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Hypoxia driven gene expression is an independent prognostic factor in stage II and III colon cancer patients

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Statement of translational relevance

Hypoxia is a well known microenvironmental factor determining tumor behavior. Despite this knowledge, its influence is never taken into account in clinical practice. We looked at gene expression changes in colon cancer cells exposed to chronic hypoxia. The hypoxia induced expression pattern correlated with the clinical outcome of more than 200 colon cancer patients in published microarray data sets confirming the detrimental role of intratumoral hypoxia on prognosis. We further describe the development of a 6 gene Colon Cancer Hypoxia Score (CCHS) which was found prognostic in two additional patient cohorts. Its value was independent of other important variables such as disease stage, differentiation grade and vascular invasion. Moreover, the CCHS performed excellent in both fresh frozen as well as in paraffin embedded material.

Abstract

Purpose – Hypoxia is considered a major microenvironmental factor influencing cancer behavior. Our aim was to develop a hypoxia-based gene score that could identify high and low risk within stage II and III colon cancer patients.

Experimental design – Differential gene expression of CaCo-2 colon cancer cells cultured in chronic hypoxia versus normoxia was tested for correlation with prognostic variables in published microarray data sets. These data sets were further used to downsize and optimize a gene score, which was subsequently determined in paraffin embedded material of 126 patients with colon cancer treated in our center.

Results – In the CaCo-2 cells, 923 genes with a 2-fold change and Limma corrected $p \leq 0.0001$ were found differentially expressed in hypoxia versus normoxia. We identified 21 genes with prognostic value and overlapping in three different training sets and (n=224). With a fourth published data set (n=177), the six gene Colon Cancer Hypoxia Score (CCHS) was developed. Patients with low CCHS showed a significant better disease free survival at three years (77.3%) compared to high CCHS patients (46.4%) (Log rank, p=0.006). This was independently confirmed in an external patient cohort of 90 stage II patients (86.9% vs 52.2%, p=0.001).

Conclusions – Hypoxia driven gene expression is associated with high recurrence rates in stage II and III colon cancer. A 6-gene score was found to be of independent prognostic value in these patients. Our findings require further validation and incorporation in the current knowledgee on molecular classification of colon cancer.

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Introduction

In Western countries, colorectal cancer (CRC) is the third most common cancer in both men and women and the second leading cause of cancer-related death, accounting for approximately 500.000 deaths annually (1). Tumor stage (American Joint Committee on Cancer - AJCC) at presentation is the main factor for therapeutic decisions and prognostic estimates. Patients with stage II disease generally undergo surgery only, whereas in stage III, adjuvant chemotherapy after resection of the affected bowel segment and attached lymph nodes is the preferred treatment option (2). However, data from historical trials shows that 60% of stage III patients do not recur following surgery only whereas 20% of stage II patients do have recurrent disease after resection (3, 4). These findings have led to an extensive search for prognostic markers that could classify CRC patients more accurately, reducing over- or undertreatment. The finding that stage II patients with a high degree of tumor microsatellite instability (MSI-H) are at very low risk of recurrence helps guiding treatment (5). Driven by the success of this approach in breast cancer (6), extensive research has led to the development of dozens of prognostic gene signatures for colon cancer (7). Although used by some oncologists, gene signatures are currently not approved by the FDA nor adopted by any CRC treatment guideline (8).

In this study, the aim was to develop a robust prognostic gene score to improve the subclassification of stage II and III colon cancer patients using a mechanism based approach previously described for hepatocellular carcinoma (9). It is now recognized that

microenvironmental factors such as hypoxia play a role in tumor behavior. Exposed to low oxygen supplies cancer cells become more aggressive, invasive and resistant to therapy (10).

In vitro, we identified the differentially expressed genes under chronic hypoxia versus normoxia. Using bioinformatics on 3 published sets of expression data with corresponding clinical information we could identify 21 genes, related to both hypoxia and prognosis. Further optimization of the model led to a Colon Cancer Hypoxia Score (CCHS) of 6 genes which we evaluated in formalin-fixed, paraffin-embedded (FFPE) tissue of our own patient cohort as well as in an external data set.

Methods

Cell culture and RNA isolation

Method details are described in the supplementary files. Briefly, as *in vitro* model we used the human adenocarcinoma cell line CaCo-2 (HTB-37; ATCC, Rockville, MD, USA). Cells were grown in a humidified incubator (Sanyo MCO-18M O2/CO2 incubator, Osaka, Japan) (5% CO_2 at 37°C) in MEM medium (InVitrogen, Merelbeke, Belgium) supplemented with 10% fetal calf serum, 2 mM L-glutamine, 100 U/ml penicillin, 100 µg/ml streptomycin and 1 mM sodium pyruvate.

For the determination of chronic hypoxia conditions by RT-PCR, cells were seeded in 25cm^2 culture flasks (10^6 cells/flask) that were placed in either 2% O_2 or 20% O_2 after 24h attachment. All culture conditions were performed in triplicate and cells were collected for RNA isolation at 0, 10, 24, 48 and 72 hours.

For microarray analysis, cells were seeded at 3x10^6 in 75 cm^2 tissue culture flasks (n=4) at 20% O_2 and were grown until 70% confluence. After reaching near-confluence, 2 flasks were placed in a humidified incubator with hypoxic conditions (2% O_2, 5% CO_2 at 37°C) for 72 h, while two other flasks remained in normoxic conditions (20% O_2).

Cells were harvested after trypsin treatment and mixed with Trizol (InVitrogen). RNA was isolated with the RNeasy Kit (Qiagen, Chatsworth, CA) according to the manufacturer's instructions.

RT-PCR

RNA was reverse transcribed into cDNA using SuperScript II reverse transcriptase and random hexamer primers (InVitrogen). The PCR reaction was carried out using TaqMan Universal PCR Master Mixture and Assays-on-Demand™ Gene Expression products (Applied Biosystems,

Foster City, California). The primers used are listed in supplementary table S1. Beta-2 microglobulin was used as housekeeping gene. Real-time PCR amplification and data analysis were performed using the A7500 Fast Real-Time PCR System (Applied Biosystems, Foster City, California). The ΔΔCt-method was used to determine relative gene expression levels.

Microarray

Samples were hybridized on dual color Agilent's Human Whole Genome Oligo Microarray (Cat# G4112F, Agilent, Diegem, Belgium) that contained 44k 60-mer oligonucleotide probes representing around 41 000 well-characterized human transcripts. Sample processing, quality control and microarray data analysis is described in detail in the supplementary methods. To identify the highly significant differentially expressed genes under chronic hypoxic conditions, probes with a 2-fold change and a corrected p-value below 0.0001 after multiple testing correction were selected (11). Results were confirmed by performing RT-PCR on selected genes.

