PROGASTRIN AND LIVER PATHOLOGIES

1. CROSS REFERENCE TO RELATED APPLICATIONS

[0001] This application claims the benefit of U.S. Provisional Application no. 61/293,557, filed January 8, 2010, the content of which is incorporated by reference in its entirety.

2. REFERENCE TO SEQUENCE LISTING

[0002] The Sequence Listing is submitted concurrently herewith.

3. BACKGROUND

[0003] The present disclosure provides methods and materials for the diagnosis and screening of liver pathologies, including proliferative or degenerative diseases of the liver; and more particularly, methods and materials for the quantification or determination of progastrin levels to diagnose liver pathologies.

3.1. Background of the Gastrin and Progastrin Hormones.

[0004] Gastrin is a gut peptide hormone that functions as a stimulant of gastric acid secretion. In the adult mammal, it is produced principally by the G cells of the gastric antrum and to a variable extent in the upper small intestine and pancreas, with barely detectable amounts in the colon. Recently, there has been increasing interest in the role of the gastrin family of peptides in colorectal carcinogenesis. In particular, there is evidence that the precursor forms of gastrin (progastrin and glycine-extended gastrin), which were previously thought to be inactive, play a role in the development of colorectal cancer.

[0005] The gastrin gene is translated into a 101-amino acid polypeptide, called preprogastrin, which contains a signal sequence (underlined) that is cleaved, giving rise to progastrin, an 80-amino acid polypeptide. In turn, progastrin is processed to provide the cleavage product G_{34}, a 34-amino acid peptide corresponding to residues 38 to 71 of progastrin. G_{34} is then extended at its carboxy terminus with a glycine residue, generating glycine-extended G_{34} (G_{34}-Gly). A by-product of progastrin cleavage is a 6-amino acid peptide, called the C-terminal flanking peptide, or CTFP, which corresponds in sequence.

[0006] Most assays for progastrin do not distinguish between progastrin and other gastrin gene products, resulting in an inaccurate measurement of full-length progastrin levels. Because progastrin levels play a role in one or more diseases, accurate means for the measurement of progastrin are desirable.

3.2. Background of Liver Pathologies.

[0007] Many liver pathologies are difficult to diagnose. For example, liver cancer cannot be diagnosed by routine blood tests. Physician screening with the tumor marker alpha-fetoprotein (AFP) is usually necessary. However, elevated AFP levels are not specific for liver cancer. In adults, high blood levels (over 500 ng/mL) of AFP are seen in three situations: liver cancer, germ cell tumors (cancers of the testes and ovaries), and metastatic cancer of the liver (a cancer originating in other organs). In addition, the sensitivity of AFP for liver cancer is about 60%. In other words, an elevated AFP blood level is seen in only about 60% of liver cancer patients; 40% of patients with liver cancer have normal AFP levels. Another difficult to diagnose liver pathology is cirrhosis, a consequence of chronic liver disease characterized by scarring of the liver and poor liver function. The gold standard of diagnosis is by way of liver biopsy, an invasive technique.

[0008] Hepatitis C is an infectious disease affecting the liver, caused by the hepatitis C virus (HCV). The infection is often asymptomatic, but once established, chronic infection can progress to scarring of the liver (fibrosis) and advanced scarring (cirrhosis). Hepatitis C is typically diagnosed by way of serological screening. Other means for detecting and/or confirming hepatitis C would be desirable.

[0009] Because the early and accurate detection of liver cancer, cirrhosis, and hepatitis C has the potential to increase the survival rate of a patient, there is a present and long-felt need for methods of diagnosing or detecting these pathologies, including instances where a patient has more than one or all of the aforementioned diseases.

4. SUMMARY

[0010] It has been discovered that whereas patients with one or two liver pathologies can exhibit elevated human progastrin (hPG) levels, patients with liver cancer, hepatitis C and cirrhosis exhibit extremely elevated levels of hPG that are more than merely additive of the hPG levels exhibited by patients with just one or two of these conditions. Based on the present disclosure, a patient's plasma or serum levels of hPG can be used to assign a

risk factor for liver pathology. Moreover, excessive serum or plasma levels of hPG can be used to diagnose a patient suffering from a liver cancer, hepatitis C and cirrhosis.

[0011] Liver cancers that can be diagnosed by the methods of the disclosure include primary liver cancers (*e.g.*, hepatocellular carcinoma, a cancer originating in the liver) and secondary liver cancers.

[0012] Methods of diagnosis are also provided wherein a patient is identified as suffering from a hepatic or liver condition if a human progastrin (hPG) level above a threshold value (for example, at least about 400, 450, 500, 550, 600, 650, 700, or 750 pM) is detected. Such methods can also be useful as a standard diagnostic in populations having a higher than average incidence of a liver disease, for example, in drug or alcohol-user populations or persons residing in geographic regions with higher than average incidences of liver disease. Accordingly, methods of detecting elevated hPG levels in such populations are also provided.

[0013] The present disclosure also provides methods for assigning a patient to a risk group for one or more liver pathologies such as liver cancer (*e.g.*, hepatocellular carcinoma), hepatitis C and cirrhosis based on the patient's hPG levels. For example, a first threshold level of hPG can indicate that the patient is at a "low risk" for liver pathology; a second threshold level of hPG can indicate that the patient is at an "elevated risk" for liver pathology; a third threshold level of hPG can indicate that the patient is at a "high risk" for liver pathology; and a fourth threshold level of hPG can indicate that the patient is at a "severe risk" for liver pathology.

[0014] Quantified hPG levels can also be used with additional biomarkers, such as alpha-fetoprotein ("AFP") to aid in the identification, diagnosis, differentiation of, or risk assignment for, liver pathologies.

[0015] The methods disclosed herein can be used to determine appropriate therapeutic courses of action. In some instances, it is useful to screen patients who are already diagnosed with a liver pathology to determine if further treatment options are warranted. Accordingly, in certain aspects, a patient who has been previously diagnosed with a liver pathology is diagnosed as suffering from a further hepatic or liver condition on the basis of hPG levels in the patient's biological sample. The previous liver pathology can be hepatitis C, cirrhosis, or liver cancer, such as hepatocellular carcinoma. Advantageously,

extremely high levels of hPG (*e.g.*, hPG concentrations of above 400, 450, 500, 550, 600, 650, 700 pM) indicate the patient has all of these conditions.

[0016] A patient whose hPG levels indicate that the patient has a liver pathology, or a patient previously diagnosed with a liver condition and whose hPG levels are indicative of greater than one liver condition, can be subjected to further testing to identify specific liver conditions. The patient can be tested for liver cancer, hepatitis C, or cirrhosis of the liver.

[0017] Patients who have been identified as having one or more liver pathologies using methods of the disclosure can be treated for their condition. Patients undergoing treatment for a hepatic pathology can also have their hPG levels monitored to evaluate disease progression and/or treatment efficacy. A patient whose hPG levels remain above a threshold value may continue to receive treatment.

[0018] In the practice of the methods disclosed herein, the particular assay for measuring hPG levels is not critical provided the measured hPG level is accurate. In some embodiments, anti-hPG antibodies are conveniently used to measure hPG levels. As noted, hPG is cleaved by the body into smaller peptides. It is preferred that hPG be detected and measured in the methods of the disclosure using assays that do not detect byproducts of progastrin processing so to avoid inaccurate hPG measurements. This can be achieved by the use of antibodies that bind to full length hPG but not to smaller hPG peptides. This can also be achieved by the use of two different antibodies that both bind to full length hPG but to the extent that the antibodies bind to smaller hPG peptides, do not both bind to the same smaller hPG peptides. In such assays, the only product bound by both antibodies is full length hPG. For example, antibodies that bind to the C- and N-terminal epitopes of hPG allow detection and measurement of full length hPG without detection and measurement of smaller hPG peptides. Thus, in certain aspects, the disclosure provides a method of diagnosing a patient wherein a biological sample is contacted with a first antibody that binds to a first epitope of hPG, preferably a C- or N-terminal epitope, and a second antibody that binds to for a different epitope of hPG, preferably an epitope at the other terminus.

[0019] In an exemplary method that utilizes an anti-hPG antibody for detection and measurement of hPG, a patient is identified as suffering from a hepatic or liver condition

by contacting a sample from the patient with at least one anti-hPG antibody; and determining whether the sample has a hPG concentration above a threshold value, for example, at least about 400, 450, 500, 550, or 600 pM, often 400, 450, or 500 pM, based on the amount of hPG bound to the antibody.

[0020] The methods of the disclosure generally include assaying a biological sample for hPG levels. The biological sample can be plasma or serum. Tissue levels can also be used; however, levels of hPG measured in tissue are expected to differ from levels of hPG found in serum or plasma but will be elevated relative to a patient's normal hPG level when a patient is suffering from liver pathologies. Thus, tissue levels of hPG can also be used to monitor a patient's risk or status of liver pathology. When a tissue sample is used, progastrin can be detected using an immunoassay performed on a cell or tissue extract, or can utilize immunohistochemical techniques employing a polyclonal or monoclonal antibody labeled with a detectable marker. Immunohistochemical techniques provide a qualitative measurement of progastrin levels. Suitable detectable markers include a radioactive label (such as radioactive iodine), a fluorescent label or a chemiluminescent label.

[0021] Diagnostic kits are also provided herein. A diagnostic kit can comprise, for example, one or more antibodies to progastrin optionally labeled with a detectable marker that can be used to screen, diagnose, or differentiate liver pathologies. A diagnostic kit can contain a first antibody having an affinity with the N-terminal peptide region of hPG and a second antibody having an affinity with a different epitope such as the C-terminal peptide region of hPG. The present disclosure also provides kits for carrying out methods of diagnosing liver pathologies, comprising an N-terminal anti-hPG monoclonal antibody and a monoclonal or polyclonal C-terminal anti-hPG antibody, each in a separate container, and suitable reagents. In some embodiments, one or both of the antibodies provided is or are labeled.

[0022] Methods of diagnosing whether a patient suffers from a plurality of hepatic or liver conditions are also provided, wherein a patient with a blood hPG concentration of at least about 400 pM is identified as having two or more of the following conditions: hepatitis C, liver cancer, or cirrhosis, *e.g.,* by testing hPG levels in serum or plasma from the patient using a biochemical assay. After such a patient is identified, the patient's liver

pathology can be further diagnosed using assays for hepatitis C, liver cancer, or cirrhosis. Hepatitis C can be diagnosed using a nucleic acid-based assay. Liver cancer can be diagnosed using a radiography or imaging technique (with or without contrast). Cirrhosis of the liver can be diagnosed by testing a sample from said patient for one or more serum markers of fibrosis including alpha-2-macroglobulin, haptoglobin, apolipoprotein A1, gamma-glutamyl transpeptidase (GGT), total bilirubin, and alanine transaminase (ALT). The patient may have other diagnostically important markers, such as elevated serum alpha-fetoprotein and/or des-gamma carboxyprothrombin, and the method of diagnosis or treatment can further comprise a step of measuring such levels.

[0023] Following diagnosis, a patient can be treated for his or her liver condition using conventional agents, for example, a patient with hepatitis C can be treated with pegylated interferon-alpha-2a, pegylated interferon-alpha-2b, and/or viral drug ribavirin; a patient with cirrhosis can be treated with liver transplant surgery and symptomatic treatment for complications of cirrhosis; and a patient diagnosed with liver cancer can be treated with radiotherapy and/or chemotherapy.

