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(71) Applicant: **ENTEROME** [FR/FR]; 94-96, Avenue Ledru Rollin, 75011 Paris (FR).

(72) Inventor: **CERVINO, Alessandra**; 3 rue de Tournezy, 77590 Bois Le Roi (FR).

(74) Agent: **REGIMBEAU**; 20, rue de Chazelles, 75847 Paris Cedex 17 (FR).

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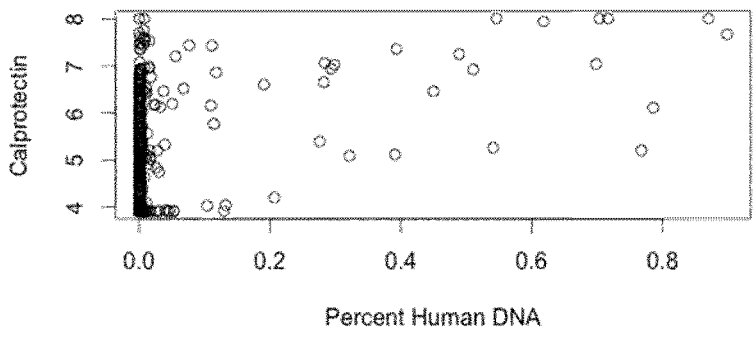
Figure 3



(57) Abstract: The present invention relates to an in vitro method for diagnosing Crohn disease in a subject comprising: a) obtaining a biological sample from said subject, b) analysing the relative abundance of the host DNA in said sample, and, c) diagnosing that said subject suffers from Crohn disease if said relative abundance is higher than a reference value. The present invention also relates to an in vitro method for diagnosing the state (stable or unstable) of the Crohn disease in a subject, comprising the steps of: a) obtaining a biological sample from said subject, b) analysing the relative abundance of the host DNA in said sample, and, c) diagnosing that said subject has a Crohn disease in an unstable state if said relative abundance is higher than another reference value.

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HOST DNA AS A BIOMARKER OF CROHN'S DISEASE

Technical field of the invention

The invention relates to a new method for diagnosing patients with Crohn disease, and/or for diagnosing the status of the disease in Crohn-suffering patients.

Background of the invention

Crohn's disease (CD) is a chronic inflammatory bowel disease (IBD) that may affect any part of the gastrointestinal tract from mouth to anus. The age of onset is generally between 15-30 years and it is equally prevalent in women and men. The highest prevalence is found in Europe and North America with just over 300 per 100.000 persons (Molodecky et al. 2012). CD generally leads to abdominal pain, severe diarrhoea and weight disorders. The disease is of unknown aetiology and multifactorial: environmental factors, host genetics and gut microbiome have all been shown to impact the risk of disease and its severity (Cho, J. H., & Brant, S. R. (2011)).

The clinical diagnosis of CD is supported by serologic, radiologic, endoscopic, and histologic findings. Currently, there are no standalone laboratory developed tests that allow the diagnostic of CD. Amongst available laboratory tests, serum CRP, faecal calprotectin and lactoferrin are the most widely used markers, but they are not specific to CD. Disease activity can be measured by the Crohn's Disease Activity index (CDAI), a score resulting from the combination of multiple parameters or the Harvey-Bradshaw index (HBI) which only consists of clinical parameters (Laas et al. 2014).

Moreover, in patients diagnosed with CD, monitoring clinical symptoms alone is not reliable enough to assess disease activity. Patients self-reporting low disease activity often present intestinal lesions during an endoscopic exam. Biological markers, such as faecal calprotectin, are useful, but nonspecific and their increase is associated with systemic/mucosal inflammation at the late onset of the flare. Endoscopy enables to detect mucosal healing, which is considered as the most robust and reliable sign of disease remission; however, routine repeated endoscopic monitoring is not feasible, because of the required bowel preparation and general anaesthesia. New imaging tools, such as MRI, have been shown to be effective, but it is expensive, time-consuming, and limited access precludes routine use. The MR Enterography, presented as the most promising approach, implies also bowel preparation and invasive colonoscopy.

The tight control of CD, through accurate surveillance and treatment adjustment, is thus a key in the management of such patients, because of the recurring and remitting nature of these disorders. Yet, none of the current diagnostic methods is satisfactory, for the above-mentioned reasons.

Patients and healthcare providers are therefore actively looking for non-invasive tools enabling evaluation of disease activity and monitoring of patients care.

More precisely, there remains a need to identify a biomarker of CD that would allow diagnosing the disease in a patient that is non-invasive, simple and accurate manner. This is precisely the subject of the present invention.

Also, there is a need for identifying a biomarker which could help in distinguishing between patients suffering from an active CD vs. from a quiescent stage of said disease. Indeed, this information could help clinicians in diagnosing the stage of CD, predicting the occurrence of said changes, in order to choose from the different treatment options (intensive or conventional), without having to perform an endoscopic analysis. This need is fulfilled by the present invention.

Increase of host DNA in stool samples of patients suffering from diseases known to induce an inflammatory state of the gut mucosa has been observed in the prior art.

In a prospective cohort of 599 hospitalized patients, a single rectal swab was obtained from each patient within 7 days of admission to the hospital. Host DNA proportions were negatively correlated with intestinal microbiota diversity. Enterococcus and Escherichia were enriched in patients excreting high quantities of human DNA, while Ruminococcus and Odoribacter were depleted. The quantification of human DNA in faeces could serve as a simple and non-invasive approach to assess bowel inflammation (Vincent et al, 2014).

Stool samples from colorectal cancer patients also contain increased concentrations of human DNA (Klaassen et al 2003).

The amount of host DNA in stool samples of Crohn suffering patients has never been assessed so far. Yet, it has been observed that stool samples from CD patients also contained increased concentrations of human DNA. This is very surprising since the total area of intestinal lesions is low in CD patients (as compared with ulcerative colitis) and visible bleeding in the faeces is quite uncommon in Crohn's disease.

The present inventors analysed, by a quantitative metagenomic analysis, but also by qPCR, the human DNA abundance in a number of stool samples that have been collected from healthy controls and CD patients. Moreover, the host DNA abundance was assessed in stool samples obtained from patients suffering from aggressive Crohn disease vs. from a quiescent stage of said disease.

In so doing, the present inventors observed that the presence and quantity of human DNA into stool samples are markers of Crohn's disease, and that patients suffering from aggressive Crohn disease vs. from a quiescent stage of said disease can be discriminated using this very same biomarker.

To sum up, they demonstrated that the presence of host DNA in the faeces of CD patients may be used as a biomarker of CD, of its activity or severity.

Figure Legends

Figure 1 discloses the Boxplots of the percent human DNA found in stool samples from healthy and NASH controls (on left) and Crohn Disease patients (on the right).

Figure 2 discloses the relative abundance levels (in %) of the Host DNA found in the studied stool samples, both for Crohn disease patients in an active phase of the disease (on the left) and in a quiescent phase (on the right). The status of the disease (active phase vs quiescent phase) were determined using the combined score of calprotectin level and HBI score.

Figure 3 discloses the percentage of human DNA versus calprotectin levels found in the studied stool samples.

Figure 4 discloses ROC curve using the percent of human DNA as a predictive score of disease activity within a CD population. The AUC has a value of 0.67 for the combined score.

Figure 5 discloses the correlation between the quantitative data of the VP5 and VP9 qPCR assays for measuring host DNA abundance.

Figure 6 discloses the correlation between the quantitative data of the VP5 qPCR assay and the percentage of human DNA relative abundance measured by Illumina sequencing.

Figure 7 discloses the correlation between the quantitative data of the VP9 qPCR assay and the percentage of human DNA relative abundance measured by Illumina sequencing.

Figure 8 discloses the correlation between mRNA level of faecal calprotectin by qPCR assay (measure of S100A8 and S100A9 mRNA level) and protein level of faecal calprotectin by ELISA (A: S100A8; B: S100A9).

Detailed description of the invention

Methods of measures, in particular to diagnose CD

In a first aspect, the present invention relates to a method for generating quantitative data for a subject having or suspected of having Crohn disease (CD), said method comprising: performing at least one assay to determine host DNA relative abundance in a biological sample from said subject, wherein the quantitative data represents host DNA relative abundance preferably compared to a reference value.

In other words, the invention relates to an *in vitro* method for analysing a biological sample from a subject having or suspected of having Crohn disease (CD), said method comprising:

a) Obtaining a biological sample from said subject,

b) Determining the relative abundance of host DNA in said sample,

and,

c) Determining if said relative abundance is higher than a reference value.

More precisely, the present invention relates to an *in vitro* method for diagnosing Crohn disease (CD) in a subject, said method comprising:

a) Obtaining a biological sample from said subject,

b) Determining the relative abundance of host DNA in said sample,

and,

c) Diagnosing that said subject suffers from Crohn disease, if said relative abundance is higher than a reference value.

This method is advantageous over the prior art diagnosis method as it is non-invasive, economically acceptable, and present a high specificity.

As used herein, the term "host DNA" refers to the DNA of the host of the gut microbiota, as opposed to microbial or viral DNA. If the tested subject is a human patient, then the term "host DNA" refers specifically to "human DNA".

DNA can be extracted from said biological sample of interest for example by using the extraction protocol described in Godon JJ. *et al,.* 1997. Other protocols can nevertheless be used and are well-known. Of note, the microbial DNA and the host DNA do not need to be physically separated for subsequent analysis.

Mammal DNA can be distinguished from microbial DNA by any conventional mean, such as detection of CpG methylation or of the bacterial 16S ribosomal DNA. It is also possible to use qPCR targeting the ALU (STR) repeat regions in human DNA, or the Beta-globulin, Beta-actin, and hTERT genes (Klaassen CHW et al, 2003; Shewale JG et al, *Journal of Forensic Science*, 2007, vol.52(2)). Nanostring technologies could be also useful.

Quantification of the host and microbial DNA can be performed by any well-known method. The most commonly used methods known in the art for the quantification of DNA strands in a sample include Northern blotting and *in situ* hybridization (Parker & Barnes, *Methods in Molecular Biology* 106:247-283 (1999)) PCR-based methods, such as quantitative polymerase chain reaction (qPCR) (Heid et al., *Genome Research* 6:986-994 (1996)), and nucleic-acid based multiplex techniques, such as multiplex PCR and DNA microoarrays. Alternatively, antibodies may be employed that can recognize sequence-specific duplexes, including DNA duplexes

or DNA-protein duplexes. Representative methods for sequencing-based analysis include chain-termination methods, shotgun sequencing methods, *de novo* sequencing, next generation sequencing methods (including Massively Parallel Signature Sequencing (MPSS), Polony sequencing, 454 pyrosequencing, Illumina (Solexa) sequencing, SOLiD sequencing, Ion semiconductor sequencing, DNA nanoball sequencing, Helioscope single molecule sequencing, Single molecule real time (SMRT) sequencing, RNAP sequencing, Nanopore DNA sequencing, Sequencing by hybridization and Microfluidic Sanger sequencing).

As shown in the examples below, it is also possible to measure host DNA from a pool of DNA by i) sequencing the DNA present in stool samples using high throughput sequencing technologies and ii) by aligning the short reads obtained by means of these sequencing technologies to the human genome. In this case, "relative abundance of host DNA" can be calculated by counting the number of reads mapped to a single reference sequence from the human genome (H) and the remaining number of reads generated (B), and normalizing the number of reads H by the total amount of reads (H+B).

As meant herein, the term "host DNA abundance" refers to the relative amount of host DNA as compared with the total amount of DNA present in said sample (including in particular bacterial and fungal DNA). In the present application, it will therefore preferably be referred to as "relative abundance" (or "relative amount") of host DNA.

Preferably, the host DNA abundance is measured by qPCR with human specific nucleic acid fragments, such as primers and/or probes.

As used herein, the term "nucleic acid", "nucleic acid sequence", "nucleotide", "nucleotide sequence", which are interchangeable, refer to a precise succession of natural nucleotides (e.g., A, T, G, C and U), corresponding to a single-stranded or double-stranded DNA such as cDNA, genomic DNA, ribosomal DNA or plasmidic

DNA, and the transcription product of said DNA, such as RNA. A nucleic acid according to the invention may be isolated and prepared by any known method including, but not limited to, any synthetic method, any recombinant method, any *ex vivo* generation method and the like, as well as combinations thereof.

