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Genes associated with metabolic syndrome predict disease-free survival in stage II colorectal cancer patients. A novel link between metabolic dysregulation and colorectal cancer

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ABSTRACT

Studies have recently suggested that metabolic syndrome and its components increase the risk of colorectal cancer. Both diseases are increasing in most countries, and the genetic association between them has not been fully elucidated. The objective of this study was to assess the association between genetic risk factors of metabolic syndrome or related conditions (obesity, hyperlipidaemia, diabetes mellitus type 2) and clinical outcome in stage II colorectal cancer patients. Expression levels of several genes related to metabolic syndrome and associated alterations were analysed by real-time qPCR in two equivalent but independent sets of stage II colorectal cancer patients. Using logistic regression models and cross-validation analysis with all tumour samples, we developed a metabolic syndrome-related gene expression profile to predict clinical outcome in stage II colorectal cancer patients. The results showed that a gene expression profile constituted by genes previously related to metabolic syndrome was significantly associated with clinical outcome of stage II colorectal cancer patients. This metabolic profile was able to identify patients with a low risk and high risk of relapse. Its predictive value was validated using an independent set of stage II colorectal cancer patients. The identification of a set of genes related to metabolic syndrome that predict survival in intermediate-stage colorectal cancer patients allows delineation of a high-risk group that may benefit from adjuvant therapy and avoid the toxic and unnecessary chemotherapy in patients classified as low risk. Our results also confirm the linkage between

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metabolic disorder and colorectal cancer and suggest the potential for cancer prevention and/or treatment by targeting these genes.

1. Introduction

Colorectal cancer (CRC) is the second most prevalent cancer worldwide (Kim et al., 2012) and the second most common cause of cancer death (Esposito et al., 2013). In Europe, CRC was the first in the ranking of cancers diagnosed in the Organization for Economic Cooperation and Development countries in 2008 (Esposito et al., 2013). Risk factors for CRC include family history of CRC and polyps, inflammatory bowel disease, and several lifestyle modifiable factors, such as obesity, lack of physical activity, cigarette smoking, and a westernized diet (Esposito et al., 2013; Kim et al., 2012).

In this sense, western dietary patterns, obesity, and physical inactivity are also risk factors for type 2 diabetes mellitus (DM) and may cause hypertension, dyslipidaemia, insulin resistance, elevated triglycerides, and low high-density lipoprotein (HDL)-cholesterol levels, all hallmarks of metabolic syndrome (MS) (Kim et al., 2007). MS has an increasing prevalence and incidence in the general population of western lifestyle countries and has become a major public health problem; the prevalence in the US population is 24% and between 24.6% and 30.9% in European countries (Pais et al., 2009).

According to the most current and widely used guideline proposed by the Third Report of the National Cholesterol Education Program Expert Panel on Detection, Evaluation, and Treatment of High Blood Cholesterol in Adults (Adult Treatment Panel III), MS is diagnosed when a patient has at least 3 of the following 5 conditions: (1) central or abdominal obesity measured by waist circumference ($\geq$102 cm [40 in] in men or $\geq$88 cm [35 in] in women); (2) fasting blood triglyceride levels $\geq$150 mg/dL (or receiving drug therapy for hypertriglyceridaemia); (3) fasting glucose $\geq$100 mg/dL (or receiving drug therapy for hyperglycaemia); (4) blood pressure $\geq$130/85 mm Hg (or receiving drug therapy for hypertension); (5) blood HDL-cholesterol levels ($<$40 mg/dL in men or $<$50 mg/dL in women, or receiving drug therapy for reduced HDL-cholesterol) (AHA, http://www.heart.org/HEARTORG/; Ishino et al., 2012).

In summary, MS is a cluster of various combinations of metabolic abnormalities suggested to play a main role in the development of cardiovascular diseases and DM (Aleksandrova et al., 2011). At the same time, it is known that DM has been associated with an increased risk for CRC in several recent epidemiological studies, particularly for right colon cancers (Kim et al., 2012). Furthermore, this association has been reinforced in different studies that suggest a beneficial effect of metformin treatment in CRC. Metformin, a biguanide that is widely used for treating hyperglycaemia and DM, has been suggested to exhibit a suppressive effect on tumourigenesis and cancer cell growth. Epidemiological, preclinical, and clinical evidence support the use of metformin as a potential anticancer agent, particularly for the treatment of cancers known to be associated with hyperinsulinaemia, such as breast and colon cancers (Dowling et al., 2011). Several clinical studies have shown significant associations between metformin exposure and improved CRC survival in a diabetic population (Garrett et al., 2012; Spillane et al., 2013). Also, metformin treatment in patients with DM with previous CRC was found to be associated with a decreased incidence of colorectal adenomas, the precursors of CRCs (Lee et al., 2012).

In line with these observations, obesity and MS have also been linked to a higher risk of developing CRC (Hull and Lagergren, 2013; Pais et al., 2009). In fact, obesity (measured by the body mass index) has been significantly associated with colon cancer recurrence and death in patients with curatively resected stage II and III cancers treated with adjuvant chemotherapy. Also, this association was more evident in patients who had severe obesity (class 2 and 3) compared with normal-weight patients. Additionally, obesity compared with normal weight was significantly associated with an increased number of lymph node metastases, an accepted worse prognostic factor in colon cancer (Sinicrope et al., 2010, 2013).

Altogether, these findings indicate a strong relationship between CRC development and metabolic disorders, such as obesity and MS, which are global epidemics in countries with western style diets. However, the role played by each component of MS on CRC risk is unknown, and the association between the disorders is often based on clinical parameters. Consequently, the genetic association between MS and CRC has never been extensively investigated.

In the present study, we aimed to determine whether genes traditionally associated with components of MS could be a risk factor for stage II CRC and hence predict the risk of recurrence in these patients. We analysed the expression levels of genes previously associated with obesity, DM, hyperlipidaemia, or directly with MS and the association with the clinical outcome in two independent groups of stage II CRC patients.

2. Material and methods

2.1. Patients

A training series of 80 patients with stage II CRC treated by curative resection at La Paz University Hospital (Madrid, Spain) between 2000 and 2004 participated in this study. Eligibility required histologically confirmed stage II primary CRC according to the American Joint Committee on Cancer/Union for International Cancer Control (AJCC/UICC) classification, long-term follow-up among survivors ($>$3 years) and age $\geq$18

years. Additional eligibility criteria included a good quality RNA sample (3 of the 80 RNA samples did not have sufficient quality and were excluded from the study). Patients who died within 30 days after surgery; patients with incompletely excised tumour, mixed histological features, or other cancers in the previous 5 years; and patients with microsatellite instability were ineligible for this study.

Additionally, we recruited a different group of 120 stage II CRC patients from La Paz University Hospital surgically treated between 2004 and 2008 before adjuvant chemotherapy as the validation series from which one sample was excluded due to reduced quality of the sample. To determine the sample size of the validation series, a statistical power of at least 0.805 at a significance level of 0.05 based on the results of the training group was applied. A diagram describing selection of the CRC patients in both cohorts is shown in Supplementary Figure 1.

