Potential of soluble CD26 as a serum marker for colorectal cancer detection

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Abstract

Colorectal cancer is characterized by a low survival rate even though the basis for colon cancer development, which involves the evolution of adenomas to carcinoma, is known. Moreover, the mortality rates continue to rise in economically transitioning countries although there is the opportunity to intervene in the natural history of the adenoma–cancer sequence through risk factors, screening, and treatment. Screening in particular accounted for most of the decline in colorectal cancer mortality achieved in the USA during the period 1975-2000. Patients show a better prognosis when the neoplasm is diagnosed early. Among the variety of screening strategies, the methods range from invasive and costly procedures such as colonoscopy to more low-cost and non-invasive tests such as the fecal occult blood test (guaiac and immunochromatographic).

As a non-invasive biological serum marker would be of great benefit because of the performance of the test, several biomarkers, including cytologic assays, DNA and mRNA, and soluble proteins, have been studied. We found that the soluble CD26 (sCD26) concentration is diminished in serum of colorectal cancer patients compared to healthy donors, suggesting the potential utility of a sCD26 immunochromatographic detection test for early diagnosis. sCD26 originates from plasma membrane CD26 lacking its transmembrane and cytoplasmic domains. Some 90%–95% of sCD26 has been associated with serum dipeptidyl peptidase IV (DPP-IV) activity. DPP-IV, assigned to the CD26 cluster, is a pleiotropic enzyme expressed mainly on epithelial cells and lymphocytes. Our studies intended to validate this test for population screening to detect colorectal cancer and advanced adenomas are reviewed here.

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EARLY DIAGNOSIS AND POPULATION SCREENING FOR COLORECTAL CANCER

Colorectal cancer (CRC) clearly meets all the required conditions for the adoption of a screening policy.

First, because it is an important issue for public health since it is one of the most common cancers (ranking third both in men and women) worldwide and because it is characterized by a low survival rate due to diagnosis in advanced stages, which leads to high mortality rates. For example, in the United States the American Cancer Society estimated that in 2010 there were 142,570 new cases and 51,370 related deaths from colon cancer\(^\text{[1]}\) and more than 1 million new cases and about 530,000 deaths worldwide\(^\text{[2]}\). Moreover, globally, while in all developed countries CRC rates have stabilized or are declining\(^\text{[3]}\), CRC incidence in economically transitioning countries continues to rise both in its incidence and in mortality because of increased exposure to risk factors\(^\text{[2], [3]}\).

The second condition is that the basis of colon cancer development is well known and involves the evolution of adenomas to carcinoma\(^\text{[4, 5]}\), therefore, individuals with a history of adenomas have a higher risk of cancer\(^\text{[6]}\) and removal of polyps results in a reduction in colon cancer incidence\(^\text{[7]}\). However, we have the opportunity to intervene in the natural history of the adenoma–cancer sequence\(^\text{[8]}\).

Third, there are precise and feasible diagnostic methods that allow detection of the disease in early stages (non-metastatic tumors), which could be surgically cured by removal (reduction in the mortality rate of CRC), as well as the identification and removal of polyps (reduction in the incidence rate of CRC)\(^\text{[9-11]}\). Moreover, treatment is more effective and patients show a better prognosis when the neoplasm is diagnosed early\(^\text{[12]}\).

Interestingly, it has recently been reported that screening accounted for 53% of the decline in CRC mortality observed between 1975-2000 in the USA (26% less mortality); the other two facts being changes in risk factors (35%) and treatment regimes (12%). Moreover, the decline in CRC mortality in the USA can be enhanced if current trends, including screening, against cancer are accelerated; for example, only approximately 50% of its population older than 50 years have been screened\(^\text{[13]}\). Needless to say that in most countries, including many developed countries, no screening strategy has been proposed.

There are a great variety of screening strategies available for the average risk population, that is, individuals of, or over, 50 years with no other known risk factors for the development of CRC. These methods range from invasive and costly procedures such as flexible sigmoidoscopy, double contrast barium enema, and colonoscopy to more low-cost and non-invasive tests such as the fecal occult blood test (FOBT). All these methods have advantages and disadvantages regarding their sensitivity, specificity, risk, availability and cost but they have been shown to decrease CRC incidence and mortality\(^\text{[14-18]}\).

Colonoscopy is the gold standard\(^\text{[19]}\) and multiple studies have provided indirect evidence regarding the higher benefits of colonoscopy compared with other methods\(^\text{[20]}\). However, the costs and risk of complications, besides discomfort, have made this and other invasive tests such as flexible sigmoidoscopy\(^\text{[21]}\), poorly accepted for screening in an asymptomatic population\(^\text{[22-25]}\).

The benefit of CRC screening using a non-invasive test for blood in stool (Hemoccult) was established in 1993\(^\text{[26]}\). Subsequently, this result was corroborated in two other randomized controlled trials, leading to recommendations in many countries for CRC screening\(^\text{[24, 25]}\). The FOBT is the simplest and least expensive non-invasive approach to CRC screening available, however, it has several disadvantages. The most common method is the non-rehydrated guaiac FOBT\(^\text{[26]}\), based on the detection of peroxidase activity in the stool sample. Consequently, reagents also bind to nonhuman hemoglobin-like substances in feces, such as animal myoglobin and plant peroxidases. As the presence of these substances in the colon and rectum are related to diet, important dietary restrictions are required to minimize false negative results\(^\text{[26, 27]}\). Notwithstanding, its sensitivity and specificity are 30%-40% and 90%-98%, respectively\(^\text{[28]}\), with lower percentages for the detection of adenomas\(^\text{[29, 30]}\).