The probes and primers required or useful to carry out the qPCR on host DNA are referred herein as "nucleic acid fragments" in the context of the invention.

By "nucleic acid fragment", it is more generally meant herein a nucleic acid hybridizing to a nucleic acid of interest. For instance, such nucleic acid fragment may be at least 10 nucleotides in length or preferably, at least 15 nucleotides in length. They may also be at least 25 or at least 50 nucleotides in length.

Nucleic acid fragments according to the invention are specific to host DNA, and preferably to human host DNA, as they allow the discrimination of host DNA from other DNA present in the biological sample (i.e. non host DNA), such as fungal and/or bacterial DNA (i.e. microbial DNA). In other words, the nucleic acid fragments of the invention will hydridize to host DNA, but not (or essentially not) bind to a substantial part of the other DNA present in the biological sample (i.e. non host DNA), such as fungal and/or bacterial DNA (i.e. microbial DNA).

In the context of the present invention, the nucleic acid fragment will preferably hybridize to the host DNA under stringent hybridization conditions. One example of stringent hybridization conditions is where attempted hybridization is carried out at a temperature from about 50°C to about 65°C, more preferably from about 55°C to about 65°C, using a salt solution which can be e.g. about 0.9 molar. However, the skilled person will be able to vary such conditions in order to take into account variables such as the nucleic acid fragment length, base composition, type of ions present, etc

A "primer" more specifically refers to a nucleic acid fragment that serves as a starting point for amplification of a nucleic acid of interest, i.e. herein of host DNA. Examples of nucleic primers of the invention include, but are not limited to, the primers of sequence SEQ ID NO:1, SEQ ID NO:2, SEQ ID NO:4 and SEQ ID NO:5. Such primers can be used in "a primer set" to amplify host DNA. Examples of primer set of the invention include, but are not limited to, the primer sets (SEQ ID NO:1, SEQ ID NO:2), and (SEQ ID NO:4, SEQ ID NO:5).

A "probe" more specifically refers to a nucleic acid fragment that can be used for detection of a nucleic acid of interest, i.e. herein of host DNA. This term encompasses various derivative forms such "fluorescent probe". When used in combination with a primer set as defined above, said probe can be used for quantification of a nucleic acid of interest. Examples of probes of the invention include, but are not limited to, the probes of sequence SEQ ID NO:3, and SEQ ID NO:6. Probes may be labelled by isotopes, radiolabels, binding moieties such as biotin, haptens such as digoxygenin, luminogenic, mass tags, phosphorescent or fluorescent moieties, or by fluorescent dyes alone (e.g., MGB, FAM, VIC, TET, NED, TAMRA, JOE, HEX, ROX, etc) or in combination with other dyes. These labels provide signals detectable by fluorescence, radioactivity, colorimetry, gravimetry, X-ray diffraction or absorption, magnetism, enzymatic activity, mass spectrometry, binding affinity and the like, and facilitate the detection or quantification of the nucleic acid of interest.

In a preferred embodiment of the invention, host DNA abundance is measured by quantitative PCR (qPCR) by using at least one nucleic acid fragment selected from the group of nucleic acid fragments of sequence SEQ ID NO:1 to SEQ ID NO:6, variants thereof and complementary sequences thereof.

More preferably, host DNA abundance is measured by quantitative PCR (qPCR) by using the primer set (SEQ ID NO:1, SEQ ID NO:2) combined with the probe of

sequence SEQ ID NO:3, and/or by using the primer set (SEQ ID NO:4, SEQ ID NO:5) combined with the probe of sequence SEQ ID NO:6.

The term "complementary" means that, for example, each nucleotide of a first nucleic acid sequence is paired with the complementary base of a second nucleic acid sequence whose orientation is reversed. Complementary nucleotides are A and T (or A and U) or C and G.

"Variants" of a nucleic acid fragment according to the present invention include, but are not limited to, nucleic acid sequences which are at least 99% identical after alignment to said nucleic acid fragment and retain their capacity to hybridize to a nucleic acid of interest, herein to host DNA. Examples of variants are degenerate nucleic acid fragments.

The methods of the invention can be applied to any subject, either human or animal. Yet, in a preferred embodiment, it is applied to a human patient, in particular to a human that is suspected of suffering from CD, or is in need of a confirmation of having CD. More precisely, the method of the invention is useful for monitoring human patients showing enhanced level of inflammation markers such as platelet count, mean platelet volume, erythrocyte sedimentation rate (ESR), serum thrombopoietin, serum erythropoietin, C-reactive protein and orosomucoid (α_1-acid glycoprotein), TNFα, Interleukins (notably IL1, IL2, IL6, IL8, IL10, IL15) as well as fecal markers of inflammation such as lactoferrin and calprotectin. Precise methods for diagnosing CD are detailed in Laas et al, 2014, which is incorporated herein by reference. More preferably, the subject is not suffering from at least one of the following pathologies: cancer or precancer, more particularly colon cancer, colorectal cancer or colorectal adenoma, ulcerative colitis, microscopic colitis (such as collagenous colitis or lymphocytic colitis), ischaemic colitis, diversion colitis, allergic colitis, Behçet's disease, colorectal polyps, celiac disease, irritable bowel syndrome (IBS), and any combination thereof.

In a preferred embodiment, the biological sample used in step a) of the method invention is a stool sample. Indeed, such a sample may be obtained by a completely harmless collection from the patient. Preferably, said stool sample is collected and stored in appropriate buffers that do not denature or affect the DNA contained in same (in this aim, one can use, e.g., the RNA later® RNA stabilization Reagent (Ambion), or the Stool DNA Stabilizer (Invitek), or a mix of EDTA and DMSO). More preferably, the samples are stored at -80°C until DNA extraction and subsequent analysis.

As used herein, the term "reference value" refers to a specific value or dataset that can be used to identify samples that are known to be poor in host DNA. This reference value is obtained for example from the historical abundance data obtained for healthy subjects. It can be a single cut-off value, such as a median or mean. It can be a single number, equally applicable to every sample. In a preferred embodiment, this reference value is a predetermined value. Typically, this predetermined value is of about 1%.

In principle, stool samples of healthy subjects are devoid of host DNA. Therefore, the presence of host DNA in the stool samples of a subject is a hint that said subject may suffer from a gut related disease. The present invention also encompasses all methods aimed at diagnosing CD in a subject, involving the detection of the presence of host DNA in stool samples. In other words, any diagnostic method involving the use of host DNA as biomarker of CD is encompassed within the present invention.

In the context of the invention, it is meant that the relative abundance of host DNA for the tested subject is “higher than a reference value” if it is superior, preferably 10 folds, and more preferably 20 folds superior to said reference value. In a preferred embodiment, it can be concluded that the tested subject is suffering from CD if the relative abundance of host DNA, as defined above, is higher than 1%, preferably higher than 10%, more preferably higher than 20%.

In other terms, the amount of host DNA is compared with a reference value. Said comparison can be done by those skilled in the art using statistical methods, in particular a ROC curve can be used to determine an optimal cut-off for sensitivity and specificity.

In a particular embodiment, the method of the invention comprises: performing at least one assay to determine host DNA relative abundance in a stool sample from a subject having or suspected of having Crohn disease (CD), wherein the quantitative data represents host DNA relative abundance preferably compared to a reference value of about 1%.

In other words, the invention relates to an *in vitro* method for analysing a biological sample from a subject having or suspected of having Crohn disease (CD), said method comprising:

a) Obtaining a stool sample from said subject,

b) Determining the relative abundance of host DNA in said sample,

and,

c) Determining if said relative abundance is higher than a reference value of about 1%.

More precisely, in this particular embodiment, the method of the invention comprises the following steps:

a) Obtaining a stool sample from a human patient,

b) Determining the relative abundance of human DNA in said sample, by any conventional means disclosed above,

and,

c) Diagnosing that said patient suffers from Crohn disease, if said relative abundance is higher than about 1%.

Methods of measures, in particular to monitor the states of CD

In another aspect, the present invention relates to a method for generating quantitative data for a subject having or suspected of having Crohn disease (CD) in an unstable state, said method comprising: performing at least one assay to determine host DNA relative abundance in a biological sample from said subject, wherein the quantitative data represents host DNA relative abundance preferably compared to a reference value.

In other words, the invention relates to an *in vitro* method for analysing a biological sample from a subject having or suspected of having Crohn disease in an unstable state, said method comprising the steps of:

a) Obtaining a biological sample from said subject,

b) Determining the relative abundance of host DNA in said sample,

and,

c) Determining if said relative abundance is higher than a reference value.

In a preferred embodiment, said reference value is of about 10%.

The present invention further relates to a method for generating quantitative data for a subject having or suspected of having Crohn disease (CD) in a stable state, said method comprising: performing at least one assay to determine host DNA relative abundance in a biological sample from said subject, wherein the quantitative data represents host DNA relative abundance preferably compared to a first reference value and to a second reference value.

In a preferred embodiment, said first reference value is of about 10%, and said second reference value is of about 1%.

In other words, the invention relates to an *in vitro* method for analysing a biological sample from a subject having or suspected of having Crohn disease in a stable state, said method comprising the steps of:

a) Obtaining a biological sample from said subject,

b) Determining the relative abundance of host DNA in said sample,

and,

c) Determining if said relative abundance is lower than a first reference value and higher than a second reference value.

In a preferred embodiment, said first reference value is of about 10%, and said second reference value is of about 1%.

More particularly, the results obtained by the inventors allowed to identify a biomarker (host DNA) allowing to distinguish between patients suffering from an inactive (quiescent state) Crohn disease from patients suffering from aggressive Crohn disease (state associated with an imminent flare period), in particular in a non-invasive manner. Their results are consequently of peculiar value with regard to monitoring the stage of this disease.

In the context of the invention, "stable" patients are defined as CD patients for whom disease activity is stable over several weeks (patient in a "stable state"). While "unstable" patients (or patient "in an unstable state") are CD patients who had their treatment changed or intensified in the following weeks, whose blood tests showed/shows elevated activity in the following weeks, and/or whose self-evaluation showed/shows decreased health and/or had/have elevated calprotectin levels in consecutive samples, and/or who had/have systemic

mucosal inflammation, more particularly systemic mucosal ulcerations. Stable or unstable can also be defined based on colonoscopical scores such as CDEIS or SES-CD.

Accordingly, the present invention more particularly targets a method aiming at diagnosing these two particular states in a subject suffering from CD.

More precisely, the present invention relates to an *in vitro* method for diagnosing the activity of the Crohn Disease in a subject, said method comprising the steps of:

a) Obtaining a biological sample from said subject,

b) Determining the relative abundance of host DNA in said sample,

and,

c) Diagnosing that said subject has a Crohn Disease in an unstable state, if said relative abundance is higher than a reference value.

The above methods are advantageous over the prior art as they are non-invasive, economically acceptable, and present high specificity.

All the embodiments disclosed above, in particular for the diagnostic method of the invention, also applies to the present methods, notably aimed at monitoring the activity of the Crohn disease.

In particular, said subject can be a human patient that is suspected of suffering from CD, or is in need of a confirmation of having CD or has been diagnosed with CD. More precisely, the methods of the invention are useful for monitoring human patients showing enhanced level of inflammation markers such as platelet count, mean platelet volume, erythrocyte sedimentation rate (ESR), serum thrombopoietin, serum erythropoietin, C-reactive protein and orosomucoid (α_1-acid glycoprotein), TNFα, Interleukins (notably IL1, IL2, IL6, IL8, IL10, IL15) as

well as fecal markers of inflammation such as lactoferrin and calprotectin. Precise methods for diagnosing CD are detailed in Laas et al, 2014, which is incorporated herein by reference.

Also, the biological sample is preferably a stool sample, more preferably handled as described above.

In a preferred embodiment, the relative abundance of host DNA is determined as disclosed above.

The present inventors have found that these methods of the invention are highly sensitive and specific when the relative abundance of host DNA is determined and compared, directly or indirectly, to a reference value.