In all cases, tumour samples were obtained with the patient's authorization and with the approval of the human research review committee of the hospital involved in the study (HULP-PI-1452). Oncologists of the La Paz University Hospital were responsible for clinical and histopathological data collection (Table 1). Patients in both groups were classified following the clinical risk criteria of the American Society of Clinical Oncology (ASCO). This clinical classifier considers patients to have a high risk of relapse if they have any of the following events: number of lymph nodes examined ≤12; T4; poor histological grade; emergency presentation with obstruction or perforation; perineural or lymphovascular invasion.

Table 1 – Clinicopathological characteristics of the stage-II CRC patients included in the study.

Variable	Training series ($n = 77$)		Validation series ($n = 119$)	
	no of patients	%	no of patients	%
Age, years				
Range	32–86		26–91	
<70	42		73	
≥70	35	45.45	46	38.66
Mean	68.22		66.08	
Median	69		66	
Localization				
Right	37	48.05	48	40.34
Left	39	50.65	71	59.66
Rectum	1	1.3	0	0
Grade				
Low	5	6.49	10	8.4
Moderate	66	85.71	95	79.8
High	5	6.49	10	8.4
Unknown	1	1.3	4	3.4
Gender				
Women	33	42.86	54	45.38
Men	44	57.14	65	54.62
No. of lymph nodes assessed				
Mean	12.09		14.2	
Range	1–29		0–43	
≤12	46	59.7	54	45.4
>12	30	39	62	52.1
Unknown	1	1.3	3	2.5
T				
T3	56	72.73	70	58.82
T4	21	27.27	49	41.18
Disease-free survival, months				
Mean	56.71		39.44	
Median	60		39	
Relapse (local, regional, distant)	22	28.57	18	15.13
Survival time				
Mean	63.57		41.82	
Median	63		42	
Death	13	16.88	11	9.24
Chemotherapy				
Yes	47	61.04	76	63.87
No	30	38.96	43	36.13

2.2. Gene expression assays

Formalin-fixed, paraffin-embedded (FFPE) tumour samples from stage II CRC patients were deparaffinated using Deparaffinization Solution (Qiagen Gmbh, Hilden, Germany). Total RNA was then purified from all samples using the RNeasy FFPE Kit (Qiagen) following the manufacturer's instructions and was reverse transcribed using the High Capacity cDNA Archive Kit (Applied Biosystems, Carlsbad, CA, USA) for 2 h at 37 °C. The gene expression profile of several genes associated with MS and related alterations (Table 2) was analysed in all stage II CRC samples and was carried out in an HT-7900 Fast Real-Time PCR System using Taq-Man Low Density Arrays (Applied Biosystems). The gene expression data were normalized using the geometric mean of the internal control genes GAPDH and B2M using real-time StatMiner software (Integromics® Inc., Madison, WI, USA).

2.3. Statistical analysis

Quantification of gene expression was calculated with the $2^{-\Delta Ct}$ method (Applied Biosystems) and presented as AQ × 10^2. Time to death was obtained for the analysis of overall survival (OS) for patients who had died, and time to last contact was obtained for living patients. Time to progression (defined as any relapse—local, distant, or local and distant) was obtained for the analysis of disease-free survival (DFS). Both parameters were defined from the time of surgical procedure.

The Kaplan–Meier method was used to estimate OS and DFS. The log-rank test for univariate Cox regression analysis was performed to test the association between DFS and individual gene expression. To assess the effect of each gene expression on survival with adjustment for potential confounding factors, proportional hazards Cox regression modelling was used. Multivariate analysis included only variables that were significant ($p < 0{\cdot}05$) in the univariate analysis and age >70 years as the main non-modifiable risk factor. Hazard ratios (HR) and 95% confidence intervals (CIs) were calculated from the Cox regression model.

The prognostic gene expression signature for stage II CRC patients was developed analysing the prediction ability for all possible Cox regression models with several genes and

Table 2 – MS-related genes analysed in stage-II CRC patients.

Gene symbol	Gene name	Disease/phenotype
ABCA1	ATP-binding cassette, sub-family A (ABC1), member 1	Low HDL cholesterol and diabetes
ACACB	Acetyl-Coenzyme A carboxylase beta	Metabolic syndrome, obesity and diabetes
ADIPOQ	Adiponectin, C1Q and collagen domain containing	Dysglycemia, obesity and insulin resistance
ADIPOR1	Adiponectin receptor 1	Diabetes and insulin resistance
AGRP	Agouti related protein homolog (mouse)	Dysglycemia
APOA1	Apolipoprotein A-I	Metabolic syndrome and hypertriglyceridemia
APOA2	Apolipoprotein A-II	Metabolic syndrome, diabetes and obesity
APOA4	Apolipoprotein A-IV	Metabolic syndrome and obesity
APOB	Apolipoprotein B	Metabolic syndrome and dyslipidemia
APOC1	Apolipoprotein C-I	Metabolic syndrome
APOC2	Apolipoprotein C-II	Hypertriglyceridemia and low HDL
APOC3	Apolipoprotein C-III	Metabolic syndrome and hypertriglyceridemia
APOD	Apolipoprotein D	Metabolic syndrome
APOE	Apolipoprotein E	Metabolic syndrome
CD36	CD36 molecule (thrombospondin receptor)	Metabolic syndrome and diabetes
FABP2	Fatty acid binding protein 2, intestinal	Metabolic syndrome
HSD11B1	Hydroxysteroid (11-beta) dehydrogenase 1	Metabolic syndrome, obesity and diabetes
INS	Insulin	Dysglycemia
LEP	Leptin	Obesity
LEPR	Leptin receptor	Obesity
LIPE	Lipase, hormone-sensitive	Dysglycemia and obesity
LIPG	Lipase, endothelial	Low HDL cholesterol
LPL	Lipoprotein lipase	Abdominal obesity
POMC	Proopiomelanocortin	Obesity
PPARG	Peroxisome proliferator-activated receptor gamma	Low HDL cholesterol, dysglycemia and obesity
PYY	Peptide YY	Metabolic syndrome and obesity
RETN	Resistin	Dysglycemia and diabetes
SLC2A4	Solute carrier family 2 (facilitated glucose transporter), member 4	Dysglycemia
UCP2	Uncoupling protein 2 (mitochondrial, proton carrier)	Obesity

selecting the multivariate model with the largest c-index using 100 times 5-fold cross validation (CV). Thus, for the performance of the risk prediction method in the context of survival data, the c-index was used (Harrell et al., 1996) implementing CV strategies. The c-index was calculated in the test sets of each partition and corrected by optimism (Harrell et al., 1996). We repeated this process 100 times with different 5-fold CV partitions, and the c-index was averaged. The expression data of each gene were categorized into a low and a high value. The cut-off value was established according to the largest prediction ability between all the univariate Cox regression models defined for each observed value of the gene level expression. All reported p values were two sided. Statistical significance was defined as $p < 0{\cdot}05$. Statistical analyses were performed with R statistical software version 2.15 (www.r-project.org).

