Human chorionic gonadotrophin protein core and sugar branches heterogeneity: basic and clinical insights

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BACKGROUND: Human chorionic gonadotrophin (hCG) is measured in serum and urine for the early detection of ectopic pregnancy, patients with higher risk of miscarriage, embryos or fetuses with chromosome abnormalities, prediction of pre-eclampsia or fetal growth restriction and identification or follow-up of trophoblast neoplasia. This review examines basic knowledge on the heterogeneity of hCG protein core and sugar branches and its relevance to assays used in a clinical setting.

METHODS: The databases Scielo and Medline/Pubmed were consulted for identification of the most relevant published papers. Search terms were gonadotrophin, glycoprotein structure, hCG structure and molecular forms of hCG.

RESULTS: The synthesis of alpha (hCGα) and beta (hCGβ) peptide chains and their further glycosylation involve the complex action of different enzymes. After assembly, hCG reaches the cell surface and is secreted as a bioactive heterodimer. The complex cascade of enzymes acting in hCG secretion results in heterogeneous molecular forms. The hCG molecules are differently metabolized by the liver, ovary and kidney, but the majority of hCG forms are excreted in the urine. Intact hCG, hCGα, hCGβ, hyperglycosylated (hCGh), nicked (hCGn) and core fragment of hCGβ (hCGβcf) forms have relevant clinical use. The immunogenicity of each hCG variant, their epitopes distribution and the available antibodies are important for the development of specific assays. Depending on the prevalent form or proportion in relation to the intact hCG, the choice of assay for measurement of a specific molecule in a particular clinical setting is paramount.

CONCLUSIONS: Measurement of hCG and/or its related molecules is useful in clinical practice, but greater awareness is needed worldwide regarding the use of new sensitive and specific assays tailored for different clinical applications.

Key words: human chorionicgonadotrophin / heterogeneity / structure / application

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Introduction

Human chorionic gonadotrophin (hCG) is mainly a product of placental syncytiotrophoblast cells. It can also be secreted by several normal non-placental tissues and trophoblastic or non-trophoblastic neoplasms (Stenman et al., 2006). Heterogeneity in structure and composition of hCG peptide chains and carbohydrate branches is a common finding in serum and urine samples, amniotic fluid and other bodily fluids (de Medeiros et al., 1992a). The heterogeneous nature of hCG has been demonstrated on the basis of charge, size, biological and immunoactivities (Hay, 1986). The clinical utility of the variant molecular forms should be viewed in the light of the extensive knowledge of their physicochemical properties. The aim of this review is to examine the structure of the native hCG molecule, to revisit the metabolic pathways involved in its secretion, to characterize the different molecular forms found in biological fluids and to correlate them with normal or abnormal conditions, with the objective of estimating their importance and usefulness clinically.

Methods

This review was structured in sections. An extensive online search of the published articles on hCG was performed. There was no restriction on the language of publication. The databases Scielo and Medline/Pubmed were consulted for identification of the most relevant papers published in the last 10 years. Earlier articles providing essential basic knowledge were also included. References of selected papers were hand-searched for additional relevant citations. Only articles or reviews published on journals with distinguished Editorial Board were consulted. Search terms included gonadotrophin, glycoprotein structure, human choriogonadotrophin, gonadotrophin heterogeneity, hCG structure and molecular forms of hCG.

Chemistry and structure of the standard hCG molecule

hCG has a molecular weight of 38 000 Da with 237 amino acids organized in two subunits, alpha and beta, each consisting of a single polypeptide chain. Seventy percentage of its structure is represented by the protein chains and 30% by carbohydrate units. The sugar branches, covalently bound to the peptide chains, are of two types: O-linked oligosaccharide containing an N-acetylgalactosamine residue linked to either a serine or a threonine residue and N-linked oligosaccharide contains an N-acetylgalactosamine residue linked to an asparagine residue (Gray, 1998).

The alpha and beta hCG subunit (hCGα and hCGβ) composition is given in Table I. hCGα is structured in three loops stabilized by disulfide bonds. These bonds, initially assigned between residues 7–31, 10–32, 28–60, 59–87 and 82–84 (Mise and Bahl, 1980), were reassigned at positions 7–31, 59–87, 10–60, 28–82 and 32–84 (Lapthorn et al., 1994; Xing et al., 2001) (Fig. 1A and B). The last three bonds comprise the cystine knot. The two N-linked complex-type carbohydrate moieties are attached to the second and third loops asparagine residues (Table I). The hCGβ has a highly glycosylated carboxyl terminal peptide (CTP) extension, rich in serine and proline, which confers the immunological and biological specificity to the whole hCG. The hCGβ disulfide bonds initially assigned at positions 9–90, 26–110, 34–88, 32–72, 38–57 and 93–100 (Mise and Bahl, 1981; Fig. 2A) were further reassigned at positions 23–72, 26–110, 34–88, 38–90, 9–57 and 93–100 (Lapthorn et al., 1994; Fig. 2B). The hCGβ molecule has two beta hairpin loops, stabilized by the disulfide bond 23–72 on one side of a central cystine knot and a long loop on the other side (Xing et al., 2001). The cystine knot is formed by a ring (residues β34–88 and

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### Table I Alpha and beta hCG composition

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<td>SA α2,3 Gal β1,4 GlcNAcβ1,2 Manα1,6 SA β1,4 GlcNAcβ1,4 GlcNAc - Asp</td>
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SA, sialic acid; gal, galactose; GlcNAc, N-acetylglucosamine; man, mannose.
**Figure 1** (A) Primary sequence of hCGα and locations of the disulfide bonds. From Mise and Bahl (1980), with permission.
(B) hCGα protein core and reassigned disulfide bonds forming three hairpin loops. From Xing et al. (2001), with permission.

**Figure 2** (A) The amino acid sequence of hCGβ showing the locations of disulfide bonds and carbohydrate moieties (CHO). From Mise and Bahl (1981), with permission.
(B) hCGβ subunit polypeptide chain (thick green line) with its seatbelt region (thick black line) and reassigned disulfide bonds. Numbers, amino acid numbers; yellow bars, cystine knots; thick blue lines, seatbelt disulfide bonds; thin blue line, small loop disulfide bond; β1, β2, β3, loops of hCGβ. From Xing et al. (2001), with permission.
Histidine at position 9–57 disulfide bridge penetrates. In its carbohydrate branches, hCG has asparagine units on both α and β subunits and unique O-glycosidically linked oligosaccharide moieties on hCGβ.

**Biosynthesis of hCG**

Both biosynthesis and processing of the hCG molecule resemble that of other glycoprotein hormones. Although the cell type determines the sort of oligosaccharide processing on alpha and beta subunits, the alpha–beta combination modulates the extension of this processing (Corless et al., 1987). The set of enzymes contained in the secretory cell determines the nature of the processing and composition of the oligosaccharides added to both subunits. Initially, each subunit is synthesized separately, in a non-balanced ratio, as a result of mRNA transcription of the two separate genes. The alpha subunit is usually synthesized in excess, and, in pregnant women, the ratio α:β subunits increases from 1:7:1.0 in the first trimester to 12.0:1.0 at term (Boothby et al., 1983). The metabolic sequence, cleavage of signal peptides, assembly of the native hCG, sequential post-translational glycosylation and formation of the disulfide bonds happens at the same time that the hCG molecules are translocated from the place of synthesis in the endoplasmic reticulum (RER) up to the cell surface (Bielinska and Boime, 1979).

**Biosynthesis and glycosylation of alpha and beta polypeptide chains**

The mechanism of synthesis of the protein portion of hCG is similar to the classical mechanism of secretory protein biosynthesis and resembles other glycoproteins. The two subunits that form the whole molecule are transcribed from separate genes. While the alpha subunit is encoded by a single gene (Fiddes and Goodman, 1981), the beta subunit is encoded by a family of at least six genes arranged in tandem in a cluster on chromosome 19 (Policastro et al., 1983). Initially, it was believed that only three beta hCG genes are functional genes encoding the correct hCGβ amino acid sequence; however, currently, it is suggested that the total amount of hCGβ gene expression rather than the expression of individual genes is important for the maintenance of normal pregnancy (Miller-Lindholm et al., 1997). The α and β genes expression may be regulated by many factors, but a key role in the control of their expression is not clear yet. Little is known about the intimate mechanisms which control hCG synthesis.

hCG secretion is under the control of a large number of factors which may act by both autocrine and paracrine mechanisms (Jameson and Hollenberg, 1993). At a cellular level, these modulators interact with specific surface receptors expressed on placental trophoblastic cells. Under stimulation of these factors, hCGα and hCGβ genes are activated by phosphorylation of a cAMP response element binding protein through the protein kinase C pathway. A second messenger is triggered in trophoblast cells. Coordinate activation of hCG synthesis might be established by a trophoblast-specific transcription, perhaps an apoprotein (AP-2) member, the ultimate regulator of hCG genes expression at the promoter region (Johnson et al., 1997). After transcription of the genetic message, the translation of the mRNA into the nascent peptide moieties of α and β subunits takes place in the membrane-bound ribosomes of the RER. Each translated product is synthesized as a slightly larger molecular weight immature peptide, named pre-alpha or pre-beta forms, containing the specific sequences and the signal peptide extension with 24 and 20 amino acids, respectively. The processing of immature subunits to the mature state involves co-translational signal peptide cleavage and removal by microsomal signal peptides while the peptide chains are still reside upon the ribosomes. Just before the oligosaccharide moieties are attached to the polypeptide chain, the newly synthesized protein is released into RER channels and transported to the Golgi (Ruddon et al., 1987).

Endocrine regulation of hCG glycosylation is still poorly understood, but some specific placental and/or extraplacental factors may influence this process. The addition of carbohydrate chains to hCG subunits is performed by the sequential action of a number of RER and Golgi enzymes (Kornfeld and Kornfeld, 1985) (Fig. 3). The N-linked oligosaccharide precursor, rich in mannose residues, is transferred ‘en block’ to each subunit, by activity of an oligosaccharyl transferase (Hubbard and Ivatt, 1981). After being attached to the nascent peptide, it undergoes a number of co-translational and post-translational processing reactions including removal of glucose and mannose residues and addition of N-acetylglucosamine, fucose, galactose and sialic acid (Table I). The process involving a well-organized sequence of steps involving the RER and the Golgi enzymes is completed only after the assembly of the dimer hCG, shortly before its secretion (Hanover et al., 1982). The O-linked oligosaccharide branch does not arise from the dolichol ester intermediate but rather occurs by the addition of one residue at a time, directly on the hCGβ polypeptide chain. The addition of the O-linked moieties takes place in the Golgi and the sequential reaction of residual incorporation, involving specific enzymes, consists of the attachment of N-acetylgalactosamine to serine residues 121, 127, 132 and 138, linkage of galactose residue to N-acetylgalactosamine and further attachment of sialic acid to galactose (Table I) (Kessler et al., 1979a).