Correlation of the hypoxia gene expression pattern with prognosis in external data sets

Biomarker development and evaluation was done following the REMARK guidelines for tumor marker studies (12). In order to determine the clinical relevance of the hypoxia gene expression pattern we compared our findings with published microarray data sets containing corresponding clinical information.

We considered four data sets of colon carcinoma patients, published in Gene Expression Omnibus (GEO) (Table 1), all based on the GeneChip Human Genome U133 Plus 2.0 array from Affymetrix. In Jorissen et al., 63 microsatellite stable (MSS) and 11 unstable (MSI) colorectal cancer samples from the Royal Melbourne Hospital were investigated (13). In Aronow et al.,

recurrence status was available for 100 colon cancer patients after exclusion of mucosa samples (14). 232 patients with colon cancer and known clinical outcome were included in the study by Smith et al. (15). This set was divided into 55 samples from Vanderbilt Medical Center (referred to as VMC) and 177 samples from the Moffitt Cancer Center (MCC).

All data sets were independent of one another, except for the data set of Aronow et al., which shows partial overlap with MCC samples (own observations). For this reason, we used the data sets of Jorissen and Aronow and the VMC subset of Smith to determine the prognostic value of the hypoxia gene expression pattern with a global test by Goeman (16).

Subsequently, overlap between the hypoxia gene expression pattern and the three data sets was used to downsize the number of genes (prognostic signature, see figure 1).

The MCC subset of Smith was used to optimize this signature by backward regression analysis using SPSS package 19 (IBM, Armonk, New York). A probability of F with a p-value of 0.05 was set as the treshhold for entry and removal. This way, we selected those genes that contributed the most to the model and enriched them with a coefficient which describes the relative contribution of the gene. The obtained score was named the "Colon Cancer Hypoxia Score"(CCHS).

Prospective evaluation of the Colon Cancer Hypoxia score

The Leuven cohort – 162 patients with stage II or III colon cancer treated in our center between 2004 and 2006 were selected to test the performance of the CCHS. Patients with missing clinical data, patients with rectal cancer and those who did not survive at least one month after surgery were excluded. For this study, we used two parallel slides of FFPE material per patient. The first slide was H&E stained, and the tumor tissue marked by a pathologist. Tumor was then

dissected from non-tumorous tissue on the second slide and subsequently deparaffinized. RNA was extracted using a modified RNeasy FFPE protocol (supplementary methods). Quantification, sizing and quality control was performed with the Bioanalyzer platform (Agilent). Only samples with at least 50% of RNA fragments longer than 200 bases were selected. Samples were analyzed for expression of selected genes by the nCounter technique (NanoString Technologies, Seattle, WA) which gives a direct read-out of transcripts without the use of enzymatic reactions (17). The used CodeSet is described in supplementary table S2.

The Amsterdam cohort – The AMC-AJCCII-90 data set consists of gene expression data on fresh frozen tumor tissue of 90 stage II patients treated in the Academic Medical Center in Amsterdam between 1997 and 2006. The Affymetrix Human Genome U133 Plus 2.0 Array was used as microarray platform (GSE33113) (18).

Statistics

All data are presented as median with range. A p-value < 0.05 was considered significant. To determine an optimal cutoff value for our Colon Cancer Hypoxia Score, a receiver operating characteristic (ROC) curve was used. For the survival analysis, Kaplan-Meier survival curves and univariate and multivariate Cox proportional hazard ratio were used. All statistics were performed using SPSS package 19 (IBM).

Results

Dynamics in gene expression in CaCo-2

To investigate the dynamics of hypoxia related gene expression in our in vitro model and to determine the optimal timing for microarray analysis we performed RT-PCR at different time points for a set of 8 representative genes known to be responsive to hypoxia. Gene expression was tested at 0 h, 10 h, 24 h, 48 h and 72 h in hypoxia (2% O_2) versus normoxia. It should be noted that when CaCo-2 cells were kept beyond 72 hours their cultures tended to become super-confluent with partly detachment of the cells resulting in an uncontrolled fluctuation of the gene expression levels (data not shown). Most of the mRNA expression levels investigated showed a dynamic curve over time at 2% O_2 with exception of *BCL2* that did not change significantly at any of the time points investigated (supplementary figure S1). These observations support the assumption that the acute hypoxic state (up to 24 h) has a different gene expression pattern compared to the more chronic state (72 hours). As such, we chose the latter condition for our microarray experiment.

Microarray: primary data analysis and development of a Colon Cancer Hypoxia Score

Using Agilent technology, in CaCo-2 cells cultured for 72 hours at either 20% oxygen or in hypoxic conditions at 2% oxygen a total of 37,707 spots showed a representative signal of which 3,389 with a ^{2}log fold change >1 or <-1 and a Limma corrected p-value <0.05. These microarray data are available at NCBI under number GSE31079.

For the development of our gene signature, we started with the top 923 differentially expressed genes (with a ^{2}log fold change >1 or <-1 and a Limma corrected p-value <0.0001): the hypoxia gene expression pattern. Of those 923 genes, 704 were up-regulated and 219 down-

regulated in hypoxic conditions. The top altered genes and significant KEGG pathways involved are listed in supplementary table S3-S4.

Subsequently, the Goeman global test showed that the overall expression pattern of these hypoxia genes was significantly related to the prognostic factor considered for each of the three training data sets (p-value <0.0001 for Jorissen, p-value = 0.00028 for Aronow and p-value = 0.0107 for Smith VMC).

Next, when only keeping the significant genes with a z-score above 1, 530 genes remained for the data set of Jorissen, 360 genes for Aronow, and 338 genes for Smith VMC. Finally, genes for which the direction of altered expression did not correspond to the direction observed in vivo in at least 2 out of 3 data sets were removed. With this approach, we were able to downsize the number of genes to twenty-one, found to overlap between the three training data sets (figure 1). In this gene set of 21 genes, 17 genes were upregulated in hypoxic conditions and 4 were downregulated (table 2).

CaCo-2 cells contain a *TP53* mutation resulting in undetectable p53 protein levels (19). As such, *TP53* was not considered early in the development of our gene signature despite being clinically of potential prognostic importance in colon cancer (20). For these reasons, *TP53* was added afterwards to the in vitro-derived 21 hypoxia cancer genes.