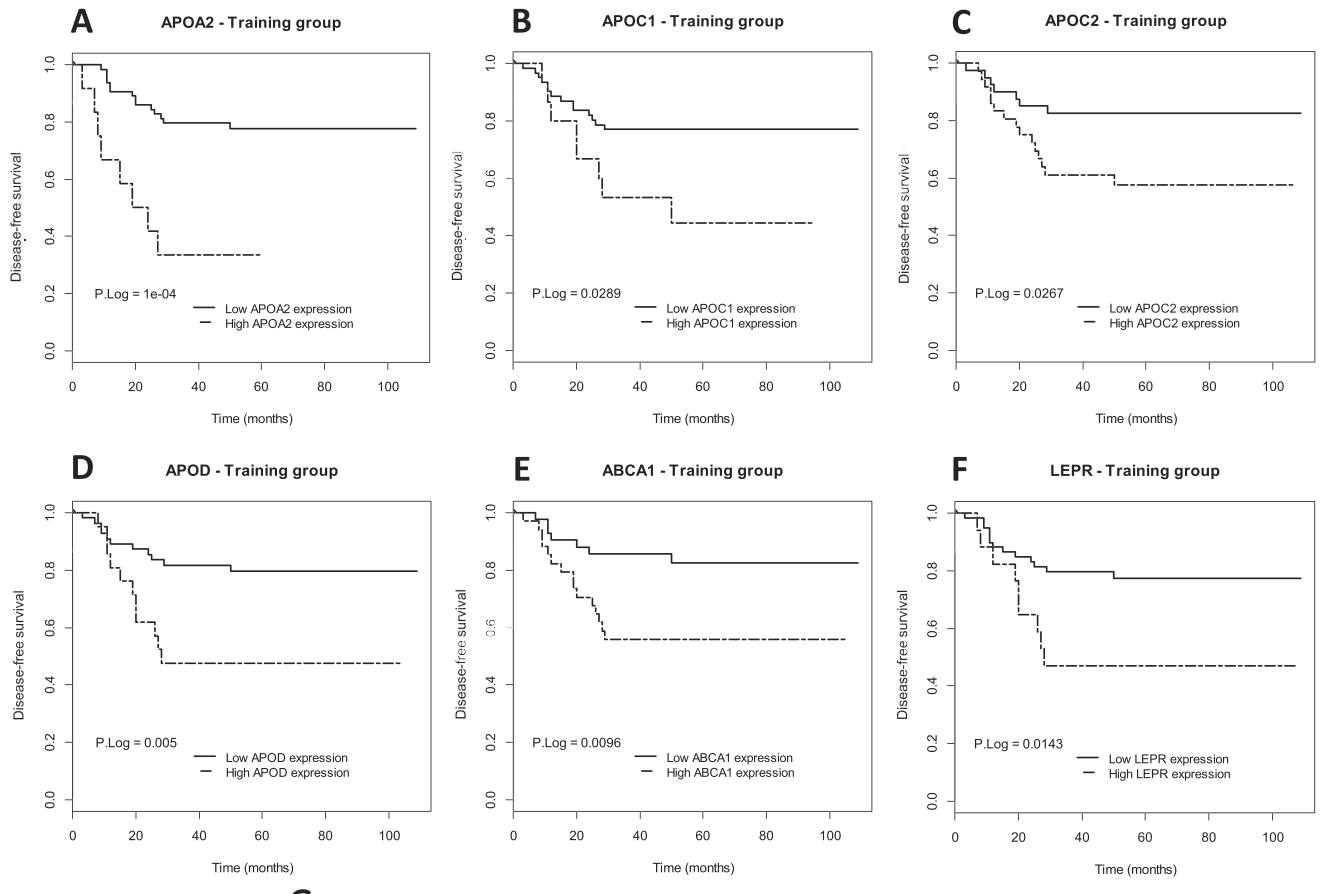
3. Results

3.1. *Identification of 6 genes related to MS and associated with colon cancer relapse*

To determine whether genes previously associated with MS or related diseases have any connection with the risk of relapse in patients with CRC, the expression levels of 29 candidate genes (Table 2) were measured by use of quantitative real-time PCR (RT-PCR) in stage II CRC patients whose tumours had been surgically resected. First, we quantified the mRNA concentrations of these genes in the 77 patients enrolled in the training series. Gene expression analysis showed that 6 out of the 29 genes assayed had a statistically significant association between mRNA concentrations and clinical prognosis. For these 6 genes—apolipoprotein A-II (*APOA2*), apolipoprotein C1 (*APOC1*), apolipoprotein C2 (*APOC2*), apolipoprotein D (*APOD*), ATP-Binding Cassette Sub-Family A Member 1 (*ABCA1*), and leptin receptor (*LEPR*)—we observed that increased gene expression levels were associated with short DFS. The Kaplan–Meier survival analysis showed a p value for the log-rank test of 0.0001, 0.0289, 0.0267, 0.005, 0.0096, and 0.0143, respectively (Figure 1). The univariate Cox regression analysis showed that the risk of relapse in patients with higher mRNA levels for these genes was significantly increased with respect to patients with lower mRNA levels, reaching an HR (95% CI) = 5.01 (2.08–12.07), $p = 0.001$ for *APOA2* (Figure 1G). Thus, 3-year DFS in patients with a high expression of *APOA2* was 33%, whereas it was 80% for patients with low levels of the enzyme. Similarly, 3-year DFS was 47%, 48%, 53%, and 56% for patients with a high expression of *LEPR*, *APOD*, *APOC1*, and *ABCA1*, respectively, whereas it was over 80% for patients with low levels of these MS-related genes.

