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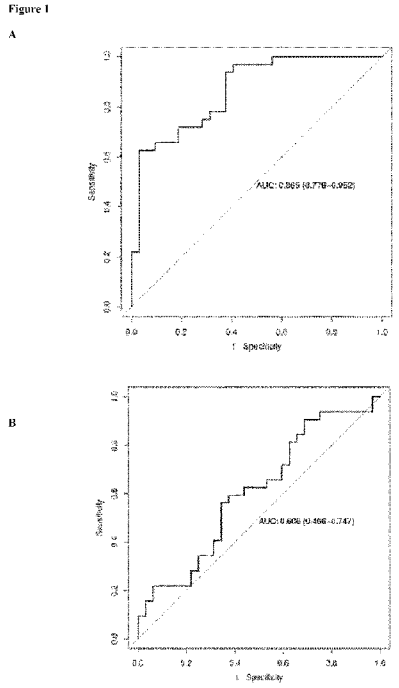
(54) Title: PROTEIN SIGNATURE FOR THE DIAGNOSIS OF COLORECTAL CANCER AND/OR PRE-CANCEROUS STAGE THEREOF

(57) Abstract: The present invention refers to an *in vitro* method for the diagnosis of colorectal cancer and/or pre-cancerous stage thereof.

Figure 1



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PROTEIN SIGNATURE FOR THE DIAGNOSIS OF COLORECTAL CANCER AND/OR PRE-CANCEROUS STAGE THEREOF

FIELD OF THE INVENTION

The present invention can be included in the medical field. Particularly, the present invention refers to an *in vitro* method for the diagnosis of colorectal cancer and/or pre-cancerous stage thereof.

STATE OF THE ART

Colorectal cancer (CRC) (also known as colon cancer, rectal cancer, or bowel cancer) is the development of cancer in the colon or rectum (parts of the large intestine). The vast majority of colorectal cancers are adenocarcinomas. This is because the colon has numerous glands within the tissue. When these glands undergo a number of changes at the genetic level, they proceed in a predictable manner as they move from benign to an invasive, malignant colon cancer. The adenomas of the colon, particularly advanced colorectal adenoma (AA), are a benign version of the malignant adenocarcinomas but still with malignant potential if not removed (they are usually removed because of their tendency to become malignant and to lead to colon cancer).

Screening is an effective way for preventing and decreasing deaths from colorectal cancer and is recommended starting from the age of 50 to 75. The best known and most frequently used screening test for colorectal cancer is called Fecal Immunochemical Test (FIT). FIT detects blood in the stool samples which can be a sign of pre-cancer or cancer. If abnormal results are obtained, usually a colonoscopy is recommended which allows the physician to look at the inside of the colon and rectum to make a diagnosis. During colonoscopy, small polyps may be removed if found. If a large polyp or tumor is found, a biopsy may be performed to check if it is cancerous. The gastroenterologist uses a colonoscopy to find and remove these adenomas and polyps to prevent them from continuing to acquire genetic changes that will lead to an invasive adenocarcinoma.

Although, as explained above, FIT is nowadays used for screening colorectal cancer, it is important to note that FIT offers a low sensitivity for AA (around 20-30% depending on literature) which means that most of said kind of patients can be wrongly classified as not having the disease. Consequently, FIT is not able to identify adenomas due to its low sensitivity. Moreover, since FIT uses stool samples, it offers a low compliance. On the other hand, the colonoscopy is an invasive technique wherein the most severe complication generally is the gastrointestinal perforation. On the other hand, colonoscopy is nowadays a procedure involving anesthesia, and the laxatives which are usually administered during the bowel preparation for colonoscopy are associated with several digestive problems.

It is important to note that the methods used today for screening general population at risk of suffering for CRC or AA are associated with a high rate of false positives. Consequently, a high amount of unnecessary follow-up colonoscopies are nowadays performed.

The present invention offers a clear solution to the problems cited above because it is focused on an *in vitro* method for identifying or screening human subjects at risk of suffering from colorectal cancer or colorectal adenomas (particularly advanced colorectal adenomas), departing from the concentration level of protein biomarkers isolated from minimally-invasive samples such as blood, serum or plasma. Since the method of the invention is based on blood, serum or plasma samples, it is expected to improve compliance to colorectal cancer screening. Moreover, the method of the invention offers high sensitivity and specificity, which means that it is a strong and cost-effective method for the detection of both colorectal cancer and colorectal adenomas.

DESCRIPTION OF THE INVENTION

The present invention refers to an *in vitro* method for diagnosing, identifying or screening human subjects at risk of suffering from colorectal cancer and/or advanced colorectal adenomas, departing from the concentration level of protein biomarkers isolated from minimally-invasive samples such as blood, serum or plasma. The method of the invention offers high sensitivity and specificity, which means that it is a strong

and cost-effective method for the detection of both colorectal cancer and colorectal adenomas.

Since the method of the invention has higher sensitivity and specificity as compared to the method used today (FIT) for screening general population at risk of suffering from CRC or AA, it is associated with a lower percentage of false positives. Consequently, the method described in the present invention clearly helps in reducing the number of follow-up colonoscopies, thus improving the way that the patients are nowadays screened or diagnosed. Once the method of the invention is performed, if it is determined that the patients might be suffering from colorectal cancer and/or precancerous stage, the result is confirmed by colonoscopy. However, if it is not determined that the patient might be suffering from colorectal cancer and/or precancerous stage, there is no need to perform a colonoscopy and routine testing with the method of the invention defined below is recommended.

Particularly, the first embodiment of the present invention refers to an *in vitro* method (hereinafter "method of the invention") for the diagnosis of colorectal cancer and/or a pre-cancerous stage thereof which comprises: a) Measuring the concentration level of at least Flt3L, in a biological sample obtained from the subject and b) wherein if a deviation or variation of the concentration level of at least Flt3L is identified, as compared with the reference concentration level measured in healthy control subjects, this is indicative that the subject is suffering from colorectal cancer and/or a pre-cancerous stage.

In a particularly preferred embodiment, the method of the invention comprises measuring the concentration level of at least the combination [Flt3L and CYFRA21-1] in a biological sample obtained from the subject.

In this regard, it is important to consider that all the most reliable signatures claimed in the present invention comprise Flt3L, preferably [Flt3L and CYFRA21-1], thus obtaining various biomarker signatures comprising Flt3L, preferably [Flt3L and CYFRA21-1], such as [Flt3L and CYFRA21-1 and AREG], [Flt3L and CYFRA21-1 and AREG and ErbB4] or [Flt3L and CYFRA21-1 and AREG and CLEC2C] with an Area Under the Curve (AUC) around 0.9 for the detection of CRC a with a good

performance also for the detection of AA (see **Table 12**). Although any of the signatures comprising Flt3L, preferably [Flt3L and CYFRA21-1], can be efficiently used according to the present invention, the following signatures comprising Flt3L, preferably [Flt3L and CYFRA21-1], are particularly preferred since they offer an AUC value above 0.9 for the detection of CRC and a good performance for the detection of AA: [Flt3L and CYFRA21-1 and AREG and ErbB4] (AUC for CRC =0.931) or [Flt3L and CYFRA21-1 and AREG and CLEC2C] (AUC for CRC = 0.915) (see **Table 12**).

The second embodiment of the present invention refers to a kit of parts comprising reagents for the determining the concentration level of any of the above cited signatures. In a preferred embodiment, the present invention refers to the *in vitro* use of a kit comprising reagents for the determination of the concentration level of Flt3L, or the combination [Flt3L and CYFRA21-1], preferably [Flt3L and CYFRA21-1 and AREG], [Flt3L and CYFRA21-1 and AREG and ErbB4] or [Flt3L and CYFRA21-1 and AREG and CLEC2C] for the diagnosis of colorectal cancer and/or a pre-cancerous stage thereof.

According to the method of the invention, after measuring the concentration level of any of the above cited combinations of biomarkers, a score value is obtained for the signature and this score value is compared with a threshold value which defines the diagnostic rule. If this score value is higher than the threshold, then the corresponding sample is classified as a positive sample, which is an indication that the patient might be suffering from colorectal cancer and/or pre-cancerous stage thereof. The threshold value has been defined in order to optimize sensitivity and specificity values. Consequently, in a preferred embodiment, the method of the invention comprises: a) Measuring the concentration level of any of the above cited combinations of biomarkers, in a biological sample obtained from the subject, b) processing the concentration values in order to obtain a risk score and c) wherein if a deviation or variation of the risk score value obtained for any of the above cited combinations of biomarkers is identified, as compared with a reference value, this is indicative that the subject is suffering from colorectal cancer and/or a pre-cancerous stage.

The third embodiment of the present invention refers to the *in vitro* use of any of the above cited biomarkers or signatures for the diagnosis of colorectal cancer and/or a pre-cancerous stage thereof.

In a preferred embodiment, the pre-cancerous stage of colorectal cancer is advanced colorectal adenoma.

In a preferred embodiment, the diagnosis of the colorectal cancer and/or a pre-cancerous stage thereof is confirmed by an image technique, preferably colonoscopy.

In a preferred embodiment the present invention refers to an *in vitro* method for detecting colorectal cancer and/or a precancerous stage thereof, said method comprising: a) obtaining a plasma sample from a human patient; and b) detecting whether any of the above cited protein biomarkers or signatures are present in the plasma sample by contacting the plasma sample with an antibody directed against said protein biomarkers or signatures and detecting binding between the proteins and the antibody.

The fourth embodiment of the present invention refers to a method for diagnosing and treating colorectal cancer or a pre-cancerous stage thereof, which comprises: a) obtaining a plasma sample from a human patient; b) detecting whether any of the above cited protein biomarkers or signatures are present in the plasma sample; c) diagnosing the patient with colorectal cancer or a pre-cancerous stage thereof when the presence of said protein biomarkers or signatures in the plasma sample is detected; and performing a colonoscopy to the patient and removing the colorectal cancer or polyps afterwards.

Alternatively, the fifth embodiment of the present invention refers to an *in vitro* method (hereinafter "method of the invention") for the diagnosis of colorectal cancer and/or a pre-cancerous stage thereof which comprises: a) Measuring the concentration level of at least AREG, in a biological sample obtained from the subject and b) wherein if a deviation or variation of the concentration level of at least AREG is identified, as compared with the reference concentration level measured in healthy control subjects, this is indicative that the subject is suffering from colorectal cancer and/or a pre-cancerous stage.

In a particularly preferred embodiment, the method of the invention comprises measuring the concentration level of at least the combination [AREG and CYFRA21-1] in a biological sample obtained from the subject.

In this regard, it is important to consider that all the most reliable signatures claimed in the present invention comprises AREG, preferably [AREG and CYFRA21-1], thus obtaining various biomarker signatures comprising AREG, preferably comprising [AREG and CYFRA21-1], with an Area Under the Curve (AUC) around 0.9 for the detection of CRC a with a good performance also for the detection of AA (see **Table 12bis**). Consequently, although any of the signatures comprising AREG, preferably comprising [AREG and CYFRA21-1], could be effectively used according to the present invention, the following signatures comprising AREG, preferably comprising [AREG and CYFRA21-1], are particularly preferred since they offer an AUC value above 0.9 for the detection of CRC and a good performance for the detection of AA: [AREG and CYFRA21-1 and Flt3L and ErbB4] (AUC for CRC =0.931) or [AREG and CYFRA21-1 and Flt3L and CLEC2C] (AUC for CRC = 0.915) (see **Table 12bis**).

So, in a particularly preferred embodiment, the method of the invention comprises measuring the concentration level of at least the combination [AREG and CYFRA21-1 and Flt3L], or the combination of [AREG and CYFRA21-1 and CLEC2C], or the combination of [AREG and CYFRA21-1 and ErbB4], or the combination [AREG and CYFRA21-1 and FasL], or the combination [AREG and CYFRA21-1 and CD147], or the combination [AREG and CYFRA21-1 and HGFR], or the combination [AREG and CYFRA21-1 and Flt3L and ErbB4], or the combination of [AREG and CYFRA21-1 and Flt3L and CLEC2C], or the combination of [AREG and CYFRA21-1 and HGFR and CD147] in a biological sample obtained from the subject.

In a particularly preferred embodiment, the method of the invention comprises measuring the concentration level of at least the combination [AREG and CD147], or the combination of [AREG and CLEC2C], or the combination of [AREG and HGFR], or the combination [AREG and CD147 and HGFR] in a biological sample obtained from the subject.