In the United States, the current recommendations include a number of screening tests in addition to Hemoccult. Immunochemical tests (iFOBT), which have not been evaluated in a randomized controlled trial, have performed similarly or even better in some studies, with generally higher compliance rates compared to Hemoccult or other guaiac-based tests\(^\text{[27, 31-34]}\), involving no dietary restriction, and resulting in fewer false positives\(^\text{[35]}\). The use of an immunochemical test in patients scheduled for colonoscopy\(^\text{[36]}\) showed the advantages of a quantitative test to determine the cutoff for positivity to adjust the screening program according to the resources available. Moreover, this test can be automated and two instead of three samples can be used for quantification\(^\text{[37]}\).

In Japan, more than 6 million people have been screened with immunochemical tests, with a positivity rate of 7.1%\(^\text{[38, 39]}\). With 60% of positive tests complying with the diagnostic protocol, the CRC detection rate was 1.6 per 1000. More than 70% of the cancers were classified as Duke’s A or Duke’s B, suggesting that the program worked well in detecting early stage cancer; CRC mortality and incidence were reduced by 72% and 59%, respectively\(^\text{[40]}\). Somewhat puzzling is the fact that guaiac is more sensitive than immunochemical for advanced adenomas (41.3% vs 29.5%)\(^\text{[41]}\), this may be because peroxidase sensitivity of the guaiac test detects lower levels of bleeding as some authors speculate. However, other explanations must also be considered.

This last study does illustrate the utility of comparing...
different tests rather than conducting long-term and expensive randomized controlled trials to evaluate each new FOBT. There are considerable data on Hemoccult, therefore comparing performance, outcome, compliance, and cost with new blood or fecal occult tests, as was done in this study, should be enough for the acceptance of new tests.

As blood could be present in the stool for other reasons, such as hemorrhoidal bleeding, iFOBT was also tested in combination with protein stool markers like hemoglobin-haptoglobin, calprotectin, carcinoembryogenic antigen, and the novel fecal markers S100A12 and tissue inhibitor of metalloproteinase-1 (TIMP-1), the latter allowing the detection of CRC at significantly higher rates than can be obtained with iFOBT alone. Genetic markers are also promising tools, such as the DNA-based stool test PreGen-Plus from EXACT Sciences, which shows a sensitivity of 51%-91% for CRC, with an average of 65%, and specificity between 93%-98%. Genetic markers are also promising tools, such as the DNA-based stool test PreGen-Plus from EXACT Sciences, which shows a sensitivity of 51%-91% for CRC, with an average of 65%, and specificity between 93%-98%.

However, non-invasive biological serum markers would be of great benefit for screening, because blood-based diagnostics can additionally classify tumors into distinct molecular subtypes and monitor disease relapse and response to treatment. Increasingly, biomarker strategies are becoming critical to identify a specific patient subpopulation that is likely to respond to a new therapeutic agent. The improved understanding of the underlying molecular features of common cancers and the availability of a multitude of recently developed technologies to interrogate the genome, transcriptome, proteome, and metabolome of tumors and biological fluids have made it possible to develop clinically applicable and cost-effective tests for many common cancers.

**Screening**

**Serum Biomarkers in CRC Screening**

Other advantages over stool testing are: sampling may be more convenient and acceptable for the patient, there is no microflora which could degrade the biomarker or hamper analysis, and sample processing may be easier. In addition, it will be commented later, information on the very early pathways of carcinogenesis, such as immune system cross-talk, can only be found in serum.

A meta-analysis evaluating blood markers for early detection of CRC reported in 2007 summarizing the performance characteristics of various approaches found that seventy different markers fulfilled the inclusion criteria with an overall sensitivity that ranged from 18% to 65%. The markers included cytopathic assays, DNA and mRNA markers, and soluble proteins.

Three studies investigated cytopathic assays, an inhibition of in vitro leukocyte adherence by incubation with tumor antigens, and the detection of circulating tumor cells by a membrane array. Sensitivity was above 70% for early stages and specificity ranged from 94% to 98%, however, the number of cases by tumor stage was very small. Notwithstanding, cellular mechanical properties have recently received increasing attention as a potential biophysical marker for cancer cells.

Four studies with DNA markers for the early detection of CRC were reported in that review. Free DNA, as well as mRNA, was isolated from circulating blood. Blood samples were analyzed for both genetic and epigenetic alterations of genes involved in the adenoma-carcinoma sequence, such as K-ras, tumor suppressor protein p53, APC (adenomatous polyposis of the colon), hMLH1 (human MutL homologue 1) or HLT (helicase-like transcription factor). Sensitivity reported for this group of markers was about 60% and lower, whereas specificity ranged from 73% to 100%. The potential of detecting adenomas was investigated only for mutations in the K-ras gene in one study which showed a sensitivity of 35% for adenomas. A recent review evaluated four commercialized biomarker tests based on that information (K-ras and B-raf mutation analyses, mismatch repair protein testing, and the Oncotype DX Colon Cancer Assay) for inclusion in the NCCN Guidelines Panel for Colon Cancer. In two cases, the available evidence was inconsistent to be included in the specific NCCN Guidelines.