3.2. *Association between common increased expression levels of lipid transport-related genes and colon cancer relapse*

Since all these genes are functionally related in connected metabolic pathways, we analysed the putative interactions



G

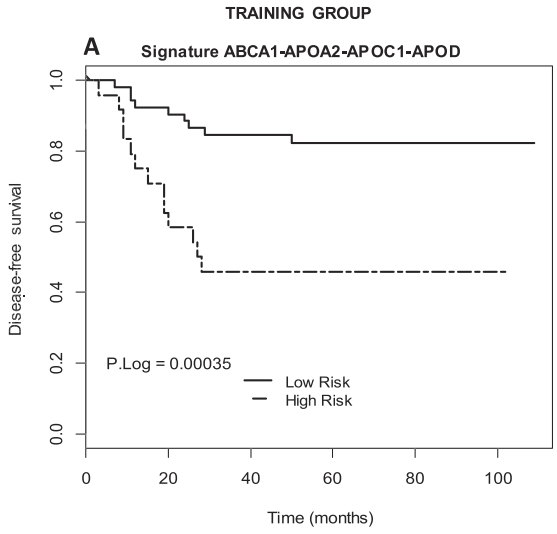
Training group

	Low Risk		High Risk		Unadjusted		Adjusted	
Gen	R	N	R	N	HR(95%CI)	P.Cox	HR(95%CI)	P.Cox
APOA2	14	65	8	12	5.01 (2.08-12.07)	0.001	4.75 (1.9-11.87)	0.002
APOC1	14	61	8	16	2.56 (1.07-6.11)	0.046	2.43 (0.96-6.14)	0.071
APOC2	7	40	15	37	2.65 (1.08-6.5)	0.027	3.25 (1.28-8.24)	0.009
APOD	11	56	11	21	3.14 (1.35-7.26)	0.009	3.21 (1.31-7.85)	0.012
ABCA1	7	43	15	34	3.08 (1.25-7.56)	0.010	3.76 (1.4-10.08)	0.006
LEPR	13	60	9	17	2.77 (1.18-6.49)	0.026	2.4 (0.96-6.01)	0.071

Figure 1 – Association between lipid transport gene expression and CRC free survival in the training set of stage II CRC patients. Kaplan–Meier plots for *APOA2* (A), *APOC1* (B), *APOC2* (C), *APOD* (D), *ABCA1* (E), and *LEPR* (F) and p log-rank value in the training group are shown. G, Univariate and multivariate Cox regression analyses for DFS of the genes *APOA2*, *APOC1*, *APOC2*, *APOD*, *ABCA1*, and *LEPR* in the training group of stage II CRC patients. HR (95% CI), hazard ratio, and corresponding 95% CI from adjusted or unadjusted Cox regression analyses; P.Cox, *p* value from adjusted or unadjusted Cox regression analyses; N, N° of patients at risk; R, N° of patients that relapsed.

among them within a genetic profile that could accurately separate CRC patients with a high risk of relapse from those with a low risk. For this purpose, an analysis of the prediction ability for DFS of all possible Cox regression models of gene expression profiles within these metabolic genes was performed using 100 times 5-fold CV. We observed that a gene expression model composed of the combination of *ABCA1–APOA2–APOC1–APOD* was able to accurately predict the risk of relapse in the training series in both univariate (HR [95% CI] = 4.2 [1.79–9.88], $p < 0.001$) and multivariate (HR [95% CI] = 4.47 [1.79–11.16], $p < 0{\cdot}001$) analysis (Figure 2). The 3-year DFS in patients from the high-risk group classified by this MS gene expression profile was 46% compared with 84% in patients from the low-risk group. The gene expression signature not only increased the HR value but also enhanced the statistical power, and its determination decreases possible experimental errors since it is constituted by individual significant genes within a biological pathway.

In order to determine the potential clinical relevance of this molecular classifier, we compared this molecular marker with the predictive value of the clinical classifier usually applied by oncologists in the management of stage II colon cancer patients. As presented in Table 3, results of the Cox regression analysis indicated that the clinical classifier



B

Training group

	Low Risk		High Risk		Unadjusted		Adjusted	
Variable	R	N	R	N	HR(95%CI)	P.Cox	HR(95%CI)	P.Cox
Genetic Signature	9	53	13	24	4.2 (1.79-9.88)	0.001	4.47 (1.79-11.16)	0.001
Clinical classifier	2	14	20	63	2.53 (0.59-10.81)	0.157		

Figure 2 – MS-related signature for predicting stage II CRC patient relapse. Kaplan–Meier plots for the proposed gene expression signature and p log-rank value in the training group are shown (A). B, Univariate and multivariate Cox regression analyses for DFS of the MS-related signature in the training group of stage II CRC patients. HR (95% CI), hazard ratio, and corresponding 95% CI from adjusted or unadjusted Cox regression analyses; P.Cox, *p* value from adjusted or unadjusted Cox regression analyses; N, N° of patients at risk; R, N° of patients that relapsed. Patients are considered high risk by clinical classifier (American Society of Clinical Oncology clinical risk criteria) if they have any of the following events: number of lymph nodes examined ≤12; T4; poor histological grade; emergency presentation with obstruction or perforation; perineural or lymphovascular invasion.

identified patients with a 2-fold increased risk of relapse (HR [95% CI] = 2.53 [0.59–10.81], $p = 0.157$), resulting our MS-related molecular classifier more powerful and accurate for (HR [95% CI] = 4.2 [1.79–9.88]) with a strong statistical significance ($p = 0.001$) that was not observed in the clinical classifier.

3.3. *Validation of the MS-related signature as a new molecular marker of prognosis for stage II CRC patients*

In order to confirm the results obtained in the training series, an additional independent set of stage II CRC patients was included in this study. A validation set of 119 patients containing 18 recurrences was analysed as determined by the statistical sample size test of detecting an HR over 4 in the validation study with a statistical power over 0.8 at a significance level of 0.05.

The individual gene expression analysis revealed a trend for individual high expression of these MS-related genes and a worse clinical outcome, with a statistically significant association between *APOA2* and *LEPR* with clinical prognosis in the multivariate analysis (HR [95% CI] = 3.1 [1.1–8.76], $p = 0.045$; HR [95% CI] = 3.27 [1.14–9.38], $p = 0.038$; Figure 3). Confirming the strength of the identified molecular signature based on MS-related genes, a common global analysis of all these genes resulted in a powerful and significant prediction. Thus, the MS-related gene expression profile was confirmed in the validation series as the most potent prognostic classifier for predicting risk of relapse in stage II CRC patients independently of clinical factors (HR [95% CI] = 5.36 [1.84–15.66], $p = 0.001$; Figure 4) and is able to add precision and accuracy to the current classifier used for early stage CRC patient management based on clinicopathological parameters (Table 3).

4. Discussion

A rapidly growing epidemic of MS is taking place in industrialized nations because of the global spread of obesity and sedentary lifestyles, and this is considered to be an emerging and worrying health problem. It is widely known that MS and its components are associated with increased risk of CRC. Previous studies have reported that individuals with three or more components of MS have a significant higher probability of developing CRC than individuals with fewer than three components (Kim et al., 2012). In this sense, obesity, dyslipidaemia (high triglyceride levels or low HDL-cholesterol levels), DM, or insulin resistance might increase the risk of CRC. Concretely it has been described a significant positive association between obesity and CRC with an increased risk of about 1.5–3 times and have found a 3% increase in the risk of CRC per one unit increase in body mass index (Kim et al., 2007).

Table 3 – Cox regression analysis of clinicopathological parameters in the two set of CRC patients included in the study.