[0024] Patients can also be monitored using the techniques of the disclosure. In one method, the disease status of a patient with a hepatic or liver disease is monitored by subjecting a patient suffering from a hepatic or liver disease and having a blood hPG concentration above a threshold amount a therapeutic regimen for said hepatic or liver disease. After treatment, it is determined whether said patient continues to have a blood hPG concentration above the threshold amount and if so, continuing the therapeutic regimen. If the patient no longer has a blood hPG concentration above the threshold amount, the patient's hPG levels can be periodically monitored to determine whether they rise above said threshold amount, allowing treatment to be reinitiated.

[0025] Systems for diagnosing a patient's liver or hepatic health status are also provided. Systems of the disclosure comprise one or more of the following components: an input that receives values of a patient's blood hPG concentration, a processor configured to compare the patient's blood hPG concentration to a reference blood hPG concentration, the processor configured to output a risk level for liver pathology for the patient based wholly or in part on the patient's blood hPG concentration, and a display configured to display the risk level for liver pathology. The systems can be adapted so that the

processor outputs low, elevated, high or severe risk level determinations for a liver pathology. The system can also comprise an input to receive values for a patient's alpha-fetoprotein (AFP) levels, wherein the processor is configured to compare the AFP level to a reference AFP level and to output a risk level for a liver pathology based on the hPG and AFP levels. Threshold (*i*) hPG and (*ii*) hPG and AFP levels for different risk groups are disclosed herein.

[0026] A computer readable storage medium is also provided that has stored therein data representing instructions executable by a programmed processor for use in diagnosing patient's liver or hepatic health status, the storage medium comprising instructions for comparing a patient's blood hPG concentration to a reference blood hPG concentration; and outputting a risk level for liver pathology for the patient based wholly or in part on the patient's blood hPG concentration. The medium can output risk levels, *e.g.,* low, elevated, high or severe risk level determinations for a liver pathology. The medium can also comprise data for comparing a patient's blood AFP level to a reference level for outputting risk level determinations. Threshold (*i*) hPG and (*ii*) hPG and AFP levels for different risk groups are disclosed herein.

5. BRIEF DESCRIPTION OF THE FIGURES

[0027] FIG. 1 provides amino acid sequences of pre-progastrin (where the signal peptide is underlined), progastrin and products of progastrin processing, including G_{34}, G_{34}-Gly, G_{17}, G_{17}-Gly, and the C-terminal flanking peptide, CTFP.

[0028] FIG. 2 provides a bar chart of hPG values indicative of certain liver pathologies.

[0029] FIG. 3A–3L provide polypeptide, and corresponding polynucleotide, sequences of VH and VL chains for exemplary murine anti-hPG monoclonal antibodies.

[0030] FIG. 3A shows the polynucleotide (SEQ ID NO:16) and polypeptide (SEQ ID NO:12) murine VH chain for anti-hPG MAb3 and **FIG. 3B** shows the polynucleotide (SEQ ID NO:17) and polypeptide (SEQ ID NO:13) murine VL chain for anti-hPG MAb3.

[0031] FIG. 3C shows the polynucleotide (SEQ ID NO:18) and polypeptide (SEQ ID NO:14) murine VH chain for anti-hPG MAb4 and **FIG. 3D** shows the polynucleotide (SEQ ID NO:19) and polypeptide (SEQ ID NO:15) murine VL chain for anti-hPG MAb4.

[0032] FIG. 3E shows the polynucleotide (SEQ ID NO:67) and polypeptide (SEQ ID NO:59) murine VH chain for anti-hPG MAb8 and **FIG. 3F** shows the polynucleotide (SEQ ID NO:71) and polypeptide (SEQ ID NO:63) murine VL chain for anti-hPG MAb8.

[0033] FIG. 3G shows the polynucleotide (SEQ ID NO:68) and polypeptide (SEQ ID NO:60) murine VH chain for anti-hPG MAb13 and **FIG. 3H** shows the polynucleotide (SEQ ID NO:72) and polypeptide (SEQ ID NO:74) murine VL chain for anti-hPG MAb13.

[0034] FIG. 3I shows the polynucleotide (SEQ ID NO:69) and polypeptide (SEQ ID NO:61) murine VH chain for anti-hPG MAb16 and **FIG. 3J** shows the polynucleotide (SEQ ID NO:73) and polypeptide (SEQ ID NO:65) murine VL chain for anti-hPG MAb16.

[0035] FIG. 3K shows the polynucleotide (SEQ ID NO:70) and polypeptide (SEQ ID NO:62) murine VH chain for anti-hPG MAb19 and **FIG. 3L** shows the polynucleotide (SEQ ID NO:74) and polypeptide (SEQ ID NO:66) murine VL chain for anti-hPG MAb19.

[0036] FIG. 4A–4Z provide the polypeptide sequences of VH and VL chains for exemplary human anti-hPG monoclonal antibodies.

[0037] FIG. 4A shows the polypeptide (SEQ ID NO:21) human VH chain for anti-hPG MAb3 and **FIG. 4B** shows the polypeptide (SEQ ID NO:22) human VL chain for anti-hPG MAb3.

[0038] FIG. 4C shows the polypeptide (SEQ ID NO:23) human VH chain for anti-hPG MAb4 and **FIG. 4D** shows the polypeptide (SEQ ID NO:24) human VL chain for anti-hPG MAb4.

[0039] FIG. 4E shows the polypeptide (SEQ ID NO:75) human VH chain for anti-hPG MAb8(a) and **FIG. 4F** shows the polypeptide (SEQ ID NO:76) human VL chain for anti-hPG MAb8(a).

[0040] FIG. 4G shows the polypeptide (SEQ ID NO:77) human VH chain for anti-hPG MAb8(b) and **FIG. 4H** shows the polypeptide (SEQ ID NO:78) human VL chain for anti-hPG MAb8(b).

[0041] FIG. 4I shows the polypeptide (SEQ ID NO:79) human VH chain for anti-hPG MAb8(c) and **FIG. 4J** shows the polypeptide (SEQ ID NO:76) human VL chain for anti-hPG MAb8(c).

[0042] FIG. 4K shows the polypeptide (SEQ ID NO:80) human VH chain for anti-hPG MAb13(a) and **FIG. 4L** shows the polypeptide (SEQ ID NO:81) human VL chain for anti-hPG MAb13(a).

[0043] FIG. 4M shows the polypeptide (SEQ ID NO:82) human VH chain for anti-hPG MAb13(b) and **FIG. 4N** shows the polypeptide (SEQ ID NO:83) human VL chain for anti-hPG MAb13(b).

[0044] FIG. 4O shows the polypeptide (SEQ ID NO:84) human VH chain for anti-hPG MAb16(a) and **FIG. 4P** shows the polypeptide (SEQ ID NO:85) human VL chain for anti-hPG MAb16(a).

[0045] FIG. 4Q shows the polypeptide (SEQ ID NO:86) human VH chain for anti-hPG MAb16(b) and **FIG. 4R** shows the polypeptide (SEQ ID NO:87) human VL chain for anti-hPG MAb16(b).

[0046] FIG. 4S shows the polypeptide (SEQ ID NO:88) human VH chain for anti-hPG MAb16(c) and **FIG. 4T** shows the polypeptide (SEQ ID NO:89) human VL chain for anti-hPG MAb16(c).

[0047] FIG. 4U shows the polypeptide (SEQ ID NO:90) human VH chain for anti-hPG MAb19(a) and **FIG. 4V** shows the polypeptide (SEQ ID NO:91) human VL chain for anti-hPG MAb19(a).

[0048] FIG. 4W shows the polypeptide (SEQ ID NO:92) human VH chain for anti-hPG MAb19(b) and **FIG. 4X** shows the polypeptide (SEQ ID NO:93) human VL chain for anti-hPG MAb19(b).

[0049] FIG. 4Y shows the polypeptide (SEQ ID NO:94) human VH chain for anti-hPG MAb19(c) and **FIG. 4Z** shows the polypeptide (SEQ ID NO:95) human VL chain for anti-hPG MAb19(c).

6. DETAILED DESCRIPTION

6.1. Definitions

[0050] Unless indicated otherwise, the following terms are intended to have their ordinary meanings, which are discussed below in the context of the present disclosure:

[0051] "Human Progastrin" or "hPG" is a polypeptide of the amino acid sequence identified as SEQ ID NO:20. As used herein, hPG is defined as comprising the primary protein product of the gastrin gene; *i.e.*, preprogastrin without the signal- or pre-peptide. See Rehfeld *et al.* (2004) *Regulatory Peptides* 120(1–3):177-183. hPG consists of three well defined regions divided by two d-Arg cleavage sites. Accordingly, unless otherwise defined, hPG consists of the products of preprogastrin processing which retain the two d-Arg sites. hPG does not include the "gastrins," *i.e.*, G_{34} and G_{17} but can comprise, for example, short truncates (up to 1, 2, 3, 4 or 5 amino acids) or variants of hPG which retain the two d-Arg sites located at or about amino acids 36/37 and 73/74. See **FIG. 1**.

[0052] "Biological marker" or "biomarker" means a characteristic that is objectively measured and evaluated as an indicator of normal biologic processes, pathogenic processes, or pharmacologic responses to a therapeutic intervention.

[0053] A "sandwich assay" refers to a specific type of immunoassay that can be used to quantify the amount of a compound that interacts with a sample. The sandwich assay is so-called because the antigen of the sample is bound between a capture antibody and a detecting or reference antibody. In the sandwich assays of the disclosure, human progastrin is bound between anti-hPG antibodies. The assay provides a quantification of hPG but not precursors or products thereof, thereby providing a more accurate measure of hPG concentration. Advantageously, the antibodies of the disclosure directed against the N-terminal or C-terminal regions of progastrin are also specific for progastrin separately. Smaller processing fragments of progastrin are therefore less likely to act as competitors to one or the other antibody thereby possibly biasing assay results.

[0054] A "subject" or "patient" is used interchangeably herein, which refers to a vertebrate, preferably a mammal, more preferably a human.

[0055] An "antibody" or "Ab" refers to an immunoglobulin molecule that specifically binds to, or is immunologically reactive with, a particular antigen, and includes polyclonal, monoclonal, genetically engineered and otherwise modified forms of

antibodies, including but not limited to chimeric antibodies, humanized antibodies, and antigen binding fragments of antibodies, including, *e.g.*, Fab', $F(ab')_2$, Fab, Fv, rIgG, and scFv fragments. In various embodiments, anti-hPG monoclonal antibodies comprise all or a portion of a constant region of an antibody. In some embodiments, the constant region is an isotype selected from: IgA (*e.g.*, IgA_1 or IgA_2), IgD, IgE, IgG (*e.g.*, IgG_1, IgG_2, IgG_3 or IgG_4), and IgM. Antibodies (including monoclonal antibodies) can be generated from any of several species including but not limited to mouse, rabbit, rat, pig, guinea pig, chicken, donkey, horse, camel, and lama.

[0056] As used herein, an antibody is "highly specific for" hPG if it binds to full-length progastrin but does not bind at all to CTFP, to amidated gastrin, or to glycine-extended gastrin, and "specific for hPG" or an "antibody specifically binds to hPG" if it exhibits at least about 5-fold greater binding to hPG than to CTFP and the other products of the gastrin gene, as measured in standard binding assays. An ELISA assay that can used to assess the specificity of a particular anti-hPG antibody is provided in Example 4.

[0057] Such specific anti-hPG antibodies (referred to herein as "anti-hPG antibodies") may be polyclonal ("anti-hPG PAbs") or monoclonal ("anti-hPG MAbs"), although for therapeutic uses and, in some instances, diagnostic or other in vitro uses, monoclonal antibodies are preferred.

