

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

Kelly Offermans¹ , Josien CA Jenniskens¹ , Colinda CJM Simons¹ , Iryna Samarska² , Gregorio E Fazzi² , Kim M Smits² , Leo J Schouten¹ , Matty P Weijnen¹ , Heike I Grabsch^{2,3†*}  and Piet A van den Brandt^{1,4†*} 

¹Department of Epidemiology, GROW School for Oncology and Developmental Biology, Maastricht University Medical Center+, Maastricht, The Netherlands

²Department of Pathology, GROW School for Oncology and Developmental Biology, Maastricht University Medical Center+, Maastricht, The Netherlands

³Pathology and Data Analytics, Leeds Institute of Medical Research at St James's, University of Leeds, Leeds, UK

⁴Department of Epidemiology, Care and Public Health Research Institute (CAPRI), Maastricht University Medical Center+, Maastricht, The Netherlands

*Correspondence to: Piet A van den Brandt, Department of Epidemiology, GROW School for Oncology and Developmental Biology, Maastricht University Medical Center+, P.O. BOX 616, 6200 MD Maastricht, The Netherlands. E-mail: pa.vandenbrandt@maastrichtuniversity.nl and Heike I Grabsch, Department of Pathology, GROW School for Oncology and Developmental Biology, Maastricht University Medical Center+, P.O. BOX 5800, 6202 AZ Maastricht, The Netherlands. E-mail: h.grabsch@maastrichtuniversity.nl

†Authors share last authorship.

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 categorised 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 tumour-node-metastasis (TNM) stage III CRC (HR_{CRC-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.

Keywords: Warburg effect; glycolysis; colorectal cancer; prognosis; survival

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Introduction

Colorectal cancer (CRC) is the third most common cancer worldwide [1,2]. Despite advances in early

detection and treatment of CRC patients, CRC remains the second most deadly cancer worldwide, accounting for more than 900,000 deaths every year [1,2]. Currently, the tumour-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 tumours 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 signalling pathway, which is regulated by the tumour suppressor PTEN [11,12]. It has been proposed that the PI3K/AKT/mTOR signalling pathway is involved in ‘rewiring’ cancer metabolism from oxidative phosphorylation towards aerobic glycolysis through the regulation of three transcription factors: HIF-1 α , MYC, and p53 [13–18].

Aerobic glycolysis, also known as the ‘Warburg effect’, is characterised by increased glucose uptake and lactate secretion, even in the presence of oxygen [16,17,19]. First, glucose uptake by tumour 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 tumour 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 [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 hypothesised 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 material, Table S1). Tumour 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