As used herein, the term "reference value" refers to a specific value or dataset that can be used to identify samples having a stable CD. This reference value is obtained for example from the historical abundance data obtained for a patient or a pool of patients having been diagnosed unambiguously for a stable CD. This reference value is for example obtained by measuring the relative abundance of the host DNA in stool samples from patients being in a stable state of CD. It can be a single cut-off value, such as a median or mean. It can be a single number, equally applicable to every sample. Said reference value may also be a predetermined value. Typically, this predetermined value is of about 10%.

In a preferred embodiment, it can be concluded that the tested patient is suffering from unstable CD if the relative abundance of host DNA, as defined above, is higher than 10%, preferably higher than 18%, more preferably higher than 20%.

In a particular embodiment, the method of the invention comprises: performing at least one assay to determine host DNA relative abundance in a stool sample from a subject having or suspected of having Crohn disease in an unstable state, wherein

the quantitative data represents host DNA relative abundance preferably compared to a reference value of about 10%.

In other words, the invention relates to an *in vitro* method for analysing a biological sample from a subject having or suspected of having Crohn disease in an unstable state, said method comprising the steps of:

a) Obtaining a stool sample from said subject,

b) Determining the relative abundance of host DNA in said sample,

and,

c) Determining if said relative abundance is higher than a reference value of about 10%.

More precisely, in this particular embodiment, the *in vitro* diagnostic method of the invention enables to diagnose an unstable state of the Crohn disease in a human patient, comprising the following steps:

a) measuring the relative abundance of human DNA in a stool sample of said patient by any of the above-mentioned methods,

and,

b) determining that said patient suffers from an unstable CD, if said relative abundance is higher than 10%.

Conversely, the present invention also allows the generation of quantitative data for a subject having or suspected of having Crohn disease (CD) in a stable state, or in other words the analysis of a biological sample from said subject, in particular for diagnosing a stable CD. In this case, it will be concluded that a subject suffers from an stable CD if the relative abundance of host DNA measured in a biological sample of said subject is higher than the reference value used for diagnosing CD

(typically 1%), but lower than the reference value used for diagnosing an unstable state of the disease (typically 10%).

The methods of the invention can include (or exclude) the steps consisting of obtaining the stool sample and extracting the nucleic acid molecule from said sample, as defined above.

In principle, stool samples of subjects being in a quiescent (inactive) CD have a relative abundance of host DNA comprised between 0 and 10% (depending on the measurement technology for example). Yet, the presence of an intermediate level of host DNA (typically between 1% and 10%) in the stool samples of a subject is a hint that said subject may suffer from CD and that said CD is in a quiescent state. Moreover, the presence of a high level of host DNA (typically superior to 10%) in the stool samples of a subject is a hint that said subject may suffer from CD and that said CD is in an active state. The present invention therefore encompasses all methods aimed at diagnosing the state of CD in a subject, involving the detection of the presence of host DNA in stool samples. In other words, any diagnostic method involving the use of host DNA as biomarker of CD state is encompassed within the present invention.

Methods of measures, in particular to design a treatment

In another embodiment, the diagnostic methods of the invention can be used for adapting a treatment for a subject suffering from the Crohn disease.

In this embodiment, the methods of the invention therefore comprise the additional step of designing a treatment for the diagnosed subject, said treatment being adapted to the particular state of CD which has been diagnosed (such as by the method of the invention).

Thus, according to this aspect, the invention relates to a method for treating a subject suffering from Crohn disease, comprising:

i) generating quantitative data for a subject having or suspected of having Crohn disease (CD) in an unstable or stable state, according to the above-mentioned method,

and

ii) treating said subject with an appropriate treatment, said appropriate treatment being chosen in those classically attributed by the practitioner.

In other words, the invention relates to a method for treating subject suffering from Crohn disease, comprising:

i) analysing a biological sample from a subject having or suspected of having Crohn disease in an unstable or stable state, according to the above-mentioned method,

and

ii) treating said subject with an appropriate treatment, said appropriate treatment being chosen in those classically attributed by the practitioner.

More preferably, the invention encompasses a method for treating a subject suffering from Crohn disease, said method comprising the following steps:

i) diagnosing the activity of CD in a subject according to the above-mentioned method,

and

ii) treating said subject with an appropriate treatment, said appropriate treatment being chosen in those classically attributed by the practitioner once said state of CD is diagnosed.

For example, if a CD patient is diagnosed in an unstable state, an adapted treatment can be a pharmacological treatment chosen in the group consisting of: azathioprine, mesalamine, abatacept, adalimumab, anakinra, certolizumab, etanercept, golimumab, infliximab, rituximab, tocilizumab, natalizumab, corticosteroids, cyclosporine, methotrexate, tacrolimus, Anti-JAK (tofacitinib), anti-integrins (Vedolizumab, rhuMAb Beta7, MAdCAM-1 Antagonist), or Anti IL12/IL23 (Ustekinumab, ABT874).

Alternatively, if a Crohn patient is diagnosed in a stable state, an adapted treatment will be lifestyle interventions, for example diets of different caloric restriction intensities and macronutrient composition (low carbohydrate, low fat, saturated fat diets).

Moreover, it is possible to use the methods of the invention for testing the efficiency of a treatment in a subject suffering from CD, in particular CD in an unstable state, or to evaluate the response of a patient to a treatment.

In this embodiment, the method of the invention comprises the following steps:

i) generating quantitative data for a subject having or suspected of having Crohn disease (CD) in an unstable state, according to the above-mentioned method, before and after the administration of a treatment,

and

ii) concluding that the treatment is efficient in said subject if the state before the administration of the treatment was unstable but becomes stable upon administration of the treatment.

In other words, the method of the invention comprises:

i) analysing a biological sample from a subject having or suspected of having Crohn disease in an unstable state, according to the above-mentioned method, before and after the administration of a treatment,

and

ii) concluding that the treatment is efficient in said subject if the state before the administration of the treatment was unstable but becomes stable upon administration of the treatment.

More precisely, the method of the invention comprises:

i) diagnosing the activity of CD before and after the administration of a treatment, according to the above-mentioned method,

and

ii) concluding that the treatment is efficient in said subject if the state before the administration of the treatment was unstable but becomes stable upon administration of the treatment.

If the Crohn patient is diagnosed to be "unstable" before the administration of the treatment and becomes "stable" upon administration of the treatment, then said patient is responding to said treatment. This efficient treatment should therefore be preferentially maintained.

Conversely, if the Crohn patient is diagnosed to be "unstable" before the administration of the treatment and remains "unstable" upon administration of the treatment, then said patient is not responding to said treatment, and it is better to replace said treatment with another one or to combine it with another treatment.

Of note, if the Crohn patient is diagnosed to be "stable" before the administration of the treatment, then it is not worth administering any chemical treatment, as lifestyle interventions could be sufficient.

Combined methods of measures, in particular for diagnosis

The present inventors furthermore propose to associate the measure of host DNA abundance with the measure of another biomarker commonly used to diagnose CD, and/or the state of CD (i.e., active vs quiescent state).

In a particular embodiment, the present invention is therefore drawn to a method for generating quantitative data for a subject having or suspected of having Crohn disease (CD), said method comprising:

a) performing at least one assay to determine host DNA relative abundance in a biological sample from said subject, wherein a first quantitative data represents host DNA relative abundance preferably compared to a reference value; and

b) performing at least one assay to determine calprotectin level, or a combined clinical score, in another biological sample from said subject, wherein a second quantitative data represents said calprotectin level preferably compared to 150 µg/mL or said combined clinical score preferably compared to a predetermined score.

In other words, the invention relates to a method for analysing a biological sample from a subject having or suspected of having Crohn disease (CD), said method comprising:

a) Obtaining a biological sample from said subject,

b) Determining the relative abundance of host DNA in said sample,

c) Determining the calprotectin level, or a combined clinical score, in another biological sample from said subject,

and,

d) Determining if said relative abundance is higher than a reference value, and if said calprotectin level is greater than 150 µg/mL or if said combined clinical score is higher than a predetermined score.

More precisely, the invention relates to a method for diagnosing Crohn disease (CD) in a subject comprising:

a) Obtaining a biological sample from said subject,

b) Determining the relative abundance of host DNA in said sample,

c) Determining the calprotectin level or a combined clinical score in another biological sample from said subject,

and,

d) Diagnosing that said subject suffers from Crohn Disease, if said relative abundance is higher than a reference value, and if said calprotectin level is greater than 150 µg/mL or if said combined clinical score is higher than a predetermined score.

In a preferred embodiment, said reference value is of about 1%.

The skilled practitioner in the art would readily understand that the calprotectin level indicated in µg/mL (or in µg/g) refers to the calprotectin protein level, or in other words to the calprotectin protein expression level. Protein expression level can be assessed by any method well-known in the art, notably reviewed by Reeves et al. (2000) and Schena (2005). Those methods generally involve contacting a biological sample of interest with one or more detectable reagents that is or are

suitable for measuring protein expression level, such as an antibody, and subsequently determining protein expression level based on the level of detected reagent, preferably after normalization. Examples of methods which generally involve the use of an antibody include, without limitation, Western blot, immunoblot, enzyme-linked immunosorbant assay (ELISA), enzyme-linked immunospot (ELISPOT), radioimmunoassay (RIA), immunohistochemistry and immunoprecipitation. Other methods suitable for measuring a protein expression level, which do not necessarily involve the use of an antibody, may be used, including, without limitation, fluorescence activated cell sorting (FACS), microscopy such as atomic force microscopy, flow cytometry, microcytometry, protein binding assay, ligand binding assay, microarray, polyacrylamide gel electrophoresis such as SDS-PAGE, surface plasmon resonance (SPR), Förster resonance energy transfer (FRET), Bioluminescence resonance energy transfer (BRET), chemiluminescence, fluorescent polarization, phosphorescence, mass spectrometry such as liquid chromatography mass spectrometry (LC-MS) or liquid chromatography/ mass spectrometry/ mass spectrometry (LC-MS-MS), matrix-assisted laser desorption/ionization time-of-flight (MALDI-TOF), surface-enhanced laser desorption/ionization time-of-flight (SELDI-TOF), and magnetic resonance imaging (MRI).

In another preferred embodiment, host DNA relative abundance and calprotectin level can be measured from the same biological sample of the subject.

Accordingly, the present invention further relates to a method for generating quantitative data for a subject having or suspected of having Crohn disease (CD), said method comprising:

a) performing at least one assay to determine host DNA relative abundance in a biological sample from said subject, wherein a first quantitative data represents host DNA relative abundance preferably compared to a reference value; and

b) performing at least one assay to determine calprotectin level, or a combined clinical score, in said sample, wherein a second quantitative data represents said calprotectin level preferably compared to 150 µg/mL or said combined clinical score preferably compared to a predetermined score.

In other words, the invention relates to a method for analysing a biological sample from a subject having or suspected of having Crohn disease (CD), said method comprising:

a) Obtaining a biological sample from said subject,

b) Determining the relative abundance of host DNA in said sample,

c) Determining the calprotectin level, or a combined clinical score, in said sample,

and,

d) Determining if said relative abundance is higher than a reference value, and if said calprotectin level is greater than 150 µg/mL or if said combined clinical score is higher than a predetermined score.

More precisely, the invention relates to a method for diagnosing Crohn disease (CD) in a subject comprising:

a) Obtaining a biological sample from said subject,

b) Determining the relative abundance of host DNA in said sample,

c) Determining the calprotectin level, or a combined clinical score, in said sample,

and,

d) Diagnosing that said subject suffers from Crohn Disease, if said relative abundance is higher than a reference value, and if said calprotectin level is greater

than 150 µg/mL or if said combined clinical score is higher than a predetermined score.

In a preferred embodiment, said reference value is of about 1%.

In a preferred embodiment, calprotectin is measured in stool samples of the tested subject. In a more preferred embodiment, host DNA and calprotectin detection as described above are performed from the same stool sample. This may nevertheless require conducting two separate types of detection, one for measuring host DNA relative abundance (e.g. by qPCR), and one for measuring calprotectin protein level (e.g. by ELISA).

Thus, in another aspect, the present invention proposes to measure the gene level of calprotectin, so that a single type of detection can be performed in the method of the invention. More preferably, such detection is performed in the same container, and even more preferably from the same biological sample, such as a stool sample.