[0058] The term "monoclonal antibody" as used herein is not limited to antibodies produced through hybridoma technology. A monoclonal antibody is derived from a single clone, including any eukaryotic, prokaryotic, or phage clone, by any means available or known in the art. Monoclonal antibodies useful with the present disclosure can be prepared using a wide variety of techniques known in the art including the use of hybridoma, recombinant, and phage display technologies, or a combination thereof. In many uses of the present disclosure, including in vivo use of the anti-hPG monoclonal antibodies in humans and in vitro detection assays, chimeric, primatized, humanized, or human antibodies can suitably be used.

[0059] The term "scFv" refers to a single chain Fv antibody in which the variable domains of the heavy chain and the light chain from a traditional antibody have been joined to form one chain.

[0060] References to "VH" refer to the variable region of an immunoglobulin heavy chain of an antibody, including the heavy chain of an Fv, scFv, or Fab. References to "VL" refer to the variable region of an immunoglobulin light chain, including the light chain of an Fv, scFv, dsFv or Fab. Antibodies (Abs) and immunoglobulins (Igs) are glycoproteins having the same structural characteristics. While antibodies exhibit binding specificity to a specific target, immunoglobulins include both antibodies and other antibody-like molecules that lack target specificity. Native antibodies and immunoglobulins are usually heterotetrameric glycoproteins of about 150,000 Daltons, composed of two identical light (L) chains and two identical heavy (H) chains. Each heavy chain has at one end a variable domain (VH) followed by a number of constant domains. Each light chain has a variable domain at one end (VL) and a constant domain at its other end.

[0061] Anti-hPG monoclonal antibodies of the disclosure comprise "complementarity-determining regions (CDRs)." CDRs are also known as hypervariable regions both in the light chain and the heavy chain variable domains. The more highly conserved portions of variable domains are called the framework (FR). As is known in the art, the amino acid position/boundary delineating a hypervariable region of an antibody can vary, depending on the context and the various definitions known in the art. Some positions within a variable domain can be viewed as hybrid hypervariable positions in that these positions can be deemed to be within a hypervariable region under one set of criteria while being deemed to be outside a hypervariable region under a different set of criteria. One or more of these positions can also be found in extended hypervariable regions. The disclosure provides antibodies comprising modifications in these hybrid hypervariable positions. The variable domains of native heavy and light chains each comprise four FR regions, largely by adopting a β-sheet configuration, connected by three CDRs, which form loops connecting, and in some cases forming part of, the β-sheet structure. The CDRs in each chain are held together in close proximity by the FR regions and, with the CDRs from the other chain, contribute to the formation of the target-binding site of antibodies (See Kabat *et al.*, *Sequences of Proteins of Immunological Interest*, National Institute of Health, Bethesda, Md. 1987).

[0062] The term "epitope" refers to any portion (determinant) of a protein that is capable of eliciting an immune response and being specifically bound by an antibody. Epitope

determinants usually consist of active surface groupings of molecules such as amino acids or GAG side chains and usually have specific three-dimensional structural characteristics, as well as specific charge characteristics. Two antibodies are said to bind to substantially the same epitope of a protein (or the overlapping epitope of a protein) if amino acid mutations in the protein that reduce or eliminate binding of one antibody also reduce or eliminate binding of the other antibody, and/or if the antibodies compete for binding to the protein, *i.e.*, binding of one antibody to the protein reduces or eliminates binding of the other antibody. The determination of whether two antibodies bind substantially to the same epitope is accomplished by the methods known in the art, such as a competition assay. In conducting an antibody competition study between a control antibody (for example, one of the anti-progastrin antibodies described herein) and any test antibody, one can first label the control antibody with a detectable label, such as, biotin, enzymatic, radioactive label, or fluorescent label to enable the subsequent identification. A test (unlabeled) antibody that binds to substantially the same epitope as the control (labeled) antibody should be able to block control antibody binding and thus should reduce control antibody binding.

[0063] An "assay specific for hPG" or an "assay specific for human progastrin" refers to an assay that distinguishes full length hPG from CTFP and the other products of the gastrin gene. In the context of an antibody-based diagnostic assay, an assay specific for hPG can utilize an antibody that specifically binds to hPG. Alternatively, an assay specific for hPG can utilize two antibodies that both bind to full length hPG but otherwise do not both bind to the same gastrin gene products, such that hPG is the only molecule produced by the gastrin gene that is recognized by both antibodies. For example, antibodies that bind to C- and N-terminal epitopes of hPG can be used in an assay that distinguishes hPG from other gastrin gene peptides.

[0064] With respect to the use of the term cancer, it is noted that in patients in whom cells from the primary (original) tumor have broken free and migrated to another location within the body, typically through the lymph or blood, via a process called "metastasis," to form another, metastatic (or secondary) tumor, the secondary or metastatic tumor is typically of the same type as the original tumor, regardless of its new location, such that the disease is referred to as metastatic cancer, and not cancer of the new resident tissue. For example, pancreatic cancer that has spread to the liver is metastatic pancreatic cancer,

not liver cancer. Accordingly, metastatic hepatocellular carcinoma refers to a cancer originating in the liver and metastasizing elsewhere. Secondary liver cancer (*i.e.*, metastatic cancer from a non-liver source that has metastasized to the liver) can also be diagnosed based on excessive levels of hPG. For example, a colorectal cancer that has metastasized (*i.e.* metastatic colorectal cancer) to the liver is a form of secondary liver cancer. In other words, cancer of the liver is detectable using hPG levels in serum, plasma, or tissue in both primary and secondary liver cancers.

6.2. Progastrin Levels and Liver Pathologies

[0065] Normal progastrin levels are generally considered to be less than 20 to 50 pM, typically between 0 and 5 pM. The present disclosure demonstrates that patients with one or two liver pathologies can exhibit elevated human progastrin (hPG) levels, and that patients with liver cancer, hepatitis C and cirrhosis exhibit extremely elevated levels of hPG.

[0066] **FIG. 2** graphically illustrates hPG levels in patients with one or more liver pathologies as determined by an ELISA-sandwich assay using anti-hPG antibodies. Outer bounds of boxed areas indicate the 25th to 75th percentile. Whiskers indicate the 5th to 95th percentile. Single lines represent the median. Dots indicate outliers. The following Table summarizes 5th to 95th percentile data:

Table 1

Progastrin Levels (pM) in Patients with Liver Pathologies (5–95 Percentile)	
Liver Cancer	**Cirrhosis**
0 to 100 (20)[†]	10 to 700 (90)
Liver Cancer and Hepatitis C	**Liver Cancer, Hepatitis C and Cirrhosis**
10 to 500 (50)	150 to 2000 (1300–1400)
[†] Values in parenthesis indicate mean hPG levels.	

[0067] Based on the foregoing, hPG levels above 50 pM or 100 pM can be indicative of one or more pathologies of the liver. hPG levels of at least about 400 pM, 500 pM or 600 pM are particularly diagnostically significant as they are strongly indicative of multiple liver pathologies, particular the presence of liver cancer and cirrhosis, liver cancer and hepatitis C, and liver cancer, cirrhosis and hepatitis C.

[0068] Plasma levels for patients with only hepatitis C have been found to be similar to healthy patients in a study measuring total progastrin levels (*i.e.* levels of full-length

progastrin and cleavage products). Konturek *et al.*, 2003, *Scand J Gastroenterol* 6:643–647. Levels of full-length progastrin only in patients with hepatitis C are expected to be very low.

[0069] Progastrin levels can be used to assign a risk point score and a corresponding disease risk probability. For an example of a model for assigning mortality risk in patients with end-stage liver disease, see Kamath *et al.* (2001) *Hepatology* 33(2):464-70.

[0070] Using empirical data correlating determined progastrin levels with liver disease states, a point score and associated disease risk probability can be derived from the levels of biomarkers, such as progastrin and optionally other biomarkers. The disease risk probability can be qualitatively described using four risk groups: low risk, elevated risk, higher risk, and severe risk.

[0071] The risk levels referred to in the present disclosure have the following meanings:

- "Low Risk" means the patient's levels of biological marker(s) do not indicate the presence of a given liver pathology.
- "Elevated Risk" means the patient's levels of biological marker(s) indicate an increased risk of a given liver pathology. The risk is of such magnitude that further testing is warranted.
- "High Risk" means the patient's levels of biological marker(s) indicate a greatly increased risk of a given liver pathology. The risk is of such magnitude that further testing is required. A tentative diagnosis based on the specificity of the biological markers might be justified.
- "Severe Risk" means the patient's levels of biological marker(s) are clearly abnormal and indicate an underlying pathology. A diagnosis, subject to the specificity of the biological marker(s), can be made.

[0072] Patients who have serum or plasma progastrin levels below 0 pM to 100 pM have a low risk for a diagnosis of both liver cancer, *e.g.*, hepatocellular carcinoma and cirrhosis, and an extremely low risk for a diagnosis of all three of liver cancer, cirrhosis and hepatitis C. Patients with normal serum or plasma levels of progastrin (*e.g.*, 0 to 5 pM) or slightly elevated levels (up to 100 pM) can nonetheless have liver cancer.

Accordingly, it is advisable to further test a patient with serum or plasma levels of hPG of 100 pM or lower to evaluate whether he or she has liver cancer.

[0073] Patients who have serum or plasma progastrin levels of between 100 pM and 400 pM are unlikely to have liver cancer without other hepatic pathologies. Such patients have a high risk of having cirrhosis (with or without liver cancer) and an elevated risk of having hepatitis C in addition. Patients who have serum or plasma progastrin levels above 400 pM are at a severe risk of having liver cancer, hepatitis C and cirrhosis.

[0074] The relationship between a patient's hPG levels and liver pathology status can be used in a variety of diagnostic methods. The diagnostic methods generally entail comparing hPG levels in a patient's sample to a normal value of hPG, for example the hPG levels from a healthy individual or a pool of healthy individual. If hPG levels are elevated, the elevated level is correlated with a likely liver pathology, for example on the basis of the risk assignments provided above. Such methods optionally include a step in which a patient provides a sample of a body fluid. The body fluid can then by analyzed for levels of hPG, preferably by a biochemical assay.

[0075] In certain applications, testing a patient's level of a cancer marker in conjunction with the patient's level of hPG can be used to increase the specificity of a liver cancer marker in a patient, or to assign a risk point score and a corresponding disease risk probability. A preferred secondary marker is alpha-fetoprotein (AFP). Serum AFP, a fetal-specific glycoprotein antigen, is the most widely used tumor marker for detecting patients with liver cancer, *e.g.,* hepatocellular carcinoma. The reported sensitivity of AFP for detecting hepatocellular carcinoma varies widely in both hepatitis B virus (HBV)-positive and HBV-negative populations, which is attributable to overlap between screening and diagnosis study designs. When AFP is used for screening of high-risk populations, a sensitivity of 39% to 97%, specificity of 76% to 95%, and a positive predictive value (PPV) of 9% to 32% have been reported. AFP is not specific for liver cancer. Titers also rise in acute or chronic hepatitis, in pregnancy, and in the presence of germ cell tumors.

[0076] The following Table indicates relative risk for a given liver pathology based on hPG levels in conjunction with AFP levels. Assignment of relative risk on the basis of

both hPG and AFP levels provides a more sensitive and accurate test for liver pathologies than on the basis of AFP alone.

Table 2

Relative Risk for Liver Pathologies at Determined Progastrin and AFP Levels				
		Progastrin Level		
		0–100 pM	**100–400 pM**	**>400 pM**
AFP Level	0 pM to 50 pM	–	–	++
	51 pM to 250 pM	–	+	+++
	251 pM to 500 pM	+	++	+++
	501 pM to 750 pM	++	+++	+++
– = low risk, + = elevated risk, ++ = high risk, and +++ = severe risk				

[0077] Other secondary markers that can be used in conjunction with progastrin include oncofetal antigens, glycoprotein antigens, enzymes and isoenzymes, gene markers, and cytokines that correlate with liver cancer. Additional suitable secondary markers include glypican-3, gamma-glutamyl transferase II, alpha-l-fucosidase, transforming growth factor-beta1, tumor-specific growth factor, gamma-glutamyl transferase mRNA, vascular endothelial growth factor, interleukin-8, and variants thereof. Exemplary markers are described in Zhou *et al.* (2006) *World Journal of Gastroenterology* 12(8):1175–1181.

