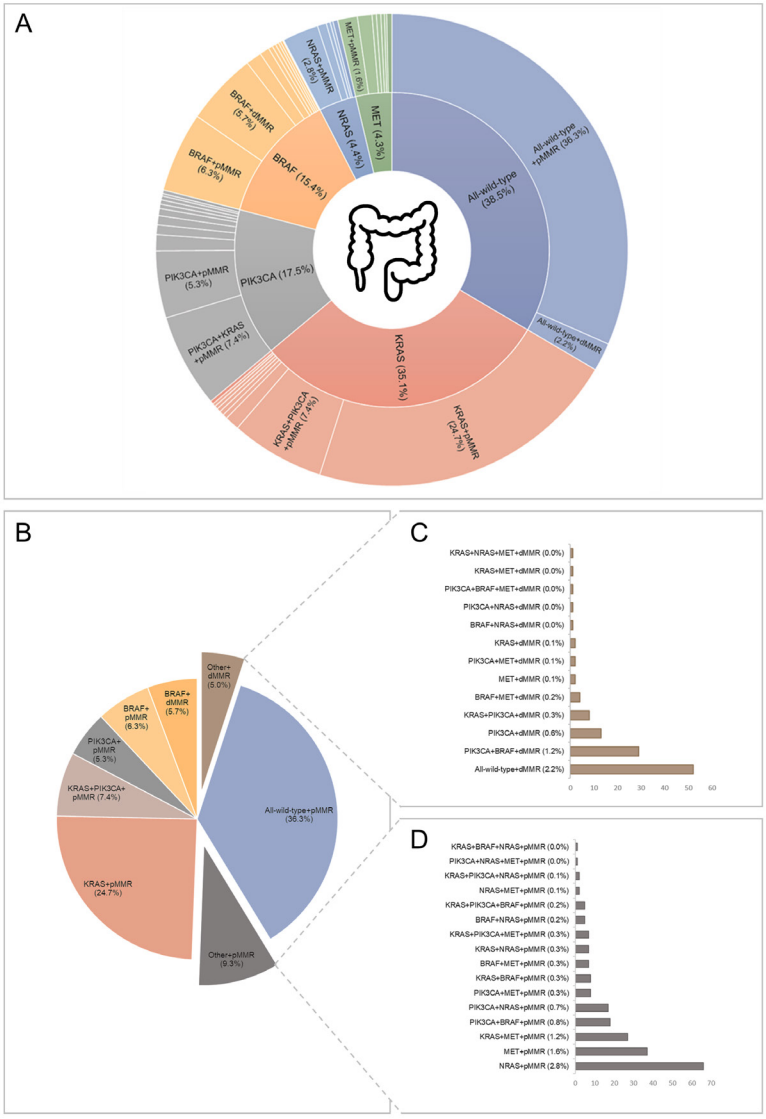


Figure 2 – Mutation frequencies and defined mutational subgroups of 2,344 CRC patients within the Netherlands Cohort Study (NLCS, 1986–2006). (**A**) Multi-layered pie chart showing the distribution and frequencies of genetic alterations in *KRAS, PIK3CA, BRAF, NRAS,* and *MET*, as well as single-, double-, and triple-mutations in combination with MMR status. The inner circle shows the total mutation frequencies of *KRAS, PIK3CA, BRAF, NRAS,* and *MET*. The outer circle shows single- double- and triple- mutations which together contribute to the total mutation frequency, in combination with MMR status. Mutations with a frequency ≤1.2% are not shown. Note: Percentages do not add up to 100% because there is some degree of overlap between mutational groups (e.g., $KRAS_{mut}$ + $PIK3CA_{mut}$). Image colon: Flaticon.com. (**B**) Pie chart showing the distribution and frequencies of the eight defined mutational subgroups: All-wild-type + pMMR, $KRAS_{mut}$ + pMMR, $KRAS_{mut}$ + $PIK3CA_{mut}$ + pMMR, $PIK3CA_{mut}$ + pMMR, $BRAF_{mut}$ + pMMR, $BRAF_{mut}$ + dMMR, other + pMMR, and other + dMMR. (**C**) Histogram showing the distributions and frequencies of combinations of markers (mutational status and MMR status) that together make up the other + dMMR subgroup. (**D**) Histogram showing the distribution and frequencies of combinations of markers (i.e., mutational status and MMR status) that together make up the other + pMMR subgroup.

RESULTS

After quality control and excluding patients with missing information on *KRAS*, *PIK3CA*, *BRAF*, *NRAS*, or *MET* mutational status (*n* = 117) or MMR status (*n* = 279), 2,344 CRC patients were available for analyses in the current study.

Mutation frequencies

All-wild-type cancers were identified in 903 (38.5%) CRC patients (**Figure 2A**). The majority of CRC patients (*n* = 1,441, 61.5%) had at least one mutation in one of the investigated genes. *KRAS*, *BRAF*, *PIK3CA*, *NRAS* or *MET* were mutated in 35.1%, 15.4%, 17.5%, 4.4%, and 4.3% of CRC, respectively (**Figure 2A**). Mutations in *HRAS* were not observed. *KRAS*, *BRAF, PIK3CA, NRAS*, and *MET* were exclusively mutated in 24.8%, 12.0%, 5.8%, 2.8%, and 1.7% of CRC, respectively (**Figure 2A**). Two or more genes were mutated in 336 (14.3%) CRC patients. Co-existing mutations in *KRAS* and *BRAF* were rare (*n* = 14, 0.6%). The most frequently observed double mutation included *KRAS* and *PIK3CA* (*n* = 181, 7.7%), whereas other double mutations were observed in less than 2% of CRC. Triple mutations were rare (*n* = 18, 0.8%). MMR deficiency (dMMR) was observed in 251 (10.7%) CRC patients. The majority of patients with dMMR CRC had a *BRAF* mutation (*n* = 134, 53.4%) or were all-wild-type (*n* = 52, 20.7%).

Mutational subgroups

Based on the observed single-, double-, or triple-mutation frequencies and MMR status, CRC patients were classified into eight mutually exclusive mutational subgroups, requiring at least 100 patients per subgroup, as: (1) All-wild-type + pMMR (*n* = 851, 36.3%), (2) $KRAS_{mut}$ + pMMR (*n* = 580, 24.7%), (3) $KRAS_{mut}$ + $PIK3CA_{mut}$ + pMMR (*n* = 173, 7.4%), (4) $PIK3CA_{mut}$ + pMMR (*n* = 124, 5.3%), (5) $BRAF_{mut}$ + pMMR (*n* = 147, 6.3%), (6) $BRAF_{mut}$ + dMMR (*n* = 134, 5.7%), (7) other + pMMR (*n* = 218, 9.3%), and (8) other + dMMR (*n* = 117, 5.0%) (**Figure 2B**). The other + dMMR subgroup mostly consisted of patients with all-wild-type CRC or patients with mutations in *BRAF* and/or *PIK3CA* (**Figure 2C**), whereas the other + pMMR subgroup mainly consisted of patients with mutations in *RAS (NRAS*, *KRAS)* and/or *MET* (**Figure 2D**). Clinical characteristics of each mutational subgroup are shown in **Table 1**. Mutational subgroups differed significantly with respect to age at diagnosis, sex, tumor location, pTNM stage, tumor extension (pT), lymph node involvement (pN), differentiation grade, and adjuvant therapy.

Table 1 – Clinical characteristics of colorectal cancer patients within the Netherlands Cohort Study (NLCS, 1986–2006) according to mutational subgroup (n = 2,344).

	Total	Mutational subgroups								
		All-wild-type + pMMR	$KRAS_{mut}$ + pMMR	$KRAS_{mut}$ + $PIK3CA_{mut}$ + pMMR	$PIK3CA_{mut}$ + pMMR	$BRAF_{mut}$		Other		P-value*
						$BRAF_{mut}$ + pMMR	$BRAF_{mut}$ + dMMR	Other + pMMR	Other + dMMR	
Number of patients, *n* (%)	2344	851 (36.3)	580 (24.7)	173 (7.4)	124 (5.3)	147 (6.3)	134 (5.7)	218 (9.3)	117 (5.0)	
Age at diagnosis in years, median (range)	74.0 (55.0-89.0)	74.0 (55.0-89.0)	74.0 (56.0-89.0)	74.0 (60.0-88.0)	72.0 (58.0-84.0)	75.0 (56.0-88.0)	76.0 (62.0-86.0)	73.0 (56.0-87.0)	74.0 (57.0-87.0)	<0.001†
Sex, *n* (%)										
Men	1311 (55.9)	542 (63.7)	311 (53.6)	94 (54.3)	79 (63.7)	62 (42.2)	41 (30.6)	129 (59.2)	53 (45.3)	<0.001
Women	1033 (44.1)	309 (36.3)	269 (46.4)	79 (45.7)	45 (36.3)	85 (57.8)	93 (69.4)	89 (40.8)	64 (54.7)	
Tumor location, *n* (%)										
Colon	1652 (70.5)	515 (60.5)	387 (66.7)	133 (76.9)	91 (73.4)	132 (89.8)	132 (98.5)	149 (68.4)	113 (96.6)	<0.001
Rectosigmoid	239 (10.2)	116 (13.6)	74 (12.8)	13 (7.5)	11 (8.9)	3 (2.0)	1 (0.8)	20 (9.2)	1 (0.9)	
Rectum	453 (19.3)	220 (25.9)	119 (20.5)	27 (15.6)	22 (17.7)	12 (8.2)	1 (0.8)	49 (22.5)	3 (2.6)	
pTNM stage, *n* (%)										
I	459 (19.6)	194 (22.8)	119 (20.5)	29 (16.8)	18 (14.5)	10 (6.8)	19 (14.2)	52 (23.9)	18 (15.4)	<0.001
II	877 (37.4)	305 (35.8)	185 (31.9)	74 (42.8)	53 (42.7)	47 (32.0)	65 (48.5)	75 (34.4)	73 (62.4)	
III	614 (26.2)	220 (25.9)	157 (27.1)	40 (23.1)	30 (24.2)	59 (40.1)	37 (27.6)	51 (23.4)	20 (17.1)	
IV	330 (14.1)	102 (12.0)	105 (18.1)	27 (15.6)	18 (14.5)	27 (18.4)	10 (7.5)	35 (16.1)	6 (5.1)	
Unknown	64 (2.7)	30 (3.5)	14 (2.4)	3 (1.7)	5 (4.0)	4 (2.7)	3 (2.2)	5 (2.3)	-	
Tumor extension (pT), *n* (%)										
T1	102 (4.4)	48 (5.6)	27 (4.7)	10 (5.8)	4 (3.2)	1 (0.7)	2 (1.5)	10 (4.6)	-	<0.001
T2	439 (18.7)	181 (21.3)	116 (20.0)	23 (13.3)	16 (12.9)	13 (8.8)	17 (12.7)	53 (24.3)	20 (17.1)	
T3	1511 (64.5)	522 (61.3)	373 (64.3)	117 (67.6)	89 (71.8)	101 (68.7)	94 (70.2)	129 (59.2)	86 (73.5)	
T4	221 (9.4)	67 (7.9)	46 (7.9)	20 (11.6)	10 (8.1)	27 (18.4)	19 (14.2)	21 (9.6)	11 (9.4)	
Unknown	71 (3.0)	33 (3.9)	18 (3.1)	3 (1.7)	5 (4.0)	5 (3.4)	2 (1.5)	5 (2.3)	-	
Lymph node involvement (pN), *n* (%)										
N0	1212 (51.7)	444 (52.2)	280 (48.3)	95 (54.9)	63 (50.8)	54 (36.7)	77 (57.5)	119 (54.6)	80 (68.4)	<0.001
N+	853 (36.4)	297 (34.9)	234 (40.3)	59 (34.1)	40 (32.3)	81 (55.1)	44 (32.8)	75 (34.4)	23 (19.7)	
Unknown	279 (11.9)	110 (12.9)	66 (11.4)	19 (11.0)	21 (16.9)	12 (8.2)	13 (9.7)	24 (11.0)	14 (12.0)	

Table 1 – Continued

	Total	Mutational subgroups								
		All-wild-type + pMMR	$KRAS_{mut}$ + pMMR	$KRAS_{mut}$ + $PIK3CA_{mut}$ + pMMR	$PIK3CA_{mut}$ + pMMR	$BRAF_{mut}$		Other		P-value*
						$BRAF_{mut}$ + pMMR	$BRAF_{mut}$ + dMMR	Other + pMMR	Other + dMMR	
Differentiation grade, *n* (%)										
Well	198 (8.5)	79 (9.3)	57 (9.8)	15 (8.7)	11 (8.9)	10 (6.8)	4 (3.0)	17 (7.8)	5 (4.3)	<0.001
Moderate	1528 (65.2)	597 (70.2)	381 (65.7)	122 (70.5)	86 (69.4)	69 (46.9)	66 (49.3)	150 (68.8)	57 (48.7)	
Poor/undifferentiated	412 (17.6)	101 (11.9)	81 (14.0)	22 (12.7)	18 (14.5)	59 (40.1)	53 (39.6)	33 (15.1)	45 (38.5)	
Unknown	206 (8.8)	74 (8.7)	61 (10.5)	14 (8.1)	9 (7.3)	9 (6.1)	11 (8.2)	18 (8.3)	10 (8.6)	
Adjuvant therapy, *n* (%)										
No	1830 (78.1)	654 (76.9)	434 (74.8)	142 (82.1)	94 (75.8)	121 (82.3)	121 (90.3)	158 (72.5)	106 (90.6)	<0.001
Yes	494 (21.1)	186 (21.9)	139 (24.0)	31 (17.9)	29 (23.4)	26 (17.7)	13 (9.7)	59 (27.1)	11 (9.4)	
Unknown	20 (0.9)	11 (1.3)	7 (1.2)	-	1 (0.8)	-	-	1 (0.5)	-	

*P-value for the χ2 test, unless otherwise specified
†P-value for the Kruskall-Wallis test
TNM, tumor-node-metastasis; pMMR, mismatch repair proficient; dMMR, mismatch repair deficient.

Patients with *BRAF*$_{mut}$ CRC had the highest median age at diagnosis ($p < 0.001$) and were more often women ($p < 0.001$), particularly those with *BRAF*$_{mut}$ + dMMR CRC. *BRAF*$_{mut}$ cancers were almost exclusively located in the colon (*BRAF*$_{mut}$ + pMMR: 89.8%, *BRAF*$_{mut}$ + dMMR: 98.5%, $p < 0.001$). In contrast, all-wild-type + pMMR cancers were more frequently located in the rectum compared to other mutational subgroups ($p < 0.001$).

Patients with *BRAF*$_{mut}$ + pMMR, *KRAS*$_{mut}$ + pMMR, *KRAS*$_{mut}$ + *PIK3CA*$_{mut}$ + pMMR, or other + dMMR CRC were more likely to be diagnosed with advanced pTNM stage ($p < 0.001$). Patients with *BRAF*$_{mut}$ + pMMR CRC more frequently had a higher depth of invasion (pT, $p < 0.001$) and lymph node involvement (pN+, $p < 0.001$). Patients with *BRAF*$_{mut}$ or other + dMMR CRC were more often diagnosed with poorly differentiated cancers ($p < 0.001$). Lastly, patients with *BRAF*$_{mut}$ + dMMR and other + dMMR CRC least often received adjuvant therapy ($p < 0.001$).

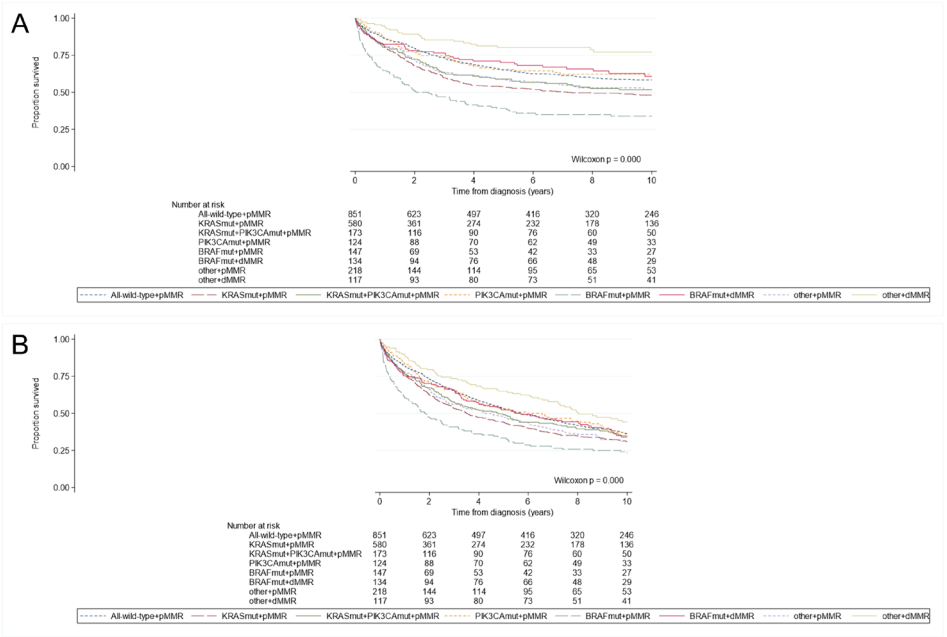


Figure 3 – Kaplan–Meier curves according to mutational subgroups (i.e., all-wild-type + pMMR, *KRAS*$_{mut}$ + pMMR, *KRAS*$_{mut}$ + *PIK3CA*$_{mut}$ + pMMR, *PIK3CA*$_{mut}$ + pMMR, B*RAF*$_{mut}$ + pMMR, *BRAF*$_{mut}$ + dMMR, other + pMMR, and other + dMMR) in colorectal cancer patients within the Netherlands Cohort Study (NLCS, 1986–2006), showing (**A**) CRC-specific survival (median survival times: *KRAS*$_{mut}$ + pMMR, 7.16 years and *BRAF*$_{mut}$ + pMMR, 2.48 years) and (**B**) overall survival (median survival times: All-wild-type + pMMR, 5.73 years; *KRAS*$_{mut}$ + pMMR, 3.49 years; *KRAS*$_{mut}$ + *PIK3CA*$_{mut}$ + pMMR, 4.79 years; *PIK3CA*$_{mut}$ + pMMR, 5.91 years; *BRAF*$_{mut}$ + pMMR, 1.83 years; *BRAF*$_{mut}$ + dMMR, 5.46 years; other + pMMR, 4.25 years; other + dMMR, 8.04 years).

Survival of CRC patients within mutational subgroups

The median (range) follow-up time since diagnosis was 4.86 years (0.0027–25.99 years). Survival analyses were restricted to 10 years of follow-up. During these first 10 years of follow-up, 1,522 (64.9%) deaths were observed, of which 961 (63.1%) were CRC-related deaths.

Univariable Kaplan–Meier curves showed statistically significant survival differences between patients for the different mutational subgroups (**Figure 3**). The poorest CRC-specific and overall-survival was observed for patients with $BRAF_{mut}$ + pMMR CRC, followed by $KRAS_{mut}$ + pMMR CRC, $KRAS_{mut}$ + $PIK3CA_{mut}$ + pMMR, or other + pMMR CRC (**Figure 3**). Multivariable-adjusted Cox-regression models showed that patients with $KRAS_{mut}$ + pMMR, $KRAS_{mut}$ + $PIK3CA_{mut}$ + pMMR, $BRAF_{mut}$ + pMMR, or other + pMMR CRC had a statistically significant worse CRC-specific and/or overall survival compared to patients with all-wild-type + pMMR CRC (**Table 2**). Patients with $BRAF_{mut}$ + pMMR CRC had the poorest survival ($HR_{CRC\text{-}specific}$ 1.88; 95% CI 1.48–2.40 and $HR_{overall}$ 1.46; 95% CI 1.18–1.81), followed by patients with $KRAS_{mut}$ + pMMR CRC ($HR_{CRC\text{-}specific}$ 1.34; 95% CI 1.14–1.58 and $HR_{overall}$ 1.19; 95% CI 1.05–1.36), other + pMMR CRC ($HR_{CRC\text{-}specific}$ 1.32; 95% CI 1.05–1.67 and $HR_{overall}$ 1.26; 95% CI 1.05–1.52), and $KRAS_{mut}$ + $PIK3CA_{mut}$ + pMMR CRC ($HR_{CRC\text{-}specific}$ 1.29; 95% CI 1.00–1.66 and $HR_{overall}$ 1.11; 95% CI 0.91–1.37) (**Table 2**). Patients with other + dMMR CRC had the most favorable CRC-specific and overall survival ($HR_{CRC\text{-}specific}$ 0.48; 95% CI 0.31–0.74 and $HR_{overall}$ 0.73; 95% CI 0.56–0.96).

When stratifying patients by tumor location, a statistically significant worse CRC-specific and overall-survival was observed for patients with $KRAS_{mut}$ + pMMR cancers and $BRAF_{mut}$ + pMMR cancers located in the colon or rectum compared to patients with all-wild-type + pMMR cancers in the colon or rectum (**Table 2**). Moreover, patients with other + pMMR cancers located in the colon had a statistically significant worse survival compared to patients with all-wild-type + pMMR cancers located in the colon. Patients with $PIK3CA_{mut}$ + pMMR cancer in the rectum showed a borderline statistically significant (possibly because of low power) worse overall survival ($HR_{overall}$ 1.62; 95% CI 0.97–2.73) compared to patients with all-wild-type + pMMR rectal cancer. No statistically significant survival differences were observed for any of the mutational subgroups in patients with cancers located in the rectosigmoid (**Table 2**).

Table 2 – Univariable and multivariable-adjusted hazard ratios for associations between mutational subgroups and survival of colorectal cancer patients within the Netherlands Cohort Study (NLCS, 1986–2006), stratified on tumor location (i.e., colon, rectosigmoid, rectum).

	N	CRC-specific survival			Overall survival		
		CRC deaths (%)	HR (95% CI)		Deaths (%)	HR (95% CI)	
			Univariable	Multivariable-adjusted*		Univariable	Multivariable-adjusted*
Colorectal							
All-wild-type + pMMR	851	316 (37.1)	1.00 (ref)	1.00 (ref)	532 (62.5)	1.00 (ref)	1.00 (ref)
$KRAS_{mut}$ + pMMR	580	274 (47.2)	1.42 (1.21-1.67)	1.34 (1.14-1.58)	395 (68.1)	1.24 (1.09-1.41)	1.19 (1.05-1.36)
$KRAS_{mut}$ + $PIK3CA_{mut}$ + pMMR	173	76 (43.9)	1.27 (0.98-1.63)	1.29 (1.00-1.66)	111 (64.2)	1.10 (0.90-1.35)	1.11 (0.91-1.37)
$PIK3CA_{mut}$ + pMMR	124	43 (34.7)	0.93 (0.68-1.28)	0.92 (0.67-1.26)	78 (62.9)	1.00 (0.79-1.27)	1.02 (0.80-1.30)
$BRAF_{mut}$ + pMMR	147	91 (61.9)	2.28 (1.81-2.89)	1.88 (1.48-2.40)	112 (76.2)	1.72 (1.40-2.11)	1.46 (1.18-1.81)
$BRAF_{mut}$ + dMMR	134	44 (32.8)	0.92 (0.67-1.26)	0.92 (0.66-1.29)	84 (62.7)	1.05 (0.83-1.32)	0.98 (0.77-1.25)
Other + pMMR	218	94 (43.1)	1.26 (1.00-1.59)	1.32 (1.05-1.67)	147 (67.4)	1.19 (0.99-1.43)	1.26 (1.05-1.52)
Other + dMMR	117	23 (19.7)	0.48 (0.32-0.74)	0.48 (0.31-0.74)	63 (53.8)	0.77 (0.59-1.00)	0.73 (0.56-0.96)
Colon							
All-wild-type + pMMR	515	202 (39.2)	1.00 (ref)	1.00 (ref)	337 (65.4)	1.00 (ref)	1.00 (ref)
$KRAS_{mut}$ + pMMR	387	183 (47.3)	1.34 (1.09-1.63)	1.30 (1.06-1.60)	264 (68.2)	1.16 (0.99-1.37)	1.16 (0.99-1.37)
$KRAS_{mut}$ + $PIK3CA_{mut}$ + pMMR	133	58 (43.6)	1.20 (0.89-1.60)	1.24 (0.92-1.66)	85 (63.9)	1.05 (0.83-1.33)	1.10 (0.87-1.40)
$PIK3CA_{mut}$ + pMMR	91	31 (34.1)	0.85 (0.58-1.24)	0.82 (0.56-1.21)	56 (61.5)	0.91 (0.69-1.21)	0.92 (0.69-1.23)
$BRAF_{mut}$ + pMMR	132	80 (60.6)	2.07 (1.60-2.68)	1.83 (1.40-2.40)	100 (75.8)	1.59 (1.27-1.99)	1.46 (1.16-1.84)
$BRAF_{mut}$ + dMMR	132	44 (33.3)	0.86 (0.62-1.20)	0.94 (0.67-1.33)	83 (62.9)	0.98 (0.77-1.24)	0.99 (0.77-1.28)
Other + pMMR	149	67 (45.0)	1.25 (0.95-1.65)	1.33 (1.01-1.76)	105 (70.5)	1.20 (0.97-1.50)	1.28 (1.03-1.60)
Other + dMMR	113	21 (18.6)	0.42 (0.27-0.66)	0.45 (0.29-0.72)	60 (53.1)	0.70 (0.53-0.93)	0.72 (0.54-0.96)
Rectosigmoid							
All-wild-type + pMMR	116	36 (31.0)	1.00 (ref)	1.00 (ref)	73 (62.9)	1.00 (ref)	1.00 (ref)
$KRAS_{mut}$ + pMMR	74	33 (44.6)	1.54 (0.96-2.47)	1.38 (0.84-2.28)	55 (74.3)	1.33 (0.93-1.89)	1.23 (0.85-1.77)
$KRAS_{mut}$ + $PIK3CA_{mut}$ + pMMR	13	5 (38.5)	1.16 (0.45-2.95)	1.55 (0.60-4.03)	7 (53.8)	0.77 (0.36-1.68)	0.87 (0.40-1.91)
$PIK3CA_{mut}$ + pMMR	11	1 (9.1)	0.24 (0.03-1.77)	0.35 (0.05-2.63)	5 (45.5)	0.57 (0.23-1.41)	0.87 (0.35-2.19)
$BRAF_{mut}$ + pMMR	3	2 (66.7)	2.42 (0.58-10.07)	2.45 (0.55-10.81)	2 (66.7)	1.25 (0.31-5.08)	1.01 (0.24-4.27)
$BRAF_{mut}$ + dMMR	1	0 (0.0)	-	-	1 (100.0)	-	-
Other + pMMR	20	9 (45.0)	1.47 (0.71-3.06)	1.71 (0.76-3.84)	13 (65.0)	1.03 (0.57-1.85)	1.33 (0.70-2.53)
Other + dMMR	1	0 (0.0)	-	-	1 (100.0)	-	-

Table 2 – Continued

	N	CRC-specific survival			Overall survival		
		CRC deaths (%)	HR (95% CI)		Deaths (%)	HR (95% CI)	
			Univariable	Multivariable-adjusted*		Univariable	Multivariable-adjusted*
Rectum							
All-wild-type + pMMR	220	78 (35.5)	1.00 (ref)	1.00 (ref)	122 (55.5)	1.00 (ref)	1.00 (ref)
$KRAS_{mut}$ + pMMR	119	58 (48.7)	1.61 (1.14-2.26)	1.51 (1.06-2.14)	76 (63.9)	1.36 (1.02-1.81)	1.29 (0.96-1.73)
$KRAS_{mut}$ + $PIK3CA_{mut}$ + pMMR	27	13 (48.1)	1.40 (0.78-2.52)	1.32 (0.73-2.39)	19 (70.4)	1.32 (0.82-2.14)	1.21 (0.74-1.97)
$PIK3CA_{mut}$ + pMMR	22	11 (50.0)	1.64 (0.87-3.09)	1.61 (0.85-3.07)	17 (77.3)	1.69 (1.01-2.80)	1.62 (0.97-2.73)
$BRAF_{mut}$ + pMMR	12	9 (75.0)	2.94 (1.47-5.86)	2.32 (1.14-4.69)	10 (83.3)	2.16 (1.13-4.12)	1.70 (0.88-3.27)
$BRAF_{mut}$ + dMMR	1	0 (0.0)	-	-	0 (0.0)	-	-
Other + pMMR	49	18 (36.7)	1.12 (0.67-1.87)	1.13 (0.67-1.90)	29 (59.2)	1.16 (0.77-1.73)	1.12 (0.74-1.70)
Other + dMMR	3	2 (66.7)	1.97 (0.48-8.03)	4.12 (0.99-17.21)	2 (66.7)	1.31 (0.32-5.31)	2.46 (0.60-10.09)

*Adjusted for age at diagnosis (years), sex (men/women), tumor location (colon/rectosigmoid/rectum), pTNM stage (I/II/III/IV/unknown), differentiation grade (well/moderate/poor/unknown), and adjuvant therapy (yes/no/unknown).
CRC, colorectal cancer; HR, hazard ratio; CI, confidence interval; pMMR, mismatch repair proficient; dMMR, mismatch repair deficient.

Next, we stratified CRC patients by pTNM stage to assess the disease stage-dependent prognostic value of the mutational subgroups (**Supplementary Table S6**). In pTNM stage I, similar associations were observed for CRC-specific survival of the mutational subgroups, whereas no statistically significant associations were observed for overall survival. Compared to patients with all-wild-type + pMMR CRC, only patients with *KRAS*$_{mut}$ + pMMR CRC (HR 1.52; 95% CI 1.07–2.15) had a significantly worse CRC-specific survival in pTNM Stage II. For pTNM stages III and IV, patients with *BRAF*$_{mut}$ + pMMR CRC had a significantly worse CRC-specific and overall survival compared to patients with all-wild-type + pMMR CRC. Moreover, patients with other + pMMR CRC had a significantly worse overall survival (HR 1.49; 95% CI 1.05–2.12) in pTNM stage III. Patients with *KRAS*$_{mut}$ + pMMR CRC had a significantly worse CRC-specific (HR 1.37; 95% CI 1.02–1.85) and overall survival (HR 1.30; 95% CI 0.98–1.73) compared to patients with all-wild-type + pMMR CRC in pTNM stage IV. Lastly, patients with other + pMMR CRC had a (borderline) significantly worse overall survival in pTNM Stage IV, whereas patients with other + dMMR CRC had a significantly better CRC-specific and overall survival in pTNM stage IV (**Supplementary Table S6**).

Relationship between mutational subgroups and Warburg-subtypes

After excluding patients with missing protein expression data on LDHA, GLUT1, MCT4, PKM2, p53, or PTEN (*n* = 76), 2,268 CRC patients with information on Warburg-subtype and mutational status were available for analyses (Warburg-low: *n* = 646, 28.5%; Warburg-moderate: *n* = 820, 36.2%; Warburg-high: *n* = 802, 35.4%).

A cross-tabulation of the mutational subgroups by Warburg-subtypes for all CRC as well as for colon, rectosigmoid and rectal cancers separately is shown in **Table 3**. All-wild-type + pMMR, *PIK3CA*$_{mut}$ + pMMR, and other + pMMR CRC were more frequently classified as Warburg-low. *BRAF*$_{mut}$ and other + dMMR CRC were more frequently classified as Warburg-high. *KRAS*$_{mut}$ + pMMR CRC were more frequently classified as Warburg-moderate or Warburg-high. Stratifying on tumor location showed similar results, except for cancers located in the rectum, where *PIK3CA*$_{mut}$ + pMMR cancers were more frequently classified as Warburg-high. When stratifying on pTNM stage (**Supplementary Table S7**) similar results were observed.

Table 3 – Frequencies of the mutational subgroups, stratified on tumor location (colon, rectosigmoid, rectum) and Warburg-subtype (Warburg-low, –moderate, –high).

	Total	Warburg-low	Warburg-moderate	Warburg-high
Colorectal				
All-wild-type + pMMR	827 (36.5)	285 (44.1)	300 (36.6)	242 (30.2)
$KRAS_{mut}$ + pMMR	554 (24.4)	128 (19.8)	226 (27.6)	200 (24.9)
$KRAS_{mut}$ + $PIK3CA_{mut}$ + pMMR	168 (7.4)	48 (7.4)	69 (8.4)	51 (6.4)
$PIK3CA_{mut}$ + pMMR	118 (5.2)	43 (6.7)	36 (4.4)	39 (4.9)
$BRAF_{mut}$ + pMMR	144 (6.4)	24 (3.7)	38 (4.6)	82 (10.2)
$BRAF_{mut}$ + dMMR	132 (5.8)	32 (5.0)	39 (4.8)	61 (7.6)
Other + pMMR	211 (9.3)	63 (9.8)	75 (9.2)	73 (9.1)
Other + dMMR	114 (5.0)	23 (3.6)	37 (4.5)	54 (6.7)
Colon				
All-wild-type + pMMR	501 (31.2)	159 (37.1)	187 (32.2)	155 (25.9)
$KRAS_{mut}$ + pMMR	374 (23.3)	81 (18.9)	154 (26.5)	139 (23.2)
$KRAS_{mut}$ + $PIK3CA_{mut}$ + pMMR	129 (8.0)	36 (8.4)	53 (9.1)	40 (6.7)
$PIK3CA_{mut}$ + pMMR	88 (5.5)	34 (7.9)	29 (5.0)	25 (4.2)
$BRAF_{mut}$ + pMMR	129 (8.0)	23 (5.4)	33 (5.7)	73 (12.2)
$BRAF_{mut}$ + dMMR	130 (8.1)	31 (7.2)	38 (6.5)	61 (10.2)
Other + pMMR	146 (9.1)	43 (10.0)	51 (8.8)	52 (8.7)
Other + dMMR	111 (6.9)	22 (5.1)	36 (6.2)	53 (8.9)
Rectosigmoid				
All-wild-type + pMMR	112 (50.2)	51 (66.2)	35 (45.5)	26 (37.7)
$KRAS_{mut}$ + pMMR	69 (30.9)	13 (16.9)	27 (35.1)	29 (42.0)
$KRAS_{mut}$ + $PIK3CA_{mut}$ + pMMR	12 (5.4)	3 (3.9)	5 (6.5)	4 (5.8)
$PIK3CA_{mut}$ + pMMR	8 (3.6)	4 (5.2)	1 (1.3)	3 (4.4)
$BRAF_{mut}$ + pMMR	3 (1.4)	1 (1.3)	-	2 (2.9)
$BRAF_{mut}$ + dMMR	1 (0.5)	1 (1.3)	-	-
Other + pMMR	18 (8.1)	4 (5.2)	9 (11.7)	5 (7.3)
Other + dMMR	-	-	-	-
Rectum				
All-wild-type + pMMR	214 (49.0)	75 (53.6)	78 (48.2)	61 (45.2)
$KRAS_{mut}$ + pMMR	111 (25.4)	34 (24.3)	45 (27.8)	32 (23.7)
$KRAS_{mut}$ + $PIK3CA_{mut}$ + pMMR	27 (6.2)	9 (6.4)	11 (6.8)	7 (5.2)
$PIK3CA_{mut}$ + pMMR	22 (5.0)	5 (3.6)	6 (3.7)	11 (8.2)
$BRAF_{mut}$ + pMMR	12 (2.8)	-	5 (3.1)	7 (5.2)
$BRAF_{mut}$ + dMMR	1 (0.2)	-	1 (0.6)	-
Other + pMMR	47 (10.8)	16 (11.4)	15 (9.3)	16 (11.9)
Other + dMMR	3 (0.7)	1 (0.7)	1 (0.6)	1 (0.7)

Table 4 – Univariable and multivariable-adjusted hazard ratios for associations between Warburg-subtypes and survival of colorectal cancer patients within the Netherlands Cohort Study (NLCS, 1986-2006) in mutational subgroups.