In this regard, the inventors have herein surprisingly discovered that calprotectin protein and gene levels correlate with one another, even though the behaviour of these types of functional entities (i.e. gene and protein encoded by said gene) cannot be predicted from each other. Indeed, it is well-known in the art that, once transcribed, a protein expression level may still be regulated at the translation level, and that the corresponding protein can be subjected to posttranslational modifications, varying half-lives, and compartmentalization.

Thus, according to this aspect, the invention relates to a method for generating quantitative data for a subject having or suspected of having Crohn disease (CD), said method comprising:

a) performing at least one assay to determine host DNA relative abundance in a biological sample from said subject, wherein a first quantitative data

represents host DNA relative abundance preferably compared to a first reference value; and

b) performing at least one assay to determine calprotectin gene level, or a combined clinical score, in said sample (preferred herein) or in another biological sample from said subject, wherein a second quantitative data represents said calprotectin gene level preferably compared to a second reference value or said combined clinical score preferably compared to a predetermined score.

In other words, the invention relates to a method for analysing a biological sample from a subject having or suspected of having Crohn disease (CD), said method comprising:

a) Obtaining a biological sample from said subject,

b) Determining the relative abundance of host DNA in said sample,

c) Determining the calprotectin gene level, or a combined clinical score, in said sample (preferred herein), or in another biological sample from said subject,

and,

d) Determining if said relative abundance is higher than a first reference value, and if the calprotectin gene level is higher than a second reference value or if said combined clinical score is higher than a predetermined score.

More precisely, the invention relates to a method for diagnosing Crohn disease (CD) in a subject comprising:

a) Obtaining a biological sample from said subject,

b) Determining the relative abundance of host DNA in said sample,

c) Determining the calprotectin gene level, or a combined clinical score, in said sample (preferred herein) or in another biological sample from said subject,

and,

d) Diagnosing that said subject suffers from Crohn Disease, if said relative abundance is higher than a first reference value, and if said calprotectin level is higher than a second reference value or if said combined clinical score is higher than a predetermined score.

In a preferred embodiment, said first reference value with regard to host DNA is as defined above, and more preferably is of about 1%.

Gene level, or gene expression level, can be measured by any method well-known in the art, such as the ones described above for measuring host and microbial DNA. Genes encoding calprotectin are well-known in the art. In particular, human calprotectin is known to form a heterodimer made of the S100 calcium binding protein A8 (S100A8, also known as calgranulin A) and the S100 calcium binding protein A9 (S100A9, also known as calgranulin B or migration inhibitory factor-related protein 14 (MRP14)). The nucleotide sequence of the human S100A8 gene is available under the Genbank accession number: CR407674, version number: CR407674.1, while the one of the human S100A9 gene is available under the NCBI Reference Sequence accession number: NM_002965, version number: NM_002965.3.

In a preferred embodiment, the second reference value is a specific value or dataset that can be used to identify samples that are known to belong to healthy subjects (i.e. not having Crohn disease). Said reference value can therefore be easily determined by the skilled practitioner. It can be a single cut-off value, such as a median or mean. It can be a single number, equally applicable to every sample. In a preferred embodiment, this reference is a predetermined value. By "higher

than a second reference value", it is thus meant herein that calprotectin gene level is superior than said reference value.

A particularly preferred technique for measuring host DNA relative abundance and/or calprotectin gene level is qPCR, using for example nucleic acid fragments (such as primers and/or probes) that are specific to the gene(s) encoding calprotectin.

By "combined clinical score", it is herein meant any score that combines biological parameters with clinical parameters to produce a score related to disease severity or mucosal healing in CD. It can be for example a combination of calprotectin levels (that are typically higher than 150µg/mL in CD suffering patients), HBI (that is typically higher than 4 in CD suffering patients), gender, age, disease duration, platelet count, albumin, platelet, CRP, rectal bleeding (Abstract OP05, 7th congress of ECCO).

By "predetermined score", it is herein meant the value resulting from the combination of these multiple parameters through any statistical or algorithmic method. It is for example 150µg/mL for Calprotectin and 4 for HBI.

In a further aspect, the present invention relates to a method for generating quantitative data for a subject having or suspected of having Crohn disease in an unstable state, said method comprising:

a) performing at least one assay to determine host DNA relative abundance in a biological sample from said subject, wherein a first quantitative data represents host DNA relative abundance preferably compared to a reference value; and

b) performing at least one assay to determine calprotectin level, or a combined clinical score, in another biological sample from said subject, wherein a second quantitative data represents said calprotectin level preferably compared to

150 µg/mL or 250 µg/g , or said combined clinical score preferably compared to a predetermined score.

In other words, the invention relates to an *in vitro* method for analysing a biological sample from a subject having or suspected of having Crohn disease in an unstable state, said method comprising the steps of:

a) Obtaining a biological sample from said subject,

b) Determining the relative abundance of host DNA in said sample,

c) Determining the calprotectin level or a combined clinical score in another biological sample from said subject,

and,

d) Determining if said relative abundance is greater than a reference value, and if said calprotectin level is greater than 150 µg/mL or 250 µg/g or if said combined clinical score is higher than a predetermined score.

More precisely, the present invention relates to an *in vitro* method for diagnosing the activity of the Crohn Disease in a subject, comprising the steps of:

a) Obtaining a biological sample from said subject,

b) Determining the relative abundance of host DNA in said sample,

c) Determining the calprotectin level or a combined clinical score in another biological sample from said subject,

and,

d) Diagnosing that said subject has a Crohn disease in an unstable state, if said relative abundance is greater than a reference value, and if said calprotectin level

is greater than 150 µg/mL or 250 µg/g or if said combined clinical score is higher than a predetermined score.

In a preferred embodiment, said reference value is of about 10%.

In another preferred embodiment, host DNA relative abundance and calprotectin level can be measured from the same biological sample of the subject.

Accordingly, the present invention further relates to a method for generating quantitative data for a subject having or suspected of having Crohn disease in an unstable state, said method comprising:

a) performing at least one assay to determine host DNA relative abundance in a biological sample from said subject, wherein a first quantitative data represents host DNA relative abundance preferably compared to a reference value; and

b) performing at least one assay to determine calprotectin level, or a combined clinical score, in said sample, wherein a second quantitative data represents said calprotectin level preferably compared to 150 µg/mL or 250 µg/g, or said combined clinical score preferably compared to a predetermined score.

In other words, the invention relates to a method for analysing a biological sample from a subject having or suspected of having Crohn disease in an unstable state, said method comprising the steps of:

a) Obtaining a biological sample from said subject,

b) Determining the relative abundance of host DNA in said sample,

c) Determining the calprotectin level or a combined clinical score, in said sample,

and,

d) Determining if said relative abundance is greater than a reference value, and if said calprotectin level is greater than 150 µg/mL or 250 µg/g, or if said combined clinical score is higher than a predetermined score.

More precisely, the present invention relates to an *in vitro* method for diagnosing the activity of the Crohn Disease in a subject, comprising the steps of:

a) Obtaining a biological sample from said subject,

b) Determining the relative abundance of host DNA in said sample,

c) Determining the calprotectin level or a combined clinical score, in said sample,

and,

d) Diagnosing that said subject has a Crohn disease in an unstable state, if said relative abundance is greater than a reference value, and if said calprotectin level is greater than 150 µg/mL or 250 µg/g, or if said combined clinical score is higher than a predetermined score.

In a preferred embodiment, said reference value is of about 10%.

In a preferred embodiment, calprotectin is measured in stool samples of the tested subject. In a more preferred embodiment, host DNA and calprotectin detection as described above are performed from the same stool sample. This may nevertheless require conducting two separate types of detection, one for measuring host DNA relative abundance (e.g. by qPCR), and one for measuring calprotectin protein level (e.g. by ELISA).

Thus, in another aspect, the present invention proposes to measure the gene level of calprotectin, so that a single type of detection can be performed in the method of the invention. More preferably, such detection is performed in the same container, and even more preferably from the same biological sample, such as a stool sample.

Thus, according to this aspect, the invention relates to a method for generating quantitative data for a subject having or suspected of having Crohn disease in an unstable state, said method comprising:

a) performing at least one assay to determine host DNA relative abundance in a biological sample from said subject, wherein a first quantitative data represents host DNA relative abundance preferably compared to a first reference value; and

b) performing at least one assay to determine calprotectin gene level, or a combined clinical score, in said sample (preferred herein) or in another biological sample from said subject, wherein a second quantitative data represents said calprotectin gene level preferably compared to a second reference value, or said combined clinical score preferably compared to a predetermined score.

In other words, the invention relates to a method for analysing a biological sample from a subject having or suspected of having Crohn disease (CD) in an unstable state, said method comprising:

a) Obtaining a biological sample from said subject,

b) Determining the relative abundance of host DNA in said sample,

c) Determining the calprotectin gene level, or a combined clinical score, in said sample (preferred herein) or in another biological sample from said subject,

and,

d) Determining if said relative abundance is higher than a first reference value, and if the calprotectin gene level is higher than a second reference value or if said combined clinical score is higher than a predetermined score.

More precisely, the invention relates to a method for diagnosing Crohn disease (CD) in an unstable state in a subject comprising:

a) Obtaining a biological sample from said subject,

b) Determining the relative abundance of host DNA in said sample,

c) Determining the calprotectin gene level, or a combined clinical score, in said sample (preferred herein) or in another biological sample from said subject,

and,

d) Diagnosing that said subject suffers from Crohn Disease in an unstable state, if said relative abundance is higher than a first reference value, and if said calprotectin gene level is higher than a second reference value or if said combined clinical score is higher than a predetermined score.

In a preferred embodiment, said first reference value with regard to host DNA is as defined above, and more preferably is of about 10%; and said second reference value with regard to calprotectin is preferably the calprotectin gene level observed in subjects having quiescent Crohn disease (i.e. having Crohn disease in a stable state).

By "combined clinical score", it is herein meant any score that combines biological parameters with clinical parameters to produce a score related to disease severity or mucosal healing in CD. It can be for example a combination of calprotectin levels, HBI, gender, age, disease duration, platelet count, albumin, platelet, CRP, rectal bleeding (Abstract OP05, 7th congress of ECCO).

By "predetermined score", it is herein meant the value resulting from the combination of these multiple parameters through any statistical or algorithmic method (see, e.g., the parameters and values mentioned in Abstract OP05, 7th congress of ECCO).

In a further aspect, the present invention relates to a method for generating quantitative data for a subject having or suspected of having Crohn disease in a stable state, said method comprising:

a) performing at least one assay to determine host DNA relative abundance in a biological sample from said subject, wherein a first quantitative data represents host DNA relative abundance preferably compared to a reference value; and

b) performing at least one assay to determine calprotectin level, or a combined clinical score, in another biological sample from said subject, wherein a second quantitative data represents said calprotectin level preferably compared to 150 µg/mL or 250 µg/g , or said combined clinical score preferably compared to a predetermined score.

In other words, the invention relates to an *in vitro* method for analysing a biological sample from a subject having or suspected of having Crohn disease in a stable state, said method comprising the steps of:

a) Obtaining a biological sample from said subject,

b) Determining the relative abundance of host DNA in said sample,

c) Determining the calprotectin level or a combined clinical score in another biological sample from said subject,

and,

d) Determining if said relative abundance is greater than a reference value, and if said calprotectin level is lower than 150 µg/mL or 250 µg/g, or if said combined clinical score is lower than a predetermined score.

More precisely, the invention relates to an *in vitro* method for diagnosing the activity of the Crohn Disease in a subject, comprising the steps of:

a) Obtaining a biological sample from said subject,

b) Determining the relative abundance of host DNA in said sample,

c) Determining the calprotectin level or a combined clinical score in another biological sample from said subject,

and,

d) Diagnosing that said subject has a Crohn disease in a stable state, if said relative abundance is greater than a reference value, and if said calprotectin level is lower than 150 µg/mL or 250 µg/g or if said combined clinical score is lower than a predetermined score.

In a preferred embodiment, said reference value is of about 1%.

In another preferred embodiment, host DNA relative abundance and calprotectin level can be measured from the same biological sample of the subject.