[0078] Liver pathologies can be confirmed by standard techniques known in the art. For example, liver cancers, such as hepatocellular carcinoma, can be confirmed by radiography or an imaging technique such as MRI, with or without biopsy and with or without quantification of a blood marker such as AFP. Hepatitis C can be confirmed by quantification of viral particles in blood, by analysis of fibrosis levels using ultrasound, using a nucleic acid-based assay and/or by fibroscan. Cirrhosis can be confirmed by diagnosis using ultrasound with or without biopsy, and/or by detecting serum markers of fibrosis. Other techniques can also be used.

6.3. Methods of Measuring Progastrin Levels

[0079] The methods of the present disclosure diagnose one or more liver pathologies and/or assign risk of liver pathology on the basis of a patient's hPG levels. Plasma and serum progastrin levels can be measured using any known analytical technique. Such techniques include, but are not limited to: ELISA, sandwich ELISA, immunoblotting (Western blotting), immunoprecipitation, BIACORE technology and the like, as well as assays based on a property of the protein including but not limited to enzymatic activity

or interaction with other protein partners. For immunoassays, a preferred class of assay, one or more anti-progastrin (anti-PG) antibodies of the disclosure (whether polyclonal or monoclonal and neutralizing or non-neutralizing) can be used.

[0080] Preferred immunoassays specifically detect progastrin as opposed to other gastrin gene products, including degradation products. Sandwich assays provide such specificity for the detection of progastrin as opposed to other gastrin gene byproducts, thereby giving a more accurate measure of serum progastrin levels. Preferred immunoassay antibodies bind an antigen/epitope comprising a terminal region unique to progastrin. For example, in some embodiments, progastrin is detected using sandwich ELISA with one anti-PG antibody targeting the N-terminus of progastrin and a second anti-PG antibody targeting the C-terminus of progastrin. Exemplary antibodies are disclosed below in Section 6.4, and a general "sandwich" technique for measuring progastrin levels using anti-PG antibodies is disclosed next.

[0081] A surface, such as the wells in a 96-well plate, is prepared to which a known quantity of a first, "capture," antibody to progastrin is bound. The capture antibody can be, for example, an anti-PG antibody that binds the C- or N-terminus of progastrin. After blocking, a test sample is applied to the surface followed by an incubation period. The surface is then washed to remove unbound antigen and a solution containing a second, "detection," antibody to progastrin is applied. The detection antibody can be any of the anti-PG monoclonal antibodies described herein, provided the detection antibody binds a different epitope from the capture antibody. For example, if the capture antibody binds a C-terminal peptide region of progastrin, then a suitable detection antibody would be one that binds an N-terminal peptide region of progastrin. Progastrin levels can then be detected either directly (if, for example, the detection antibody is conjugated to a detectable label) or indirectly (through a labeled secondary antibody that binds the detection anti-PG antibody).

[0082] In a specific embodiment, human progastrin (hPG) levels are measured from a biological test sample as described in Example 1.

[0083] Receiver Operating Characteristic (ROC) curves have been generated based on plasma hPG levels as determined by a sandwich assay and demonstrate that measurement

of hPG levels provides a diagnostically useful test for distinguishing between patients with one or liver pathologies and patients having other cancers and/or healthy individuals.

[0084] Exemplary antibodies for the antibody-based methods of measuring hPG levels are disclosed in the following section.

6.4. Anti-hPG Antibodies

[0085] An immunoassay for measuring hPG levels can utilize one or more polyclonal or monoclonal anti-hPG antibodies or an antigen-binding fragment thereof.

[0086] Various procedures known in the art can be used for the production of polyclonal antibodies to hPG. In a particular embodiment, rabbit polyclonal antibodies can be obtained. For the production of antibody, various host animals can be immunized by injection with hPG, including but not limited to rabbits, mice, rats, *etc.* Various adjuvants can be used to increase the immunological response, depending on the host species, and including but not limited to Freund's (complete and incomplete), mineral gels such as aluminum hydroxide, surface active substances such as lysolecithin, pluronic polyols, polyanions, peptides, oil emulsions, keyhole limpet hemocyanins, and dinitrophenol.

[0087] Monoclonal antibodies are preferably used in the methods of the disclosure. Monoclonal antibodies, which are homogeneous populations of antibodies to a particular antigen, may be obtained by any technique which provides for the production of antibody molecules by continuous cell lines in culture. These include, but are not limited to, the hybridoma technique of Kohler and Milstein, the human B-cell hybridoma technique, and the EBV-hybridoma technique. See, *e.g.*, Kohler and Milstein (1975) *Nature* 256:495-497; Kozbor *et al.* (1983) *Immunology Today* 4:72; Cole *et al.* (1985) pp. 77-96 in Reisfeld and Sell (1985) *Monoclonal Antibodies and Myeloma Therapy* Liss; Coligan (1991) *Current Protocols in Immunology* Lippincott; Harlow and Lane; *Antibodies: A Laboratory Manual* (1988) CSH Press; and Goding (1986) *Monoclonal Antibodies: Principles and Practice* (2d ed.) Academic Press. Such antibodies may be of any immunoglobulin class including IgG, IgM, IgE, IgA, IgD and any subclass thereof.

[0088] Recently, it has been discovered that, at least for monoclonal anti-hPG antibodies, the selection of antigen used to raise the anti-hPG antibodies may be important (see, International Application No. PCT/EP2010/006329 filed October 15, 2010 and U.S. application No. 12/906,041 filed October 15, 2010, the disclosures and specifically

disclosed anti-hPG antibodies of which are incorporated herein by reference; hereinafter referred to as the '329 and '041 applications, respectively). As disclosed in the '329 and '041 applications, not all antigens derived from hPG stimulate production of monoclonal antibodies that specifically bind hPG under physiological conditions. Indeed, certain antigens that have been used to successfully raise polyclonal anti-hPG antibodies, such as full-length recombinant hPG (see, *e.g.*, WO 08/076454 to Singh) and a peptide corresponding to the last ten amino acids at the C-terminal end of hPG (see WO 07/135542 to Hollande *et al.*) failed to generate monoclonal antibodies.

[0089] In one preferred embodiment, antibodies specific for the C- and N-terminal epitopes of hPG may be used to detect and measure hPG levels with specificity, meaning only full-length hPG is detected. As noted in the '329 and '041 applications, antigenic N-terminal and C-terminal sequences within the hPG sequence have been identified that can be used to generate monoclonal antibodies that specifically bind hPG. Interestingly, the antigenic sequence need not be limited to regions of the hPG sequence that are unique to it. Peptide antigens having regions of sequence in common with other products of the gastrin gene, for example, G_{17}, G_{34} and CTFP, yield monoclonal antibodies that not only bind hPG, but bind it specifically.

[0090] Anti-hPG antibodies obtainable using a peptide antigen having a sequence corresponding to an N-terminal region of hPG and/or that bind an N-terminal region of hPG are referred to herein as "N-terminal anti-PG antibodies." A specific exemplary antigenic region of hPG that can be used to construct an immunogen suitable for obtaining both polyclonal and monoclonal antibodies specific for hPG corresponds to residue 1 to 14 of hPG: SWKPRSQQPDAPLG (SEQ ID NO:25). Exemplary immunogens useful for obtaining N-terminal anti-hPG antibodies, as well as CDR and VH and VL sequences of N-terminal anti-hPG monoclonal antibodies obtained with these exemplary immunogens, are provided in Table 3A, below, and the Example sections:

	VL CDR 1.4	QSLVHSSGVTY	(SEQ ID NO:10)	mVL.4	(SEQ ID NO:13)	hVL.4	(SEQ ID NO:24)
	VL CDR 2.4	KVS	(SEQ ID NO:5)				
	VL CDR 3.4	SQSTHVPPT	(SEQ ID NO:11)				
MAb15							
MAb16	VH CDR 1.16	GYTFTSYY	(SEQ ID NO:39)	mVH.16	(SEQ ID NO:61)	hVH.16a	(SEQ ID NO:84)
	VH CDR 2.16	INPSNGGT	(SEQ ID NO:43)			hVH.16b	(SEQ ID NO:86)
	VH CDR 3.16	TRGGYYPFDY	(SEQ ID NO:47)			hVH.16c	(SEQ ID NO:88)
	VL CDR 1.16	QSLLDSDGKTY	(SEQ ID NO:50)	mVL.16	(SEQ ID NO:65)	hVL.16a	(SEQ ID NO:85)
	VL CDR 2.16	LVS	(SEQ ID NO:53)			hVL.16b	(SEQ ID NO:87)
	VL CDR 3.16	WQGTHSPYT	(SEQ ID NO:57)			hVL.16c	(SEQ ID NO:89)
MAb17							
MAb18							
MAb19	VH CDR 1.19	GYSITSDYA	(SEQ ID NO:40)	mVH.19	(SEQ ID NO:62)	hVH.19a	(SEQ ID NO:90)
	VH CDR 2.19	ISFSGYT	(SEQ ID NO:44)			hVH.19b	(SEQ ID NO:92)
	VH CDR 3.19	AREVNYGDSYHFDY	(SEQ ID NO:48)			hVH.19c	(SEQ ID NO:94)
	VL CDR 1.19	SQHRTYT	(SEQ ID NO:51)	mVL.19	(SEQ ID NO:66)	hVL.19a	(SEQ ID NO:91)
	VL CDR 2.19	VKKDGSH	(SEQ ID NO:54)			hVL.19b	(SEQ ID NO:93)
	VL CDR 3.19	GVGDAIKGQSVFV	(SEQ ID NO:58)			hVL.19c	(SEQ ID NO:95)
MAb20							

s-BSA, also represented as (SEQ ID NO:25)-Ahx-Cys-BSA;
s-KLH, also represented as (SEQ ID NO:25)-Ahx-Cys-KLH

nted using conventional N→C orientation. For each immunogen, the progastrin peptide was synthesized with a C-terminal linker of one aminohexanoic acid e, which was then conjugated to a either a bovine serum albumin ("BSA") or keyhole limpet hemocyanin ("KLH") carrier *via* the Cys linker residue.

[0091] Anti-hPG antibodies obtainable using a peptide antigen having a sequence corresponding to a C-terminal region of hPG, and/or that bind a C-terminal region of hPG, are referred to herein as "C-terminal anti-hPG antibodies." A specific exemplary antigenic region that can be used to construct an immunogen useful for obtaining both polyclonal and monoclonal C-terminal anti-hPG antibodies corresponds to residues 55 to 80 of hPG: QGPWLEEEEEAYGWMDFGRRSAEDEN (SEQ ID NO:27). Exemplary immunogens including this antigen useful for obtaining C-terminal anti-hPG antibodies, as well as CDR and VH and VL sequences of C-terminal anti-hPG monoclonal antibodies obtained with these exemplary immunogens, are provided in Table 3B, below, and the Examples section.

b9						
b10						
b11						
b12						
b13	VH CDR 1.13	GFIFSSYG	(SEQ ID NO:38)	mVH.13	(SEQ ID NO:60)	hVH.13a (SEQ ID NO:80)
	VH CDR 2.13	INTFGDRT	(SEQ ID NO:42)			hVH.13b (SEQ ID NO:82)
	VH CDR 3.13	ARGTGTY	(SEQ ID NO:46)			
	VL CDR 1.13	QSLLDSDGKTY	(SEQ ID NO:50)	mVL.13	(SEQ ID NO:64)	hVL.13a (SEQ ID NO:81)
	VL CDR 2.13	LVS	(SEQ ID NO:53)			hVL.13b (SEQ ID NO:83)
	VL CDR 3.13	WQGTHFPQT	(SEQ ID NO:56)			
b14						
b21						
b22						
b23						

EEEEEAYGWMDFGRRSAEDEN, also represented as KLH-Cys-Ahx-Ahx-(SEQ ID NO:27)
EEEEAYGWMDFGRRSAEDEN, also represented as DT-Cys-Ahx-Ahx-(SEQ ID NO:27)

ented using conventional N→C orientation. For each immunogen, the progastrin peptide was synthesized with an N-terminal Ahx-Ahx-Cys linker, which hemocyanin ("KLH") or a diphtheria toxin ("DT") carrier *via* the Cys linker residue.