	N	CRC-specific survival			Overall survival		
		CRC deaths (%)	HR (95% CI)		Deaths (%)	HR (95% CI)	
			Univariable	Multivariable-adjusted*		Univariable	Multivariable-adjusted*
Total							
Warburg-low	646	241 (37.3)	1.00 (ref)	1.00 (ref)	393 (60.8)	1.00 (ref)	1.00 (ref)
Warburg-moderate	820	343 (41.8)	1.15 (0.98-1.36)	1.07 (0.91-1.26)	526 (64.1)	1.09 (0.96-1.24)	1.05 (0.92-1.20)
Warburg-high	802	346 (43.1)	1.26 (1.07-1.49)	1.16 (0.98-1.37)	550 (68.6)	1.25 (1.10-1.43)	1.20 (1.05-1.36)
All-wild-type + pMMR							
Warburg-low	285	97 (34.0)	1.00 (ref)	1.00 (ref)	173 (60.7)	1.00 (ref)	1.00 (ref)
Warburg-moderate	300	112 (37.3)	1.07 (0.82-1.41)	0.99 (0.75-1.31)	177 (59.0)	0.95 (0.77-1.17)	0.94 (0.76-1.16)
Warburg-high	242	93 (38.4)	1.22 (0.92-1.62)	0.98 (0.72-1.32)	163 (67.4)	1.22 (0.98-1.51)	1.10 (0.88-1.38)
$KRAS_{mut}$ + pMMR							
Warburg-low	128	55 (43.0)	1.00 (ref)	1.00 (ref)	81 (63.3)	1.00 (ref)	1.00 (ref)
Warburg-moderate	226	104 (46.0)	1.08 (0.78-1.50)	1.06 (0.76-1.48)	149 (65.9)	1.06 (0.81-1.39)	1.00 (0.76-1.32)
Warburg-high	200	105 (52.5)	1.39 (1.00-1.92)	1.31 (0.94-1.84)	147 (73.5)	1.35 (1.03-1.78)	1.27 (0.96-1.68)
$KRAS_{mut}$ + $PIK3CA_{mut}$ + pMMR							
Warburg-low	48	22 (45.8)	1.00 (ref)	1.00 (ref)	30 (62.5)	1.00 (ref)	1.00 (ref)
Warburg-moderate	69	31 (44.9)	1.04 (0.60-1.80)	1.25 (0.70-2.23)	46 (66.7)	1.11 (0.70-1.76)	1.30 (0.81-2.11)
Warburg-high	51	21 (41.2)	0.95 (0.52-1.72)	0.95 (0.48-1.88)	32 (62.7)	1.03 (0.63-1.70)	1.07 (0.62-1.86)
$PIK3CA_{mut}$ + pMMR							
Warburg-low	43	16 (37.2)	1.00 (ref)	1.00 (ref)	22 (51.2)	1.00 (ref)	1.00 (ref)
Warburg-moderate	36	14 (38.9)	1.14 (0.56-2.34)	0.63 (0.28-1.43)	25 (69.4)	1.57 (0.88-2.79)	1.22 (0.65-2.27)
Warburg-high	39	12 (30.8)	0.88 (0.41-1.85)	0.92 (0.39-2.17)	27 (69.2)	1.50 (0.86-2.64)	1.59 (0.85-2.97)
$BRAF_{mut}$ + pMMR							
Warburg-low	24	12 (50.0)	1.00 (ref)	1.00 (ref)	18 (75.0)	1.00 (ref)	1.00 (ref)
Warburg-moderate	38	27 (71.1)	1.49 (0.76-2.95)	1.22 (0.59-2.50)	31 (81.6)	1.15 (0.64-2.05)	0.99 (0.53-1.83)
Warburg-high	82	51 (62.2)	1.16 (0.62-2.18)	1.42 (0.74-2.71)	61 (74.4)	0.92 (0.54-1.55)	1.13 (0.65-1.95)
$BRAF_{mut}$ + dMMR							
Warburg-low	32	10 (31.3)	1.00 (ref)	1.00 (ref)	16 (50.0)	1.00 (ref)	1.00 (ref)
Warburg-moderate	39	13 (33.3)	1.03 (0.45-2.35)	1.46 (0.61-3.54)	24 (61.5)	1.18 (0.63-2.22)	1.52 (0.78-2.96)
Warburg-high	61	21 (34.4)	1.13 (0.53-2.40)	1.41 (0.60-3.31)	42 (68.9)	1.44 (0.81-2.56)	1.54 (0.83-2.87)

Table 3 – Continued

	N	CRC-specific survival			Overall survival		
		CRC deaths (%)	HR (95% CI)		Deaths (%)	HR (95% CI)	
			Univariable	Multivariable-adjusted*		Univariable	Multivariable-adjusted*
Other + pMMR							
Warburg-low	63	28 (44.4)	1.00 (ref)	1.00 (ref)	42 (66.7)	1.00 (ref)	1.00 (ref)
Warburg-moderate	75	33 (44.0)	1.06 (0.64-1.75)	1.18 (0.70-1.99)	54 (72.0)	1.19 (0.79-1.78)	1.30 (0.85-1.97)
Warburg-high	73	30 (41.1)	0.91 (0.55-1.53)	0.95 (0.55-1.64)	48 (65.8)	0.98 (0.65-1.48)	1.02 (0.66-1.58)
Other + dMMR							
Warburg-low	23	1 (4.3)	1.00 (ref)	1.00 (ref)	11 (47.8)	1.00 (ref)	1.00 (ref)
Warburg-moderate	37	9 (24.3)	7.18 (0.91-56.75)	5.03 (0.59-42.62)	20 (54.1)	1.55 (0.74-3.24)	1.37 (0.63-2.99)
Warburg-high	54	13 (24.1)	7.09 (0.93-54.27)	8.13 (0.93-71.34)	30 (55.6)	1.61 (0.80-3.21)	1.65 (0.78-3.50)

*Adjusted for age at diagnosis (years), sex (men/women), tumor location (colon/rectosigmoid/rectum), pTNM stage (I/II/III/IV/unknown), differentiation grade (well/moderate/poor/unknown), and adjuvant therapy (yes/no/unknown).
CRC, colorectal cancer; HR, hazard ratio; CI, confidence interval; pMMR, mismatch repair proficient; dMMR, mismatch repair deficient

Survival of Warburg-subtypes within mutational subgroups

Univariable Kaplan–Meier curves showed no statistically significant survival differences between Warburg-subtypes within any of the mutational subgroups (**Supplementary Figure S1**).

Multivariable-adjusted analyses showed that, compared to patients with Warburg-low CRC, patients with Warburg-high CRC had a (borderline) statistically significant worse CRC-specific (HR 1.16; 95% CI 0.98–1.37) and overall survival (HR 1.20; 95% CI 1.05–1.36) (**Table 4**). Further analyses according to mutational subgroups showed no statistically significant associations with survival across Warburg-subtypes within any of the mutational subgroups. A worse, though not statistically significant, CRC-specific and overall survival was observed for the Warburg-high subtype as compared to the Warburg-low subtype in patients with $KRAS_{mut}$ + pMMR CRC ($HR_{CRC\text{-}specific}$ 1.31; 95% CI 0.94–1.84 and $HR_{overall}$ 1.27; 95% CI 0.96–1.68), $BRAF_{mut}$ + pMMR CRC ($HR_{CRC\text{-}specific}$ 1.42; 95% CI 0.74–2.71 and $HR_{overall}$ 1.13; 95% CI 0.65–1.95), and $BRAF_{mut}$ + dMMR CRC ($HR_{CRC\text{-}specific}$ 1.41; 95% CI 0.60–3.31 and $HR_{overall}$ 1.54; 95% CI 0.83–2.87) (**Table 4**). In contrast, the Warburg-high subtype was not associated with CRC-specific or overall survival in patients with all-wild-type + pMMR CRC, $KRAS_{mut}$ + $PIK3CA_{mut}$ + pMMR CRC, and $PIK3CA_{mut}$ + pMMR CRC (**Table 4**).

Sensitivity analyses

In sensitivity analyses, excluding CRC patients with unknown pTNM stage, differentiation grade, or missing information with respect to adjuvant therapy yielded similar results, except for a statistically significant worse overall survival for patients with $KRAS_{mut}$ + pMMR CRC in pTNM stage III (HR 1.32; 95% CI 1.02–1.71) and a borderline statistically significant difference in CRC-specific survival for patients with $KRAS_{mut}$ + pMMR CRC in pTNM stage IV (HR 1.30; 95% CI 0.96–1.78) (*data not shown*). Furthermore, a statistically significant positive association was found between the Warburg-high subtype and overall- and CRC-specific survival (HR 1.49; 95% CI 1.04–2.13 and HR 1.44; 95% CI 1.07–1.94, respectively) in patients with $KRAS_{mut}$ + pMMR CRC (*data not shown*).

DISCUSSION

In this large population-based series of colorectal cancer (CRC) patients, we have investigated the association between mutational subgroups and patient survival. Moreover, we investigated the relationship between previously identified Warburg-subtypes[23] and survival within these mutational subgroups to examine whether Warburg-subtypes provide additional prognostic value.

CRC patients were classified into eight mutually exclusive mutational subgroups, based on the presence of somatic mutations in *RAS* (*KRAS*, *NRAS*, *HRAS*), *BRAF*, *PIK3CA*, *MET*, as well as, patients' mismatch repair (MMR) status: (1) all-wild-type + pMMR, (2) $KRAS_{mut}$ + pMMR, (3) $KRAS_{mut}$ + $PIK3CA_{mut}$ + pMMR, (4) $PIK3CA_{mut}$ + pMMR, (5) $BRAF_{mut}$ + pMMR, (6) $BRAF_{mut}$ + dMMR, (7) other + pMMR, and (8) other + dMMR. The other + dMMR subgroup largely consisted of patients with all-wild-type CRC or patients with mutations in *BRAF* and/or *PIK3CA*, whereas, the other + pMMR subgroup mainly consisted of patients with mutations in *RAS* (*NRAS*, *KRAS*) and/or *MET*.

We found important survival differences across mutational subgroups, independent of known prognostic factors like pTNM stage. Compared to patients with all-wild-type + pMMR CRC, patients with $KRAS_{mut}$ + pMMR, $KRAS_{mut}$ + $PIK3CA_{mut}$ + pMMR, $BRAF_{mut}$ + pMMR or other + pMMR CRC had a worse survival. Patients with $BRAF_{mut}$ + pMMR CRC had the poorest survival, whereas patients with other + dMMR CRC had the most favorable survival. Furthermore, our results indicate that $BRAF_{mut}$, $KRAS_{mut}$ + pMMR, and other + dMMR CRC may be related to the Warburg-high subtype. Lastly, we did not observe statistically significant survival differences for the Warburg-subtypes within mutational subgroups.

Mutation frequencies of *RAS* (*KRAS*, *NRAS*, *HRAS*), *BRAF*, *PIK3CA*, and *MET*, as well as the frequency of dMMR in this study are similar to those reported previously[33-35] and those described in the COSMIC database.[17, 36] Moreover, our results confirm previous reports that *BRAF* mutations occur frequently in dMMR CRC, whereas co-existence of *KRAS* mutations and *BRAF* mutations or dMMR are rare.[37, 38] In addition, our study confirms that *PIK3CA* mutations often co-exist with other mutations, and especially with *KRAS* mutations, as reported previously.[34]

In the present study, we found that compared to patients with all-wild-type + pMMR CRC, patients with $KRAS_{mut}$ + pMMR CRC had a poor survival. No significant association with survival was observed for patients with $PIK3CA_{mut}$ + pMMR CRC whereas patients with $KRAS_{mut}$ + $PIK3CA_{mut}$ + pMMR had a worse CRC-specific survival, suggesting that *KRAS* mutations may drive the worse survival observed for this subgroup. The survival of patients with *BRAF*-mutated CRC was highly dependent on MMR status. Patients with $BRAF_{mut}$ + pMMR CRC had the poorest survival, whereas no difference in survival was found for patients with $BRAF_{mut}$ + dMMR CRC. These results suggest that dMMR may 'override' the negative prognostic potential of BRAF mutations. In addition, our results indicate that patients with other + pMMR CRC have a poor survival. The most favorable survival was observed for patients with other + dMMR CRC, again highlighting the favorable prognostic value of dMMR.

Many studies have investigated the prognostic value of MMR status, *KRAS*-, *BRAF*-, or *PIK3CA*-mutations in CRC in the past. However, most studies did not evaluate these mutations exclusively (e.g., patients with a *KRAS*-mutant or *KRAS* wild-type cancer may have had another mutation in a different gene),[39] which could have potentially diluted their results. Studies assessing the prognostic value of CRC subgroups based on combinations of frequently occurring mutations (*RAS*, *BRAF*, *PIK3CA*) and/or MMR status are very limited and rarely evaluate all markers at the same time.[40-43]

MSI status is most consistently associated with CRC survival.[40] It has been shown that patients with MSI high (MSI-H) CRC have a better overall survival compared to patients with microsatellite stable (MSS) CRC.[40] Mutations in *BRAF* have also consistently been associated with poor survival in CRC.[44,45] In contrast, the prognostic significance of mutations in *KRAS* and/or *PIK3CA* is unclear, as results of previous studies are inconsistent.[42, 46, 47] More recently, several studies have investigated the association between combinations of markers and CRC survival. Various studies have reported on the association between MMR status in combination with *BRAF* or *KRAS* mutations and CRC survival. In line with our results, it has been shown that the adverse effect of mutant *BRAF* on survival is limited to MSS CRC.[20, 22, 43, 48] In addition, a poorer survival was reported for patients with MSS and a *KRAS* mutation, compared to the reference group (i.e., MSS, $BRAF_{wild\text{-}type}$, and $KRAS_{wild\text{-}type}$).[40, 43, 49] These and our results suggest a complex interplay between these markers and highlight the importance of evaluating multiple markers at the same time.

Even though future studies – with higher numbers of CRC patients within each of the subgroups – are necessary to validate our findings and to investigate the biological basis for the observed differences in subgroup-specific survival, a potential mechanism may be the involvement of the Warburg-effect.

It has been suggested that mutations in *RAS* (*KRAS*, *NRAS*, *HRAS*), *BRAF*, and *PIK3CA* promote the Warburg-effect through activation of the *PI3K/AKT/mTOR* and *RAS/RAF/MEK/ERK* oncogenic pathways.[12-14] We have previously shown that patients with Warburg-high CRC (i.e., a high probability of the presence of the Warburg-effect) had a worse survival compared to patients with Warburg-low CRC, especially in patients with rectal cancers or pTNM stage III CRC.[23] To our knowledge, this is the first study to investigate the relationship between mutational subgroups and these previously defined Warburg-subtypes, and to examine whether Warburg-subtypes provide additional prognostic value within mutational subgroups in CRC. The results of the present study suggest that $BRAF_{mut}$, $KRAS_{mut}$ + pMMR, and other + dMMR subgroups may be related to the Warburg-high subtype in cancers located in the colon and rectum. In addition, the $PIK3CA_{mut}$ + pMMR subgroup seems to be related to the Warburg-high subtype in cancers located in the rectum. We did not find statistically significant survival differences across Warburg-subtypes within mutational

subgroups. This might be due to limited statistical power when subclassifying based on mutational subgroups and Warburg-subtypes despite investigating a very large cohort of CRC. Similarly, associations may be concealed overall as we did not have enough power to stratify our analyses on tumor location or pTNM stage.

The main strengths of this study include the use of a large population-based series of incident CRC patients, the nearly complete follow-up, the fact that patients were mainly treated with surgery, and the availability of DNA and tumor material for a large number of CRC patients. Our study has some limitations. First, the ColoCarta panel that was used includes assays for most known *KRAS* (99%) and *BRAF* (98%) mutations, but only 78% of known *PIK3CA* mutations.[35] Second, we determined MMR status as a proxy for MSI status, which might have led to misclassification of some CRC patients. However, it has been described that IHC analysis of MLH1 and MSH2 expression is a reliable method for the detection of the vast majority of patients with MSI CRC.[50] Third, our study did not have a validation cohort available to confirm the observed associations. Fourth, we made no adjustments for multiple testing which may have potentially resulted in chance findings. Therefore, our results should be interpreted with caution, and validation of the current findings is required. Fifth, we did not have detailed clinical information available regarding the exact type, duration or dosage of treatment. Lastly, other limitations with regard to Warburg-subtyping have been described previously.[23]

CONCLUSION

In this large, population-based series of CRC patients, we have shown that mutational subgroups, based on the observed mutation frequencies of *RAS* (*KRAS*, *NRAS*, *HRAS*), *BRAF*, *PIK3CA*, and *MET*, as well as patients' MMR status, are associated with important differences in survival. Our results suggest that $BRAF_{mut}$, $KRAS_{mut}$ + pMMR, and other + dMMR subgroups may be related to the Warburg-high subtype in cancers located in the colon or rectum. However, no statistically significant survival differences were observed for the Warburg-subtypes within mutational subgroups. All in all, our results highlight the prognostic value of mutational subgroups in CRC. In the future, CRC-subtyping based on mutational subgroups may be used for risk stratification, the design of (combined) targeted therapies, and to improve therapeutic outcomes of CRC patients. Future, larger-scale prospective studies or pooled studies are necessary to validate our findings, to further explore the potential prognostic value of Warburg-subtypes, and to examine the potential clinical utility of CRC subtyping based on mutational subgroups.

REFERENCES

1. Ferlay J, Ervik M, Lam F, et al. Global Cancer Observatory: Cancer Today. Lyon, France: International Agency for Research on Cancer. https://gco.iarc.fr/today. Published 2020. Accessed 15 February, 2021.
2. Mo S, Zhou Z, Li Y, et al. Establishment and validation of a novel nomogram incorporating clinicopathological parameters into the TNM staging system to predict prognosis for stage II colorectal cancer. *Cancer Cell Int.* 2020;20:285.
3. Bensinger SJ, Christofk HR. New aspects of the Warburg effect in cancer cell biology. *Semin Cell Dev Biol.* 2012;23(4):352-361.
4. Kato Y, Maeda T, Suzuki A, Baba Y. Cancer metabolism: New insights into classic characteristics. *Jpn Dent Sci Rev.* 2018;54(1):8-21.
5. Warburg O, Wind F, Negelein E. The metabolism of tumors in the body. *The Journal of general physiology.* 1927;8(6):519-530.
6. Sakashita M, Aoyama N, Minami R, et al. Glut1 expression in T1 and T2 stage colorectal carcinomas: its relationship to clinicopathological features. *Eur J Cancer.* 2001;37(2):204-209.
7. Pavlova NN, Thompson CB. The emerging hallmarks of cancer metabolism. *Cell metabolism.* 2016;23(1):27-47.
8. Hutton JE, Wang X, Zimmerman LJ, et al. Oncogenic KRAS and BRAF drive metabolic reprogramming in colorectal cancer. *Molecular & cellular proteomics.* 2016;15(9):2924-2938.
9. Liu M, Liu W, Qin Y, et al. Regulation of metabolic reprogramming by tumor suppressor genes in pancreatic cancer. *Experimental Hematology & Oncology.* 2020;9(1):1-16.
10. van Geel RM, Beijnen JH, Bernards R, Schellens JH. Treatment individualization in colorectal cancer. *Current colorectal cancer reports.* 2015;11(6):335-344.
11. Haluska F, Pemberton T, Ibrahim N, Kalinsky K. The RTK/RAS/BRAF/PI3K pathways in melanoma: biology, small molecule inhibitors, and potential applications. Paper presented at: Seminars in oncology2007.
12. Papa S, Choy PM, Bubici C. The ERK and JNK pathways in the regulation of metabolic reprogramming. *Oncogene.* 2019;38(13):2223-2240.
13. Kang S, Bader AG, Vogt PK. Phosphatidylinositol 3-kinase mutations identified in human cancer are oncogenic. *Proceedings of the National Academy of Sciences.* 2005;102(3):802-807.
14. Feron O. Pyruvate into lactate and back: from the Warburg effect to symbiotic energy fuel exchange in cancer cells. *Radiotherapy and oncology.* 2009;92(3):329-333.
15. Chandel V, Raj S, Choudhari R, Kumar D. Role of c-Met/HGF Axis in Altered Cancer Metabolism. In: *Cancer Cell Metabolism: A Potential Target for Cancer Therapy.* Springer; 2020:89-102.
16. Guo F, Gong H, Zhao H, et al. Mutation status and prognostic values of KRAS, NRAS, BRAF and PIK3CA in 353 Chinese colorectal cancer patients. *Scientific reports.* 2018;8(1):1-11.
17. Tate JG, Bamford S, Jubb HC, et al. COSMIC: the catalogue of somatic mutations in cancer. *Nucleic acids research.* 2019;47(D1):D941-D947.
18. Mendoza MC, Er EE, Blenis J. The Ras-ERK and PI3K-mTOR pathways: cross-talk and compensation. *Trends in biochemical sciences.* 2011;36(6):320-328.
19. De Luca A, Maiello MR, D'Alessio A, Pergameno M, Normanno N. The RAS/RAF/MEK/ERK and the PI3K/AKT

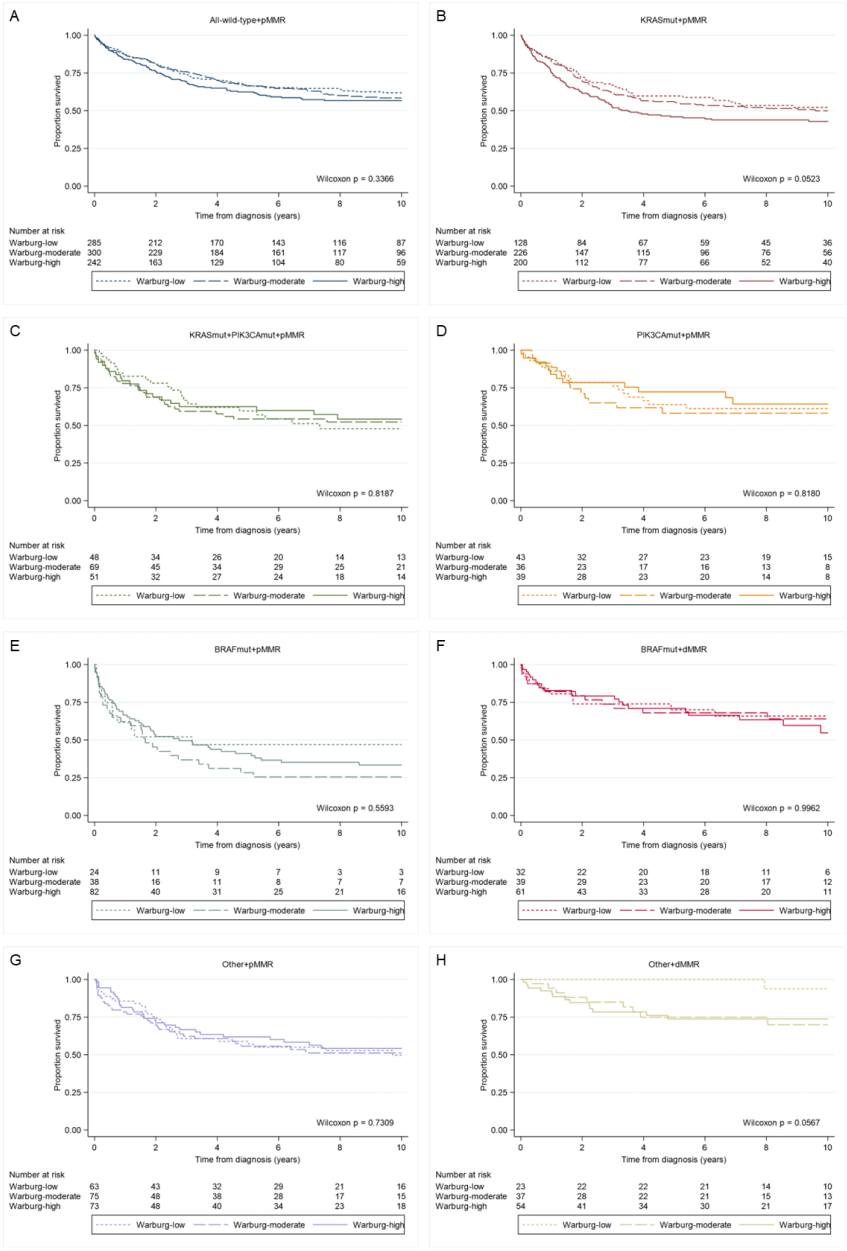
signalling pathways: role in cancer pathogenesis and implications for therapeutic approaches. *Expert opinion on therapeutic targets.* 2012;16(sup2):S17-S27.

20. Wu M, Kim YS, Ryu HS, et al. MSI status is associated with distinct clinicopathological features in BRAF mutation colorectal cancer: A systematic review and meta-analysis. *Pathol Res Pract.* 2020;216(1):152791.
21. Lochhead P, Kuchiba A, Imamura Y, et al. Microsatellite instability and BRAF mutation testing in colorectal cancer prognostication. *J Natl Cancer Inst.* 2013;105(15):1151-1156.
22. Seppälä T, Böhm J, Friman M, et al. Combination of microsatellite instability and BRAF mutation status for subtyping colorectal cancer. *British journal of cancer.* 2015;112(12):1966-1975.
23. Offermans K, Jenniskens JC, Simons CC, et al. Expression of proteins associated with the Warburg-effect and survival in colorectal cancer. *The Journal of Pathology: Clinical Research.* 2021.
24. van den Brandt PA, Goldbohm RA, van 't Veer P, Volovics A, Hermus RJ, Sturmans F. A large-scale prospective cohort study on diet and cancer in The Netherlands. *J Clin Epidemiol.* 1990;43(3):285-295.
25. Casparie M, Tiebosch AT, Burger G, et al. Pathology databanking and biobanking in The Netherlands, a central role for PALGA, the nationwide histopathology and cytopathology data network and archive. *Cell Oncol.* 2007;29(1):19-24.
26. van den Brandt PA, Schouten LJ, Goldbohm RA, Dorant E, Hunen PM. Development of a record linkage protocol for use in the Dutch Cancer Registry for Epidemiological Research. *Int J Epidemiol.* 1990;19(3):553-558.
27. Goldbohm RA, van den Brandt PA, Dorant E. Estimation of the coverage of municipalities by cancer registries and PALGA using hospital discharge data. *Tijdschr Soc Gezondheidsz.* 1994;72:80-84.
28. van den Brandt PA. Molecular pathological epidemiology of lifestyle factors and colorectal and renal cell cancer risk. Maastricht Pathology 2018. 11th Joint Meeting of the British Division of the International Academy of Pathology and the Pathological Society of Great Britain & Ireland, 19-22 June 2018. . *J Pathol 2018;246 Suppl 1: S9.*
29. Jenniskens JC, Offermans K, Samarska I, et al. Validity and reproducibility of immunohistochemical scoring by trained non-pathologists on Tissue MicroArrays. *Cancer Epidemiology and Prevention Biomarkers.* 2021.
30. Yurgelun MB, Hampel H. Recent Advances in Lynch Syndrome: Diagnosis, Treatment, and Cancer Prevention. *Am Soc Clin Oncol Educ Book.* 2018;38:101-109.
31. Schoenfeld D. Partial residuals for the proportional hazards regression model. *Biometrika.* 1982;69(1):239-241.
32. Sobin LH, Compton CA, Gospodarowicz M, et al. 'Evidence-based medicine: the time has come to set standards for staging'. Is a radical overhaul really needed? *The Journal of pathology.* 2010;221(4):361-362.
33. Zhang J, Zheng J, Yang Y, et al. Molecular spectrum of KRAS, NRAS, BRAF and PIK3CA mutations in Chinese colorectal cancer patients: analysis of 1,110 cases. *Sci Rep.* 2015;5:18678.
34. Poulsen TS, de Oliveira DVNP, Espersen MLM, Klarskov LL, Skovrider-Ruminski W, Hogdall E. Frequency and coexistence of KRAS, NRAS, BRAF and PIK3CA mutations and occurrence of MMR deficiency in Danish colorectal cancer patients. *Apmis.* 2021;129(2):61-69.
35. Fumagalli D, Gavin PG, Taniyama Y, et al. A rapid, sensitive, reproducible and cost-effective method for mutation profiling of colon cancer and metastatic lymph nodes. *BMC cancer.* 2010;10(1):1-14.
36. Catalogue of Somatic Mutations in Cancer, release version 94. http://cancer.sanger.ac.uk/cosmic. Accessed 17 October 2021.
37. Sanchez J, Krumroy L, Plummer S, et al. Genetic and epigenetic classifications define clinical phenotypes and

determine patient outcomes in colorectal cancer. *Journal of British Surgery.* 2009;96(10):1196-1204.

38. Cushman-Vokoun AM, Stover DG, Zhao Z, Koehler EA, Berlin JD, Vnencak-Jones CL. Clinical utility of KRAS and BRAF mutations in a cohort of patients with colorectal neoplasms submitted for microsatellite instability testing. *Clinical colorectal cancer.* 2013;12(3):168-178.
39. De Roock W, Claes B, Bernasconi D, et al. Effects of KRAS, BRAF, NRAS, and PIK3CA mutations on the efficacy of cetuximab plus chemotherapy in chemotherapy-refractory metastatic colorectal cancer: a retrospective consortium analysis. *The lancet oncology.* 2010;11(8):753-762.
40. Phipps AI, Limburg PJ, Baron JA, et al. Association between molecular subtypes of colorectal cancer and patient survival. *Gastroenterology.* 2015;148(1):77-87 e72.
41. Zlobec I, Bihl MP, Foerster A, Rufle A, Terracciano L, Lugli A. Stratification and prognostic relevance of Jass's molecular classification of colorectal cancer. *Frontiers in oncology.* 2012;2:7.
42. Luo Q, Chen D, Fan X, Fu X, Ma T, Chen D. KRAS and PIK3CA bi-mutations predict a poor prognosis in colorectal cancer patients: A single-site report. *Translational oncology.* 2020;13(12):100874.
43. Alwers E, Jia M, Kloor M, Bläker H, Brenner H, Hoffmeister M. Associations between molecular classifications of colorectal cancer and patient survival: a systematic review. *Clinical Gastroenterology and Hepatology.* 2019;17(3):402-410. e402.
44. Sanz-Garcia E, Argiles G, Elez E, Tabernero J. BRAF mutant colorectal cancer: prognosis, treatment, and new perspectives. *Annals of Oncology.* 2017;28(11):2648-2657.
45. Safaee Ardekani G, Jafarnejad SM, Tan L, Saeedi A, Li G. The prognostic value of BRAF mutation in colorectal cancer and melanoma: a systematic review and meta-analysis. 2012.
46. Mei ZB, Duan CY, Li CB, Cui L, Ogino S. Prognostic role of tumor PIK3CA mutation in colorectal cancer: a systematic review and meta-analysis. *Ann Oncol.* 2016;27(10):1836-1848.
47. Kwak MS, Cha JM, Yoon JY, et al. Prognostic value of KRAS codon 13 gene mutation for overall survival in colorectal cancer: direct and indirect comparison meta-analysis. *Medicine.* 2017;96(35).
48. Formica V, Sera F, Cremolini C, et al. KRAS and BRAF Mutations in Stage II/III Colon Cancer: A Systematic Review and Meta-Analysis. *JNCI: Journal of the National Cancer Institute.* 2021.
49. Sinicrope FA, Shi Q, Smyrk TC, et al. Molecular markers identify subtypes of stage III colon cancer associated with patient outcomes. *Gastroenterology.* 2015;148(1):88-99.
50. Lanza G, Gafà R, Maestri I, Santini A, Matteuzzi M, Cavazzini L. Immunohistochemical pattern of MLH1/MSH2 expression is related to clinical and pathological features in colorectal adenocarcinomas with microsatellite instability. *Modern Pathology.* 2002;15(7):741-749.