Novel data on genetic and epigenetic mechanisms of CRC and how these alterations relate to emerging biomarkers for early detection, risk stratification, prognosis and prediction of treatment responses, are reviewed in . Potential markers waiting to undergo clinical validation for response to therapies are hypermethylation of septin-9 and DPYD (dihydropyrimidine dehydrogenase) genes.

Many relevant studies applied reverse transcription-PCR to detect mRNA expressed in circulating tumor cells. Blood samples were analyzed for mRNA molecules coding for CEA, cytokeratins (CK) 8, 9, and 20, human telomerase reverse transcriptase (hTERT), guanylyl cyclase C (GCC), carcinoembryonic gene member 2 (CGM2), melanoma-associated antigen family A (uMAGE-A), tumor-associated antigen L6, mucins (MUC) 1 and 2, protease M (ProtM), and thymidylate synthase. The most promising performance characteristics in this group of markers were reported for GCC mRNA, showing above 80% sensitivity for early stages.

Recent research has shed light on the biological importance of microRNAs (miRNAs). Their association with formation, angiogenesis, metastasis, and chemotherapy resistance of tumors has become one of the core issues in epigenetics of cancer, including CRC. miRNAs serve as micromanagers, negatively regulating gene expression. The potential utility of miRNAs in the preclinical stage has been explored, since manipulation of miRNAs may offer an alternative therapy for chemo- and radio-resistant CRCs.

The discovery that aberrantly expressed miRNAs vary among different tumor types and some of them are secreted in highly stable, cell-free form into blood led to the hypothesis that circulating (and fecal) miRNAs might potentially serve as non-invasive markers for early diagnosis.
of CRC\textsuperscript{83}. For example, 69 miRNAs were detected in CRC but not in control group sera; of these, 12 were not found in the serum of lung cancer patients\textsuperscript{84}. Circulating miRNAs are packed in complexes, either called exosomes or microvesicles, and emerging evidence has indicated that such external miRNAs are involved in cell-to-cell signal transduction and genetic information exchange\textsuperscript{85,86}. Two miRNAs significantly elevated in plasma and CRC tissues, but reduced in postoperative samples when compared with preoperative samples are miR-17-3p and miR-92a, both belonging to the miR-17-92 cluster. At a cut-off value of 3.6 for miR-17-3p (relative expression in comparison with RN6B), the sensitivity was 64% and the specificity was 70% at a cut-off value of 240 for miR-92a, the sensitivity was 89% and the specificity was 70%. In addition, miR-92a can distinguish CRC from other gastrointestinal cancers and inflammatory bowel diseases as well as advanced adenoma from normal controls, with a sensitivity of 64.9% and a specificity of 81.4%, whereas its expression levels were not correlated with tumor-node-metastasis (TNM) stages\textsuperscript{87,88}.

Fifty two protein markers in the meta-analysis of 2007\textsuperscript{67} were analyzed by common standard procedures, still more easy-to-use and quicker than the nucleic acid methods, like ELISA, RIA, or activity assays, or by chromatographic and mass spectrometric (MS) assays based on surface-enhanced laser desorption/ionization time-of-flight (SELDI-TOF) MS, and matrix assisted laser desorption/ionization time-of-flight (MALDI-TOF) MS. This group of markers can be further subdivided into carbohydrate antigens, carcinoembryonic antigens, other antigens, antibodies, cytokines, and other proteins. Sometimes, different markers were analyzed in parallel. For example, combinations including carbohydrate antigens and carcinoembryonic antigens were very common.

Carcinoembryonic antigen (CEA) was the first blood marker proposed in connection with CRC\textsuperscript{78,80}. Although overall sensitivity ranged between 43% and 69%, there was a clear increase in sensitivity by tumor stage, ranging from 8% for Duke's A up to 89% for Duke's D. Specificity was above 90% in nineteen studies.

Carbohydrate antigens (defined by monoclonal antibodies against colon carcinoma cell lines) include CA 19-9, CA 195, CA M26, CA M29, CA 50, CA 72-4, CA M43 and CA 242. Many studies evaluated CA 19-9, with overall sensitivity ranging between 43% and 69%, there were no correlations with tumor-node-metastasis (TNM) stages\textsuperscript{89,90}. As a summary of these and more recent studies, the more promising results, for both sensitivity and specificity, were observed with u-PA (76% and 96% respectively\textsuperscript{61}, M2-PA\textsuperscript{100} (69% and 90%), TPA-M (70% and 96%), CP (cancer procoagulant; 86% and 82%)\textsuperscript{102}, sCD26 (soluble cluster of differentiation 26) (90% and 90%)\textsuperscript{93}, fibrin degradation (DR-70) (80% and 93%)\textsuperscript{104}, prolactin (77% and 98%)\textsuperscript{105}, laminin (89% and 88%)\textsuperscript{106}, BSP (bone sialoprotein; 88% and 100%), although similar results were found in breast and prostate cancers\textsuperscript{107} and CC52A-2 (78% and 97%), but sCD26 has been the most studied, as commented in later subheadings\textsuperscript{108-111}.