[0092] The specific epitopes bound by the exemplary anti-hPG monoclonal antibodies MAb1-MAb23 provided in Tables 4A and 4B were mapped using the SPOT technique and alanine scanning, as described in Laune *et al.* (2002) *J. Immunol. Methods* 267:53–70 and Laune (1997) *J. Biol. Chem.* 272:30937–30944, respectively (see also, Example 6 of the '329 application).

[0093] In the SPOT technique, 15 amino acid peptide sequences spanning a putative epitope are generated and spotted onto a nitrocellulose membrane which is then probed with the test antibody to determine the minimal epitope sequence recognized by the antibody. Alanine scanning is used to determine residues within an epitope that are critical for antibody binding. Each residue within a putative epitope is mutated, one by one, to an alanine, and the alanine-containing peptides are then probed with the test antibody.

[0094] For N-terminal anti-hPG monoclonal antibodies MAbs #1-4 and 15-20, epitopes comprise at least the following sequences: DAPLG (SEQ ID NO:28), PDAPLG (SEQ ID NO:29), PRSQQPD (SEQ ID NO:30), WKPRSQQPD (SEQ ID NO:31), or WKPRSQQPDAPLG (SEQ ID NO:32), as shown in Table 4A below.

Table 4A

MAb #	PG peptide antigen: SWKPRSQQPDAPLG	SEQ ID NO:
MAb2	WKPRSQQPDAPLG	32
MAb4	WKPRSQQPDAPLG	32
MAb1	PDAPLG	29
MAb3	DAPLG	28
MAb17	WKPRSQQPD	31
MAb18	WKPRSQQPD	31
MAb19	WKPRSQQPD	31
MAb20	WKPRSQQPD	31
MAb15	PRSQQPD	30
MAb16	PRSQQPD	30

[0095] For C-terminal anti-hPG monoclonal antibodies MAbs #5-7, 9-12, 14 and 21-23, epitopes comprise at least the following sequences: FGRR (SEQ ID NO:33), MDFGR (SEQ ID NO:34), AEDEN (SEQ ID NO:35), and GWMDFGRR (SEQ ID NO:36), as shown in Table 4B, below.

Table 4B		
MAb #	**PG peptide antigen: QGPWLEEEEEAYGWMDFGRRSAEDEN**	**SEQ ID NO:**
MAb14	GWMDFGRR	36
MAb11	MDFGR	34
MAb5	FGRR	33
MAb6	FGRR	33
MAb7	FGRR	33
MAb9	FGRR	33
MAb10	FGRR..E	33
MAb12	FGRR	33
MAb23	AEDEN	35

[0096] The epitope mapping experiments reveal that anti-hPG MAb2 and MAb4 bind the same epitope; anti-hPG MAb1 and MAb3 bind approximately the same epitope; MAb17, MAb18, MAb19, and MAb20 bind approximately the same epitope; MAb15 and MAb16 bind approximately the same epitope; anti-hPG MAb5, MAb6, MAb7, MAb9, and MAb12 bind the same epitope and bind approximately the same epitope as anti-hPG MAb10; and anti-hPG MAb11 and MAb14 bind approximately the same epitope.

[0097] Specific embodiments of N-terminal anti-PG antibodies useful in the methods and kits described herein include antibodies that bind an epitope that includes residues 10 to 14 of hPG (SEQ ID NO:28), residues 9 to 14 of hPG (SEQ ID NO:29), residues 4 to 10 of hPG (SEQ ID NO:30), residues 2 to 10 of hPG (SEQ ID NO:31), or residues 2 to 14 of hPG (SEQ ID NO:32).

[0098] Specific embodiments of C-terminal anti-PG antibodies useful in the methods and kits described herein include antibodies that bind an epitope that includes residues 71 to 74 of hPG (SEQ ID NO:33), residues 69 to 73 of hPG (SEQ ID NO:34), residues 76 to 80 of hPG (SEQ ID NO:35), or residues 67 to 74 of hPG (SEQ ID NO:36).

[0099] N-terminal and C-terminal anti-hPG antibodies useful in the methods and kits disclosed herein in addition to those provided in Tables 4A & 4B can be identified in competitive binding assays with exemplary MAbs 1-23, or with other reference antibodies that bind N- or C- terminal epitopes, as will be described in more detail in a later section.

[0100] Several of the hybridomas useful for obtaining the antibodies were deposited on October 6, 2010 with the Collection Nationale de Cultures de Microorganismes (CNCM)

in accordance with the Treaty of Budapest. The designated names of the hybridomas producing anti-hPG MAbs1–23 and the depository registration numbers of those hybridomas deposited are provided in Tables 4A & 4B. In addition, for several of the antibodies, the amino acid sequences of their variable heavy chains (VH), variable light chains (VL), VL complementarity determining regions (CDRs) and VH CDRs have been determined. These amino acid sequences, and the shorthand nomenclature used to reference them throughout the disclosure, are also provided in Tables 4A & 4B. Briefly, murine heavy and light chain variable domains are referred to herein as mVH and mVL followed by the number of the corresponding monoclonal antibody, for example mVH.3 and mVL.3 for the variable light and variable heavy chains of anti-hPG MAb3, respectively. Similarly, human heavy and light chain variable domains are referred to herein as hVH and hVL followed by the number of the corresponding monoclonal antibody. The three variable heavy chain CDRs and three variable light chain CDRs are referred to as VH CDR 1, 2, or 3, and VL CDR 1, 2, or 3, respectively, followed by the number of the specific anti-hPG monoclonal antibody. For example, VH CDR 1 of MAb3 is denoted VH CDR 1.3 and VL CDR 1 of MAb3 is denoted VL CDR 1.3. VH CDR 2 of MAb3 is denoted VH CDR 2.3, and VL CDR 2 of MAb3 is denoted VL CDR 2.3.

[0101] It is expected that corresponding CDRs and/or VH and VL chains of anti-hPG monoclonal antibodies that bind approximately the same epitopes could be interchanged to yield new anti-hPG monoclonal antibodies useful in the methods and kits described herein. For example, as noted above, exemplary anti-hPG monoclonal antibodies MAb5 and MAb6 bind the same epitope. An anti-hPG monoclonal antibody can be designed that includes, in its VL chain, various combinations of the VL CDRs of these two antibodies, and/or in its VH chain various combinations of the VH CDRs of these two antibodies. As a specific non-limiting example to illustrate the various combinations possible, such an antibody could include in its VL chain, CDRs 1 and 2 of MAb5 (VL CDR 1.5 and VL CDR 2.5, respectively) and CDR 3 of MAb6 (VL CDR 3.6), and in its VH chain, CDR 1 of MAb6 (VH CDR 1.6) and CDRs 2 and 3 of MAb5 (VH CDR 2.5 and VH CDR 3.5, respectively). Amino acid sequences of CDRs of antibodies produced by hybridomas that have been deposited can be obtained using conventional means. See,

e.g., Coligan (1996) *Current Protocols in Immunology*, Vol. 3, New York: John Wiley and Sons.

[0102] With reference to Table 3A, specific embodiments of N-terminal anti-hPG antibodies useful in the methods and kits described herein include, but are not limited to, the following:

(a) antibodies having VL CDRs that correspond in sequence to the VL CDRs of MAb1, MAb2, MAb3, MAb4, MAb15, MAb16, MAb17, MAb18, MAb19 or MAb20, and VH CDRs that correspond in sequence to the VH CDRs of MAb1, MAb2, MAb3, MAb4, MAb15, MAb16, MAb17, MAb18, MAb19 or MAb20;

(b) antibodies having VL CDRs and VH CDRs that correspond in sequence to the VL and VH CDRs of MAb1, MAb2, MAb3, MAb4, MAb15, MAb16, MAb17, MAb18, MAb19 or MAb20;

(c) antibodies in which:

(i) VL CDR 1 is selected from QSIVHSNGNTY ("VL CDR 1.3"; SEQ ID NO:4), QSLVHSSGVTY ("VL CDR 1.4"; SEQ ID NO:10), QSLLDSDGKTY ("VL CDR 1.16"; SEQ ID NO:50), and SQHRTYT ("VL CDR 1.19"; SEQ ID NO:51);

(ii) VL CDR2 is selected from KVS ("VL CDR 2.3" and ("VL CDR 2.4"; SEQ ID NO:5), LVS ("VL CDR 2.16"; SEQ ID NO:53), and VKKDGSH ("VL CDR 2.19"; SEQ ID NO:54);

(iii) VL CDR3 is selected from FQGSHVPFT ("VL CDR 3.3"; SEQ ID NO:6), SQSTHVPPT ("VL CDR 3.4"; SEQ ID NO:11), WQGTHSPYT ("VL CDR 3.16"; SEQ ID NO:57), and GVGDAIKGQSVFV ("VL CDR 3.19"; SEQ ID NO:58);

(iv) VH CDR1 is selected from GYIFTSYW ("VH CDR 1.3"; SEQ ID NO:1), GYTFSSSW ("VH CDR 1.4"; SEQ ID NO:7), GYTFTSYY ("VH CDR 1.16"; SEQ ID NO:39), and GYSITSDYA ("VH CDR 1.19"; SEQ ID NO:40);

(v) VH CDR2 is selected from FYPGNSDS ("VH CDR 2.3"; SEQ ID NO:2), FLPGSGST ("VH CDR 2.4"; SEQ ID NO:8), INPSNGGT ("VH CDR 2.16"; SEQ ID NO:43), and ISFSGYT ("VH CDR 2.19"; SEQ ID NO:44); and

(vi) VH CDR3 is selected from TRRDSPQY ("VH CDR 3.3"; SEQ ID NO:3), ATDGNYDWFAY ("VH CDR 3.4" SEQ ID NO:9), TRGGYYPFDY ("VH CDR 3.16"; SEQ ID NO:47), and AREVNYGDSYHFDY ("VH CDR 3.19"; SEQ ID NO:48);

(d) antibodies having a VL that corresponds in sequence to the VL of MAb1, MAb2, MAb3, MAb4, MAb15, MAb16, MAb17, MAb18, MAb19 or MAb20 and a VH that corresponds in sequence to the VH of MAb1, MAb2, MAb3, MAb4, MAb15, MAb16, MAb17, MAb18, MAb19 or MAb20; and

(e) antibodies having a VL and a VH that corresponds in sequence to the VL and VH of MAb1, MAb2, MAb3, MAb4, MAb15, MAb16, MAb17, MAb18, MAb19 or MAb20.