Using a backward linear regression analysis with the 21 genes and *TP53* as independent variables and the AJCC disease stage of patients in a fourth data set (Smith MCC) as dependent variable, six genes showed the highest contribution to the model. These genes with their corresponding coefficient form the **Colon Cancer Hypoxia Score** (figure 1). The score consists of

the expression value of *BCCIP*, *GADD45B*, *INSIG2*, *BNIP3L*, *MPHOSPH6* and *TP53*. The latter three have an inverse effect on the score value. All genes are involved in processes related to cell cycle, apoptosis and DNA repair.

Prospective evaluation of CCHS

Of the 162 stage II/III patients treated in our center between 2004 and 2006, 129 were selected for nCounter analysis after exclusion of clinical abberant cases and quality control (figure 2). Gene expression level of the CCHS genes was successfully obtained in 126 samples (97.7%). The known clinical and pathological features of these patients are summarized in table 3. Estimated median disease free survival times were 66.2 months (95% CI 60 – 71.5) and 46 months (95% CI 37.5 – 54.5) for stage II and stage III patients, respectively.

Using an optimal cut-off (see supplementary figure S2), patients with a low CCHS showed to have significant better disease free survival (DFS) at three years compared to those with a high CCHS (77.3% vs 46.4% respectively, p=0.006). These DFS rates remained unchanged at five years. Median disease free survival was 62,2 months (95% CI 56.6-67.7) and 42.7 months (95% CI 33.4 – 52) for CCHS low and CCHS high patients respectively (p=0.006) (figure 3a). Patients with a favorable CCHS were more likely to be alive at three years (estimated overall survival 81.5% vs 66.6%, p = 0.044) and at five years (71.9% vs 59.1%, p = 0.036).

When disease AJCC stage was taken into account, survival analysis could identify three groups with significant difference in survival (figure 3b). Patients with stage II disease and unfavorable CCHS had almost similar disease behavior as stage III patients with a good (low) Colon Cancer Hypoxia Score. Moreover, the CCHS could identify a subgroup of stage III patients with an

estimated recurrence rate at three years of 75% and a median disease free survival of only 28.2 months (95% CI 16 – 40.4).

The characteristics of the 90 patients of the Amsterdam cohort are listed in table 3. Estimated disease free survival time for the whole cohort was 95.5 months (95% CI 85.8 – 105.2). Similar to the Leuven patient cohort, the CCHS identified two subgroups of stage II patients with different DFS at three years (86.9% vs 52.2%, $p < 0.001$) and five years (86.9% vs 52.2%, $p < 0.001$). Median disease free survival was 102.5 months (95% CI 93.1 – 111.9) vs. 55.4 months (95% CI 38.7 – 72) (p=0.001) (figure 3c). Hazard ratio for recurrence in CCHS high versus CCHS low patients was 3.970 (95% CI 1.6 – 9.8) ($p = 0.004$).

Subsequently, a univariate Cox regression analysis was performed on the Leuven cohort analyzing all known clinical and pathological variables. Four variables with significant prognostic value were included in a multivariate analysis. The multivariate Cox regression model confirmed that the Colon Cancer Hypoxia Score is a predictor for recurrence independent of disease stage, venous invasion and the number of examined lymph nodes (table 4).

Discussion

Colon cancer is associated with high morbidity and mortality and prognostic subclassification of stage II and III patients remains an ongoing clinical challenge. We describe the development and testing of a mechanism based prognostic gene score. Hypoxia as microenvironmental factor is well known for its influence on cancer behavior. If hypoxia does not induce cell death, it increases tumor aggressiveness, invasiveness and metastatic potential mainly through activation of the hypoxia-inducible factor (HIF) pathway (10). Despite this knowledge, hypoxia is

seldom taken into account in the clinical setting in part due to the lack of good endogenous hypoxia markers (21). HIF overexpression has been proven associated with prognosis, but HIF itself is not considered to be a hypoxia marker exclusively due to the fact that it can be activated in various settings including oncogene drive (22). Our method shows a correlation between the altered expression of hundreds of genes under hypoxic conditions in vitro, with that of tumors in published microarray data sets with clinical data available. This confirms the role of intratumoral hypoxia in disease behavior as was previously shown in HCC (9).

Colon cancer is subject to extensive global scientific research and many gene signatures and scores have been developed in the past mostly based on hierarchical clustering. Some of these signatures have made it to commercially available tests. Although a promising approach to capture the complexity of cancer biology, few signatures show good power in external patient cohorts (7). We tried to address these limitations by using a mechanism-driven approach and involving different data sets in the development of our signature. This resulted in a workable amount of genes derived from data of over 400 patient samples. Moreover, we hypothesized that the optimization process with a backward regression model has added power to the prognostic value of the genes.

p53 is a known important tumor suppressor in colon cancer and its pathway is modulated by hypoxia (23). This is further supported by our findings that, in vitro under hypoxic conditions, the p53 pathway is a highly significant altered KEGG pathway (supplementary table S4 and figure S3). For this reason and those stated above, *TP53* was appended to the gene list in the knowledge that the backward regression analysis would eliminate this gene immediately in the

absence of an added value to the model. The fact that it was withheld in the CCHS suggests that *TP53* gene expression is of prognostic value for colon cancer patients.

Evaluation of the CCHS was done in formalin-fixed paraffin-embedded material. For mRNA level measurements, this type of material often presents a challenge as it is subjected to RNA degradation. After strict quality control (figure 2), the nCounter platform showed excellent performance and reliability for determining the expression levels of all 6 CCHS genes. Independent of other clinical and pathological parameters, the CCHS was found to predict relapse in stage II and III colon cancer. In stage II patients, it identified a high risk subgroup that might benefit from adjuvant therapy. Furthermore, stage III patients with unfavorable CCHS suffer from high recurrence rates.

Recently, progress has been made in the field of molecular classification of colon cancer based on gene expression clustering, epigenetic markers and therapy response. This approach led to the proposition of 3 to 6 CRC subtypes (18, 24, 25). De Sousa *et al* identified two classes which seem to represent the known subgroups of MSI/CIMP and chromosomal-instable (CIN) tumors respectively (18). A third class, previously unidentified and related to serrated polyps, was associated with a poor prognosis. Tumors of this subgroup have stemness features, which is in line with others who have isolated a stem cell or epithelial-to-mesenchymal transition (EMT) associated subgroup of colon cancer patients with high recurrence rates (24-26). We see a striking overlap between the KEGG pathways involved in adapting to hypoxic conditions and those pathways attributed to the stem cell-like phenotype proposed by Marisa *et al* (25) (supplementary table S4). Further prospective validation in a larger patient cohort is needed to

clarify whether a high CCHS is indicative for the stemness subtype. If so, the limited number of genes together with the performance in FFPE samples render our CCHS a potential clinical tool.