**SOLUBLE CD26**

Dipeptidyl peptidase IV (DPP-IV), assigned to the CD26 cluster, is a multifunctional or pleiotropic protein expressed particularly on epithelial cells and lymphocytes. CD26/DPP-IV has been consistently associated with...
cancer since it was known as ADCP, or the ADA-2 / Large isoform [112,113]. Many reviews have discussed the non-enzymatic role of CD26/DPP-IV as an extracellular anchorage for ADA in cancer and the potential usefulness of this protein in therapeutics and diagnostics [114-119]. The ADA-CD26 complexes may participate in cell-to-cell contacts [120-122] or, more probably in this context, through the catalysis of adenosine to inosine [121,122,123]. Proliferating cells accumulate high extracellular concentrations of adenosine, a purine nucleoside found within the interstitial fluid of solid tumors, which may be toxic or influence the proliferative potential of a cell, depending on the relative expression and type of adenosine receptor (AR). Therefore, the different levels of the cell-surface CD26-ADA complex and relative expression of ARs on a tumor cell may lead to the generation of tumor subclones, as well as its participation in the well-known adenosine inhibition of cell-mediated immune responses to tumor cells [115,116,119,124-127]. Other pro-oncogenic activities may be related to the recently described CD26-ADA-plasminogen ternary complex. Binding of plasminogen to cell-surface receptors promotes its conversion to plasmin, which is required for proteolysis of the ECM in several physiological and pathological processes, including cell migration, tumor cell invasion and metastasis [127].

CD26, also present at the invadopodia, together with other ecto-proteases and metallocproteinases (MMPs) [128-130], can participate in malignant transformation and cancer progression through its ability to bind collagen and fibronectin [116,117,128,129,132-134], MMPs and FAPα (a CD26 homologous protein expressed in tumor cells) digestion of ECM components will allow passage of the malignant cells through basement membranes and stromal barriers. This pro-oncogenic behavior is thus consistent with the non-enzymatic interactions with cell-surface ADA and plasminogen mentioned above, and the formation of FAP-CD26 heterodimers [129,133].

However, there is a fundamental difference between CD26 and the other proteases involved in cancer development and progression as executors of ECM degradation: CD26 is constitutively expressed in the tissues mentioned at the beginning of this heading, and its enzymatic activity regulates the biological activity of regulatory peptides, such as incretins secreted by the enteroendocrine system. DPP-IV has therefore become a novel therapeutic target for inhibitors that extend the endogenously-produced insulin half-life in diabetes [114,115,119,144], and similarly the half-life of growth factors and chemokines [115].

In addition, glypican-3 has recently been reported as the first natural inhibitor of CD26/DPP-IV enzymatic activity, in in vitro experiments [116]. Glypicans are basically absent in adult tissues, but up-regulated in many tumor tissues [145,146]. If glypican-3-dependent local DPP-IV inhibition can be confirmed in a physiological context, this indicates a natural protective role for the enzyme that should be blocked in the tumorigenic process. This anti-oncogenic role, first contrasted in 1999 by Houghton’s group [145-148], together with many data

-differences in the cellular staining pattern with respect to the normal tissue, significant intratumor heterogeneity and changes in CD26 expression linked to the transition of tumor stages- already reviewed [118], indicate a quite complex situation in the physiological microenvironment of cancer niches. The possibility that the tumorigenic process may manipulate the functions of CD26/DPP-IV, for example evading the immune system by modifying local chemokine gradients (and therefore, immune cell homing), and by modulating cytokines and angiogenic or immunosuppressive factors [90,106-109] deserve to be studied in more detail [142].

In this context, the role of serum DPP-IV activity, first discovered in 1968 by Nagatsu’s group in Japan [90], is not known. Within normal plasma/serum, some 90%-95% of DPP-IV activity has been associated with a relatively high concentration of serum (or soluble, in contrast to transmembrane) CD26 (sCD26) in human serum (570 μg L⁻¹) [115,118,155-157]. Since sCD26 is heavily glycosylated, its molecular weight is similar to that of transmembrane CD26 [136,138] although it lacks transmembrane and cytoplasmic domains (the sequence starting at the 39th position) [115].

There is no direct correlation between serum CD26 protein concentrations and serum enzymatic activity assays, for three reasons: (1) There are some circulating proteins other than CD26 with DPP-IV activity (DPP-II, FAPα,…); (2) sialylation (a type of glycosylation) of sCD26 [114,118,155-157] is strongly enhanced in elderly individuals [159], and certain type of hypersialylation can inhibit DPP-IV activity [160], consistent with the fact that serum/plasma DPP-IV enzymatic activity tends to decrease with age [154,158] (3) it has recently been suggested that the serum protein attractin, which enhances the enzymatic activity of trolloid proteases [151,162], may regulate the DPP-IV activity of CD26/sCD26 in the same way [119]. Serum attractin is actually frequently co-purified with sCD26 [153-156].

Iwaki-Egawa et al. [156,157], suggested that sCD26 must be shed from any plasma membrane on CD26 expressing cells that are in contact with blood, by proteolytic cleavage. The fact that only one CD26 mRNA form is usually reported [119,171], and that it is transported from its site of synthesis in the rough endoplasmic reticulum to the microvillar membrane of enterocytes, and in some cell lines in a membrane-bound state [133,134,172-174], also suggest that it is not secreted. It must be pointed out that the shedding of most integral membrane proteins is often regulated by a PKC-dependent mechanism [175,176].

However, CD26 has been found to be soluble in the lumen of secretory granules, undergoing exocytosis to the interstitial space of endocrine pancreatic A cells, where sCD26 may act on secretory products of neighbouring islet cells [178,179]. Autolysis of the protein by the acidic pH conditions inside the granules has been observed in vitro [179,180]. In addition, another possibility related to the intracellular sorting is the secretion of soluble proteins through MPP-dependent shedding from exosomes. Exosomes are small membrane vesicles derived from...
intracellular multivesicular bodies that can undergo constitutive and regulated secretion from cells upon fusion with the PM[181-183]. Exosomes with CD26/DPP-IV have been found in human saliva, released at the basolateral surface of enterocytes, and in ram epididymal fluid[184-186]. In addition, the origin of sCD26 is also unknown.