The sixth embodiment of the present invention refers to the *in vitro* use of any of the above cited signatures for the diagnosis of colorectal cancer and/or a pre-cancerous stage thereof.

According to the method of the invention, after measuring the concentration level of any of the above cited combinations of biomarkers, a score value is obtained for the signature and this score value is compared with a threshold value which defines the diagnostic rule. If this score value is higher than the threshold, then the corresponding sample is classified as a positive sample, which is an indication that the patient might be suffering from colorectal cancer and/or pre-cancerous stage thereof. The threshold value has been defined in order to optimize sensitivity and specificity values. Consequently, in a preferred embodiment, the method of the invention comprises: a) Measuring the concentration level of any of the above cited combinations of biomarkers, in a biological sample obtained from the subject, b) processing the concentration values in order to obtain a risk score and c) wherein if a deviation or variation of the risk score value obtained for any of the above cited combinations of biomarkers is identified, as compared with a reference value, this is indicative that the subject is suffering from colorectal cancer and/or a pre-cancerous stage.

The seventh embodiment of the present invention refers to a kit of parts comprising reagents for the determining the concentration level of any of the above cited signatures. In a preferred embodiment, the present invention refers to the *in vitro* use of a kit comprising reagents for the determination of the concentration level of any of the above cited combinations of biomarkers for the diagnosis of colorectal cancer and/or a pre-cancerous stage thereof.

In a preferred embodiment, the pre-cancerous stage of colorectal cancer is advanced colorectal adenoma.

In a preferred embodiment, the diagnosis of the colorectal cancer and/or a pre-cancerous stage thereof is confirmed by an image technique, preferably colonoscopy.

In a preferred embodiment the present invention refers to an *in vitro* method for detecting colorectal cancer and/or a precancerous stage thereof, said method

comprising: a) obtaining a plasma sample from a human patient; and b) detecting whether any of the above cited protein biomarkers or signatures are present in the plasma sample by contacting the plasma sample with an antibody directed against said protein biomarkers or signatures and detecting binding between the proteins and the antibody.

The last embodiment of the present invention refers to a method for diagnosing and treating colorectal cancer or a pre-cancerous stage thereof, which comprises: a) obtaining a plasma sample from a human patient; b) detecting whether any of the above cited protein biomarkers or signatures are present in the plasma sample; c) diagnosing the patient with colorectal cancer or a pre-cancerous stage thereof when the presence of said protein biomarkers or signatures in the plasma sample is detected; and performing a colonoscopy to the patient and removing the colorectal cancer or polyps afterwards.

For the purpose of the present invention the following terms are defined:

- The term "colorectal cancer" is a medical condition characterized by cancer of cells of the intestinal tract below the small intestine (i.e., the large intestine (colon), including the cecum, ascending colon, transverse colon, descending colon, sigmoid colon, and rectum).
- The expression “colorectal adenoma” refers to adenomas of the colon, also called adenomatous polyps, which is a benign and pre-cancerous stage of the colorectal cancer but still with high risk of progression to colorectal cancer.
- The expression “advanced colorectal adenoma” refers to adenomas having a size of at least 10 mm or histologically having high grade dysplasia or a villous component higher than 20%.
- The expression “minimally-invasive biological sample” refers to any sample which is taken from the body of the patient without the need of using harmful instruments, other than fine needles used for taking the blood from the patient, and consequently without being harmfully for the patient. Specifically, minimally-invasive biological sample refers in the present invention to: blood, serum, or plasma samples.

- The expression “reference concentration level measured in healthy control subjects”, refer to a “reference value” of the concentration level of the proteins. If a deviation of the concentration level of the proteins is determined with respect to said “reference concentration level measured in healthy control subjects”, this is an indication of colorectal cancer or pre-cancerous stage thereof. Particularly, if the concentration level of the biomarkers or signatures of the present invention are significantly higher or lower with respect to said “reference value” this is an indication of colorectal cancer or pre-cancerous stage thereof.
- The expression “risk score” refers to a risk value obtained after processing one or more concentration values into a single value (or risk value), which represents the probability of disease for the individual. This risk value will be compared with a reference value to evaluate if the patient might be suffering from colorectal cancer and/or pre-cancerous stage thereof.
- A “reference value” can be a threshold value or a cut-off value. Typically, a "threshold value" or "cut-off value" can be determined experimentally, empirically, or theoretically. A threshold value can also be arbitrarily selected based upon the existing experimental and/or clinical conditions, as would be recognized by a person of ordinary skilled in the art. The threshold value has to be determined in order to obtain the optimal sensitivity and specificity according to the function of the test and the benefit/risk balance (clinical consequences of false positive and false negative). Preferably, the person skilled in the art may compare the biomarker levels (or scores) obtained according to the method of the invention with a defined threshold value. Typically, the optimal sensitivity and specificity (and so the threshold value) can be determined using a Receiver Operating Characteristic (ROC) curve based on experimental data. For example, after determining the levels of the biomarkers in a group of reference, one can use algorithmic analysis for the statistic treatment of the measured concentrations of biomarkers in biological samples to be tested, and thus obtain a classification standard having significance for sample classification. The full name of ROC curve is receiver operator characteristic curve, which is also known as receiver operation characteristic curve. It is mainly used for clinical biochemical diagnostic tests. ROC curve is a comprehensive indicator that reflects the continuous variables of true positive rate (sensitivity) and false

positive rate (1-specificity). It reveals the relationship between sensitivity and specificity with the image composition method. A series of different cut-off values (thresholds or critical values, boundary values between normal and abnormal results of diagnostic test) are set as continuous variables to calculate a series of sensitivity and specificity values. Then sensitivity is used as the vertical coordinate and specificity is used as the horizontal coordinate to draw a curve. The higher the area under the curve (AUC), the higher the accuracy of diagnosis. On the ROC curve, the point closest to the far upper left of the coordinate diagram is a critical point having both high sensitivity and high specificity values. The AUC value of the ROC curve is between 1.0 and 0.5. When AUC>0.5, the diagnostic result gets better and better as AUC approaches 1. When AUC is between 0.5 and 0.7, the accuracy is low. When AUC is between 0.7 and 0.9, the accuracy is good. When AUC is higher than 0.9, the accuracy is quite high. This algorithmic method is preferably done with a computer. Existing software or systems in the art may be used for the drawing of the ROC curve, such as: MedCalc 9.2.0.1 medical statistical software, SPSS 9.0.

- By "comprising" it is meant including, but not limited to, whatever follows the word "comprising". Thus, use of the term "comprising" indicates that the listed elements are required or mandatory, but that other elements are optional and may or may not be present.
- By "consisting of" it is meant "including, and limited to", whatever follows the phrase "consisting of". Thus, the phrase "consisting of" indicates that the listed elements are required or mandatory, and that no other elements may be present.

For the purpose of the present invention the following proteins are identified according to Uniprot data base:

Abbreviation	Protein Name	Uniprot ID.
AREG	Amphiregulin	P15514
CD147	Basigin	P35613
CLEC2C	Early activation antigen CD69	Q07108
CYFRA21-1	Cytokeratin fragment antigen 21-1	N/A

ErbB4	Receptor tyrosine-protein kinase erbB-4	Q15303
FasL	Tumor necrosis factor ligand superfamily member 6	P48023
Flt3L	Fms-related tyrosine kinase 3 ligand	P49771
HGFR	Hepatocyte growth factor receptor	P08581
IFNgamma	Interferon gamma	P01579

Brief description of the figures

Figure 1. Receiver-operating-characteristic (ROC) curve for: **A)** [Flt3L and CYFRA21-1] in colorectal cancer. Area Under Curve (AUC) = 0.865. **B)** [Flt3L and CYFRA21-1] in advanced colorectal adenoma. Area Under Curve (AUC) = 0.606. X axis represents Specificity. Y axis represents Sensitivity.

Figure 2. Receiver-operating-characteristic (ROC) curve for: **A)** [Flt3L and CYFRA21-1 and AREG] in colorectal cancer. Area Under Curve (AUC) = 0.899. **B)** [Flt3L and CYFRA21-1 and AREG] in advanced colorectal adenoma. Area Under Curve (AUC) = 0.720. X axis represents Specificity. Y axis represents Sensitivity.

Figure 3. Receiver-operating-characteristic (ROC) curve for: **A)** [Flt3L and CYFRA21-1 and AREG and ErbB4] in colorectal cancer. Area Under Curve (AUC) = 0.931. **B)** [Flt3L and CYFRA21-1 and AREG and ErbB4] in advanced colorectal adenoma. Area Under Curve (AUC) = 0.707. X axis represents Specificity. Y axis represents Sensitivity.

Figure 4. Receiver-operating-characteristic (ROC) curve for: **A)** [Flt3L and CYFRA21-1 and AREG and CLEC2C] in colorectal cancer. Area Under Curve (AUC) = 0.915. **B)** [Flt3L and CYFRA21-1 and AREG and CLEC2C] in advanced colorectal adenoma. Area Under Curve (AUC) = 0.727. X axis represents Specificity. Y axis represents Sensitivity.

Figure 5. Receiver-operating-characteristic (ROC) curve for: **A)** [AREG and CYFRA21-1] in colorectal cancer. Area Under Curve (AUC) = 0.878. **B)** [AREG and

CYFRA21-1] in advanced colorectal adenoma. Area Under Curve (AUC) = 0.722. X axis represents Specificity. Y axis represents Sensitivity.

Figure 6. Receiver-operating-characteristic (ROC) curve for: **A)** [AREG and CYFRA21-1 and Flt3L and ErbB4] in colorectal cancer. Area Under Curve (AUC) = 0.931. **B)** [AREG and CYFRA21-1 and Flt3L and ErbB4] in advanced colorectal adenoma. Area Under Curve (AUC) = 0.707. X axis represents Specificity. Y axis represents Sensitivity.

Figure 7. Receiver-operating-characteristic (ROC) curve for: **A)** [AREG and CYFRA21-1 and Flt3L and CLEC2C] in colorectal cancer. Area Under Curve (AUC) = 0.915. **B)** [AREG and CYFRA21-1 and Flt3L and CLEC2C] in advanced colorectal adenoma. Area Under Curve (AUC) = 0.727. X axis represents Specificity. Y axis represents Sensitivity.

Figure 8. Receiver-operating-characteristic (ROC) curve for: **A)** [AREG and CYFRA21-1 and CD147 and HGFR] in colorectal cancer. Area Under Curve (AUC) = 0.888. **B)** [AREG and CYFRA21-1 and CD147 and HGFR] in advanced colorectal adenoma. Area Under Curve (AUC) = 0.769. X axis represents Specificity. Y axis represents Sensitivity.

Detailed description of the invention

Example 1. MATERIAL AND METHODS.

Example 1.1. Population of study.

A total of 96 subjects from eight Spanish hospitals (Hospital de Burgos, Hospital de Vigo, Hospital de Donosti, Hospital de Ourense, Hospital del Bierzo, Hospital de Beltvigte and Hospital de Zaragoza) were prospectively included in this study: 64 patients newly diagnosed with sporadic colorectal neoplasia (32 with CRC and 32 with AA) and 32 healthy individuals without personal history of any cancer and with a recent colonoscopy confirming the lack of colorectal neoplastic lesions. Patients with AA were those with adenomas having a size of at least 10 mm or histologically having high grade dysplasia or >20% villous component. The characteristics of participants are shown in **Table 1**. Blood samples were collected prior to endoscopy or surgery in all individuals.

Table 1

Cases	Control (CTL)	AA	CRC	TOTAL
Mean age (SD)	64,3(44-86)	65,1(52-88)	71,6(54-85)	67(44-88)
GENDER				
Male	13	18	18	49 (51%)
Female	19	14	14	47 (49%)
COLORECTAL FEATURES				
TNM stage				
I			4	
II			9	
III			10	
IV			6	
Unknown			3	
Location				
Ascending colon and cecum			10	
Descending colon and sigma			12	
Transverse colon			3	
Rectum			3	
Unknown			4	
ADVANCED COLORECTAL ADENOMA FEATURES				
Size = > 10 mm		28		
Small AA (<=15mm)		19		
Big AA (>15mm)		13		
Mean size (mm) (SD)		18,8		
No. AAs Mean (SD)		2,3		
High-grade dysplasia				
Yes		9		
No		21		
Unknown		2		
Villous component				
Yes		13		
No		17		
Unknown		2		

Clinical-pathological characteristics of the study cohort

The study was approved by the Institutional Ethics Committee of each Hospital, and written informed consent was obtained from all participants in accordance with the Declaration of Helsinki.