**Assembly of hCG**

The association of hCGα:hCGβ subunits form the complete dimer and both subunits still contain a high content of mannose. The two subunits are intimately associated with each other along much of their surfaces, each subunit having similar folds with two hairpin loops at one end and a single loop at the other (Wu et al., 1994). The assembly of hCG in the RER is made by threading the glycosylated end of hCGα loop 2 beneath a hole formed in a disulfide latched strand of the β-subunit named seatbelt (Xing et al., 2004). The CTP of hCGβ is in contact with hCGα in the native dimer and forms the seatbelt around the hCGα (residues 89–110) that stabilizes the heterodimer. The final closing of the β26–110 bridge locks the seatbelt and secures the αβ dimer, preventing disassembly (Ruddon et al., 1996).

**Role of the carbohydrate and peptide chains on the hCG assembly**

Folding and assembly of the subunits are dependent on the carbohydrate moieties and their specific positions on the peptide chains. Although the carbohydrates are not obligatory for the formation of
the correct tertiary structure of alpha subunit during the folding process, they may prevent the formation of non-active disulfide bonds and ensure that certain portions of the polypeptide chain remain on the surface of the molecule during the folding reaction (Bielinska et al., 1989). Although the O-linked oligosaccharides do not appear to affect neither the assembly nor secretion of hCG, the N-linked oligosaccharide is important for both processes. The oligosaccharide branches from Asn-52 and Asn-78 assures normal secretion of hCG \( \alpha \) and its removal reduces correct dimerization (Matzuk and Boime, 1988; Feng et al., 1995). The Asn-30 oligosaccharide on the hCG \( \beta \) is important for secretion but not assembly, and the Asn-13 branch influences mainly the assembly (Fares, 2006). The polypeptide sequences \( \alpha_{27-40} \) and \( \alpha_{38-42} \) have an important role in the dimer formation. Whereas \( \alpha_{\text{Tyr-37}} \) is a critical residue for proper combination of \( \alpha \beta \), the \( \alpha_{\text{Tyr-65}} \) is involved in holding both subunits in native conformation. In addition, the single substitution of \( \alpha_{\text{Thr 39}} \) with Phe or Ala eliminates or reduces \( \alpha \beta \) dimerization (Xia et al., 1994). The residues \( \beta_{90-111} \) wrap around a helical loop of the hCG\( \alpha \) and play an important role in subunits assembly, but the whole hCG\( \beta \)-CTP (\( \beta_{115-145} \)) extension is not required for subunit association. Besides the sugar branches and the polypeptide composition of \( \alpha \) and \( \beta \) subunits, the final conformation of the hCG dimer is stabilized by disulfide bonds and by proper readjustment after subunits assembly. All non-cysteine residues within the hCG cystine knot are required for the formation and assembly of the dimer. Disulfide bonds \( \alpha_{7-31}, \alpha_{59-87} \) and \( \alpha_{10-32} \) are not essential for the hCG\( \alpha \) combination with the hCG\( \beta \), but the hCG\( \beta \) disulfide bonds \( \beta_{9-57}, \beta_{34-88} \) and \( \beta_{38-90} \) are essential for heterodimer formation (Mishra et al., 2003). The disulfide bond \( \beta_{26-110} \) is formed only after \( \alpha \beta \) assembly (Huth et al., 1992). The \( \alpha \beta \) subunits are aligned ‘head-to-toe’ such that \( \alpha_{2} \) loop is adjacent to \( \beta_{1}, \beta_{3} \) and \( \beta_{2} \) is adjacent to \( \alpha_{1}, \)

**Figure 3** Schematic pathway of oligosaccharide processing on hCG molecule.

Arabian numbers represent: 1, oligosaccharyltransferase; 2, \( \alpha \)-glucosidase I; 3, \( \alpha \)-glucosidase II; 4, RER \( \alpha_{1,2} \)-mannosidase I; 5, golgi \( \alpha \)-mannosidase I; 6, N-acetylglucosaminytranferase I; 7, golgi \( \alpha \)-mannosidase II; 8, N-acetylgalactosaminyl transferase II; 9, fucosyltransferase; 10, galactosyltransferase; 11, sialyltransferase. Roman numbers represent: I, N-acetylglucosaminylphosphotransferase; II, N-acetylglucosaminylphosphotransferase-I-phosphodiester \( \alpha \)-N-acetylgalactosaminidase. The symbols represent: ■ N-acetylglucosamine, ▲ glucose, ● galactose, ○ mannose, † fucose and ♂ sialic acid. From Kornfeld and Kornfeld (1985), with permission.
The cystine knot, with three disulfide bonds, creates a ring that includes the intervening polypeptide backbone, and a third bond penetrates this ring (Fig. 4B). The additional residues of hCGβ, termed seatbelt, surround the hCGα to stabilize the heterodimer (Lapthorn et al., 1994).

**Secretion of hCG**

The secretion of hCG shows spontaneous pulse-like bursts with irregular amplitudes and frequencies. The fine-tuned and dynamic pattern of hCG secretion may involve an up- and down-regulation of the GnRH receptor (Lin et al., 1995). The process involves a series of steps in which cells make up and release hCG, and hCGα/hCGβ free subunits. There is no releasing factor specific to hCG, although cAMP activates the genetic transcription of DNA sequences for synthesis of the polypeptide chains. The processing of the carbohydrate chains, their transport to the Golgi for αβ combination and release of hCG from the cell surface in circulation are not completely clear. The primary signal for enzyme expression can be stimulated by the cAMP analogs, epidermal growth factor, tumor necrosis factor-α, gonadotrophin-releasing hormone, estradiol, insulin, and glucocorticoids (Chardonnens et al., 1999; Morrish et al., 2007). The principal modulators with inhibitory activity are progesterational steroids and prolactin (Yuen et al., 1980; Szilágyi et al., 1992). Table II shows several regulator factors that seem to be involved in the hCG secretion.

**Figure 4** (A) Assembly of α and β subunits to form hCG dimer. Thick red line, hCGα polypeptide chains; thick green line, hCGβ polypeptide chains; numbers, amino acid numbers; Lα, loops of hCGα; Lβ, loops of hCGβ; yellow bars, cystine knots on α and β subunits; thick black line of hCGβ, seatbelt region; thick blue lines, seatbelt disulfide bonds; thin blue line, small loop disulfide bond of β-seatbelt; grey lines, other disulfide bonds. From Xing et al. (2001), with permission. (B) Spatial representation of αβ assembly in which the hCGα carboxyl terminal extension penetrates the hCGβ and is locked by the β-seatbelt portion. Adapted from Moyle et al. (1998).

α3 (Wu et al., 1994; Moyle et al., 1998; Fig. 4A). The cystine knot, with three disulfide bonds, creates a ring that includes the intervening polypeptide backbone, and a third bond penetrates this ring (Fig. 4B). The additional residues of hCGβ, termed seatbelt, surround the hCGα to stabilize the heterodimer (Lapthorn et al., 1994).

**Biological activity of hCG**

hCG binds to the specific receptor on the membrane of the target cell. The hCG/LH receptor is encoded by a single gene, located on human chromosome 2p21 and belongs to superfamily of G protein-coupled seven transmembrane (TM) domain receptors (Rousseau-Merck et al., 1990). The gene codes for protein receptor containing 701 amino acids structured in three distinct domains: a large N-terminal extracellular domain which binds hCG, a serpentine TM containing seven TM repeats connected by three extra- and intracellular loops (TM region) and a C-terminal tail under cellular membrane. This receptor is expressed early in life, prior to the time at which normal luteal regression would occur (Duncan et al., 1996). The early hCG secretion by developing trophoblast prevents its own receptor downregulation. The current knowledge of the hCG/LH receptor and its tertiary structure enhanced our comprehension of its endocrine function. The extracellular domains of hCG and other gonadotrophin receptors are members of the leucine-rich repeat (LRR) protein superfamily and are responsible for the high-affinity binding. hCGα and hCGβ subunits share a receptor-binding region and an agonist activity to adjacent areas of the molecule (Willey and Leidenberger, 1989). The tertiary model shows that the contact between hCG and its receptor is made by interacting residues in the curved portion of the extracellular domain of the receptor and the grooved in the hormone formed by the apoposis of the α loop 2 and β loops 1 and 3 (Moyle et al., 1995) (Fig. 5). The conformational
changes and the signal transduction led by hCG binding result from influence of hCG on the portion between the arms of the extracellular domains coupled to multiple sites to the TM domain. The extension of the hCG biological activity is dependent on the structure, proper conformational modifications in one or both subunits, and on specific regions of the protein chain and certain carbohydrate residues. Even though the ligand-binding portion of the molecule to the receptor is located on the surface of hCG, the Tyr-Tyr-His-Lys-Ser of the CTP portion of hCGα also are important for receptor binding (Chen et al., 1992). Alpha His-94 is more involved with receptor binding and α His-82 may be involved in the biological activation of the target cell. Similarities between hCG and serine proteases implicate an enzymatic cascade in the hCG activation of the cell (Willey and Leidenberger, 1989). The α15–17 and α73–75 sequences contact the second extracellular loop of the hCG receptor and promote signal transduction (Couture et al., 1996). The α39–41 sequence contained in the long loop 2 which interfaces with loops 1 and 3 of the hCGβ subunit implicates in recognition for the receptor (Jackson et al., 1999). The CTP residues α87–92 also are important for receptor binding and biological expression (Chen et al., 1992). There appears to exist three major biological roles for the hCGα subunit after hCG formation: to carry some sites necessary for receptor binding, to induce active conformation of hCGβ and to stabilize the hormone-receptor complex (Milius et al., 1983). There are at least two peptide regions located between cysteine residues β38–57 and β93–100 (Prasad et al., 2007) named receptor-determined loops, within the hCGβ which confer receptor specificity. The surface able to activate the hCG receptor would include the majority of the hCGα and the Asp-99 residue contained in loops β93–100 (Bernard et al., 2004). The CTP hCGβ, not important for receptor binding or in vitro signal transduction, is critical for in vivo biological response (Chen and Puett, 1991). The N-terminal region of the hCGβ and the C-terminal of the hCGα also appear to be involved in receptor binding (El-Deiry et al., 1989). There is no general

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</table>

EGF, epidermal growth factor; LIF, leukemia inhibiting factor; TNF, tumor necrosis factor; M-CSF, macrophage-colony-stimulating factor; TGF, transforming growth factor; GABA, gamma amino butyric acid; PTH, parathyroid hormone; HGH, human growth hormone.
Agreement regarding which receptor regions are in contact with hCG. N-terminal peptides 7–40, central peptide 102–121 and CTP 259–273 of the ectodomain seem to bind hCG surface (Moyle et al., 1995). Sequences Arg21-Pro38, Arg102-Thr115, Try253-Phe272 and Lys513-Lys583 of the extracellular domain may also be important in receptor binding (Bhowmick et al., 1996). Then, binding of hCG to its receptor induces a conformational change in the cytoplasmic domain and generates a conventional signal transduction through the activation of the associated heterotrimeric G-protein with an increase in cAMP and consequent activation of protein kinase A (PKA) upon activation of the adenylyl cyclase (AC) pathway (Fig. 5) and an increase in the intracellular calcium through inositol triphosphate/phospholipase A2 pathway (Ryu et al., 1998). Alternatively, in endometrium, hCG induces phosphorylation of the extracellular signal-regulated kinase (ERK 1/2) in a PKA-independent manner and is involved in processes of proliferation, growth and differentiation (Cameo et al., 2004). hCG receptor selectivity is given by the N-terminal three-fifth of the exodomain, which includes a specific cysteine-rich cluster (NCR), flanked by LRR6 sequence on the surface of hCGR molecule. This sequence is able to distinguish the positive-charged seatbelt loop of hCG, between cys10 and cys11, from other gonadotrophins (Bogerd, 2007). Therefore, hCG receptor is not activated by other glycoprotein hormones. The antagonist effect of trypsin, chymotrypsin and of the serum protease inhibitor protinin suggests that these inhibitors bind to the binding site of the hCG receptor or to the hCG-binding site (Grewal et al., 1997). The carbohydrate moieties of hCG also have relevant roles in its biological activity. The N-linked carbohydrates on alpha, beta or both subunits have little binding effect and the removal of certain sugar residues even increase the affinity of hCG for its receptor.