This study has some limitations. Unfortunately, at the time of study onset, extensive clinical data were not available for some of the published data sets used for CCHS development. This forced us to use surrogate parameters such as microsatellite instability which could influence the power of the obtained score. During study design, after careful evaluation of the available information, considerable overlap between different data sets published in the Gene Expression Omnibus was noticed. For example: most samples of the data set provided by Aronow et al. (GSE5206) have also been included in both GSE14333 and GSE17536 (own observations). This requires great caution when designing studies with this widely used published data. In our case, we compared the individual patient data of the data sets and found that there was a partial overlap between the database by Aronow and Smith MCC. In our study design, the latter was only involved in optimization of the gene signature and this overlap should therefore not affect our conclusions.

When testing the performance of the CCHS we excluded rectal cancer. Despite the obvious similarities between colon and rectal cancer, we wanted to exclude the differences in treatment modalities as a confounder. As such, our results cannot be extrapolated to these patients.

Finally, our study was designed to evaluate the CCHS as a prognostic marker. Further research is needed to determine whether the score has predictive abilities, ie. potency to identify patients likely to benefit from adjuvant treatment.

In conclusion, we discovered that the gene expression pattern of CaCo-2 cells cultured in hypoxia shows good correlation with clinical parameters of colon cancer patients in published microarray data sets. This confirms the importance of intratumoral hypoxia in disease behavior. From this point, a 6 gene Colon Cancer Hypoxia Score was developed which proved to be an independent prognostic biomarker for relapse in stage II and III colon cancer.

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Legends to tables and figures

Table 1: Clinical overview of published microarray data sets used for global performance testing of the hypoxia expression pattern and for development and optimization of the Colon Cancer Hypoxia Score.

Abbreviations: NA, not available – MSS, microsatellite stable – MSI, microsatellite instable

Table 2: Overview of the 21 gene signature with their relative direction in vitro under hypoxia and cellular processes involved. *TP53* was added to the signature and eventually incorporated in the CCHS.

Abbreviations: CCHS, colon cancer hypoxia score

Table 3: Clinical and pathological features of the Leuven cohort as well as the AMC-AJCCII-90 data set (16). Abbreviations: NA, not available – Median FU, median follow-up defined as time to relapse or last contact.

Table 4: Univariate and multivariate Cox regression analysis on all clinical and pathological variables in the Leuven cohort. The Colon Cancer Hypoxia Score (CCHS) is an independent predictor of disease recurrence.

Abbreviations: CI, Confidence Interval

Figure 1: Process to obtain the Colon Cancer Hypoxia Score. Overlap between three published data sets and the hypoxia gene expression pattern in CaCo-2 cells was used to downsize the

number of genes to 21. After adding *TP53*, a backward regression analysis was performed to obtain a six gene score.

Figure 2: Inclusion and selection process of the stage II/III colon cancer patients in our center. Of the 162 colon cancer patients included in this study, after clinical and technical selection, the expression levels of 126 patients was successfully determined.

Figure 3a: Kaplan-Meier curve for disease free survival in the Leuven cohort (n = 126).

Figure 3b: AJCC disease stage and Colon Cancer Hypoxia Score (CCHS) devide patients in three groups with significant differences in disease free survival. When the patients of AJCC Patients with stage II disease and a high CCHS have comparable outcome to patients with stage III disease and a low CCHS.

Figure 3c: Kaplan-Meier curve for disease free survival in the Amsterdam cohort (n = 90).

Table 1

Author	**Jorissen**	**Aronow**	**Smith**	
Reference	13	14	15	
			VMC database	MCC database
Data set ID	GSE13294	GSE5206	GSE17537	GSE17536
Used for	Training signature	Training signature	Training signature	Developing score
Array type	Affymetrix Human Genome U133 Plus 2.0 Array			
Sample type	Fresh frozen	Fresh frozen	Fresh frozen	Fresh frozen
N samples	74	105	55	177
N Colon cancer	74	100	55	177
Rectal cancer included	Yes	Yes	Yes	Yes
N other		5 normal mucosa		
Age (median) (yrs)	NA	66	62	66
Min		26	23	26
Max		92	94	92
Sex (M/F)	NA			
Male		46 (46%)	26 (47%)	96 (54%)
Female		54 (54%)	29 (53%)	81 (46%)
AJCC stage	NA			
I		15 (15%)	4 (7%)	24 (14%)
II		29 (29%)	15 (27%)	57 (32%)
III		33 (33%)	19 (35%)	57 (32%)
IV		20 (20%)	17 (31%)	39 (22%)
NA		3 (3%)	0	0
Differentiation	NA			
Good		8 (8%)	1 (2%)	16 (9%)
Moderate		78 (78%)	32 (58%)	134 (76%)
Poor		10 (10%)	3 (5%)	27 (15%)
NA		4 (4%)	19 (35%)	0
Recurrence	NA			
Yes		23 (23%)	19 (34%)	36 (24%)
No		77 (77%)	36 (66%)	109 (62%)
NA				32 (18%)
Median FU (months)	NA	NA	50.2	42.3

Min			0.4	0.9
Max			111	142.5
Microsatellite instability		NA	NA	NA
MSS	63 (85%)			
MSI	11 (15%)			