Example 1.2. Sample preparation.

Ten mL of whole blood from each participant were collected in EDTA K2 containing tubes. Blood samples were placed at 4°C until plasma separation. Samples were centrifuged at 1,600 x g for 10 min at 4°C to spin down blood cells, and plasma was transferred into new tubes, followed by further centrifugation at 16,000 x g for 10 minutes at 4°C to completely remove cellular components.

Example 1.3. Molecular analysis.

The concentration of the biomarkers in plasma samples was established using commercial ELISA (Enzyme-linked immunosorbent assay) and CLIA (Chemiluminescence immunoassay) test and following their corresponding instruction manual. HGFR and ErbB4 was analyzed with ELISA kit from Cloud clone Corp. Level of CD147, CLEC2C, Flt3L, and FasL was measured using ELISA Kit form Elabscience. In the case of the IFNgamma, ELISA kit from Abcam was used. Related to CLIA test, CYFRA21-1 and AREG were analyzed with CLIA test from Cloud Clone Corp.

Example 1.4. Data Quantification.

For the protein quantification step the samples were processed with the corresponding kit (ELISA/CLIA) and distributed in experimental plates. Each plate contained also control data used to construct a standard curve. Fluorescence data obtained from each run (expressed as integer numbers) have been background corrected for each sample and quantified using a standard curve generated using a 2-degree polynomial regression model.

Example 1.5. Statistical analysis.

Three groups of individuals were considered in the analysis. CRC (Individuals diagnosed with colorectal cancer), AA (Individuals diagnosed with advanced adenoma) and CTL (Individuals with no disease).

Raw quantification data have been transformed by applying square root function, and then centering and scaling so that, after the transformation, each protein measure have mean 0 and standard deviation 1. Quantification values are summarized in **Table 2** and **Table 3**, where each protein is described as median and interquartile range in the different groups considered.

Non-normality of the data was confirmed by Shapiro-Wilk test and, consequently, Wilcoxon rank-sum test was used to compare either CRC cases or AA cases against CTL individuals.

Diagnostic performance for the individual proteins and some of their combinations has been assessed by their receiver operating characteristic (ROC) curves, and the area under the ROC curves (AUC). Moreover, sensitivities, specificities, positive predictive

and negative predictive values (PPV and NPV) for the different tests were calculated at the optimal cutoff point defined by the best Youden's Index (or equivalently, the point of the ROC that maximizes the sum of sensitivity and specificity).

Scores used for deriving the ROC-AUCs and the rest of performance values were obtained using univariate logistic regression model for the individual proteins and multivariate logistic regression models for the different combination of proteins considered. 95% CI for the AUCs was obtained with the DeLong methodology both in individual markers and combination of them.

Example 2. RESULTS.

Example 2.1. Individual marker results.

Different metrics to evaluate the individual proteins were determined, also perming the following comparisons: CRC/AA vs CTL. It can be seen that AREG, CYFRA21-1 and Flt3L are significantly different between CRC and CTL groups and their AUC are significantly different from 0.5 (as their 95% confidence interval do not include 0.5).In the case of AA group, AREG also shows statistically differences compared to CTL group.

Table 2 and **Table 3** shows metrics for individual proteins, including p-value from Wilcoxon test (p.Wilc), area under the ROC curve (AUC), and Sensitivity (Sens.), Specificity (Spec.), and positive (VPP) and negative (VPN) predictive values computed in the cut-off point of the ROC curve with the best Youden´s index. The sign column indicates, for the biomarkers with p-value < 0.25, whether high levels of the marker increase or decrease the risk of disease (+ and – respectively).

Table 2

	CRC.vs.CTL						
	p.Wilc.	**Sign**	**AUC**	**Sens.**	**Spec.**	**VPP**	**VPN**
AREG	0.0008	+	0.744 (0.619,0.868)	65.62	81.25	77.78	70.27
CD147	0.5280		0.546 (0.402,0.691)	53.12	62.50	58.62	57.14
CLEC2C	0.2021	+	0.593 (0.452,0.734)	93.75	28.12	56.60	81.82
CYFRA21-1	0.0000	+	0.795 (0.682,0.909)	90.62	68.75	74.36	88.00
ErbB4	0.1177	-	0.614 (0.47,0.758)	53.12	75.00	68.00	61.54
FasL	0.1859	+	0.598 (0.454,0.741)	54.84	68.75	62.96	61.11

Flt3L	0.0191	-	0.67 (0.531,0.809)	56.25	84.38	78.26	65.85
HGFR	0.1729	+	0.6 (0.452,0.747)	65.62	68.75	67.74	66.67
IFNgamma	0.1537	+	0.604 (0.464,0.745)	68.75	53.12	59.46	62.96

Table 3

	AA.vs.CTL						
	p.Wilc.	**Sign**	**AUC**	**Sens.**	**Spec.**	**VPP**	**VPN**
AREG	0.0286	+	0.66 (0.525,0.795)	87.50	40.62	59.57	76.47
CD147	0.1772	+	0.599 (0.458,0.739)	87.50	31.25	56.00	71.43
CLEC2C	0.7831		0.521 (0.376,0.665)	100.00	9.38	52.46	100.00
CYFRA21-1	0.1412	+	0.607 (0.467,0.748)	90.62	37.50	59.18	80.00
ErbB4	0.3270		0.572 (0.427,0.716)	50.00	71.88	64.00	58.97
FasL	0.5730		0.542 (0.394,0.689)	74.19	46.88	57.50	65.22
Flt3L	0.8155		0.518 (0.372,0.663)	43.75	75.00	63.64	57.14
HGFR	0.0678	+	0.633 (0.493,0.773)	68.75	62.50	64.71	66.67
IFNgamma	0.8467		0.515 (0.37,0.659)	81.25	31.25	54.17	62.50

Example 2.2. Best combinations of biomarkers.

With the aim of improving individual diagnostic capability, combinations of proteins have been explored with the following procedure: Based on markers with $p<0.25$ either in CRC.vs.CTL (**Table 3**) or AA.vs.CTL (**Table 4**) comparisons, we used multivariate logistic regression to explore all possible combinations of two, three and four of these proteins taken at the same time.

Table 4, Table 5, Table 5 bis, Table 6 and **Table 6 bis** show the AUC achieved for the combinations of two, three and four biomarkers respectively, discriminating CRC vs CTL.

Table 4

Combinations of two biomarkers CRC vs CTL	**AUC**
AREG,CYFRA21-1	0.8779297
Flt3L,CYFRA21-1	0.8652344
CYFRA21-1,ErbB4	0.8359375
AREG,CLEC2C	0.8349609
CYFRA21-1,IFNgamma	0.8017578

CYFRA21-1,FasL	0.7993952
CLEC2C,CYFRA21-1	0.7988281
CYFRA21-1,HGFR	0.7919922
AREG,Flt3L	0.7832031
AREG,HGFR	0.7626953
AREG,IFNgamma	0.7431641
AREG,ErbB4	0.7324219
AREG,FasL	0.7247984
Flt3L,HGFR	0.6982422
CLEC2C,Flt3L	0.6953125
ErbB4,Flt3L	0.6914062
FasL,Flt3L	0.6814516
Flt3L,IFNgamma	0.6708984
ErbB4,IFNgamma	0.6318359
ErbB4,HGFR	0.6142578
CLEC2C,IFNgamma	0.6083984
CLEC2C,ErbB4	0.6064453
FasL,HGFR	0.6018145
HGFR,IFNgamma	0.5869141
CLEC2C,FasL	0.5866935
ErbB4,FasL	0.5856855
FasL,IFNgamma	0.5836694
CLEC2C,HGFR	0.5810547

Table 5

Combinations of three biomarkers for CRC vs CTL supporting the combination Flt3L+CYFRA21-1	**AUC**
Flt3L,CYFRA21-1,AREG	0.8994141
AREG,CLEC2C,CYFRA21-1	0.8955078
AREG,CYFRA21-1,ErbB4	0.8955

	078
AREG,CYFRA21-1,FasL	0.8931452
Flt3L,CYFRA21-1	0.8847656
AREG,CYFRA21-1,IFNgamma	0.8837891
AREG,CYFRA21-1,HGFR	0.8759766
Flt3L,CYFRA21-1,CLEC2C	0.8681641
Flt3L,CYFRA21-1,FasL	0.8679435
Flt3L,CYFRA21-1,IFNgamma	0.8671875
Flt3L,CYFRA21-1,HGFR	0.8662109
AREG,CLEC2C,Flt3L	0.8427734
CYFRA21-1,ErbB4,HGFR	0.8388672
CYFRA21-1,ErbB4,IFNgamma	0.8359375
CLEC2C,CYFRA21-1,ErbB4	0.8349609
AREG,CLEC2C,HGFR	0.8330078
CYFRA21-1,ErbB4,FasL	0.8326613
AREG,CLEC2C,FasL	0.8316532
AREG,CLEC2C,ErbB4	0.8300781

AREG,CLEC2C,IFNgamma	0.8173828
AREG,Flt3L,HGFR	0.8066406
CLEC2C,CYFRA21-1,IFNgamma	0.8066406
CYFRA21-1,FasL,IFNgamma	0.8054435
CYFRA21-1,HGFR,IFNgamma	0.8037109
CYFRA21-1,FasL,HGFR	0.8014113
CLEC2C,CYFRA21-1,FasL	0.7973790
CLEC2C,CYFRA21-1,HGFR	0.7968750
AREG,ErbB4,Flt3L	0.7841797
AREG,Flt3L,IFNgamma	0.7832031
AREG,FasL,Flt3L	0.7782258
AREG,ErbB4,HGFR	0.7705078
AREG,HGFR,IFNgamma	0.7666016
AREG,FasL,HGFR	0.7641129
AREG,ErbB4,IFNgamma	0.7451172
AREG,FasL,IFNgamma	0.7358871
AREG,ErbB4,FasL	0.7197

	581
ErbB4,Flt3L,HGFR	0.7119141
CLEC2C,FasL,Flt3L	0.7076613
CLEC2C,ErbB4,Flt3L	0.7031250
ErbB4,FasL,Flt3L	0.7006048
CLEC2C,Flt3L,HGFR	0.6962891
FasL,Flt3L,HGFR	0.6935484
CLEC2C,Flt3L,IFNgamma	0.6933594
Flt3L,HGFR,IFNgamma	0.6894531
ErbB4,Flt3L,IFNgamma	0.6875000
FasL,Flt3L,IFNgamma	0.6814516
ErbB4,FasL,IFNgamma	0.6250000
ErbB4,HGFR,IFNgamma	0.6230469
CLEC2C,ErbB4,IFNgamma	0.6191406
ErbB4,FasL,HGFR	0.6139113
CLEC2C,ErbB4,HGFR	0.6123047
CLEC2C,ErbB4,FasL	0.6068548

CLEC2C,FasL,IFNgamma	0.6048387
FasL,HGFR,IFNgamma	0.5997984
CLEC2C,FasL,HGFR	0.5967742
CLEC2C,HGFR,IFNgamma	0.5966797

Table 5 bis

Combinations of three biomarkers for CRC vs CTL supporting the combination AREG+CYFRA21-1	**AUC**
AREG,CYFRA21-1,Flt3L	0.8994141
AREG,CYFRA21-1,CLEC2C	0.8955078
AREG,CYFRA21-1,ErbB4	0.8955078
AREG,CYFRA21-1,FasL	0.8931452
CYFRA21-1,ErbB4,Flt3L	0.8847656
AREG,CYFRA21-1,IFNgamma	0.8837891
AREG,CYFRA21-1,HGFR	0.8759766
CLEC2C,CYFRA21-1,Flt3L	0.8681641
CYFRA21-1,FasL,Flt3L	0.8679435
CYFRA21-1,Flt3L,IFNgamma	0.8671875