**Figure 5** Model of hCG signal transduction showing the signaling specificity domain (SSD) on the extracellular surface of the transmembrane domain (TMD) and the leucine-rich domain (LRD) near the SSD–TMD complex.

The NH2- and COOH-terminal portions of the LRD contact the ends of the SSD and TMD helices. hCG binding increases the distance between the top of the SSD and the top of LRD, promotes the rotation of LRD and a gate-like movement of the LRD and creates a binding pocket for TMD rearrangement and signaling. After binding, hCG activates its receptor and the heterotrimeric G-protein-coupled receptor is formed. GDP is released from the G-protein and is replaced by GTP. This leads to dissociation of the G-protein subunits into α-subunit and βγ dimer. Ga activates adenylyl cyclase, which leads to an increase in intracellular cAMP levels, stimulation of PKA expression of steroidogenic acute regulatory protein (StAR), cholesterol uptake, and steroidogenic enzymes activation (P450scc, 3β-HSD, P450c17). Adapted from Moyle et al. (2004).
(Thotakura et al., 1990). The N-linked oligosaccharides αN78, and βN30 do not play a significant role in the function, but the αN52 is crucial for signal transduction (Matzuk et al., 1989). The Asn 13 oligosaccharide of hCGβ seems to play an important role in steroidogenesis. The mannose moieties also are essential in stimulating cAMP accumulation and steroidogenesis (Wang et al., 1989). The Asn 52 on hCGα also is critical for both the cAMP response and the steroidogenesis by maintaining the stability of the dimer and proper conformation of hCG and assuring dimer secretion (Heikoop et al., 1998). In addition, αAsn52 helps to position hCG in a favorable orientation for signal transduction (Matzuk et al., 1989). The sialic acid content of hCG has major significance in the receptor-binding ability and biological activity. Although the biological activity of hCG diminishes with the gradual desialylation or partial or complete removal of carbohydrate units internal to the sialic acid, the removal of certain residues of carbohydrates increases the affinity of hCG for its receptor (O’Connor et al., 1994). The β-O-linked branches, not involved in bioactivity, contribute to the longer half-life (Kalyan and Bahl, 1983). Although regular hCG functions promoting progesterone production at the corpus luteum hCG receptor, hyperglycosylated hCG (hCGH) has an autocrine rather than an endocrine function in growth, invasion and tumor formation (Elliott et al., 1997) via inhibition of apoptosis in cancer cells seemingly through the transforming growth factor TGFβ-I receptor (Cole et al., 2007). The hypothesis that some variant forms could act in other receptors, such as TGFβ-IIR, could explain the role of these molecules in certain abnormal pregnancy conditions or cancer tissues.

In addition to the maintenance of corpus luteum function, hCG is an important autocrine and paracrine regulator of epidermal growth factor, transforming growth factor, and leukemic inhibitory factor for increasing placental syncytium formation (Yang et al., 2003) and in blastocyst implantation (Licht et al., 2007). It also mediates glycolysis in human placental villi (Demers et al., 1973), stimulates prostaglandin synthesis by placental tissue and inhibits myometrium contractility (Ticcioni et al., 2007). The presence of hCG receptors on a variety of non-gonadal tissues suggests other functions. In cord blood, and amniotic fluid, hCG may regulate the vascular tone (Rao and Lei, 2007), and attenuates the vascular response to angiotensin II (Hermsteiner et al., 2002). In its free form, hCGβ seems to exert proper functions, with inhibitory or stimulatory activity in the cellular growth (Gillot et al., 1996). The free hCGα is linked with proactin secretion and control of endometrial cell differentiation (Blithe et al., 1991). In addition, free hCGα potentiates progesterone-mediated decidualization of endometrial stromal cells during normal menstrual cycles (Nemansky et al., 1998a).

**Immunological properties of hCG**

Three major types of epitopes have been defined on hCG, according to their localization on either subunit or on the hCG dimer. Most of epitopes on hCG are conformation-specific or discontinuous, made up of amino acids that juxtapose in the native conformation (Lund and Delves, 1998). Major immunogenic sites on both free hCGα and hCGβ subunits are determined by the first and third adjacent loops protruding from the cystine knot. A single epitope cluster in the junction between the cystine knot loop 2 to hCGβ and loop 1 of the hCGα distinguishes the dimer hCG from its free subunits (Norman et al., 1987). The second opposing loop is not immunogenic. The assembled hCGβ possesses four spatially distinct antigenic domains (Table III). Three are highly specific for hCG but are poorly immunogenic. The fourth domain has high immunogenicity and it is determined by the hCGβcf (epitopes B2–B3) (Bidart et al., 1993).

Several groups have investigated the hCG immune response. The group of Columbia University has delineated six domains on hCG: sites I, II, III, IV, V and VI (O’Connor et al., 1994). Four polyclonal antibodies, R141, R252, R529 and R561, bind to site I at the CTP (β115–145). Whereas R525 and R529 antibodies recognize carbohydrate-containing and measure intact hCG, free hCGβ and their desialylated preparations. R141 antibody recognizes desialylated hCG or βCTP in which the O-linked sugars terminate with galactose residues (Birken et al., 1988a). Several polyclonal and monoclonal antibodies bind to site II. This site is heterogeneous and either may contain residues in both α and β subunits or be a conformational epitope which is present only after the dimer formation. Site II is the most potent antigen site and recognizes heterodimeric hCG, hCGβ and the hCGβ core fragment (hCGβcf). Three monoclonal antibodies (B101, B107 and B109) bind to site III (β38–56) on dimeric hCG and measure exclusively intact hCG. Site IIIα recognizes antibody A102 which has affinity to free hCGα and antibody A102 which bind almost exclusively hCGα on hCG. Site IV (β100–109), binding B201, B202, B204, B205, B208 and B210 antibodies, is present on hCGβcf and on free hCGβ. B201 antibody does not discriminate between free hCGβ and hCGβcf, but it does not bind to dimeric hCG. B204 and B205 antibodies bind the hCGβcf with an affinity 10 times higher than that for free hCGβ. B210 antibody binds nearly exclusively hCGβcf, cross-reacting <0.1% with free hCGβ (O’Connor et al., 1994).

**Table III Localization of the immunogenic epitopes on hCG**

<table>
<thead>
<tr>
<th>Conformation-specific</th>
<th>Epitopes</th>
<th>Specificity</th>
<th>Immunogenicity</th>
</tr>
</thead>
<tbody>
<tr>
<td>hCGα</td>
<td>loop 1, loop 3; cystine knot</td>
<td>High</td>
<td>High</td>
</tr>
<tr>
<td>hCGβ</td>
<td>loop 1, loop 3; cystine knot</td>
<td>High</td>
<td>High</td>
</tr>
<tr>
<td>hCG</td>
<td>cystine knot loop 2 hCGβ + loop 1 hCGα</td>
<td>High</td>
<td>High</td>
</tr>
<tr>
<td></td>
<td>β seatbelt + C-terminus + loop 2 hCGα</td>
<td>High</td>
<td>High</td>
</tr>
<tr>
<td>Spatially antigenic</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>domain</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Assembled hCGα</td>
<td>α13–18</td>
<td>High</td>
<td>High</td>
</tr>
<tr>
<td></td>
<td>α17–22</td>
<td>—</td>
<td>—</td>
</tr>
<tr>
<td></td>
<td>α33–42</td>
<td>Mild</td>
<td>—</td>
</tr>
<tr>
<td></td>
<td>α87–92</td>
<td>—</td>
<td>—</td>
</tr>
<tr>
<td>Free hCGα</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Assembled hCGβ</td>
<td>Arg10, Arg60, Glu 89</td>
<td>High</td>
<td>Poor</td>
</tr>
<tr>
<td></td>
<td>β141–144</td>
<td>High</td>
<td>Poor</td>
</tr>
<tr>
<td></td>
<td>β113–116</td>
<td>High</td>
<td>Poor</td>
</tr>
<tr>
<td>Free hCGβ</td>
<td>β20–25</td>
<td>—</td>
<td>—</td>
</tr>
<tr>
<td></td>
<td>β68–77</td>
<td>Mild</td>
<td>—</td>
</tr>
<tr>
<td>HCGβcf</td>
<td>β20–25</td>
<td>Mild</td>
<td>High</td>
</tr>
<tr>
<td></td>
<td>β45–52</td>
<td>Mild</td>
<td>High</td>
</tr>
</tbody>
</table>

hCGβcf, hCGβ core fragment.
Two monoclonal antibodies (A₁₀₃ and A₁₀₅) bind to site V (α₅₀–₅₃) and recognize both intact hCG and free hCGα. Site VI, present only on the hCGβcf, is recognized by a few polyclonal (RW37 and DeM₃) or monoclonal (B₂₀₁, B₂₀₈ and B₂₁₀) antibodies (Wehmann et al., 1990; De Medeiros et al., 1992c).