Accordingly, the present invention further relates to a method for generating quantitative data for a subject having or suspected of having Crohn disease in a stable state, said method comprising:

a) performing at least one assay to determine host DNA relative abundance in a biological sample from said subject, wherein a first quantitative data represents host DNA relative abundance preferably compared to a reference value; and

b) performing at least one assay to determine calprotectin level, or a combined clinical score, in said sample, wherein a second quantitative data represents said calprotectin level preferably compared to 150 µg/mL or 250 µg/g , or said combined clinical score preferably compared to a predetermined score.

In other words, the invention relates to a method for analysing a biological sample from a subject having or suspected of having Crohn disease (CD) in a stable state, said method comprising:

a) Obtaining a biological sample from said subject,

b) Determining the relative abundance of host DNA in said sample,

c) Determining the calprotectin level or a combined clinical score, in said sample,

and,

d) Determining if said relative abundance is greater than a reference value, and if said calprotectin level is lower than 150 µg/mL or 250 µg/g or if said combined clinical score is lower than a predetermined score.

More precisely, the invention relates to a method for diagnosing Crohn disease (CD) in a stable state in a subject comprising:

a) Obtaining a biological sample from said subject,

b) Determining the relative abundance of host DNA in said sample,

c) Determining the calprotectin level or a combined clinical score in said sample,

and,

d) Diagnosing that said subject has a Crohn disease in a stable state, if said relative abundance is greater than a reference value, and if said calprotectin level is lower than 150 µg/mL or 250 µg/g, or if said combined clinical score is lower than a predetermined score.

In a preferred embodiment, said reference value is of about 1%.

In a preferred embodiment, calprotectin is measured in stool samples of the tested subject. In a more preferred embodiment, host DNA and calprotectin detection as described above are performed from the same stool sample. This may nevertheless require conducting two separate types of detection, one for measuring host DNA relative abundance (e.g. by qPCR), and one for measuring calprotectin protein level (e.g. by ELISA).

Thus, in another aspect, the present invention proposes to measure the gene level of calprotectin, so that a single type of detection can be performed in the method of the invention. More preferably, such detection is performed in the same container, and even more preferably from the same biological sample, such as a stool sample.

Thus, according to this aspect, the invention relates to a method for generating quantitative data for a subject having or suspected of having Crohn disease in a stable state, said method comprising:

a) performing at least one assay to determine host DNA relative abundance in a biological sample from said subject, wherein a first quantitative data represents host DNA relative abundance preferably compared to a first reference value; and

b) performing at least one assay to determine calprotectin gene level, or a combined clinical score, in said sample (preferred herein) or in another biological sample from said subject, wherein a second quantitative data represents said calprotectin level preferably compared to a second reference value and/or to a third reference value, or said combined clinical score preferably compared to a predetermined score.

In other words, the invention relates to a method for analysing a biological sample from a subject having or suspected of having Crohn disease (CD) in a stable state, said method comprising:

a) Obtaining a biological sample from said subject,

b) Determining the relative abundance of host DNA in said sample,

c) Determining the calprotectin gene level, or a combined clinical score, in said sample (preferred herein), or in another biological sample from said subject,

and,

d) Determining if said relative abundance is higher than a first reference value, and if the calprotectin gene level is lower than a second reference value and/or higher than a third reference value or if said combined clinical score is lower than a predetermined score.

More precisely, the invention relates to a method for diagnosing Crohn disease (CD) in a stable state in a subject comprising:

a) Obtaining a biological sample from said subject,

b) Determining the relative abundance of host DNA in said sample,

c) Determining the calprotectin gene level, or a combined clinical score, in said sample (preferred herein), or in another biological sample from said subject,

and,

d) Diagnosing that said subject suffers from Crohn Disease in a stable state, if said relative abundance is higher than a first reference value, and if said calprotectin level is lower than a second reference value and/or higher than a third reference value or if said combined clinical score is lower than a predetermined score.

In a preferred embodiment, said first reference value with regard to host DNA is as defined above for a stable state (1%); said second reference value with regard to calprotectin is preferably the calprotectin gene level observed in subjects having active Crohn disease (i.e. having Crohn disease in an unstable state); and/or said

third reference value with regard to calprotectin is preferably the calprotectin gene level observed in healthy subjects (i.e. not having Crohn disease).

By "combined clinical score", it is herein meant any score that combines biological parameters with clinical parameters to produce a score related to disease severity or mucosal healing in CD. It can be for example a combination of calprotectin levels, HBI, gender, age, disease duration, platelet count, albumin, platelet, CRP, rectal bleeding (Abstract OP05, 7th congress of ECCO).

By "predetermined score", it is herein meant the value resulting from the combination of these multiple parameters through any statistical or algorithmic method (see, e.g., the parameters and values mentioned in Abstract OP05, 7th congress of ECCO).

These methods can be applied to any subject, either human or animal. Yet, in a preferred embodiment, they are applied to a human patient, in particular to a human that is suspected of suffering from CD, or is in need of a confirmation of having CD, or has been diagnosed for CD.

The biological sample used in the method of the invention is preferably a stool sample.

In a preferred embodiment, the relative DNA abundance is determined by using profiling methods based on hybridization analysis of polynucleotides, and/or sequencing of polynucleotides described above.

As indicated above, the calprotectin level is measured according to any method commonly known by the one of skill in the art. Preferably, calprotectin protein level can be expressed in µg/mL, or in µg/g.

These methods have significant advantages over the prior art, in particular compared with those involving the measure of calprotectin level in stool samples

alone. Indeed, as known from the one of skill in the art, such diagnostic methods are not sensitive enough, and give false positive results.

Moreover, it has been observed by the Inventors that the two measures (step b) and step c)) do not reflect a simple correlation: the percent of human DNA is significantly increased in the samples with calprotectin higher than 150 µg/mL reflecting the fact that there is more human DNA present in the stool of patients having signs of gut inflammation. Therefore, although the two measures relate they do not seem to capture exactly the same clinical characteristics of clinical disease.

A preferred technique for measuring host DNA relative abundance and calprotectin gene level is qPCR.

Kits for use in the methods of the invention

The methods described above may be performed, for example, by using prepackaged kits, comprising or consisting of the nucleic acid fragments of the invention.

The invention is thus directed to a kit for use in any method of the invention, said kit comprising, or consisting of:

a) at least one nucleic acid fragment hydridizing specifically with host DNA; and

b) instructions for performing said method.

As used herein, the term "instructions" refers to a publication, a recording, a diagram, or any other medium which can be used to communicate how to perform a method of the invention. Said instructions can, for example, be affixed to a

container which contains said kit. Preferably, the instructions for using said kit include a reference value.

According to a preferred embodiment, said nucleic acid fragment hydridizing specifically with host DNA is selected from the group of the nucleic acid fragments of sequence SEQ ID NO:1 to SEQ ID NO:6, variants thereof and complementary sequences thereof,

More preferably, said kit comprises, or consists of:

a) the primer set (SEQ ID NO:1, SEQ ID NO:2) and the probe of sequence SEQ ID NO:3; and/or the primer set (SEQ ID NO:4, SEQ ID NO:5) and the probe of sequence SEQ ID NO:6; and
b) instructions for performing said method.

Yet, even more preferably, the above kit can further comprise:

c) at least one reagent capable of specifically determining calprotectin protein or gene level.

The term "reagent capable of specifically determining calprotectin level" or "reagent capable of specifically determining calprotectin expression level" designates a reagent or a set of reagents which specifically recognizes calprotectin and allows for the quantification of the expression level thereof, at the protein or gene level. These reagents can be for example antibodies, aptamers or affibodies specifically recognizing the protein calprotectin, or nucleic acid fragments such as primers and/or probes recognizing the gene(s) encoding calprotectin. In the context of the present invention, such reagent is said to be "specific" for calprotectin or "recognizes specifically" calprotectin if it 1) exhibits a threshold level of binding and/or hybridizing activity, and/or 2) does not significantly cross-react with target molecules known to be related to calprotectin. The binding affinity of such reagent can be easily determined by one skilled in the art, for example, by Scatchard analysis. Cross-reactivity of a reagent can as well be easily

determined by one skilled in the art, and thus need to be further detailed herein. Examples of reagents capable of specifically determining the expression level of calprotectin include, without limitation, anti-calprotectin antibodies (such as the MAC387 IgG1 from Invitrogen) and nucleic acid fragments hydridizing specifically with gene(s) encoding calprotectin, such as the S100A8 and/or S100A9 genes as described above.

The invention further relates to the use (in particular *in vitro* use) of the kit as described above, in any method of the invention.

Examples

EXAMPLE 1

1. *Material and Methods*

1.1. Cohort

1.1.1 CD cohort

All participants were part of the "CrohnOmeter 1" study, which aim was to collect stool samples from a diverse population of Crohn Disease patients to investigate their gut microbiome. The inclusion criteria into the study were a clinical diagnosis of Crohn Disease and the participants signed an informed consent form. CrohnOmeter 1 is a longitudinal study, on average each participant provided 8 stool samples over an 8 months period of time. A total of 99 participants were included and provided stool samples. Out of the 99 participants, 68 had their stool samples sequenced. In total 438 samples were sequenced.

Each study participant filled in a questionnaire each time a stool sample was provided into the study. The questionnaire captured information on the patient's health and stool characteristics. In particular the following information was used to evaluate the state of disease activity/inflammatory status:

- The calprotectin level (dosed in patient stools) was measured (calprotectin is a protein marker highlighting inflammation);

- The Harvey-Bradshaw index (HBI) of each patient is recorded. HBI is a composite auto-evaluated index reflective of the general health status of the patient. The score is based on an evaluation of general well-being, an evaluation of abdominal pain, the number of liquid stools per day, the presence of abdominal mass and the presence of complications. It is widely spread for the evaluation of Crohn patient status.

1.1.2 Healthy control cohort

A control group of individuals was assembled from healthy individuals. Main exclusion criteria were the use of prescription medication and history of significant disease. Multiple samples were collected from the same individuals leading to a total of 137 samples.

1.1.3 NASH control cohort

An additional two NASH patients from a larger study were sequenced. The aim of the larger NASH study, called NASH2, was to collect stool samples from a diverse population of NASH patients to investigate their gut microbiome and identify differences between NASH and simple steatosis patients. 6 samples were available from those 2 patients. This cohort although small, was selected as a control population for an inflammatory disease not localized in the gut.

1.2. Sample collection and preparation

1.2.1. Fecal sampling

The subjects from the CD cohort were provided dedicated collection kits containing a DNA stabilizer and written instructions every three weeks for the collection of a stool sample from their home. Upon collection of two, approx. 1 gram aliquots in a validated DNA preservation buffer (typically RNA later®), the tubes containing the samples were shipped by regular post to the laboratory. One tube was directly stored at -80°C as a stool suspension backup. The second tube was used for DNA extraction: three aliquots were prepared from the stool material using high speed centrifugation. These three aliquots were then stored at -80°C before DNA extraction.

The same collection kit was used for the control cohorts.

1.2.2. DNA extraction

A frozen aliquot of each fecal sample was suspended in 250µL of guanidine thiocyanate 0.1 M Tris (pH 7.5) and 40 µL of 10% N-lauroyl sarcosine. The suspension was then submitted to vigorous bead-beating to release DNA from microbial cells and DNA extraction was conducted using standard protocol (Godon et al, 1997). The DNA integrity and concentration were evaluated by Nanodrop and Agilent and on agarose gel electrophoresis.

1.3. Illumina sequencing

The sequencing was performed at an ISO17025 accredited laboratory on a HiSeq 2500 Illumina sequencer. They used ISO 17025-accredited method HSHOv4 PE100. DNA library preparation followed the manufacturer's instruction (Illumina). The workflow indicated by the sequencing device provider was used to perform the different steps: cluster generation, template hybridization, isothermal amplification, linearization, blocking and denaturing and hybridization of the sequencing primers. The base-calling was performed using the provider's pipeline. The target of 40 million minimum paired-end reads were generated for each sample. Sequencing read length was 100bp.