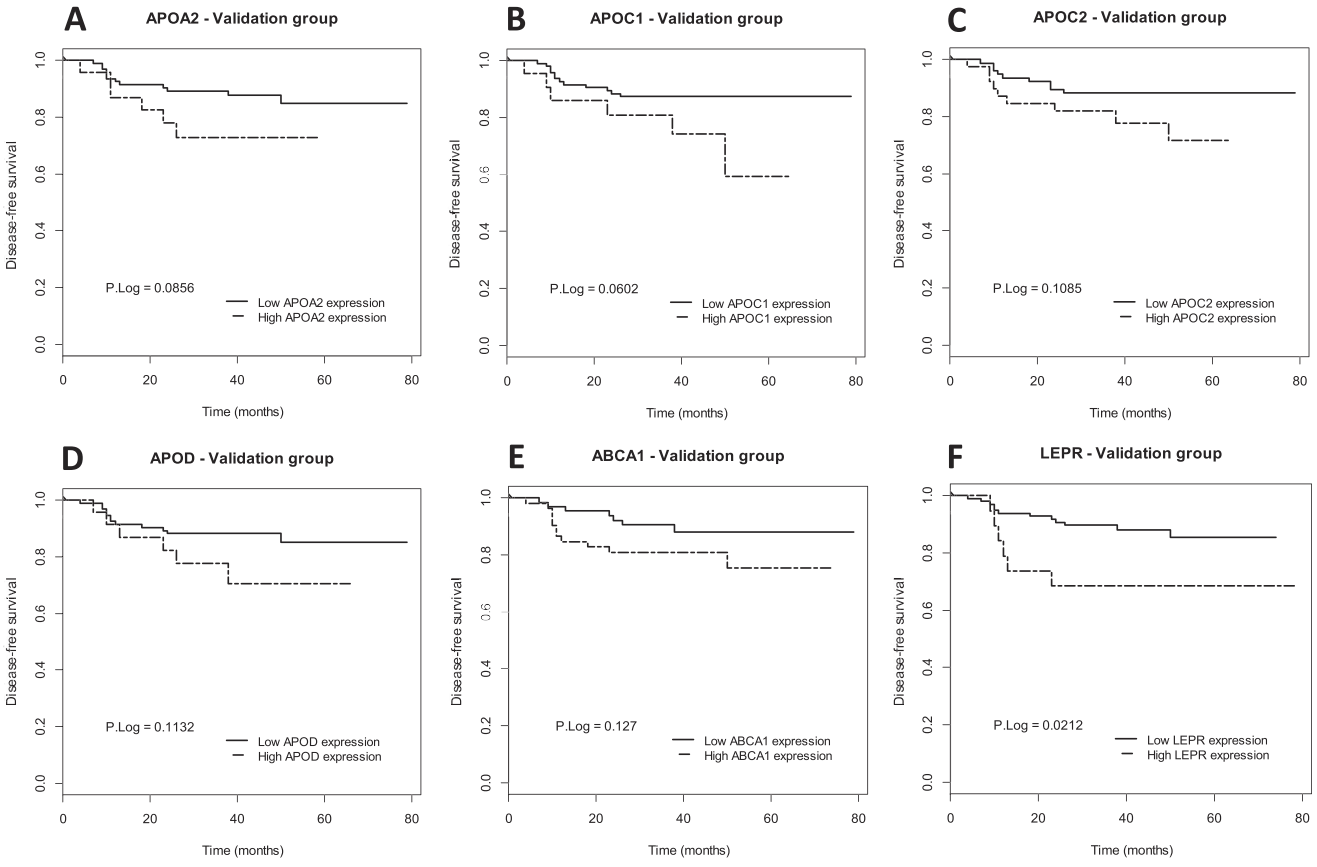
Variable	Univariate analysis			
	Training series		Validation series	
	HR (95% CI)	P	HR (95% CI)	P
Age (continuous)	1 (0.96–1.05)	0.839	1.01 (0.98–1.05)	0.492
Age, $> v \leq 70$	1.08 (0.46–2.52)	0.863	1.37 (0.53–3.54)	0.525
Localization		0.636		0.6
Right v Left	1.22 (0.52–2.85)		1.3 (0.49–3.45)	
Right v Rectum	NA		NA	
Grade		0.585		0.457
Moderate v High	2.07 (0.48–8.92)		1.51 (0.34–6.68)	
Moderate v Low	0.63 (0.08–4.72)		2.31 (0.66–8.1)	
Gender, men v women	1.86 (0.76–4.56)	0.163	1.48 (0.57–3.82)	0.413
Lymph nodes assessed				
Mean (continuous)	1.01 (0.95–1.08)	0.759	0.97 (0.91–1.04)	0.435
Range, $>v \leq 12$	1.74 (0.76–4.02)	0.196	0.56 (0.22–1.46)	0.23
T (T4 v T3)	1.75 (0.73–4.17)	0.223	3.19 (1.2–8.49)	0.016
Chemotherapy, yes v no	1.03 (0.43–2.45)	0.952	1.27 (0.45–3.58)	0.642
Vascular invasion, yes v no	1.08 (0.44–2.65)	0.866	3.08 (1.22–7.78)	0.02
Perineural invasion, yes v no	1.16 (0.39–3.43)	0.793	3.04 (1.2–7.72)	0.025
Bowel obstruction/perforation, yes v no	3.07 (1.19–7.87)	0.034	1.59 (0.63–4)	0.328
Clinical classifier (ASCO risk[a]), high v low risk	2.53 (0.59–10.81)	0.157	4.35 (0.6–554.13)	0.187

Abbreviations: HR (95% CI), hazard ratio and corresponding 95% confidence interval from univariate cox proportional hazards analysis; P, *p* value from univariate cox regression analysis; NA, not available; ASCO, American Society of Clinical Oncology.
a Definition of High risk group by ASCO (American Society of Clinical Oncology): patients with any of the following events: high differentiated tumor; numbers of lymph nodes assessed ≤ 12; T stage = T4; Lymphovascular or perineural invassion; bowel obstruction or perforation.

In fact, epidemiological studies have reported that around 11% of CRC cases can be attributed to overweight and obesity in Europe. These data suggest that obesity is associated with a worse cancer outcome, such as recurrence of the primary cancer or mortality (Bardou et al., 2013).