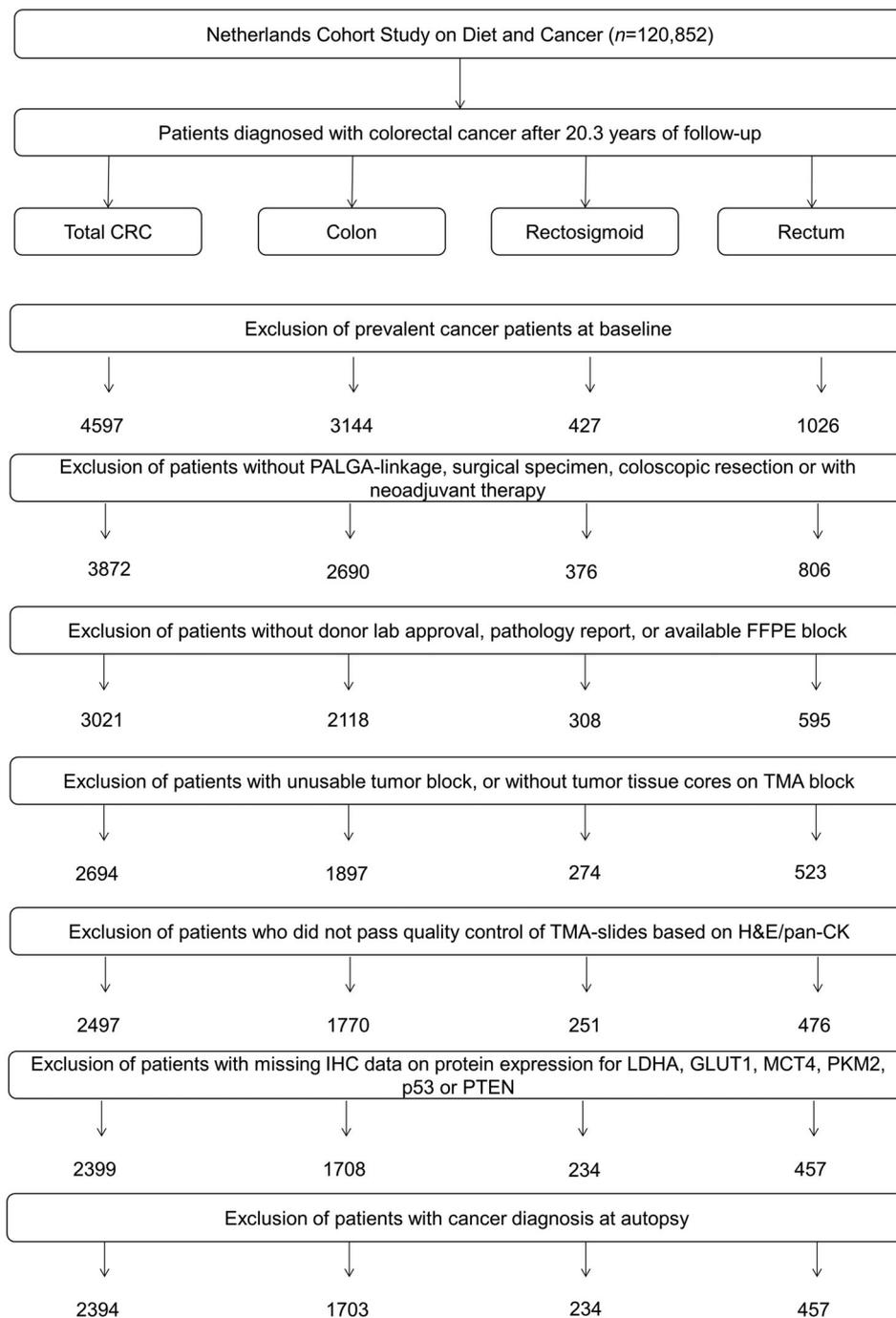


Figure 1. Flow diagram of the number of CRC patients available for analyses in the Netherlands Cohort Study (NLCS), 1986–2006.

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 tumour density were marked. Three 0.6 mm diameter cores with tumour and three cores with normal epithelium were sampled per patient (TMA-Grandmaster, 3D-Histech, Hungary). After excluding patients with unusable

FFPE blocks, tumour cores from 2,694 CRC patients were successfully assembled in 78 TMA blocks (Figure 1).

Immunohistochemistry

Five micrometre 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 material, 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 recognising 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 tumour core per patient, tumour cores of 2,497 CRC patients passed quality control (Figure 1).

After quality control, all tumour cores were scored by at least two observers, independently and blinded for patient characteristics (see supplementary material, 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 material, Table S4. In brief, the expression of p53 in the tumour 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 tumour cytoplasm); weak (staining of tumour cytoplasm weaker than adjacent stroma); moderate (similar staining intensity in tumour cytoplasm and adjacent stroma); or strong (staining of tumour cytoplasm stronger than adjacent stroma), according to the protocol of Richman *et al* [28]. GLUT1 and MCT4 expression were scored as negative; 1–10% tumour cells with membranous staining; 11–50% positive tumour cells; and >50% positive tumour cells. LDHA was evaluated according to the protocol of Koukourakis *et al* [29], with minor adaptations. LDHA expression was scored in the tumour cells as negative/weak cytoplasmic staining; 1–50% tumour cells strong cytoplasmic staining; >50% tumour cells strong cytoplasmic staining. PKM2 expression was scored in the tumour cells as negative/weak cytoplasmic staining; moderate cytoplasmic staining; 1–50%

tumour cells strong cytoplasmic staining; and >50% tumour cells strong cytoplasmic staining.

Supplementary material, 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 tumour cores (1–3 tumour cores per patient) were averaged and the value was rounded to the nearest scoring category. The average score was categorised 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 material, 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 categorised 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 material, Figure S1).

DNA mismatch repair status

DNA mismatch repair (MMR) status was assessed by IHC for MLH1 and MSH2 proteins (see supplementary material, 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 tumour served as internal positive controls. Tumours with loss of either MLH1 or MSH2 expression, in the presence of internal positive controls, were considered MMR deficient (dMMR). Tumours that expressed both MLH1 and MSH2 were considered MMR proficient (pMMR).