SUPPLEMENTARY MATERIAL



Supplementary Figure S1 – Kaplan-Meier curves showing CRC-specific survival of Warburg-subtypes, according to mutational subgroup: (**A**) all-wild-type + pMMR, (**B**) *KRAS*$_{mut}$ + pMMR, (**C**) *KRAS*$_{mut}$ + PIK3CA$_{mut}$ + pMMR, (**D**) *PIK3CA*$_{mut}$ + pMMR, (**E**) *BRAF*$_{mut}$ + pMMR, (**F**) *BRAF*$_{mut}$ + dMMR, (**G**) other + pMMR, (**H**) other + dMMR.

Supplementary Table S1 – Details primary antibodies and staining protocols.

Antibody	Clone	Source	Dilution	Staining procedure	Antigen retrieval	Incubation time	Visualisation system	Chromogen
Pan-CK	AE1/AE3	DAKO (GA053)	RTU	DAKO Autostainer[a]	PT high[b]	10 minutes	EnVision FLEX[f]	DAB
MLH1	ES05	DAKO (M3640)	RTU	DAKO Autostainer[a]	PT high[b]	40 minutes	EnVision FLEX[f]	DAB
MSH2	FE11	DAKO (M3639)	RTU	DAKO Autostainer[a]	PT high[b]	40 minutes	EnVision FLEX[f]	DAB
p53	DO-7	DAKO (M7001)	RTU	DAKO Autostainer[a]	PT high[b]	20 minutes	EnVision FLEX[f]	DAB
PTEN	6H2.1	DAKO (M3627)	1:100	DAKO Autostainer[a]	PT high[b]	20 minutes	EnVision FLEX[f]	DAB
GLUT1	-	TFS (RB-9052-P1)	1:200	DAKO Autostainer[a]	PT low[c]	20 minutes	EnVision FLEX[f]	DAB
LDHA	E-9	SCBT (sc-137243)	1:800	Manual	HIER high[d]	Overnight, 4°C	REAL EnVision[g]	DAB
MCT4	D-1	SCBT (sc-376140)	1:100	Manual	HIER high[d]	Overnight, 4°C	REAL EnVision[g]	DAB
PKM2	C-11	SCBT (sc-365684)	1:100	Manual	HIER low[e]	1hr, 37°C	LSAB2 Kit/HRP[h]	DAB

[a]DAKO Autostainer Link 48
[b]High pH retrieval (K8004) for 20 minutes on the Dako PT link (Agilent Technologies)
[c]Low pH retrieval (K8005) for 20 minutes on the Dako PT link (Agilent Technologies)
[d]Heat-induced antigen retrieval using a solution of Tris/EDTA (pH 9.0)
[e]Heat-induced antigen retrieval using a solution of sodium citrate (pH 6.0)
[f]EnVision FLEX Visualization Kit (K8008, DAKO)
[g]REAL EnVision Detection System (K5007, DAKO)
[h]Universal LSAB2 kit/HRP (K0675, Agilent)
TFS, Thermo Fisher Scientific; SBCT, Santa Cruz Biotechnology; RTU, ready-to-use; DAB, 3,3'-diaminobenzide;

Supplementary Table S2 – Scoring protocols and kappa values with 95% confidence intervals for inter- and intra-observer agreement of the six proteins incorporated in the Warburg-subtypes

	p53	PTEN	GLUT1	LDHA	MCT4	PKM2
Localization[a]	**Nucleus**	**Cytoplasmb**	**Membrane**	**Cytoplasm**	**Membrane**	**Cytoplasm**
Scoring protocol						
Low						
Category 1	(1) negative	(1) negative	(1) negative	(1) negative/weak	(1) negative	(1) negative/weak
Category 2	(2) 1-10% positive		(2) 1-10% positive		(2) 1-10% positive	
Moderate						
Category 2		(2) weak		(2) 1-50% strong positive		(2) moderate positive
Category 3	(3) 11-50% positive	(3) moderate	(3) 11-50% positive		(3) 11-50% positive	(3) 1-50% strong positive
High						
Category 3				(3) >50% strong positive		
Category 4	(4) 51-90% positive	(4) strong	(4) >50% positive		(4) >50% positive	(4) >50% strong positive
Category 5	(5) >90% positive					
Scoring agreement						
Inter-observer agreement[c]	κ (95%-CI)	κ (95%-CI)	κ (95%-CI)	κ (95%-CI)	κ (95%-CI)	κ (95%-CI)
Final score[d] vs pathologist						
Weighted kappa[e]	0.75 (0.72-0.79)	0.58 (0.53-0.62)	0.71 (0.67-0.74)	0.65 (0.60-0.69)	0.74 (0.71-0.77)	0.65 (0.61-0.69)
Non-weighted kappa	0.63 (0.58-0.68)	0.47 (0.41-0.52)	0.61 (0.57-0.66)	0.59 (0.54-0.64)	0.63 (0.59-0.68)	0.56 (0.51-0.60)
Intra-observer agreement[c, f]						
Non-pathologist assessor 1						
Weighted kappa[e]	0.83 (0.80-0.86)	0.69 (0.65-0.74)	0.82 (0.79-0.85)	0.78 (0.74-0.82)	0.86 (0.83-0.88)	0.70 (0.67-0.74)
Non-weighted kappa	0.73 (0.69-0.77)	0.63 (0.58-0.69)	0.75 (0.72-0.79)	0.76 (0.71-0.80)	0.79 (0.75-0.82)	0.58 (0.53-0.62)
Non-pathologist assessor 2						
Weighted kappa[e]	0.87 (0.84-0.90)	0.69 (0.64-0.74)	0.75 (0.72-0.78)	0.77 (0.73-0.81)	0.83 (0.81-0.86)	0.72 (0.68-0.75)
Non-weighted kappa	0.80 (0.76-0.84)	0.65 (0.60-0.70)	0.65 (0.60-0.69)	0.73 (0.69-0.78)	0.75 (0.71-0.79)	0.62 (0.58-0.67)

[a]Only immunoreactivity in reported cellular localization was considered positive staining.
[b]For PTEN scoring, staining intensity of tumour cells was compared with that of stromal cells: (1) no PTEN staining in the tumour cells; (2) staining intensity in the tumour cells weaker than in the stromal cells; (3) similar staining intensity in tumour and stromal cells; (4) staining intensity in the tumour cells stronger than in the stromal cells.
[c]Based on a random 10% of TMA sections
[d]The final score is based on at least two non-pathologists, with discrepancies replaced by a consensus score or pathologist's score.
[e]Weight of 0.5 for adjacent categories and 0 for non-adjacent categories.
[f]10% of TMA sections were scored for a second time after at least 2 months.

Supplementary Table S3 – ColoCarta panel genes and mutations

Gene	Assay	Mutation
BRAF	15/16	V600E/K/L/M/R
	9	D594G/V
KRAS	1	G12A/D/V
	2	G12C/R/S
	4	G13D/V
	5	A59T
	7	Q61L/P/R
	8	Q61H_A/H_G
PIK3CA	1	R88Q
	3	C420R
	5	E542K
	6	E545K
	7	Q546K
	8	H701P
	9	H1047L/R
NRAS	1	G12A/D/V
	2	G12C/R/S
	3	G13A/D/V
	4	G13C/R/S
	7	Q61H
	8	Q61E/K
HRAS	6	Q61L/P/R
MET	1	R970C
	2	T992I

Supplementary Table S4 – Mutational subgroups, based on the observed frequencies of (combinations of) tumor markers (i.e., KRAS, BRAF, PIK3CA, NRAS, MET, MMR).

Mutational subgroups	KRAS	BRAF	PIK3CA	NRAS	MET	MMR
All-wild-type + pMMR	wild-type	wild-type	wild-type	wild-type	wild-type	proficient
$KRAS_{mut}$ + pMMR	mutant	wild-type	wild-type	wild-type	wild-type	proficient
$KRAS_{mut}$ + $PIK3CA_{mut}$ + pMMR	mutant	wild-type	mutant	wild-type	wild-type	proficient
$PIK3CA_{mut}$ + pMMR	wild-type	wild-type	mutant	wild-type	wild-type	proficient
$BRAF_{mut}$ + pMMR	wild-type	mutant	wild-type	wild-type	wild-type	proficient
$BRAF_{mut}$ + dMMR	wild-type	mutant	wild-type	wild-type	wild-type	deficient
Other + pMMR	wild-type/mutant	wild-type/mutant	wild-type/mutant	wild-type/mutant	wild-type/mutant	proficient
Other + dMMR	wild-type/mutant	wild-type/mutant	wild-type/mutant	wild-type/mutant	wild-type/mutant	deficient

Supplementary Table S5 – TNM classification of colorectal cancer, according to incidence year.

Topography	**153.0-154.1 or C18-C20**		
Histology	**Epithelial cancers (M8010-8580)**		
Incidence years	1988-2002		
TNM versions	4.1-5		
Stage	T	N	M
I	1-2	0/X	0/X
II	3-4	0/X	0/X
III	Any T	1-3	0/X
IV	Any T	Any N	1
X	X	0/X	0/X

Incidence years	**2003-2009**		
TNM versions	**6**		
Stage	T	N	M
I	1-2	0/X	0/X
IIA	3	0/X	0/X
IIB	4	0/X	0/X
III	X	1	0/X
IIIA	1-2	1	0/X
IIIB	3-4	1	0/X
IIIC	Any T	2	0/X
IV	Any T	Any N	1
X	X	0/X	0/X

Supplementary Table S6 – Univariable and multivariable-adjusted hazard ratios for associations between mutational subgroups and survival of colorectal cancer patients within the Netherlands Cohort Study (NLCS, 1986-2006), stratified on pTNM stage.

	N	CRC-specific survival			Overall survival		
		CRC deaths (%)	HR (95% CI)		Deaths (%)	HR (95% CI)	
			Univariable	Multivariable-adjusted[a]		Univariable	Multivariable-adjusted[a]
pTNM stage I							
All-wild-type + pMMR	194	27 (13.9)	1.00 (ref)	1.00 (ref)	86 (44.3)	1.00 (ref)	1.00 (ref)
$KRAS_{mut}$ + pMMR	119	29 (24.4)	1.85 (1.09-3.12)	2.06 (1.21-3.50)	56 (47.1)	1.13 (0.80-1.58)	1.18 (0.84-1.66)
$KRAS_{mut}$ + $PIK3CA_{mut}$ + pMMR	29	6 (20.7)	1.63 (0.67-3.94)	1.93 (0.79-4.73)	16 (55.2)	1.38 (0.81-2.36)	1.31 (0.76-2.26)
$PIK3CA_{mut}$ + pMMR	18	4 (22.2)	1.72 (0.60-4.91)	1.73 (0.60-5.01)	9 (50.0)	1.21 (0.61-2.41)	1.40 (0.70-2.81)
$BRAF_{mut}$ + pMMR	10	4 (40.0)	3.52 (1.23-10.08)	3.34 (1.12-9.94)	7 (70.0)	2.21 (1.02-4.77)	1.44 (0.66-3.18)
$BRAF_{mut}$ + dMMR	19	1 (5.3)	0.41 (0.06-2.99)	0.42 (0.06-3.21)	8 (42.1)	1.01 (0.49-2.09)	0.91 (0.43-1.94)
Other + pMMR	52	7 (13.5)	1.00 (0.44-2.30)	1.07 (0.46-2.46)	22 (42.3)	0.98 (0.61-1.56)	1.08 (0.67-1.73)
Other + dMMR	18	1 (5.6)	0.37 (0.05-2.72)	0.33 (0.04-2.49)	7 (38.9)	0.81 (0.38-1.76)	0.67 (0.30-1.49)
pTNM stage II							
All-wild-type + pMMR	305	68 (22.3)	1.00 (ref)	1.00 (ref)	167 (54.8)	1.00 (ref)	1.00 (ref)
$KRAS_{mut}$ + pMMR	185	60 (32.4)	1.52 (1.08-2.16)	1.52 (1.07-2.15)	111 (60.0)	1.17 (0.92-1.49)	1.11 (0.87-1.41)
$KRAS_{mut}$ + $PIK3CA_{mut}$ + pMMR	74	21 (28.4)	1.29 (0.79-2.11)	1.25 (0.76-2.04)	39 (52.7)	0.97 (0.69-1.38)	0.92 (0.65-1.31)
$PIK3CA_{mut}$ + pMMR	53	10 (18.9)	0.81 (0.42-1.57)	0.79 (0.40-1.54)	30 (56.6)	1.00 (0.68-1.47)	0.98 (0.66-1.44)
$BRAF_{mut}$ + pMMR	47	17 (36.2)	1.64 (0.96-2.78)	1.51 (0.87-2.62)	25 (53.2)	0.97 (0.64-1.48)	0.90 (0.58-1.38)
$BRAF_{mut}$ + dMMR	65	14 (21.5)	0.95 (0.53-1.68)	0.85 (0.47-1.55)	36 (55.4)	1.02 (0.71-1.46)	0.87 (0.59-1.27)
Other + pMMR	75	24 (32.0)	1.48 (0.93-2.35)	1.40 (0.87-2.24)	46 (61.3)	1.18 (0.85-1.63)	1.15 (0.82-1.60)
Other + dMMR	73	13 (17.8)	0.79 (0.44-1.43)	0.73 (0.40-1.35)	38 (52.1)	0.94 (0.66-1.33)	0.89 (0.61-1.28)
pTNM stage III							
All-wild-type + pMMR	220	116 (52.7)	1.00 (ref)	1.00 (ref)	158 (71.8)	1.00 (ref)	1.00 (ref)
$KRAS_{mut}$ + pMMR	157	78 (49.7)	1.06 (0.80-1.42)	1.14 (0.85-1.53)	114 (72.6)	1.15 (0.90-1.46)	1.21 (0.94-1.55)
$KRAS_{mut}$ + $PIK3CA_{mut}$ + pMMR	40	22 (55.0)	1.10 (0.70-1.74)	1.18 (0.75-1.87)	27 (67.5)	0.98 (0.65-1.48)	1.06 (0.70-1.60)
$PIK3CA_{mut}$ + pMMR	30	14 (46.7)	0.89 (0.51-1.55)	0.97 (0.55-1.69)	20 (66.7)	0.93 (0.58-1.48)	1.04 (0.65-1.67)
$BRAF_{mut}$ + pMMR	59	41 (69.5)	2.06 (1.44-2.95)	2.06 (1.41-3.02)	49 (83.1)	1.82 (1.32-2.51)	1.76 (1.25-2.48)
$BRAF_{mut}$ + dMMR	37	18 (48.6)	1.14 (0.70-1.88)	1.14 (0.67-1.95)	27 (73.0)	1.26 (0.84-1.89)	1.16 (0.74-1.80)
Other + pMMR	51	29 (56.9)	1.30 (0.86-1.95)	1.42 (0.94-2.14)	40 (78.4)	1.33 (0.94-1.89)	1.49 (1.05-2.12)
Other + dMMR	20	6 (30.0)	0.56 (0.25-1.27)	0.54 (0.23-1.26)	12 (60.0)	0.80 (0.44-1.44)	0.78 (0.43-1.44)

Supplementary Table S6 – Continued

	N	CRC-specific survival			Overall survival		
		CRC deaths (%)	HR (95% CI)		Deaths (%)	HR (95% CI)	
			Univariable	Multivariable-adjusted[a]		Univariable	Multivariable-adjusted[a]
pTNM stage IV							
All-wild-type + pMMR	102	92 (90.2)	1.00 (ref)	1.00 (ref)	102 (100.0)	1.00 (ref)	1.00 (ref)
$KRAS_{mut}$ + pMMR	105	98 (93.3)	1.25 (0.94-1.66)	1.37 (1.02-1.85)	104 (99.0)	1.18 (0.90-1.56)	1.30 (0.98-1.73)
$KRAS_{mut}$ + $PIK3CA_{mut}$ + pMMR	27	26 (96.3)	1.37 (0.88-2.11)	1.36 (0.86-2.13)	27 (100.0)	1.28 (0.84-1.96)	1.26 (0.81-1.95)
$PIK3CA_{mut}$ + pMMR	18	15 (83.3)	0.87 (0.50-1.51)	1.09 (0.62-1.93)	17 (94.4)	0.84 (0.50-1.42)	1.06 (0.62-1.81)
$BRAF_{mut}$ + pMMR	27	25 (92.6)	2.19 (1.41-3.42)	1.97 (1.25-3.10)	27 (100.0)	2.14 (1.40-3.28)	1.91 (1.24-2.96)
$BRAF_{mut}$ + dMMR	10	9 (90.0)	1.16 (0.58-2.32)	0.91 (0.44-1.88)	10 (100.0)	1.20 (0.62-2.32)	0.97 (0.48-1.93)
Other + pMMR	35	31 (88.6)	1.23 (0.82-1.85)	1.50 (0.99-2.27)	35 (100.0)	1.25 (0.85-1.84)	1.53 (1.03-2.26)
Other + dMMR	6	3 (50.0)	0.23 (0.07-0.74)	0.13 (0.04-0.42)	6 (100.0)	0.40 (0.17-0.93)	0.23 (0.09-0.54)

Supplementary Table S7 – Frequencies of the mutational subgroups, stratified on pTNM stage and Warburg-subtype (Warburg-low, -moderate, -high).

	Colorectal cancer			
	Total	**Warburg-low**	**Warburg-moderate**	**Warburg-high**
Colorectal				
All-wild-type + pMMR	827 (36.5)	285 (44.1)	300 (36.6)	242 (30.2)
$KRAS_{mut}$ + pMMR	554 (24.4)	128 (19.8)	226 (27.6)	200 (24.9)
$KRAS_{mut}$ + $PIK3CA_{mut}$ + pMMR	168 (7.4)	48 (7.4)	69 (8.4)	51 (6.4)
$PIK3CA_{mut}$ + pMMR	118 (5.2)	43 (6.7)	36 (4.4)	39 (4.9)
$BRAF_{mut}$ + pMMR	144 (6.4)	24 (3.7)	38 (4.6)	82 (10.2)
$BRAF_{mut}$ + dMMR	132 (5.8)	32 (5.0)	39 (4.8)	61 (7.6)
Other + pMMR	211 (9.3)	63 (9.8)	75 (9.2)	73 (9.1)
Other + dMMR	114 (5.0)	23 (3.6)	37 (4.5)	54 (6.7)
pTNM stage I				
All-wild-type + pMMR	192 (43.2)	75 (46.9)	66 (39.8)	51 (43.2)
$KRAS_{mut}$ + pMMR	111 (25.0)	38 (23.8)	46 (27.7)	27 (22.9)
$KRAS_{mut}$ + $PIK3CA_{mut}$ + pMMR	26 (5.9)	9 (5.6)	11 (6.6)	6 (5.1)
$PIK3CA_{mut}$ + pMMR	17 (3.8)	8 (5.0)	3 (1.8)	6 (5.1)
$BRAF_{mut}$ + pMMR	10 (2.3)	3 (1.9)	2 (1.2)	5 (4.2)
$BRAF_{mut}$ + dMMR	19 (4.3)	4 (2.5)	9 (5.4)	6 (5.1)
Other + pMMR	51 (11.5)	15 (9.4)	22 (13.3)	14 (11.9)
Other + dMMR	18 (4.1)	8 (5.0)	7 (4.2)	3 (2.5)
pTNM stage II				
All-wild-type + pMMR	298 (34.9)	99 (42.5)	109 (37.1)	90 (27.6)
$KRAS_{mut}$ + pMMR	179 (21.0)	40 (17.2)	72 (24.5)	67 (20.6)
$KRAS_{mut}$ + $PIK3CA_{mut}$ + pMMR	73 (8.6)	18 (7.7)	32 (10.9)	23 (7.1)
$PIK3CA_{mut}$ + pMMR	50 (5.9)	18 (7.7)	12 (4.1)	20 (6.1)
$BRAF_{mut}$ + pMMR	45 (5.3)	7 (3.0)	9 (3.1)	29 (8.9)
$BRAF_{mut}$ + dMMR	65 (7.6)	16 (6.9)	16 (5.4)	33 (10.1)
Other + pMMR	72 (8.4)	23 (9.9)	24 (8.2)	25 (7.7)
Other + dMMR	71 (8.3)	12 (5.2)	20 (6.8)	39 (12.0)
pTNM stage III				
All-wild-type + pMMR	214 (36.0)	71 (45.5)	88 (39.5)	55 (25.5)
$KRAS_{mut}$ + pMMR	150 (25.2)	29 (18.6)	63 (28.3)	58 (26.9)
$KRAS_{mut}$ + $PIK3CA_{mut}$ + pMMR	39 (6.6)	13 (8.3)	11 (4.9)	15 (6.9)
$PIK3CA_{mut}$ + pMMR	30 (5.0)	11 (7.1)	10 (4.5)	9 (4.2)
$BRAF_{mut}$ + pMMR	58 (9.8)	8 (5.1)	16 (7.2)	34 (15.7)
$BRAF_{mut}$ + dMMR	35 (5.9)	9 (5.8)	11 (4.9)	15 (6.9)
Other + pMMR	50 (8.4)	13 (8.3)	17 (7.6)	20 (9.3)
Other+dMMR	19 (3.2)	2 (1.3)	7 (3.1)	10 (4.6)
pTNM stage IV				
All-wild-type + pMMR	96 (30.0)	27 (34.2)	28 (24.4)	41 (32.5)
$KRAS_{mut}$ + pMMR	104 (32.5)	19 (24.1)	40 (34.8)	45 (35.7)
$KRAS_{mut}$ + $PIK3CA_{mut}$ + pMMR	27 (8.4)	7 (8.9)	14 (12.2)	6 (4.8)
$PIK3CA_{mut}$ + pMMR	17 (5.3)	5 (6.3)	9 (7.8)	3 (2.4)
$BRAF_{mut}$ + pMMR	27 (8.4)	6 (7.6)	8 (7.0)	13 (10.3)
$BRAF_{mut}$ + dMMR	10 (3.1)	3 (3.8)	2 (1.7)	5 (4.0)
Other + pMMR	33 (10.3)	11 (13.9)	11 (9.6)	11 (8.7)
Other + dMMR	6 (1.9)	1 (1.3)	3 (2.6)	2 (1.6)

CHAPTER 5

Association between long-term energy balance-related factors and survival in colorectal cancer overall and by metabolic Warburg-subtypes

EMBARGOED

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Manuscript in preparation

CHAPTER 6

Association between adjuvant therapy and survival in colorectal cancer patients according to metabolic Warburg-subtypes

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ABSTRACT

Purpose

Tumor location and tumor-node-metastasis (TNM) stage guide treatment decisions in colorectal cancer (CRC) patients. However, patients with the same disease stage do not benefit equally from adjuvant therapy. Hence, there remains an urgent clinical need to identify prognostic and/or predictive biomarker(s) to personalize treatment decisions. In this exploratory study, we investigated whether our previously defined metabolic Warburg-subtypes can predict which CRC patients might derive survival benefit from adjuvant therapy.

Methods

Information regarding treatment (surgery only: *n* = 1,451; adjuvant radiotherapy: *n* = 82; or adjuvant chemotherapy: *n* = 260) and Warburg-subtype (Warburg-low: *n* = 485, -moderate: *n* = 641, or –high: *n* = 667) was available for 1,793 CRC patients from the Netherlands Cohort Study (NLCS). Kaplan-Meier curves and Cox regression models were used to investigate survival benefit from adjuvant therapy compared to surgery only for the different Warburg-subtypes.

Results

Patients with Warburg-moderate CRC ($HR_{CRC\text{-}specific}$ 0.64; 95% CI 0.47-0.86, $HR_{overall}$ 0.61; 95% CI 0.47-0.80), and possibly Warburg-high CRC ($HR_{CRC\text{-}specific}$ 0.86; 95% CI 0.65-1.14, $HR_{overall}$ 0.82; 95% CI 0.64-1.05), had survival benefit from adjuvant therapy. No survival benefit was observed for patients with Warburg-low CRC ($HR_{CRC\text{-}specific}$ 1.07; 95% CI 0.76-1.52, $HR_{overall}$ 0.95; 95% CI 0.70-1.30). There was a significant interaction between Warburg-subtype and adjuvant therapy for CRC-specific survival (p = 0.049) and overall survival (p = 0.035).

Conclusion

Our results suggest that Warburg-subtypes may predict survival benefit from adjuvant therapy in CRC patients. A survival benefit from adjuvant therapy was observed for patients with Warburg-moderate and possibly Warburg-high CRC, but not for patients with Warburg-low CRC. Future prospective studies are necessary to validate our findings.

INTRODUCTION

Colorectal cancer (CRC) is the third most commonly diagnosed cancer and the second leading cause of cancer-related death worldwide, accounting for more than 900,000 deaths in 2020[1,2]. Currently, tumor location and tumor-node-metastasis (TNM) stage guide treatment decisions in CRC patients[3, 4]. However, patients with the same disease stage can have different survival and response to adjuvant therapy[3-7]. This may be due to heterogeneity in patient or tumor characteristics[3-7].

Currently, there is only a limited number of biomarkers to identify patients who are most likely to benefit from adjuvant therapy[5]. Molecular classification of CRC may identify patient subgroups at high risk for recurrence and death, thereby facilitating the selection of patients for (personalized) therapy[4, 7]. However, to date, only assessment of DNA mismatch repair (MMR) status and *RAS* and *BRAF* mutation status have been integrated into routine clinical practice to select patients for specific therapies[8, 9]. Hence, there remains an urgent clinical need to identify novel prognostic and/or predictive biomarker(s) to improve survival and quality of life in CRC patients[5, 8].

Metabolic reprogramming is one of the recognized hallmarks of cancer[10]. Otto Warburg first described, in the 1920s, that cancer cells increase their glucose uptake and lactate secretion, even in the presence of oxygen[11-14]. This phenomenon of aerobic glycolysis, also known as the "Warburg-effect", has since been observed in a variety of cancer types, including CRC[15, 16]. In a previous study, we classified CRC as Warburg-low (i.e., low probability of the presence of the Warburg-effect), Warburg-moderate, or Warburg-high using a pathway-based sum score based on the expression levels of six glycolytic proteins and transcriptional regulators indicative of the Warburg-effect (LDHA, GLUT1, MCT4, PKM2, p53, PTEN)[17-19]. Our previous results, based on the total series of CRC patients, indicated that the Warburg-high subtype was associated with a poor survival in CRC, independent of known prognostic factors like TNM stage[17].

Many studies have investigated the relationship between cellular metabolism and therapy resistance in CRC[20]. The majority of studies suggest that the Warburg-effect promotes tumor characteristics that contribute to adjuvant therapy resistance[20-26]. However, most current evidence is based on *in vitro* cell culture studies, whereas – to the best of our knowledge – evidence from prospective cohort studies is lacking.

We hypothesized that patients with Warburg-high CRC will not derive a survival benefit from adjuvant chemo- or radiotherapy, whereas patients with Warburg-low CRC will derive survival benefit from adjuvant therapy. In this exploratory study, we therefore aimed to investigate whether our previously defined Warburg-subtypes can be used to predict survival benefit from adjuvant therapy in CRC patients.

METHODS

Design and study population

The population-based series of colorectal cancer (CRC) patients in this study was derived from the prospective Netherlands Cohort Study (NLCS), which has been described in detail previously[27]. Briefly, the NLCS was initiated in September 1986 and included 120,852 men and women, aged 55-69 years old, who completed a mailed, self-administered questionnaire on diet and other cancer risk factors at baseline[27]. Participants agreed to participate in the study by completing and returning the questionnaire.

The entire prospective cohort was followed-up for cancer incidence by annual record linkage with the Netherlands Cancer Registry and PALGA, the nationwide Dutch Pathology Registry[28, 29], covering 20.3 years of follow-up (September 17, 1986 until January 1, 2007). The completeness of cancer incidence follow-up was estimated to be >96%[30]. After excluding patients who reported a history of cancer (excluding non-melanoma skin cancer) at baseline, 4,597 incident CRC patients were available (**Figure 1**).

The NLCS was approved by the institutional review boards of the TNO Quality of Life Research Institute (Zeist, the Netherlands) and Maastricht University (Maastricht, the Netherlands). Ethical approval for this study was obtained from the Medical Ethical Committee (METC) of Maastricht University Medical Center+.

Defining Warburg-subtypes based on Immunohistochemistry

Formalin-fixed paraffin-embedded (FFPE) tissue blocks from CRC resection specimens, excluding CRC patients who received neo-adjuvant chemotherapy (*n* = 10), were collected as part of the Rainbow-Tissue MicroArray (TMA) project[31]. Details regarding TMA construction have been described previously[17].

In total, 78 TMA blocks were constructed containing three 0.6mm cores from tumor and three from normal epithelium of 2,694 CRC patients (**Figure 1**). Serial sections (5 µm) were subjected to immunohistochemistry (IHC) for Warburg-related proteins (LDHA, GLUT1, MCT4, PKM2, p53, PTEN) and mismatch-repair (MMR)-related proteins (MLH1, MSH2), as described previously[17-19, 32].

Requiring at least one tumor core per patient, 2,497 CRC patients passed quality control (**Figure 1**). Multiple core-level IHC scores were combined into patient-level Warburg-subtypes as described previously[17-19]. After excluding patients with missing IHC data, 2,394 CRC patients were categorized as "Warburg-low" (*n* = 695, 29.0%), "Warburg-moderate" (*n* = 858, 35.8%) or "Warburg-high" (*n* = 841, 35.1%).

Clinical characteristics and follow-up

Follow-up for vital status of the CRC patients was carried out through linkage to the Central Bureau of Genealogy and the municipal population registries until December 31, 2012. Patients who were found to have CRC at autopsy (*n* = 5), patients with incomplete data regarding initial treatment (*n* = 21), patients who did not receive any treatment (no surgery, chemo- or radiotherapy; *n* = 8), patients who received another type of therapy (*n* = 7), or patients who received neoadjuvant radiotherapy (*n* = 143) were excluded. Furthermore, patients with TNM stage I CRC (*n* = 422), who were mostly treated with surgery only (*n* = 412, 97.6%), were excluded from analyses to ensure that patients in the surgery only subgroup had similar clinical characteristics as patients in the adjuvant therapy subgroup. Hence, 1,793 CRC patients were available for analyses (**Figure 1**).

Causes of death were retrieved from Statistics Netherlands. CRC-specific deaths included patients with an underlying cause attributed to malignant neoplasms of the colon, rectosigmoid junction, or rectum. Overall vital status was available for 1,792 (99.9%) patients and CRC-specific vital status for 1,765 (98.4%) patients.

Information about age at diagnosis, pTNM stage, tumor location, tumor differentiation grade, and primary adjuvant therapy (i.e., treatments included in the initial treatment plan drawn up after diagnosis) was retrieved from the cancer registry or PALGA histopathology reports. The cancer registry only registers information regarding the primary treatment that was performed.

Statistical Analyses

Descriptive statistics were calculated for clinical characteristics, using mean (standard deviation) or median (range) for continuous data and frequencies (percentage) for categorical data. For categorical variables, differences across treatment subgroups (i.e., surgery only, surgery and adjuvant radiotherapy, surgery and adjuvant chemotherapy) were evaluated using Chi-squared (χ^2) tests. For continuous variables, the distributions across groups were evaluated using Kruskal-Wallis tests.

The primary outcomes were CRC-specific survival (time from CRC diagnosis to CRC-related death or end of follow-up) and overall survival (time from CRC diagnosis to death from any cause or end of follow-up). Survival analyses were restricted to 10 years of follow-up because of the limited number of events in the later period (CRC-specific deaths: *n* = 22; overall deaths: *n* = 175). Kaplan-Meier curves were estimated to examine survival benefit from adjuvant therapy for the different Warburg-subtypes (Warburg-low, Warburg-moderate, and Warburg-high). Differences between survival curves were investigated using Wilcoxon tests.

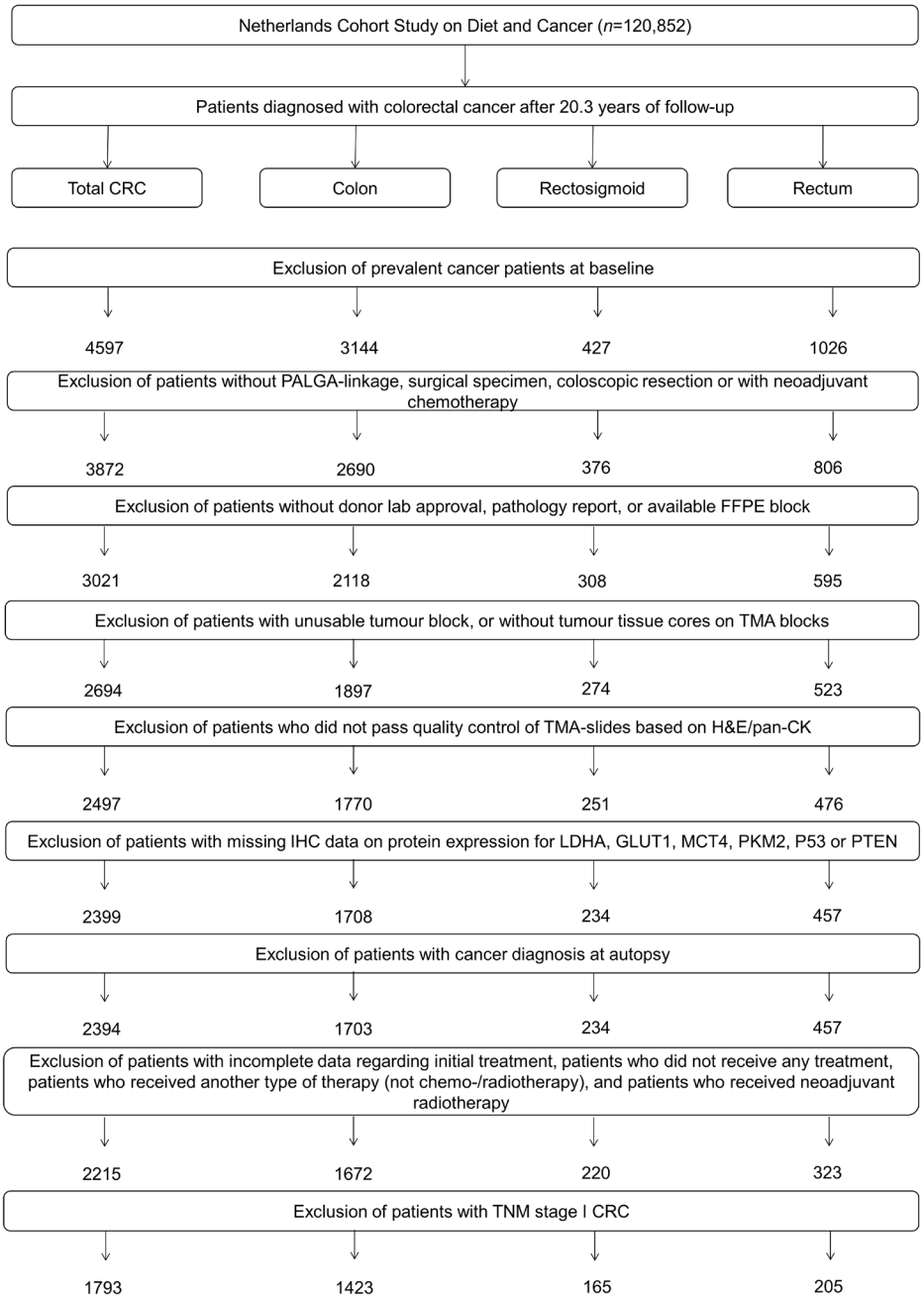

Figure 1 – Flow diagram of the number of CRC patients available for analyses in the Netherlands Cohort Study (NLCS), 1986-2006. Abbreviations: CRC, colorectal cancer; PALGA, Netherlands pathology database; TMA, Tissue MicroArray.