[0103] With reference to Table 3B, specific embodiments of C-terminal anti-hPG antibodies useful in the methods and kits described herein include, but are not limited to, the following:

(f) antibodies having VL CDRs that correspond in sequence to the VL CDRs of MAb5, MAb6, MAb7, MAb8, MAb9, MAb10, MAb11, MAb12, MAb13, MAb14, MAb21, MAb22or MAb23 and VH CDRs that correspond in sequence to the VH CDRs of MBb5, MAb6, MAb7, MAB8, MAB9, MAb10, MAb11, MAb12, MAb13, MAb14, MAb21, MAb22 or MAb23;

(g) antibodies having VL CDRs and VH CDRs that correspond in sequence to the VL and VH CDRs of MAb5, MAb6, MAb7, MAb8, MAb9, MAb10, MAb11, MAb12, MAb13, MAb14, MAb21, MAb22 or MAb23;

(h) antibodies in which:

(i) VL CDR1 is selected from KSLRHTKGITF ("VL CDR 1.8"; SEQ ID NO:49) and QSLLDSDGKTY ("VL CDR 1.13"; SEQ ID NO:50);

(ii) VL CDR2 is selected from QMS ("VL CDR 2.8"; SEQ ID NO:52) and LVS ("VL CDR 2.13"; SEQ ID NO:53);

(iii) VL CDR3 is selected from AQNLELPLT ("VL CDR 3.8"; SEQ ID NO:55) and WQGTHFPQT ("VL CDR 3.13"; SEQ ID NO:56);

(iv) VH CDR1 is selected from GFTFTTYA ("VH CDR 1.8"; SEQ ID NO:37) and GFIFSSYG ("VH CDR 1.13"; SEQ ID NO:38);

(v) VH CDR2 is selected from ISSGGTYT ("VH CDR 2.8"; SEQ ID NO:41) and INTFGDRT ("VH CDR 2.13"; SEQ ID NO:42); and

(vi) VH CDR3 is selected from ATQGNYSLDF ("VH CDR 3.8"; SEQ ID NO:45) and ARGTGTY ("VH CDR 3.13"; SEQ ID NO:46);

(i) antibodies having a VL that corresponds in sequence to the VL of MAb5, MAb6, MAb7, MAb8, MAb9, MAb10, MAb11, MAb12, MAb13, MAb14, MAb21, MAb22or MAb23 and a VH that corresponds in sequence to the VH of MAb5, MAb6, MAb7, MAb8, MAb9, MAb10, MAb11, MAb12, MAb13, MAb14, MAb21, MAb22 or MAb23; and

(j) antibodies having a VL and a VH that correspond in sequence to the VL and VH that correspond in sequence to the VL and VH of MAb5, MAb6, MAb7, MAb8, MAb9, MAb10, MAb11, MAb12, MAb13, MAb14, MAb21, MAb22 or MAb23.

[0104] As noted in Tables 4A & 4B, several N-terminal and C-terminal monoclonal anti-hPG antibodies have been identified. All of these antibodies are specific for hPG, and all but MAb14 exhibit neutralizing activity on colorectal cancer cells. Although neutralizing activity may be important for therapeutic applications, it is not necessary for the diagnostic purposes of this disclosure. Thus, both non-neutralizing and neutralizing antibodies that specifically bind hPG are useful for the various diagnostic methods described herein.

[0105] The affinity of an anti hPG antibody is not critical to the diagnostic methods of the disclosure, but high affinity antibodies improve the sensitivity of progastrin detection. Furthermore, high affinity antibodies are necessary for therapeutic applications. Accordingly, there may be advantages to using antibodies exhibiting affinities of at least about 1 nM; for example, an affinity of at least about 90 nM, 80 nM, 70 nM, 60 nM, 50 nM, 40 nM, 30 nM, 20 nM, 15 nM, 10 nM, 7 nM, 6 nM, 5 nM, 4 nM, 3 nM, 2 nM, 1 nM, 0.1 nM, 0.01 nM, 0.001 nM or even greater.

[0106] The measured affinities of the anti-hPG monoclonal antibodies identified in Tables 4A & 4B range from 10^{-6} to 10^{-12} M, as noted in Table 5, below:

Table 5

Monoclonal Antibody	Affinity constant measured KD (M)
Anti-hPG MAb 1	2.5 μM (2.5 x10^{-6}M)
Anti-hPG MAb 2	185 nM (1.85 x10^{-7}M)
Anti-hPG MAb 3	6.4 nM (6.4 x10^{-9}M)
Anti-hPG MAb 4	3.5 nM (3.5 x10^{-9}M)
Anti-hPG MAb 5	13 pM (1.30 x10^{-11}M)
Anti-hPG MAb 6	0.6 nM (6.38 x10^{-10}M)
Anti-hPG MAb 7	58 pM (5.84 x10^{-11}M)
Anti-hPG MAb 8	0.1 nM (1.08 x10^{-10}M)
Anti-hPG MAb 10	3.6 nM (3.62 x10^{-9}M)
Anti-hPG MAb 11	0.3 nM (3.12 x10^{-10}M)
Anti-hPG MAb 12	0.4 nM (4.43 x10^{-10}M)
Anti-hPG MAb 13	0.6 nM (6.12 x10^{-10}M)
Anti-hPG MAb 14	6.8 pM (6.86 x10^{-12}M)
Anti-hPG MAb 15	0.2 nM (2.11 x10^{-10}M)
Anti-hPG MAb 16	0.2 nM (2.78 x10^{-10}M)
Anti-hPG MAb 17	8.3 nM (8.29 x10^{-9}M)
Anti-hPG MAb 18	1.2 nM (1.24 x10^{-9}M)
Anti-hPG MAb 19	0.7 nM (7.79 x10^{-10}M)
Anti-hPG MAb 20	0.2 nM (2.47 x10^{-10}M)
Anti-hPG MAb 21	3.9 nM (3.90 x10^{-9}M)
Anti-hPG MAb 22	5 nM (4.94 x10^{-9}M)
Anti-hPG MAb 23	0.4 μM (3.99 x10^{-7}M)

[0107] An anti-PG monoclonal antibody having an affinity especially suited for a particular desired application can be readily selected from amongst these, or generated or designed using the various immunogens, complementarity determining region (CDR) sequences, variable heavy (VH) and variable light (VL) chain sequences of anti-hPG antibodies described herein. The affinity of any particular anti-PG monoclonal antibody

can be determined using techniques well known in the art or described herein, such as for example, ELISA, isothermal titration calorimetry (ITC), BIAcore, or fluorescent polarization assays. A specific assay is provided in Example 5.

[0108] As will be recognized by skilled artisans, anti-hPG antibodies having specific binding properties, such as the ability to bind a specific epitope of interest, can be readily obtained using the various antigens and immunogens described herein and assessing their ability to compete for binding hPG with a reference antibody of interest. Any of the anti-hPG antibodies described herein can be utilized as a reference antibody in such a competition assay. A specific assay useful for assessing the ability of an antibody to compete for binding hPG with a biotinylated reference anti-hPG antibody of interest is provided in Example 6.

[0109] In conducting an antibody competition study between a reference anti-hPG antibody and any test antibody (irrespective of species or isotype), one may first label the reference with a label detectable either directly, such as, for example, a radioisotope or fluorophore, or indirectly, such as, for example biotin (detectable via binding with fluorescently-labeled streptavidin) or an enzyme (detectable via an enzymatic reaction), to enable subsequent identification. In this case, a labeled reference anti-hPG antibody (in fixed or increasing concentrations) is incubated with a known amount of hPG, forming a hPG-labeled anti-hPG antibody complex. The unlabeled test antibody is then added to the complex. The intensity of the complexed label is measured. If the test antibody competes with the labeled reference anti-hPG antibody for hPG by binding to an overlapping epitope, the intensity of the complexed label will be decrease relative to a control experiment carried out in the absence of test antibody.

[0110] Numerous methods for carrying out binding competition assays are known and can be adapted to yield results comparable to the assay described above and in Example 6.

[0111] An antibody is considered to compete for binding hPG with a reference anti-hPG antibody, and thus considered to bind approximately the same or an overlapping epitope of hPG as the reference anti-hPG antibody, if it reduces binding of the reference anti-hPG antibody to hPG in a competitive binding assay, and specifically the competitive binding assay of Example 6, by at least 50%, at a test antibody concentration in the range of 0.01

to 100 μg/mL (*e.g.*, 0.01 μg/mL, 0.08 μg/mL, 0.4 μg/mL, 2 μg/mL, 10 μg/mL, 50 μg/mL or 100 μg/mL or other concentration within the stated range), although higher levels of reduction, for example, 60%, 70%, 80%, 90% or even 100%, may be desirable

[0112] As will be appreciated by skilled artisans, anti-hPG antibodies useful in the diagnostic methods can be of any origin, including, for example, mammalian (*e.g.*, human, primate, rodent, goat or rabbit), non-mammalian, chimeric in nature (derived from more than one species of origin) and/or CDR-grafted (*e.g.*, humanized).

[0113] Methods for humanizing antibodies, including methods for designing humanized antibodies, are also well-known in the art. See, *e.g.*, Lefranc *et al.* (2003) *Dev. Comp. Immunol.* 27:55-77; Lefranc *et al.* (2009) *Nucl. Acids Res.* 37:D1006-1012; Lefranc (2008) *Mol. Biotechnol.* 40:101-111; Riechmann *et al.* (1988) *Nature* 332:323-7; U.S. Patent Nos. 5,530,101, 5,585,089, 5,693,761, 5,693,762 and 6,180,370 to Queen *et al.*; EP239400; PCT publication WO 91/09967; U.S. Patent No. 5,225,539; EP592106; EP519596; Padlan (1991) *Mol. Immunol.* 28:489-498; Studnicka *et al.* (1994) *Prot. Eng.* 7:805-814; Roguska *et al.* (1994) *Proc. Natl. Acad. Sci.* 91:969-973; and U.S. Patent No. 5,565,332, the disclosures of which are hereby incorporated by reference in their entireties.

[0114] Humanized versions of antibodies having CDR sequences corresponding to the CDRs of non-human anti-hPG antibodies, including by way of example and not limitation, the various N-terminal anti-hPG monoclonal antibodies provided in Table 3A and the various C-terminal anti-hPG monoclonal antibodies provided in Table 3B, can be obtained using these well-known methods. Projected sequences for humanized VL and VH chains of selected anti-hPG antibodies are provided in Tables 4A and 4B. Either murine or humanized antibodies may be used for the diagnostic purposes of the disclosure. Specific examples of humanized antibodies include antibodies comprising:

(a) any three VL CDRs and any three VH CDRs disclosed herein;

(b) a heavy chain variable region comprising an amino acid sequence corresponding to SEQ ID NO:21 and a light chain variable region comprising an amino acid sequence corresponding to SEQ ID NO:22;

(c) a heavy chain variable region comprising an amino acid sequence corresponding to SEQ ID NO:23 and a light chain variable region comprising an amino acid sequence corresponding to SEQ ID NO:24;

(d) a heavy chain variable region comprising an amino acid sequence selected from the group consisting of SEQ ID NO:75, 77, and 79 and a light chain variable region comprising an amino acid sequence selected from the group consisting of SEQ ID NO:76 and 78;

(e) a heavy chain variable region comprising an amino acid sequence selected from the group consisting of SEQ ID NO:80 and 82 and a light chain variable region comprising an amino acid sequence selected from the group consisting of SEQ ID NO:81 and 83;

(f) a heavy chain variable region comprising an amino acid sequence selected from the group consisting of SEQ ID NO:84, 86, and 88 and a light chain variable region comprising an amino acid sequence selected from the group consisting of SEQ ID NO:85, 87, and 89; and

(g) a heavy chain variable region comprising an amino acid sequence selected from the group consisting of SEQ ID NO:90, 92, and 94 and a light chain variable region comprising an amino acid sequence selected from the group consisting of SEQ ID NO:91, 93, and 95.