Using several monoclonal antibodies, it was possible to recognize at least nine epitopes on hCGβ molecule, epitopes B₁–B₇ (Berger et al., 1996). The first hCGβ loop accommodates epitope B₆ (β₂₀–₂₅) and the third one the B₁ (β₆₆–₇₇) (Jackson et al., 1996). While epitopes β₆ and β₇ are specific for free hCGβ (Table III), epitopes B₁–B₅, B₆ and B₇ are accessible both on the free and assembled forms of hCGβ. Epitopes B₁, B₃, B₅ and B₆ are specific to the βCTP, epitopes B₂, B₃, B₄ and B₅ cross-react with LH and LHβ molecules, but their location has not been determined yet. In addition, there exist four epitopes specific for hCGβcf (B₁₀–B₁₃) not shared by free or assembled hCGβ (Dinhofe et al., 1994a; Berger et al., 1996). The major immunogenic regions on hCGα are provided by loops 1 and 3. The antigenic surface of hCGα could be differentiated into seven epitopes (A₁–A₇), arranged in four spatially distinct antigenic domains. One of these clusters to loop α₁ and includes sequences α₁₃–₂₂ (A₁ and A₂). The second epitope (A₂), located on loop 3 α₆₅–₆₈, clusters on the intact hCG, is conformation sensitive. The third epitope (A₃), portion α₃₂–₄₁, specific for the free hCGα, is masked by the hCGβ in the native hormone (Dinhofe et al., 1994b). The sequences α₁₃–₁₈, α₁₇–₂₂, α₃₃–₄₂ and α₈₇–₉₂ form the epitopes A₁, A₄, A₅ and A₇, respectively, and are dependent on the tertiary structure. The sequence α₆–₇ is specific for the free hCGα subunit. The free hCGβ epitope A₆, α₃₃–₄₂, is used as a target in various hCGα specific immunoassays (Troalen et al., 1988).

Metabolism of hCG
After miscarriage or delivery, the clearance of hCG follows a triphasic model with median half-lives of 3.6, 18 and 53 h (Korhonen et al., 1997). hCGβ is cleared more slowly than hCG with half-lives of 1, 23 and 94 h. The half-life of hCGα is even shorter than those of hCG and hCGβ, with half-lives of 0.6, 6 and 22 h. Studies including healthy volunteers show that recombinant hCG, compared with urinary hCG, has linear bi-exponential model and similar pharmacokinetics and pharmacodynamics (Trinchard-Lugan et al., 2002). After single intravenous injection of recombinant hCG, the majority of the molecules are excreted in urine in ~30–36 h. It suggests quicker renal clearance rates and shorter half-life, possibly reflecting either a variation in the glycosylation of the recombinant hCG or different abilities of the immunoassays to detect the various isoforms of urinary hCG (Norman et al., 2000). About 22% of the hCG molecules are excreted without any modification. The remaining 78% are retained in the body, taken by other tissues or excreted as metabolic products of the primary molecule (Nisula et al., 1989). In pregnant women, the mechanisms that modulate the glomerular filtration and tubular intake of hCG change with gestational age. The microheterogeneity in the carbohydrate chains, variable according to the period of gestation, responds for the type of the excreted hCG molecule. Later in pregnancy, the hCG becomes more acidic and more easily crosses the glomerular membrane (Hay, 1986).

A large proportion of the hCG molecules is modified by the renal parenchyma before being excreted into urine. In the kidney, the hCG is internalized by proximal renal tubule cells and degraded to small fragments (Markkanen and Rejaniemi, 1979), but a significant part is excreted unaltered by passage through the tubule to the collecting duct. The molecules more rich in sialic acid are eliminated more quickly (Amr et al., 1985). Desialylated hCG passes through the glomerules to the urine, or alternatively, the tubule cells take them up more quickly (Nisula et al., 1989). Owing to proteolytic processing of hCG as it passes through the kidney, the urine contains a much greater variety of forms of hCG than does the blood.

Liver accumulates the hCG molecules as soon as 2 h after injection of the gonadotrophin. The concentration of hCG in the liver is about 5-folds lower than that in the kidney (Markkanen and Rejaniemi, 1979). Removal of sialic acid increases the clearance of hCG up to 200 times (Rosa et al., 1984). A large proportion of hCG does not contain the sialic acid, and these desialylated forms exposing galactose residues bind to high affinity hepatocyte receptors for galactose-terminated oligosaccharides, being further distributed in the interior of the hepatocytes (Lefort et al., 1984). Native hCG is taken up primary by Kupffer cells in liver tissue and distributed along the sinusoids (Nisula et al., 1989). In the ovary, variable amounts of hCG molecules are internalized by granulosa/theca lutein cells (Ziminsky et al., 1982). These cells may degrade it to small fragments (Conn et al., 1978). Initially, it was thought that the uptake of hCG was limited to the availability of specific receptor, but further studies have found that in ovarian tissue, hCG can be degraded either after binding specific receptor or following other alternative mechanism of uptake (Campbell et al., 1981). The intracellular fate of hCG in granulosa cells is not completely clear; it is probably transported to lysosomes and degraded to small molecules or fragments there (Amsterdam et al., 1979). Human granulosa cells incorporate and degrade intact hCG to hCGβcf, even after being previously exposed to hCG in vivo (De Medeiros et al., 1992a). In addition, after injection of hCG, this fragment can be detected in follicular fluid 34–36 h later, suggesting either the fragment of hCGβ accumulates in this biological compartment or represents a pool of fragments of the different gonadotrophins cross-reacting with the hCGβcf assays (De Medeiros et al., 1992c).

Clinical importance of hCG and hCG-variant molecules
The variant forms of hCG or its subunits are summarized in Table IV. In addition to intact hCG, different forms are present on normal pregnancy serum or urine samples (Fig. 6). hCG can be synthesized in many tissues or clinical conditions. Some of these tissues are incapable of synthesizing the complete carbohydrate chains, resulting in hypoglycosylated molecular forms. Other tissues, on the other hand, incapable of trimming the highly glycosylated precursors of hCG secrete hyperglycosylated forms. Microheterogeneity in carbohydrate branches can still result from local modulators or the existing hormonal environment at the moment of synthesis (Fares, 2006). In serum, and urine, there is a marked variability in hCG sugar moieties without affecting the protein backbone (Sutton, 2004) (Tables V and VI). The variant molecular forms of hCG have different plasma half-life,
receptor-binding affinity and bioactivity. Their clinical effect, if any, is still unclear. Different degrees in oligosaccharides heterogeneity respond for their physiological action and receptor affinity. Fine tuning knowledge of how the carbohydrate units affect in vivo bioactivity in the clinical setting is missing. It is also assumed that heterogeneity in terminal oligosaccharides implies in different signaling responses. Current immunoassays for the hCG-variant forms, or variant combinations, provide useful information on the clinical utility of these markers for the early detection of pregnancy, pregnancy-related disorders, placental neoplasia, and several male or female tumors. It is possible to choose a specific assay that best attends a specific clinical condition (Table VII). It follows a basic and clinical analysis of the principal forms of hCG, especially those with potential clinical utility.

**Intact human chorionic gonadotrophin**

Intact hCG molecules appears between 2 and 8 days after fertilization in the embryo’s culture medium (Lachlan and Lopata, 1988), hCG can be detected in maternal serum 7 days after fertilization and 8 days following ovulation (Hay and Lopata, 1988). hCG rises 3-fold between the day of detection and the next day, decreasing thereafter and reaching 1.6-fold between Days 6 and 7 (Nepomnaschy et al., 2008). hCG can be absorbed into the blood through the uterine cavity and may be detected in urine even in the absence of implantation (Chang et al., 1998). The intact hCG concentration increases exponentially in the first trimester, doubling every 31–48 h and reaches greater amounts between 11 and 13 weeks (Pittaway et al., 1985). In the second trimester, at 20 weeks gestation, it diminishes to 80% and remains in this concentration until the term. The intact hCG is more negatively charged in the early than in the latter stage of gestation and this change in charge occurs around Week 13, when the placental production of estradiol and progesterone increases. hCG molecules from the first trimester have greater molecular size and bioactivity (Hay, 1985). Heterogeneity in charge, size and chemical composition of intact hCG has been demonstrated in serum and urine of different patients (Weintraub and Rosen, 1973) or tissues (Papapetrou and Nicopoulos, 1986). Quantitative differences in carbohydrate content account for the heterogeneity of hCG observed in several clinical conditions (Tables V and VI). Variable degrees of desialylation or fucosylation have also been demonstrated both in highly purified or crude preparations of hCG, hCG from placental extracts, urine and serum from normal pregnant women, urine and semen from patients with gestational trophoblastic neoplasia (GTN) (Norman et al., 1990a).

**Table IV** Intact hCG and variant molecular forms

<table>
<thead>
<tr>
<th>Molecular form</th>
<th>Source</th>
<th>References</th>
</tr>
</thead>
<tbody>
<tr>
<td>hCG</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Intact, standard</td>
<td>Pregnancy, tumors</td>
<td>Cole (2007)</td>
</tr>
<tr>
<td>Hyperglycosylated</td>
<td>Pregnancy, tropoblastic neoplasia</td>
<td>Cole et al. (1991)</td>
</tr>
<tr>
<td>Nicked</td>
<td>Pregnancy, tumors</td>
<td>Dufau et al. (1972)</td>
</tr>
<tr>
<td>Hypoglycosylated</td>
<td>Pregnancy, tumors</td>
<td>Mizuochi et al. (1983)</td>
</tr>
<tr>
<td>Asialo-hCG</td>
<td>Choriocarcinoma, gestational thyrotoxicosis</td>
<td>Cassels et al. (1989)</td>
</tr>
<tr>
<td>Acidic hCG</td>
<td>Trophoblast tumor, testicular tumor</td>
<td>Odell and Griffin (1989), Braunstein (2002)</td>
</tr>
<tr>
<td>Pituatory hCG</td>
<td>Non-pregnant women</td>
<td>Manjunath and Saram (1982)</td>
</tr>
<tr>
<td>Deglycosylated hCG</td>
<td>?</td>
<td>Maruo et al. (1980)</td>
</tr>
<tr>
<td>Large hCG</td>
<td>Placenta extracts</td>
<td></td>
</tr>
<tr>
<td>hCGβ</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Free hCGβ</td>
<td>Pregnancy, tumors</td>
<td>Cole (1998)</td>
</tr>
<tr>
<td>Pré-beta form</td>
<td>Placenta</td>
<td>Hussa et al. (1986)</td>
</tr>
<tr>
<td>Nicked</td>
<td>Pregnancy, tumors</td>
<td>Birken et al. (2001)</td>
</tr>
<tr>
<td>hCGα</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Free hCGα</td>
<td>Pregnancy, tumors</td>
<td>Weintraub et al. (1977)</td>
</tr>
<tr>
<td>Hyperglycosylated</td>
<td>Pregnancy, tumors</td>
<td>Nemansky et al. (1998b)</td>
</tr>
<tr>
<td>Pre-alpha form</td>
<td>Placenta</td>
<td>Sakakibara et al. (1986)</td>
</tr>
<tr>
<td>Big hCGα</td>
<td>Pituatory, pregnancy, choriocarcinoma</td>
<td>Bliithe and Nisula (1985)</td>
</tr>
<tr>
<td>Small hCGα</td>
<td>Pregnancy, non-tropoblastic tumors</td>
<td>Bielinska et al. (1989)</td>
</tr>
<tr>
<td>Other fragmented forms</td>
<td></td>
<td></td>
</tr>
<tr>
<td>hCGβ missing CTP</td>
<td>Trophoblastic, non-trophoblastic tumors</td>
<td>Berger et al. (1993)</td>
</tr>
<tr>
<td>Asialo β-CTP</td>
<td>Choriocarcinoma, pregnancy</td>
<td>Amr et al. (1985)</td>
</tr>
<tr>
<td>hCGβ peptide 48–145</td>
<td>Pregnancy, tropoblastic disease</td>
<td>Nishimura et al. (1998)</td>
</tr>
<tr>
<td>hCGβ missing CTP</td>
<td>Trophoblastic neoplasia</td>
<td>Wang et al. (1989)</td>
</tr>
</tbody>
</table>
Most hCG assays currently available do not distinguish hCG and free hCGβ molecules. Two assays are indicated when measurement of only the dimer hCG is preferred: an enzyme spectrometry assay that cross-reacts <2% with LH (Cole and Sutton, 2004) and a fluorooimmunoassay highly specific for intact hCG (Lemay et al., 1995). Either high or diminished concentrations of intact hCG have been associated with maternal or embryo–placenta abnormalities. In the clinical setting, its measurement becomes a useful method to detect higher risk of miscarriage, ectopic pregnancy, predict pre-eclampsia, intrauterine fetal growth restriction (IUGR), fetal hydrops or identify trisomy.