Table 2

Official gene symbol	Direction In vitro	Gene name	Processes involved
BCCIP	▼	BRCA2 and CDKN1A-interacting protein isoform C	DNA metabolic / repair
BNIP3L	▲	BCL2/adenovirus E1B 19 kDa protein-interacting protein 3-like	apoptosis
BTBD14A	▲	nucleus accumbens-associated protein 2	unknown
DDIT4	▲	DNA damage-inducible transcript 4 protein	apoptosis / hypoxia
ERRFI1	▲	ERBB receptor feedback inhibitor 1	GTPase regulation
FAM46C	▼	hypothetical protein LOC54855	unknown
GADD45B	▲	growth arrest and DNA damage-inducible protein GADD45 beta	apoptosis
HBP1	▲	HMG box-containing protein 1	cell cycle
ING5	▼	inhibitor of growth protein 5	DNA metabolic / repair
INSIG2	▲	insulin-induced gene 2 protein	ER-nuclear signaling
KLF7	▲	Krueppel-like factor 7	cell morphogenisis
MPHOSPH6	▼	M-phase phosphoprotein 6	cell cycle
NNMT	▲	nicotinamide N-methyltransferase	N-methyltransferase
P4HA1	▲	prolyl 4-hydroxylase subunit alpha-1 isoform 1 precursor	metabolic processes
P4HA2	▲	prolyl 4-hydroxylase subunit alpha-2 isoform 1 precursor	metabolic processes
PLAUR	▲	urokinase plasminogen activator surface receptor isoform 3 precursor	metabolic processes
RHOU	▲	rho-related GTP-binding protein RhoU	cell cycle
TMCC1	▲	transmembrane and coiled-coil domains protein 1 isoform a	transmembrane signaling
UBE2H	▲	ubiquitin-conjugating enzyme E2 H isoform 1	proteolysis
ULBP2	▲	NKG2D ligand 2 precursor	transmembrane signaling
XIAP/BIRC4	▲	baculoviral IAP repeat-containing protein 4	apoptosis
TP53	NA	tumor protein p53	apoptosis/cell cycle

Used for CCHS
✓
✓
✓
✓
✓
✓

Table 3

	Leuven Cohort			AMC-AJCC-II
	All patients	Stage II	Stage III	
Patient number	126	71	55	90
Age (median) (yrs)	71.2	70	72.9	73.4
Min (yrs)	35.9	35.9	38.8	34.6
Max (yrs)	92.9	92.9	86.2	95.1
Sex				
Male	67 (53%)	34 (48%)	33 (60%)	42 (47%)
Female	59 (47%)	37 (52%)	22 (40%)	48 (53%)
Localisation				
Right sided tumor	63 (50%)	39 (55%)	24 (43,5%)	48 (53%)
Left sided tumor	63 (50%)	32 (45%)	31 (56,5%)	42 (47%)
T-stage				NA
T2	4 (3%)	0 (0%)	4 (7%)	
T3	109 (86,5%)	67 (94%)	42 (76,5%)	
T4	13 (10,5%)	4 (6%)	9 (16,4%)	
N-stage				NA
N0	71 (56%)	71 (100%)	0 (0%)	
N1	39 (31%)	0 (0%)	39 (71%)	
N2	16 (13%)	0 (0%)	16 (29%)	
AJCC stage				
II	71 (56%)	71 (100%)	0 (0%)	90 (100%)
III	55 (44%)	0 (0%)	55 (100%)	0 (0%)
Mucinous type				NA
Yes	36 (29%)	22 (31%)	41 (74,5%)	
No	90 (71%)	49 (69%)	14 (25,5%)	
Differentiation				NA
Good	14 (11%)	7 (10%)	7 (13%)	
Moderate	63 (50%)	40 (56,5%)	23 (42%)	
Poor	45 (36%)	20 (28%)	25 (45%)	
Undifferentiated	1 (1%)	1 (1,5%)	0 (0%)	
Not available	3 (2%)	3 (4%)	0 (0%)	

Peritoneal invasion				NA
Yes	1 (1%)	1 (1%)	0 (0%)	
No	125 (99%)	70 (99%)	55 (100%)	
Extramural venous invasion				NA
Yes	36 (28,6%)	11 (15%)	25 (45%)	
No	81 (64,3%)	59 (84%)	22 (40%)	
NA	9 (7,1%)	1 (1%)	8 (15%)	
Resection margin				NA
Positive	1 (1%)	0 (0%)	1 (2%)	
Negative	124 (98%)	71 (100%)	53 (96%)	
NA	1 (1%)	0 (0%)	1 (2%)	
Bowel perforation				NA
Yes	5 (4%)	3 (4%)	2 (4%)	
No	121 (96%)	68 (96%)	53 (96%)	
Examined lymph nodes				NA
12 or more	83 (66%)	49 (69%)	34 (62%)	
Less than 12	42 (33%)	22 (31%)	20 (36%)	
NA	1 (1%)	0 (0%)	1 (2%)	
Adjuvant chemotherapy				NA
Yes	63 (50%)	50 (70,4%)	42 (76,5%)	
No	63 (50%)	21 (29,6%)	13 (23,5%)	
Relapse				
Yes	35 (28%)	12 (17%)	23 (42%)	19 (21%)
No	91 (72%)	59 (83%)	32 (58%)	71 (79%)
Median FU (months)	45.42	49.14	23.1	39.3
Min (months)	1.3	1.3	1.5	1.6
Max (months)	76.4	76.4	73.9	120

Table 4

	Univariate Cox regression analysis					Multivariate Cox regression analys			
	Beta coefficient	p-value	Hazard Ratio	95,0% CI Lower	Upper	Beta coefficient	p-value	Hazard Ratio	95,0 Lower
Age	0.03	0.070	1.03	1.00	1.07				
Location (left vs right)	0.13	0.701	1.14	0.59	2.21				
Stage (III vs II)	1.23	**0.001**	3.41	1.69	6.86	0.95	**0.017**	2.58	1.19
Peritoneal invasion (yes vs no)	1.30	0.203	3.66	0.50	26.89				
Extramural venous invasion (yes vs no)	1.60	**< 0,001**	4.95	2.43	10.07	1.17	**0.003**	3.21	1.50
Resection margin (positive vs negative)	1.47	0.149	4.36	0.59	32.24				
Bowel perforation (yes vs no)	0.70	0.336	2.02	0.48	8.45				
Mucinous type (yes vs no)	-0.27	0.506	0.76	0.35	1.68				
Differentiation		0.856							
Differentiation (moderate vs good)	0.38	0.541	1.46	0.43	4.97				
Differentiation (poor vs good)	0.66	0.296	1.94	0.56	6.77				
Adjuvant chemotherapy (yes vs no)	0.19	0.587	1.20	0.62	2.35				
Colon Cancer Hypoxia Score (high vs low)	0.91	**0.008**	2.49	1.27	4.86	0.95	**0.013**	2.58	1.23
Lymph nodes examined (12 or more vs less than 12)	-0.73	**0.034**	0.48	0.25	0.95	-0.40	0.283	0.67	0.33