1.4 Bioinformatics processing

The raw reads were processed using Enterome's in house pipeline. Briefly the pipeline is based on MOCAT (Kultima et al. , 2012) and a compilation of internal scripts. It consists of quality controls, mapping and calculation of gene abundance using MOCAT v1.3, including list of Illumina adapters and human genome (hg19). The number of reads mapping to the human genome is based on 95% identity on 90% of the length and are returned after the quality controls steps that includes trimming bases with a low quality score. The percent of human reads in a sample is

calculated using 1- number of reads mapping to hg19/number of reads after trimming.

2. *Results*

2.1. Comparison between controls versus Crohn Disease patients (CD)

Using the Wilcoxon rank sum test, we compared the 137 samples from healthy controls, 6 from NASH patients to the 438 samples from CD patients. The p-value was highly significant for CD versus healthy controls (p-value = 1.667e-12), Figure 1. Summary statistics for the two cohorts are provided in Table 1.

Cohort	Min.	1stQu.	Median	Mean	3rdQu.	Max.
CD	0.0000282	0.0003732	0.0009794	0.0318600	0.0036770	0.8986000
Healthy	1.278e-05	1.822e-04	3.998e-04	8.975e-04	7.883e-04	1.012e-02
NASH	0.0007524	0.0009039	0.0011470	0.0021420	0.0014250	0.0074630

Table 1: Summary statistics for percent of reads mapping to the Human genome HG19.

99% of samples from healthy controls had all less than 1% human DNA in their total stool DNA, compared to 84% of Crohn samples. Thus with a cutoff value of 1%, 16% of Crohn samples could be captured. The presence of DNA in the stool is thus highly specific.

2.2. Association to disease severity in Crohn Disease patients (CD)

Since there is a highly significant difference between Crohn Disease patients and healthy controls in terms of percent of human DNA in the stool sample, the relation

to disease severity was studied, to assess whether the measure of host DNA relative abundance could be used as a biomarker of disease activity.

To that end, patients were classified into disease active groups according to three criteria: 1) whether they had a calprotectin level above 150,

2) whether they had an HBI score above 4,

3) whether they had an HBI score above 4 or their calprotectin level above 150 (combined score)

The difference, based on the Wilcoxon rank sum test, between percent (relative abundance) of human DNA in stool samples from patients in a quiescent (n=227) versus in stool samples of patients in an active phase of CD (n=211) was highly significant (see figure 2,P-val=1.034e-09).

95.5% of the samples with more than 20% human DNA were from active patients.

90% of the samples with more than 10% human DNA were from active patients.

80% of the samples with more than 1% human DNA were from active patients.

The ROC curve (Figure 4) is a visual representation, indicating the number of true and false positives based on various cut offs. As can be seen in the bottom left corner, there is a very high specificity (100%, but a low sensitivity, only about 10% of patients are captured). The straight line represents a “non informative” score.

Looking at calprotectin levels on its own and comparing it to human DNA (Figure 3), the two measures do not reflect a simple correlation however, the percent of human DNA is significantly increased in the samples with calprotectin higher than 150 µg/ml (P-value=1.041e-07) reflecting the fact that there is more Human DNA present in the stool of patients having signs of gut inflammation. Interestingly, although the two measures relate they do not seem to capture exactly the same

clinical characteristics of clinical disease since, as can be seen from the figure 3, some samples have very high calprotectin levels and no human DNA.

EXAMPLE 2

1. *Material and Methods*

1.1. Cohort

Participants were part of the "CrohnOmeter 1" study, as described above. In total 11 stool samples were sequenced. The cohort fulfilled the same criteria as the one of Example 1.

1.2. Sample collection and preparation

1.2.1. Fecal sampling and DNA extraction

Fecal sampling and DNA extraction were performed, according to a similar procedure as the one detailed in Example 1 above.

1.2.2. qPCR performed on host DNA

The eleven samples were analyzed with the ValidPrime® assay (TATAA Biosciences) and run in triplicates. ValidPrime is highly optimized and specific to a non-transcribed locus of genomic DNA that is present in exactly one copy per haploid normal genome.

The primers were run in a final concentration of 400 nM and probe had a final concentration of 200 nM.

A standard curve spanning 100 000 copies to 6.10 copies per reaction was run together with the samples, dilution factor between standards was 4x. Samples were normalized to a concentration of 4.84 ng/µl which was at least a 10x dilution.

Primers and probes	Nucleotide sequence from 5' to 3' (SEQ ID NO:)
VP5 forward	AACTTGGTGCGGAGGT (SEQ ID NO:1)
VP5 reverse	ATCGCTTCTGATGGACAC (SEQ ID NO:2)
VP5 probe	CCGCCAGACTGCAATCCATCAATGACA (SEQ ID NO:3)
VP9 forward	GCGGAAACACAAGGGAA (SEQ ID NO:4)
VP9 reverse	TTAGAGGCAAAAGCAAAGAA (SEQ ID NO:5)
VP9 probe	ACAGCTAATTAAAATTGCACAGTTCCT (SEQ ID NO:6)

Table 2. Primers and probes for qPCR quantification of host DNA

Data, Cq-Values, from CFX manager software (Bio-Rad) was generated using threshold method. Threshold was set to 230. Standard curves were obtained from CFX manager software (Bio-Rad). Sample concentrations were calculated in the CFX manager using the standard curves.

1.2.3. Statistical analysis of qPCR data

The percentage of human DNA estimated was calculated based on the number of reads mapping to the human genome divided by the total number of reads in the sample. This percentage was correlated to the quantification of human DNA using the ValidPrime assays.

The ability to predict the value of a variable (human DNA) using the values of another variable (qPCR assay) was typically determined from a linear regression analysis of the data, assuming there is a linear response between the two variables. The statistical analysis was performed in R.

2. *Results*

2.1. Quantification of host DNA abundance by qPCR specific primers

Figure 5 demonstrates a statistically significant correlation in the quantitative data generated between the qPCR assays performed using the VP5 and VP9 primers and probes (correlation = 0.964).

Figures 6 and 7 show a statistically significant correlation in the quantitative data generated by the qPCR assay performed using the VP5 (Figure 6) or the VP9 (Figure 7) primers and probes, and the percentage of human DNA measured by the method proposed in Example 1 (i.e. Illumina sequencing) (correlation = 0.909 for VP5 assay, and 0.927 for VP9 assay).

Accordingly, the measure of host DNA abundance by qPCR, using the VP5 and VP9 primers and probes described above in Table 2, can allow for the diagnosis of Crohn disease, and monitoring of the activity of said disease.

EXAMPLE 3

1. *Material and Methods*

1.1. Cohort

Participants were part of the "CrohnOmeter 1" study, as described above. In total 15 stool samples were sequenced. The cohort fulfilled the same criteria as the one of Example 1.

1.2. Sample collection and preparation

1.2.1. Fecal sampling and RNA extraction

Fecal sampling was performed, according to the procedure detailed in Example 1 above

The stool samples were then extracted with PowerMag® Microbiome RNA/DNA Isolation Kit (Cat No 27500-4-EP, MOBIO Laboratories, Inc) according to manufacturer's instructions, with a few modifications. Briefly, 650 µl lysis buffer and 100 µl phenol:chloroform:isoamyl alcohol were added directly to the stool samples. As much as possible was transferred to the glass bead plate. A homogenizer (Tissuelyser II, Qiagen) was run at 30 Hz for 2 x 5 min. After transferring the supernatant, an extra bead beating step was performed. The volumes added to the pellet were 300 µl lysis buffer and 45 µl phenol:chloroform:isoamyl alcohol. 220 µl of inhibitor removal solution was added to the pooled supernatant and 450 µl total sample volume was further processed with KingFisher Flex (Thermo Scientific).

1.2.2. RNA quality and normalization

The absorbance and purity of the 15 extracted RNA/DNA samples were analyzed on a spectrophotometer (DropSense96, Trinean nv). The quality of the RNA was measured in RIN-values on gel electrophoresis (BioAnalyzer, Agilent Technologies). The samples were normalized to approximately 66.67 ng/ µl based on the absorbance measurement.

1.2.3. cDNA synthesis

All samples were reverse transcribed into cDNA using TATAA GrandScript cDNA Synthesis kit #A103 (TATAA Biocenter AB). Prior to cDNA synthesis a DNase treatment was performed using Heat&Run gDNA removal Kit (Cat No 80200-50, ArticZymes) according to manufacturer's instructions. Maximum load of RNA was added to the reaction to be able to retrieve as high Cq-values as possible. The reagents were mixed. Reverse transcription was performed in 20 µl reaction volume on T100 (Bio-Rad Laboratories, Inc). The temperature program in table 3 was applied for the cDNA synthesis.

1.2.4. qPCR

The 15 samples were diluted 9X after reverse transcription. qPCR was performed with TATAA Probe® GrandMaster Mix #TA02 (TATAA Biocenter AB) and the reagents were mixed. All samples including genomic DNA and a negative control were run in triplicates in 10 µl reactions on CFX384 platform (Bio-Rad). The samples were run on using 2 genes of interest (See Table 3) , ValidPrime (for genomic DNA background correction) and B2M medium and short assays (for control of physical fragmentation, large delta Cq between those two assays will indicate degradation see Björkman, et al. (2016). The pipetting was performed by a pipetting robot (EpMotion 5070, Eppendorf, Germany). A 2-step temperature program was applied and detection was performed in the FAM channel.

qPCR was performed using primers and probes designed to amplify the S100A8 and S100A9 genes, which encode protein that form the heterodimer of calprotectin. Said primers and probes can be easily designed by the skilled practitioner based on the publicly available nucleotide sequence of these genes.

Gene Symbol	Protein encoded by said gene	Gene sequence
S100A8	S100 calcium binding protein A8	Genbank CR407674.1
S100A9	S100 calcium binding protein A9	NCBI RefSeq NM_002965.3

Table 3. Genes encoding the S100A8 and S100A9 proteins

Raw data was generated using threshold method on the CFX manager software (Bio-Rad). qPCR data was analyzed with GenEx software (MultiD Analyses AB)

using reference gene validation with geNorm and NormFinder to evaluate the most stably expressed genes.

1.2.5. Statistical analysis

For each gene, triplicate values were averaged. The Cq values along with the values normalized by house-keeping genes (delta Cq values), were compared to the log10 transformed ELISA values obtained for measuring calprotectin protein level, (see Example 1, value of 150 µg/mL). The spearman correlation was computed. Additionally a cutoff of 250µg/g was used to classify patients as inflamed or non inflammed (Dhaliwal et al., 2015) and a Wilcoxon rank test was performed on the Cq values for each gene to compare the two groups. The statistical analysis was performed in R.

2. *Results*

2.1. Quantification of calprotectin gene level by qPCR specific primers

S100A8 and S100A9 genes had Cq values for all 12 (S100A9) to 15 (S100A8) of the tested samples. Stati

Gene Symbol	Spearman Correlation	P-value
S100A8	-0.72	0.0025
S100A9	-0.59	0.039

Table 4. Statistical data

Figure 8 shows a statistically significant correlation between faecal mRNA levels in genes S100A8 and S100A9 and faecal calprotectin level (protein level).

Accordingly, the measure of calprotectin gene level by qPCR, and more particularly of the S100A8 and/or S100A9 gene level, can allow for the diagnosis of Crohn disease, and more particularly for the monitoring of the activity of said disease, when preferably combined with the measure of host DNA relative abundance.

This test can further be easily performed in combination with the qPCR test for measuring host DNA abundance described in Example 2, preferably in a single test tube.

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Claims

1. An *in vitro* method for diagnosing the activity of the Crohn disease (CD) in a subject suffering therefrom, said method comprising the steps of:

a) Obtaining a stool sample from said subject,

b) Determining the relative abundance of host DNA in said sample,

and,

c) Diagnosing that said subject has a Crohn disease in an unstable state, if said relative abundance is higher than a reference value.

2. An *in vitro* method for diagnosing the activity of the Crohn Disease in a subject, comprising the steps of:

a) Obtaining a stool sample from said subject,

b) Determining the relative abundance of host DNA in said sample,

c) Determining the calprotectin level, in said sample or in another biological sample from said subject,

and,

d) Diagnosing that said subject has a Crohn disease in an unstable state, if said relative abundance is greater than a reference value, and if said calprotectin level is greater than 150 µg/mL or 250 µg/g.