Urinary hCG levels <10 000 mUI/ml between 8 and 16 weeks of pregnancy indicate a poor prognosis in patients with threatened miscarriage and levels over 20 000 mUI/ml are associated with a good pregnancy outcome (Nygren et al., 1973). An increase in hCG of <35–50% in 2 days suggests a non-viable or ectopic pregnancy (Seeber et al., 2006). When intact hCG reaches 6000–7000 mUI/ml, in the absence of an intrauterine gestational sack, the probability of ectopic pregnancy is high. On the other side, increased hCG levels in the second trimester are predictive of poor pregnancy results in the third trimester, including maternal hypertension, fetal growth restriction and preterm delivery (Ilagan et al., 2004). Levels of hCG of 5.3 MoM, associated with abnormalities in placental ultrasound and uterine artery Doppler, predict preterm delivery, intraterine fetal death and IUGR with sensitivity between 75% and 100% (Alkazaleh et al., 2006). Levels between 2.0 and 3.0 MoM are considered positive predictors for development of pre-eclampsia or HELLP syndrome (hemolytic anemia, elevated liver enzymes, low platelet count, Shenah et al., 2003) and is a potential predictive marker of this condition in the second and third trimesters (Lambert-Messerlian et al., 2000; Roiz-Hernandez et al., 2006). Despite the good correlation between the levels of hCG and hydrogen peroxide,

**Figure 6** hCG and variant molecules in the placenta, blood and urine. Thick lines, polypeptide chains; numbers, amino acid numbers; thin lines, disulfide bonds; N, asparagines-linked oligosaccharides; O, serine-linked oligosaccharides; solid arrows, nicking, dissociation and degradation pathways. [Adapted from Cole (1997) with permission.]
<table>
<thead>
<tr>
<th>N-linked oligosaccharide</th>
<th>Clinical condition</th>
<th>Prevalent hCG form</th>
<th>Mechanism</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sα2,3 Galβ1,4 GlcNAc β1,2 Man</td>
<td>Normal pregnancy</td>
<td>hCG, free hCGα</td>
<td>Glucosidases normal expression</td>
</tr>
<tr>
<td>Sα2,3 Galβ1,4 GlcNAcβ1,2 Manα1,6</td>
<td>Normal pregnancy</td>
<td>hCG, hCGβ (Asp13), hCGα (Asn78)</td>
<td>Normal expression of glucosidases and sialyl fucosyltransferases</td>
</tr>
<tr>
<td>Sα2,3 Galβ1,4 GlcNAcβ1,2 Manα1,6</td>
<td>Normal pregnancy</td>
<td>hCG, hCGβ (Asp 30)</td>
<td>Normal expression of glucosidases and sialyl fucosyltransferases</td>
</tr>
<tr>
<td>Sα2,3 Galβ1,4 GlcNAcβ1,2 Manα1,3</td>
<td>Normal pregnancy</td>
<td>hCG</td>
<td>↑ Expression α1-6 fucosyltransferase</td>
</tr>
<tr>
<td>Sα2,3 Galβ1,4 GlcNAcβ1,2 Manα1,3</td>
<td>Normal pregnancy</td>
<td>hCG, hCGα (Asp52)</td>
<td>↑ N-acetylglucosaminyl transferase IV</td>
</tr>
<tr>
<td>Sα2,3 Galβ1,4 GlcNAcβ1,2 Manα1,3</td>
<td>Normal pregnancy</td>
<td>Invasive mole</td>
<td>↑ α1–6 fucosyl IV and α1–4 N-acetylglucosaminyl transferases</td>
</tr>
<tr>
<td>Sα2,3 Galβ1,4 GlcNAcβ1,4</td>
<td>Gestational diabetes</td>
<td>hCG</td>
<td>↑ N-acetylglucosaminyl transferase IV</td>
</tr>
<tr>
<td>Sα2,3 Galβ1,4 GlcNAcβ1,2</td>
<td>Early pregnancy</td>
<td>hCG, hCGβ</td>
<td>↑ Expression N-acetylglucosaminyl transferase IV ↑ (1 → 6f → 6 branches) β1 → 4 galactosyltransferase</td>
</tr>
<tr>
<td>Sα2,3 Galβ1,4 GlcNAcβ1,4</td>
<td>Choriocarcinoma</td>
<td>hCG</td>
<td>↑ N-acetylglucosaminyl transferase V (1 → 6f → 3 branches)</td>
</tr>
<tr>
<td>Sα2,3 Galβ1,4 GlcNAcβ1,1,4 Manβ1,4 GlcNAcβ1,4 Manα1,6 Fuc</td>
<td>Choriocarcinoma</td>
<td>hCG</td>
<td>↑ α1–6 fucosyltransferase IV β1–4 galactosyltransferase (1 → 6f → 6 branches)</td>
</tr>
<tr>
<td>Sα2,3 Galβ1,4 GlcNAcβ1,1,4 Manβ1,4 GlcNAcβ1,4 Manα1,6 Fuc</td>
<td>Choriocarcinoma</td>
<td>hCG</td>
<td>↑ N-acetylglucosaminyl transferases IV and V</td>
</tr>
</tbody>
</table>

SA: sialic acid; Gal: galactose; GlcNAc: N-acetyl glucosamine; man: mannose.
the clinical applicability of the intact hCG measurement to evaluate the placental oxidative stress, and the prognosis of pre-eclampsia, requires more studies (Towner et al., 2006). Low levels of hCG can be also involved in cases that result in IUGR (Crocker et al., 2004).

Intact hCG measurement may discriminate normal gestations from gestations with Down syndrome, in which hCG is increased between 11 and 14 weeks (Spencer et al., 2002a). In trisomy 21, cytotrophoblast cells do not fuse and differentiate normally, and secrete an

Table VI  O-linked oligosaccharide types found in standard hCG and/or hCG-variant molecular forms

<table>
<thead>
<tr>
<th>O-linked oligosaccharide</th>
<th>Clinical condition</th>
<th>Prevalent hCG form</th>
<th>Mechanism</th>
</tr>
</thead>
<tbody>
<tr>
<td>core 1 SA α2,3Galβ1, 3GalNAc -Ser</td>
<td>Normal pregnancy</td>
<td>hCG</td>
<td>Glucosidases normal expression</td>
</tr>
<tr>
<td>SA α2,3Gal β1,3GalNAc -Ser</td>
<td>Normal pregnancy</td>
<td>hCG</td>
<td>Glucosidases normal expression</td>
</tr>
<tr>
<td>SA α2,6GalNAc -Ser</td>
<td>Normal pregnancy</td>
<td>hCG</td>
<td>β3-galactosyltransferase</td>
</tr>
<tr>
<td>SA α2,3Galβ1,4GlcNAc -Ser</td>
<td>Very early pregnancy (6th–7th week), gestational trophoblastic neoplasm, choriocarcinoma, germ cells malignancy</td>
<td>hCGh (ser 132)</td>
<td>β6-glucosaminyltransferase</td>
</tr>
<tr>
<td>SA a2,3Galβ1, 3GalNAc β1 -Ser</td>
<td>Gestational trophoblastic neoplasm, choriocarcinoma, germ cells malignancy</td>
<td>hCG, hCGh</td>
<td>β6-glucosaminyltransferase ↓ α-2 sialyltransferase</td>
</tr>
</tbody>
</table>

SA, sialic acid; Gal, galactose; GlcNAc, N-acetyl glucosamine.

Table VII  Selected assays for hCG and/or hCG-related molecules according to the different clinical conditions

<table>
<thead>
<tr>
<th>Clinical condition</th>
<th>Prevalent molecule</th>
<th>Preferred assay</th>
<th>Available assays/source</th>
</tr>
</thead>
<tbody>
<tr>
<td>Normal pregnancy, early detection (Weeks 1–5)</td>
<td>hCGh</td>
<td>Intact hCG, hCGh, hCGhDelfia</td>
<td>Baxter—Dade-Stratus II, IFMA, American Dade Diagnostics</td>
</tr>
<tr>
<td>Normal single pregnancy detection, diagnosis and follow-up of ectopic pregnancy or early pregnancy loss</td>
<td>Dimer hCG</td>
<td>Total hCG (hCG + hCGβ), hCGn</td>
<td>Elecsys hCG, hCG β, ECLIA, Roche Diagnostics Tandem R hCG, IRMA, Hybritech, Inc. Immulite 2000, IRMA DPC, Inc.</td>
</tr>
<tr>
<td>Hydatiform mole</td>
<td>hCG, hCGβ, CTP-hCG</td>
<td>hCGβ, CTP-hCG</td>
<td>hCGβ Delfia assay, CLIA, Pharmacia Wallac Immulite 2500 free βhCG, IRMA, DPC, Inc. UBI MAGNIVEL free βhCG, ELISA</td>
</tr>
<tr>
<td>Placental site trophoblastic tumor</td>
<td>hCGβ</td>
<td>Free hCGβ</td>
<td>Immulite 2500 free βhCG, IRMA, DPC, Inc.</td>
</tr>
<tr>
<td>Choriocarcinoma</td>
<td>hCG, hCGh, hCGβ</td>
<td>Total hCG, hCGh, hCGβ</td>
<td>Immulite 2000, IRMA DPC, Inc.</td>
</tr>
<tr>
<td>GTD after evacuation/chemotherapy</td>
<td>hCGn</td>
<td>hCGn, hCGβn</td>
<td>hCGn, IRMA, not commercially available/Dr J.C. O’Connor; Irving Center for Clinical Research, Columbia University</td>
</tr>
<tr>
<td>Down’s syndrome screening Pre-eclampsia prediction</td>
<td>hCG, hCGβ, hCGh</td>
<td>Total hCG, hCGh, hCGβcf (urine)</td>
<td>Baxter-Dade-Stratus II, IFMA, hCGh Nichols Assay</td>
</tr>
<tr>
<td>Germ cell tumors</td>
<td>hCG, hCGβ</td>
<td>Total hCG, hCGβ, hCGβcf (urine)</td>
<td>UFG-EIA Toa-EIA, Toagosl Co. Ltd, Tokyo, Japan</td>
</tr>
<tr>
<td>Gynecologic tumors</td>
<td>hCGβ</td>
<td>hCGβ, hCGβcf (urine)</td>
<td>Immulite 2500 free βhCG, IRMA, UFG-EIA Toa-EIA</td>
</tr>
</tbody>
</table>