Cox proportional hazards regression was used to estimate Hazard ratios (HRs) and 95% confidence intervals (CIs) for associations between adjuvant therapy and survival by Warburg-subtype. The proportional hazards assumption was tested using the scaled Schoenfeld residuals[33], by evaluating -log-log transformed survival curves or by introducing time-covariate interactions into the models. HRs were adjusted for a set of *a priori* selected prognostic factors: age at diagnosis (years); sex (men, women); tumor location (colon, rectosigmoid, rectum); pTNM stage (I, II, III, IV); differentiation grade (well, moderate, poor/undifferentiated); and MMR deficiency (no, yes). Year of diagnosis and pTNM version were considered as potential confounders, and were retained in the models if they altered HRs by more than 10%[34, 35]. A separate category ('unknown') was used for patients with unknown clinical information regarding pTNM stage, differentiation grade or MMR status, to enable inclusion of these patients in the Cox proportional hazards models.

Disease stage was based on the pTNM classification according to the edition valid at the time of cancer diagnosis, resulting in the use of five different TNM editions (UICC TNM editions 3-6), as described previously[17]. However, the main TNM stage groupings (I/II/III/IV) have remained essentially unchanged[36].

In sensitivity analyses, excluding CRC patients with unknown clinical information regarding TNM stage and differentiation grade (n = 143) yielded similar results (*data not shown*).

All analyses were conducted in Stata Statistical Software: Release 16 (StataCorp., College Station, TX). Two-sided p-values < 0.05 were considered significant.

RESULTS

Clinical characteristics

Clinical characteristics of the 1,793 included colorectal cancer (CRC) patients according to adjuvant therapy are presented in **Table 1**. The large majority (n = 1,451, 80.9%) of CRC patients from the prospective Netherlands Cohort Study (NLCS) were treated with surgery only, while 82 (4.6%) and 260 (14.5%) patients were treated with adjuvant radio- or chemotherapy, respectively. The use of adjuvant chemotherapy increased over time (from 1.3% in 1986-1988 to 13.4% in 2004-2006), whereas the administration of adjuvant radiotherapy decreased (from 10.5% in 1986-1988 to 0.0% in 2004-2006; p < 0.001).

CRC patients treated with adjuvant radio- or chemotherapy were younger compared to patients treated with surgery only (median age at diagnosis 69.0 and 72.0 versus 75.0, respectively; p < 0.001). Men were more frequently treated with adjuvant radio- or chemotherapy compared to women (5.4% and 16.4% of men versus 3.6% and 12.2% of women, respectively;

p = 0.004). Patients with colon cancers were more often treated with surgery only compared to patients with rectosigmoid or rectal cancers (84.4% versus 75.8% and 61.0%, respectively; p < 0.001). Furthermore, patients with rectal cancers were more often treated with adjuvant radiotherapy compared to patients with rectosigmoid or colon cancers (28.8% versus 7.9% and 0.7%, respectively). Patients with pTNM stage III or IV CRC more often received adjuvant chemotherapy compared to patients with pTNM stage II CRC (27.3% and 25.8% versus 2.2%, respectively; p < 0.001). Patients who were treated with adjuvant radio- or chemotherapy were, in retrospect, more likely to have MMR proficient CRC ($MMR_{proficient}$ 5.1% and 15.3% versus $MMR_{deficient}$ 0.9% and 9.4%, respectively; p = 0.002).

Table 1 – Clinical characteristics of colorectal cancer patients (*n* = 1,793) within the Netherlands Cohort Study (NLCS, 1986-2006), according to adjuvant therapy (surgery, surgery and radiotherapy, surgery and chemotherapy).

Clinical characteristics	Total CRC (*n* = 1,793)	Surgery only (*n* = 1,451)	Adjuvant therapy		P-value[a]
			Surgery + RT (*n* = 82)	Surgery + CHT (*n* = 260)	
Year of diagnosis, *n* (%)					
1986-1988	76 (4.2)	67 (88.2)	8 (10.5)	1 (1.3)	
1989-1991	149 (8.3)	118 (79.2)	18 (12.1)	13 (8.7)	
1992-1994	243 (13.6)	190 (78.2)	19 (7.8)	34 (14.0)	
1995-1997	336 (18.7)	262 (78.0)	20 (6.0)	54 (16.1)	<0.001
1998-2000	330 (18.4)	256 (77.6)	16 (4.9)	58 (17.6)	
2001-2003	323 (18.0)	267 (82.7)	1 (0.3)	55 (17.0)	
2004-2006	336 (18.7)	291 (86.6)	-	45 (13.4)	
Age at diagnosis in years, median (range)	74.0 (55.0-89.0)	75.0 (55.0-89.0)	69.0 (56.0-79.0)	72.0 (60.0-86.0)	<0.001[b]
Sex, n (%)					
Men	980 (54.7)	766 (78.2)	53 (5.4)	161 (16.4)	0.004
Women	813 (45.3)	685 (84.3)	29 (3.6)	99 (12.2)	
Tumor location, *n* (%)					
Colon	1423 (79.4)	1201 (84.4)	10 (0.7)	212 (14.9)	
Rectosigmoid	165 (9.2)	125 (75.8)	13 (7.9)	27 (16.4)	<0.001
Rectum	205 (11.4)	125 (61.0)	59 (28.8)	21 (10.2)	
pTNM stage, *n* (%)					
II	860 (48.0)	806 (93.7)	35 (4.1)	19 (2.2)	
III	578 (32.2)	379 (65.6)	41 (7.1)	158 (27.3)	<0.001
IV	322 (18.0)	236 (73.3)	3 (0.9)	83 (25.8)	
Unknown	33 (1.8)	30 (90.9)	3 (9.1)	-	
Tumor extension (pT), *n* (%)					
T1	8 (0.5)	5 (62.5)	1 (12.5)	2 (25.0)	
T2	69 (3.9)	41 (59.4)	7 (10.1)	21 (30.4)	
T3	1448 (80.6)	1188 (82.0)	62 (4.3)	198 (13.7)	<0.001
T4	229 (12.8)	182 (79.5)	9 (3.9)	38 (16.6)	
Unknown	39 (2.2)	35 (89.7)	3 (7.7)	1 (2.6)	
Lymph node involvement (pN), *n* (%)					
N0	817 (45.6)	752 (92.0)	32 (3.9)	33 (4.0)	
N+	813 (45.3)	546 (67.2)	44 (5.4)	223 (27.4)	<0.001
Unknown	163 (9.1)	153 (93.9)	6 (3.7)	4 (2.5)	

Table 1 – Continued

Clinical characteristics	Total CRC (*n* = 1,793)	Surgery only (*n* = 1,451)	Adjuvant therapy		P-value[a]
			Surgery + RT (*n* = 82)	Surgery + CHT (*n* = 260)	
Differentiation grade, *n* (%)					
Well	133 (7.4)	112 (84.2)	3 (2.3)	18 (13.5)	0.100
Moderate	1165 (65.0)	943 (80.9)	62 (5.3)	160 (13.7)	
Poor/undifferentiated	267 (20.5)	286 (77.9)	14 (3.8)	67 (18.3)	
Unknown	128 (7.2)	110 (85.9)	3 (2.3)	15 (11.7)	
dMMR, ***n*** (%)					
No	1560 (87.0)	1241 (79.6)	80 (5.1)	239 (15.3)	0.002
Yes	214 (11.9)	192 (89.7)	2 (0.9)	20 (9.4)	
Unknown	19 (1.1)	18 (94.7)	-	1 (5.3)	
Warburg-subtype, *n* (%)					
Warburg-low	485 (27.1)	395 (81.4)	23 (4.7)	67 (13.8)	0.950
Warburg-moderate	641 (35.8)	518 (80.8)	31 (4.8)	92 (14.4)	
Warburg-high	667 (37.2)	538 (80.7)	28 (4.2)	101 (15.1)	

[a]P-value for the $\chi 2$ test, unless otherwise specified
[b]P-value for the Kruskall-Wallis test
Abbreviations: CRC, colorectal cancer; RT, radiotherapy; CHT, chemotherapy; TNM, tumor node metastasis; dMMR, mismatch repair deficient.

Warburg-subtypes and survival after adjuvant therapy

The median follow-up time since diagnosis was 3.72 years (range: 0.0027 to 25.49 years). Survival analyses were restricted to 10 years of follow-up, because of the limited number of events in the later period. During these first 10 years of follow up, 1,243 (69.3%) deaths were observed of which 848 (68.2%) were CRC-related deaths.

Association between adjuvant therapy and survival within Warburg-subtype

In patients with Warburg-low CRC, univariable Kaplan-Meier curves showed significant differences in CRC-specific survival ($p_{CRC\text{-}specific}$ = 0.047), but not overall survival ($p_{overall}$ = 0.394), between treatment groups (**Figure 2A**, **Figure 3A**). Patients with Warburg-low CRC treated with adjuvant (chemo)therapy had a significantly worse CRC-specific survival compared to patients with Warburg-low CRC treated with surgery only ($HR_{adjuvant\ therapy}$ 1.63; 95% CI 1.20-2.20 and $HR_{adjuvant\ chemotherapy}$ 1.75; 95% CI 1.25-2.45; **Table 2**). These associations with survival disappeared after adjustment for confounders in multivariable-adjusted analyses ($HR_{adjuvant\ therapy}$ 1.07; 95% CI 0.76-1.52 and $HR_{adjuvant\ chemotherapy}$ 1.03; 95% CI 0.70-1.51; **Table 2**).

In patients with Warburg-moderate CRC, univariable Kaplan-Meier curves showed significant differences in overall survival ($p_{overall}$ = 0.041), but not CRC-specific survival ($p_{CRC\text{-}specific}$ = 0.397), between treatment groups (**Figure 2B**, **Figure 3B**). Patients with Warburg-moderate CRC treated with adjuvant (chemo)therapy had a better overall survival compared to patients with Warburg-moderate CRC treated with surgery only ($HR_{adjuvant\ therapy}$ 0.81;

95% CI 0.64-1.03 and $HR_{adjuvant\ chemotherapy}$ 0.77; 95% CI 0.58-1.02; **Table 2**). In multivariable-adjusted analyses, these inverse associations with survival became even stronger and reached statistical significance for both CRC-specific ($HR_{adjuvant\ therapy}$ 0.64; 95% CI 0.47-0.86 and $HR_{adjuvant\ chemotherapy}$ 0.53; 95% CI 0.38-0.75; **Table 2**) and overall survival ($HR_{adjuvant\ therapy}$ 0.61; 95% CI 0.47-0.80 and $HR_{adjuvant\ chemotherapy}$ 0.50; 95% CI 0.37-0.67; **Table 2**).

In patients with Warburg-high CRC, univariable Kaplan-Meier curves showed significant differences in CRC-specific survival ($p_{CRC\text{-}specific}$ = 0.019), but not overall survival ($p_{overall}$ = 0.288), between treatment groups (**Figure 2B**, **Figure 3B**). Patients with Warburg-high CRC treated with adjuvant (chemo)therapy had a significantly worse CRC-specific ($HR_{adjuvant\ therapy}$ 1.58; 95% CI 1.23-2.02, $HR_{adjuvant\ chemotherapy}$ 1.67; 95% CI 1.27-2.18) and overall survival ($HR_{adjuvant\ therapy}$ 1.31; 95% CI 1.05-1.62, $HR_{adjuvant\ chemotherapy}$ 1.31; 95% CI 1.03-1.67) compared to patients with Warburg-high CRC treated with surgery only (**Table 2**). In multivariable-adjusted analyses, these associations with survival changed direction but did not reach statistical significance (CRC-specific survival: $HR_{adjuvant\ therapy}$ 0.86; 95% CI 0.65-1.14; overall survival: $HR_{adjuvant\ therapy}$ 0.82; 95% CI 0.64-1.05; **Table 2**). However, the association between adjuvant chemotherapy and overall survival did reach statistical significance ($HR_{adjuvant\ chemotherapy}$ 0.75; 95% CI 0.57-0.98; **Table 2**).

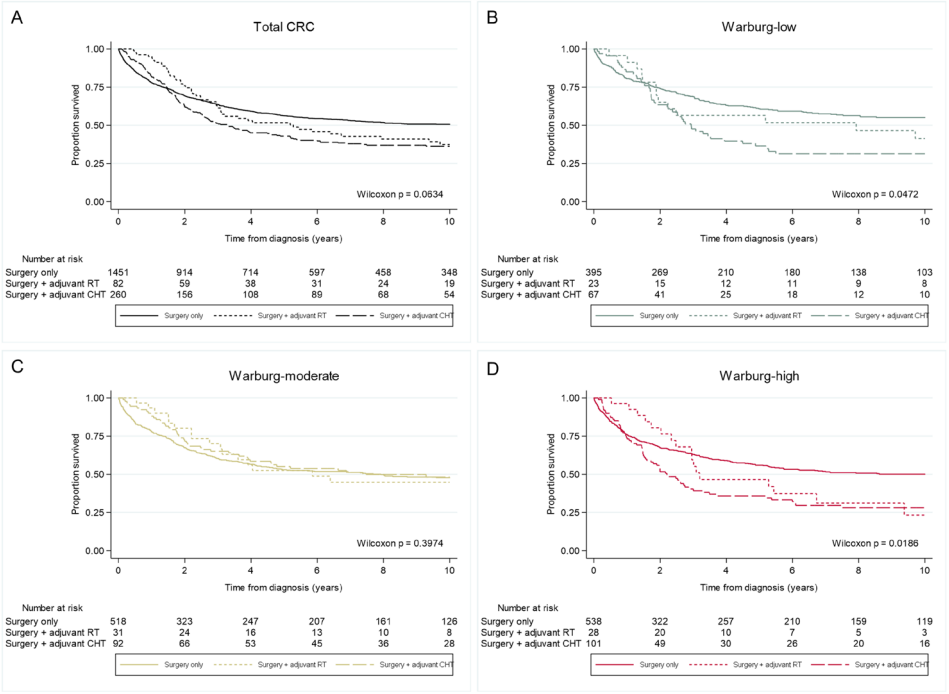

Figure 2 – Univariable Kaplan-Meier curves showing CRC-specific survival of colorectal cancer patients within the Netherlands Cohort Study (NLCS, 1986-2006) for (**A**) Total CRC, (**B**) Warburg-low CRC, (**C**) Warburg-moderate CRC, or (**D**) Warburg-high CRC, according to the treatment received (surgery only, surgery and adjuvant radiotherapy, surgery adjuvant chemotherapy). Abbreviations: RT, radiotherapy; CHT, chemotherapy.

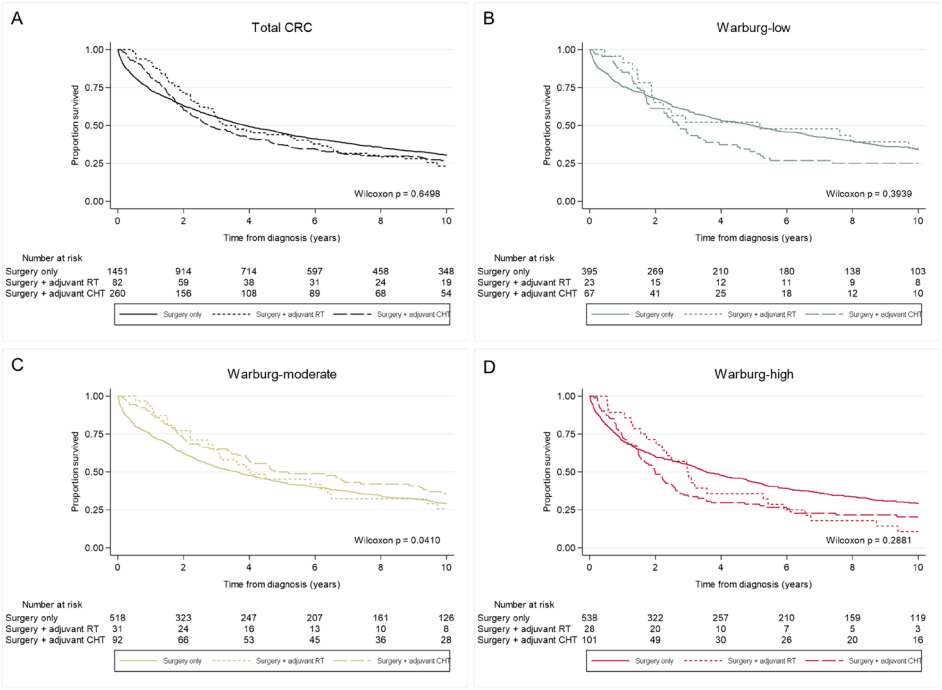

Figure 3 – Univariable Kaplan-Meier curves showing overall survival of colorectal cancer patients within the Netherlands Cohort Study (NLCS, 1986-2006) for (**A**) Total CRC, (**B**) Warburg-low CRC, (**C**) Warburg-moderate CRC, or (**D**) Warburg-high CRC, according to the treatment received (surgery only, surgery and adjuvant radiotherapy, surgery and adjuvant chemotherapy). Abbreviations: RT, radiotherapy; CHT, chemotherapy.

Table 2 – Univariable and multivariable-adjusted hazard ratios for associations between adjuvant therapy (surgery, surgery plus radiotherapy, surgery plus chemotherapy) and CRC-specific and overall survival for the Warburg-subtypes (Warburg-low, Warburg-moderate, and Warburg-high) within the Netherlands Cohort Study (NLCS, 1986-2006).

	N	CRC-specific survival			Overall survival		
		CRC deaths (%)	HR (95% CI)		Deaths (%)	HR (95% CI)	
			Univariable	Multivariable-adjusted[a]		Univariable	Multivariable-adjusted[a]
Total CRC							
Surgery only	1451	644 (44.4)	1.00 (ref)	1.00 (ref)	992 (68.4)	1.00 (ref)	1.00 (ref)
Surgery + adjuvant therapy	342	204 (59.6)	1.31 (1.12-1.53)	0.80 (0.68-0.96)	251 (73.4)	1.07 (0.94-1.23)	0.78 (0.67-0.91)
Surgery + adjuvant RT	*82*	*46 (56.1)*	*1.15 (0.85-1.55)*	*1.14 (0.81-1.61)*	*63 (76.8)*	*1.05 (0.81-1.35)*	*1.22 (0.91-1.63)*
Surgery + adjuvant CHT	*260*	*158 (60.8)*	*1.37 (1.15-1.63)*	*0.74 (0.61-0.90)*	*188 (72.3)*	*1.08 (0.93-1.27)*	*0.69 (0.58-0.82)*
Warburg-low							
Surgery only	395	159 (40.3)	1.00 (ref)	1.00 (ref)	255 (64.6)	1.00 (ref)	1.00 (ref)
Surgery + adjuvant therapy	90	57 (63.3)	1.63 (1.20-2.20)	1.07 (0.76-1.52)	65 (72.2)	1.20 (0.91-1.57)	0.95 (0.70-1.30)
Surgery + adjuvant RT	*23*	*13 (56.5)*	*1.31 (0.74-2.30)*	*1.26 (0.67-2.36)*	*15 (65.2)*	*0.93 (0.55-1.57)*	*1.17 (0.66-2.09)*
Surgery + adjuvant CHT	*67*	*44 (65.7)*	*1.75 (1.25-2.45)*	*1.03 (0.70-1.51)*	*50 (74.6)*	*1.31 (0.97-1.78)*	*0.90 (0.63-1.27)*
Warburg-moderate							
Surgery only	518	245 (47.3)	1.00 (ref)	1.00 (ref)	362 (69.9)	1.00 (ref)	1.00 (ref)
Surgery + adjuvant therapy	123	62 (50.4)	0.92 (0.69-1.21)	0.64 (0.47-0.86)	81 (65.9)	0.81 (0.64-1.03)	0.61 (0.47-0.80)
Surgery + adjuvant RT	*31*	*16 (51.6)*	*0.94 (0.57-1.56)*	*1.23 (0.67-2.24)*	*23 (74.2)*	*0.93 (0.61-1.42)*	*1.32 (0.80-2.18)*
Surgery + adjuvant CHT	*92*	*46 (50.0)*	*0.91 (0.66-1.25)*	*0.53 (0.38-0.75)*	*58 (63.0)*	*0.77 (0.58-1.02)*	*0.50 (0.37-0.67)*
Warburg-high							
Surgery only	538	240 (44.6)	1.00 (ref)	1.00 (ref)	375 (69.7)	1.00 (ref)	1.00 (ref)
Surgery + adjuvant therapy	129	85 (65.9)	1.58 (1.23-2.02)	0.86 (0.65-1.14)	105 (81.4)	1.31 (1.05-1.62)	0.82 (0.64-1.05)
Surgery + adjuvant RT	*28*	*17 (60.7)*	*1.29 (0.79-2.12)*	*1.22 (0.67-2.21)*	*25 (89.3)*	*1.29 (0.86-1.93)*	*1.25 (0.77-2.03)*
Surgery + adjuvant CHT	*101*	*68 (67.3)*	*1.67 (1.27-2.18)*	*0.81 (0.60-1.09)*	*80 (79.2)*	*1.31 (1.03-1.67)*	*0.75 (0.57-0.98)*

[a]Adjusted for age at diagnosis (years), sex (male/female), tumour location (colon/rectosigmoid/rectum), TNM stage (II, III, IV, unknown), differentiation grade (well/moderate/poor/undifferentiated/unknown), MMR deficiency (no/yes/unknown), and year of diagnosis (per 3 years). Abbreviations: CRC, colorectal cancer; HR, hazard ratio; CI, confidence interval; RT, radiotherapy; CHT, chemotherapy.

The interaction between Warburg-subtype and adjuvant therapy as calculated in a multivariable-adjusted Cox proportional hazard model, adjusted for age at diagnosis, sex, tumor location, TNM stage, differentiation grade, MMR status and year of diagnosis was statistically significant for CRC-specific survival (p = 0.049) and overall survival (p = 0.035). In stratified analyses according to disease stage (**Supplementary Table S1**), similar trends were observed for patients with pTNM stage III CRC. However, in patients with pTNM stage II CRC, no significant association between adjuvant therapy and survival was observed for any of the Warburg-subtypes. In contrast, in patients with pTNM stage IV CRC, a better survival was observed for CRC patients receiving adjuvant (chemo)therapy compared to patients who received surgery only, independent of Warburg-subtype. In stratified analyses according to tumor location (**Supplementary Table S2**), a significantly better survival was observed for patients with Warburg-moderate or Warburg-high cancers located in the colon who received adjuvant (chemo)therapy compared to patients who received surgery only. Furthermore, a significant survival benefit was observed for patients with Warburg-moderate cancers located in the rectum who received adjuvant (radio)therapy.

DISCUSSION

In this large, population-based series of colorectal cancer (CRC) patients, we investigated whether our previously defined immunohistochemistry (IHC)-based Warburg-subtypes can be used to predict survival in patients treated with adjuvant therapy. Our results indicate that Warburg-subtypes may predict treatment benefit in CRC patients. While in general patients with stage II-IV CRC who received adjuvant (chemo)therapy had a significantly favorable CRC-specific and overall survival compared to patients who received surgery only, this benefit was only observed in patients with Warburg-moderate CRC. Patients with Warburg-high CRC also seemed to benefit from adjuvant therapy, but associations did not reach statistical significance. In contrast, no benefit from adjuvant (chemo)therapy was found for patients with Warburg-low CRC.

Since the 1950s, 5-fluorouracil (5-FU)-based chemotherapy remains the main pharmacological treatment modality for patients with CRC[37]. Although the administration of chemotherapy can improve the survival of cancer patients, chemotherapy resistance remains a major problem[20]. In CRC, 5-FU-based chemotherapy remains ineffective in approximately 30% of patients[26]. Hence, there remains an urgent clinical need to identify novel prognostic and/or predictive biomarker(s) to improve survival and quality of life in CRC patients[5, 8].

To the best of our knowledge, we are the first to prospectively investigate whether Warburg-subtypes are associated with adjuvant (chemo)therapy resistance in a large

population-based cohort of CRC patients. Nevertheless, many studies have investigated the relationship between cellular metabolism and therapy resistance *in vitro*[20]. Moreover, one retrospective study has investigated the relation between expression patterns of proteins related to the Warburg-effect and response to therapy in patient tissue samples[26]. On the one hand, the majority of studies suggest that aerobic glycolysis promotes tumor characteristics that contribute to adjuvant therapy resistance[20-26]. On the other hand, there are studies that suggest that therapy resistance is accompanied by a metabolic shift from aerobic glycolysis towards oxidative phosphorylation (OXPHOS)[38-40]. Assuming that the Warburg-high subtype represents CRC that rely mainly on aerobic glycolysis to meet their metabolic demands, whereas the Warburg-low subtype represents a more oxidative metabolic phenotype (i.e., OXPHOS), our results are in contrast with those of the majority of previous studies which showed that aerobic glycolysis is associated with adjuvant therapy resistance[20-25].

Even though future studies are necessary to validate our results and to further investigate the biological mechanisms, the discrepancy in results might be explained by the fact that previous reports were mostly based on *in vitro* cell culture studies[25] or were conducted retrospectively[26]. It has been reported that *in vitro* conditions differ drastically from the conditions found *in vivo* in the tumor microenvironment[41, 42]. Furthermore, it has been suggested that the effect of therapy might differ depending on the environment in which the cancer cells reside[43]. For example, research suggests that cancer cells may be sensitive to chemotherapy in cell culture, but become resistant when transplanted into animal models[44].

A potential explanation for the observation that patients with Warburg-low CRC had no survival benefit from adjuvant (chemo)therapy has been described by Vellinga *et al.*[39]. Normally, the amount of adenosine 5'-triphosphate (ATP) that is generated by aerobic glycolysis is sufficient to support tumor cell growth and basal DNA repair activity[39, 45]. However, when chemotherapy is administered, the cellular ATP demand in cancer cells increases significantly as many enzymes involved in DNA repair, drug efflux, and drug detoxification require ATP to function[39, 45]. As OXPHOS is the most efficient way to generate ATP[46], cancer cells may switch from aerobic glycolysis to OXPHOS at times of high ATP demand[39]. In line with our results, this may suggest that patients with Warburg-low CRC (i.e., patients with cancers that rely mainly on oxidative metabolism) are more capable of repairing DNA damage and regulating drug metabolism compared to patients with Warburg-moderate and Warburg-high CRC (i.e., patients with cancers that rely mainly on aerobic glycolysis), rendering them more resistant to adjuvant therapy.

Our results suggest that the predictive value of Warburg-subtypes may be limited to TNM stage III CRC. In TNM stage II, no survival benefit from adjuvant (chemo)therapy

was observed for any of the Warburg-subtypes, while in TNM stage IV all CRC patients had survival benefit from adjuvant (chemo)therapy regardless of Warburg-subtype. As adjuvant chemotherapy is the standard of care for TNM stage III CRC[47], and chemotherapy resistance is still a major problem in clinical practice[20, 26], Warburg-subtypes may in the future help to determine which stage III CRC patients will benefit most from adjuvant (chemo)therapy.

The main strengths of the present study include the use of a large population-based series of incident CRC patients, the prospective design, the nearly complete follow-up, and the availability of tumor material for a large number of CRC patients. Our study has some limitations. First, we did not have a validation cohort available to confirm the observed associations. Second, we did not have any detailed clinical information available regarding the dosage, duration or exact type of treatment. Third, we did not adjust for multiple testing which may have potentially resulted in chance findings. Fourth, in the Netherlands Cohort Study (NLCS) the large majority of CRC patients were treated with surgery only, resulting in a relatively small number of patients that were treated with adjuvant therapy, thereby limiting the power of our analyses. However, the limited number of patients treated with adjuvant therapy was representative for this time period (1986-2006)[48]. Lastly, limitations with regard to Warburg-subtyping were described in detail previously[17].

CONCLUSION

In conclusion, Warburg-subtypes may predict treatment benefit in CRC patients. Our results suggest that survival benefit from adjuvant (chemo)therapy in patients with CRC may depend on Warburg-subtype. Opposite to expectation, a survival benefit from adjuvant (chemo)therapy was observed for patients with Warburg-moderate and possibly also Warburg-high CRC, but not for patients with Warburg-low CRC.

All in all, our results highlight the importance of molecular classification of CRC based on Warburg-related proteins, in addition to TNM stage and tumor location, to identify subgroups of patients who are more likely to benefit from adjuvant (chemo)therapy. However, as this is an exploratory study, our results should be interpreted with caution and future prospective studies are necessary to validate our findings.

REFERENCES

1. Rawla P, Sunkara T, Barsouk A. Epidemiology of colorectal cancer: incidence, mortality, survival, and risk factors. Prz Gastroenterol 2019;14:89-103.
2. Ferlay J, Ervik M, Lam F, et al. Global Cancer Observatory: Cancer Today. Volume 2021: Lyon, France: International Agency for Research on Cancer, 2020.
3. Roelands J, Kuppen PJ, Vermeulen L, et al. Immunogenomic classification of colorectal cancer and therapeutic implications. International journal of molecular sciences 2017;18:2229.
4. Kawakami H, Zaanan A, Sinicrope FA. Microsatellite instability testing and its role in the management of colorectal cancer. Current treatment options in oncology 2015;16:1-15.
5. Ji D, Zhan T, Li M, et al. Enhancement of sensitivity to chemo/radiation therapy by using miR-15b against DCLK1 in colorectal cancer. Stem cell reports 2018;11:1506-1522.
6. Zhai Z, Yu X, Yang B, et al. Colorectal cancer heterogeneity and targeted therapy: clinical implications, challenges and solutions for treatment resistance, In Seminars in cell & developmental biology, Elsevier, 2017.
7. Sinicrope FA, Okamoto K, Kasi PM, et al. Molecular biomarkers in the personalized treatment of colorectal cancer. Clinical Gastroenterology and Hepatology 2016;14:651-658.
8. Ten Hoorn S, de Back TR, Sommeijer DW, et al. Clinical value of consensus molecular subtypes in colorectal cancer: a systematic review and meta-analysis. JNCI: Journal of the National Cancer Institute 2022;114:503-516.
9. Fontana E, Eason K, Cervantes A, et al. Context matters—consensus molecular subtypes of colorectal cancer as biomarkers for clinical trials. Annals of Oncology 2019;30:520-527.
10. Hanahan D, Weinberg RA. Hallmarks of cancer: the next generation. cell 2011;144:646-674.
11. Warburg O, Wind F, Negelein E. The metabolism of tumors in the body. The Journal of general physiology 1927;8:519-530.
12. Wolpaw AJ, Dang CV. Exploiting Metabolic Vulnerabilities of Cancer with Precision and Accuracy. Trends Cell Biol 2018;28:201-212.
13. Bensinger SJ, Christofk HR. New aspects of the Warburg effect in cancer cell biology. Semin Cell Dev Biol 2012;23:352-61.
14. Kato Y, Maeda T, Suzuki A, et al. Cancer metabolism: New insights into classic characteristics. Jpn Dent Sci Rev 2018;54:8-21.
15. Sakashita M, Aoyama N, Minami R, et al. Glut1 expression in T1 and T2 stage colorectal carcinomas: its relationship to clinicopathological features. Eur J Cancer 2001;37:204-9.
16. Potter M, Newport E, Morten KJ. The Warburg effect: 80 years on. Biochemical Society Transactions 2016;44:1499-1505.
17. Offermans K, Jenniskens JC, Simons CC, et al. Expression of proteins associated with the Warburg-effect and survival in colorectal cancer. The Journal of Pathology: Clinical Research 2021.
18. Jenniskens JC, Offermans K, Simons CC, et al. Energy balance-related factors in childhood and adolescence and risk of colorectal cancer expressing different levels of proteins involved in the Warburg-effect. International Journal of Cancer 2022;150:1812-1824.
19. Jenniskens JC, Offermans K, Simons CC, et al. Energy balance-related factors and risk of colorectal cancer

expressing different levels of proteins involved in the Warburg-effect. Cancer Epidemiology and Prevention Biomarkers 2021.
20. Liu C, Jin Y, Fan Z. The Mechanism of Warburg Effect-Induced Chemoresistance in Cancer. Frontiers in oncology 2021:3408.
21. Dong S, Liang S, Cheng Z, et al. ROS/PI3K/Akt and Wnt/β-catenin signalings activate HIF-1α-induced metabolic reprogramming to impart 5-fluorouracil resistance in colorectal cancer. Journal of Experimental & Clinical Cancer Research 2022;41:1-27.
22. Zaal EA, Berkers CR. The influence of metabolism on drug response in cancer. Frontiers in oncology 2018;8:500.
23. Desbats MA, Giacomini I, Prayer-Galetti T, et al. Metabolic plasticity in chemotherapy resistance. Frontiers in oncology 2020;10:281.
24. Zhong J-T, Zhou S-H. Warburg effect, hexokinase-II, and radioresistance of laryngeal carcinoma. Oncotarget 2017;8:14133.
25. Morandi A, Indraccolo S. Linking metabolic reprogramming to therapy resistance in cancer. Biochimica et Biophysica Acta (BBA)-Reviews on Cancer 2017;1868:1-6.
26. Kitazawa M, Hatta T, Sasaki Y, et al. Promotion of the Warburg effect is associated with poor benefit from adjuvant chemotherapy in colorectal cancer. Cancer science 2020;111:658.
27. van den Brandt PA, Goldbohm RA, van 't Veer P, et al. A large-scale prospective cohort study on diet and cancer in The Netherlands. J Clin Epidemiol 1990;43:285-95.
28. Casparie M, Tiebosch AT, Burger G, et al. Pathology databanking and biobanking in The Netherlands, a central role for PALGA, the nationwide histopathology and cytopathology data network and archive. Cell Oncol 2007;29:19-24.
29. van den Brandt PA, Schouten LJ, Goldbohm RA, et al. Development of a record linkage protocol for use in the Dutch Cancer Registry for Epidemiological Research. Int J Epidemiol 1990;19:553-8.
30. Goldbohm RA, van den Brandt PA, Dorant E. Estimation of the coverage of municipalities by cancer registries and PALGA using hospital discharge data. Tijdschr Soc Gezondheidsz 1994;72:80-4.
31. van den Brandt PA. Molecular pathological epidemiology of lifestyle factors and colorectal and renal cell cancer risk. Maastricht Pathology 2018. 11th Joint Meeting of the British Division of the International Academy of Pathology and the Pathological Society of Great Britain & Ireland, 19-22 June 2018. . J Pathol 2018;246 Suppl 1: S9.
32. Jenniskens JC, Offermans K, Samarska I, et al. Validity and reproducibility of immunohistochemical scoring by trained non-pathologists on Tissue MicroArrays. Cancer Epidemiology and Prevention Biomarkers 2021.
33. Schoenfeld D. Partial residuals for the proportional hazards regression model. Biometrika 1982;69:239-241.
34. Alexander LK, Lopes B, Ricchetti-Masterson K, et al. Confounding bias, part II and effect measure modification. UNC CH Department of Epidemiology 2015.
35. Kamangar F. Confounding variables in epidemiologic studies: basics and beyond. Arch Iran Med 2012;15:508-16.
36. Sobin LH, Compton CA, Gospodarowicz M, et al. 'Evidence-based medicine: the time has come to set standards for staging'. Is a radical overhaul really needed? The Journal of pathology 2010;221:361-362.
37. Van der Jeught K, Xu HC, Li YJ, et al. Drug resistance and new therapies in colorectal cancer. World J Gastroenterol 2018;24:3834-3848.