Clinical characteristics and follow-up

Information on patient and tumour characteristics, such as incidence date, TNM stage, tumour location, tumour differentiation grade, and initial treatment information was retrieved from the cancer registry or PALGA histopathology reports. Patients who were diagnosed at autopsy were excluded ($n = 5$) because the date of diagnosis was not known, 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.

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 tumour 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, tumour 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 material, 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 $\geq 10\%$ 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 material, 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 tumour location,

Table 1. Clinical characteristics of the colorectal cancer patients within the Netherlands Cohort Study (NLCS, 1986–2006, total $n = 2394$) according to Warburg-subtypes.

Clinical characteristics	Total CRC patients ($n = 2394$)	Warburg-subtypes			P value*
		Warburg- low ($n = 695$)	Warburg- moderate ($n = 858$)	Warburg- high ($n = 841$)	
Year of diagnosis					
1986–1988	109 (4.6)	38 (5.5)	35 (4.1)	36 (4.3)	0.211
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)	
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 [†]
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)	
Tumour location, n (%)					
Colon	1703 (71.1)	467 (67.2)	608 (70.9)	628 (74.7)	0.027
Rectosigmoid	234 (9.8)	81 (11.7)	81 (9.4)	72 (8.6)	
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)	0.001
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)	
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)	
Tumour extension (pT), n (%)					
T1	101 (4.2)	39 (5.6)	35 (4.1)	27 (3.2)	0.007
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)	
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 involvement (pN), n (%)					
N0	1247 (52.1)	377 (54.2)	450 (52.5)	420 (49.9)	0.006
N+	870 (36.3)	220 (31.7)	314 (36.6)	336 (40.0)	
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)	<0.001
Moderate	1571 (65.6)	463 (66.6)	565 (65.9)	543 (64.6)	
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)	0.181
Yes	499 (20.8)	137 (19.7)	185 (21.6)	177 (21.1)	
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)	0.001
Yes	254 (10.6)	58 (8.4)	79 (9.2)	117 (13.9)	
Unknown	24 (1.0)	9 (1.3)	4 (0.5)	11 (1.3)	

*P value for the Chi-square test, unless otherwise specified.

†P value for the Kruskal–Wallis test.

TNM stage, tumour 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 tumours located in the colon, whereas the Warburg-low and -moderate subtypes were more often observed in tumours located in the rectum or rectosigmoid ($p = 0.027$). Furthermore, the Warburg-high subtype was more common in TNM stage IV tumours, whereas Warburg-low and -moderate subtypes were more common in TNM stage I tumours ($p = 0.001$). The Warburg-high subtype was more frequently observed in tumours with a higher primary tumour extension (pT, $p = 0.007$) and tumours with lymph node involvement (pN, $p = 0.006$). Lastly, Warburg-high tumours were more often poorly differentiated ($p < 0.001$) and MMR deficient ($p < 0.001$) compared to Warburg-low and -moderate tumours.

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 tumours 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 tumours 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% CI 1.05–1.35 respectively) (Table 2). No significant difference