CYFRA21-1,Flt3L,HGFR	0.8662109
AREG,CLEC2C,Flt3L	0.8427734
CYFRA21-1,ErbB4,HGFR	0.8388672
CYFRA21-1,ErbB4,IFNgamma	0.8359375
CLEC2C,CYFRA21-1,ErbB4	0.8349609
AREG,CLEC2C,HGFR	0.8330078
CYFRA21-1,ErbB4,FasL	0.8326613
AREG,CLEC2C,FasL	0.8316532
AREG,CLEC2C,ErbB4	0.8300781
AREG,CLEC2C,IFNgamma	0.8173828
AREG,Flt3L,HGFR	0.8066406
CLEC2C,CYFRA21-1,IFNgamma	0.8066406
CYFRA21-1,FasL,IFNgamma	0.8054435
CYFRA21-1,HGFR,IFNgamma	0.8037109
CYFRA21-1,FasL,HGFR	0.8014113
CLEC2C,CYFRA21-1,FasL	0.7973790
CLEC2C,CYFRA21-1,HGFR	0.7968

	750
AREG,ErbB4,Flt3L	0.7841797
AREG,Flt3L,IFNgamma	0.7832031
AREG,FasL,Flt3L	0.7782258
AREG,ErbB4,HGFR	0.7705078
AREG,HGFR,IFNgamma	0.7666016
AREG,FasL,HGFR	0.7641129
AREG,ErbB4,IFNgamma	0.7451172
AREG,FasL,IFNgamma	0.7358871
AREG,ErbB4,FasL	0.7197581
ErbB4,Flt3L,HGFR	0.7119141
CLEC2C,FasL,Flt3L	0.7076613
CLEC2C,ErbB4,Flt3L	0.7031250
ErbB4,FasL,Flt3L	0.7006048
CLEC2C,Flt3L,HGFR	0.6962891
FasL,Flt3L,HGFR	0.6935484
CLEC2C,Flt3L,IFNgamma	0.6933594

Flt3L,HGFR,IFNgamma	0.6894531
ErbB4,Flt3L,IFNgamma	0.6875000
FasL,Flt3L,IFNgamma	0.6814516
ErbB4,FasL,IFNgamma	0.6250000
ErbB4,HGFR,IFNgamma	0.6230469
CLEC2C,ErbB4,IFNgamma	0.6191406
ErbB4,FasL,HGFR	0.6139113
CLEC2C,ErbB4,HGFR	0.6123047
CLEC2C,ErbB4,FasL	0.6068548
CLEC2C,FasL,IFNgamma	0.6048387
FasL,HGFR,IFNgamma	0.5997984
CLEC2C,FasL,HGFR	0.5967742
CLEC2C,HGFR,IFNgamma	0.5966797

Table 6

Combinations of four biomarkers for CRC vs CTL supporting the combination Flt3L+CYFRA21-1	**AUC**

Flt3L,CYFRA21-1,ErbB4,AREG	0.9306641
Flt3L,CYFRA21-1,AREG,CLEC2C	0.9150391
AREG,CLEC2C,CYFRA21-1,ErbB4	0.9023438
AREG,CYFRA21-1,ErbB4,FasL	0.9022177
AREG,CLEC2C,CYFRA21-1,IFNgamma	0.9003906
Flt3L,CYFRA21-1, HGFR,AREG,	0.9003906
AREG,CLEC2C,CYFRA21-1,FasL	0.9002016
AREG,CYFRA21-1,ErbB4,IFNgamma	0.8994141
Flt3L,CYFRA21-1,IFNgamma,AREG	0.8984375
Flt3L,CYFRA21-1,FasL,AREG	0.8981855
AREG,CYFRA21-1,FasL,IFNgamma	0.8951613
AREG,CYFRA21-1,ErbB4,HGFR	0.8945312
AREG,CLEC2C,CYFRA21-1,HGFR	0.8935547
AREG,CYFRA21-1,FasL,HGFR	0.8901210
Flt3L,CYFRA21-1,ErbB4,HGFR	0.8867188
Flt3L,CYFRA21-1,ErbB4,IFNgamma	0.8867188
AREG,CYFRA21-1,HGFR,IFNgamma	0.8847

	656
Flt3L,CYFRA21-1,ErbB4,CLEC2C	0.8847656
Flt3L,CYFRA21-1,ErbB4,FasL	0.8830645
Flt3L,CYFRA21-1,FasL,IFNgamma	0.8689516
Flt3L,CYFRA21-1,HGFR,IFNgamma	0.8671875
Flt3L,CYFRA21-1,FasL,HGFR	0.8669355
Flt3L,CYFRA21-1,IFNgamma,CLEC2C	0.8662109
Flt3L,CYFRA21-1,FasL,CLEC2C	0.8649194
Flt3L,CYFRA21-1,HGFR,CLEC2C	0.8632812
AREG,CLEC2C,Flt3L,HGFR	0.8564453
AREG,CLEC2C,ErbB4,Flt3L	0.8457031
AREG,CLEC2C,FasL,Flt3L	0.8447581
AREG,CLEC2C,Flt3L,IFNgamma	0.8447266
CYFRA21-1,ErbB4,HGFR,IFNgamma	0.8417969
CLEC2C,CYFRA21-1,ErbB4,HGFR	0.8398438
AREG,CLEC2C,ErbB4,HGFR	0.8378906
CLEC2C,CYFRA21-1,ErbB4,IFNgamma	0.8359375

AREG,CLEC2C,ErbB4,FasL	0.8346774
AREG,CLEC2C,FasL,HGFR	0.8326613
CLEC2C,CYFRA21-1,ErbB4,FasL	0.8326613
CYFRA21-1,ErbB4,FasL,IFNgamma	0.8326613
CYFRA21-1,ErbB4,FasL,HGFR	0.8316532
AREG,CLEC2C,HGFR,IFNgamma	0.8271484
AREG,CLEC2C,ErbB4,IFNgamma	0.8251953
AREG,CLEC2C,FasL,IFNgamma	0.8245968
AREG,ErbB4,Flt3L,HGFR	0.8203125
CLEC2C,CYFRA21-1,FasL,IFNgamma	0.8074597
AREG,Flt3L,HGFR,IFNgamma	0.8066406
CYFRA21-1,FasL,HGFR,IFNgamma	0.8064516
CLEC2C,CYFRA21-1,HGFR,IFNgamma	0.8056641
AREG,FasL,Flt3L,HGFR	0.7993952
CLEC2C,CYFRA21-1,FasL,HGFR	0.7983871
AREG,ErbB4,Flt3L,IFNgamma	0.7861328
AREG,ErbB4,HGFR,IFNgamma	0.7802

	734
AREG,ErbB4,FasL,Flt3L	0.7802419
AREG,FasL,Flt3L,IFNgamma	0.7772177
AREG,ErbB4,FasL,HGFR	0.7721774
AREG,FasL,HGFR,IFNgamma	0.7681452
AREG,ErbB4,FasL,IFNgamma	0.7419355
ErbB4,FasL,Flt3L,HGFR	0.7358871
CLEC2C,ErbB4,FasL,Flt3L	0.7167339
CLEC2C,FasL,Flt3L,HGFR	0.7086694
CLEC2C,FasL,Flt3L,IFNgamma	0.7056452
CLEC2C,ErbB4,Flt3L,HGFR	0.7050781
ErbB4,Flt3L,HGFR,IFNgamma	0.7050781
CLEC2C,ErbB4,Flt3L,IFNgamma	0.7011719
ErbB4,FasL,Flt3L,IFNgamma	0.6985887
CLEC2C,Flt3L,HGFR,IFNgamma	0.6933594
FasL,Flt3L,HGFR,IFNgamma	0.6895161
CLEC2C,ErbB4,FasL,IFNgamma	0.6290323

CLEC2C,ErbB4,FasL,HGFR	0.6239919
CLEC2C,ErbB4,HGFR,IFNgamma	0.6220703
ErbB4,FasL,HGFR,IFNgamma	0.6118952
CLEC2C,FasL,HGFR,IFNgamma	0.6088710

Table 6 bis

Combinations of four biomarkers for CRC vs CTL supporting the combination AREG+CYFRA21-1	**AUC**
AREG,CYFRA21-1, Flt3L, ErbB4	0.9306641
AREG,CYFRA21-1, Flt3L CLEC2C	0.9150391
AREG,CYFRA21-1,ErbB4,CLEC2C	0.9023438
AREG,CYFRA21-1,ErbB4,FasL	0.9022177
AREG,CYFRA21-1,CLEC2C,IFNgamma	0.9003906
AREG,CYFRA21-1,Flt3L,HGFR	0.9003906
AREG,CYFRA21-1,CLEC2C,FasL	0.9002016
AREG,CYFRA21-1,ErbB4,IFNgamma	0.8994141
AREG,CYFRA21-1,Flt3L,IFNgamma	0.8984375
AREG,CYFRA21-1, Flt3L, FasL,	0.8981855

AREG,CYFRA21-1,FasL,IFNgamma	0.8951613
AREG,CYFRA21-1,ErbB4,HGFR	0.8945312
AREG,CYFRA21-1,HGFR,CLEC2C,	0.8935547
AREG,CYFRA21-1,FasL,HGFR	0.8901210
CYFRA21-1,ErbB4,Flt3L,HGFR	0.8867188
CYFRA21-1,ErbB4,Flt3L,IFNgamma	0.8867188
AREG,CYFRA21-1,HGFR,IFNgamma	0.8847656
CLEC2C,CYFRA21-1,ErbB4,Flt3L	0.8847656
CYFRA21-1,ErbB4,FasL,Flt3L	0.8830645
CYFRA21-1,FasL,Flt3L,IFNgamma	0.8689516
CYFRA21-1,Flt3L,HGFR,IFNgamma	0.8671875
CYFRA21-1,FasL,Flt3L,HGFR	0.8669355
CLEC2C,CYFRA21-1,Flt3L,IFNgamma	0.8662109
CLEC2C,CYFRA21-1,FasL,Flt3L	0.8649194
CLEC2C,CYFRA21-1,Flt3L,HGFR	0.8632812
AREG,CLEC2C,Flt3L,HGFR	0.8564453
AREG,CLEC2C,ErbB4,Flt3L	0.8457

	031
AREG,CLEC2C,FasL,Flt3L	0.8447581
AREG,CLEC2C,Flt3L,IFNgamma	0.8447266
CYFRA21-1,ErbB4,HGFR,IFNgamma	0.8417969
CLEC2C,CYFRA21-1,ErbB4,HGFR	0.8398438
AREG,CLEC2C,ErbB4,HGFR	0.8378906
CLEC2C,CYFRA21-1,ErbB4,IFNgamma	0.8359375
AREG,CLEC2C,ErbB4,FasL	0.8346774
AREG,CLEC2C,FasL,HGFR	0.8326613
CLEC2C,CYFRA21-1,ErbB4,FasL	0.8326613
CYFRA21-1,ErbB4,FasL,IFNgamma	0.8326613
CYFRA21-1,ErbB4,FasL,HGFR	0.8316532
AREG,CLEC2C,HGFR,IFNgamma	0.8271484
AREG,CLEC2C,ErbB4,IFNgamma	0.8251953
AREG,CLEC2C,FasL,IFNgamma	0.8245968
AREG,ErbB4,Flt3L,HGFR	0.8203125
CLEC2C,CYFRA21-1,FasL,IFNgamma	0.8074597

AREG,Flt3L,HGFR,IFNgamma	0.8066406
CYFRA21-1,FasL,HGFR,IFNgamma	0.8064516
CLEC2C,CYFRA21-1,HGFR,IFNgamma	0.8056641
AREG,FasL,Flt3L,HGFR	0.7993952
CLEC2C,CYFRA21-1,FasL,HGFR	0.7983871
AREG,ErbB4,Flt3L,IFNgamma	0.7861328
AREG,ErbB4,HGFR,IFNgamma	0.7802734
AREG,ErbB4,FasL,Flt3L	0.7802419
AREG,FasL,Flt3L,IFNgamma	0.7772177
AREG,ErbB4,FasL,HGFR	0.7721774
AREG,FasL,HGFR,IFNgamma	0.7681452
AREG,ErbB4,FasL,IFNgamma	0.7419355
ErbB4,FasL,Flt3L,HGFR	0.7358871
CLEC2C,ErbB4,FasL,Flt3L	0.7167339
CLEC2C,FasL,Flt3L,HGFR	0.7086694
CLEC2C,FasL,Flt3L,IFNgamma	0.7056452
CLEC2C,ErbB4,Flt3L,HGFR	0.7050

	781
ErbB4,Flt3L,HGFR,IFNgamma	0.7050781
CLEC2C,ErbB4,Flt3L,IFNgamma	0.7011719
ErbB4,FasL,Flt3L,IFNgamma	0.6985887
CLEC2C,Flt3L,HGFR,IFNgamma	0.6933594
FasL,Flt3L,HGFR,IFNgamma	0.6895161
CLEC2C,ErbB4,FasL,IFNgamma	0.6290323
CLEC2C,ErbB4,FasL,HGFR	0.6239919
CLEC2C,ErbB4,HGFR,IFNgamma	0.6220703
ErbB4,FasL,HGFR,IFNgamma	0.6118952
CLEC2C,FasL,HGFR,IFNgamma	0.6088710

Table 7, Table 8 and **Table 9** show the AUC achieved for the combinations of two, three and four biomarkers respectively, discriminating AA vs CTL.