38. Denise C, Paoli P, Calvani M, et al. 5-fluorouracil resistant colon cancer cells are addicted to OXPHOS to survive and enhance stem-like traits. Oncotarget 2015;6:41706.
39. Vellinga TT, Borovski T, de Boer VC, et al. SIRT1/PGC1α-dependent increase in oxidative phosphorylation supports chemotherapy resistance of colon cancer. Clinical cancer research 2015;21:2870-2879.
40. Taniguchi K, Sakai M, Sugito N, et al. PKM1 is involved in resistance to anti-cancer drugs. Biochemical and biophysical research communications 2016;473:174-180.
41. Vermeersch KA, Wang L, McDonald JF, et al. Distinct metabolic responses of an ovarian cancer stem cell line. BMC systems biology 2014;8:1-14.
42. Pampaloni F, Reynaud EG, Stelzer EH. The third dimension bridges the gap between cell culture and live tissue. Nature reviews Molecular cell biology 2007;8:839-845.
43. Jo Y, Choi N, Kim K, et al. Chemoresistance of cancer cells: requirements of tumor microenvironment-mimicking in vitro models in anti-cancer drug development. Theranostics 2018;8:5259.
44. Trédan O, Galmarini CM, Patel K, et al. Drug resistance and the solid tumor microenvironment. Journal of the National Cancer Institute 2007;99:1441-1454.
45. Gottesman MM, Fojo T, Bates SE. Multidrug resistance in cancer: role of ATP–dependent transporters. Nature reviews cancer 2002;2:48-58.
46. Vander Heiden MG, Cantley LC, Thompson CB. Understanding the Warburg effect: the metabolic requirements of cell proliferation. science 2009;324:1029-1033.
47. Kornmann M, Formentini A, Ette C, et al. Prognostic factors influencing the survival of patients with colon cancer receiving adjuvant 5-FU treatment. European Journal of Surgical Oncology (EJSO) 2008;34:1316-1321.
48. Van Steenbergen L, Elferink M, Krijnen P, et al. Improved survival of colon cancer due to improved treatment and detection: a nationwide population-based study in The Netherlands 1989–2006. Annals of oncology 2010;21:2206-2212.

Supplementary Table S1 – Univariable and multivariable-adjusted hazard ratios for associations between adjuvant therapy (surgery, surgery plus radiotherapy, surgery plus chemotherapy) and CRC-specific and overall survival for the Warburg-subtypes (Warburg-low, Warburg-moderate, and Warburg-high) within the Netherlands Cohort Study (NLCS, 1986-2006), stratified on TNM stage (II/III/IV).

	N	CRC-specific survival			Overall survival		
		CRC deaths (%)	HR (95% CI)		Deaths (%)	HR (95% CI)	
			Univariable	Multivariable-adjusted[a]		Univariable	Multivariable-adjusted[a]
TNM stage II							
Warburg-low							
Surgery only	230	59 (25.7)	1.00 (ref)	1.00 (ref)	126 (54.8)	1.00 (ref)	1.00 (ref)
Surgery + adjuvant therapy	15	5 (33.3)	1.28 (0.51-3.19)	1.63 (0.60-4.45)	8 (53.3)	0.94 (0.46-1.93)	1.39 (0.65-2.98)
Surgery + adjuvant RT	*9*	*4 (44.4)*	*1.86 (0.67-5.12)*	*2.71 (0.77-9.47)*	*6 (66.7)*	*1.31 (0.58-2.98)*	*2.54 (0.98-6.56)*
Surgery + adjuvant CHT	*6*	*1 (16.7)*	*0.57 (0.08-4.11)*	*0.72 (0.10-5.39)*	*2 (33.3)*	*0.51 (0.13-2.07)*	*0.63 (0.15-2.62)*
Warburg-moderate							
Surgery only	278	80 (28.8)	1.00 (ref)	1.00 (ref)	155 (55.8)	1.00 (ref)	1.00 (ref)
Surgery + adjuvant therapy	19	6 (31.6)	0.99 (0.43-2.26)	1.39 (0.55-3.51)	10 (52.6)	0.85 (0.45-1.60)	1.10 (0.54-2.23)
Surgery + adjuvant RT	*14*	*6 (42.9)*	*1.43 (0.62-3.28)*	*1.91 (0.73-4.99)*	*9 (64.3)*	*1.14 (0.58-2.23)*	*1.57 (0.73-3.40)*
Surgery + adjuvant CHT	*5*	-	-	-	*1 (20.0)*	*0.26 (0.04-1.83)*	*0.32 (0.04-2.39)*
Warburg-high							
Surgery only	298	76 (25.5)	1.00 (ref)	1.00 (ref)	167 (56.0)	1.00 (ref)	1.00 (ref)
Surgery + adjuvant therapy	20	9 (45.0)	1.91 (0.95-3.80)	1.74 (0.76-3.96)	14 (70.0)	1.36 (0.79-2.34)	1.49 (0.79-2.78)
Surgery + adjuvant RT	*12*	*7 (58.3)*	*2.94 (1.35-6.41)*	*3.08 (1.16-8.21)*	*11 (91.7)*	*2.30 (1.25-4.24)*	*2.65 (1.25-5.61)*
Surgery + adjuvant CHT	*8*	*2 (25.0)*	*0.85 (0.21-3.48)*	*0.75 (0.17-3.27)*	*3 (37.5)*	*0.54 (0.17-1.70)*	*0.63 (0.19-2.03)*
TNM stage III							
Warburg-low							
Surgery only	103	45 (43.7)	1.00 (ref)	1.00 (ref)	69 (67.0)	1.00 (ref)	1.00 (ref)
Surgery + adjuvant therapy	49	31 (63.3)	1.33 (0.84-2.10)	1.72 (0.95-3.11)	34 (69.4)	0.96 (0.64-1.45)	1.07 (0.64-1.79)
Surgery + adjuvant RT	*11*	*8 (72.7)*	*1.61 (0.76-3.41)*	*1.96 (0.76-5.05)*	*8 (72.3)*	*1.05 (0.50-2.18)*	*1.31 (0.54-3.17)*
Surgery + adjuvant CHT	*38*	*23 (60.5)*	*1.25 (0.76-2.07)*	*1.67 (0.90-3.11)*	*26 (68.4)*	*0.94 (0.60-1.48)*	*1.02 (0.59-1.76)*
Warburg-moderate							
Surgery only	137	74 (54.0)	1.00 (ref)	1.00 (ref)	107 (78.1)	1.00 (ref)	1.00 (ref)
Surgery + adjuvant therapy	77	31 (40.3)	0.53 (0.35-0.80)	0.46 (0.29-0.75)	45 (58.4)	0.51 (0.36-0.72)	0.49 (0.33-0.73)

Supplementary Table S1 – Continued

	N	CRC-specific survival			Overall survival		
		CRC deaths (%)	HR (95% CI)		Deaths (%)	HR (95% CI)	
			Univariable	Multivariable-adjusted[a]		Univariable	Multivariable-adjusted[a]
Surgery + adjuvant RT	*15*	*8 (53.3)*	*0.82 (0.40-1.71)*	*1.01 (0.37-2.75)*	*12 (80.0)*	*0.85 (0.47-1.54)*	*1.36 (0.59-3.16)*
Surgery + adjuvant CHT	*62*	*23 (37.1)*	*0.47 (0.29-0.75)*	*0.40 (0.23-0.67)*	*33 (53.2)*	*0.44 (0.30-0.65)*	*0.39 (0.25-0.62)*
Warburg-high							
Surgery only	139	78 (56.1)	1.00 (ref)	1.00 (ref)	111 (79.9)	1.00 (ref)	1.00 (ref)
Surgery + adjuvant therapy	73	44 (60.3)	0.87 (0.60-1.25)	0.82 (0.54-1.25)	55 (75.3)	0.76 (0.55-1.05)	0.80 (0.55-1.16)
Surgery + adjuvant RT	*15*	*10 (66.7)*	*0.99 (0.51-1.92)*	*0.58 (0.24-1.39)*	*13 (86.7)*	*0.92 (0.52-1.63)*	*0.66 (0.31-1.39)*
Surgery + adjuvant CHT	*58*	*34 (58.6)*	*0.84 (0.56-1.25)*	*0.90 (0.57-1.41)*	*42 (72.4)*	*0.72 (0.51-1.03)*	*0.85 (0.57-1.27)*
TNM stage IV							
Warburg-low							
Surgery only	55	51 (92.7)	1.00 (ref)	1.00 (ref)	55 (100.0)	1.00 (ref)	1.00 (ref)
Surgery + adjuvant therapy	24	21 (87.5)	0.56 (0.34-0.94)	0.39 (0.20-0.80)	23 (95.8)	0.56 (0.34-0.92)	0.40 (0.21-0.79)
Surgery + adjuvant RT	*1*	*1 (100.0)*	*0.55 (0.08-4.00)*	*0.50 (0.06-4.51)*	*1 (100.0)*	*0.51 (0.07-3.71)*	*0.37 (0.04-3.17)*
Surgery + adjuvant CHT	*23*	*20 (87.0)*	*0.56 (0.33-0.95)*	*0.38 (0.18-0.82)*	*22 (95.7)*	*0.56 (0.34-0.93)*	*0.41 (0.20-0.85)*
Warburg-moderate							
Surgery only	90	83 (92.2)	1.00 (ref)	1.00 (ref)	90 (100.0)	1.00 (ref)	1.00 (ref)
Surgery + adjuvant therapy	27	25 (92.6)	0.58 (0.37-0.92)	0.38 (0.21-0.68)	26 (96.3)	0.56 (0.36-0.87)	0.36 (0.20-0.64)
Surgery + adjuvant RT	*2*	*2 (100.0)*	*0.74 (0.18-3.01)*	*0.38 (0.03-4.19)*	*2 (100.0)*	*0.70 (0.17-2.86)*	*0.48 (0.05-4.57)*
Surgery + adjuvant CHT	*25*	*23 (92.0)*	*0.57 (0.36-0.91)*	*0.38 (0.20-0.70)*	*24 (96.0)*	*0.55 (0.35-0.87)*	*0.36 (0.20-0.65)*
Warburg-high							
Surgery only	91	82 (90.0)	1.00 (ref)	1.00 (ref)	91 (100.0)	1.00 (ref)	1.00 (ref)
Surgery + adjuvant therapy	35	32 (91.4)	0.66 (0.44-1.01)	0.69 (0.40-1.18)	35 (100.0)	0.65 (0.43-0.96)	0.63 (0.38-1.05)
Surgery + adjuvant RT	-	-	-	-	-	-	-
Surgery + adjuvant CHT	*35*	*32 (91.4)*	*0.66 (0.44-1.01)*	*0.69 (0.40-1.18)*	*35 (100.0)*	*0.65 (0.43-0.96)*	*0.63 (0.38-1.05)*

[a]Adjusted for age at diagnosis (years), sex (male/female), tumor location (colon/rectosigmoid/rectum), differentiation grade (well/moderate/poor/undifferentiated/unknown), MMR deficiency (no/yes), year of diagnosis (per 3 years)

Supplementary Table S2 – Univariable and multivariable-adjusted hazard ratios for associations between adjuvant therapy (surgery, surgery plus radiotherapy, surgery plus chemotherapy) and CRC-specific and overall survival for the Warburg-subtypes (Warburg-low, Warburg-moderate, and Warburg-high) within the Netherlands Cohort Study (NLCS, 1986-2006), stratified on tumor location (colon/rectosigmoid/rectum).

	N	CRC-specific survival			Overall survival		
		CRC deaths (%)	HR (95% CI)		Deaths (%)	HR (95% CI)	
			Univariable	Multivariable-adjusted[a]		Univariable	Multivariable-adjusted[a]
Colon							
Warburg-low							
Surgery only	319	130 (40.8)	1.00 (ref)	1.00 (ref)	206 (64.6)	1.00 (ref)	1.00 (ref)
Surgery + adjuvant therapy	56	34 (60.7)	1.54 (1.05-2.25)	0.93 (0.60-1.45)	41 (73.2)	1.22 (0.87-1.71)	0.88 (0.60-1.30)
Surgery + adjuvant RT	*4*	*2 (50.0)*	*0.98 (0.24-3.98)*	*0.73 (0.17-3.08)*	*3 (75.0)*	*0.95 (0.30-2.97)*	*0.82 (0.25-2.65)*
Surgery + adjuvant CHT	*52*	*32 (61.5)*	*1.59 (1.08-2.35)*	*0.95 (0.60-1.49)*	*38 (73.1)*	*1.25 (0.88-1.77)*	*0.89 (0.60-1.32)*
Warburg-moderate							
Surgery only	429	203 (47.3)	1.00 (ref)	1.00 (ref)	299 (69.7)	1.00 (ref)	1.00 (ref)
Surgery + adjuvant therapy	82	43 (52.4)	0.96 (0.69-1.33)	0.54 (0.37-0.78)	55 (67.1)	0.83 (0.62-1.10)	0.52 (0.38-0.72)
Surgery + adjuvant RT	*4*	*3 (75.0)*	*1.55 (0.50-4.85)*	*3.57 (1.06-11.95)*	*4 (100.0)*	*1.49 (0.56-4.02)*	*2.65 (0.93-7.49)*
Surgery + adjuvant CHT	*78*	*40 (51.3)*	*0.93 (0.66-1.30)*	*0.49 (0.33-0.71)*	*51 (65.4)*	*0.80 (0.59-1.08)*	*0.47 (0.34-0.66)*
Warburg-high							
Surgery only	453	199 (43.9)	1.00 (ref)	1.00 (ref)	312 (68.9)	1.00 (ref)	1.00 (ref)
Surgery + adjuvant therapy	84	54 (64.3)	1.58 (1.17-2.14)	0.77 (0.55-1.08)	65 (77.4)	1.27 (0.97-1.66)	0.73 (0.54-0.99)
Surgery + adjuvant RT	*2*	*1 (50.0)*	*0.79 (0.11-5.64)*	*0.88 (0.12-6.47)*	*1 (50.0)*	*0.49 (0.07-3.45)*	*0.51 (0.07-3.72)*
Surgery + adjuvant CHT	*82*	*53 (64.6)*	*1.61 (1.19-2.19)*	*0.77 (0.54-1.08)*	*64 (78.0)*	*1.30 (0.99-1.70)*	*0.74 (0.54-1.00)*
Rectosigmoid							
Warburg-low							
Surgery only	35	10 (28.6)	1.00 (ref)	1.00 (ref)	25 (71.4)	1.00 (ref)	1.00 (ref)
Surgery + adjuvant therapy	13	8 (61.5)	2.55 (1.00-6.50)	2.98 (0.40-22.21)	9 (69.2)	1.20 (0.56-2.57)	1.36 (0.31-6.05)
Surgery + adjuvant RT	*3*	-	-	-	*1 (33.3)*	*0.34 (0.05-2.52)*	-
Surgery + adjuvant CHT	*10*	*8 (80.0)*	*3.95 (1.53-10.19)*	*4.12 (0.53-31.87)*	*8 (80.0)*	*1.76 (0.78-3.94)*	*2.15 (0.45-10.20)*
Warburg-moderate							
Surgery only	41	13 (31.7)	1.00 (ref)	1.00 (ref)	26 (63.4)	1.00 (ref)	1.00 (ref)

Supplementary Table S2 – Continued

	N	CRC-specific survival			Overall survival		
		CRC deaths (%)	HR (95% CI)		Deaths (%)	HR (95% CI)	
			Univariable	Multivariable-adjusted[a]		Univariable	Multivariable-adjusted[a]
Surgery + adjuvant therapy	15	7 (46.7)	1.44 (0.57-3.62)	0.71 (0.18-2.79)	10 (66.7)	1.08 (0.52-2.25)	0.97 (0.38-2.46)
Surgery + adjuvant RT	*5*	*3 (60.0)*	*2.26 (0.64-8.02)*	*3.17 (0.46-22.00)*	*5 (100.0)*	*2.21 (0.83-5.86)*	*2.67 (0.73-9.82)*
Surgery + adjuvant CHT	*10*	*4 (40.0)*	*1.14 (0.37-3.48)*	*0.33 (0.07-1.65)*	*5 (50.0)*	*0.72 (0.28-1.88)*	*0.45 (0.13-1.56)*
Warburg-high							
Surgery only	49	26 (53.1)	1.00 (ref)	1.00 (ref)	38 (77.6)	1.00 (ref)	1.00 (ref)
Surgery + adjuvant therapy	12	9 (75.0)	1.34 (0.62-2.89)	1.00 (0.39-2.52)	11 (91.7)	1.21 (0.61-2.39)	1.09 (0.48-2.45)
Surgery + adjuvant RT	*5*	*4 (80.0)*	*1.53 (0.53-4.42)*	*0.82 (0.15-4.32)*	*5 (100.0)*	*1.44 (0.56-3.72)*	*0.94 (0.22-4.01)*
Surgery + adjuvant CHT	*7*	*5 (71.4)*	*1.23 (0.47-3.21)*	*1.11 (0.34-3.54)*	*6 (85.7)*	*1.07 (0.45-2.54)*	*1.18 (0.43-3.25)*
Rectum							
Warburg-low							
Surgery only	41	19 (46.3)	1.00 (ref)	1.00 (ref)	24 (58.5)	1.00 (ref)	1.00 (ref)
Surgery + adjuvant therapy	21	15 (71.4)	1.55 (0.79-3.06)	1.90 (0.54-6.63)	15 (71.4)	1.25 (0.65-2.38)	1.89 (0.61-5.80)
Surgery + adjuvant RT	*16*	*11 (68.8)*	*1.58 (0.75-3.32)*	*2.86 (0.78-10.52)*	*11 (68.8)*	*1.25 (0.61-2.56)*	*2.60 (0.80-8.40)*
Surgery + adjuvant CHT	*5*	*4 (80.0)*	*1.49 (0.50-4.39)*	*0.52 (0.08-3.45)*	*4 (80.0)*	*1.24 (0.43-3.59)*	*0.79 (0.15-4.15)*
Warburg-moderate							
Surgery only	48	29 (60.4)	1.00 (ref)	1.00 (ref)	37 (77.1)	1.00 (ref)	1.00 (ref)
Surgery + adjuvant therapy	26	12 (46.2)	0.62 (0.32-1.22)	0.42 (0.14-1.25)	16 (61.5)	0.63 (0.35-1.14)	0.33 (0.12-0.87)
Surgery + adjuvant RT	*22*	*10 (45.5)*	*0.62 (0.30-1.27)*	*0.45 (0.14-1.41)*	*14 (63.6)*	*0.66 (0.36-1.23)*	*0.37 (0.13-1.03)*
Surgery + adjuvant CHT	*4*	*2 (50.0)*	*0.63 (0.15-2.63)*	*0.34 (0.06-1.97)*	*2 (50.0)*	*0.47 (0.11-1.96)*	*0.21 (0.04-1.12)*
Warburg-high							
Surgery only	36	15 (41.7)	1.00 (ref)	1.00 (ref)	25 (69.4)	1.00 (ref)	1.00 (ref)
Surgery + adjuvant therapy	33	22 (66.7)	1.78 (0.92-3.46)	0.83 (0.34-2.07)	29 (87.9)	1.45 (0.84-2.48)	1.13 (0.53-2.41)
Surgery + adjuvant RT	*21*	*12 (57.1)*	*1.36 (0.63-2.92)*	*0.72 (0.23-2.22)*	*19 (90.5)*	*1.34 (0.74-2.45)*	*1.42 (0.61-3.32)*
Surgery + adjuvant CHT	*12*	*10 (83.3)*	*2.88 (1.28-6.51)*	*1.00 (0.31-3.29)*	*10 (83.3)*	*1.69 (0.81-3.56)*	*0.73 (0.24-2.23)*

[a]Adjusted for age at diagnosis (years), sex (male/female), TNM stage (II, III, IV, unknown), differentiation grade (well/moderate/poor/undifferentiated/unknown), MMR deficiency (no/yes), year of diagnosis (per 3 years)

CHAPTER 7

General discussion

Despite advances in the early detection and treatment, colorectal cancer (CRC) remains the world's second most deadly cancer (more than 900,000 deaths in 2020)[1]. To date, the tumor-node-metastasis (TNM) staging system remains the most important clinically used factor to predict patient prognosis and guide treatment decisions in colorectal cancer (CRC)[2-5]. However, patients with the same disease stage may have different survival and response to adjuvant therapy most likely due to heterogeneity in patient or tumor characteristics[4-8]. In recent years, numerous biomarkers have been studied as tools to improve the prognostication of CRC patients and predict which patients are most likely to benefit from adjuvant therapy[9]. However, to date, only a limited number of biomarkers have been translated to clinical practice[6, 9]. Hence, there remains an urgent clinical need to identify novel prognostic and/or predictive (bio)marker(s) to improve the survival of CRC patients[6, 9, 10]. Prospective cohort studies, such as the Netherlands Cohort Study[11], are very suitable to explore the potential prognostic and/or predictive value of markers because of their high statistical power and their prospective design[12].

Previous research indicated that the expression of proteins related to the Warburg-effect may have prognostic value and may predict survival benefit from adjuvant therapy in CRC[13-20]. However, to date, the evidence is very limited and results are inconsistent. In this large population-based series of CRC patients (*n* = 2,394) originating from the prospective Netherlands Cohort Study (NLCS), our principal aim was to investigate whether Warburg-subtyping, based on the estimated presence of the Warburg-effect in tumor cells, had prognostic value in CRC and was able to predict survival benefit from adjuvant therapy. To this end, we have categorized patients into three Warburg-subtypes based on the immunohistochemical (IHC) expression of six glycolytic proteins and transcriptional regulators related to the Warburg-effect (LDHA, GLUT1, MCT4, PKM2, p53, PTEN).

In this chapter (**Chapter 7**), we will first provide a summary of the main findings of this thesis. Then, we will elaborate on our interpretation of the obtained results and put them further into perspective by relating them to previously published research. Next, the methodological considerations related to the research presented in this thesis are discussed. Finally, this chapter ends with our recommendations for future research and concluding remarks.

1. MAIN FINDINGS

In **Chapter 2**, we found that trained non-pathologists were able to generate reproducible IHC results that are similar to those of an experienced pathologist. A combination score of at least two non-pathologists was found to yield optimal results. In **Chapter 3**, we classified CRC patients in the NLCS into three "Warburg-subtypes" (i.e., "Warburg-low", "Warburg-

moderate", or "Warburg-high") and found that CRC patients with Warburg-high tumors had a worse CRC-specific and overall survival compared to patients with Warburg-low tumors, independent of known prognostic factors such as TNM stage. In **Chapter 4**, we found important survival differences across mutually exclusive mutational subgroups based on the observed mutational frequencies of *RAS*, *BRAF*, *PIK3CA*, and *MET*, as well as MMR status. In addition, we found that *BRAF*-mutated proficient mismatch repair (pMMR) CRC, *KRAS*-mutated pMMR CRC, and deficient (d)MMR CRC may be related to the Warburg-high subtype. No statistically significant survival differences were observed across Warburg-subtypes within these mutational subgroups. In **Chapter 5**, we found that of all studied long-term energy balance-related factors, only increased adult body mass index (BMI) was associated with a worse overall survival in the total series of CRC patients. Stratified analyses showed that associations with survival differed significantly according to our Warburg-subtypes for adult BMI, weight change since age 20 years, energy restriction during childhood and adolescence, and potentially adult-attained height. Weight gain since age 20 years and increased adult-attained height were associated with a poor prognosis only in patients with Warburg-high CRC, whereas increased adult BMI was associated with a poor prognosis only in patients with Warburg-moderate CRC. Lastly, in **Chapter 6** we found that only patients with Warburg-moderate and possibly Warburg-high CRC had survival benefit from adjuvant (chemo)therapy. In contrast, no survival benefit from adjuvant (chemo)therapy was observed for patients with Warburg-low CRC.

2. INTERPRETATION OF MAIN RESULTS

To be able to interpret the results presented in this thesis and to put them further into perspective, we compared the survival rate of patients diagnosed with CRC in the NLCS (*n* = 2,356) to that of patients diagnosed with CRC in the general Dutch population during the same time period (1986-2006)[21]. We found that the CRC-specific survival rates for our series of CRC patients were comparable to the survival rates of patients diagnosed with CRC in the general Dutch population (1981-1990: 59.3% versus 52%, 1991-2000: 60.4% versus 54%, and 2001-2010: 59.7% versus 60%[21]).

2.1. Warburg-subtypes

The expression of proteins related to the Warburg-effect may have prognostic value in CRC[13]. However, results remain inconsistent which may be due to the fact that previous research focused on investigating single proteins involved in the Warburg-effect, while this pathway is much more complicated. For this research, we therefore categorized CRC patients into Warburg-subtypes based on the expression of several proteins involved in different steps of the Warburg-effect pathway. These steps included: (1) upstream regulation of the Warburg-effect (PTEN, p53), (2) glucose import (GLUT1), (3) glycolysis

(PKM2), (4) increased lactate production (LDHA), and (5) lactate secretion (MCT4). We have done this using a comprehensive methodology that can be reproduced in future studies (also see methodological considerations paragraph below). Moreover, we investigated the reproducibility and validity of IHC assays and IHC scoring protocols (**Chapter 2**, also see paragraph on methodological considerations).

To the best of our knowledge, we were the first to categorize patients into metabolic Warburg-subtypes in CRC and to investigate their prognostic value in a large population-based series of CRC patients (**Chapter 3**). However, there have been some studies investigating the existence and prognostic value of metabolic subtypes in other cancer types. Assuming that our Warburg-high subtype represents a glycolytic subtype, our results were in line with previously published research in breast cancer[22], pancreatic ductal adenocarcinoma (PDAC)[23], and cutaneous melanoma[24]. In these studies, patients with tumors having a glycolytic phenotype had a poorer overall survival[22-24]. Furthermore, our results confirmed the findings of a meta-analysis by Yu et al.[13] who reported that glycolysis-related markers were associated with a poor survival in various cancer types, including CRC. In addition, in line with our results, a previous study by Zhu *et al.*[25] showed that a glycolysis-related risk score based on messenger RNA (mRNA) sequencing data from The Cancer Genome Atlas (TCGA) and the Gene Expression Omnibus (GEO) databases was associated with a poor prognosis in CRC.

Altogether, the results presented in **Chapter 3** of this thesis, in combination with the previously published literature, suggest that the presence of the Warburg-effect (i.e., glycolytic phenotype) is associated with a poor survival in CRC. A potential explanation by which the Warburg-effect is thought to contribute to a poor prognosis in cancer patients is the acidification of the tumor microenvironment, resulting from the increased lactate export by cancer cells[26, 27]. It has been suggested that this acidification of the tumor microenvironment may contribute to tumor invasion and metastasis, therapy resistance, and immunosuppression[26, 28, 29].

2.2. Mutational subgroups

Previous research has shown that metabolic reprogramming from oxidative phosphorylation (OXPHOS) towards the Warburg-effect is influenced by two major oncogenic signaling pathways: the *PI3K/AKT/mTOR* pathway and the *RAS/RAF/MEK/ERK* pathway[30-33]. Key genes involved in these pathways (*RAS*, *BRAF*, *PIK3CA* and *MET*) are frequently mutated in human cancers, including CRC[34-36]. In addition, MMR deficiency, a surrogate of microsatellite instability (MSI)[37], is a common molecular characteristic of CRC[38-40]. Mutations in *RAS*, *BRAF*, *PIK3CA* and *MET,* as well as MMR deficiency have been associated with the presence of the Warburg-effect[34-36, 38-41].

Many studies have investigated the prognostic value of MMR status, *KRAS*-, *BRAF*-, or *PIK3CA*-mutations in CRC. However, most studies did not evaluate these mutations exclusively (e.g. patients with *KRAS*-mutated or *KRAS* wild-type cancers may have had another mutation in a different gene)[42], which might have diluted the results. Moreover, studies assessing the prognostic value of CRC subgroups based on combinations of frequently occurring molecular characteristics (*RAS, BRAF, PIK3CA* and/or MMR status) are very limited and rarely evaluated all markers at the same time[43-46].

In **Chapter 4**, we found that, compared to patients with all-wild-type pMMR CRC, patients with *BRAF*-mutated pMMR CRC had the poorest survival. In contrast, no association with survival was observed for patients with *BRAF*-mutated dMMR CRC compared to patients with all-wild-type pMMR CRC (**Chapter 4**). These results indicate that dMMR 'overrides' the negative prognostic potential of *BRAF* mutations, which supports the findings of previous studies that reported that the adverse effect of mutant *BRAF* was limited to microsatellite stable (MSS) CRC[46-49]. Furthermore, we found that patients with *KRAS*-mutated or *KRAS* and *PIK3CA*-mutated pMMR had a poor prognosis compared to patients with all-wild-type pMMR CRC, whereas no significant association was observed for patients with *PIK3CA*-mutated pMMR cancers (**Chapter 4**). To date, the prognostic significance of *PIK3CA* mutations in CRC is uncertain as results of previous studies are inconsistent[45, 50, 51]. In contrast, previous research suggests that patients with *KRAS*-mutated pMMR CRC have a poor survival in CRC[43, 46, 52]. Our results confirm the findings of previous research, and suggest that *KRAS* mutations most likely drive the poor survival observed in the subgroup of patients with *KRAS* and *PIK3CA*-mutated pMMR CRC. Lastly, our results confirm that CRC patients with dMMR cancers have a better prognosis compared to patients with pMMR cancers[43] (**Chapter 4**).

Furthermore, our results suggested that patients with Warburg-high CRCs are more likely to have *BRAF* mutations, dMMR, or *KRAS* mutations in combination with pMMR (**Chapter 4**). In addition, rectal cancer patients with Warburg-high cancers were more likely to have *PIK3CA* mutations in combination with pMMR (**Chapter 4**). As described in the first part of this paragraph, this was in line with what was expected based on previous research with regard to molecular features driving the Warburg-effect[30-36, 38-41]. We did not find statistically significant survival differences across Warburg-subtypes within mutational subgroups (**Chapter 4**). This might be due to limited statistical power as a result of subclassifying patients on both mutational subgroups and Warburg-subtypes. Furthermore, we did not have enough power to stratify our analyses on tumor location or pathological (p)TNM stage, which may have concealed associations as Warburg-subtypes particularly had prognostic value in pTNM stage III CRC and cancers located in the rectum (Chapter 3). Nevertheless, our results suggest that the Warburg-high subtype may provide additional prognostic information in patient subgroups with *KRAS*-mutated pMMR CRCs or *BRAF*-

-mutated pMMR or dMMR CRCs (**Chapter 4**). It may therefore be of interest to further examine the additional prognostic value of Warburg-subtypes within these mutational subgroups in a larger prospective cohort study or pooled study.