is
% CI
Upper
5.61
6.89
5.43
1.39

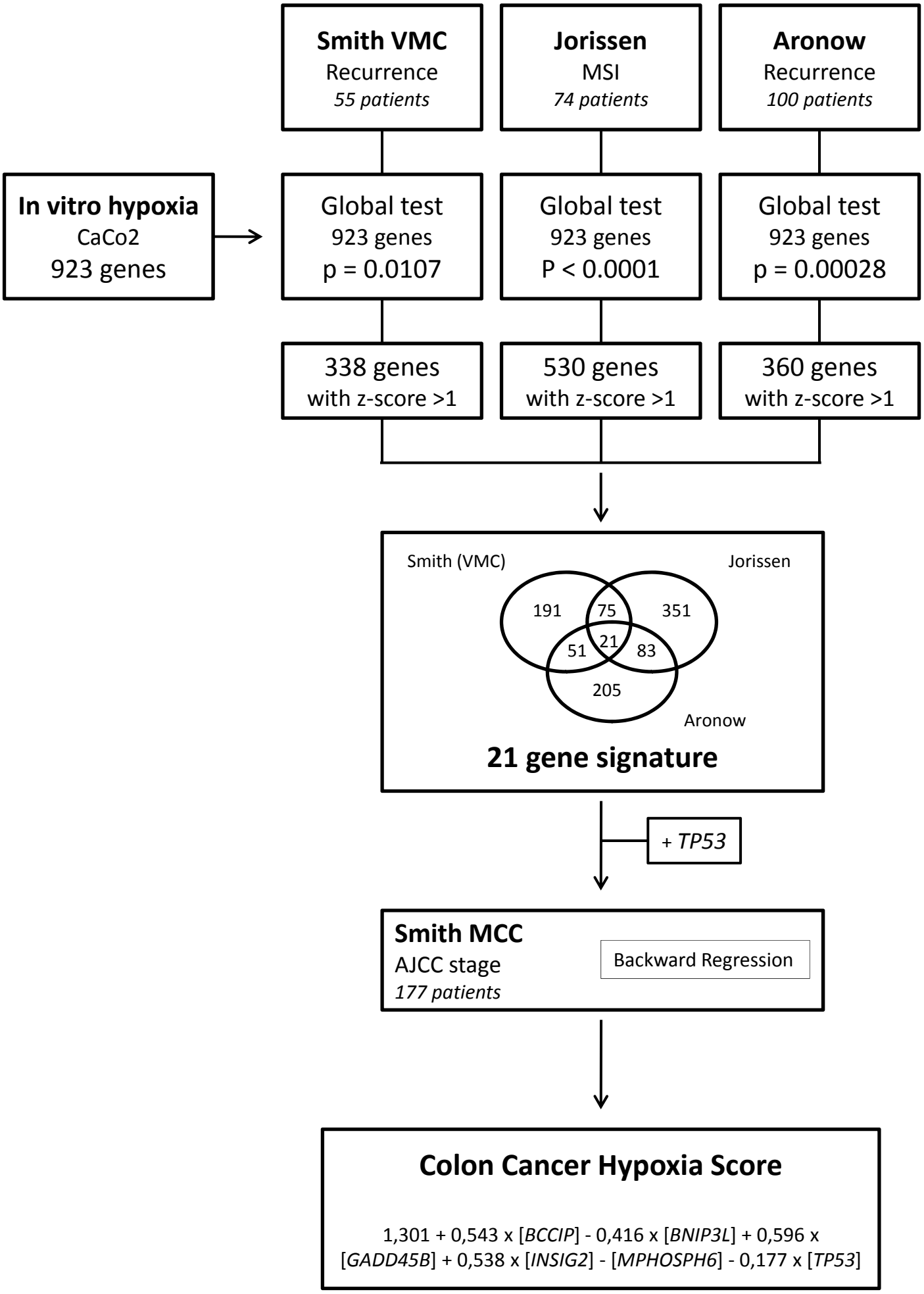
Smith VMC
Recurrence
55 patients
Jorissen
MSI
74 patients
Aronow
Recurrence
100 patients
In vitro hypoxia
CaCo2
923 genes
Global test
923 genes
p = 0.0107
Global test
923 genes
P < 0.0001
Global test
923 genes
p = 0.00028
338 genes
with z-score >1
530 genes
with z-score >1
360 genes
with z-score >1
Smith (VMC)
Jorissen
Aronow
191
75
351
51
21
83
205
21 gene signature
+ TP53
Smith MCC
AJCC stage
177 patients
Backward Regression
Colon Cancer Hypoxia Score
1,301 + 0,543 x [BCCIP] - 0,416 x [BNIP3L] + 0,596 x [GADD45B] + 0,538 x [INSIG2] - [MPHOSPH6] - 0,177 x [TP53]

Figure 1

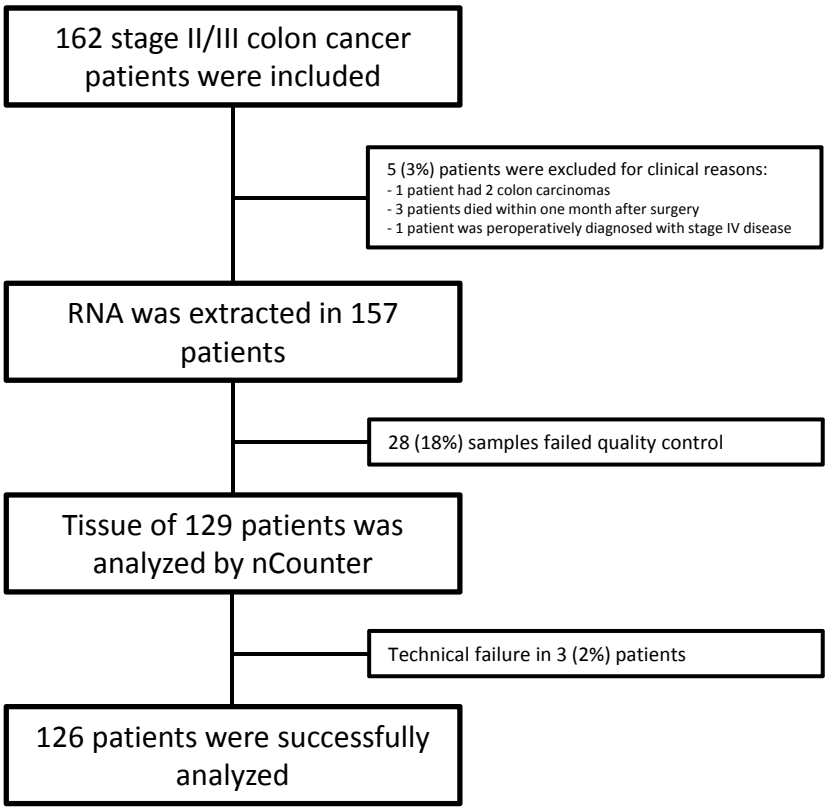
162 stage II/III colon cancer patients were included
5 (3%) patients were excluded for clinical reasons:
- 1 patient had 2 colon carcinomas
- 3 patients died within one month after surgery
- 1 patient was peroperatively diagnosed with stage IV disease
RNA was extracted in 157 patients
28 (18%) samples failed quality control
Tissue of 129 patients was analyzed by nCounter
Technical failure in 3 (2%) patients
126 patients were successfully analyzed