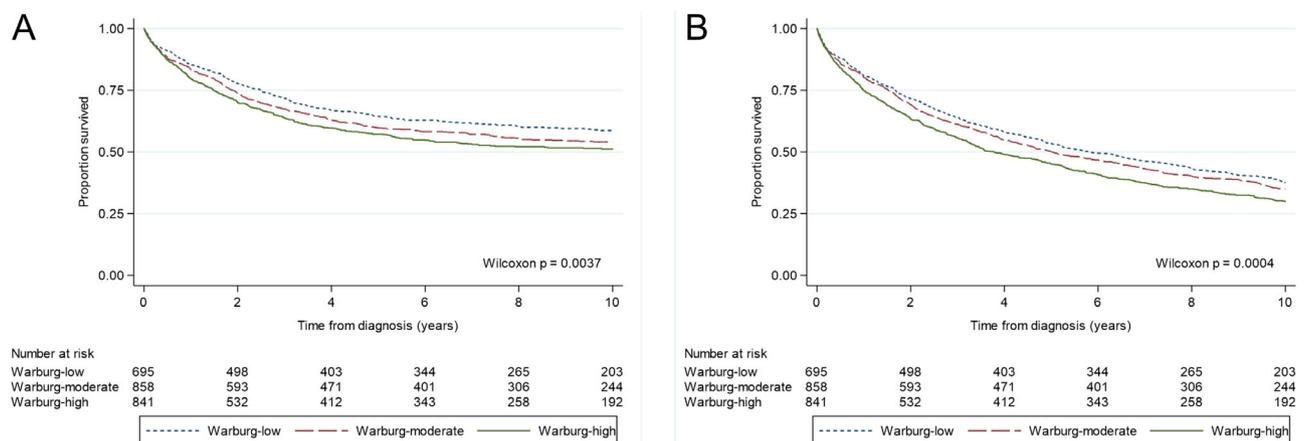


Figure 2. Kaplan–Meier curves according to metabolic subtypes (i.e. ‘Warburg-low’, ‘Warburg-moderate’, ‘Warburg-high’) in colorectal cancer patients within the Netherlands Cohort Study (NLCS, 1986–2006, total $n = 2394$), showing (A) CRC-specific survival and (B) overall survival.

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

	<i>n</i>	CRC-specific survival			Overall survival		
		CRC deaths (%)	HR (95% CI)		Deaths (%)	HR (95% CI)	
			Univariable	Multivariable-adjusted*		Univariable	Multivariable-adjusted*
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)

*Adjusted for age at diagnosis, sex (men/women), tumour 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).

in survival was observed for CRC patients with Warburg-moderate compared to Warburg-low tumours.

Univariable and multivariable-adjusted HRs for other relevant prognostic factors included in the model are shown in supplementary material, Table S7. In multivariable-adjusted analyses, age at diagnosis (per year), TNM stage, and tumour 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 tumour location and CRC-specific or overall survival.

Next, we stratified CRC patients by TNM stage to assess the disease stage-dependent prognostic value of

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*		Univariable	Multivariable-adjusted*
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)

*Adjusted for age at diagnosis, sex (men/women), tumour location (colon/rectosigmoid/rectum), differentiation grade (well/moderate/poor/unknown), adjuvant therapy (yes/no/unknown) and MMR deficiency (yes/no/unknown).

Table 4. Tumour 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*		Univariable	Multivariable-adjusted*
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)

*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).

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 material, Figure S2). Univariable Cox regression analyses revealed that patients with Warburg-high tumours 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 tumours 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).

In addition, CRC-specific survival differed between Warburg-subtypes in patients with tumours located in the rectosigmoid ($p = 0.0003$) (supplementary material, Figure S3). Patients with Warburg-high tumours located in the rectosigmoid or rectum had a significantly worse CRC-specific and overall survival compared to patients with Warburg-low tumours (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 tumours 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 tumours in the rectum after multivariable-adjustment (*data not shown*).

Discussion

To our knowledge, this is the first study to identify the prognostic value of immunohistochemistry (IHC)-based Warburg-subtypes in colorectal cancer (CRC), in a large population-based series of CRC patients. Warburg-subtypes were characterised 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 tumours 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 tumours 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, cholesterologenic and mixed) in pancreatic ductal adenocarcinoma (PDAC), based on RNA-sequencing data of glycolytic and cholesterologenic 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 tumour, non-glycolytic stroma); (2) the null type (non-glycolytic tumour, non-glycolytic stroma); (3) the mixed type; and (4) the reverse Warburg type (non-glycolytic tumour, 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 tumour 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 tumour 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 tumour 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 tumour 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 tumour 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 was 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 tumour 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 categorise 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.

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Author contributions statement

PAvdB, HIG, CJMS, JCAJ and KO conceived the study. PvdB designed the methodology. PvdB and LJS collected the data. KO performed the formal analysis and investigation. PvdB, HIG, JCAJ and KO wrote the

original manuscript. All authors critically reviewed the manuscript and approved the final version. PAVdB and HIG acquired the financial support for the project leading to this publication and supervised the study.

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SUPPLEMENTARY MATERIAL ONLINE

Figure S1. Flow diagram of the process of combining core-level scores into patient-level Warburg-subtypes

Figure S2. Kaplan–Meier curves showing CRC-specific survival of Warburg subtypes within TNM stage I, II, III, and IV

Figure S3. Kaplan–Meier curves showing CRC-specific survival of Warburg subtypes within colon, rectosigmoid and rectum

Table S1. Acknowledgements for the Rainbow-TMA project

Table S2. Details of primary antibodies and staining protocols

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

Table S4. Scoring protocols and kappa values for inter- and intra-observer agreement of the six proteins incorporated in the Warburg-subtypes

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

Table S6. Individual protein expression data of colorectal cancer patients from the Netherlands Cohort Study according to tumour location and Warburg-subtypes

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