Table 7

Biomarker combination AA vs CTL	AUC
AREG,CD147	0.7548828
AREG,HGFR	0.7460938
AREG,CYFRA21-1	0.7221680
CYFRA21-1,HGFR	0.6250000
CD147,HGFR	0.6191406
CD147,CYFRA21-1	0.6152344

5

Table 8

Biomarker combination AA vs CTL	AUC
AREG,CD147,CYFRA21-1	0.7617188
AREG,CYFRA21-1,HGFR	0.7607422
AREG,CD147,HGFR	0.7539062
CD147,CYFRA21-1,HGFR	0.6337891

Table 9

Biomarker combination AA vs CTL	AUC
AREG,CYFRA21-1,HGFR,CD147	0.7685547

Based on their respective AUCs, the best models have been selected. **Table 10** and **Table 10bis** show the best results for CRC. **Table 11** and **Table 11bis** show the best results for AA. The metrics for the best combinations of proteins are included, comprising area under the ROC curve (AUC), Sensitivity (Sens.), Specificity (Spec.), and positive (PPV) and negative (NPV) predictive values computed in the cut-off point of the ROC curve with the best Youden´s index.

Table 10

	CRC.vs.CTL				
Biomarker combination supporting Flt3L+CYFRA21-1	**AUC (95%CI)**	**Sens**	**Spec**	**PPV**	**NPV**
Flt3L,CYFRA21-1	0.865(0.779,0.952)	0.625	0.969	0.952	0.721
Flt3L,CYFRA21-1,AREG	0.899(0.821,0.978)	0.781	0.938	0.926	0.811
Flt3L,CYFRA21-1,AREG,ErbB4	0.931(0.861,1)	0.875	0.938	0.933	0.882
Flt3L,CYFRA21-1,AREG,CLEC2C	0.915(0.848,0.982)	0.844	0.844	0.844	0.844

Table 10bis

	CRC.vs.CTL				
Biomarker combination supporting AREG+CYFRA21-1	**AUC (95%CI)**	**Sens**	**Spec**	**PPV**	**NPV**
AREG,CYFRA21-1	0.878(0.789,0.966)	0.875	0.844	0.848	0.871
AREG,CLEC2C	0.835(0.729,0.941)	0.906	0.812	0.829	0.897
AREG,HGFR	0.763(0.643,0.883)	0.656	0.812	0.778	0.703
AREG,CD147	0.842(0.734,0.95)	0.844	0.844	0.844	0.844
AREG,CYFRA21-1,Flt3L	0.899(0.821,0.	0.78	0.93	0.92	0.81

	978)	1	8	6	1
AREG,CYFRA21-1,CLEC2C	0.896(0.814,0.977)	0.875	0.844	0.848	0.871
AREG,CYFRA21-1,ErbB4	0.896(0.812,0.979)	0.875	0.844	0.848	0.871
AREG,CYFRA21-1,FasL	0.893(0.81,0.976)	0.903	0.812	0.824	0.897
AREG,CYFRA21-1,CD147	0.886(0.797,0.975)	0.875	0.844	0.848	0.871
AREG,CYFRA21-1,HGFR	0.876(0.787,0.965)	0.875	0.844	0.848	0.871
AREG,CD147,HGFR	0.843(0.735,0.951)	0.844	0.844	0.844	0.844
AREG,CYFRA21-1,ErbB4,Flt3L	0.931(0.861,1)	0.875	0.938	0.933	0.882
AREG,CYFRA21-1,Flt3L, CLEC2C	0.915(0.848,0.982)	0.844	0.844	0.844	0.844
AREG,CYFRA21-1,CD147,HGFR	0.888(0.801,0.975)	0.875	0.844	0.848	0.871

Table 11

		AA.vs.CTL			
Biomarker combination supporting Flt3L+CYFRA21-1	**AUC (95%CI)**	**Sens**	**Spec**	**PPV**	**NPV**
Flt3L,CYFRA21-1	0.606(0.466,0.747)	0.906	0.312	0.569	0.769
Flt3L,CYFRA21-1,AREG	0.72(0.591,0.848)	0.812	0.625	0.684	0.769
Flt3L,CYFRA21-1,AREG,ErbB4	0.707(0.578,0.836)	0.656	0.75	0.724	0.686
Flt3L,CYFRA21-1,AREG,CLEC2C	0.727(0.6,0.853)	0.812	0.625	0.684	0.769

Table 11bis

		AA.vs.CTL			
Biomarker combination supporting AREG+CYFRA21-1	**AUC (95%CI)**	**Sens**	**Spec**	**PPV**	**NPV**
AREG,CYFRA21-1	0.722(0.594,0.85)	0.844	0.625	0.692	0.8
AREG,CLEC2C	0.738(0.613,0.864)	0.781	0.688	0.714	0.759
AREG,HGFR	0.746(0.621,0.871)	0.531	0.938	0.895	0.667
AREG,CD147	0.755(0.633,0.877)	0.656	0.844	0.808	0.711
AREG,CYFRA21-1,Flt3L	0.72(0.591,0.848)	0.812	0.625	0.684	0.769
AREG,CYFRA21-1,CLEC2C	0.728(0.601,0.854)	0.812	0.625	0.684	0.769

AREG,CYFRA21-1,ErbB4	0.71(0.582,0.838)	0.656	0.75	0.724	0.686
AREG,CYFRA21-1,FasL	0.711(0.581,0.84)	0.774	0.625	0.667	0.741
AREG,CYFRA21-1,CD147	0.762(0.642,0.882)	0.844	0.656	0.711	0.808
AREG,CYFRA21-1,HGFR	0.761(0.634,0.887)	0.75	0.75	0.75	0.75
AREG,CD147,HGFR	0.754(0.632,0.876)	0.562	0.906	0.857	0.674
AREG,CYFRA21-1, Flt3L, ErbB4,	0.707(0.578,0.836)	0.656	0.75	0.724	0.686
AREG,CYFRA21-1, Flt3L, CLEC2C	0.727(0.6,0.853)	0.812	0.625	0.684	0.769
AREG,CYFRA21-1,CD147,HGFR	0.769(0.65,0.888)	0.781	0.719	0.735	0.767

Finally, **Table 12** and **Table 12bis** have been designed to show the overlapping of the most important signatures claimed in the present invention. It is clearly shown that all the best signature signatures comprise [Flt3L and CYFRA21-1] and [AREG and CYFRA21-1].

Table 12

AREG	CLEC2C	CYFRA21-1	Flt3L	ErbB4	CRC vs CTL	AA vs CTL
X		**X**	**X**	X	**0.931**	0.707
X	X	**X**	**X**		0.915	**0.727**
X		**X**	**X**		0.899	0.720
		X	**X**		0.865	0.606

Table 12 bis

CD147	FasL	CLEC2C	AREG	CYFRA21-1	Flt3L	ErbB4	HGFR	CRC	AA
			X	**X**	X	X		**0.931**	0.707
		X	**X**	**X**	X			0.915	0.727
			X	**X**	X			0.899	0.720
		X	**X**	**X**				0.896	0.728
			X	**X**		X		0.896	0.710
	X		**X**	**X**				0.893	0.711
X			**X**	**X**			X	0.888	**0.769**
X			**X**	**X**				0.886	0.762
			X	**X**				0.878	0.722
			X	**X**			X	0.876	0.761
X			X				X	0.843	0.754
X			X					0.842	0.755
		X	X					0.835	0.738
			X				X	0.763	0.746

CLAIMS

1. *In vitro* method for the diagnosis of colorectal cancer and/or a pre-cancerous stage thereof which comprises: a) Measuring the concentration level of the combination [Flt3L and CYFRA21-1] in a biological sample obtained from the subject and b) wherein if a deviation or variation of the concentration level of at least the combination [Flt3L and CYFRA21-1] is identified, as compared with the reference concentration level measured in healthy control subjects, this is indicative that the subject is suffering from colorectal cancer and/or a pre-cancerous stage.
2. *In vitro* method for the diagnosis of colorectal cancer and/or a pre-cancerous stage thereof, according to claim 1, which comprises: a) Measuring the concentration level of at least the combination [Flt3L and CYFRA21-1 and AREG], or the combination [Flt3L and CYFRA21-1 and AREG and ErbB4] or the combination [Flt3L and CYFRA21-1 and AREG and CLEC2C] in a biological sample obtained from the subject and b) wherein if a deviation or variation of the concentration level of at least one combination cited in step a) is identified, as compared with the reference concentration level measured in healthy control subjects, this is indicative that the subject is suffering from colorectal cancer and/or a pre-cancerous stage.
3. *In vitro* method, according to any of the previous claims, wherein the pre-cancerous stage of colorectal cancer is advanced colorectal adenoma.
4. *In vitro* use of the combination [Flt3L and CYFRA21-1] in a biological sample obtained from the patient for the diagnosis of colorectal cancer and/or a pre-cancerous stage thereof.
5. *In vitro* use of the combination [Flt3L and CYFRA21-1 and AREG], or [Flt3L and CYFRA21-1 and AREG and ErbB4], or [Flt3L and CYFRA21-1 and AREG and CLEC2C] in a biological sample obtained from the patient, according to claim 4, for the diagnosis of colorectal cancer and/or a pre-cancerous stage thereof.
6. Kit of parts comprising reagents for the determination of the concentration level of at least a signature selected from the list: [Flt3L and CYFRA21-1], or [Flt3L

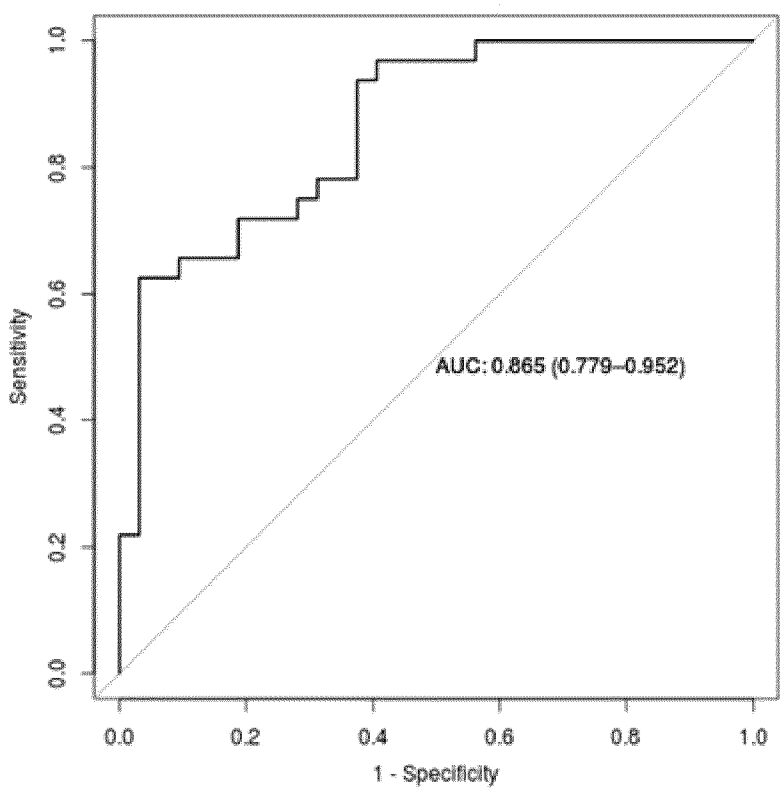
and CYFRA21-1 and AREG], or [Flt3L and CYFRA21-1 and AREG and ErbB4], or [Flt3L and CYFRA21-1 and AREG and CLEC2C].