Figure 2

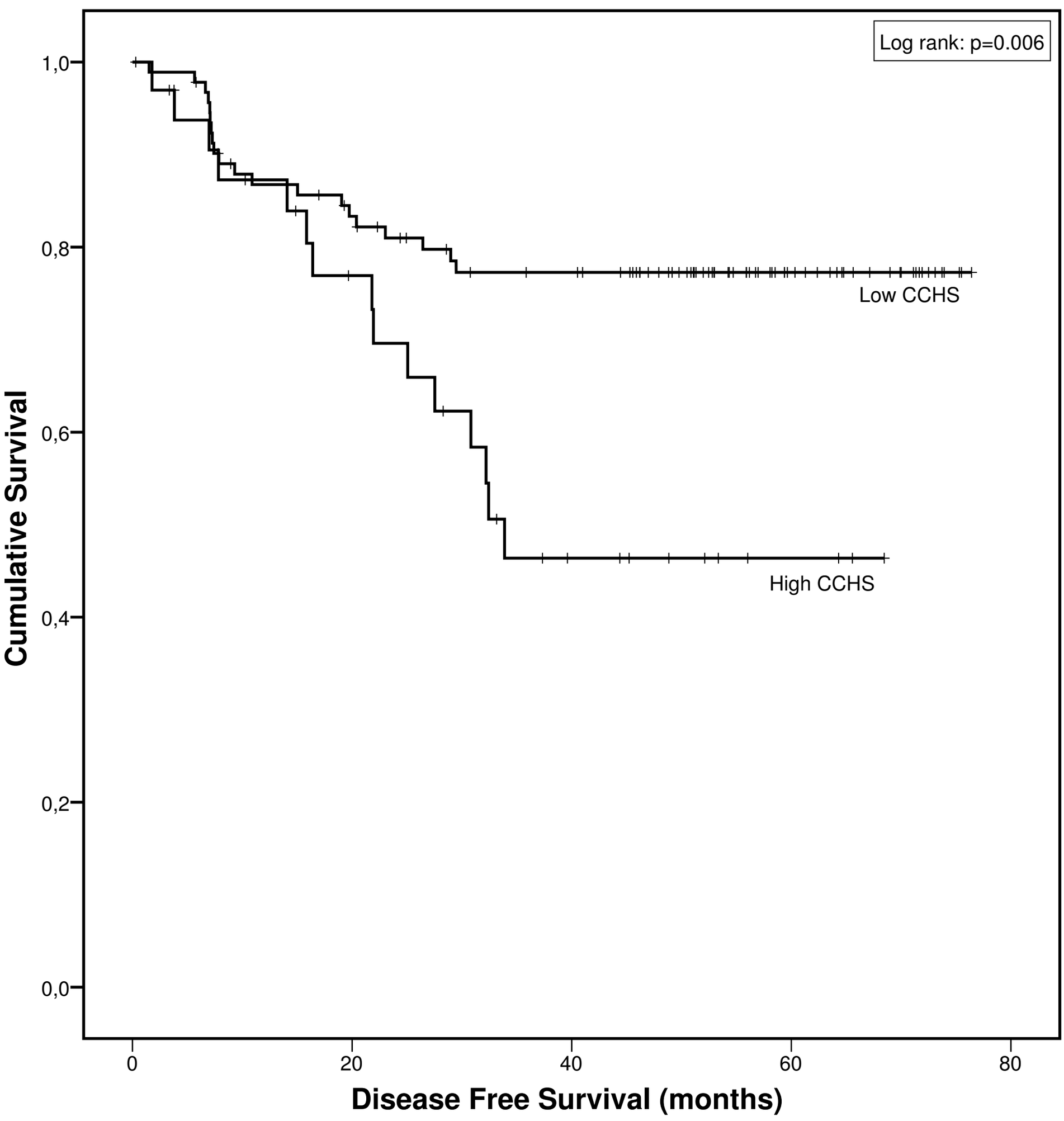
Log rank: p=0.006
Low CCHS
High CCHS
Cumulative Survival
1,0
0,8
0,6
0,4
0,2
0,0
0
20
40
60
80
Disease Free Survival (months)