GTD, gestational trophoblastic disease; hCGh, hyperglycosylated hCG; hCGn, nicked hCG.
abnormal weakly bioactive hCG molecule. In addition, in this syndrome, there is a marked decrease in the number of hCG receptors expressed on cytotrophoblast cells (Pidoux et al., 2007). Diminished expression of mature hCGR in Down syndrome suggests a lack of utilization of circulating hCG, explaining, at least in part, the elevation of hCG (Banerjee et al., 2005). Despite its high level, serum intact hCG does not seem to be the best biochemical marker for Down syndrome at this stage (Goshen, 1999). The current assays for intact hCG are capable of identifying only 41–63% of cases, false-positive rate of 5% (Wald et al., 2003). The results, in MoM, vary between 1.11 and 1.91 in the first (Nicolaides, 2005) and second (Canick and MacRae, 2005) trimesters. Although in trisomy 13 the intact hCG levels are increased, they are diminished in trisomy 18, probably secondary to the poor differentiation of the cytotrophoblast (Banerjee et al., 2005).

Either in the diagnosis or follow-up of tumors, intact hCG must be measured in serum or plasma, not in urine samples (Norman et al., 1990a). As tumors of worse prognosis secrete hCGβ in higher proportion, the current assays should discriminate the hCG dimer from this subunit. Tumors of female or male germinative cells express hCG in high enough levels for detection in serum of 15–72% of patients (Hoshi et al., 2000). Levels of hCG over 100–1000 mU/l/ml in these tumors indicate greater risk and worse prognosis (Mead and Stennin, 1993). Cancer of bladder, kidney, prostate, liver, lung, breast and colon-rectum expresses hCG at different levels (Stenman et al., 2004). Elevated hCG levels can also be found in other non-tumoral conditions such as cirrhosis, inflammatory bowel disease and peptic ulcer disease (Randeva et al., 2001).

**Hyperglycosylated human chorionic gonadotrophin**

A large (38 500–40 000 Da) hyperglycosylated molecular form of hCG (hCGh), with additional monosaccharides in its carbohydrate chains, and higher bioactivity has been investigated. Both N- and O-linked carbohydrate chains on hCGh differ from those present in standard hCG by containing 100% tetrasaccharide core structures on serine residues in its CTP (Tables V and VI). Therefore, the differences in glycosylation at ser121, 127, 132 and 138 are the greatest discriminator of regular hCG and hCGh (Cole et al., 2007). hCGh is produced early by a still undifferentiated invasive trophoblast cells in an independent way of the intact hCG molecule. In the first 3 or 4 weeks of pregnancy hCGh is the predominant form (Kovalevskaya et al., 2002), but it is soon replaced with native hCG during a few weeks after implantation, when it declines from more than 80% to 50% of total hCG forms. In fact, from 4 weeks it diminishes quickly, remaining in small amount during the rest of pregnancy (Cole et al., 2007). This shift in the glycosylation pattern results from production initially by cytotrophoblasts gradually shifting to that by syncytiotrophoblast. hCGh is a good marker of invasive trophoblast cells function in early pregnancy and its carbohydrate chains may reduce trophoblast cell binding and fixation to the basement membrane by its autocrine role in trophoblast invasion (Cole, 1998; Handschuh et al., 2007). hCGh, but not regular hCG, has an autocrine rather than an endocrine function and directly modulates the cell growth, tumor formation and cytotrophoblast invasion in early pregnancy, choriocarcinoma and testicular germ cell malignancy (Cole and Khanlian, 2007; Kelly et al., 2007).

Historically, the majority of the available assays to measure intact hCG also recognized this hyperglycosylated molecule in different proportions, and its presence was a potential source of quantitative discordance seen with the different commercial kits (Cole et al., 2006). Currently, an immunoradiometric assay, capable of quantifying only this hyperglycosylated form is available. The assay uses the monoclonal antibodies B152 that detect the presence of biantennary oligosaccharides at serine 132 in the middle of the variable three O-linked oligosaccharides as capture antibody, and B207, directed to hCGβcf, designated radiolabeled detection antibody (Kovalevskaya et al., 1999a). This assay detects hCGh with 0% cross-reactivity with pure regular hCG and 60% with the hCGh free β-subunit. The assay was further improved and, using the same antibodies, an automated chemiluminometric procedure with short incubation time and cross-reacting <4.5% with hCGn and <3.5% with any other hCG form has been developed (Pandian et al., 2003).

The hyperglycosylated form, produced by the stem cytotrophoblast cells, by initiating growth and invasion of cytotrophoblast cells and modulation of apoptosis, favors implantation in normal pregnancy (Cole, 2007). hCGh is the preferable form for the early diagnosis of pregnancy or its complications (Birken, 2005). In low levels, it could explain implantation failure. It is also possible that low levels of hCG could reduce expression of progesterone receptor and disturb myometrial contractility inhibition (Ticconi et al., 2007). Further studies are needed to confirm this hypothesis, also taking into account the different types of hCG. Therefore, hCGh discriminates naturally conceived pregnancies which will carry to term and those destined for early pregnancy loss (Kovalevskaya et al., 2002). In pregnancies leading to failure, the proportion of hCGh/hCG decreases from 88% to 44% (Sasaki et al., 2008). In another study, hCGh/hCG was found in 81% of normal pregnancies and in only 36–73% of pregnancies with tendency to early loss at a 2.9% false-positive rate using serum and at a 15% false-positive rate using urine (Sutton-Riley et al., 2006). A simple single point cut-off of 13 ng/ml hCGh in serum could be used between 4 and 7 weeks of pregnancy to differentiate a failure outcome (<13 ng/ml) from term outcome (>13 ng/ml). Thus, measuring this hCG form may be useful to identify those pregnancies with higher risk of miscarriage (Kovalevskaya et al., 2007).

hCGh is also the most common hCG-variant form found in malignant trophoblastic disease (Kovalevskaya et al., 2002), with 100% sensitivity and specificity in discriminating malignant and pre-malignant disease. In choriocarcinoma, hCGh initiates the invasive activity of the tumor cells (Cole et al., 2006) and accounts for 30–100% of the hCG immunoreactivity. In this condition, it can be used to differentiate a preinvasive from an invasive form (Elliott et al., 1997). In addition, it is the principal hCG-related molecule produced by testicular and other male and female germ cell malignancies and responds for the invasive behavior of these cells (Kovalevskaya et al., 2002; Cole et al., 2004). Additionally, it can be found in the serum or urine of patients with a variety of other malignant conditions including cervical, colon, bladder and lung cancer (Kelly et al., 2007).

hCGh isoform, deficient in sialic acid, tends to be higher in the first and second trimesters of pregnant women with Down syndrome (Pandian et al., 2004), due to delayed differentiation of the cytotrophoblast into a syncytiotrophoblast (Frendo et al., 2000). In this
syndrome, its levels, given in MoM, are 3.5–9.5 higher when compared with those levels verified in normal pregnancies. hCG may detect up to 78% of Down syndrome cases at a 5–8% false-positive rate (Palomaki et al., 2005), but in the first trimester its performance is lower, with a reported 63% Down syndrome detection rate at a 10% false-positive rate (Weinans et al., 2005). The measurement of urinary hCGh as a test for tracking the Down syndrome seems promising (Wald et al., 2003). The decreased detection rate with the Nichols hCGh test reflects the poor detection of sialic acid-deficient hCG molecule with this assay. A specific assay to detect hCGh poor in sialic acid is still awaited. Currently, no marker, and even the use of multiple tests, reaches the sensitivity observed with hCGh to detect trisomy 21 (Bahado-Singh et al., 2000). However, this glycoprotein has no value in the identification of embryos with trisomy 18.

As pre-eclampsia occurs as a consequence of abnormal invasion by the trophoblast and the uterine spiral arteries in human pregnancy, low maternal mid-trimester hCGh levels predict pre-eclampsia development. Decrease in the concentrations of hCGh of 0.9 to 0.1 MoM, between 14 and 21 weeks of pregnancy, indicates 10 times higher risk of pre-eclampsia (Bahado-Singh et al., 2002a). This decrease in hCGh concentrations is attributed to the fast reduction in its production, resulting in low capacity or failure of trophoblast invasion seen in this condition.

Nicked human chorionic gonadotrophin

This hCG variant either lacks or has very little steroidogenic activity and may even act as an hCG antagonist (Cole et al., 1991). The nicked hCG form (hCGn) suffers cleavage between peptides β47 and β48 of the polypeptidic chain of the dimer hCG; less frequently the nicking occurs between residues β42 and β43, β43 and β44 or β44 and β45, as seen in normal pregnancy and some cases of choriocarcinoma (Elliot et al., 1997). The nicking in the middle of the molecule, one of the principal αβ subunits interaction sites (Fig. 7), leads to rapid dissociation of hCG molecules, releasing a nicked free hCGβ (hCGβn) (Cole et al., 1993). With the bond cleavages it loses immunoactivity, and is not detected by many hCG immunoassays. As the percentage of peptide bond nicking in the various hCG standard preparations has been shown to be as high as 10–20% (Birken et al., 1991), significant discrepancy can be seen with the different commercially available assays. Most of the commercial assays currently available measure this isofrom (Cole et al., 2004), but an assay able to distinguish it from the intact hCG has been developed. The assay uses the monoclonal antibodies B151 for detection and B604 for capture in an immunoradiometric assay cross-reacting with the intact hCG in only 2.5%, with hCGβ in 3.7%, and with non-nicked hCG in only 2.5% (Kovalevskaya et al., 1999b).

hCGn is more abundant in urine than in serum samples. In the serum, the concentrations of this molecular form represent a ratio of 9–10% in relation to the non-nicked molecules (Birken et al., 1991). In the second month of pregnancy, this ratio increases up to 21%, remaining in this range until term. The ratio hCGn:hCG in urine increases from 8% to 31% during the pregnancy (Cole et al., 1991). hCGn abnormal secretion is due to hypoxia change and leukocyte activation in trophoblast tissue (Lunghi et al., 2007). The hCGn form is the result of metalloprotease activities in macrophages and T-lymphocyte helper cells to inactivate hCG function in pregnancy and cancer tissues (Kardana and Cole, 1994). It is expected that clinical conditions with an increased trophoblast expression of protease enzymes, such as pre-eclampsia or cancer tissue, would present higher levels of this low bioactive molecule variant. In addition, in a clinical setting, pregnancies that will not carry to term have an easily recognized difference in production of hCGn (O’Connor et al., 1998). It has been found to be higher in serum and urine of individuals with pre-eclampsia, trophoblast disease, Down syndrome and male or female patients with testicular or bladder cancer. It is, still, the principal molecular form of hCG found in the weeks following molar evacuation or chemotherapy for hydatiform mole or choriocarcinoma (Cole and Sutton, 2003).