In addition, it has been reported that DM contributes to the mortality and incidence of CRC. In line with these observations, different hypotheses describe the mechanisms of carcinogenesis in obesity or DM through insulin resistance, oxidative stress, and obesity-related inflammation (Kim et al., 2007). Obesity and other components of MS might confer a model of visceral fat deposition that is associated with insulin resistance and high levels of insulin-like growth factor-1 (IGF-I), which can regulate the tumourigenesis process by inhibiting apoptosis and stimulating cell proliferation (Kim et al., 2007). Immune signalling in adipose tissue has also been suggested to explain many of the observed associations between obesity, inflammation, insulin resistance, and cancer development and/or progression (Yang et al., 2013); dysregulation of cytokines and growth signals (including IGF-I, insulin, and adipokines) could be responsible for the association between MS and CRC in the tumourogenic process. Visceral adipose tissue, in addition to its role in fat storage, is an endocrine tissue that releases a wide range of inflammatory cytokines and other secreted proteins, such as C-reactive proteins, tumour necrosis factors, interleukin-6, adiponectin, and leptin (Kim et al., 2012). Most of these cytokines and growth signals associated with insulin resistance and central obesity have also been described as prognostic factors for CRC (Chung et al., 2006; Guadagni et al., 2009; Knupfer and Preiss, 2010; Koike et al., 2008; Nikiteas et al., 2005; Peters et al., 2003; Sharma et al., 2008). Although a large number of epidemiological studies have supported the association between individual components of MS and the risk of CRC (Pais et al., 2009), the underlying mechanisms linking both diseases are still a subject of debate. Moreover, most of these studies are based completely on the association among clinical data, without considering the genetic differences among individuals. For this reason, we aimed to assess the association between the expression level of genes previously associated with components of MS and CRC outcome following a common approach in order to identify an accurate signature for clinical prediction. We selected a wide range of genes directly related to MS or any of its components, such as diabetes, obesity, insulin resistance, dysglycaemia, hypertriglyceridaemia, or HDL-cholesterol and analysed the expression levels of these genes in stage II CRC patients.

Among all the MS-related genes analysed, *APOA2* was found to be the gene with the best individual value of prognosis prediction for stage II-CRC patients, suggesting a relevant role of the enzyme in the progression of the disease. ApoA2, the second most abundant apolipoprotein in HDL, is found in plasma as a monomer, homodimer, or heterodimer with apolipoprotein D and modulates cholesterol transport. Defects in this gene may result in apolipoprotein A-II deficiency or hypercholesterolaemia (NCBI, http://www.ncbi.nlm.nih.gov/). *APOA2* plays an undefined role in the susceptibility of several diseases, such as insulin resistance, obesity, and atherosclerosis. Polymorphisms in the *APOA2* gene have been associated with body mass index or obesity in the presence of high-saturated fat intake in Mediterranean and Asian populations, supporting the specificity



G

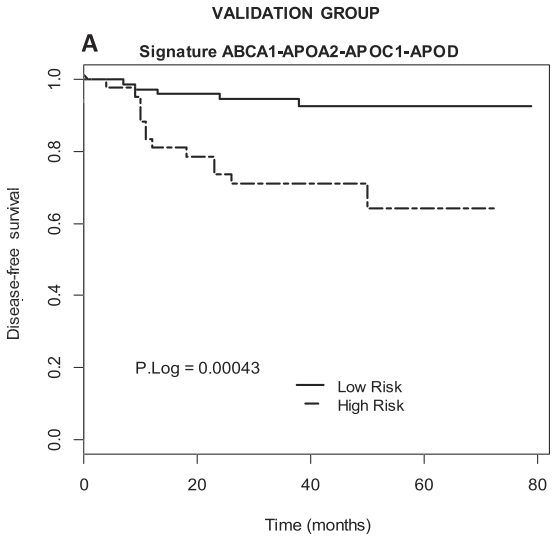
Validation group

	Low Risk		High Risk		Unadjusted		Adjusted	
Gen	R	N	R	N	HR(95%CI)	P.Cox	HR(95%CI)	P.Cox
APOA2	12	95	6	24	2.31 (0.86-6.19)	0.115	3.1 (1.1-8.76)	0.045
APOC1	12	97	6	22	2.48 (0.93-6.62)	0.087	1.86 (0.66-5.27)	0.254
APOC2	9	78	9	41	2.09 (0.83-5.28)	0.121	1.78 (0.65-4.87)	0.268
APOD	12	96	6	23	2.16 (0.81-5.77)	0.143	1.97 (0.68-5.67)	0.225
ABCA1	7	66	11	53	2.06 (0.8-5.33)	0.128	2.27 (0.84-6.14)	0.099
LEPR	12	99	6	20	3.02 (1.13-8.08)	0.042	3.27 (1.14-9.38)	0.038

Figure 3 – Association between lipid transport gene expression and CRC free survival in the validation set of stage II CRC patients. Kaplan–Meier plots for *APOA2* (A), *APOC1* (B), *APOC2* (C), *APOD* (D), *ABCA1* (E), and *LEPR* (F) and p log-rank value in the validation group are shown. G, Univariate and multivariate Cox regression analyses for DFS of the genes *APOA2*, *APOC1*, *APOC2*, *APOD*, *ABCA1*, and *LEPR* in the validation group of stage II CRC patients. HR (95% CI), hazard ratio, and corresponding 95% CI from adjusted or unadjusted Cox regression analyses; P.Cox, *p* value from adjusted or unadjusted Cox regression analyses; N, N° of patients at risk; R, N° of patients that relapsed.

of saturated fat as a driver of this interaction (Corella et al., 2011). Furthermore, the APOA2 gene (located in chromosome 1q23) has been described as a potential candidate for DM and has been linked in several studies to chromosome 1q21–24 (Corella et al., 2011). Additionally, ApoA2 has been related to various types of cancer in different clinical studies (Podzielinski et al., 2013; Vermaat et al., 2010). ApoA2 has been described as being discriminatory between renal cancer patients and healthy controls (Vermaat et al., 2010). The ApoA2 protein was also detected by Surface Enhanced Laser Desorption Ionization Time Of Flight Mass Spectrometry (SELDI-TOF MS) and enzyme-linked immunosorbent assay (ELISA) analysis in ovarian cyst fluids and was able to discriminate malignant, borderline, and benign tumours. These results demonstrate that ApoA2 concentration is uncorrelated and higher in malignant ovarian tumours and is an independent classifier of malignant ovarian tumours, yielding 55.1% sensitivity, 95% specificity, and 88.1% accuracy to discern malignant from benign and borderline tumours. This suggests a dysregulation of lipoprotein metabolism in ovarian cancer (Podzielinski et al., 2013).

Likewise, *LEPR* has also been associated with metabolic disorders and several types of cancer. Several studies have documented that leptin signalling plays a key function in



B

Validation group

	Low Risk		High Risk		Unadjusted		Adjusted	
Variable	R	N	R	N	HR(95%CI)	P.Cox	HR(95%CI)	P.Cox
Genetic Signature	5	76	13	43	5.28 (1.88-14.83)	0.001	5.36 (1.84-15.66)	0.001
Clinical classifier	0	13	18	106	4.35 (0.6-554.13)	0.187		

Figure 4 – Validation of the MS-related signature for predicting stage II CRC patient relapse. Kaplan–Meier plots for the proposed gene expression signature and p log-rank value in the validation group are shown (A). B, Univariate and multivariate Cox regression analyses for DFS of the MS-related signature in the validation group of stage II CRC patients. HR (95% CI), hazard ratio, and corresponding 95% CI from adjusted or unadjusted Cox regression analyses; P.Cox, *p* value from adjusted or unadjusted Cox regression analyses; N, N° of patients at risk; R, N° of patients that relapsed. Patients are considered high risk by clinical classifier (American Society of Clinical Oncology clinical risk criteria) if they have any of the following events: number of lymph nodes examined ≤12; T4; poor histological grade; emergency presentation with obstruction or perforation; perineural or lymphovascular invasion.