Figure 3a

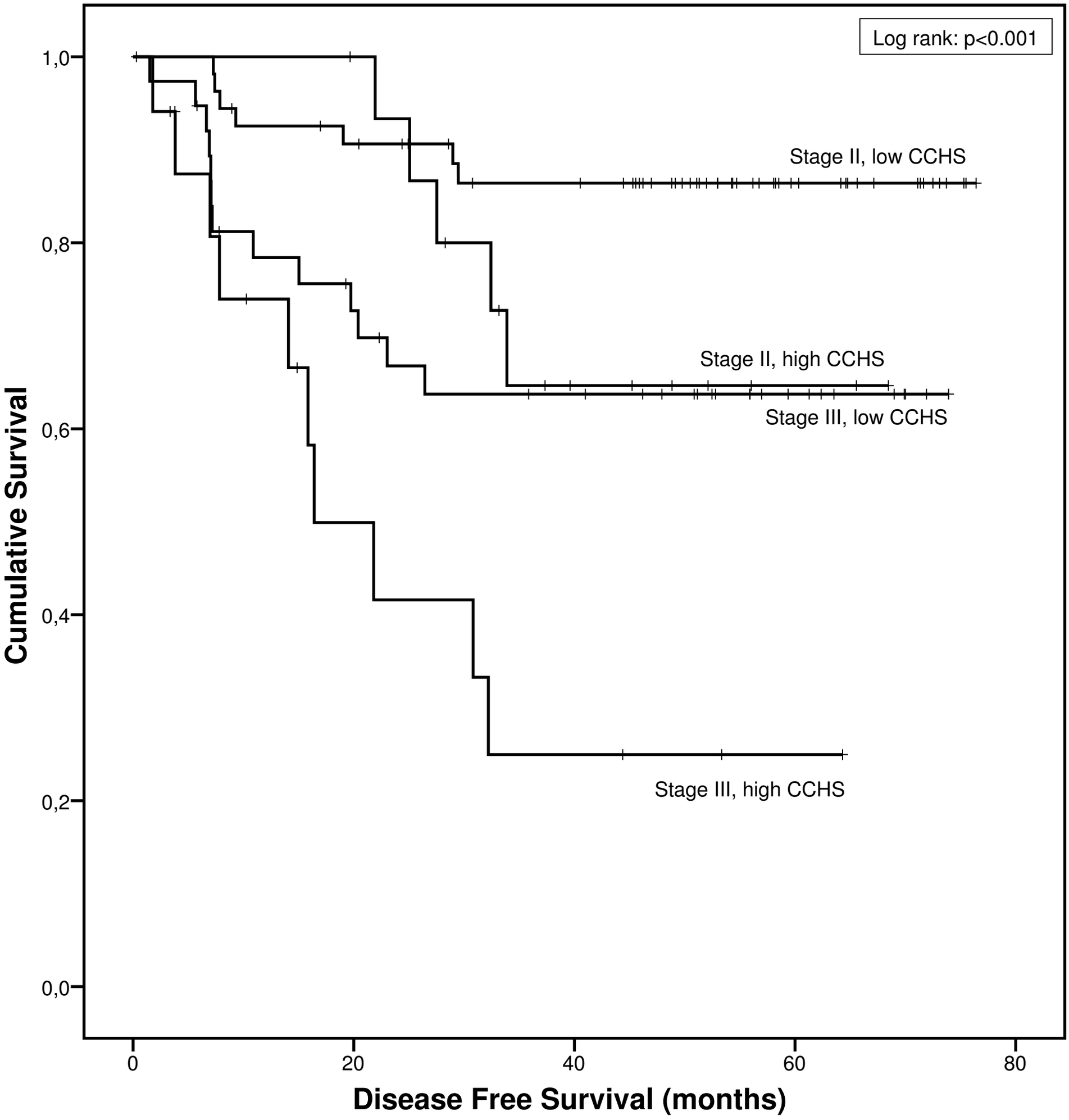
Log rank: p<0.001
Stage II, low CCHS
Stage II, high CCHS
Stage III, low CCHS
Stage III, high CCHS
Cumulative Survival
1,0
0,8
0,6
0,4
0,2
0,0
0
20
40
60
80
Disease Free Survival (months)

Figure 3b

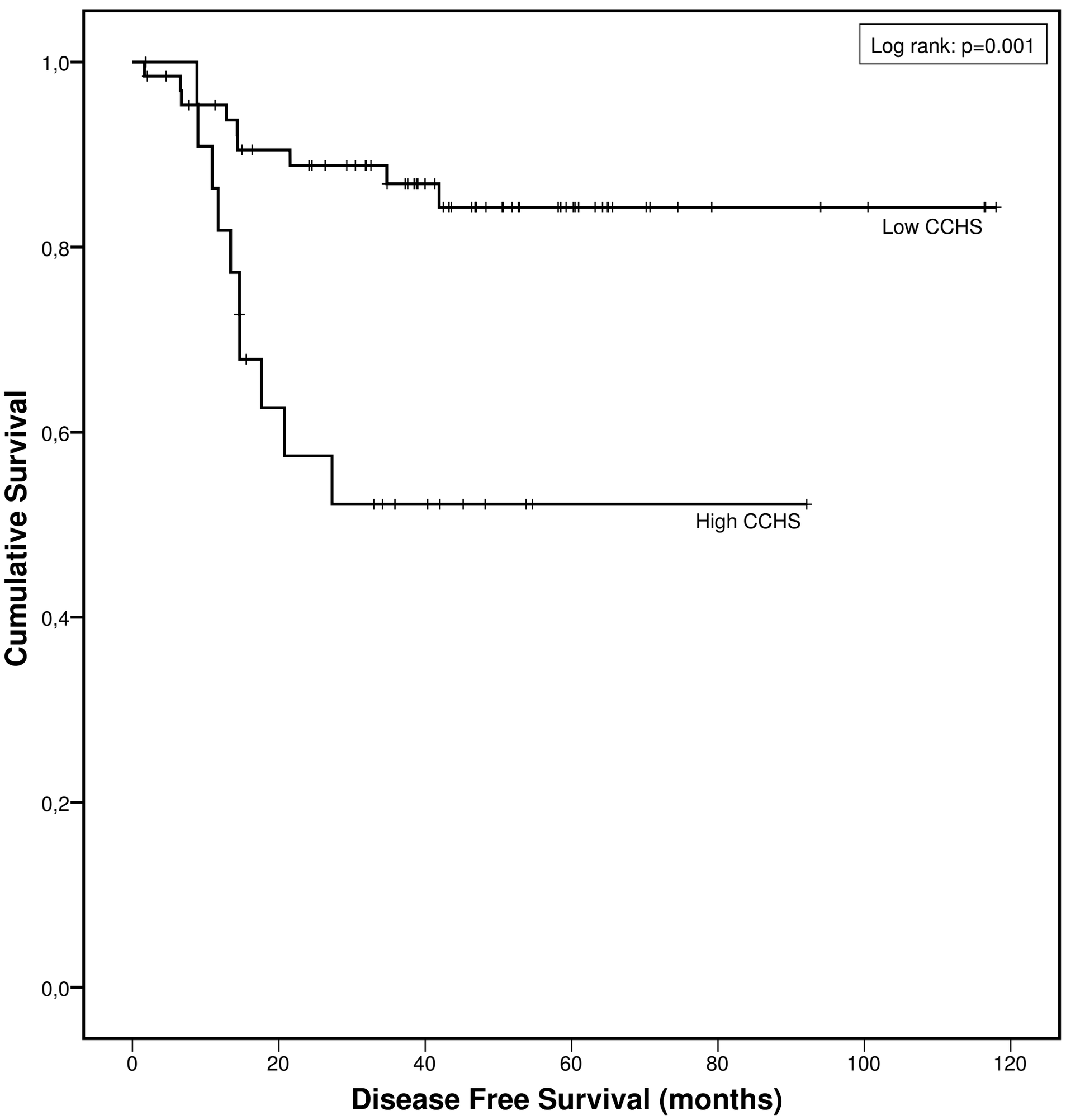
Log rank: p=0.001
Low CCHS
High CCHS
Cumulative Survival
1,0
0,8
0,6
0,4
0,2
0,0
0
20
40
60
80
100
120
Disease Free Survival (months)

Figure 3c