7. Use of the kit according to claim 6 for the diagnosis of colorectal cancer and/or a pre-cancerous stage thereof.

8. Use of the kit, according to claim 7, wherein the pre-cancerous stage of colorectal cancer is advanced colorectal adenoma.

Figure 1

A


Sensitivity
1 - Specificity
AUC: 0.865 (0.779–0.952)
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0.2
0.4
0.6
0.8
1.0

B

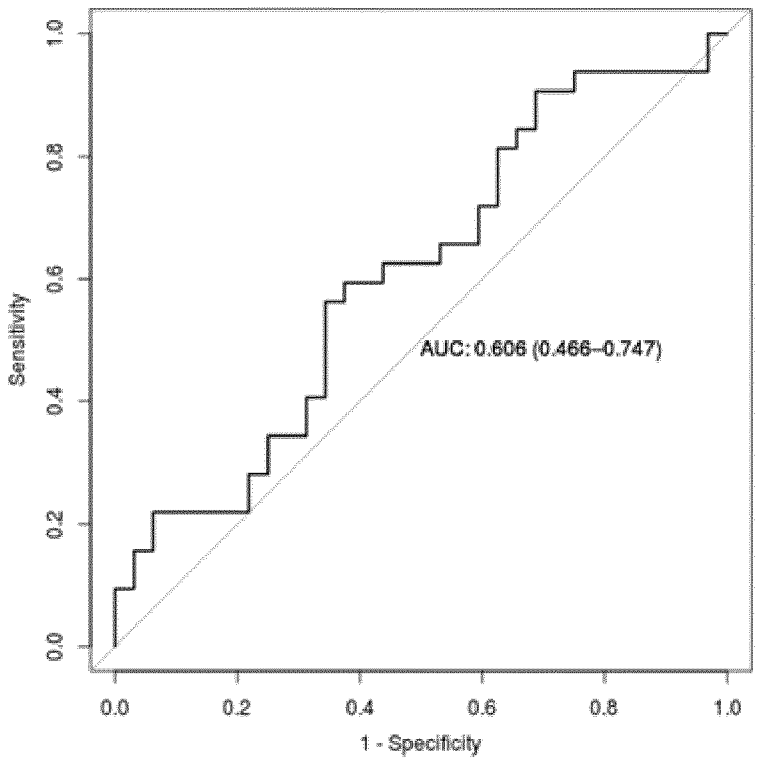
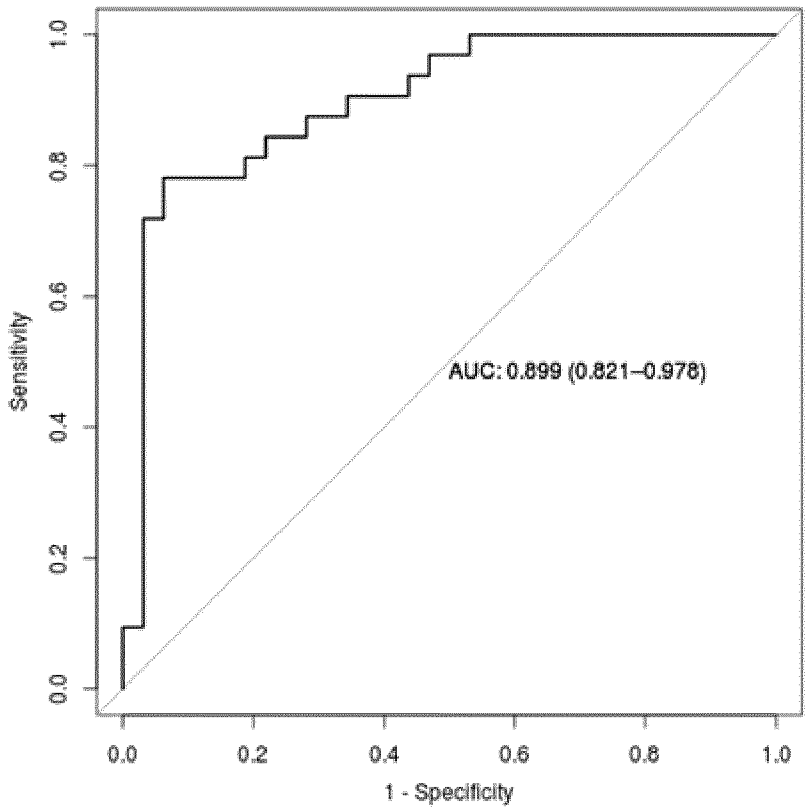

Sensitivity
1 - Specificity
AUC: 0.606 (0.466–0.747)
0.0
0.2
0.4
0.6
0.8
1.0

Figure 2

A


Sensitivity
1 - Specificity
AUC: 0.899 (0.821–0.978)
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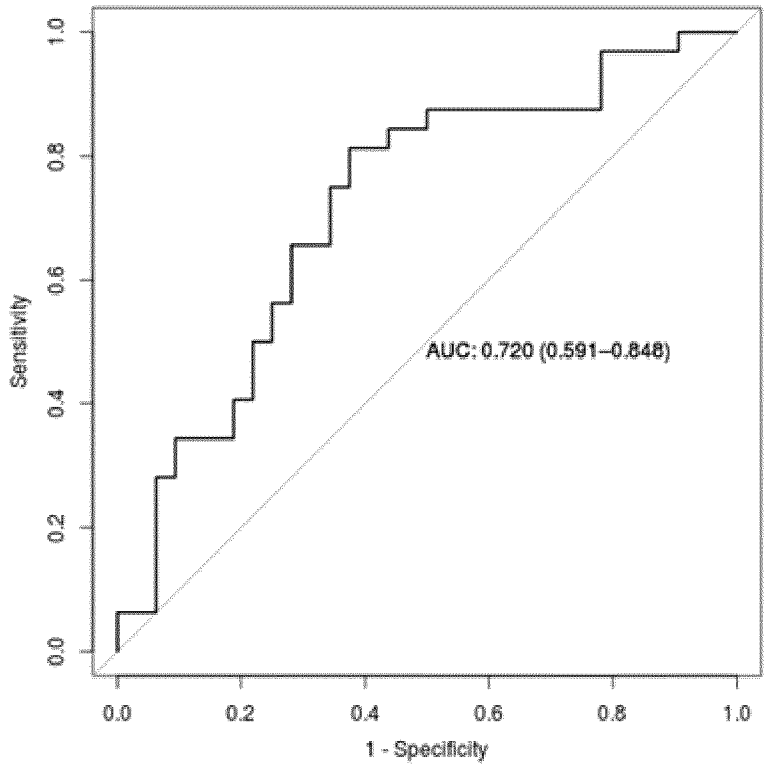
0.2
0.4
0.6
0.8
1.0

B


Sensitivity
1 - Specificity
AUC: 0.720 (0.591–0.848)
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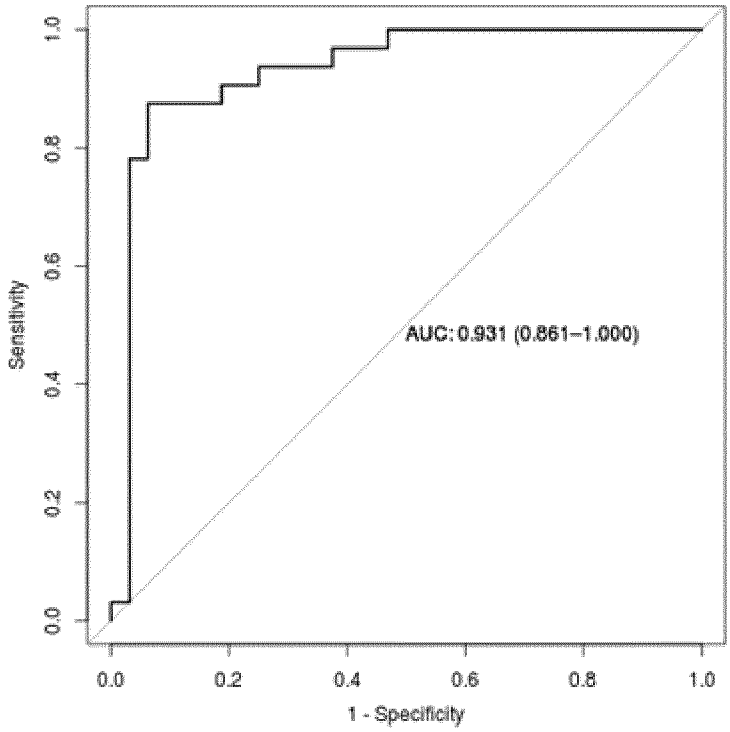
0.2
0.4
0.6
0.8
1.0

Figure 3

A


Sensitivity
1 - Specificity
AUC: 0.931 (0.861–1.000)
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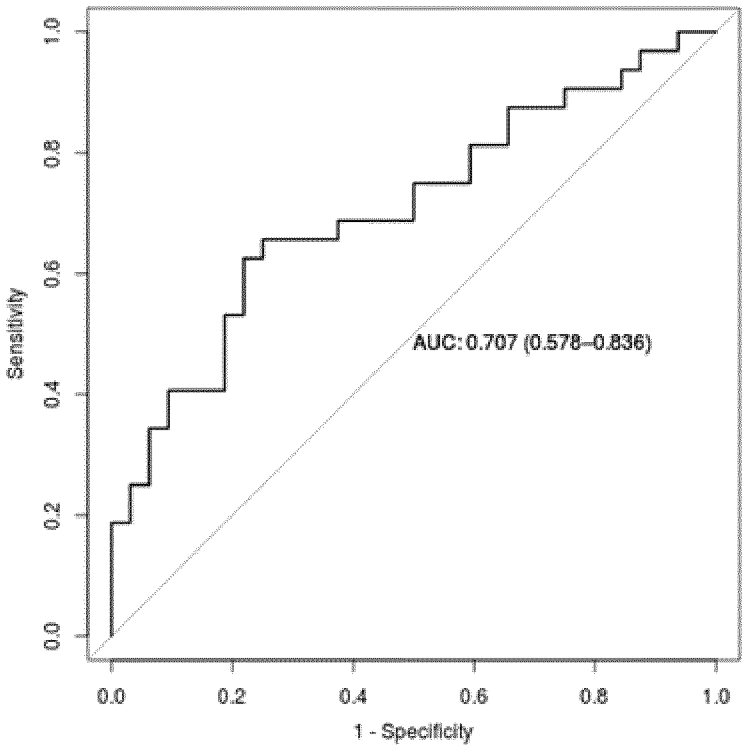
0.2
0.4
0.6
0.8
1.0

B


Sensitivity
1 - Specificity
AUC: 0.707 (0.578–0.836)
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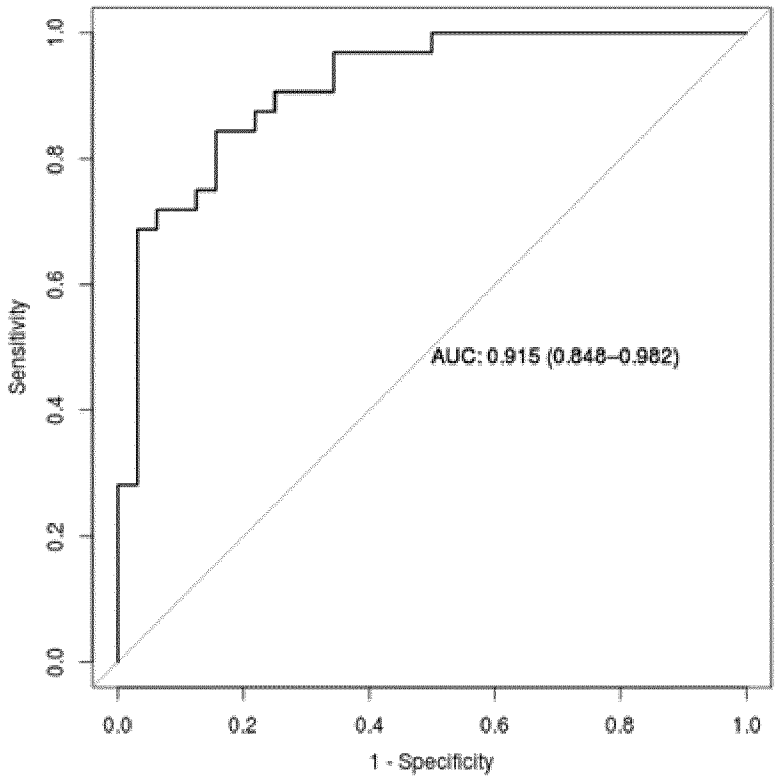
0.2
0.4
0.6
0.8
1.0