High levels of hCGn in pregnancies with Down’s syndrome suggest that in this condition there is an increase in the nicking of hCG and reduced inhibitory feedback mechanism of hCG on its own production. Because of increased leukocyte activation, the serum levels of hCGn are ~30–40% higher in pregnant women with pre-eclampsia (Lee et al., 1997). Higher concentrations of hCGn have also been detected in individuals with both seminomatous and non-seminomatous testicular cancer (Hoermann et al., 1994). hCGn, and its dissociation product hCGβn, become the major or sole molecules in serum and urine when hCG results fall below 100 mU/ml in patients with trophoblast disease or germ cell malignancies (Kohorn and Cole, 2000).

Free alpha subunit of human chorionic gonadotrophin

The heterogeneity of this subunit reflects mainly excessive or poor glycosylation (Fein et al., 1980). A free variant form (hCGαx) is created when additional carbohydrate is placed on the molecule so that the association of the αβ dimer cannot take place (Weintraub et al., 1977). The glycosylation of free hCGα from the third trimester of pregnancy is different from that from the first trimester (Nemansky
et al., 1999b). Its bigger size is the result of higher content of fucose and sialic acid and a greater proportion of monosialylated carbohydrate branches with biantennary and triantennary structures (Blithe and Nisula, 1985; Nemansky et al., 1998b). Free hCGx can itself be heterogeneous; about 70% of the molecules produced in normal placentas have their amino acid chains with the sequence Ala-Pro-Asp-NH2, 20% initiate with Val-NH2 and 10% with Asp-Va-NH2. hCGx isolated from pooled human pregnancy urine contains 73–83% of the chains starting alanine residue 1, 7–11% commencing at aspartic acid residue 3 and 12–20% commencing at valine residue 4. About 7–8% of the hCGx subunits as part of the dimer is nicked between residues α70 and α71 (Birken et al., 1978).

The free mature form of hCGx has been detected in hCG commercial preparations, urine and serum from pregnant women, placenta explants, pituitary and tumors. The free hCGx levels increase continuously to about 10-fold up to term (Skarulis et al., 1992). In relation to hCG, its ratio is lower than 10% in the first trimester and reaches 30–60% at term (Benvensite and Scromigna, 1981). A hyperglycosylated hCGx form unable to combine with hCGβ was found in normal pregnancy and individuals with tumors. A big mature molecular form of hCGx (hCGxb) with longer peptide chain was detected in commercial hCG preparations, pregnancy urine, serum, placenta, pituitary, choriocarcinoma, carcinoid gastric cells and various neoplasms or tumor cell culture systems (Blithe and Nisula, 1985). A small hCGx (hCGxa), with less amino acids in the peptide chain, was found in normal pregnancy and individuals with non-trophoblast tumors (Bielinska et al., 1989).

The current assays have demonstrated higher levels of hCGx in patients with pre-eclampsia after Week 30; although its measurement was not found to be useful for predicting this condition (Moodley et al., 1995). In non-pregnant women, and men, hCGx subunit is produced by the pituitary and reflects the pooled production of hCGx, FSHα, LHα and TSHα. In post-menopause women, the levels of hCGx increase about five times (Norman et al., 1987). About 10–20% of the patients with testicular cancer have elevated serum levels of hCGx and individuals with tumor carcinoids show even a much higher hCGx concentration (Alfthan and Stenman, 1996). Because it is produced in high levels, the measurement of hCGx can be useful for early detection of tumors in pituitary, lung, testes, insulinasomas and gastric or pancreatic carcinoid (Braunstein et al., 1979). At least in a lung tumor cell line, hCGx seems to exert a paracrine growth-stimulating function (Rivera et al., 1989). Even though assays to quantify only the hCGx are available, the clinical use of this subunit as tumor marker is limited.

Free beta subunit of human chorionic gonadotrophin

The concentrations of hCGβ, nicked or non-nicked forms, parallel the concentrations of hCG, reaching higher levels around Week 10. In serum of normal pregnancy, the concentrations of hCG are 200 times lesser than the dimer concentration, but in urine the hCGβ is one of the predominant forms (McChesney et al., 2005). In a molar ratio basis, the hCGβ concentration is only 0.9% of that of hCG in the second month of pregnancy, declining to 0.5% at term. Higher ratios of hCGβ, between 9% and 40%, can be found in urine. Stronger heterogeneity of free hCGβ is found in the molecules secreted by abnormal trophoblast or other tissues, usually malignant. Besides the free hCGβ molecules found in normal pregnancy and non-trophoblast tumors, the other hCGβ forms with clinical importance are the immature beta form found in placenta, the hCG beta beta monodimer (hCGββ), and hyperglycosylated hCGβ (hCGβh) forms, found in pregnancy or trophoblast tumors (Butler and Iles, 2004; Roy et al., 2007). Practically, all the hCGβ molecules in urine are nicked (Birken et al., 1991). The principal alterations detected in the protein core of hCGβ molecules are a decrease in disulfide bonds, unfold or misfold forms, presence of additional peptide residues, existence of alternative amino acid sequences, deletion of amino acid residues at the CTP extension and deletion of the whole CTP (Cole et al., 1982; Ruddon et al., 1996). In addition, forms of free hCGβ with heterogeneous oligosaccharide side chains are common. hCGβ molecules with large O-linked oligosaccharide chain at the CTP extension, high mannose or sialic acid content and desialylated forms have been reported (Stenman et al., 2006). Monoclonal antibodies which have a cross-reactivity of only 0.23% with intact hCG have been developed (Cole et al., 1994a). Assays to measure only the hCGβ are available and used to identify the free molecule as a marker of a number of abnormal conditions (Cole 1998; Spencer, 2005).

The relevance of the clinical use of hCGβ as a predictive marker of the ongoing pregnancy has been examined in a few centers and it seems to be limited to measurement before Week 14. Low serum levels of this molecule in the first trimester was associated with ectopic pregnancy (Okamoto et al., 1987), spontaneous miscarriage before 22–24 weeks (Dugoff et al., 2004), development of pre-eclampsia, IUGR and premature birth (Ong et al., 2000; Yaron et al., 2004). An hCGβ concentration below the 25th percentile of the values seen in singleton pregnancies has shown sensitivity of 100% and capability to predict ectopic pregnancy as soon as 12 days after the embryo transfer in nearly 70% of cases (Okamoto et al., 1987). In fact, hCGβ concentrations are systematically lower in ectopic pregnancies than in singleton normal ones. Pregnant women in whom free hCGβ levels increase 66% over a 48 h interval, with an indeterminate pelvic ultrasound examination is predictive of ectopic pregnancy (Dart et al., 1999). Higher levels of hCGβ can also be found early in pregnancies with pre-eclampsia (Luckas et al., 1998). A strong association was found between high maternal serum hCGβ levels in the third trimester with pregnancy-induced hypertension, mainly when proteinuria was present (Yadav et al., 1997). The discriminative cut-off value to detect higher risk to develop pre-eclampsia or not is 2.0 MoM (Roiz-Hernandez et al., 1997). The detection of hCGβ in serum over 3% of the total hCG level may be indicative of GTN (van Trommel et al., 2005). In addition, even in low levels, it is the predominant form in cases of placental site trophoblast tumors (PSTT) (Cole et al., 2006; Plura et al., 2007). hCGβ to hCG proportion is even higher in choriocarcinoma. Non-gynecological tumors also produce hCGβ, mainly in lung, urinary tract, sarcoma, gastrointestinal system, vulva and cervical cancer (Demirtas et al., 2007). In addition, hCGβ has a strong correlation with tumor aggressiveness and invasion. The levels of hCGβ in malignancy are quite variable from positive immunohistochemistry only to a serum level as high as 10 000 mU/ml.

Free hCGβ is also high in triploidy of paternal origin (diandric), and low in triploidy of maternal origin (digynic) (Yaron et al., 2004).
The serum levels change from 8.04 MoM in the case of diandric triploidy to 0.18 MoM in triploidy of digenic origin (Spencer et al., 2002). When the free hCGB is associated with other markers, it can identify more than 90% of embryos with triploidy of both phenotypes up to the second trimester of pregnancy (Evans et al., 2007). It seems that free hCGB can identify up to 95% of the affected fetuses, following a model with a false-positive rate fixed in 5% (Falcon et al., 2006). In trisomy 21, the levels of hCGB remain between 1.40 and 3.5 MoM, higher up to the second trimester (Hallahan et al., 1998; Hsu et al., 1999; Malone et al., 2005). A free nicked hCGB form is also high in Down’s syndrome and, as a potential marker, levels higher than 4.7 MoM were found (Rotmensch et al., 1996). An hCGB intermediate which exposes hCGBcf epitopes may be released to a higher degree upon cell death in Down syndrome than in normal pregnancy. It is possible that the Down syndrome trophoblast cells secrete an hCGB molecule that is cleaved more easily, resulting in high levels of hCGBn form (Roig et al., 2007). In cases of trisomy 18, the levels of hCGB are low in the first trimester and diminish yet more in the second trimester (Krantz et al., 2004). In trisomy 13, the levels of hCGB are diminished to similar extents in both the two initial trimesters (Spencer et al., 2005).

**Beta-core fragment of human chorionic gonadotrophin**

The beta-core fragment of hCG (hCGBcf) results from the removal of most of loop 2 of the hCG subunit as well as the entire seatbelt portion of the molecule. This 14 000 Da fragment, containing 73 amino acids in its protein core, is structured in two small polypeptide chains, residues 6–40 and 55–92 of hCGB, linked by five non-covalent disulfide bonds and lacking the CTP extension (Birken et al., 1988b). The carbohydrates attached to the peptides Asn-13 and Asn-30 are also degraded and quite different from those of the hCGB, and contain only 5–11 residues of sugar. In addition, 30% of the hCGBcf molecules are completely deglycosylated (de Medeiros et al., 1993). The fragment has no proved function yet. The availability of highly purified preparations of hCGBcf allowed the development of specific assays for its quantification without cross-reaction with other related hCG molecules (de Medeiros et al., 1992c; Krivchevsky et al., 1994). A new WHO standard for hCGBcf measurement was recently purified hCGBcf 99/708 (Bristow et al., 2005). Commercially available, the urine beta-core assay UGF-EIA Toa Kit (Toagosli Co. Ltd., Minato-Ku, Tokyo, Japan) has been shown to be specific for the hCGBcf (Cole et al., 1999).