The hepatobiliary system was the first to be suggested[187]. Liver epithelium is often cited as the most likely potential source[113, 116, 137, 188-192] and at least in some conditions, sCD26 originates from the brush border of hepatocytes[190]. However, CD26 is predominantly located in the bile canaliculi[190, 193, 194], and a recent study found that in chronic hepatitis C and other liver viral infections, DPP-IV activity levels were not correlated with several markers of bile duct injury or hepatocyte injury[190]. These authors suggested that the increased activity in these diseases may originate directly from its shedding from the peripheral blood T cells involved in the control of viral infections or, indirectly, by stimulating other cells such as hepatic stellate cells. The involvement of T cells had already been suggested in studies of liver regeneration[183,194]. In fact, Kasahara et al[197] suggested a possible origin of sCD26 from the immune system, although they also identified serum isoforms from liver, spleen or kidney. Kidney, an obvious potential source because it contains large amounts of CD26, was rejected early on[156] because anephric individuals have normal amounts of sCD26, and because sCD26 contains approximately twice as much sialic acid as kidney CD26. However, several data suggest that serum CD26 is at least partly shed from T cells[158, 159, 161, 194, 198-204] sCD26 PATHOPHYSIOLOGY AND CRC DIAGNOSIS

Many studies have demonstrated altered serum levels of enzymatic DPP-IV activity (see review[142]) and soluble CD26 protein in several diseases. Some studies show contradictory results, probably related to the stage of the disease considered (or in which a particular patient has been recruited)[103,110,111,158, 228-230]. However, other discrepancies between enzymatic activity and protein concentration measurements can be explained by putative changes in the glycosylation pattern (leading to a lack of immunorecognition of sCD26), the putative presence of the DPP-IV activator attractin, inhibitor glypican-3 or the secretion of other dipeptidyl peptidases such as DPP-II or soluble FAPα (DASH). For example, in myocardial infarction patients treated with streptokinase, the enzyme concentration is reduced to more than 50% after 90 d of therapy, while measurements of DPP-IV enzymatic activity did not change during that period[211]. On the contrary, the same authors found that there was no change in sCD26 concentrations between healthy donors and patients with rheumatoid arthritis and lupus erythematosus, although a lower enzymatic activity was detected[158]. Reference values of DPP-IV specific activity show no differences in serum and plasma[118,142,187], but most reports do not use the same assay conditions or the same definition of specific activity -the same applies to the units of catalytic activity-, making it difficult to compare results even from the same authors. However, the amount of sCD26 antigen found in normal serum with the most commonly used commercial ELISA kit (Bender MedSystems), corresponds well with the expected values based on the specific activity of purified serum DPP-IV[118,142]. Together, these findings support the use of immunodetection techniques for the quantification of these molecules because they are more specific.

DPP-IV activity is high in patients with hepatic cancer, hepatitis, osteoporosis, cholestasis and other liver diseases. On the other hand, the mean DPP-IV activity remains unchanged in metastatic bone disease, esophagus, gall bladder, chronic myelocytic leukemia or leiomyosarcoma cancers, in allergic asthma, celiac disease, and adult T-cell leukemia, although serum DPP-IV in the latter is strongly correlated with the percentage of CD26+ T cells. However, decreased levels of DPP-IV were observed in patients with acute lymphocytic leukemia, thyroid and oral cancer, advanced gastric carcinoma, HCV infections, inflammatory bowel diseases, type II diabetes, in healthy smokers, in pregnancy, and in alcoholics and patients suffering from major depression.
A reduction in DPP-IV activity has been related to symptoms of depression and anxiety under certain circumstances. Contradictory results were reported for psychologically-related eating disorders such as anorexia or bulimia, CRC, rheumatoid arthritis, lupus erythematosus and Sjögren syndrome.

Many studies have used sCD26 as a soluble marker of Th1 cellular immune activation, together with sCD30 and sometimes sCD23 as markers of Th2 (humoral response) [231-234]. The concentration of sCD26 increases in HIV-1 patients, leishmaniasis, myocardial infarction and atopic dermatitis. It does not change in asthmatics, osteoarthritics and gastric cancers. In many, but not all studies, it decreases in rheumatoid arthritis and particularly in lupus erythematosus and Sjögren syndrome.

We were the first to report reduced levels of sCD26, using immunodetection, in the serum of CRC patients, compared with healthy donors [103]. Reduced levels of enzymatic activity were reported for a small group of patients in 1987 [233], although other authors found increased DPP-IV activity in a cohort of CRC patients comparable to ours [229]. We have already made some putative explanations to clarify this lack of correlation. From a biological point of view, further research studies are needed, but this issue does not affect the focus of this article. Our most important finding was that lower concentrations were found particularly in the early stages of the disease. Sensitivities higher than 80% (Table 1) were found for Dukes’ stages A, B and C, whereas it was impaired in Dukes’ stage D, in which CEA levels diagnosed better. Interestingly, it was in stage D where the DPP-IV activity actually increased in the study mentioned [229]. In this first study, we also found that sCD26 as a variable is not related with Dukes’ stage classification, age, gender, tumor location or degree of differentiation, which also suggested the potential usefulness of this molecule for early diagnosis of CRC. We also showed preliminary data on the potential prognostic value with a follow-up of 2 years until recurrence; additional data has not yet been published. Moreover, we did not find changes in related diseases such as gastric-tract carcinomas, and in two of four blood cell cancers the concentration was raised (q = 4); impaired levels of sCD26 were observed only in some cases with gastric tract benign pathology and with Crohn’s disease [110, 228].