[0115] The anti-PG monoclonal antibodies and antibody fragments used in the methods of the present disclosure can be derivatized, *e.g.*, covalently modified or conjugated to other molecules.

[0116] In certain embodiments, the anti-PG antibodies or fragments thereof are conjugated to a diagnostic agent. Detection of an anti-PG antibody-bound hPG can be facilitated by coupling the antibody to a substance that can be detected directly or indirectly. Examples of detectable substances include various enzymes, prosthetic groups, fluorescent materials, luminescent materials, bioluminescent materials, radioactive materials, positron emitting metals using various positron emission tomographies, and nonradioactive paramagnetic metal ions.

[0117] The detectable substance may be coupled or conjugated either directly to the antibody (or fragment thereof) or indirectly, through an intermediate (such as, for example, a linker known in the art) using techniques known in the art.

[0118] Examples of suitable enzymes include horseradish peroxidase, alkaline phosphatase, beta-galactosidase, or acetylcholinesterase. Bioluminescent, chemiluminescent and/or chromogenic substrates for these enzymes are known in the art. For example, when the enzyme is alkaline phosphatase, the substrate may include chemiluminescent substrates such as AMPPD® (3-(2′-spiroadamantane)-4-methoxy-4-(3″-phosphoryloxy)phenyl-1,2-dioxetane), CDP-star® (disodium 4-chloro-3-(methoxyspiro{1,2-dioxetane-3,2′-(5′-chloro)tricyclo[3.3.1.1^{3,7}]decan}-4-yl)phenyl phosphate) and CSPD® (disodium 3-(4-methoxyspiro{1,2-dioxetane-3,2-(5′-chloro)tricyclo[3.3.1.1^{3,7}]decan}-4-yl)phenyl phosphate); chromogenic substrates such as p-nitrophenyl phosphate, 5-bromo-4-chloro-3-indolyl-phosphate (BCIP), 4-nitroblue tetrazolium chloride (NBT) and iodo nitro tetrazolium (INT) and the like.

[0119] Examples of metal ions that can be conjugated to anti-hPG antibodies for use in the diagnostic methods of the disclosure are disclosed in U.S. Pat. App. No. 4,741,900. Examples of suitable prosthetic group complexes include streptavidin/biotin and avidin/biotin. Examples of suitable fluorescent materials include umbelliferone, fluorescein, fluorescein isothiocyanate, rhodamine, dichlorotriazinylamine fluorescein, dansyl chloride or phycoerythrin. An example of a luminescent material is luminol. Examples of bioluminescent materials include luciferase, luciferin, and aequorin. Examples of suitable radioactive material include ^{125}I, ^{131}I, ^{111}In or ^{99}Tc.

[0120] Methods of coupling antibodies to detectable substrates are known in the art. Typical techniques are described by Kennedy *et al.*, (1976) *Clin. Chim. Acta* 70:1-31); Schurs *et al.* (1977) *Clin. Chim Acta* 81:1-40; *Antibodies: A Laboratory Manual* Harlow & Lane, eds., Cold Spring Harbor Laboratory Press (1988) at ch. 9; *Bioconjugate Techniques*, Hermanson, Academic Press (2008).

[0121] Antibodies may also be attached to solid supports, which are particularly useful for immunoassays or purification of hPG. Such solid supports include, but are not limited to, glass, cellulose, polyacrylamide, nylon, polystyrene, polyvinyl chloride or polypropylene.

[0122] Although the various anti-hPG antibodies useful in the methods and kits described herein have been exemplified with full length antibodies, skilled artisans will appreciate that binding fragments, or surrogate antibodies designed or derived from full-length antibodies or binding fragments, may also be used. Suitable fragments, surrogates, *etc.*, include, but are not limited to, Fab', $F(ab')_2$, Fab, Fv, vIgG, scFv fragments and surrobodies.

6.5. Kits

[0123] In an aspect of the disclosure, kits are provided for use in diagnostic and research applications as suggested above. In some embodiments, a kit is provided comprising anti-hPG antibodies and reagents necessary to detect and/or quantify hPG in a sample and can include assay reagents and buffers. In addition, the kits can include instructional materials containing instructions (*e.g.*, protocols) for the practice of diagnostic methods. While the instructional materials typically comprise written or printed materials, they are not limited to such. A medium capable of storing such instructions and communicating them to an end user is contemplated by this invention. Such media include, but are not limited to, electronic storage media (*e.g.*, magnetic discs, tapes, cartridges, chips), optical media (*e.g.*, CD ROM), and the like. Such media can include addresses to Internet sites that provide such instructional materials.

[0124] The kits can be adapted for the diagnosis or screening of patients already diagnosed with one or more conditions. For example, the kit can contain instructional materials for patients diagnosed with hepatitis C, so that a diagnosis of additional liver pathologies based on hPG levels can be made. The materials can also be directed to patients diagnosed with other liver conditions such as cirrhosis and/or liver cancer. The kits can also be adapted for the screening of high-risk populations, including populations where hepatitis C or other liver pathologies are more prevalent.

6.6. Automated Methods and Implementations

[0125] In some embodiments of the disclosure, the method of diagnosing or determining an indicated risk for one or more liver pathologies is implemented in whole or in part by a machine. For example, the method can be practiced by a diagnostic unit comprising a blood sample input. The blood sample is then manipulated by the machine to produce a progastrin level. Such manipulation can include binding of progastrin in the blood

sample with one or more antibodies. The progastrin-antibody sample can comprise a detectable marker. Measurement of the detectable marker provides a data output that can be delivered to a display. Alternatively, the data output can be correlated by a computer according to the progastrin level with one or more liver pathologies. In another embodiment, the data output can be compared with a threshold value of hPG, for example 400, 450, 500, 550, 600, 650, or 700 pM. If the determined hPG level is above the threshold, a diagnosis of one or more liver pathologies is made.

[0126] The computer can further comprise a database of statistical or experiential information. The information can be used to correlate the quantified progastrin level and additional data which can be inputted by a user to provide a more detailed or accurate diagnosis or indication of risk of one or more liver pathologies. The database can also comprise information as to recommended treatment or further diagnostic options. Accordingly, the methods of the disclosure can be implemented in the form of a computerized medical diagnostic method. Exemplary automated systems useful for implementing the assay diagnostics of the present disclosure include those described in U.S. Pat. Nos. 6,063,026, 6,063,340, and 7,381,370.

[0127] In general, such automated systems can comprise one or more of the following: blood or sample input units, blood or sample manipulation units, hPG and/or hPG marker detection units, a CPU with storage for one or more databases, a display and/or communication unit, or units combining the functionality of any one of the previous units. The units are integrated to receive a sample, process the sample using reagents to provide a detectable marker indicative of hPG levels, detect the level of marker, correlate the level of marker with a hPG level, and provide an output of the information, namely the determined hPG level and optionally a diagnosis based on the determined hPG level. The units can also integrate with a CPU and database to provide additional information such as a diagnosis of liver cancer, hepatitis C, cirrhosis, or a combination thereof based on the determined hPG level and optional additional information provided by the automated assay or inputted by a user. In some aspects, the units can integrate with non-automated methods. For example, a partially automated method can comprise units for receiving and manipulating sample. The manipulated sample can then be submitted to testing by way of a non-automated method.

7. EXAMPLES

Example 1: Quantification of Plasma Progastrin

[0128] 96-well microtiter plates are coated with between 0.5 and 10 μg/mL of a C-terminal anti-hPG antibody, for example, a rabbit C-terminal anti-hPG polyclonal antibody, and then incubated overnight. Plates are then washed three times in PBS-Tween (0.05%) and blocked with 2% (w/v) nonfat dried milk in PBS-Tween (0.05%). Separately, test samples, control samples (blank or PG-negative plasma or serum samples), and between about 5 pM (0.5×10^{-11} M) and about 0.1 nM (1×10^{-10} M) of an hPG reference standard (lyophilized hPG diluted in PG-negative plasma or serum) are prepared in an appropriate diluent (*e.g.*, PBS-Tween 0.05%). Samples are incubated on the coated plates for between 2 and 4 hours at 37°C, or alternatively between 12 and 16 hours at 21°C. After incubation, plates are washed three times with PBS-Tween (0.05%) and incubated with between 0.001 and 0.1 μg/mL of an N-terminal anti-hPG monoclonal antibody as described herein, coupled to horseradish peroxidase (HRP) (Nakane *et al.*, 1974, *J. Histochem. Cytochem.* 22(12):1084–1091) for 30 minutes at 21°C. Plates are then washed three times in PBS-Tween (0.05%) and HRP substrate is added for 15 minutes at 21°C. The reaction is stopped by added 100 μL of 0.5M sulfuric acid and an optical density measurement is taken at 405 nm. Test sample hPG levels are determined by comparison to a standard curve constructed from the measurements derived from the hPG reference standard.

[0129] Other assays for competition are known and can be adapted to yield comparable results to the assay described above.

Example 2: Identification of an Increased Risk of or Diagnosis of One or More Liver Pathologies in a Patient

[0130] A patient who is suspected of having a liver pathology, or a patient who is undergoing screening, *e.g.*, a screening done as part of a routine physical, provides a biological sample such as plasma, serum or blood. The progastrin levels are quantified, for example, as shown in Example 1, to produce a number that corresponds to the progastrin concentration in the blood sample. In the methods of the disclosure, the number is generally within 1% to 5% (or less) of the true progastrin number, as assays specific for progastrin and not its precursors or products are used. This number is then

compared to a listing of liver pathologies associated with progastrin levels within a defined range. In other words, progastrin levels indicative of certain liver pathologies are compared to the quantified number. Certain sub-ranges can indicate differing levels of risk. For example, a low but elevated level of progastrin can indicate a moderate risk of one or more pathologies, whereas a high level of progastrin can indicate a high risk of one or more pathologies. The level can be compared with a threshold value, thereby producing a positive or negative test result. Optionally, a second biomarker can be tested to confirm a diagnosis or indication. For example, a patient who presents with a moderate risk of a liver pathology, but not a high risk, can have a second biomarker tested. If the second biomarker also indicates the same liver pathology, then a diagnosis of that pathology is more likely to be accurate. The means for determining progastrin levels can be provided in a kit. The kit can also provide statistical or other data correlating progastrin levels with one or more pathologies, particularly liver pathologies, and as optional supplemented by one or more additional biomarkers.

[0131] Patients who have already been diagnosed with a liver pathology can also undergo hPG testing. For example, a patient diagnosed with liver cancer can undergo hPG testing to determine whether the patient also has additional liver pathologies such as hepatitis C and cirrhosis. Because hepatitis C levels are not normally elevated except in the case where a patient presents with multiple liver pathologies, *e.g.*, liver cancer, cirrhosis, and hepatitis C, progastrin is a useful biomarker for the diagnosis of hepatitis C in such patients. Likewise, patients already diagnosed with hepatitis C or cirrhosis can be screened for additional pathologies by determining whether hPG levels are abnormal.

[0132] hPG levels can be used in some instances to confirm a diagnosis. For example, a patient who presents with abnormal test results for one or more biomarkers indicative of liver cancer or another liver pathology, can be screened for abnormally high levels of hPG to confirm the initial diagnosis. hPG levels can be quantified in at-risk populations as part of routine screening program. For example, because very high hPG levels are correlated with multiple liver pathologies and because patients presenting with hepatitis C typically co-present with at least one other liver pathology, populations with high levels of hepatitis C are likely candidates for hPG screening. hPG levels can also be used to

monitor the course of treatment of one or more liver pathologies. Decreasing hPG levels would be correlated with effective treatment.