All in all, the results presented in **Chapter 4** of this thesis, in combination with evidence from previously published studies, suggest that mutually exclusive subgroups based on mutations in *RAS* (*KRAS, NRAS, HRAS*), *BRAF*, *PIK3CA* and *MET,* as well as MMR status, have prognostic value in CRC. Furthermore, our results suggest that Warburg-subtyping may have additional prognostic value in patients with *KRAS* or *BRAF* mutations independent of known prognostic factors like TNM stage.

2.3. Long-term energy balance-related factors

Long-term energy balance-related factors, including increased adult BMI[53], weight gain since adolescence[54], and increased adult-attained height[55, 56] have been associated with an increased risk of developing CRC. In contrast, physical activity[57, 58] and energy restriction during childhood and adolescence[59, 60] have been associated with a decreased risk of developing CRC. However, the impact of these long-term energy balance-related factors on survival after CRC diagnosis remains to be clarified, as the number of studies to date is very limited.

In **Chapter 5**, we found that of all investigated long-term energy balance-related factors, only an increased adult BMI was associated with a poor prognosis in the total series of CRC patients. In line with our results, the majority of previous studies reported that pre-diagnostic obesity was associated with a significantly poorer survival in CRC[61-64], and found that every 5 kg/m^2 increase in pre-diagnostic BMI was associated with a higher risk of mortality[63, 65]. Contrary to our results (**Chapter 5**), the majority of previously published studies reported that pre-diagnostic non-occupational physical activity was associated with a more favourable prognosis in CRC[66-70]. With regard to pre-diagnostic weight change, our results (**Chapter 5**) confirmed those of a previous study who reported that adult weight change (per 5 kg increase) was associated with a marginal (non-significant) higher risk of overall mortality [65]. In addition, our results (**Chapter 5**) were in line with those of a previous study which reported that adult-attained height was not associated with survival in CRC patients[63]. To the best of our knowledge, we were the first to investigate the association between energy restriction during childhood and adolescence and survival in a large series of CRC patients, and therefore cannot compare our results to previously published studies. It has previously been proposed that associations between energy balance-related factors and survival in CRC may be modified by biomarkers related to the PI3K/AKT/mTOR pathway[71]. More specifically, it has been suggested that disruption of this pathway drives these associations, as the PI3K/AKT/mTOR pathway integrates signals from growth factors, nutrients, and hormones to induce cancer-related characteristics (e.g., cell proliferation,

resistance to apoptosis, autophagy, metabolic reprogramming). However, evidence that supports this hypothesis is currently lacking. In **Chapter 5**, we found that associations with survival for increased adult BMI, weight gain since age 20 years, and potentially adult-attained height, differed significantly according to Warburg-subtype. Increased adult BMI was associated with worse survival only in patients with Warburg-moderate CRC, whereas weight gain since age 20 years and potentially adult-attained height were associated with a worse survival only in patients with Warburg-high CRC. In addition, our results showed that associations between energy restriction proxies (i.e., place of residence during the Dutch Hunger Winter (1944-1945) or World War II (1940-1945), and father's employment status during the Dutch Economic Depression (1932-1940)) and survival differed according to Warburg-subtype. However, inconsistent associations with survival were observed for the three energy restriction proxies according to Warburg-subtype. Hence, we refrain from drawing any conclusions. The association between non-occupational physical activity and survival did not differ according to Warburg-subtype.

All in all, the results presented in **Chapter 5** of this thesis corroborate previous findings that maintaining a healthy pre-diagnostic BMI may be beneficial for survival after CRC diagnosis. Moreover, our results suggest that associations between adult BMI, weight gain since age 20 years, energy restriction during childhood or adolescence, and potentially adult-attained height and survival after CRC diagnosis differ according to Warburg-subtype.

2.4. Warburg-subtypes and survival after adjuvant therapy

Many *in vitro* studies have investigated the relationship between cellular metabolism and therapy resistance in CRC[15]. The majority of these studies showed that the Warburg-effect promotes tumor characteristics that contribute to therapy resistance (e.g., increased drug efflux and DNA damage repair, metabolic inactivation of drugs, epigenetic alterations, mutations in drug targets, activation of survival pathways, evasion of apoptosis)[14-20, 72]. To the best of our knowledge, we were the first to prospectively investigate whether Warburg-subtyping can be used as biomarker to predict survival benefit from adjuvant therapy in a large population-based series of CRC patients. We found that while in general patients with stage II-IV CRC who received adjuvant (chemo)therapy had a significantly favourable CRC-specific and overall survival compared to patients who received surgery only, this benefit was only observed in patients with Warburg-moderate and possibly Warburg-high CRC. No survival benefit from adjuvant (chemo)therapy was observed for patients with Warburg-low CRC.

These results were not in line with what we would have expected based on the results of previously published studies. This might be explained by the fact that previous evidence was mostly based on *in vitro* cell culture studies[19] (i.e., it has been described that *in vitro* conditions differ drastically from *in vivo* conditions[73-75]) or (retrospective) case-control

studies[20] (i.e., this study design is more prone to various biases, including selection bias and confounding bias). However, as mentioned in **Chapter 6**, a potential explanation for the observation that patients with Warburg-low CRC showed no survival benefit from adjuvant (chemo)therapy has been described by Vellinga *et al.*[76]. Usually, the amount of adenosine 5'-triphosphate (ATP) that is generated by aerobic glycolysis is sufficient to support tumour cell growth and DNA repair[76, 77]. When adjuvant (chemo)therapy is administered, however, the cellular ATP demand increases tremendously as many proteins involved in DNA repair, drug efflux, and drug detoxification are dependent on ATP to exert their function[76, 77]. As OXPHOS is more efficient in terms of ATP generation, cancer cells may switch their metabolism from aerobic glycolysis towards OXPHOS[76]. Applied to our results, this may suggest that patients with Warburg-low CRC (i.e., tumours with a more oxidative metabolism) are more capable of repairing DNA damage and regulating drug efflux and metabolism compared to patients with Warburg-moderate or Warburg-high CRC (i.e., tumours with a more glycolytic metabolism), hence making them more resistant to adjuvant (chemo)therapy.

Altogether, the results presented in **Chapter 6** of this thesis suggest that Warburg-subtyping may be used to predict survival benefit from adjuvant (chemo)therapy in CRC patients. Patients with Warburg-low CRC had no survival benefit from adjuvant (chemo) therapy, whereas patients with Warburg-moderate and possibly Warburg-high CRC did show survival benefit from adjuvant (chemo)therapy.

2.5. Warburg-subtypes and other classification systems

Several other classification systems for CRC have been described in the past few years[78-83]. Recently, the CRC Subtyping Consortium (CRCSC) was established, aiming to elucidate potential overlaps between these existing classification systems and to develop a more unified taxonomy[84, 85]. Using a network-based meta-analysis of the six existing subtyping systems, four consensus molecular subtypes (CMS) with distinguishing features were identified[84, 85]. These four CMS subtypes included the CMS1-MSI immune subtype (characterized by hypermutation, MSI, CpG island methylator phenotype (CIMP), immune activation and *BRAF* mutations), CMS2-canonical subtype (characterized by epithelial features, activated WNT and c-Myc signaling pathways, chromosomal instability (CIN), and MSS), CMS3-metabolic subtype (characterized by deregulation of metabolic pathways, *KRAS* mutations, and mixed CIN-MSI status), and the CMS4-mesenchymal subtype (characterized by upregulation of epithelial-to-mesenchymal transition (EMT), transforming growth factor (TGF)-β activation, MSS, CIN, angiogenesis, and stromal invasion)[84-86]. In spite of the early enthusiasm about this CMS system, however, translation to clinical practice has thus far been challenging as a result of several major challenges[84]. These challenges include: (1) the CMS classification is impractical in the clinical setting (as a result of the high costs, turnaround time, and reliance on bioinformatic expertise associated with gene

expression profiles), (2) the accuracy of the CMS classification on FFPE samples remains questionable as it was developed using fresh frozen samples, (3) there is still a lack of biomarkers predictive of CMS that can be comprehensible and conducted by pathologists and clinicians[84]. Although the latter challenge has been addressed in two recent studies which aimed to develop a clinically practical IHC-based CMS classifier consisting of four markers, concordance between the IHC and RNA-sequencing based CMS classifiers was still suboptimal (71.4%)[87, 88]. In addition to these well-known CMS subtypes, other subtypes of CRC have been described, including, but not limited to, subtypes based on the three major molecular pathways involved in the development of CRC (i.e., CIN, CIMP, MSI)[89, 90] or clustering analyses[78, 83]. However, a definitive and comprehensive subtype classification for CRC has not been implemented in clinical practice to date.

As can be seen from the characteristics of the four CMS subtypes provided above, our Warburg-high subtype would most likely overlap with the CMS3-metabolic subtype[84-86]. However, the Warburg-high subtype was also found to be related to dMMR cancers (**Chapter 2 and 3**)[38, 91] and *BRAF*-mutated cancers (**Chapter 3**)[91], and therefore also shows some overlap with characteristics of the CMS1-MSI immune subtype[84-86]. Unfortunately, at this time we do not have sufficient information on characteristics related to the CMS subtypes available for (a subset of) our patient population. However, we believe that it would certainly be of interest to investigate this in the future as integrating our IHC-based Warburg-subtypes into the existing CMS classification may improve the overall accuracy of prognostication in CRC.

3. METHODOLOGICAL CONSIDERATIONS

The results presented in this thesis are based on a combination of observational and molecular data. As a result, they may be subject to various sources of bias, which may have influenced the validity of our findings. In the following sections, several methodological considerations and their consequences for the interpretation of our results are discussed.

3.1. The Netherlands Cohort Study

All results presented in this thesis are based on observational data from the NLCS. The NLCS has several strengths, including its large population-based prospective cohort design with coverage throughout the Netherlands, its long-term and nearly complete (≥95%) follow-up for cancer incidence, the availability of tumor material for a large number of CRC patients, the large amount of information on cancer risk factors and potential confounders, and the long-term mortality follow-up. All in all, the design of this cohort study greatly reduces the risk of information and selection bias. Furthermore, the large majority (>80%) of CRC patients in the NLCS have only been treated with surgery and did

not receive adjuvant therapy, thereby limiting the risk of confounding by indication (i.e., the choice of adjuvant therapy might be associated with assumed prognosis, which might be associated with the prognostic subgroups that we are studying).

The large number of participants included in the NLCS in combination with its long follow-up resulted in a large population-based series of CRC patients, which enabled us to study the prognosis of CRC patients in metabolic "Warburg-subtypes" (**Chapter 3-5**). In addition to this, the NLCS offers the unique opportunity to study the relationship between early-life energy restriction and CRC prognosis (**Chapter 5**), as participants were children or adolescents during World War II (1940-1944), the Dutch Hunger winter (1944-1945) and the Dutch Economic Depression (1932-1940).

A methodological consideration related to the design of the NLCS that should be taken into account is that information on long-term energy balance-related factors was obtained from a self-administered questionnaire at baseline. This may have influenced the results presented in **Chapter 5** of this thesis in several ways. First, misclassification of exposure data may have occurred as the baseline questionnaire was self-reported. As the NLCS has a prospective cohort design, it is unlikely that the degree of misclassification is different among participants who developed CRC and those who did not (i.e., differential misclassification)[92]. Nevertheless, non-differential misclassification may have occurred. However, the associations with survival for pre-diagnostic BMI and adult-attained height reported in this thesis were similar to those of studies in which these exposures were assessed by non-self-reported measures[61-63, 93]. Second, exposure data was only measured at baseline and no repeated measurements were available. During the long time that passed between exposure measurement at baseline and CRC diagnosis changes in exposures may have occurred. This may have resulted in underestimation of associations because of misclassification bias. However, it is most likely that participants of the NLCS had relatively stable (dietary) habits because of their older age at baseline[11]. Third, CRC patients may have changed their overall lifestyle after CRC diagnosis, which may have biased our results. However, it has previously been reported that CRC patients only marginally change their overall lifestyle following diagnosis[94]. Furthermore, the associations we observed for pre-diagnostic BMI, pre-diagnostic weight gain, and adult-attained height were comparable to those reported by previous studies[61-65].

3.2. Tissue MicroArrays and Immunohistochemistry

The Tissue MicroArray (TMA) technology was introduced in 1998 by Kononen et al.[95], thereby enabling high-throughput analysis of cancer biomarkers in large-scale patient cohorts using formalin-fixed paraffin embedded (FFPE) tissue blocks[96]. The TMA technology has many advantages over whole tissue slides. First, IHC on TMAs is more time-efficient and cost-effective compared to IHC on whole tissue sections[97-102]. Second, the TMA technology

ensures a higher level of assay standardization as all tissue cores assembled on a TMA are exposed to the same experimental conditions during, for example, IHC[97, 99, 100, 102-104]. Third, using TMAs preserves tissue resources and maximizes the number of IHC stainings or other experiments that can be performed with the material present in one paraffin block[97, 99, 100].

The TMA technology also has some limitations. Concerns have been raised about the representativeness of TMAs for the whole tissue specimen[97]. However, it has been shown previously that IHC results obtained from TMA sections and whole tumor sections have a high degree of concordance when three 0.6-mm tissue cores per patient were sampled[105-109], as was done for the present study. Another methodological consideration is that, due to their small size, TMA cores are more prone to tissue loss during processing compared to whole tissue sections[97]. To substantially reduce the total number of 'lost patients' in the present study, three tumor tissue cores per patient were sampled during TMA construction, as suggested previously[97]. An additional concern with regard to TMAs is that FFPE tissue blocks from different time periods and different hospitals are assembled on the same TMA block[97]. It has been described that differences in fixation protocols and/or storage time may affect the quality of IHC staining protocols[97]. In the present study, we therefore used internal controls whenever possible as a control measure to assure the quality of IHC stainings. In addition, we have ensured sufficient quality and specificity of the IHC stainings by optimizing (existing) staining protocols on multi-tissue paraffin blocks (i.e., paraffin blocks containing tissue sections from different organs) and spare TMA sections containing tumor cores as well as normal epithelium from CRC patients.

Another methodological consideration with regard to IHC in general is the validity and quality of the used antibodies. For use in an IHC assay, the antibody must be sensitive and specific to the target antigen[110]. Preferably, monoclonal antibodies should be selected as they are highly specific and consistent between experiments[110]. However, for an average scientist or pathologist, a good monoclonal antibody for IHC assays can be hard to find, as there are many antibodies available (both commercially and research-developed) that target the same antigen[110]. In 2005, the first version of the Human Protein Atlas portal (www.proteinatlas.org) was released, containing 275 internally generated antibodies and 443 commercially available antibodies[111, 112]. Currently, the Human Protein Atlas covers 15,323 genes (i.e., 76% of all protein-coding genes) for which antibodies are available[112, 113]. In the present study, we have used optimized and validated IHC assays that are currently used in routine clinical practice at the Department of Pathology at Maastricht University Medical Centre (MUMC+, Maastricht) whenever possible. When no validated IHC assay was available, we have considered the Human Protein Atlas or previously published literature to search for potential antibodies (preferably monoclonal antibodies). As described above, protocols for these antibodies were then optimized and validated using multi-tissue FFPE blocks and spare TMA sections containing tumor as well as normal tissue cores from CRC

patients. Unfortunately, after several attempts, we have not been able to find antibodies of sufficient quality for HIF-1α, c-Myc, and PDK1.

Lastly, the subjectivity of IHC scoring should be considered[114, 115]. IHC scoring is very prone to substantial inter- and intra-observer variability[115]. Hence, it has been recommended previously that IHC scoring should be performed by multiple assessors to improve reproducibility[116]. In the present study, we therefore ensured that all tumor tissue cores were scored independently by at least two trained non-pathologist assessors (**Chapter 2**). Furthermore, the quality of the generated IHC scoring results was investigated by assessing the inter-observer agreement between the scores generated by the trained non-pathologists and the scores generated by an experienced pathologist (**Chapter 2**).

3.3. Warburg-subtypes

For the present study, CRC patients were categorized into Warburg-subtypes using a pathway-based sum score of six proteins indicative of the presence of the Warburg-effect (i.e., LDHA, GLUT1, MCT4, PKM2, p53, PTEN; **Chapter 3-6**). In this section, the methodological considerations with regard to defining Warburg-subtypes are discussed.

First, the six proteins that we considered in the present study only represent a small selection of proteins known to be involved in the Warburg-effect. Still, we believe that our defined Warburg-subtypes give a good indication of the presence of the Warburg-effect, as we ensured that proteins involved in the different levels of the Warburg-effect pathway (i.e., upstream regulators, glucose import, glycolysis, and lactate secretion) were included. Nevertheless, we could have added more proteins to our sum score, including the well-known regulators of the Warburg-effect hypoxia-inducible factor (HIF)-1α, c-Myc, and pyruvate dehydrogenase kinase 1 (PDK1)[117, 118]. These three proteins were originally planned to be included in our Warburg-subtypes, but unfortunately the quality of the IHC stainings did not meet our standards. Furthermore, capturing the complete Warburg-effect pathway was nearly impossible considering the time and budgetary constraints of this project.

Second, there are several ways that could be considered to combine the obtained core-level IHC data into patient-level data, which may have affected our results. Based on previous literature, potential possibilities to combine core-level data into patient-level data include (1) taking the highest or the lowest score (depending on the protein under investigation)[119-121] or (2) taking the average score of all available cores[122-125]. After carefully considering both options, we have chosen for the 'average score'-approach because we believed that this was the best way to account for the potential intra-tumoral heterogeneity that would be considered when assessing whole tissue sections.

Third, defining subtypes for patient stratification implies the introduction of cut-offs[126]. The choice of these cut-offs is not straightforward, and may eventually influence the obtained results[126]. For this project, we have chosen a two-step approach, in which we first generated a (unweighted) pathway-based sum score and then categorized patients into three subtypes based on predefined cut-off values. To generate these cut-off values, we have considered two options. The first option encompassed a more restricted 'Warburg-low' and 'Warburg-high' category, and was therefore statistically less efficient. The second option encompassed more equally-sized patient subgroups (i.e., ~30% of patients per subtype) but was less restricted, which enhanced the statistical efficiency but might result in attenuation of associations in analyses (i.e., there may be patients included in the Warburg-high subtype that are not showing high expression for the majority of proteins). For this study, we have chosen for the second option as this would provide us with the greatest statistical efficiency and allowed us to perform stratified analyses if necessary. Choosing this option may, as described above, have resulted in attenuation of associations in our analyses.

3.4. Mutational subgroups

Next to our IHC-based Warburg-subtypes, we also categorized patients into mutational subgroups based on mutations in genes that have been associated with the Warburg-effect (*RAS, BRAF*, *PIK3CA*, *MET*) as well as MMR status (**Chapter 4**). Several methodological considerations should be taken into account here, especially with regard to measurement error and misclassification.

Mutational status of CRC patients was assessed using the commercially available ColoCarta panel (Agena Bioscience, Hamburg)[127]. The ColoCarta panel does not include all regulators of the Warburg-effect, such as *mTOR* or *AKT1*[128]. However, due to budgetary constraints as a result of the large number of CRC patients included in this study, the ColoCarta panel was our best available option. The ColoCarta panel is a validated panel that contains assays for mutations in the most clinically relevant CRC genes (i.e., 99%, 98% and 78% of all known mutations in *KRAS, BRAF* or *PIK3CA*, respectively)[127] and, in addition, contains assays for other mutations in genes that intersect with the same pathways (e.g., *HRAS, NRAS,* and *MET*)[127]. The frequencies of the measured mutations within our cohort were in line with previous literature[127, 129, 130] and the Catalogue of Somatic Mutations in Cancer (COSMIC) database[131, 132].

MMR status was determined using IHC for mutL homolog 1 (MLH1) and mutS homolog 2 (MSH2) as a proxy for microsatellite instability (MSI). This may have resulted in misclassification of some CRC patients, as we did not stain for the MMR proteins mutS homolog 6 (MSH6) and PMS1 homolog 2 (PMS2). However, it has been described previously that IHC analysis of MLH1 and MSH2 expression is a reliable method for the detection of the vast majority of patients with MSI CRC[133]. In addition, the frequency of MMR deficiency in our cohort was in line with what was reported in previous literature[134, 135].

4. RECOMMENDATIONS FOR FUTURE RESEARCH

The results presented in this thesis indicate that Warburg-subtypes have prognostic value in CRC, modify the association between long-term energy balance-related factors and survival, and may potentially be used to predict survival after adjuvant therapy. As we were the first to investigate this in a large population-based series of CRC patients, future large(r)-scale prospective studies or pooled studies are required to validate our findings. In addition to replicating the current results, we propose several other directions for future research.

First, it would be of interest to investigate the exact biological mechanisms that are responsible for the differences in survival we observed across Warburg-subtypes. Potential suggestions for future research are to further investigate the relationship between the Warburg-effect, acidification of the tumor microenvironment, and tumor invasion, metastasis, and tumor immunity[26-29, 136]. The exact biological mechanism(s) through which the Warburg-effect, and subsequent acidification of the microenvironment, influences cancer cell invasion, migration, metastasis and the functioning of immune cells, may be further investigated in animal models or *in vitro* models. In addition, it may be of interest to examine the association between our Warburg-subtypes and anti-tumor immune response in patient tissue samples, as it has been suggested that increased tumor glycolytic metabolism is related to decreased T-cell infiltration and activity[137].

Furthermore, as our results indicate that our comprehensive way of Warburg-subtyping has prognostic value in CRC, it may be of interest to investigate its prognostic value in other cancer types. For example, the Warburg-effect has been observed in a variety of cancer cells, including hepatic cancer, pancreatic cancer, breast cancer, esophageal cancer, brain cancer, renal cancer, lung cancer, melanoma, pancreatic cancer, endometrial cancer, ovarian cancer, and cervical cancer[138, 139].

In addition, we recommend considering other ways to estimate the presence of the Warburg-effect, as our defined Warburg-subtypes do not include all proteins known to be involved in the Warburg-effect. For example, one might consider adding other important proteins that we did not or could not measure in the present study, such as c-Myc, HIF-1α, or PDK1[117, 118]. Furthermore, other techniques may be considered in order to measure the presence of the Warburg-effect in tumor samples, such as (multi-)omics techniques[140]. However, the translation of omics techniques to clinical practice has been challenging, due to several significant barriers, including the lack of clinician knowledge and ethical considerations (e.g. overdiagnosis)[141]. Therefore, we believe that our Warburg-subtyping may be easier to translate to a clinical setting, as this is a comprehensive and transparent way of subtyping based on IHC (a technique that is already widely used in clinical practice).

In addition, we believe that it would be worthwhile to investigate less invasive techniques, such as plasma metabolites and proteins derived from the Warburg-effect (e.g. glucose, lactate, GLUT1) or fluorodeoxyglucose (FDG)-uptake by primary tumors, as those may be used for both prognosis and diagnosis of CRC[142].

Moreover, as it takes a considerable amount of time to score all IHC-stained sections to define Warburg-subtypes we believe that it would be of great interest to consider the application of artificial intelligence. For example, it has been shown previously that MSI status can be predicted from hematoxylin and eosin (H&E)-stained tissue sections using artificial intelligence[143,144]. We believe that it would be of interest to explore whether artificial intelligence can be used to predict the presence of the IHC-based Warburg-effect or the mutational status of *RAS, BRAF*, or *PIK3CA* from H&E-stained tissue sections.

Also, we recommend that future research investigates whether our Warburg-subtyping adds prognostic or predictive information beyond that of other promising newly developed markers, such as tumor budding[145], immunoscore®[146], tumor/stroma ratio[147], neutrophil/lymphocyte ratio[148], and CMS[85]. It may therefore be of interest to study the prognostic value of Warburg-subtyping in a large prospective cohort of CRC patients, while adjusting for TNM stage as well as for these 'new' biomarkers.

Besides, we recommend that future research investigates why patients with Warburg-low CRC show no survival benefit from adjuvant chemotherapy. Future research may investigate whether patients with Warburg-low CRC (i.e., patients with cancers that rely mainly on oxidative metabolism) are more capable of repairing DNA damage and regulating drug metabolism compared to patients with Warburg-moderate and Warburg-high CRC (i.e., patients with cancers that rely on aerobic glycolysis). As our results did not match the results obtained in the majority of *in vitro* studies, we suggest that this should preferably be investigated in a prospective study that includes more CRC patients that received adjuvant chemo- or radiotherapy, to increase power. These future studies may therefore also consider to measure OXPHOS metabolism, preferably in a similar way as was used to define our Warburg-subtypes (using IHC for OXPHOS-related markers, such as complex I-V or porin[149]) for comprehensiveness and reproducibility. Furthermore, it could be considered to include pre-treatment and post-treatment tissue samples of (a sample of) CRC patients to examine the changes in cellular metabolism after administration of adjuvant therapy.

Lastly, as mentioned before, the Warburg-high subtype shows similar characteristics as the CMS1-MSI subtype and CMS3-metabolic subtype[84-86]. Therefore, we believe that it would be of interest to investigate the relationship between our Warburg-subtypes and the previously described CMS subtypes[84-86] as integrating our IHC-based Warburg-subtypes

into the existing CMS classification may improve the overall accuracy of prognostication in CRC.

5. CONCLUDING REMARKS

In this thesis, we aimed to investigate whether Warburg-subtyping, based on the estimated presence of the Warburg-effect in tumor cells, has prognostic value in CRC and is able to predict survival benefit from adjuvant therapy. To this end, we classified 2,394 CRC patients in the Netherlands Cohort Study (NLCS) into Warburg-subtypes, based on the IHC expression of six glycolytic proteins and transcriptional regulators related to the Warburg-effect (LDHA, GLUT1, MCT4, PKM2, p53, PTEN). Overall, our results indicate that our defined Warburg-subtypes can be used for prognostication and to predict survival benefit from adjuvant (chemo)therapy in CRC patients, independent of known prognostic and predictive factors such as TNM stage. More specifically, we found that the presence of the Warburg-effect (i.e., Warburg-high CRC; a more glycolytic phenotype) is associated with poor survival in CRC patients, whereas the absence of the Warburg-effect (i.e., Warburg-low CRC; a more oxidative phenotype) is associated with a poor survival benefit from adjuvant (chemo) therapy. In addition, we found that mutational subgroups based on molecular features that have been related to the Warburg-effect (i.e., *RAS*, *BRAF*, *PIK3CA*, and MMR status) have prognostic value in CRC, and that Warburg-subtypes may potentially provide additional prognostic information within these mutational subgroups. Furthermore, our results indicate that associations between long-term energy balance-related factors and survival in CRC may differ according to Warburg-subtype. As we were the first to categorize a large population-based series of CRC patients into metabolic subtypes and to investigate their prognostic and predictive value, validation in large(r)-scale prospective studies is required.

REFERENCES

1. Ferlay J, Ervik M, Lam F, et al. Global Cancer Observatory: Cancer Today. Volume 2021: Lyon, France: International Agency for Research on Cancer, 2020.
2. Wei XL, Wang DS, Xi SY, et al. Clinicopathologic and prognostic relevance of ARID1A protein loss in colorectal cancer. World J Gastroenterol 2014;20:18404-12.
3. Mo S, Zhou Z, Li Y, et al. Establishment and validation of a novel nomogram incorporating clinicopathological parameters into the TNM staging system to predict prognosis for stage II colorectal cancer. Cancer Cell Int 2020;20:285.
4. Roelands J, Kuppen PJ, Vermeulen L, et al. Immunogenomic classification of colorectal cancer and therapeutic implications. International journal of molecular sciences 2017;18:2229.
5. Kawakami H, Zaanan A, Sinicrope FA. Microsatellite instability testing and its role in the management of colorectal cancer. Current treatment options in oncology 2015;16:1-15.
6. Ji D, Zhan T, Li M, et al. Enhancement of sensitivity to chemo/radiation therapy by using miR-15b against DCLK1 in colorectal cancer. Stem cell reports 2018;11:1506-1522.
7. Zhai Z, Yu X, Yang B, et al. Colorectal cancer heterogeneity and targeted therapy: clinical implications, challenges and solutions for treatment resistance, In Seminars in cell & developmental biology, Elsevier, 2017.
8. Sinicrope FA, Okamoto K, Kasi PM, et al. Molecular biomarkers in the personalized treatment of colorectal cancer. Clinical Gastroenterology and Hepatology 2016;14:651-658.
9. Ogunwobi OO, Mahmood F, Akingboye A. Biomarkers in colorectal cancer: current research and future prospects. International Journal of Molecular Sciences 2020;21:5311.
10. Ten Hoorn S, de Back TR, Sommeijer DW, et al. Clinical value of consensus molecular subtypes in colorectal cancer: a systematic review and meta-analysis. JNCI: Journal of the National Cancer Institute 2022;114:503-516.
11. van den Brandt PA, Goldbohm RA, van 't Veer P, et al. A large-scale prospective cohort study on diet and cancer in The Netherlands. J Clin Epidemiol 1990;43:285-95.
12. Bosch RJ, Zhang X, Sandler NG. Study design issues in evaluating immune biomarkers. Current Opinion in HIV and AIDS 2013;8:147.
13. Yu M, Chen S, Hong W, et al. Prognostic role of glycolysis for cancer outcome: evidence from 86 studies. J Cancer Res Clin Oncol 2019;145:967-999.
14. Dong S, Liang S, Cheng Z, et al. ROS/PI3K/Akt and Wnt/β-catenin signalings activate HIF-1α-induced metabolic reprogramming to impart 5-fluorouracil resistance in colorectal cancer. Journal of Experimental & Clinical Cancer Research 2022;41:1-27.
15. Liu C, Jin Y, Fan Z. The Mechanism of Warburg Effect-Induced Chemoresistance in Cancer. Frontiers in oncology 2021:3408.
16. Zaal EA, Berkers CR. The influence of metabolism on drug response in cancer. Frontiers in oncology 2018;8:500.
17. Desbats MA, Giacomini I, Prayer-Galetti T, et al. Metabolic plasticity in chemotherapy resistance. Frontiers in oncology 2020;10:281.
18. Zhong J-T, Zhou S-H. Warburg effect, hexokinase-II, and radioresistance of laryngeal carcinoma. Oncotarget 2017;8:14133.

19. Morandi A, Indraccolo S. Linking metabolic reprogramming to therapy resistance in cancer. Biochimica et Biophysica Acta (BBA)-Reviews on Cancer 2017;1868:1-6.
20. Kitazawa M, Hatta T, Sasaki Y, et al. Promotion of the Warburg effect is associated with poor benefit from adjuvant chemotherapy in colorectal cancer. Cancer science 2020;111:658.
21. (IKNL) IkN. NKR Cijfers. Volume 2022: IKNL, 2022.
22. Choi J, Jung WH, Koo JS. Metabolic interaction between cancer cells and stromal cells according to breast cancer molecular subtype. Breast cancer research 2013;15:1-20.
23. Karasinska JM, Topham JT, Kalloger SE, et al. Altered gene expression along the glycolysis–cholesterol synthesis axis is associated with outcome in pancreatic cancer. Clinical Cancer Research 2020;26:135-146.
24. Zhang L, Chen Y, Wang Y, et al. A Novel Glycolysis-Related Gene Signature Predicts Prognosis For Cutaneous Melanoma. Combinatorial chemistry & high throughput screening.
25. Zhu J, Wang S, Bai H, et al. Identification of Five Glycolysis-Related Gene Signature and Risk Score Model for Colorectal Cancer. Frontiers in Oncology 2021;11:211.
26. Spencer NY, Stanton RC. The Warburg effect, lactate, and nearly a century of trying to cure cancer, In Seminars in nephrology, Elsevier, 2019.
27. Jiang B. Aerobic glycolysis and high level of lactate in cancer metabolism and microenvironment. Genes & diseases 2017;4:25-27.
28. Schuurbiers OC, Kaanders JH, van der Heijden HF, et al. The PI3-K/AKT-pathway and radiation resistance mechanisms in non-small cell lung cancer. J Thorac Oncol 2009;4:761-7.
29. Peppicelli S, Bianchini F, Calorini L. Extracellular acidity, a "reappreciated" trait of tumor environment driving malignancy: perspectives in diagnosis and therapy. Cancer and Metastasis Reviews 2014;33:823-832.
30. Hutton JE, Wang X, Zimmerman LJ, et al. Oncogenic KRAS and BRAF drive metabolic reprogramming in colorectal cancer. Molecular & cellular proteomics 2016;15:2924-2938.
31. Liu M, Liu W, Qin Y, et al. Regulation of metabolic reprogramming by tumor suppressor genes in pancreatic cancer. Experimental Hematology & Oncology 2020;9:1-16.
32. van Geel RM, Beijnen JH, Bernards R, et al. Treatment individualization in colorectal cancer. Current colorectal cancer reports 2015;11:335-344.
33. Haluska F, Pemberton T, Ibrahim N, et al. The RTK/RAS/BRAF/PI3K pathways in melanoma: biology, small molecule inhibitors, and potential applications, In Seminars in oncology, Elsevier, 2007.
34. Papa S, Choy PM, Bubici C. The ERK and JNK pathways in the regulation of metabolic reprogramming. Oncogene 2019;38:2223-2240.
35. Kang S, Bader AG, Vogt PK. Phosphatidylinositol 3-kinase mutations identified in human cancer are oncogenic. Proceedings of the National Academy of Sciences 2005;102:802-807.
36. Feron O. Pyruvate into lactate and back: from the Warburg effect to symbiotic energy fuel exchange in cancer cells. Radiotherapy and oncology 2009;92:329-333.
37. Yurgelun MB, Hampel H. Recent Advances in Lynch Syndrome: Diagnosis, Treatment, and Cancer Prevention. Am Soc Clin Oncol Educ Book 2018;38:101-109.
38. Offermans K, Jenniskens JC, Simons CC, et al. Expression of proteins associated with the Warburg-effect and survival in colorectal cancer. The Journal of Pathology: Clinical Research 2021.
39. Boland CR, Goel A. Microsatellite instability in colorectal cancer. Gastroenterology 2010;138:2073-2087. e3.