These last results on specificity agreed with published works, and Crohn’s disease data may be irrelevant for screening since these patients should have been detected years before the CRC screening procedure. To establish the feasibility of the sCD26 test for the diagnosis of CRC, we decided to perform a first pilot case-finding study that tested 170 persons of both genders at average risk for CRC (older than 50 years and asymptomatic for bowel disease), excluding individuals with a family history of CRC, or colorectal polyps, or personal history of CRC. From 29 individuals positive for the marker (with serum levels below or at the cut-off of 410 ng/mL), as previously studied [113], 21 underwent the colonoscopic procedure with colorectal findings in ten individuals (47.6%) against 3 out of ten individuals negative for the marker (30%) [113], showing an additional value of sCD26 for the detection of premalignant lesions (Table 1).

The aim of a later case-finding study in a large cohort (2754 presumably healthy individuals) was to evaluate its association with epidemiologic parameters as well as certain common digestive-related symptoms or pathologies [109]. Personal questionnaires were completed for data such as personal and familial history of colorectal polyps or cancer, bowel diseases (non-inflammatory benign pathologies: anal fissure, hemorrhoids, diverticula, irritable bowel syndrome and spastic colon; and inflammatory bowel diseases: colitis

| n | CRC risk | sCD26 cut-off (ng/mL) | CRC in sCD26 + patients | Advanced adenomas in sCD26 + patients | Polyps in sCD26 + patients | Other findings in sCD26 + patients | Sensitivity (%) | Specificity (%) |
|---|---|---|---|---|---|---|---|---|
| 1st Case-control study | 175 | Diagnosed with CRC | 410 | 99/110 | - | - | 6/110 Crohn’s | 90% (CRC) | 90% (CRC) |
| 1st Case-finding study | 170 | Average-risk | 410 | - | - | 8/21 | 2/21 diverticula | - | - |
| 2nd Case-finding study | 2673 | Average- and increased-risk | 410 | 2/140 | 4/140 | 146/140 | 12/140 diverticula | 100% (CRC) | 89.9% (CRC) |
| 2nd Case-control study | 299 | Increased-risk | 460 | 27/110 | 20/110 | 13/110 | 18/110 IBD; 18/110 non-IBD; 14/110 anemia, diarrhoea, rectal bleeding | 81.8% (CRC) | 72.3% (CRC) |

Table 1 Performance characteristics of the sCD26 test

n: Number of individuals; CR: Colorectal; CRC: Colorectal cancer; CN: Colorectal neoplasms including CRC and advanced adenomas; GC: Gastric cancer; IBD (inflammatory bowel diseases, includes colitis and Crohn’s); non-IBD (includes hemorrhoids and diverticula). ‘No data on polyps’ pathology could be obtained; 1: Data obtained after one-year follow-up for the detection of interval cancers. 2: Pathological anatomy information obtained only for 16 of 46 polyps. The four advanced adenomas are also included in the polyps’ column.
or Crohn’s disease), symptoms (rectal bleeding or fecal blood and changes in bowel habits), and smoking status. Individuals with a personal history of CRC, personal history of a cancer other than CRC, personal history of colorectal polyps, and familial history of cancer and/or colorectal polyps were excluded.

The mean sCD26 concentration in this cohort corresponded to 555.9 ± 181.7 ng/mL, similar to that previously reported for 52 healthy donors (559.7 ± 125.5 ng/mL). However, the range in this large cohort was considerably broad compared to that of the healthy donors (118–3062 ng/mL and 273–863 ng/mL, respectively)\[109\]. Information concerning the smoking status was also obtained. 63.8% of the individuals were non-smokers, 27.8% were current smokers, while 8.4% were former smokers. Former smokers showed statistically significant higher values of sCD26, and current smokers lower than non-smokers, the latter fact correlating with data on enzymatic activity\[236-240\]. However, this small difference (20 and 10 ng/mL, respectively) was not statistically significant when grouped by the number of cigarettes per day.

According to the cut-off point 410 ng/mL, 273 individuals (10.2%) were sCD26-\[109\]. To extend the validation of sCD26 as an early biomarker for CRC, a colonoscopic procedure was recommended to these individuals. Among the 140 individuals that underwent colonoscopy, one case of CRC was diagnosed, resulting in a very high prevalence (0.7%) for this cohort. In addition, there were 46 cases of colorectal polyps (32.9%), 12 cases of colorectal diverticula (8.6%) and 81 individuals without apparent colorectal pathology (Table 1). The PPV for the sCD26 test considering all the findings was 42.1%\[111\]. The sCD26+ individual diagnosed with CRC after colonoscopy received surgery after three months, finding a tumor in Dukes’ stage B. Interestingly this patient had a negative FOBT two weeks before the measurement of sCD26. Another case was a sCD26+ individual who had a second positive test three months afterwards, and later was diagnosed by colonoscopy with a 1-cm villous polyp in the transverse colon, which was not extirpated. After seven months, the patient was diagnosed and operated on for a moderately differentiated adenocarcinoma at Dukes’ stage A (data not published). The 46 individuals diagnosed with colorectal polyps represent a percentage similar to those diagnosed in the first case-finding study, also elevated considering the average risk\[109\]. Information regarding the pathological anatomy of polyps was obtained only for 16 cases. Of these, 75% presented neoplastic histology (adenomas). Trying to explain the high number of colorectal polyps diagnosed, no differences were found between the mean age of the individuals with and without polyps (data not published).