regulating food intake (control of appetite), energy balance, and body weight control (Liu et al., 2013; Rene Gonzalez et al., 2009). Genetic studies have demonstrated the association between *LEPR* gene polymorphisms and obesity, insulin resistance, and dyslipidaemia, and they are also associated with metabolic diseases among different specific ethnic groups (Li et al., 2013; Wang et al., 2012). In this sense, evidence suggests a relevant role for leptin signalling in the link between obesity and cancer, including breast and CRC (Liu et al., 2013; Rene Gonzalez et al., 2009). It is known that the pleiotropic effects of leptin are linked to diverse processes related to tumourigenesis, such as proliferation, anti-apoptosis, angiogenesis, changes in the components of extracellular membranes, and metastasis. In fact, leptin signalling can upregulate the expression of the anti-apoptotic Bcl-2 protein, resulting in increased tumour cell survival (Rene Gonzalez et al., 2009). Moreover, studies have documented that *LEPR* is associated with enhanced in vitro cancer cell proliferation and tumour angiogenesis in breast cancers (Wang et al., 2012). Different studies have shown that *LEPR* is elevated in breast cancer cells compared with normal mammary cells (Rene Gonzalez et al., 2009) and is significantly overexpressed in human CRC with respect to normal colonic mucosa (Uddin et al., 2009). Additionally, *LEPR* is positively related to the expression of hypoxia-inducible factor 1, a pro-neoplastic transcriptional regulator that produces a more advanced tumour phenotype (Liu et al., 2013). In vivo studies have shown that colon tumour growth was inhibited in leptin-deficient and leptin-receptor-deficient mice and in leptin-deficient tumours, whereas stimulation with leptin led to phosphorylation of p42/44 mitogen-activated protein kinase and increased cell proliferation in vitro and in vivo (Liu et al., 2013). Elevated leptin concentrations have been shown to stimulate the proliferation of colorectal epithelial cells by interaction with its receptor, whereas *LEPR*-deficient mice presented increased susceptibility to azoxymethane-induced tumours (Liu et al., 2013). Genetic studies also support the role of *LEPR* in tumour growth and cancer development. Variants in the *LEPR* gene have been associated with an increased risk for CRC, breast cancer, non-small cell lung cancer, and oral squamous cell carcinoma (Liu et al., 2013; Slattery et al., 2008).

In this study we observed that increased gene expression levels of *APOA2*, *APOC1*, *APOC2*, *APOD*, *ABCA1*, and *LEPR* were associated with DFS of CRC patients. However, as reported in previous studies, the adjusted model showed a moderate decrease in statistical power in the association between DFS and individual mRNA levels of these genes. In particular, we demonstrated a statistical association between expression levels of *APOA2* and *LEPR* genes and CRC outcome in two independent series of stage II CRC patients, further supporting their particular relevance in tumour aggressiveness. However, although these results suggest that overexpression of these

genes and the consequent activation of lipid metabolism is related to tumour progression, the lack of accuracy in the individual determinations makes their introduction as specific biomarkers in the clinical setting difficult. By contrast, as might be expected from the special requirements of tumoural cells, lipid transport and signalling are over activated at different levels, and the common determination of these four genes results in an accurate prediction of CRC patient outcome, potentiating the information obtained by analysing each gene individually. In addition, the reduced number of genes included in the signature, together with the fact that all the genes constituting this biomarker are individually associated with a worse clinical outcome, might result in limited experimental failure reducing uncertain clinical interpretations. Thus, although studies are still needed to further confirm its clinical utility, our results suggest that this MS-related signature might constitute a useful molecular biomarker that is helpful for decision making regarding stage II CRC patient management.

On the other hand, previous studies suggest the potential of *APOA2* or leptin-signalling inhibition as a novel treatment for CRC. This approach could open a new therapeutic strategy for the treatment of patients with CRC, similar to that of metformin (a biguanide widely used for treating hyperglycaemia and DM), which has been suggested to exhibit a suppressive effect in CRC (Dowling et al., 2011). In this sense, several leptin receptor antagonists have recently been synthesized and selected to exhibit inhibitory effects in pro-neoplastic leptin activity in vitro and in vivo in breast and CRC cells (Beccari et al., 2013; Gonzalez et al., 2006; Otvos et al., 2011). This anti-proliferative action was associated with the inhibition of several leptin-induced pathways, including JAK/STAT3, MAPK/ERK1/2 and PI3K/AKT, cyclin D1, and E-cadherin, playing a relevant role in cancer progression (Beccari et al., 2013). These results further support this hypothesis and might also provide a method for identifying patients that might benefit most from this new anticancer strategy.

After a global analysis of the gene expression profile of MS-related genes in patients with low-stage CRC, we identified a multivariate model with the highest predictive power using 100 times 5-fold CV. This model, composed of *ABCA1*, *APOA2*, *APOC1*, and *APOD*, was able to strongly predict DFS in the training group of stage II CRC patients. Here, for the first time, we report a gene expression profile that suggests a genetic relationship between components of MS and CRC and constitutes a potential accurate molecular biomarker within lipid metabolism activation with a strong predictive value independent of clinicopathological parameters. Together with apolipoproteins involved in lipid transport, we find *ABCA1*, which is involved in the transport of cellular cholesterol, identified as a major regulator of phospholipid homeostasis.

Several antitumoural compounds have been reported as indirect inhibitors of *ABCA1*, including the mammalian target of rapamycin (mTOR) inhibitor rapamycin. In addition, cyclosporine A (CsA), an immunosuppressant drug widely used in organ transplantation to prevent rejection, and PSC833, a non-immunosuppressive analog of CsA, also inhibit *ABCA1* via direct binding (Nagao et al., 2013). CsA reduces cell-cycle progression and induces necroptosis of human carcinoma cell lines. CsA may also target transformed colon and oesophageal carcinoma cells without affecting nontransformed cells, promoting beneficial tumouristatic effects (Werneck et al., 2012). Although the specific mechanism involved in this effect has not been well documented, it has been demonstrated that PSC833 inhibits colorectal tumour multiplicity, tumour burden, and tumour progression to colorectal adenocarcinoma in experimental colorectal carcinogenesis initiated with 1,2-dimethylhydrazine in rats (Kankesan et al., 2006).