3. The method of claim 1 or 2, wherein said reference value is of about 10%.

4. An *in vitro* method for diagnosing the activity of the Crohn Disease in a subject, comprising the steps of:

a) Obtaining a stool sample from said subject,

b) Determining the relative abundance of host DNA in said sample,

c) Determining the calprotectin gene level, in said sample or in another biological sample from said subject,

and,

d) Diagnosing that said subject has a Crohn disease in an unstable state, if said relative abundance is greater than a first reference value, and if said calprotectin gene level is greater than a second reference value.

5. The method of claim 4, wherein said first reference value is of about 10%.

6. An *in vitro* method for testing the efficiency of a treatment in a subject suffering from CD, or to evaluate the response of a patient to a treatment, comprising the following steps:

i) diagnosing the activity of CD before and after the administration of a treatment, according to the method of any one of claims 1 to 5, preferably according to the method of claim 1,

and

ii) concluding that the treatment is efficient in said subject if the state before the administration of the treatment was unstable but becomes stable upon administration of the treatment.

7. An *in vitro* method for diagnosing Crohn Disease (CD) in a subject comprising:

a) Obtaining a stool sample from said subject,

b) Determining the relative abundance of host DNA in said sample,

and,

c) Diagnosing that said subject suffers from Crohn disease, if said relative abundance is higher than a reference value.

8. The method of claim 7, wherein said subject is suspected of suffering from the Crohn Disease.

9. The method of claim 7 or 8, wherein said reference value is of about 1%.

10. An *in vitro* method for diagnosing Crohn Disease (CD) in a subject comprising:

a) Obtaining a stool sample from said subject,

b) Determining the relative abundance of host DNA in said sample,

c) Determining the calprotectin level, in said sample or in another biological sample from said subject,

and,

d) Diagnosing that said subject suffers from Crohn Disease, if said relative abundance is higher than a reference value, and if said calprotectin level is greater than 150 μg/mL.

11. The method of claim 10, wherein said reference value is of about 1%.

12. An *in vitro* method for diagnosing Crohn Disease (CD) in a subject comprising:

a) Obtaining a stool sample from said subject,

b) Determining the relative abundance of host DNA in said sample,

c) Determining the calprotectin gene level, in said sample or in another biological sample from said subject,

and,

d) Diagnosing that said subject suffers from Crohn Disease, if said relative abundance is higher than a first reference value, and if said calprotectin gene level is higher than a second reference value.

13. The method of claim 12, wherein said reference value is of about 1%.

14. The method of any one of claims 1 to 13, wherein said relative abundance is the relative amount of host DNA as compared with the total amount of DNA present in said sample.

15. The method of any one of claims 1 to 14, wherein said subject is a human patient.

16. The method of any one of claims 1 to 15, wherein said abundance is measured by quantitative PCR.

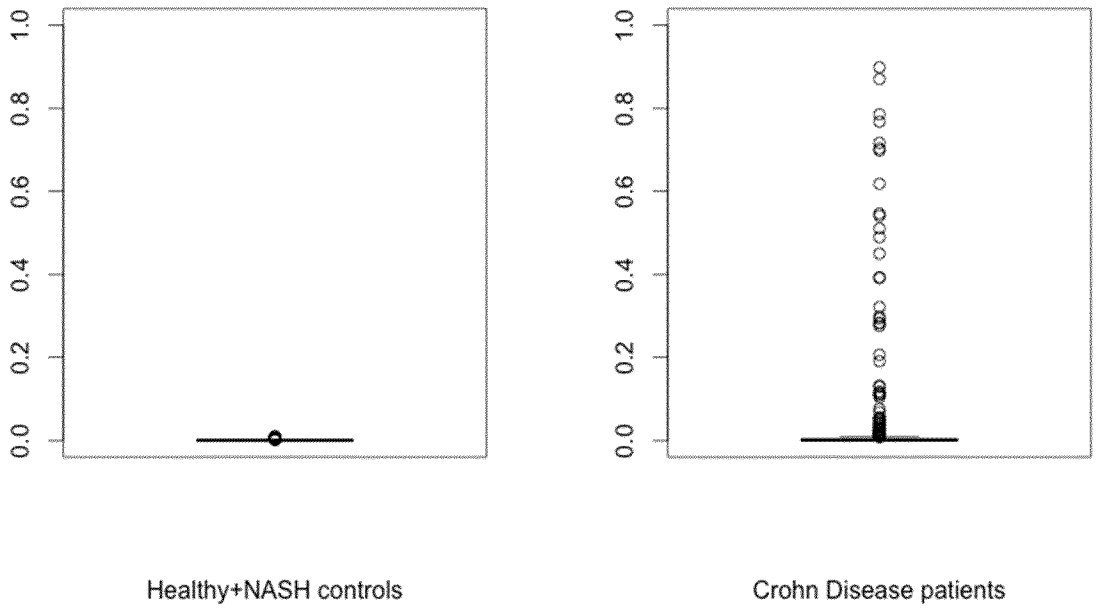
17. The method of claim 16, wherein said abundance is measured by using at least one nucleic acid fragment selected from the group of nucleic acid fragments of sequence SEQ ID NO:1 to SEQ ID NO:6, variants thereof and complementary sequences thereof.

18. A kit for use in any method of any one of claims 1 to 17, comprising:

a) at least one nucleic acid fragment hydridizing specifically with host DNA, said nucleic acid fragment being as defined in claim 17; and

b) instructions for performing said method.

Figure 1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Healthy+NASH controls
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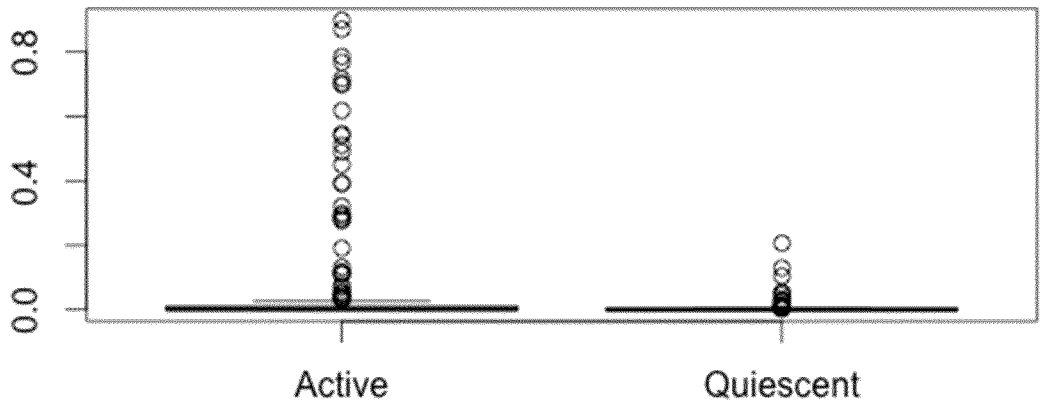
Figure 2



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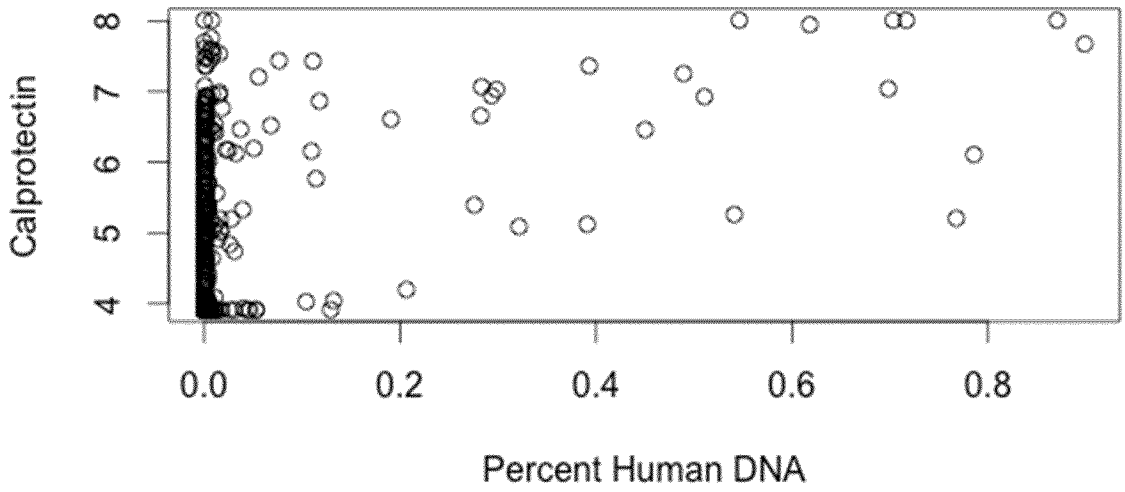
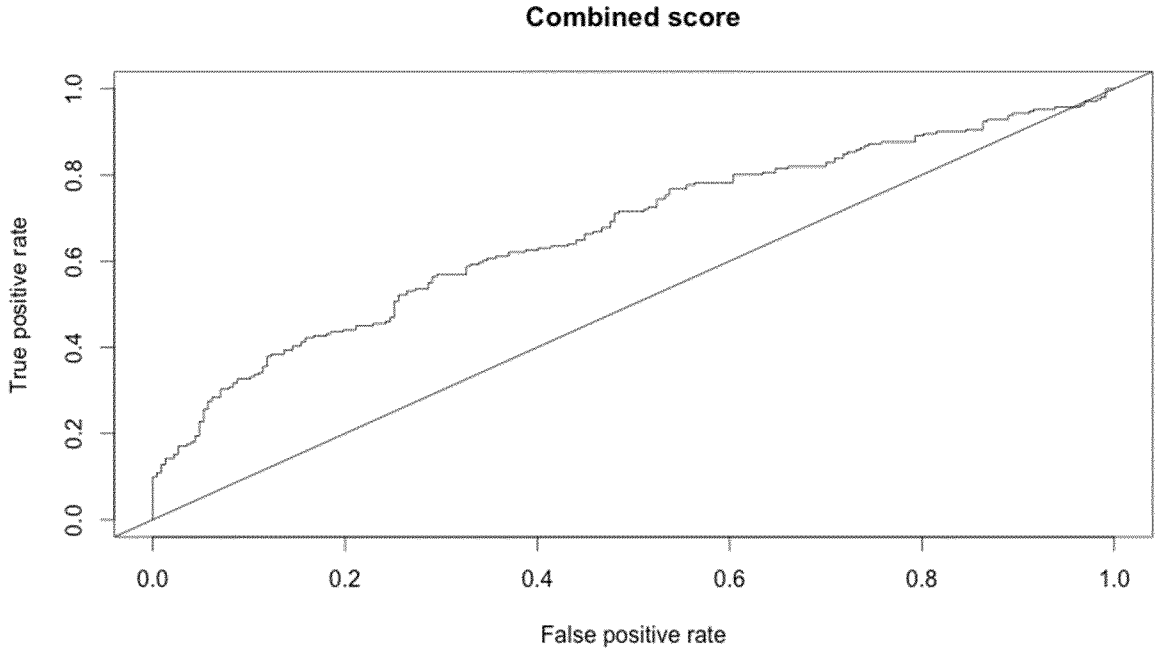