Although the most accurate means of measuring sensitivity and specificity is to perform colonoscopies in all the screened patients regardless of the test result, when this is not possible, several authors use a follow-up period to detect interval cancers\[24,214,241\]. Therefore, it is assumed that a false negative becomes clinically apparent through subsequent screening or the appearance of symptoms. According to this approach, with a one year follow-up of our individuals, a sensitivity of 100% and a specificity of 89.9% for CRC were obtained (Table 1)\[111\].

These results, with special interest on the absence of correlation among all the parameters analyzed, particularly the personal and familial history of CRC and polyps together with rectal bleeding and changes in bowel habits, proved that the sCD26 test can be easily offered and evaluated in a large population cohort. Additional data also support the usefulness of serum sCD26 levels for patient monitoring because four of the patients diagnosed with polyps requested a second sCD26 test after polypectomy, which showed normalized values (> 410 ng/mL) in the new measurement in all patients\[111\].

However, accurate clinical values suggesting that a serum CD26 test is an improvement on the current non-invasive screening tests recommended was lacking. Therefore a case-control study with 299 symptomatic and asymptomatic patients, who were to undergo colonoscopy, was performed\[108\]. Colonoscopy indication was mostly due to symptoms such as rectal bleeding, abdominal pain, diarrhea, constipation, anemia, colorectal polyp or cancer surveillance, and CRC screening. Patients were classified into groups as follows: no colorectal pathology (symptomatic with rectal bleeding, abdominal pain, diarrhea, anemia, constipation, or asymptomatic with personal history of polyps or CRC, and family history of polyps); non-IBD (hemorrhoids and diverticula); IBD (colitis or Crohn’s disease); colorectal polyps (hyperplastic polyps, non-advanced adenomas and advanced adenomas); and CRC.

The average sCD26 level for the group of patients with no colorectal pathology or benign colorectal pathology was 641.2 ± 241.2 ng/mL, higher than the cut-off point obtained with healthy donors as the control cohort. Therefore, we chose to calculate a new cut-off, 460 ng/mL. According to this, the sCD26 test has a sensitivity and specificity of 81.8% and 72.3%, respectively, for CRC (Table 1), (a specificity of 79.3% when the group of symptomatic patients with no colorectal pathology was considered). The mean sCD26 concentration decreased, although non-significantly, as the pathology diagnosed was more severe, that is, from no colorectal pathology to CRC, with a noticeable decrease in the group with IBD and anemia. Interestingly, individuals with anemia showed a substantially elevated sCD26 positivity rate (71.4%), as well as the IBD group (69.2%), both responsible for the specificity value. IBD is associated with at least a 5-fold increased risk for CRC, representing one of the highest risk groups based on the inflammation-dysplasia-carcinoma sequence\[242\]. However, these individuals are usually diagnosed at the age of the potential CRC screening procedure, as commented, and its impact on the specificity data can be avoided. When considering only asymptomatic individuals, specificity increases to 90%, which agrees with our previously published results\[103,104,113\].
On the other hand, as no carcinomas in situ were detected in the patients included in the study, the decrease in sensitivity in this context is probably related to altered frequencies in CRC stages (sCD26 is a poorer marker in Duke’s A than in B or C stages)\[103\].

In this study, we also analyzed the relationship of this biomarker with advanced adenomas. Defining advanced adenomas as those larger than 10 mm, with tubulovillous or villous histology, or with high-grade dysplasia, and classifying patients with more than one polyp according to the most advanced lesion, sensitivity for the detection of CRC and advanced adenomas was 58.0%, with a specificity of 75.5% (Table 1). We found no statistical differences, according to the sCD26 positivity rate, with regard to the number of polyps, their size, location, morphology or histology, but differences closely significant were observed with the grade of dysplasia, a morphological marker of neoplastic lesions. The positivity rate increased gradually with the degree of dysplasia: 22.2% for non-dysplastic polyps, 32.5% for low-grade dysplastic adenomas and almost double (60.0%) for high-grade dysplastic adenomas. Concerning advanced adenomas, a term commonly used to group adenomas that have an increased likelihood of malignant transformation, the sCD26 positivity rate was statistically significant.

As commented, iFOBT is now preferentially offered for average-risk screening. A highly sensitive FOBT (guaiac-based) test (Hemoccult SENSA®) reached 71-79% sensitivity with single testing, and 85% with multiple testing, with corresponding specificities of 86% and 95%\[241-243\], although these parameters are probably overestimated as these studies lacked colonoscopic examination of the negative cases. For both pathologies together (CRC and advanced adenomas) in an asymptomatic high-risk cohort, however, higher sensitivity, 65.3% (and 87.5% for specificity), resulted with Hemoccult SENSA®\[244\] compared to iFOGT (33.1% sensitivity and specificity of 97.5%, parameters obtained with flexible sigmoidoscopy). For other experimental serum biomarkers, the CCSA-2 has shown 97.3% sensitivity and 78.4% specificity, although hyperplastic polyps and non-advanced adenomas were considered as findings, while IBD patients were absent in their cohort\[109\]. Therefore, sCD26 seems to perform adequately as a blood biomarker for CRC and advanced adenomas, and is independent of the frequent but intermittent bleeding, unlike guaiac FOBT or iFOBT.