Figure 4

A


Sensitivity
1 - Specificity
AUC: 0.915 (0.848–0.982)
0.0
0.2
0.4
0.6
0.8
1.0

B

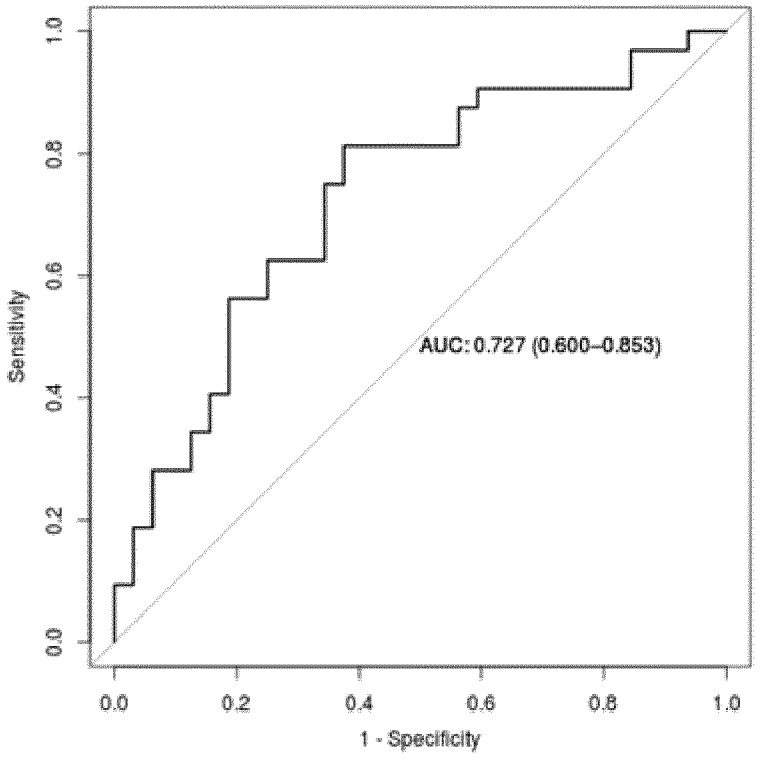
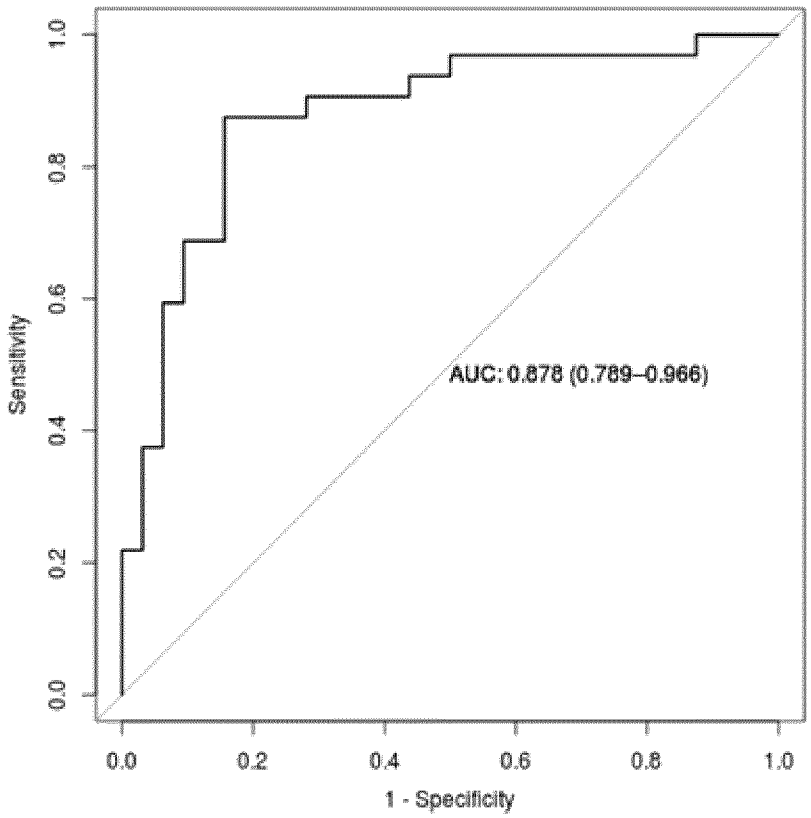

Sensitivity
1 - Specificity
AUC: 0.727 (0.600–0.853)
0.0
0.2
0.4
0.6
0.8
1.0

Figure 5

A


Sensitivity
1 - Specificity
AUC: 0.878 (0.789–0.966)
0.0
0.2
0.4
0.6
0.8
1.0

B

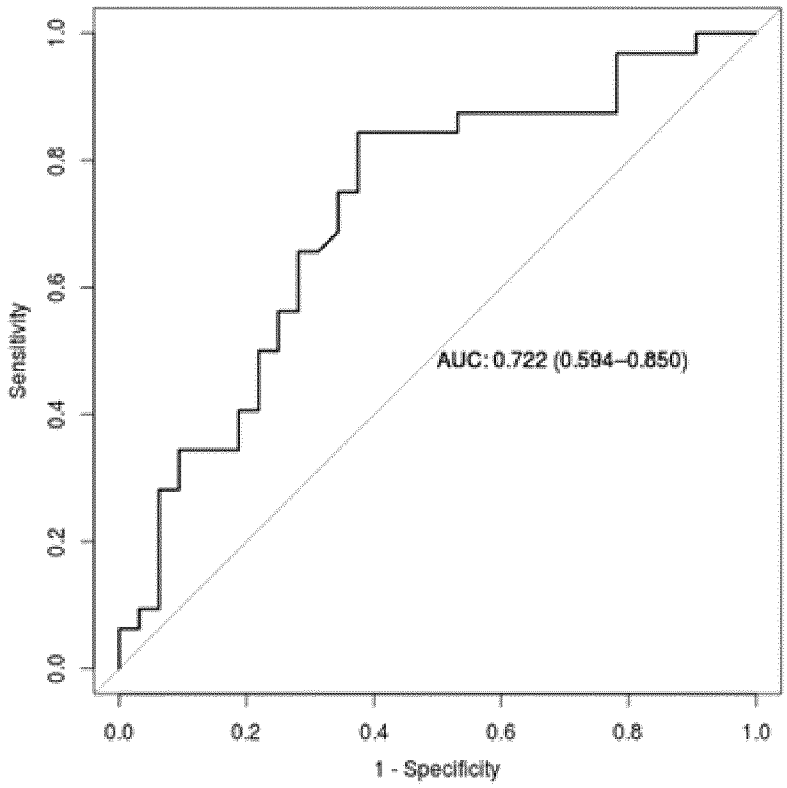
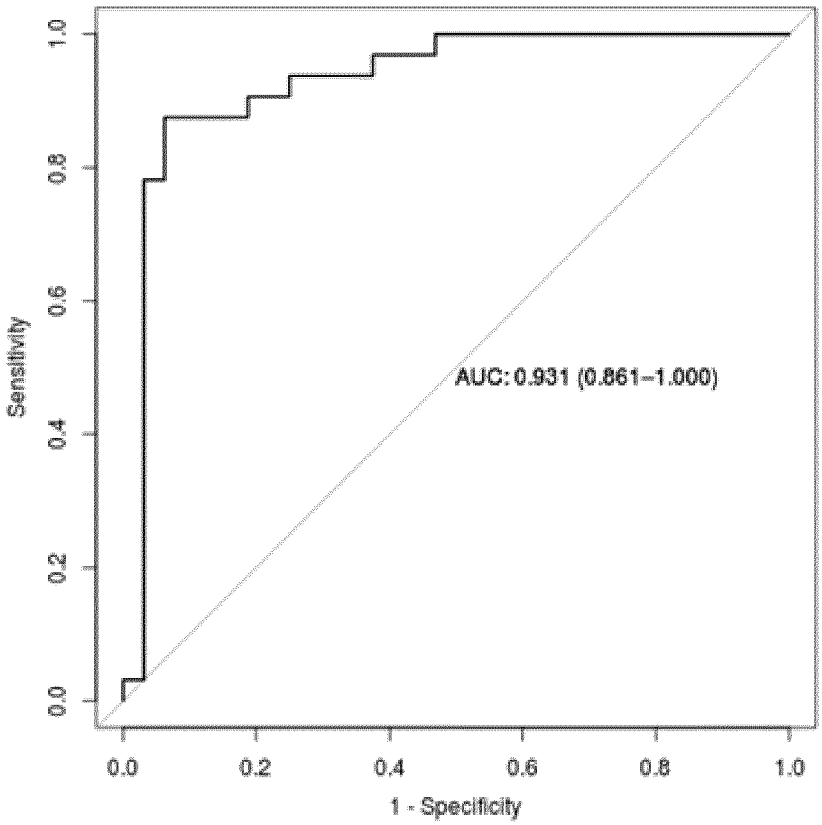

Sensitivity
1 - Specificity
AUC: 0.722 (0.594–0.850)
0.0
0.2
0.4
0.6
0.8
1.0

Figure 6

A


Sensitivity
1 - Specificity
AUC: 0.931 (0.861–1.000)
0.0
0.2
0.4
0.6
0.8
1.0

B

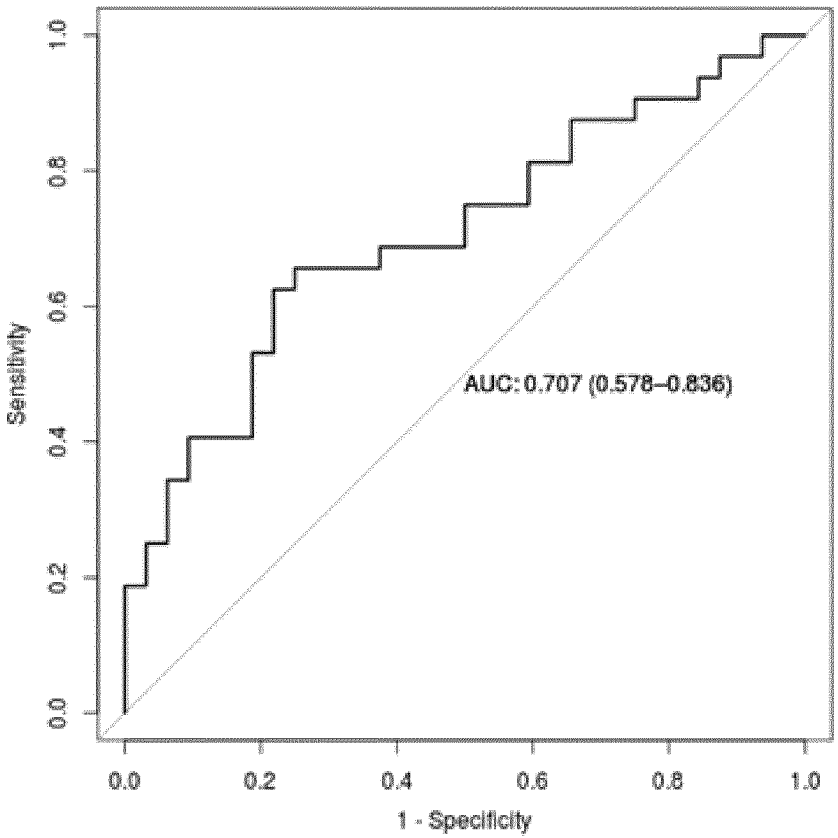
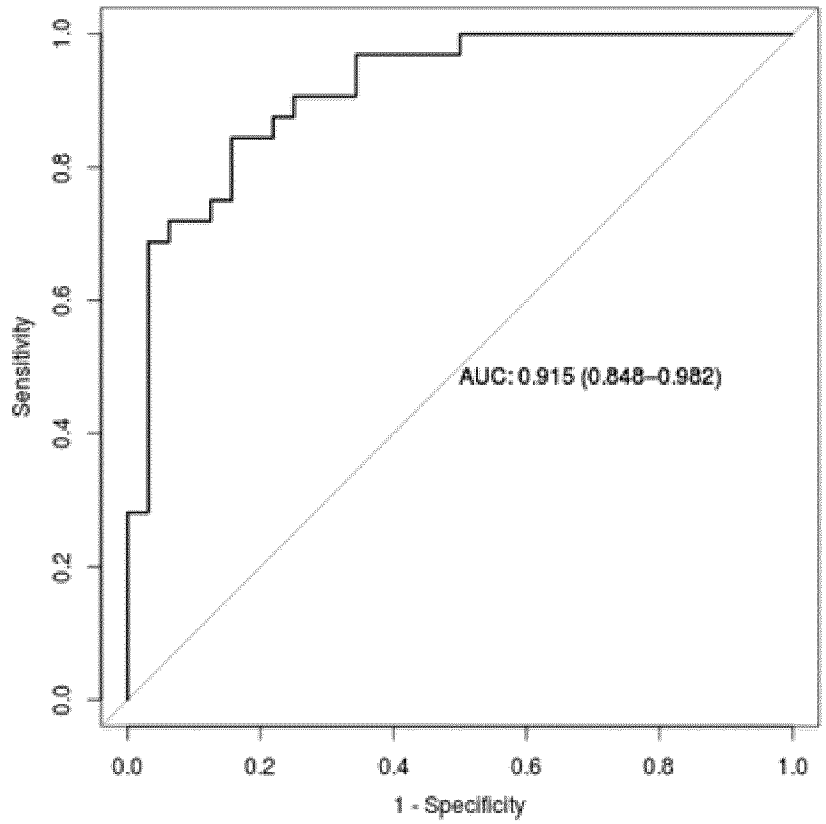