Example 3: Kits, Units, Tests, and Diagnostics for the Diagnosis of One or More Liver Pathologies.

[0133] Articles comprising one or more diagnostics can be utilized in the methods of diagnosis described herein. For example, a blotter or chromatographic strip can be adapted to change color upon binding with blood or blood components from a patient wherein the progastrin level is above a threshold value. Alternatively, the kit can comprise reagents for binding a marker to progastrin in blood. Such a marker can be, for example, a radioisotope or chromophore. Detection and quantification of radiation (induced by excitation or otherwise) can then be used to generate a positive test result if the marker emission is of sufficient intensity to indicate progastrin levels above a threshold. Test results can be adapted to indicate ranges, such as risk values. For example, a kit can comprise a test adapted to display a first signal for one range of progastrin levels and additional signals for other ranges of progastrin levels.

[0134] In a particular example, a diagnostic unit or system comprises one or more sample chambers for manipulation of a blood sample, either by a technician or by an automated mechanism. The unit or system further comprises one or more reagents, such as antibodies that specifically bind to progastrin. In a unit or system for the specific assaying of progastrin, a sandwich assay can be performed using a plurality of antibodies provided in the unit or system. The system or unit can comprise a detector for detecting the amount of a marker used to quantify the level of bound-progastrin. The system or unit can further comprise a processor such as a computer programmed with instructions to correlate the level of detected marker with a level of bound-progastrin and to correlate a level of bound-progastrin with a diagnosis. Finally, the system or unit can comprise a unit for displaying or communicating the test results and/or diagnosis. In an alternative unit or system, the unit comprises a blood sample repository and one or more reagents that bind to progastrin. For example, a test strip impregnated or coated with reagents which contact progastrin and result in a color change can be used to provide a one-drop liver pathology test.

Example 4: ELISA Assay for Assessing Specificity of Anti-hPG Antibodies

[0135] Specificity of anti-hPG antibodies can be conveniently determined using an ELISA assays as follows. 96-well plates are incubated overnight at 4°C with appropriate concentration(s) of test polypeptide (*e.g.*, 25 and 50 ng recombinant human PG, and 50 and 250 ng CTFP or other gastrin-derived gene products) in Phosphate-Buffered Saline (PBS), after which the wells are washed three times with wash solution (PBS and 0.1% Tween-20), and then incubated for 2 hours at 22°C with 100 µL blocking solution (PBS, 0.1% Tween-20, 0.1% Bovine Serum Albumin or casein hydrolysate) per well. After blocking, the wells are washed three times and the antibody to be assayed (test antibody) is added. 100 µL of the test antibody (at 0.3 to 1 ng/mL) in PBS and 0.1% Tween-20 are added to each well. Plates are then incubated for 2 hours at 22°C, after which the test antibody solution is discarded and replaced, after a wash step (3X 100 µL wash solution, as noted above), with blocking solution containing a secondary antibody, a goat anti-mouse IgG (Fc) antibody coupled to horseradish peroxidase. After a 1-hour incubation with secondary antibody, 100 µL of substrate solution (*e.g.*, Fast OPD, or O-phenylenediamine dihydrochloride, available from Sigma-Aldrich Co., prepared according to manufacturer's directions) is added to each well and incubated in the dark for 20 minutes at 22°C. The reaction is stopped by adding 50 µL of 4N sulfuric acid and the amount of substrate catalyzed determined by measuring the optical density (O.D.) at 492 nm. Substrate conversion is proportional to the amount of primary (test) antibody bound to the antigen. Experiments are run in duplicate and OD measurements plotted as a function of antigen concentration. Test antibodies are scored as highly specific for PG if the measured O.D. is between 0.2 and 1.5 for hPG and there is no statistically significant signal above background with CTFP or any of the other gastrin-gene derived peptides, where the background is the average signal from control wells containing only PBS.

Example 5: Assay for Assessing Affinity of an Anti-hPG Antibody

[0136] Affinity constants of anti-hPG antibodies can be measured using the Proteon Technique (BioRad), according to Nahshol *et al.* (2008) *Analytical Biochemistry* 383:52–60, hereby incorporated by reference in its entirety. Briefly, for murine anti-PG

antibodies, an anti-mouse IgG antibody (50 μg/mL) is first coated on a sensor chip, making sure that the signal detected by the chip after injection of the antibody falls between 10,000 and 11,500 response units (RU). The murine anti-hPG antibody of interest (test antibody) is then injected (at a typical concentration of 30 μg/mL). If the test antibody binds sufficiently, and additional signal of at least 500 RU will be observed. A time-course of binding between test antibody and hPG is then obtained by injecting varying concentrations of hPG, for example 200 nM, 100 nM, 50 nM, 25 nM, and 12.5 nM, and detecting the level of association. Typically, several channels are available to test multiple antibodies in parallel in a single experiment, making it possible to assay binding of a single test antibody at different concentrations of hPG in parallel. One channel should be injected with a murine monoclonal antibody that is not specific to hPG as a control for non-specific binding and another channel should be injected with dilution buffer alone as a baseline for the background signal. Generally, no binding is detectable in the channel injected with non-specific murine antibody. Antibodies displaying a high level of association in this setting, which may result in saturation of the trapped monoclonal antibody by hPG, can be tested against lower hPG concentrations (50 nM, 25 nM, 12.5 nM, 6.25 nM and 3.125 nM), allowing for a more refined measurement.

[0137] Affinity constants (K_D) are calculated as the ratio between the dissociation constant (k_d) and the association constant (k_a). Experimental values can be validated by analyzing the statistically relevant similarity between experimental curves based on binding measurements and theoretical profiles.

[0138] Affinity constants of non-murine anti-hPG antibodies can be assessed in a similar format using an IgG specific for the species of origin of the anti-hPG test antibody.

Example 6: Assay for Assessing Competitive Binding With a Reference Anti-hPG Antibody

[0139] An assay with specificity for assessing whether an antibody of interest (test antibody) competes for binding hPG with a biotinylated reference anti-hPG antibody can be performed as follows. 96-well plates are coated with a capture anti-hPG antibody (polyclonal or monoclonal antibody recognizing an N-or C-terminal region of hPG that differs from the epitope recognized by the biotinylated reference anti-hPG antibody), at a concentration to be chosen within the range of 1–10 μg/mL, overnight at 4°C (0.1 to 1

μg/well). After blocking with blocking buffer (0.1% Tween-20, 0.1% BSA in PBS) for 2 hr at 22°C, recombinant hPG is added at a concentration ranging between 10 pM to 1 nM (10 to 1000 pg/well) and incubated for 2 hr at 22°C. Thereafter, the biotinylated reference anti-hPG antibody (or a mixture containing the biotinylated reference anti-hPG antibody) is added, along with increasing concentrations of unlabeled test antibody, and incubated for 1 hr at 22°C. After washing to remove unbound antibodies, detection of bound labeled reference anti-hPG antibody is performed by incubating the mixture with 50 ng/mL steptavidin-HRP for 1 hr at 22°C, followed by incubation with a fluorogenic substrate for horseradish peroxidase and quantification of the relative light units (RLU) in a luminometer. Assays are performed in duplicate.

[0140] Antibodies that compete with a reference anti-hPG antibody inhibit the binding of the reference antibody to hPG. An antibody that binds to substantially the same epitope, or with an overlapping epitope, as the reference antibody significantly reduces (for example, by at least 50%) the amount of reference anti-hPG antibody bound, as evidenced by a reduction observed RLUs.

[0141] A high control value is obtained from a control experiment carried out by incubating the labeled reference antibody with recombinant hPG without test antibody. A low control value is obtained from a control experiment carried out by incubating the labeled reference antibody with recombinant hPG in the presence of excess concentrations of the unlabeled reference antibody (the unlabeled reference antibody thus competing with the labeled antibody for binding to hPG). The capacity of test antibodies to compete with the reference anti-hPG antibody is then determined by incubating the labeled reference antibody with recombinant hPG in the presence of increasing concentrations of the unlabeled test antibody

[0142] In a test assay, a significant reduction in the observed RLUs in the presence of a test antibody indicates that the test antibody recognizes substantially the same epitope as the reference anti-hPG antibody.

[0143] The inhibition of binding can be expressed as an inhibition constant, or K_i, which is calculated according to the following formula:

$$K_i = \frac{IC_{50}}{1+[\frac{R_c}{K_d}]}$$

where "IC_{50}" is the concentration of test antibody that yields a 50% reduction in binding of the reference antibody, R_c is the reference anti-hPG Ab concentration and K_D is the dissociation constant of the reference anti-hPG antibody, a measure of its affinity for hPG. Useful test antibodies that compete with a reference anti-hPG antibody (for example, one of the anti-hPG antibodies described herein) will typically have K_is ranging from 10 pM to 100 nM under assay conditions described herein.

[0144] All publications, patents, patent applications and other documents cited in this application are hereby incorporated by reference in their entireties for all purposes to the same extent as if each individual publication, patent, patent application or other document were individually indicated to be incorporated by reference for all purposes. While various specific embodiments have been illustrated and described, it will be appreciated that various changes can be made without departing from the spirit and scope of the invention(s).

The claims defining the invention are as follow:

1. A method of diagnosing whether a patient is susceptible to present multiple liver pathologies, wherein a patient has been diagnosed with a first liver pathology, comprising the step of determining the human progastrin (hPG) level in a sample from said patient, wherein a progastrin level of at least 400pM is indicative that the patient is susceptible to present multiple liver pathologies.

2. The method according to claim 1, wherein said first liver pathology is selected from the group consisting of: liver cancer, cirrhosis, and hepatitis C.

3. The method according to claim 1 or 2, wherein said multiple liver pathologies are selected from the group consisting of:

- liver cancer and cirrhosis,
- liver cancer and hepatitis C,
- liver cancer, cirrhosis and hepatitis C.

4. The method according to any one of claims 1 to 3, wherein the step of determining the hPG level is carried out by an antibody that specifically binds hPG.

5. The method according to any one of claims 1 to 4, wherein the progastrin level is at least 500pM.

6. The method according to any one of claims 1 to 5, wherein said patient belongs to a population having a higher than average incidence of hepatitis C or said patient resides in a geographical region having a higher than average incidence of hepatitis C or said patient has a drug or alcohol dependency condition.

7. The method according to claim 1, comprising the steps:

(a) identifying a patient with a blood hPG concentration of at least about 400pM,

(b) testing said patient for at least two of the following conditions:

(i) liver cancer, using a radiography or imaging technique;

(ii) hepatitis C virus, using a nucleic acid-based assay; and

(iii) cirrhosis of the liver, by testing a blood sample from said patient for one or more serum markers of fibrosis,

thereby diagnosing whether the patient suffers from multiple liver pathologies.

8. A method of determining a patient's risk for a plurality of liver conditions, comprising:

(a) determining by a biochemical assay a patient's serum or plasma hPG levels; and

(b) on the basis of (a), assigning the patient a relative risk for one or more liver pathologies, wherein:

(i) an hPG level of below 100pM indicates that the patient is at a low risk for one or more liver pathologies;

(ii) an hPG level of between 100pM and 400pM indicates that a patient is at a high risk for having cirrhosis with or without liver cancer, and at an elevated risk for also having hepatitis C, and

(iii) an hPG level of above 400pM indicates that a patient is at a severe risk for having liver cancer, cirrhosis, and hepatitis C;

(c) wherein said patient is optionally tested using other diagnostic means to confirm that said patient does not have liver cancer if said patient has hPG levels below 100pM; and

(d) wherein said patient is optionally further tested using other diagnostic means for liver cancer and/or hepatitis C if the patient has hPG levels between 100pM and 400pM.