40. Vasaikar S, Huang C, Wang X, et al. Proteogenomic analysis of human colon cancer reveals new therapeutic opportunities. Cell 2019;177:1035-1049. e19.
41. Chandel V, Raj S, Choudhari R, et al. Role of c-Met/HGF Axis in Altered Cancer Metabolism. Cancer Cell Metabolism: A Potential Target for Cancer Therapy: Springer, 2020:89-102.
42. De Roock W, Claes B, Bernasconi D, et al. Effects of KRAS, BRAF, NRAS, and PIK3CA mutations on the efficacy of cetuximab plus chemotherapy in chemotherapy-refractory metastatic colorectal cancer: a retrospective consortium analysis. The lancet oncology 2010;11:753-762.
43. Phipps AI, Limburg PJ, Baron JA, et al. Association between molecular subtypes of colorectal cancer and patient survival. Gastroenterology 2015;148:77-87 e2.
44. Zlobec I, Bihl MP, Foerster A, et al. Stratification and prognostic relevance of Jass's molecular classification of colorectal cancer. Frontiers in oncology 2012;2:7.
45. Luo Q, Chen D, Fan X, et al. KRAS and PIK3CA bi-mutations predict a poor prognosis in colorectal cancer patients: A single-site report. Translational oncology 2020;13:100874.
46. Alwers E, Jia M, Kloor M, et al. Associations between molecular classifications of colorectal cancer and patient survival: a systematic review. Clinical Gastroenterology and Hepatology 2019;17:402-410. e2.
47. Seppälä T, Böhm J, Friman M, et al. Combination of microsatellite instability and BRAF mutation status for subtyping colorectal cancer. British journal of cancer 2015;112:1966-1975.
48. Formica V, Sera F, Cremolini C, et al. KRAS and BRAF Mutations in Stage II/III Colon Cancer: A Systematic Review and Meta-Analysis. JNCI: Journal of the National Cancer Institute 2021.
49. Wu M, Kim YS, Ryu HS, et al. MSI status is associated with distinct clinicopathological features in BRAF mutation colorectal cancer: A systematic review and meta-analysis. Pathol Res Pract 2020;216:152791.
50. Mei ZB, Duan CY, Li CB, et al. Prognostic role of tumor PIK3CA mutation in colorectal cancer: a systematic review and meta-analysis. Ann Oncol 2016;27:1836-48.
51. Kwak MS, Cha JM, Yoon JY, et al. Prognostic value of KRAS codon 13 gene mutation for overall survival in colorectal cancer: direct and indirect comparison meta-analysis. Medicine 2017;96.
52. Sinicrope FA, Shi Q, Smyrk TC, et al. Molecular markers identify subtypes of stage III colon cancer associated with patient outcomes. Gastroenterology 2015;148:88-99.
53. Harriss D, Atkinson G, George K, et al. Lifestyle factors and colorectal cancer risk (1): systematic review and meta-analysis of associations with body mass index. Colorectal disease 2009;11:547-563.
54. Han X, Stevens J, Truesdale KP, et al. Body mass index at early adulthood, subsequent weight change and cancer incidence and mortality. International journal of cancer 2014;135:2900-2909.
55. Hughes LA, Simons CC, van den Brandt PA, et al. Body size and colorectal cancer risk after 16.3 years of follow-up: an analysis from the Netherlands Cohort Study. American journal of epidemiology 2011;174:1127-1139.
56. Song X, Gong X, Zhang T, et al. Height and risk of colorectal cancer: a meta-analysis. European Journal of Cancer Prevention 2018;27:521-529.
57. Samad A, Taylor R, Marshall T, et al. A meta-analysis of the association of physical activity with reduced risk of colorectal cancer. Colorectal disease 2005;7:204-213.
58. Clinton SK, Giovannucci EL, Hursting SD. The World Cancer Research Fund/American Institute for Cancer Research third expert report on diet, nutrition, physical activity, and cancer: impact and future directions. The Journal of nutrition 2020;150:663-671.

59. Hughes LA, van den Brandt PA, Goldbohm RA, et al. Childhood and adolescent energy restriction and subsequent colorectal cancer risk: results from the Netherlands Cohort Study. International journal of epidemiology 2010;39:1333-1344.
60. Svensson E, Møller B, Tretli S, et al. Early life events and later risk of colorectal cancer: age-period-cohort modelling in the Nordic countries and Estonia. Cancer Causes & Control 2005;16:215-223.
61. Wang N, Khankari NK, Cai H, et al. Prediagnosis body mass index and waist–hip circumference ratio in association with colorectal cancer survival. International journal of cancer 2017;140:292-301.
62. Laake I, Larsen IK, Selmer R, et al. Pre-diagnostic body mass index and weight change in relation to colorectal cancer survival among incident cases from a population-based cohort study. BMC cancer 2016;16:1-10.
63. Fedirko V, Romieu I, Aleksandrova K, et al. Pre-diagnostic anthropometry and survival after colorectal cancer diagnosis in Western European populations. International journal of cancer 2014;135:1949-1960.
64. Taghizadeh N, Boezen HM, Schouten JP, et al. BMI and lifetime changes in BMI and cancer mortality risk. PloS one 2015;10:e0125261.
65. Campbell PT, Newton CC, Newcomb PA, et al. Association between body mass index and mortality for colorectal cancer survivors: overall and by tumor molecular phenotype. Cancer Epidemiology and Prevention Biomarkers 2015;24:1229-1238.
66. Hardikar S, Newcomb PA, Campbell PT, et al. Prediagnostic Physical Activity and Colorectal Cancer Survival: Overall and Stratified by Tumor CharacteristicsPhysical Activity and Colorectal Cancer Survival. Cancer Epidemiology, Biomarkers & Prevention 2015;24:1130-1137.
67. Jayasekara H, English DR, Haydon A, et al. Associations of alcohol intake, smoking, physical activity and obesity with survival following colorectal cancer diagnosis by stage, anatomic site and tumor molecular subtype. International journal of cancer 2018;142:238-250.
68. Walter V, Jansen L, Knebel P, et al. Physical activity and survival of colorectal cancer patients: Population-based study from Germany. International journal of cancer 2017;140:1985-1997.
69. Arem H, Pfeiffer RM, Engels EA, et al. Pre-and postdiagnosis physical activity, television viewing, and mortality among patients with colorectal cancer in the National Institutes of Health–AARP diet and health study. Journal of Clinical Oncology 2015;33:180.
70. Boyle T, Fritschi L, Platell C, et al. Lifestyle factors associated with survival after colorectal cancer diagnosis. British journal of cancer 2013;109:814-822.
71. Weijenberg MP, Hughes LA, Bours MJ, et al. The mTOR Pathway and the Role of Energy Balance Throughout Life in Colorectal Cancer Etiology and Prognosis: Unravelling Mechanisms Through a Multidimensional Molecular Epidemiologic Approach. Curr Nutr Rep 2013;2:19-26.
72. Icard P, Shulman S, Farhat D, et al. How the Warburg effect supports aggressiveness and drug resistance of cancer cells? Drug Resistance Updates 2018;38:1-11.
73. Vermeersch KA, Wang L, McDonald JF, et al. Distinct metabolic responses of an ovarian cancer stem cell line. BMC systems biology 2014;8:1-14.
74. Pampaloni F, Reynaud EG, Stelzer EH. The third dimension bridges the gap between cell culture and live tissue. Nature reviews Molecular cell biology 2007;8:839-845.
75. Jo Y, Choi N, Kim K, et al. Chemoresistance of cancer cells: requirements of tumor microenvironment-mimicking in vitro models in anti-cancer drug development. Theranostics 2018;8:5259.

76. Vellinga TT, Borovski T, de Boer VC, et al. SIRT1/PGC1α-dependent increase in oxidative phosphorylation supports chemotherapy resistance of colon cancer. Clinical cancer research 2015;21:2870-2879.
77. Gottesman MM, Fojo T, Bates SE. Multidrug resistance in cancer: role of ATP–dependent transporters. Nature reviews cancer 2002;2:48-58.
78. De Sousa E Melo F, Wang X, Jansen M, et al. Poor-prognosis colon cancer is defined by a molecularly distinct subtype and develops from serrated precursor lesions. Nature medicine 2013;19:614-618.
79. Schlicker A, Beran G, Chresta CM, et al. Subtypes of primary colorectal tumors correlate with response to targeted treatment in colorectal cell lines. BMC medical genomics 2012;5:1-15.
80. Budinska E, Popovici V, Tejpar S, et al. Gene expression patterns unveil a new level of molecular heterogeneity in colorectal cancer. The Journal of pathology 2013;231:63-76.
81. Roepman P, Schlicker A, Tabernero J, et al. Colorectal cancer intrinsic subtypes predict chemotherapy benefit, deficient mismatch repair and epithelial-to-mesenchymal transition. International journal of cancer 2014;134:552-562.
82. Marisa L, de Reyniès A, Duval A, et al. Gene expression classification of colon cancer into molecular subtypes: characterization, validation, and prognostic value. PLoS medicine 2013;10:e1001453.
83. Sadanandam A, Lyssiotis CA, Homicsko K, et al. A colorectal cancer classification system that associates cellular phenotype and responses to therapy. Nature medicine 2013;19:619-625.
84. Wang W, Kandimalla R, Huang H, et al. Molecular subtyping of colorectal cancer: Recent progress, new challenges and emerging opportunities, In Seminars in cancer biology, Elsevier, 2019.
85. Guinney J, Dienstmann R, Wang X, et al. The consensus molecular subtypes of colorectal cancer. Nature medicine 2015;21:1350-1356.
86. Martini G, Dienstmann R, Ros J, et al. Molecular subtypes and the evolution of treatment management in metastatic colorectal cancer. Therapeutic Advances in Medical Oncology 2020;12:1758835920936089.
87. Li X, Larsson P, Ljuslinder I, et al. A modified protein marker panel to identify four consensus molecular subtypes in colorectal cancer using immunohistochemistry. Pathology-Research and Practice 2021;220:153379.
88. Ten Hoorn S, Trinh A, Jong Jd, et al. Classification of colorectal cancer in molecular subtypes by immunohistochemistry. Colorectal Cancer: Springer, 2018:179-191.
89. Phipps AI, Limburg PJ, Baron JA, et al. Association between molecular subtypes of colorectal cancer and patient survival. Gastroenterology 2015;148:77-87. e2.
90. Jass J. Classification of colorectal cancer based on correlation of clinical, morphological and molecular features. Histopathology 2007;50:113-130.
91. Offermans K, Jenniskens JC, Simons CC, et al. Association between mutational subgroups, Warburg-subtypes, and survival in patients with colorectal cancer. Cancer Medicine 2022.
92. Kesmodel US. Information bias in epidemiological studies with a special focus on obstetrics and gynecology. Acta Obstetricia et Gynecologica Scandinavica 2018;97:417-423.
93. Kuiper JG, Phipps AI, Neuhouser ML, et al. Recreational physical activity, body mass index, and survival in women with colorectal cancer. Cancer Causes & Control 2012;23:1939-1948.
94. van Zutphen M, Boshuizen HC, Kok DE, et al. Colorectal cancer survivors only marginally change their overall lifestyle in the first 2 years following diagnosis. Journal of Cancer Survivorship 2019;13:956-967.
95. Kononen J, Bubendorf L, Kallionimeni A, et al. Tissue microarrays for high-throughput molecular profiling of

tumor specimens. Nature medicine 1998;4:844-847.
96. Montaser-Kouhsari L, Knoblauch NW, Oh E-Y, et al. Image-guided coring for large-scale studies in molecular pathology. Applied Immunohistochemistry & Molecular Morphology 2016;24:431.
97. Parsons M, Grabsch H. How to make tissue microarrays. Diagnostic histopathology 2009;15:142-150.
98. Tawfik El-Mansi M, Williams AR. Validation of tissue microarray technology using cervical adenocarcinoma and its precursors as a model system. Int J Gynecol Cancer 2006;16:1225-33.
99. Ilyas M, Grabsch H, Ellis IO, et al. Guidelines and considerations for conducting experiments using tissue microarrays. Histopathology 2013;62:827-39.
100. Camp RL, Neumeister V, Rimm DL. A decade of tissue microarrays: progress in the discovery and validation of cancer biomarkers. J Clin Oncol 2008;26:5630-7.
101. Domeny-Duarte P, Niero L, Domingues MAC. Tissue microarrays of bone marrow aspirate clot allow assessment of multiple samples. Pathol Res Pract 2020;216:152721.
102. Shergill IS, Shergill NK, Arya M, et al. Tissue microarrays: a current medical research tool. Curr Med Res Opin 2004;20:707-12.
103. Bubendorf L, Nocito A, Moch H, et al. Tissue microarray (TMA) technology: miniaturized pathology archives for high-throughput in situ studies. J Pathol 2001;195:72-9.
104. Al Kuraya K, Simon R, Sauter G. Tissue microarrays for high-throughput molecular pathology. Ann Saudi Med 2004;24:169-74.
105. Boone J, van Hillegersberg R, van Diest PJ, et al. Validation of tissue microarray technology in squamous cell carcinoma of the esophagus. Virchows Arch 2008;452:507-14.
106. Zhang D, Salto-Tellez M, Putti TC, et al. Reliability of tissue microarrays in detecting protein expression and gene amplification in breast cancer. Mod Pathol 2003;16:79-84.
107. Hassan S, Ferrario C, Mamo A, et al. Tissue microarrays: emerging standard for biomarker validation. Curr Opin Biotechnol 2008;19:19-25.
108. Hoos A, Urist MJ, Stojadinovic A, et al. Validation of tissue microarrays for immunohistochemical profiling of cancer specimens using the example of human fibroblastic tumors. Am J Pathol 2001;158:1245-51.
109. Jourdan F, Sebbagh N, Comperat E, et al. Tissue microarray technology: validation in colorectal carcinoma and analysis of p53, hMLH1, and hMSH2 immunohistochemical expression. Virchows Archiv 2003;443:115-121.
110. MacNeil T, Vathiotis IA, Martinez-Morilla S, et al. Antibody validation for protein expression on tissue slides: a protocol for immunohistochemistry. Biotechniques 2020;69:460-468.
111. Berglund L, Björling E, Oksvold P, et al. A genecentric Human Protein Atlas for expression profiles based on antibodies. Molecular & cellular proteomics 2008;7:2019-2027.
112. Thul PJ, Lindskog C. The human protein atlas: a spatial map of the human proteome. Protein Science 2018;27:233-244.
113. Capuano N, Foggia P, Greco L, et al. A Linked Data Application for Harmonizing Heterogeneous Biomedical Information. Applied Sciences 2022;12:9317.
114. Chung GG, Kielhorn EP, Rimm DL. Subjective differences in outcome are seen as a function of the immunohistochemical method used on a colorectal cancer tissue microarray. Clinical Colorectal Cancer 2002;1:237-242.
115. Peck AR, Girondo MA, Liu C, et al. Validation of tumor protein marker quantification by two independent

automated immunofluorescence image analysis platforms. Modern Pathology 2016;29:1143-1154.
116. Adams E, Green J, Clark A, et al. Comparison of different scoring systems for immunohistochemical staining. Journal of clinical pathology 1999;52:75-77.
117. Atas E, Oberhuber M, Kenner L. The implications of PDK1–4 on tumor energy metabolism, aggressiveness and therapy resistance. Frontiers in oncology 2020;10:583217.
118. Dang C. The interplay between MYC and HIF in the Warburg effect. Oncogenes Meet Metabolism 2008:35-53.
119. Kim Y, Choi J-W, Lee J-H, et al. Expression of lactate/H+ symporters MCT1 and MCT4 and their chaperone CD147 predicts tumor progression in clear cell renal cell carcinoma: immunohistochemical and The Cancer Genome Atlas data analyses. Human pathology 2015;46:104-112.
120. Boström P, Thoms J, Sykes J, et al. Hypoxia marker GLUT-1 (glucose transporter 1) is an independent prognostic factor for survival in bladder cancer patients treated with radical cystectomy. Bladder Cancer 2016;2:101-109.
121. Antonarakis ES, Keizman D, Zhang Z, et al. An immunohistochemical signature comprising PTEN, MYC, and Ki67 predicts progression in prostate cancer patients receiving adjuvant docetaxel after prostatectomy. Cancer 2012;118:6063-6071.
122. Yan S, Coffing BN, Li Z, et al. Diagnostic and prognostic value of ProEx C and GLUT1 in melanocytic lesions. Anticancer Research 2016;36:2871-2880.
123. Stackhouse BL, Williams H, Berry P, et al. Measurement of glut-1 expression using tissue microarrays to determine a race specific prognostic marker for breast cancer. Breast cancer research and treatment 2005;93:247-253.
124. Winter JL, Stackhouse BL, Russell GB, et al. Measurement of PTEN expression using tissue microarrays to determine a race-specific prognostic marker in breast cancer. Archives of pathology & laboratory medicine 2007;131:767-772.
125. Hodgson A, Xu B, Downes MR. p53 immunohistochemistry in high-grade urothelial carcinoma of the bladder is prognostically significant. Histopathology 2017;71:296-304.
126. Van Calster B, Van Hoorde K, Froyman W, et al. Practical guidance for applying the ADNEX model from the IOTA group to discriminate between different subtypes of adnexal tumors. Facts, views & vision in ObGyn 2015;7:32.
127. Fumagalli D, Gavin PG, Taniyama Y, et al. A rapid, sensitive, reproducible and cost-effective method for mutation profiling of colon cancer and metastatic lymph nodes. BMC cancer 2010;10:1-14.
128. Robey RB, Hay N. Is Akt the "Warburg kinase"?—Akt-energy metabolism interactions and oncogenesis, In Seminars in cancer biology, Elsevier, 2009.
129. Zhang J, Zheng J, Yang Y, et al. Molecular spectrum of KRAS, NRAS, BRAF and PIK3CA mutations in Chinese colorectal cancer patients: analysis of 1,110 cases. Sci Rep 2015;5:18678.
130. Poulsen TS, de Oliveira DVNP, Espersen MLM, et al. Frequency and coexistence of KRAS, NRAS, BRAF and PIK3CA mutations and occurrence of MMR deficiency in Danish colorectal cancer patients. Apmis 2021;129:61-69.
131. Tate JG, Bamford S, Jubb HC, et al. COSMIC: the catalogue of somatic mutations in cancer. Nucleic acids research 2019;47:D941-D947.
132. Catalogue of Somatic Mutations in Cancer, release version 94.
133. Lanza G, Gafà R, Maestri I, et al. Immunohistochemical pattern of MLH1/MSH2 expression is related to clinical and pathological features in colorectal adenocarcinomas with microsatellite instability. Modern Pathology

2002;15:741-749.
134. Saeed OA, Mann SA, Luchini C, et al. Evaluating mismatch repair deficiency for solid tumor immunotherapy eligibility: Immunohistochemistry versus microsatellite molecular testing. Human Pathology 2021;115:10-18.
135. Amonkar M, Lorenzi M, Zhang J, et al. Structured literature review (SLR) and meta-analyses of the prevalence of microsatellite instability high (MSI-H) and deficient mismatch repair (dMMR) in gastric, colorectal, and esophageal cancers: American Society of Clinical Oncology, 2019.
136. Xiao C, Tian H, Zheng Y, et al. Glycolysis in tumor microenvironment as a target to improve cancer immunotherapy. Frontiers in Cell and Developmental Biology 2022;10.
137. Siska PJ, Singer K, Evert K, et al. The immunological Warburg effect: Can a metabolic-tumor-stroma score (MeTS) guide cancer immunotherapy? Immunological Reviews 2020;295:187-202.
138. Bensinger SJ, Christofk HR. New aspects of the Warburg effect in cancer cell biology. Semin Cell Dev Biol 2012;23:352-61.
139. Lebelo MT, Joubert AM, Visagie MH. Warburg effect and its role in tumourigenesis. Archives of pharmacal research 2019;42:833-847.
140. Satoh K, Soga T. Multi-omics-based approach to colorectal cancer metabolism. Cancer Research 2017;77:2513-2513.
141. D'Adamo GL, Widdop JT, Giles EM. The future is now? Clinical and translational aspects of "Omics" technologies. Immunology and cell biology 2021;99:168-176.
142. Nava GM, Madrigal Perez LA. Metabolic profile of the Warburg effect as a tool for molecular prognosis and diagnosis of cancer. Expert Review of Molecular Diagnostics 2022;22:439-447.
143. Kather JN, Pearson AT, Halama N, et al. Deep learning can predict microsatellite instability directly from histology in gastrointestinal cancer. Nature medicine 2019;25:1054-1056.
144. Echle A, Laleh NG, Schrammen PL, et al. Deep learning for the detection of microsatellite instability from histology images in colorectal cancer: a systematic literature review. ImmunoInformatics 2021:100008.
145. Lugli A, Kirsch R, Ajioka Y, et al. Recommendations for reporting tumor budding in colorectal cancer based on the International Tumor Budding Consensus Conference (ITBCC) 2016. Modern pathology 2017;30:1299-1311.
146. Fridman WH, Sautès-Fridman C, Galon J. The immune contexture in human tumours: impact on clinical outcome. Nature Reviews Cancer 2012;12:298-306.
147. Mesker WE, Junggeburt J, Szuhai K, et al. The carcinoma–stromal ratio of colon carcinoma is an independent factor for survival compared to lymph node status and tumor stage. Analytical Cellular Pathology 2007;29:387-398.
148. Li MX, Liu XM, Zhang XF, et al. Prognostic role of neutrophil-to-lymphocyte ratio in colorectal cancer: A systematic review and meta-analysis. International journal of cancer 2014;134:2403-2413.
149. Özsoy M, Zimmermann FA, Feichtinger RG, et al. Changes in the expression of oxidative phosphorylation complexes in the aging intestinal mucosa. Experimental Gerontology 2020;135:110924.

CHAPTER 8

Impact

Colorectal cancer (CRC) is the world's third most common cancer, with more than 1.9 million newly diagnosed patients in 2020[1]. The global incidence of CRC has more than doubled over the past 30 years[2]. Its incidence is expected to increase even further in the coming years to 3.2 million new CRC cases in 2040[3], as more and more countries adopt a so-called "western" lifestyle (e.g., high consumption of animal fats, processed meats, low physical activity)[4, 5]. Even though screening and treatment has improved significantly over the past few decades[6, 7], CRC is still the second most deadly cancer worldwide, accounting for more than 900,000 deaths in 2020[1]. In addition, CRC places a significant economic burden on populations and healthcare systems[8], estimated to be around €19.1 billion in Europe in 2015[9].

Disease stage at diagnosis, as assessed by the American Joint Committee on Cancer (AJCC)[10] and the Union for International Cancer Control (UICC)[11] tumor-node-metastasis (TNM) staging system, remains the most important determinant of prognosis and guides clinical management in patients with CRC[12]. In the Netherlands, five-year survival for TNM stage I is 95% and drops to only 12% for TNM stage IV CRC patients[13]. However, the survival of individual CRC patients diagnosed with the same disease stage may differ substantially, often due to the heterogeneous nature of the disease[12]. Furthermore, it has been described that under-treatment or over-treatment of some patient groups may arise when using the TNM staging system for treatment allocation[14]. Hence, there remains an urgent clinical need to identify novel prognostic and/or predictive biomarkers in CRC[15]. However, despite the great interest and immense amount of research invested in the development of additional prognostic and/or predictive markers[12, 16-20], only few markers have been implemented in clinical practice to date[12, 21].

Metabolic reprogramming is a recognized hallmark of cancer cells[22-24]. The most commonly known metabolic abnormality in cancer cells is the so-called Warburg-effect (named after Dr. Otto H. Warburg, who was the first to describe the altered metabolism of cancer cells in the 1920s[25]), a phenomenon characterized by increased glycolysis even in the presence of oxygen[24]. It has been proposed that the Warburg-effect promotes the malignant potential of cancer cells[26], thereby potentially affecting patient prognosis and response to therapy[27-34]. However, to date, the evidence is very limited and results are inconsistent[27]. This inconsistency in results may be explained by the fact that previous prognostic studies mostly focused on investigating a single protein involved in the Warburg-effect, while this pathway is much more complicated. The predictive value of the Warburg-effect has mostly been investigated in *in vitro* cell culture studies[29]. To the best of our knowledge, only one study to date has investigated the association between response to 5-fluorouracil (5-FU) therapy and the expression of proteins related to metabolism in human tissue samples[34]. However, this was investigated in a (retrospective) case-control design, which is prone to various biases (e.g., selection bias, confounding bias).

Targeting the Warburg-effect has become a major area of focus in the development of new anti-cancer drugs[35], as inhibition of the Warburg-effect may reduce tumor cell proliferation and metastasis[36]. Various inhibitors of glycolytic enzymes and transporters (e.g., GLUT, PKM2, LDHA, MCT1) are currently in (pre)clinical development[37, 38]. Unfortunately, there has been little clinical success to date[37, 38]. It has been proposed that this lack of clinical success may result from the limited knowledge on the metabolic pathways involved in CRC, as no metabolic profiling is currently performed before initiation of therapy[38]. Even though the Warburg-effect is a common phenomenon observed a variety of cancers, it is not a universal trait of all tumor cells[38, 39].

The principal aim of this thesis was to examine whether Warburg-subtyping, based on the estimated presence of the Warburg-effect in cancer cells, has prognostic value and can be used to predict survival benefit from adjuvant therapy in CRC. Our research provides evidence that Warburg-subtyping may have prognostic value in CRC, independent of known prognostic factors such as TNM stage (**Chapter 3**)[40]. Furthermore, our research indicates that of all subgroups based on molecular characteristics that have been associated with the presence of the Warburg-effect (mutations in *RAS, BRAF, PIK3CA,* and *MET*, as well as MMR deficiency), especially *BRAF*-mutated proficient (p)MMR CRC, *KRAS*-mutated pMMR CRC, and deficient (d)MMR CRCs were related to the Warburg-high subtype in our patient series (**Chapter 3-4**). In addition, our results suggest that Warburg-subtyping may provide additional prognostic information in patient subgroups with *KRAS*-mutated pMMR CRC or *BRAF*-mutated pMMR or dMMR CRC (**Chapter 4**)[41]. Furthermore, our research indicates that associations between adult BMI, weight change since age 20 years, energy restriction during childhood and adolescence, and potentially adult-attained height and survival in CRC differ according to Warburg-subtype (**Chapter 5**). Lastly, our research provides evidence that Warburg-subtyping may predict survival benefit from adjuvant (chemo)therapy in CRC patients (**Chapter 6**).

As we were the first to investigate the potential prognostic and/or predictive value of Warburg-subtyping in a large population-based series of CRC patients, validation of the current findings in other cohort studies is required. Nevertheless, our results indicate that Warburg-subtyping may have prognostic value and may be used to predict survival benefit from adjuvant (chemo)therapy in CRC, independent of known clinical factors such as TNM stage. Although caution is warranted in drawing conclusions based on the results presented in this thesis alone, our results are promising and indicate that Warburg-subtyping may in the future be used for risk stratification of CRC patients, and the design and tailoring of Warburg-targeted therapies. This may, in the future, potentially improve the survival of CRC patients.

Next to the potential future clinical impact of our Warburg-subtyping, our research also impacts academic endeavors. As mentioned before, many previous studies have investigated the prognostic value of the Warburg-effect in CRC using one (or multiple) immunohistochemistry (IHC) markers, showing conflicting results. In addition, the predictive value of the Warburg-effect in tumor cells has, to the best of our knowledge, mostly been investigated in *in vitro* cell culture studies[29], with the exception of one (retrospective) case-control study[34]. In this thesis, we have developed and described a transparent and comprehensive methodology for Warburg-subtyping on formalin-fixed paraffin-embedded (FFPE) tissue samples of CRC patients. Using a pathway-based sum score, based on the IHC expression of six glycolytic proteins and transcriptional regulators involved in different steps of the Warburg-effect pathway, we attempted to capture the presence of the Warburg-effect in tumor cells in a large population-based series of CRC patients (n = 2,347). Furthermore, we have attempted to increase the reproducibility of the current results as well as to enhance the applicability of our methodology in future research by (i) optimizing and describing all used IHC staining and scoring protocols (**Chapter 2-3**), (ii) evaluating and describing the validity and reproducibility of IHC scoring results (**Chapter 2**), (iii) describing how to combine the IHC scores on core-level to patient-level scores (**Chapter 3-6**), and (iv) by developing and describing a comprehensive and transparent way of Warburg-subtyping based on a pathway-based sum score and pre-defined cut-off values. (**Chapter 3**).

Knowledge transfer

To ensure knowledge transfer, the scientific research summarized in this thesis has been, or will be, shared with fellow researchers through publication in open access, international peer-reviewed scientific journals. In addition, our results were presented at various national and international conferences and symposia covering a broad audience consisting of both pathologists and epidemiologists. Presentations were given at the virtual annual meeting of the American Association of Cancer Research (2021), the online Dutch Epidemiological Conference (WEON) (2021), the virtual conference of the Pathological Society of Great Britain and Ireland (PathSoc, Manchester Pathology) (2021), the Science Day of the Maastricht University Medical Centre+ (2021), and the online GROW Science Day of Maastricht University (2021).

Conclusion

In this thesis, we aimed to investigate whether Warburg-subtyping, based on the estimated presence of the Warburg-effect in cancer cells, has prognostic value and can be used to predict survival benefit from adjuvant therapy in CRC. Altogether, the results presented in this thesis suggest that Warburg-subtyping has prognostic value in CRC, independent of known prognostic factors such as TNM stage, and may be used to predict survival benefit from adjuvant (chemo)therapy. As we were the first to investigate this in a large

population-based series of CRC patients, validation of the results presented in this thesis is necessary. Nevertheless, our results are promising and may suggest that Warburg-subtyping could in the future be used for risk stratification of CRC patients and the design and tailoring of Warburg-targeted therapies, thereby potentially (eventually) improving the survival of CRC patients.

REFERENCES

1. Ferlay J, Ervik M, Lam F, et al. Global Cancer Observatory: Cancer Today. Volume 2021: Lyon, France: International Agency for Research on Cancer, 2020.
2. Sharma R, Abbasi-Kangevari M, Abd-Rabu R, et al. Global, regional, and national burden of colorectal cancer and its risk factors, 1990–2019: a systematic analysis for the Global Burden of Disease Study 2019. The Lancet Gastroenterology & Hepatology 2022.
3. Morgan E, Arnold M, Gini A, et al. Global burden of colorectal cancer in 2020 and 2040: incidence and mortality estimates from GLOBOCAN. Gut 2022.
4. Dekker E, Tanis PJ, Vleugels JL, et al. Pure-AMC. Lancet 2019;394:1467-80.
5. Sawicki T, Ruszkowska M, Danielewicz A, et al. A review of colorectal cancer in terms of epidemiology, risk factors, development, symptoms and diagnosis. Cancers 2021;13:2025.
6. Araghi M, Soerjomataram I, Jenkins M, et al. Global trends in colorectal cancer mortality: projections to the year 2035. International journal of cancer 2019;144:2992-3000.
7. Schreuders EH, Ruco A, Rabeneck L, et al. Colorectal cancer screening: a global overview of existing programmes. Gut 2015;64:1637-1649.
8. Bhimani N, Wong GY, Molloy C, et al. Cost of colorectal cancer by treatment type from different health economic perspectives: A systematic review. European Journal of Surgical Oncology 2022.
9. Henderson RH, French D, Maughan T, et al. The economic burden of colorectal cancer across Europe: a population-based cost-of-illness study. The Lancet Gastroenterology & Hepatology 2021;6:709-722.
10. Edition S, Edge S, Byrd D. AJCC cancer staging manual. AJCC cancer staging manual 2017.
11. UfIC C. UICC and the TNM Classification of Malignant Tumours. 2021.
12. Marks K, West N, Morris E, et al. Clinicopathological, genomic and immunological factors in colorectal cancer prognosis. Journal of British Surgery 2018;105:e99-e109.
13. (IKNL) IkN. NKR Cijfers. Volume 2022: IKNL, 2022.
14. Reimers MS, Zeestraten EC, Kuppen PJ, et al. Biomarkers in precision therapy in colorectal cancer. Gastroenterology report 2013;1:166-183.
15. Lee JK, Chan AT. Molecular prognostic and predictive markers in colorectal cancer: current status. Current colorectal cancer reports 2011;7:136-144.
16. Lugli A, Kirsch R, Ajioka Y, et al. Recommendations for reporting tumor budding in colorectal cancer based on the International Tumor Budding Consensus Conference (ITBCC) 2016. Modern pathology 2017;30:1299-1311.
17. Fridman WH, Sautès-Fridman C, Galon J. The immune contexture in human tumours: impact on clinical outcome. Nature Reviews Cancer 2012;12:298-306.
18. Mesker WE, Junggeburt J, Szuhai K, et al. The carcinoma–stromal ratio of colon carcinoma is an independent factor for survival compared to lymph node status and tumor stage. Analytical Cellular Pathology 2007;29:387-398.
19. Li MX, Liu XM, Zhang XF, et al. Prognostic role of neutrophil-to-lymphocyte ratio in colorectal cancer: A systematic review and meta-analysis. International journal of cancer 2014;134:2403-2413.
20. Guinney J, Dienstmann R, Wang X, et al. The consensus molecular subtypes of colorectal cancer. Nature medicine 2015;21:1350-1356.