Sensitivity
1 - Specificity
AUC: 0.707 (0.578–0.836)
0.0
0.2
0.4
0.6
0.8
1.0

Figure 7

A


Sensitivity
1 - Specificity
AUC: 0.915 (0.848–0.982)
0.0
0.2
0.4
0.6
0.8
1.0

B

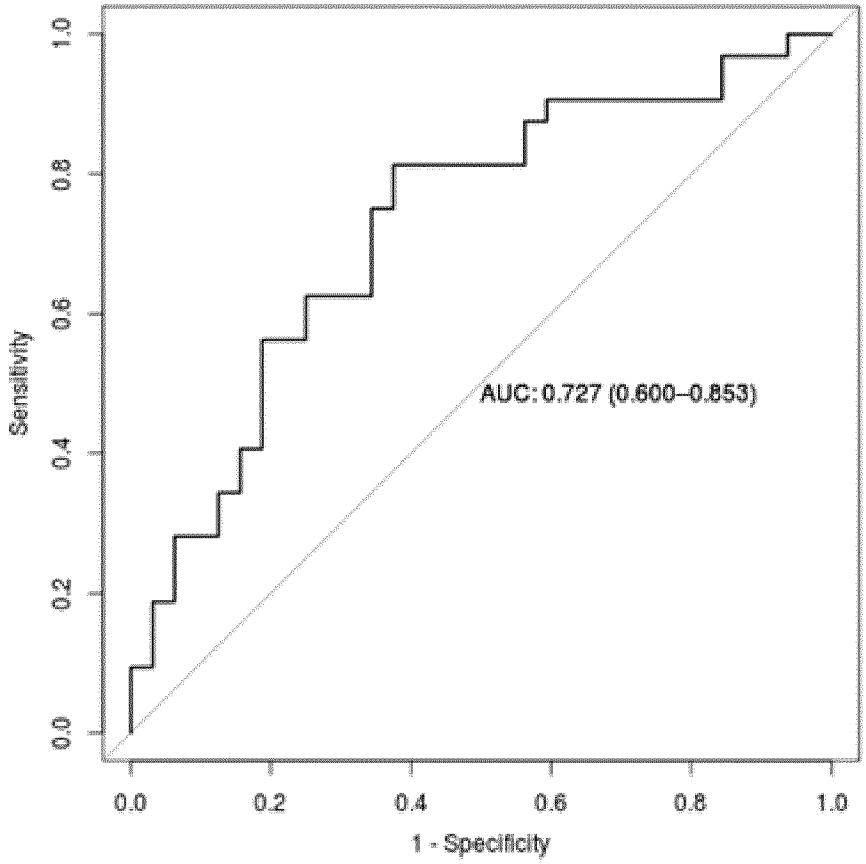
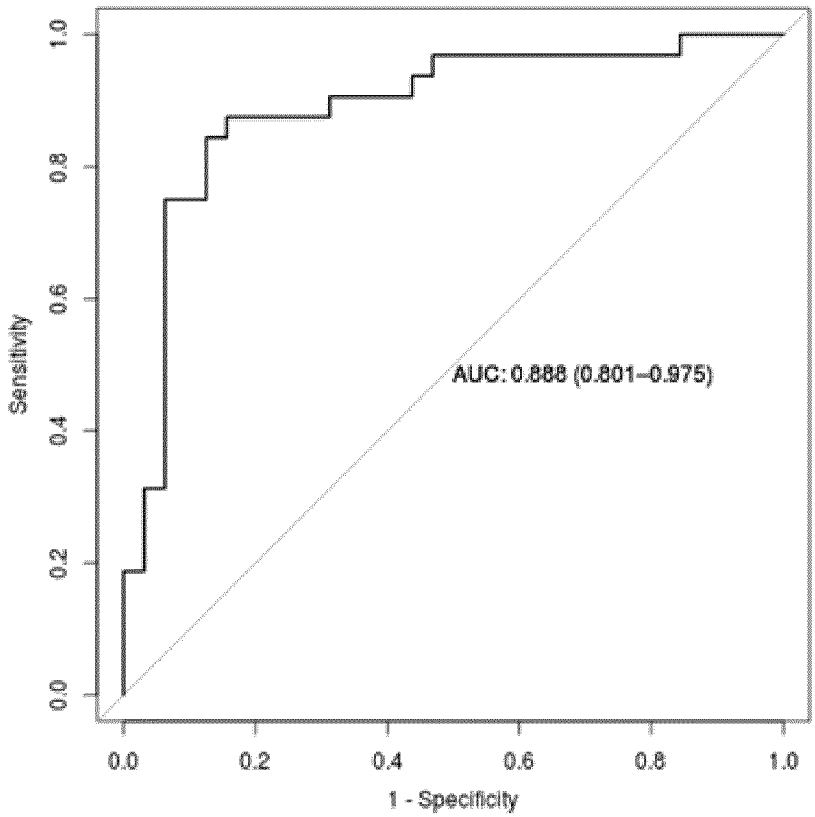
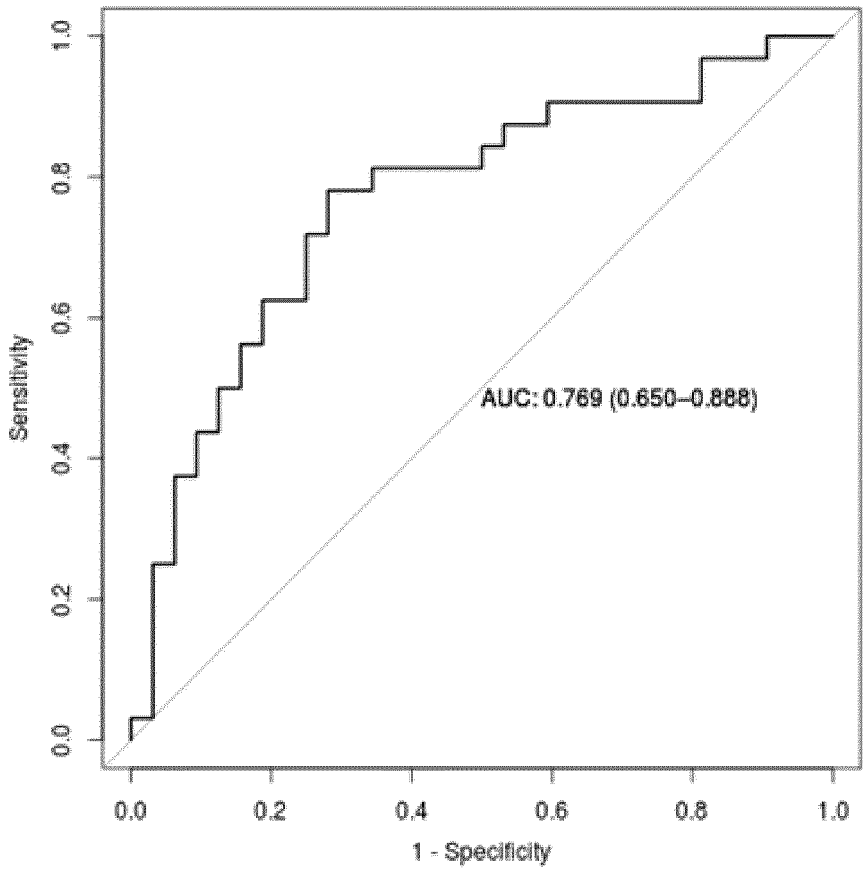

Sensitivity
1 - Specificity
AUC: 0.727 (0.600–0.853)
0.0
0.2
0.4
0.6
0.8
1.0

Figure 8

A


Sensitivity
1 - Specificity
AUC: 0.888 (0.801–0.975)
0.0
0.2
0.4
0.6
0.8
1.0

B


Sensitivity
1 - Specificity
AUC: 0.769 (0.650–0.888)
0.0
0.2
0.4
0.6
0.8
1.0

INTERNATIONAL SEARCH REPORT

International application No
PCT/EP2020/055277

A. CLASSIFICATION OF SUBJECT MATTER
INV. G01N33/574
ADD.

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
G01N

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

EPO-Internal, BIOSIS, EMBASE, WPI Data

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	HONGDA CHEN ET AL: "Development and validation of a panel of five proteins as blood biomarkers for early detection of colorectal cancer", CLINICAL EPIDEMIOLOGY, vol. Volume 9, 1 October 2017 (2017-10-01) , pages 517-526, XP055512097, DOI: 10.2147/CLEP.S144171 the whole document abstract ----- -/--	1-8

[X] Further documents are listed in the continuation of Box C.

[X] See patent family annex.

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"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

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"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art

"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
6 April 2020	16/04/2020

Name and mailing address of the ISA/	Authorized officer
European Patent Office, P.B. 5818 Patentlaan 2 NL - 2280 HV Rijswijk Tel. (+31-70) 340-2040, Fax: (+31-70) 340-3016	Jenkins, Gareth

1

Form PCT/ISA/210 (second sheet) (April 2005)

C(Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	H. CHEN ET AL: "Head-to-Head Comparison and Evaluation of 92 Plasma Protein Biomarkers for Early Detection of Colorectal Cancer in a True Screening Setting", CLINICAL CANCER RESEARCH, vol. 21, no. 14, 26 May 2015 (2015-05-26), pages 3318-3326, XP055512107, US ISSN: 1078-0432, DOI: 10.1158/1078-0432.CCR-14-3051 the whole document abstract table 2	1-8
A	CHEN HONGDA ET AL: "Empirical evaluation demonstrated importance of validating biomarkers for early detection of cancer in screening settings to limit the number of false-positive findings", JOURNAL OF CLINICAL EPIDEMIOLOGY, PERGAMON, GB, vol. 75, 2 February 2016 (2016-02-02), pages 108-114, XP029616862, ISSN: 0895-4356, DOI: 10.1016/J.JCLINEPI.2016.01.022 the whole document abstract table 2	1-8
A	QIAN JING ET AL: "Biomarker discovery study of inflammatory proteins for colorectal cancer early detection demonstrated importance of screening setting validation", JOURNAL OF CLINICAL EPIDEMIOLOGY, PERGAMON, GB, vol. 104, 1 August 2018 (2018-08-01), pages 24-34, XP085523437, ISSN: 0895-4356, DOI: 10.1016/J.JCLINEPI.2018.07.016 the whole document abstract table 3	1-8
X	US 2014/220006 A1 (AGHVANYAN ANAHIT [US] ET AL) 7 August 2014 (2014-08-07)	6
A	the whole document paragraph [0044]	1-5,7,8

1

INTERNATIONAL SEARCH REPORT

Information on patent family members

International application No
PCT/EP2020/055277

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
US 2014220006 A1	07-08-2014	US 2014220006 A1	07-08-2014
		US 2017160281 A1	08-06-2017