Figure 4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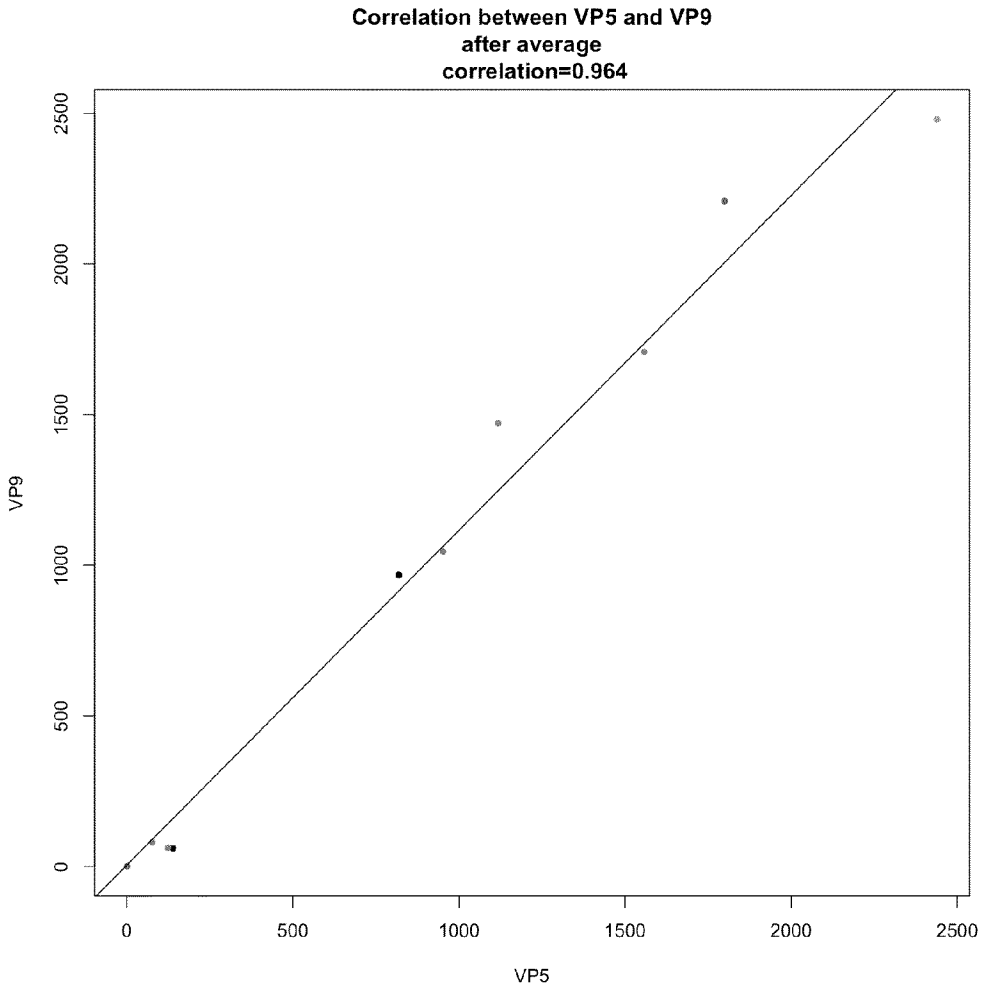
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Figure 6

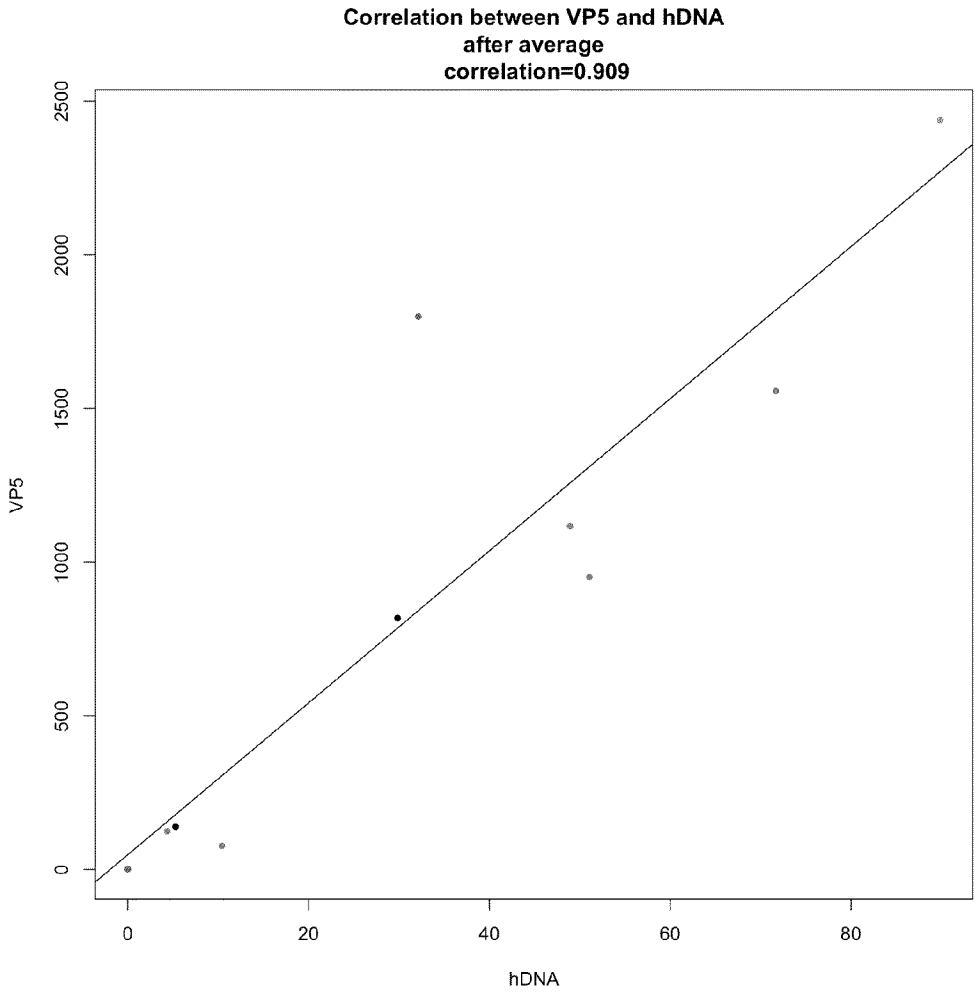
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Figure 7

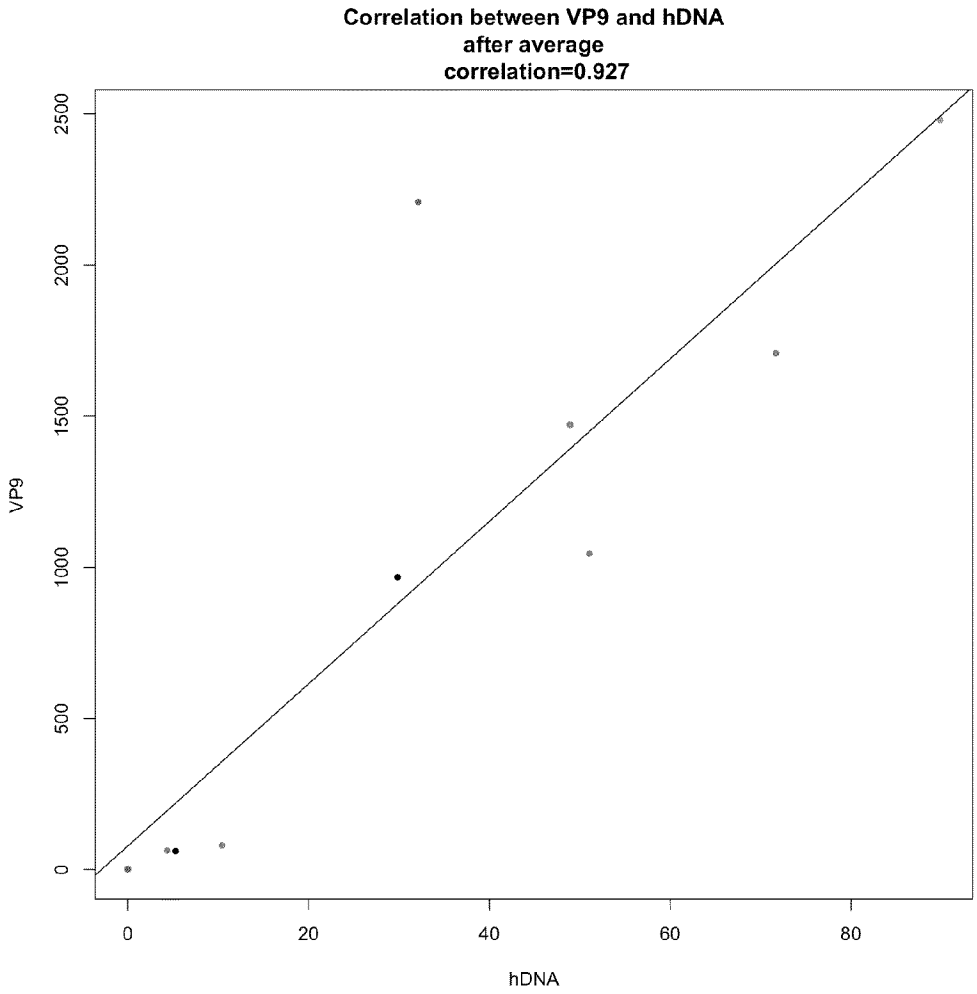
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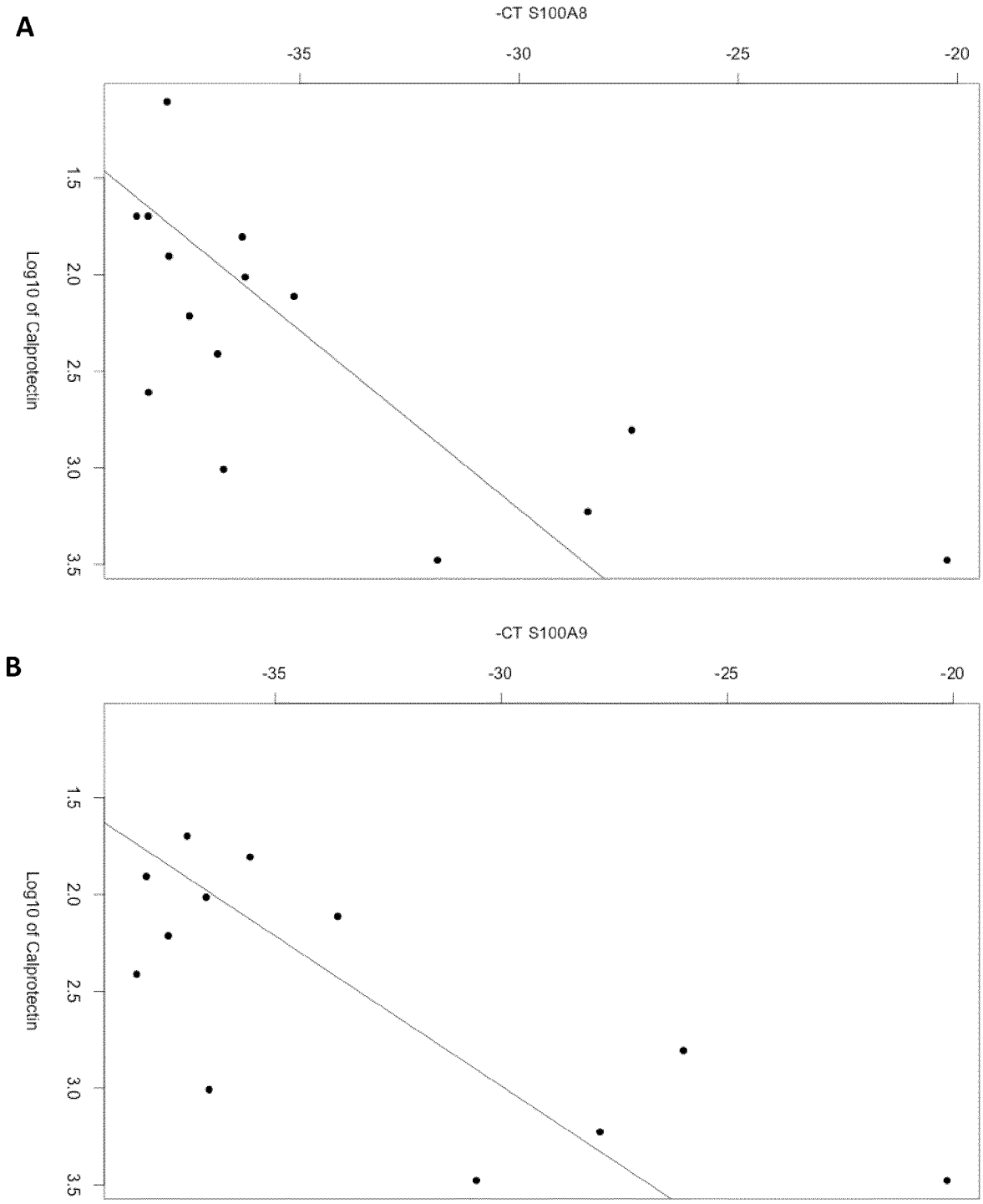
Figure 8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