In hepatocarcinoma, a loss of membrane CD26 is correlated with higher DPP-IV levels. This fact is not seen in CRC, as almost all CRC patients show reduced serum levels of sCD26\[108,111\], but loss of membrane CD26 expression only occurs in 11% of colorectal tumor\[245\]. In conclusion, for CRC, sCD26 is not correlated with cell proliferation, or with the alteration of CD26 expression in CRC tumor cells. Nor is there any direct correlation between sCD26 levels and tumor location, degree of histological differentiation, type of metastasis or Dukes’ stages of CRC\[246\], which may affect the hepatic production of sCD26. Therefore, sCD26 is also independent, if not of the tumorigenic locus, at least of the tumorigenic tissue. In addition, as it seems immune-related\[142\], the sCD26 decrease in the plasma of patients should appear sooner in the adenoma-carcinoma development compared to the presence of fecal blood.

CONCLUSION
As commented previously, it has recently been reported that screening accounted for 53% of the decline in CRC mortality observed during 1975-2000 in the USA (26% less mortality). Moreover, decline in CRC mortality in the USA could be enhanced if current trends against cancer were accelerated\[151\]. Therefore, any kind of screening strategy should be proposed in advanced and developing countries. For FOBT Hemoccult, which is a non-invasive and relatively cheap test, there are considerable data from many prospective studies in different countries of the world; however, not many countries include this screening method in their public health systems.

In the context of this review, dealing with an experimental CRC screening test that is easier to monitor in the health system, or with a better clinical value, the idea of comparing different tests rather than conducting long-term and expensive randomized controlled trials to evaluate each new test is very important, as suggested by Mandel\[173\] in the commentary on the Allison study that compared performance, outcome, compliance, and cost of guaiac FOBT and iFOBT\[245\] fecal occult blood tests. In this way, it will be much easier to study and promote new fecal or blood tests. With this aim, we are currently initiating a multicentric, prospective, double-blinded study in an average-risk population, where the performance of the quantitative iFOBT and the sCD26 assay will be assessed and compared with the gold standard colonoscopy.

Another important idea, as we have previously proposed\[108\], is the combination of biomarkers for the management of cancer, since it is difficult to achieve a simple test to detect early-stage tumors that is useful for screening purposes. For example, we have tested sCD26 levels, α-L-fucosidase activity and CEA in the same patients\[109\], and while, at a specificity of 100%, α-L-fucosidase activity did not enhance the sensitivity value obtained with sCD26 alone in TNM stage II patients, the sensitivity obtained from the combination of both markers was 65% versus 33% for sCD26 alone in TNM stage I patients. In the same way, a very recent work assessed the combination of CEA with three other biomarkers, sCD26, DR-70 and MMP-9, previously selected from 26 candidates, for the detection of CRC\[246\]. This study confirmed that sCD26 and DR-70 (fibrin and fibrinogen degradation products\[108\]) are the more promising of the available serum markers, although DR-70 showed a significant correlation with age. Values of the area under the ROC curve, and sensitivity and specificity for sCD26 were similar to our latest study mentioned above\[108\] using a similar cohort of case-control patients who attended...
colonoscopy. The same study also showed that a combination of sCD26, DR-70 and CEA detected CRC, particularly at the early stage of disease, significantly better than CEA alone or other biomarker combinations at certain specifications. Nevertheless, our data on sCD26 for the detection of the earliest stages were much better (and data of CEA worst). This discrepancy perhaps may be due to the differences in the cohort composition in each study, to the statistical method employed for the combination of biomarkers, or to a development of the ELISA for the measurement of CEA levels (the kits used for CEA, but not for sCD26, were different in each study).

Therefore, the approach tries to increase the clinical value of each biomarker or yield a test more able to distinguish between patients and healthy individuals, and ideally also among different kinds of tumors, in the way we have tested for head and neck cancer versus non-small cell lung cancer at a low scale, and others for the screening of Alzheimer disease, or to identify lymph node metastases in non-small cell lung cancer patients. In this case, a panel of six serum biomarkers classified the patients better than conventional clinical methods.

To measure several biomarkers at a time, ELISAs for key serum markers are being arrayed or multiplexed based on immunoblot technology or flow cytometric beads. These techniques, in relation with other genomic or proteomic techniques, are more transferable to practical application in clinical decision-making. In this sense, it is interesting to note that the multiplexed diagnostics market has grown rapidly and generated sales of approximately $2.4 billion in 2009, and is expected to tip in favor of continued rapid growth, reaching almost $5.8 billion in 2015. Moreover, the multivariate data obtained from such a test can easily be managed with new statistical methods already developed for the fields of genomics and proteomics in general.

However, as multiple cancer screening tests are being advocated for the general population, clinicians and patients are not always well-informed of screening burdens. For example, in the ongoing Prostate, Lung, Colorectal, and Ovarian (PLCO) Cancer Screening Trial, a randomized controlled trial to determine the effects of prostate, lung, colorectal, and ovarian cancer screening on disease-specific mortality, an individual has an approximately 50% or greater risk of a false-positive finding by the 14th test. Physicians should educate patients about the likelihood of false positives and resulting diagnostic interventions when counseling on cancer screening.

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