21. Specialisten FM. Richtlijnendatabase. URL: https://richtlijnendatabase.nl/richtlijn/colorectaal_carcinoom_crc/gemetastaseerd_colorectaalcarcinoom_crc.html 2020.
22. Hanahan D, Weinberg RA. Hallmarks of cancer: the next generation. cell 2011;144:646-674.
23. Hanahan D. Hallmarks of cancer: new dimensions. Cancer discovery 2022;12:31-46.
24. Wu W, Zhao S. Metabolic changes in cancer: beyond the Warburg effect. Acta biochimica et biophysica Sinica 2013;45:18-26.
25. Warburg O. The metabolism of carcinoma cells. The Journal of Cancer Research 1925;9:148-163.
26. Lu J, Tan M, Cai Q. The Warburg effect in tumor progression: mitochondrial oxidative metabolism as an anti-metastasis mechanism. Cancer letters 2015;356:156-164.
27. Yu M, Chen S, Hong W, et al. Prognostic role of glycolysis for cancer outcome: evidence from 86 studies. J Cancer Res Clin Oncol 2019;145:967-999.
28. Dong S, Liang S, Cheng Z, et al. ROS/PI3K/Akt and Wnt/β-catenin signalings activate HIF-1α-induced metabolic reprogramming to impart 5-fluorouracil resistance in colorectal cancer. Journal of Experimental & Clinical Cancer Research 2022;41:1-27.
29. Liu C, Jin Y, Fan Z. The Mechanism of Warburg Effect-Induced Chemoresistance in Cancer. Frontiers in oncology 2021:3408.
30. Zaal EA, Berkers CR. The influence of metabolism on drug response in cancer. Frontiers in oncology 2018;8:500.
31. Desbats MA, Giacomini I, Prayer-Galetti T, et al. Metabolic plasticity in chemotherapy resistance. Frontiers in oncology 2020;10:281.
32. Zhong J-T, Zhou S-H. Warburg effect, hexokinase-II, and radioresistance of laryngeal carcinoma. Oncotarget 2017;8:14133.
33. Morandi A, Indraccolo S. Linking metabolic reprogramming to therapy resistance in cancer. Biochimica et Biophysica Acta (BBA)-Reviews on Cancer 2017;1868:1-6.
34. Kitazawa M, Hatta T, Sasaki Y, et al. Promotion of the Warburg effect is associated with poor benefit from adjuvant chemotherapy in colorectal cancer. Cancer science 2020;111:658.
35. Li Y, Wang Y, Liu Z, et al. Atractylenolide I Induces Apoptosis and Suppresses Glycolysis by Blocking the JAK2/STAT3 Signaling Pathway in Colorectal Cancer Cells. Front Pharmacol 2020;11:273.
36. Nenkov M, Ma Y, Gassler N, et al. Metabolic Reprogramming of Colorectal Cancer Cells and the Microenvironment: Implication for Therapy. Int J Mol Sci 2021;22.
37. Le A. The Heterogeneity of Cancer Metabolism: Springer Nature, 2021.
38. Vanhove K, Graulus G-J, Mesotten L, et al. The metabolic landscape of lung cancer: new insights in a disturbed glucose metabolism. Frontiers in oncology 2019;9:1215.
39. Correa PN, Correa AN. A unitarian biochemical and bioenergetic theory of adaptive oncogenesis: from hypoxia and energy starvation (aerobic and ambipolar) to the roles of HIF-1, IGF-I, and vitamins C and D. Journal of Biophysics, Hematology and Oncology 2010;1:1-93.
40. Offermans K, Jenniskens JC, Simons CC, et al. Expression of proteins associated with the Warburg-effect and survival in colorectal cancer. The Journal of Pathology: Clinical Research 2021.
41. Offermans K, Jenniskens JC, Simons CC, et al. Association between mutational subgroups, Warburg-subtypes, and survival in patients with colorectal cancer. Cancer Medicine 2022.

ADDENDUM

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SUMMARY

Colorectal cancer (CRC) is the third most commonly diagnosed cancer worldwide. Despite advances in the early detection and treatment of CRC, it remains the second leading cause of cancer-related mortality worldwide, accounting for more than 900,000 deaths in 2020. To date, the tumor-node-metastasis (TNM) staging system remains the most important clinically used factor to predict patient prognosis and guide treatment decisions in CRC. However, even patients within the same TNM stage can have a significantly different prognosis and response to adjuvant therapy, most likely due to heterogeneity in patient and tumor characteristics.

The *PI3K/AKT/mTOR* signaling pathway is one of the most frequently activated molecular pathways in CRC. It has been proposed that this signaling pathway rewires cancer cell metabolism from oxidative phosphorylation towards aerobic glycolysis, a phenomenon known as the "Warburg-effect". Previous research suggests that the Warburg-effect increases the malignant potential of tumor cells and may even contribute to therapy resistance. However, evidence to date is scarce and results remain inconsistent.

In this thesis, we therefore aimed to investigate whether Warburg-subtyping, based on the estimated presence of the Warburg-effect, has prognostic value and is able to predict benefit from adjuvant therapy in CRC patients. Furthermore, using a molecular pathological epidemiology (MPE) approach, we investigated (i) the potential additional prognostic value of Warburg-subtyping in subgroups based on mutations in oncogenes and tumor suppressor genes that have been associated with the presence of the Warburg-effect (i.e., *RAS (KRAS, NRAS, HRAS), PIK3CA,* and *BRAF* mutations) as well as mismatch repair (MMR) status, and (ii) whether associations between long-term energy balance-related factors and survival in CRC differed according to Warburg-subtype. To this end, CRC patients were classified as having Warburg-low (i.e., low probability of the presence of the Warburg-effect), Warburg-moderate, or Warburg-high CRC using a pathway-based sum score based on the protein expression levels of six glycolytic proteins and transcriptional regulators indicative of the Warburg-effect (i.e., LDHA, GLUT1, MCT4, PKM2, p53, PTEN).

All results presented in this thesis were based on observational data from the Netherlands Cohort Study (NLCS) on diet and cancer. This large, population-based prospective cohort study was initiated in 1986, and included 120,852 men and women aged 55-69 at baseline. Information with regard to, but not limited to, long-term energy balance-related factors were collected at baseline through a mailed, self-administered questionnaire. Follow-up for cancer incidence was established by annual record linkage with the Netherlands Cancer Registry and PALGA, the nationwide Dutch Pathology Registry, covering 20.3 years of follow up (September 17, 1986 until January 1, 2007). After this follow-up period of

20.3 years, 4,597 incident CRC cases had occurred. Follow-up for vital status was carried out through linkage with the Central Bureau of Genealogy and the municipal population registries, until December 31, 2012, and causes of death were retrieved from Statistic Netherlands.

In 2012, the Rainbow-TMA project was initiated, aiming to enrich cohorts with Tissue MicroArrays (TMAs) and DNA. Tumor and normal formalin-fixed paraffin-embedded (FFPE) tissue blocks from CRC patients were retrieved from pathology laboratories throughout the Netherlands. For TMA construction, pathologists reviewed Hematoxylin & Eosin (H&E)-stained sections and marked areas with the highest tumor density. From these areas, three 0.6 mm diameter tumor cores and three normal tissue cores were sampled and assembled in TMA blocks.

For the current thesis, serial TMA sections were subjected to immunohistochemistry (IHC) for Warburg-related proteins (i.e., LDHA, GLUT1, MCT4, PKM2, p53, PTEN), as well as mismatch repair (MMR)-related proteins (MLH1, MSH2). Stained sections were scored by three trained non-pathologist assessors and a random 10% was additionally scored by an experienced pathologist. Expression levels of the Warburg-related proteins were combined into a pathway-based sum score and, based on this sum score, patients were categorized into three Warburg-subtypes (Warburg-low, -moderate, and -high). In addition, available tumor DNA from CRC patients was screened for *RAS (KRAS, NRAS, HRAS)*, *PIK3CA*, *BRAF*, and *MET* mutations. Patients were then classified into eight mutually exclusive mutational subgroups, based on observed mutation (mut) frequencies and MMR status (i.e., all-wild-type + $MMR_{proficient}$, $KRAS_{mut}$ + $MMR_{proficient}$, $KRAS_{mut}$ + $PIK3CA_{mut}$ + $MMR_{proficient}$, $PIK3CA_{mut}$ + $MMR_{proficient}$, $BRAF_{mut}$ + $MMR_{proficient}$, $BRAF_{mut}$ + $MMR_{deficient}$, other + $MMR_{proficient}$, and other + $MMR_{deficient}$).

After excluding patients who did not pass IHC quality control, 2,394 CRC patients with complete IHC expression data for Warburg-subtyping (**Chapter 3 and 5**) and 2,344 patients with complete data for mutational subgroups were available for analyses (**Chapter 4**). In addition, the relationship between adjuvant therapy and survival could be analyzed for 1,793 CRC patients (**Chapter 6**). Kaplan-Meier curves and Cox regression models were used to investigate associations with survival for Warburg-subtypes alone, and in combination with mutational subgroups, long-term energy balance-related factors and adjuvant therapy.

In **Chapter 2**, we investigated whether non-pathologists can generate valid and reproducible IHC scoring results. This was done by assessing inter-observer agreement between trained non-pathologists and an experienced histopathologist for three IHC markers with different subcellular localization (i.e., nucleus, membrane, cytoplasm). In addition, intra-observer

agreement among trained non-pathologists was assessed. Our results indicated that adequately trained non-pathologists were able to produce similar IHC scoring results as an experienced histopathologists. Combining the scores of at least two non-pathologists yielded the most optimal results.

In **Chapter 3**, we studied whether Warburg-subtyping has prognostic value in CRC patients. We found that patients with Warburg-high CRC had a worse survival compared to patients with Warburg-low CRC, independent of known prognostic factors such as TNM stage.

In **Chapter 4**, we investigated whether mutational subgroups based on somatic mutations in *RAS, BRAF, PIK3CA* and *MET*, as well as MMR status, hold prognostic value in CRC. Moreover, we investigated whether Warburg-subtyping had additional prognostic value within these mutational subgroups. We found that compared to patients with all-wild-type + $MMR_{proficient}$ CRC, patients with $KRAS_{mut}$ + $MMR_{proficient}$, $KRAS_{mut}$ + $PIK3CA_{mut}$ + $MMR_{proficient}$, $BRAF_{mut}$ + $MMR_{proficient}$, or other + $MMR_{proficient}$ CRC had a worse survival. Patients with $BRAF_{mut}$ + $MMR_{proficient}$ CRC had the worst survival, while patients with other + $MMR_{deficient}$ CRC had the most favorable survival. Furthermore, we found that $BRAF_{mut}$, $KRAS_{mut}$ + $MMR_{proficient}$ and other + $MMR_{deficient}$ CRC may be related to the Warburg-high subtype. No statistically significant survival differences were observed across Warburg-subtypes within mutational subgroups.

In **Chapter 5**, we investigated the association between long-term energy balance-related factors (i.e., adult BMI, non-occupational physical activity, weight change since age 20 years, adult-attained height, and exposure to energy restriction during childhood and adolescence) and survival in CRC. Moreover, we evaluated whether associations between long-term energy balance-related factors and survival differed according to Warburg-subtype. We found that of all studied long-term energy balance-related factors, only increased adult (pre-diagnostic) BMI was associated with a worse survival in the total series of CRC patients. In stratified analyses, we found that associations with survival for increased adult BMI, weight gain since age 20 years, energy restriction during childhood and adolescence and potentially increased adult-attained height differed according to Warburg-subtype. Weight gain since age 20 years and adult-attained height were associated with a worse overall survival only in patients with Warburg-high CRC. Increased adult BMI was associated with a worse survival only in patients with Warburg-moderate CRC. Associations between energy restriction proxies (i.e., place of residence during World War II or the Dutch Hunger winter, employment of father during the Dutch Economic Depression) and survival did not show consistent patterns when stratified on Warburg-subtype

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In **Chapter 6**, we explored whether Warburg-subtypes can predict survival benefit from adjuvant therapy in patients with CRC. We found that while in general patients with TNM stage II-IV CRC who received adjuvant (chemo)therapy had a significantly favorable survival compared to patients who received surgery only, this survival benefit was limited to patients with Warburg-moderate and potentially Warburg-high CRC. No survival benefit from adjuvant (chemo)therapy was found for patients with Warburg-low CRC.

In **Chapter 7**, this thesis was concluded by a summary of the main findings, interpretation of the study results, a discussion of methodological considerations, and recommendations for future research. All in all, the results presented in this thesis suggest that Warburg-subtyping has prognostic value in CRC and may be used to predict survival benefit from adjuvant (chemo)therapy.

NEDERLANDSTALIGE SAMENVATTING

Jaarlijks krijgen ruim 1.9 miljoen mensen wereldwijd de diagnose dikkedarmkanker, ook wel aangeduid met de term 'colorectaal carcinoom' (CRC). Het is daarmee de derde meest voorkomende maligniteit na borst- en longkanker. Ondanks verbeteringen in de screening en behandeling van CRC, sterven er jaarlijks nog steeds meer dan 900.000 mensen aan deze ziekte. Daarmee is het de op één na belangrijkste oorzaak van kanker-gerelateerde sterfte wereldwijd. Tot op heden wordt het TNM-stadiëringssysteem (TNM-classificatie van maligne tumoren) gezien als de belangrijkste prognostische factor bij CRC. Classificatie op basis van het TNM-stadiëringssysteem lijkt echter onvoldoende nauwkeurig. Zo kunnen er bij tumoren met hetzelfde TNM-stadium grote onderlinge verschillen bestaan in prognose en respons op adjuvante therapie. Het is aangetoond dat dit hoogstwaarschijnlijk het gevolg is van verschillen op zowel het niveau van de patiënt als op het niveau van tumorbiologie. De *PI3K/AKT/mTOR*-pathway is een van de meest frequent geactiveerde moleculaire signaleringsroutes bij CRC. Onderzoek heeft uitgewezen dat deze pathway het metabolisme van kankercellen kan herprogrammeren van oxidatieve fosforylering naar aerobe glycolyse. Dit fenomeen staat ook wel bekend als het "Warburg-effect". Eerder onderzoek suggereert dat het Warburg-effect kwaadaardige tumoreigenschappen kan versterken en kan bijdragen aan therapieresistentie, maar tot op heden is het bewijs hiervoor schaars en zijn resultaten inconsistent.

In dit proefschrift hebben we daarom onderzocht of Warburg-subtypering prognostische waarde heeft bij CRC. Ook hebben we bekeken of Warburg-subtypering kan worden gebruikt om te achterhalen voor welke patiënten behandeling met adjuvante chemo- of radiotherapie het meeste overlevingsvoordeel oplevert. Daarnaast hebben we met behulp van een moleculaire pathologische epidemiologische (MPE) benadering bekeken (i) of Warburg-subtypering additionele prognostische waarde heeft binnen patiëntsubgroepen gebaseerd op moleculaire karakteristieken die gerelateerd zijn aan het Warburg-effect, en (ii) of associaties tussen factoren die aan de energiebalans zijn gerelateerd (op de lange termijn) en overleving verschillen tussen Warburg-subtypen.

Om dit te onderzoeken hebben we CRC-patiënten geclassificeerd als Warburg-laag (de geschatte kans dat het Warburg-effect aanwezig is in de tumor is laag), Warburg-matig, of Warburg-hoog (de geschatte kans dat het Warburg-effect aanwezig is in de tumor is hoog). Deze classificatie kwam tot stand met behulp van een somscore op basis van de eiwitexpressieniveaus van zes glycolytische eiwitten en transcriptiefactoren in tumorweefsel die indicatief zijn voor de aanwezigheid van het Warburg-effect (LDHA, GLUT1, MCT4, PKM2, p53, PTEN).

Alle resultaten gepresenteerd in dit proefschrift zijn gebaseerd op observationele data van de Nederlandse Cohortstudie naar voeding en kanker (NLCS), een groot prospectief cohortonderzoek dat in 1986 is gestart onder 120.852 mannen en vrouwen in de leeftijd van 55-69 jaar. Informatie met betrekking tot factoren die verband houden met de energiebalans op de lange termijn werd bij aanvang verzameld door middel van een per post verstuurde, zelf in te vullen, vragenlijst. Het volledige cohort werd, door middel van een koppeling met de Nederlandse Kankerregistratie en PALGA (Pathologisch-Anatomisch Landelijk Geregistreerd Archief), opgevolgd voor het optreden van incidente kankergevallen. Na een follow-up periode van 20.3 jaar (17 september 1986 tot 1 januari 2007), werd colorectale kanker vastgesteld bij 4,597 deelnemers. Door koppeling met het Centraal Bureau voor Genealogie (CBG) en de Gemeentelijke Basisadministratie (GBA) kon de vitale status en overlijdensdatum van alle deelnemers worden vastgesteld tot en met 31 december 2012. Doodsoorzaakgegevens werden opgevraagd bij het Centraal Bureau voor de Statistiek (CBS).

In 2012 werd het "Rainbow-TMA project" gestart, met als doel om bestaande cohorten te verrijken met zogenaamde Tissue MicroArray's (TMA's) en DNA. Formaline gefixeerd en in paraffine ingebedde (FFPE) blokken met tumorweefsel en het bijbehorende normale weefsel van CRC-patiënten werden opgevraagd bij pathologielaboratoria in heel Nederland. Van deze FFPE-blokken werden weefselcoupes gesneden en gekleurd met hematoxyline en eosine (H&E). Deze H&E-gekleurde weefselcoupes werden beoordeeld door pathologen, en de gebieden met de hoogste tumordichtheid werden gemarkeerd voor TMA-constructie. Vanuit deze gemarkeerde gebieden werden drie 0.6 mm grote tumorkernen en drie kernen van bijbehorend normaal weefsel bemonsterd en verzameld in TMA-blokken.

Voor het onderzoek gepresenteerd in dit proefschrift werden weefselcoupes van deze TMA-blokken onderworpen aan immunohistochemie (IHC). Het doel hiervan was het aantonen van eiwitten in tumorweefsel die aan het Warburg-effect gerelateerd zijn (LDHA, GLUT1, MCT4, PKM2, p53, PTEN), evenals het aantonen van mismatch repair (MMR)-gerelateerde eiwitten (MLH1, MSH2). De met IHC gekleurde weefselcoupes werden beoordeeld door drie getrainde niet-pathologen. Daarnaast werd een willekeurige 10% van alle gekleurde weefselcoupes beoordeeld door een ervaren histopatholoog. Expressieniveaus (laag, matig, hoog) van de aan het Warburg-effect gerelateerde eiwitten werden gecombineerd door middel van een somscore. Op basis van deze somscore werden CRC-patiënten onderverdeeld in drie Warburg-subtypen: Warburg-laag, Warburg-matig, en Warburg-hoog. Ook werd het beschikbare tumor DNA van CRC-patiënten gescreend op mutaties in *RAS* (*KRAS, NRAS, HRAS*), *PIK3CA*, *BRAF*, en *MET*. Op basis van deze mutaties (mut) en MMR-status werden patiënten onderverdeeld in acht mutatiesubgroepen: (1) wildtype + $MMR_{proficiënt}$, (2) $KRAS_{mut}$ + $MMR_{proficiënt}$, (3) $KRAS_{mut}$ + $PIK3CA_{mut}$ + $MMR_{proficiënt}$, (4) $PIK3CA_{mut}$ + $MMR_{proficiënt}$, (5) $BRAF_{mut}$ + $MMR_{proficiënt}$, (6) $BRAF_{mut}$ + $MMR_{deficiënt}$, (7) andere (combinaties

van) mutaties + $MMR_{proficiënt}$, en (8) andere (combinaties van) mutaties + $MMR_{deficiënt}$.

Na exclusie van patiënten waarvan het tumorweefsel niet voldeed aan de IHC-kwaliteitscontrole, waren er 2.394 CRC-patiënten beschikbaar voor Warburg-subtypering (**Hoofdstuk 3 en 5**). Voor 2.344 patiënten was er complete data beschikbaar voor subgroepering op basis van mutatiedata en MMR-status (**Hoofdstuk 4**). De relatie tussen adjuvante therapie en prognose kon worden onderzocht voor 1.793 CRC-patiënten (**Hoofdstuk 6**). Kaplan-Meier curves en Cox-regressieanalyses werden gebruikt om associaties tussen Warburg-subtypen en kanker-specifieke en algehele overleving te onderzoeken; al dan niet in combinatie met mutatiesubgroepen, factoren die aan energiebalans zijn gerelateerd op de lange termijn, of adjuvante therapie.

In **Hoofdstuk 2** hebben we onderzocht of getrainde niet-pathologen valide en reproduceerbare IHC-resultaten kunnen genereren. Dit werd onderzocht door de interbeoordelaarsbetrouwbaarheid tussen getrainde niet-pathologen en een ervaren histopatholoog te berekenen voor drie IHC-markers met verschillende subcellulaire lokalisaties (i.e., nucleus, membraan, cytoplasma). Daarnaast hebben we ook de intrabeoordelaarsbetrouwbaarheid onderzocht voor getrainde niet-pathologen. Onze resultaten wijzen erop dat adequaat getrainde niet-pathologen vergelijkbare IHC-resultaten kunnen genereren als een ervaren histopatholoog. Een combinatiescore van ten minste twee niet-pathologen resulteerde in de meest optimale resultaten, en werd dus gebruikt voor verder onderzoek.

In **Hoofdstuk 3** hebben we bestudeerd of Warburg-subtypering prognostische waarde heeft bij CRC. We hebben gevonden dat patiënten met Warburg-hoge CRC een slechtere kanker-specifieke en algehele overleving hebben vergeleken met patiënten met Warburg-lage CRC. Dit resultaat bleek onafhankelijk van bekende prognostische factoren, zoals de TNM-stadiëring.

In **Hoofdstuk 4** hebben we onderzocht of mutatiesubgroepen, gebaseerd op somatische mutaties in *RAS, BRAF, PIK3CA* en *MET*, evenals MMR status, geassocieerd zijn met kanker-specifieke en algehele overleving bij CRC. Daarnaast hebben we ook bestudeerd of onze Warburg-subtypering additionele prognostische waarde had binnen deze mutatiesubgroepen. Vergeleken met patiënten met wildtype + $MMR_{proficiënte}$ CRC, bleken patiënten met $KRAS_{mut}$ + $MMR_{proficiënte}$, $KRAS_{mut}$ + $PIK3CA_{mut}$ + $MMR_{proficiënte}$, $BRAF_{mut}$ + $MMR_{proficiënte}$, en andere (combinaties van) mutaties + $MMR_{proficiënte}$ CRC een slechtere kanker-specifieke en algehele overleving te hebben. Patiënten met $BRAF_{mut}$ + $MMR_{proficiënte}$ CRC bleken de slechtste overlevingskans te hebben, terwijl patiënten met andere (combinaties van) mutaties + $MMR_{deficiënte}$ CRC de beste overlevingskans bleken te hebben. Daarnaast hebben we gevonden dat $BRAF_{mut}$, $KRAS_{mut}$ + $MMR_{proficiënte}$, en andere (combinaties van)

mutaties + $MMR_{deficiënte}$ CRC gerelateerd kunnen zijn aan het Warburg-hoge subtype. Er werden geen statistisch significante verschillen gevonden tussen Warburg-subtypen binnen de mutatiesubgroepen.

In **Hoofdstuk 5** hebben we de associatie tussen factoren die aan energiebalans zijn gerelateerd op de lange termijn (i.e., BMI op volwassen leeftijd, niet-beroepsmatige fysieke activiteit, gewichtsverandering vanaf de leeftijd van 20 jaar, lichaamslengte, en blootstelling aan energierestrictie tijdens de kindertijd en adolescentie) en kanker-specifieke en algehele overleving bij CRC bestudeerd. Verder hebben we in gestratificeerde analyses onderzocht of deze associaties verschilden tussen de Warburg-subtypen. We hebben gevonden dat van alle lange termijn factoren die aan energiebalans zijn gerelateerd, alleen BMI op volwassen leeftijd geassocieerd was met een slechtere algehele overleving na CRC-diagnose. Verder hebben we in gestratificeerde analyses gezien dat associaties met overleving verschilden tussen Warburg-subtypen voor BMI op volwassen leeftijd, gewichtstoename vanaf de leeftijd van 20 jaar, energierestrictie tijdens de kindertijd en adolescentie, en wellicht voor lichaamslengte. Gewichtstoename vanaf de leeftijd van 20 jaar en een toename in lichaamslengte waren geassocieerd met een slechtere algehele overleving in patiënten met Warburg-hoge CRC. Een verhoogd BMI op volwassen leeftijd was alleen in patiënten met Warburg-matige CRC geassocieerd met een slechtere overlevingskans. Associaties tussen energierestrictie proxy's (i.e., woonplaats tijdens de Tweede Wereldoorlog of de Nederlandse Hongerwinter, werkloosheid van de vader tijdens de Nederlandse Economische Depressie) en overleving vertoonden geen consistente patronen wanneer analyses gestratificeerd werden op Warburg-subtype.

In **Hoofdstuk 6** hebben we onderzocht of Warburg-subtypering kan worden gebruikt om te voorspellen voor welke patiënten het meeste overlevingsvoordeel valt te behalen bij behandeling met adjuvante chemo- or radiotherapie naast chirurgische resectie. We hebben gezien dat alleen patiënten met Warburg-matige en mogelijk Warburg-hoge CRC een overlevingsvoordeel hadden van behandeling met adjuvante (chemo)therapie naast chirurgische resectie. Er werd geen overlevingsvoordeel gevonden na adjuvante (chemo) therapie bij patiënten met Warburg-lage CRC.

Dit proefschrift wordt in **Hoofdstuk 7** afgesloten met een samenvatting van de belangrijkste bevindingen, een algemene interpretatie van de onderzoeksresultaten, een bespreking van de methodologische overwegingen, en aanbevelingen voor toekomstig onderzoek. Al met al suggereren de resultaten gepresenteerd in dit proefschrift dat Warburg-subtypering prognostische waarde heeft bij CRC, onafhankelijk van bekende prognostische factoren zoals het TNM-stadiëringssysteem. Daarnaast lijkt Warburg-subtypering ook te kunnen worden gebruikt om te voorspellen voor welke patiënten het meeste overlevingsvoordeel kan worden behaald bij behandeling met adjuvante (chemo)therapie.

DANKWOORD

Nu zijn we dan aangekomen op de laatste, en misschien wel meest belangrijke, pagina's van mijn (!!!) proefschrift. En wat ben ik trots dat me dit gelukt is! Maar.. promoveren doe je natuurlijk niet alleen, en een leuke en leerzame tijd hebben al helemaal niet. Daarom wil ik iedereen die op een of andere manier aan dit proefschrift heeft bijgedragen graag bedanken, en een aantal mensen in het bijzonder.

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CURRICULUM VITAE

Kelly Offermans was born on 25th October 1992 in Voerendaal, the Netherlands. After completing secondary school (Sintermeertencollege, Heerlen, the Netherlands) in 2011, she studied Biomedical Sciences at Maastricht University. Kelly obtained her bachelor degree in 2014.

During her master's education, Kelly specialized in the field of Clinical Molecular Science. As a junior intern, she investigated the effect of oxidative phosphorylation (OXPHOS) inhibition on CAIX expression and HIF-1α stabilization at the Department of Radiation Oncology (MAASTRO, Maastricht University). During her senior master internship, Kelly investigated the role of PARP1 activity and NAD+ depletion in the development of mitochondrial dysfunction in skeletal muscle cells following chemotherapeutic treatment at the Department of Pharmacology and Toxicology, Maastricht University.

From 2018 to 2022, Kelly was employed as a PhD student in the Department of Epidemiology at Maastricht University. Under the supervision of Prof. dr. Piet A. van den Brandt, Prof. dr. Matty P. Weijenberg, and Dr. Kim M. Smits she investigated the potential prognostic value of metabolic Warburg-subtypes in colorectal cancer, using data from the Netherlands Cohort Study (NLCS).

As of November 2022, Kelly works as a postdoctoral fellow in the department of Epidemiology at Maastricht University.

LIST OF PUBLICATIONS

Publications in scientific journals

Van Gisbergen MW, **Offermans K**, Voets AM, Lieuwes NG, Biemans R, Hoffmann RF, Dubois LJ, Lambin P. Mitochondrial dysfunction inhibits hypoxia-induced HIF-1α stabilization and expression of its downstream targets. Frontiers Oncol. 2020 May;10:770.

Echle A, Grabsch HI, Quirke P, van den Brandt PA, West NP, Hutchins GGA, Heij LR, Tan X, Richman SD, Krause J, Alwers E, Jenniskens J, **Offermans K**, Gray R, Brenner H, Chang-Claude J, Trautwein C, Pearson AT, Boor P, Luedde T, Gaisa NT, Hoffmeister M, Kather JN. Clinical-grade detection of microsatellite instability in colorectal tumors by deep learning. Gastroenterology. 2020 Oct;159(4):1406-16.

Krause J, Grabsch HI, Kloor M, Jendrusch M, Echle A, Buelow RD, Boor P, Luedde T, Brinker TJ, Trautwein C, Pearson AT, Quirke P, Jenniskens J, **Offermans K**, van den Brandt PA, Kather JN. Deep learning detects mutations in cancer histology generated by adversarial networks. J Pathol. 2021 May;254(1):70-79.

Jenniskens JCA, **Offermans K**, Samarska I, Fazzi GE, Simons CCJM, Smits KM, Schouten LJ, Weijenberg MP, van den Brandt PA, Grabsch HI. Validity and reproducibility of immunohistochemical scoring by trained non-pathologists on Tissue MicroArrays. Cancer Epidemiol Biomarker Prev. 2021 Oct;30(10):1867-74.

Offermans K, Jenniskens JCA, Simons CCJM, Samarska I, Fazzi GE, Smits KM, Schouten LJ, Weijenberg MP, Grabsch HI, van den Brandt PA. Expression of proteins associated with the Warburg-effect and survival in colorectal cancer. J Pathol Clin Res. 2022 Mar;8(2):169-80.

Jenniskens JCA, **Offermans K**, Simons CCJM, Samarska I, Fazzi GE, Smits KM, Schouten LJ, Weijenberg MP, Grabsch HI, van den Brandt PA. Energy balance-related factors and risk of colorectal cancer expressing different levels of proteins involved in the Warburg-effect. Cancer Epidemiol Biomarker Prev. 2022 Mar;31(3): 633-46.

Echle A, Ghaffari Laleh N, Quirke P, Grabsch HI, Muti HS, Saldanha OL, Brockmoeller SF, van den Brandt PA, Hutchins GGA, Richman SD, Horisberger K, Galata C, Ebert MP, Eckardt M, Boutros M, Horst D, Reissfelder C, Alwers E, Brinker TJ, Langer R, Jenniskens JCA, **Offermans K**, Mueller W, Gray R, Gruber SB, Greenson JK, Rennert G, Bonner JD, Schmolze D, Chang-Claude J, Brenner H, Trautwein C, Boor P, Jaeger D, Gaisa NT, Hoffmeister M, West NP, Kather JN. Artificial intelligence for detection of microsatellite instability in colorectal cancer – a multicentric analysis of a pre-screening tool for clinical application. ESMO Open. 2022 Apr; 7(2):100400.

Jenniskens JCA, **Offermans K**, Simons CCJM, Samarska I, Fazzi GE, Smits KM, Schouten LJ, Weijenberg MP, Grabsch HI, van den Brandt PA. Energy balance-related factors in childhood and adolescence and risk of colorectal cancer expressing different levels of proteins involved in the Warburg-effect. Int J Cancer. 2022 Jun;150(11):1812-24.

Jenniskens JCA, **Offermans K**, Simons CCJM, Samarska I, Fazzi GE, van der Meer JRM, Smits KM, Schouten LJ, Weijenberg MP, Grabsch HI, van den Brandt PA. Energy balance-related factors and risk of colorectal cancer based on KRAS, PIK3CA, and BRAF mutations and MMR status. J Cancer Res Clin Oncol. 2022 Oct;148(10):2723-42.

Jenniskens JCA, **Offermans K**, Simons CCJM, Samarska I, Fazzi GE, van der Meer JRM, Smits KM, Schouten LJ, Weijenberg MP, Grabsch HI, van den Brandt PA.. Energy balance-related factors in childhood and adolescence and risk of colorectal cancer based on KRAS, PIK3CA, and BRAF mutations and MMR status. Mol Carcinog. 2022 Dec;61(12):1099-115.

Offermans K, Jenniskens JCA, Simons CCJM, Samarska I, Fazzi GE, van der Meer J, Smits KM, Schouten LJ, Weijenberg MP, Grabsch HI, van den Brandt PA. Association between mutational subgroups, Warburg-subtypes, and survival in patients with colorectal cancer. Cancer Med 2023 Jan;12(2):1137-56.

Offermans K, Jenniskens JCA, Simons CCJM, Samarska I, Fazzi GE, Smits KM, Schouten LJ, Weijenberg MP, Grabsch HI, van den Brandt PA. Association between Warburg-subtypes and survival in colorectal cancer patients treated with adjuvant therapy. J. Cancer Res. Clin. Oncol 2023 Feb.

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Offermans K, Jenniskens JCA, Simons CCJM, Samarska I, Fazzi GE, Smits KM, Schouten LJ, Weijenberg MP, Grabsch HI, van den Brandt PA. Association between long-term energy balance-related factors, Warburg-subtypes, and survival in patients with colorectal cancer.

Wagner JW, Reisenbüchler D, West NP, Niehues JM, Veldhuizen GP, Quirke P, Grabsch HI, van den Brandt PA, Hutchins GGA, Richman SD, Yuan T, Langer R, Jenniskens JCA, **Offermans K**, Mueller W, Gray R, Gruber SB, Greenson JK, Rennert G, Bonner JD, Schmolze D, James JA, Loughrey MB, Salto-Tellez M, Brenner H, Hoffmeister M, Truhn D, Schnabel JA, Boxberg M, Peng T, Kather JN. Fully transformer-based biomarker prediction from colorectal cancer histology: A large-scale multicentric study.

Steeghs JPJM, **Offermans K**, Jenniskens JCA, Samarska I, Fazzi GE, van den Brandt PA, Grabsch HI. Relationship between the Warburg effect in tumour cells and the tumour microenvironment in colorectal cancer patients: results from a large multicentre study.