A high concentration of Apo C, a basic apolipoprotein principally produced by the liver and found in plasma as a component of HDL and triglyceride-rich lipoproteins, is associated with increased triglycerides in men with MS (van der Ham et al., 2009). In addition, a polymorphism located downstream of the *APOC1* gene is associated with elevated plasma glucose, atherogenic dyslipidaemia, vascular inflammation, and central obesity, suggesting a novel loci associated with MS phenotype domains (Avery et al., 2011). ApoC1 has also been related to various types of cancer in different clinical and in vitro studies, including gastric and pancreatic cancers (Claerhout et al., 2011; Takano et al., 2008) where it has been suggested as being a plasmatic biomarker of tumour malignancy. Takano et al. showed the usefulness of the ApoC1 serum level as a potentially prognostic marker for pancreatic cancer and suggested that ApoC1 might be essential for pancreatic cancer cell survival (Takano et al., 2008).

Finally, together with these genes, the model is also constituted by Apo D, a small glycoprotein of 24 kD, member of the lipocalin family, and originally purified from blood as a minor apoprotein component of HDL particles (Liu et al., 2001; Soiland et al., 2007). However, Apo D does not show sequence similarity to other apolipoproteins, and its biological function as well as the identity of its putative ligand remain to be completely described. It has been postulated that Apo D could act as a transporter for diverse molecules structurally similar to haem-related molecules, such as biliverdin and porphyrins, and other ligands, including arachidonic acid, cholesterol, and steroid hormones, such as progesterone and pregnenolone (Liu et al., 2001; Soiland et al., 2007).

Molecules that regulate food intake, nutrient metabolism, and body weight, such as glucocorticoids, progesterone, and testosterone, are reported to stimulate the expression and secretion of Apo D in cultured cells, whereas oestrogens have the opposite effect on Apo D expression, suggesting a potential function for Apo D in energy homeostasis and body weight control. This idea is supported by genetic studies that have associated a polymorphism in the *APOD* gene to obesity and hyperinsulinaemia (Liu et al., 2001). Furthermore, it has been described that *APOD* is overexpressed in different tumours, such as breast, prostate, ovarian, and endometrial carcinomas (Alvarez et al., 2003). *APOD* has also been suggested as a prognostic factor in breast cancer, and numerous cellular mechanisms have been postulated to explain the association between *APOD* expression and adverse outcome in breast cancer. Due to its potential cytoprotective action, *APOD* overexpression could increase the survival time of the malignant cells and the clinical aggressiveness of the tumours (Soiland et al., 2009). Moreover, the reported poor prognosis of breast cancer patients with 3q chromosome gain could be explained by a high expression of *APOD* located on 3q26.2-qter (Soiland

et al., 2009). Of note, Apo D interacts with ApoA2 (found in plasma as a heterodimer with apolipoprotein D) (NCBI, http://www.ncbi.nlm.nih.gov/) and specifically with the cytoplasmic portion of LEPR (Liu et al., 2001; Yang et al., 1994), further supporting a relevant biological role of the combination of genes identified as an MS-related biomarker.

Complex gene expression profiles have been previously described to predict prognosis in CRC patients, but neither of them were focused on genes related to MS (Agesen et al., 2012; Eschrich et al., 2005; Salazar et al., 2011; Sveen et al., 2012). By contrast, most of these studies suggest that prognostic genes are randomly found. In this sense, efforts have tried to bring together different molecular classifications systems, suggesting that different signatures might be related to defining different aspects of more aggressive malignant progression (Sadanandam et al., 2014). In addition, most of the proposed molecular biomarkers are constituted by a larger number of genes (i.e., the OncotypeDx Colon Cancer test is based on the mRNA expression of 12 target genes (Gray et al., 2011; Quasar Collaborative et al., 2007), or ColoPrint that uses a set of 18 genes taken from a microarray platform (Salazar et al., 2011)) that might complicate both the experimental performance and the interpretation of the results by deviating from expected results. This signature is based on a combination of only 4 genes, all related to MS, that not only facilitate experimental application due to the small number of genes involved but also reflect a putative metabolic advantage of these tumoural cells. This suggests that additional metabolic alterations might also be found in these tumours and further explains their increased aggressiveness. Furthermore, although additional studies are needed, our results also suggest that these patients could benefit most from a therapeutic approach, including drugs used for metabolic alterations.

In summary, several differentially expressed genes previously linked to MS or related conditions were identified and associated with prognosis in stage II CRC patients. Furthermore, we identified a gene expression profile constituted of 3 apolipoproteins (ApoA2, ApoC1, and ApoD) and one cholesterol transporter (ABCA1), with relevant roles in metabolic disorders as an accurate biomarker for stage II CRC patients. This gene expression profile was a powerful predictor of prognosis independent of clinicopathological factors in the stage II CRC patients of both the training and validation groups. It also increased the robustness of prognosis prediction compared to the individual prognosis value of single genes and compared to ASCO clinical risk criteria, usually applied as a prognostic clinicopathological classifier.

It is known that this histopathological criteria is a good predictor of survival in patients with TNM stage I and IV. However, for TNM stage II and III, patients within the same tumour stage often show a different prognosis, indicating that traditional well-established staging procedures may be complemented with molecular biomarkers to precisely predict intermediate-stage CRC patients for which chemotherapy administration is a management option. In fact, adjuvant chemotherapy with oxaliplatin plus 5-fluorouracil (5-FU) reduces the risk of relapse in stage III CRC patients, despite its effectiveness in stage II CRC patients being subject to controversy. Moreover, this treatment produces adverse toxicity effects that frequently endure in the long term. Obviously, the need to better stratify and correctly prescribe the best treatment for patients to optimize outcome, reduce adverse toxicity events, reduce cost-effectiveness ratios, and rationalize resources is essential for early stage CRC patients and current health systems.

Our results showed that the commonly applied clinical classifier distinguished patients at high risk of relapse without statistical significance. Thus, the clinical classifier grouped patients in excess in the high-risk group in both sets of stage II CRC patients; this could result in unnecessary overtreatment of some patients. By contrast, our genetic signature was able to improve the prognostic ability in these groups of stage II CRC patients (Figures 2 and 4), suggesting its potential relevance as a complementary approach in clinical decision making for avoiding an excessive high-risk determination.

The impact of our findings is clinically relevant. These apolipoproteins are detectable in serum, and although further work is needed, the common determination of their expression could be easily and economically implemented in any hospital to quantify them in a rapid and noninvasive manner. Consequently, in future studies it will be necessary to confirm the correlation between levels of mRNA of these apolipoproteins in tissue samples and levels of protein detected in serum.

These findings could help us understand the molecular mechanisms linking CRC and MS. In addition, determination of these genes could constitute a new prognosis biomarker for detection, disease monitoring, and therapeutics in cancer patients. In addition to identifying a molecular marker of malignancy for intermediate-stage CRC patients, this study also provides a molecular basis for the association between MS and colon cancer progression. Since the identified genes linking both malignancies are metabolic "druggable" enzymes that currently constitute targets of metabolic diseases, this work establishes a molecular rationale for the potential application of this approach to colon cancer patients.

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Appendix A. Supplementary data

Supplementary data related to this article can be found at http://dx.doi.org/10.1016/j.molonc.2014.05.015.

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