The hCGBcf can be detected in large amounts in urine of normal pregnant or non-pregnant women, trophoblast neoplasia, amniotic fluid, vesicles of hydatiform mole, ovarian follicular fluid after use of hCG, newborn’s urine, semen, placenta and extracts of many normal or abnormal tissues (de Medeiros et al., 1992a, b; Udagawa et al., 1998; Khan et al., 2000). Very small amounts of this fragment could be detected in serum of pregnant or non-pregnant individuals (Alfthan and Stenman, 1990). In urine of pregnant women, its concentration is between 2 and 10 times higher than the intact molecule of hCG reflecting the active role of the kidney in hCG catabolism (de Medeiros et al., 1992b; Norman et al., 2000). Besides the renal production, placenta, hydatiform mole, chorionicarcinoma and ovarian cancer cells secrete hCGBcf (de Medeiros et al., 1992a; Okamoto et al., 2001). The hCGBcf is a stable molecule (de Medeiros et al., 1991) but suffers diurnal modifications, in a way that higher concentrations are found in the morning and lower levels in the afternoon (Rotmensch et al., 2001). This diurnal variation of urinary hCGBcf was not seen in patients with gynecological neoplasmas, however (Neven, et al., 1994). In the second half of pregnancy, the hCGBcf concentration represents 15–75% of the total hCG immunoreactivity, either when estimated by assay using antibodies that also recognize free hCGB or intact hCG (de Medeiros et al., 1992c) or only the fragment (Birken et al., 1988a). Urine samples throughout the pregnancy show higher concentrations of hCGBcf between the 8th and the 15th weeks, decreasing later between the 20th and the 29th (de Medeiros et al., 1992b). In relation to the intact hCG, the proportion of hCGBcf in pregnancy is always in excess of about 1.6–9.6-fold between Week 6 and Week 41. The secretion hCGBcf emerge as the predominant form in 5 weeks after-conception.

In the first trimester, hCGBcf measurement has been used to discriminate between normal intrauterine and ectopic pregnancy, where it has shown higher capability to differentiate these two conditions, when compared with the use of intact hCG or hCGB (Cole et al., 1994b). In MoM normalized for creatinine, the values of hCGBcf vary from 0.15 to 0.008 between 2nd and 5th weeks. However, despite 100% of sensitivity, this is at the expense of a specificity of only 48% (Borrelli et al., 2003). When hCGBcf is used to predict spontaneous miscarriage, the results may change from 0.110 to 0.016 MoM, showing a positive predictive value of 76% (Cole et al., 1997). Because the levels of hCGBcf show positive correlation with gestational age between Week 4 and Week 6 in pregnancies with normal evolution, the measurement of hCGBcf has been considered the method of choice in this phase of pregnancy (Cardwell et al., 1997). In the second trimester, the fragment was also tested to predict fetal growth and its levels were higher in those gestations with small fetus, standardized for gestational age; sensitivity of 78% and specificity of 70% (Bahado-Singh et al., 2002b). Midtrimester increase in hCGBcf may be a marker of hyperplacentosis which is a compensatory response to trophoblastic damage (Bahado-Singh et al., 1998). Thus, any condition with trophoblast disturbance would result in hCGBcf increase. The decrease in trophoblast oxygen pressure which occurs in pre-eclampsia increases the hCG production and hCGBcf form (Roiz-Hernandez et al., 2006), probably as a consequence of increased H2O2 signaling (Aris et al., 2007). The relative risk of pre-eclampsia is 2.1 times higher in gestations with levels of hCGBcf ≥2.0 ≤4.0 MoM and 5.2 times in cases where the hCGBcf level is above 4.0 MoM, when compared with normal gestation.

The measurement of hCGBcf in the first 3 months is of no value for detecting Down syndrome (Kornman et al., 1997), but detection rates of this syndrome between 60% and 80% have been reached with the use of this marker in the second trimester (Isozaki et al., 1997). The proportion of true Down’s cases having an hCGBcf value >95th centile of the controls may vary from 61% to 93%, far superior to any single serum marker (Iles, 1996). Between 15 and 24 weeks of gestation, its measurement identifies fetuses with trisomy 21 when its levels are ≥97th percentile; sensitivity of 61% and false-positive rate of 3.2% (Bahado-Singh et al., 1998). The studies examining the effectiveness of hCGBcf measurement in the detection of Down syndrome show sensitivity between 41% and 93% and specificity between 90%
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and 95% (Bahado-Singh et al., 1999). In MoM, the values of hCGβcf have been found between 1.06 and 2.91 in the first trimester and 1.06 and 12.89 in the second trimester (Hallahan et al., 1998; Hsu et al., 1999). In combination with maternal age and total estriol levels, the measurement of this fragment for screening Down syndrome in the second trimester results in a detection rate of 75–81% with a 5% false-positive rate (Kellner et al., 1997). A combination algorithm consisting of nuchal thickness, maternal age and hCGβcf showed a sensitivity of 86% at a 4.9% false-positive rate (Bahado-Singh et al., 1999).

hCGβcf shows relevance in the follow-up of pregnant women with trophoblastic disease or individuals with cancer, either gynecological or not (Norman et al., 1993). As PSTT produces low levels of hCG, urine hCGβcf is an alternative marker for this condition (Seki et al., 2004). hCGβcf identifies ~11–18% of individuals with intraepithelial cervical neoplasia (Norman et al., 1993). It is possible to detect hCGβcf in 48% of the patients with cancer of cervix, 38% in endometrial cancer and 84% in ovarian carcinoma (Nishimura et al., 1998). Urinary hCGβcf measurement has a sensitivity of 74%, specificity of 92% for some gynecological cancers and may be a more sensitive marker of hCG production by tumors than serum hCG (Norman et al., 1990b). In vulvar and cervix cancers, the measurement of the hCGβcf has shown sensitivity of 51–84% (Ngan et al., 1995) and, when present, it indicates worse prognosis (Carter et al., 1994). In lung cancer, the use of hCGβcf as biochemical marker shows sensitivity of 48% in the early stages and of 72% in most advanced ones (Yoshimura et al., 1994).

Other native forms or fragments of hCG/hCGβ molecules

hCG missing the carboxyl terminal extension (CTP-hCG) of ~43 000 Da in size and with activity ~50–80% of the native hCG is found in serum of patient with GTN (Berger et al., 1993). In this situation, assays requiring the β-CTP can give misleading results (Cole et al., 2004). Only the DPC immulite/2000 and the United Kingdom RIA (Tosoh AIA 600) assays detect this hCG variant. Urinary forms of hCG with oligosaccharides deficient in sialic acid content, named asialo hCG (hCGas), have been reported in patients with gestational thyrotoxicosis or choriocarcinoma in greater amounts than in healthy pregnant women (Tsuruta et al., 1995). An assay, designed as a lectin-immunoradiometric assay, specific and sensitive for measurement of hCGas in urine samples was developed but not applied in large scale yet. Another highly acidic variant of hCG (hCGav) with reduced metabolic clearance has been identified in patients with trophoblastic or testicular tumors (Cassels et al., 1989), comprising up to 45% of the total hCG content in these conditions. A human pituitary chorionic gonadotrophin form (hCGp), isolated from acetone-preserved human pituitary glands, was recently characterized and its amino acid and CTP extension is similar to those found in hCG from urine of pregnant women (Odell and Griffin, 1987; Birken et al., 1996). This pituitary hCG molecular form has an altered carbohydrate structure that results in a more rapid disappearance from the bloodstream (Braunstein, 2002) and on a molar basis has about half of the sialic acid found in urinary hCG. This hCG form increases in perimenopausal and older women (Odell and Griffin, 1987), with ranges from 2 to 31 mUl/ml and increases after the administration of hormone therapy (Cole et al., 2007). The biological activity of the sulfated pituitary hCG has only 50–65% of the urinary hCG activity (Birken et al., 1996). Currently, its measurement is of no clinical utility. The presence of non-sulfated hCG after menopause may be derived from hCG-secreting tumors and not from the pituitary gland (McCudden et al., 2008).

Concluding remarks

The occurrence of modifications in both structure and composition of the carbohydrate and peptide chains of the hCG and related molecules in biological fluids is common. The individual identification of the dimer hCG, and the variant forms, such as free subunits and fragments, has remarkable clinical application in predicting the ongoing pregnancy evolution, tracking occurrences of ectopic pregnancy, pre-eclampsia and intrauterine growth fetal restriction, detection of chromosome anomalies and identification or follow-up of trophoblast neoplasia. The available assays employ standards contaminated, in different proportions, with the different hCG molecule variants, and one should always keep in mind that the purest standards essentially devoid of contaminants used as calibrators are still derived from an urine pool. In addition, the assays use antibodies with different abilities to bind one or more of the hCG molecules, and have different designs. As a consequence, the hCG assays can measured different amounts of the same variants. Furthermore, either normal or abnormal trophoblastic or non-trophoblastic tissues secrete the hCG molecules in different ratios. In the clinical setting, to match a specific assay with the prevalent secreted molecule in a specific clinical condition is of paramount importance. In measuring hCG in serum or urine, the result will always be an amount of hormone that represents a pool of hCG and hCG-variant molecules. Because in normal pregnancy, the intact hCG dimer is the most prevalent molecule after a few weeks post-implantation, these difficulties may not represent a trouble for the clinician in monitoring normal pregnant women from 6 to 7 weeks of gestation until term. However, it may be a problem in monitoring patients with abnormal pregnancy, benign trophoblastic disease, choriocarcinoma and non-trophoblastic tumors. Even keeping in mind these difficulties, it is possible to choose a specific assay that best addresses a desired specific clinical condition.

A rationale might be the use of total hCG assays, able to measure intact hCG plus free hCGβ (or other variants) for diagnosis and follow-up of normal single pregnancy, suspected of ectopic pregnancy and risk of early pregnancy loss. For the diagnosis of pregnancy from the implantation to Week 5, the hCGβ variant should be preferred because the proportion of this variant at this gestational time is higher than 80%. Thus, this assay is preferable in all reproductive medicine units in which assisted reproduction techniques are used. Unfortunately, although sensitive, this assay is not available worldwide. Assays designed for the free hCGβ subunit detection are first choice in hydatiform mole, because in this situation this subunit is secreted in higher proportion than in normal pregnancy. CTP-hCG assay can also be used, since free hCGβ may be the major molecule present and is missed in the majority of assays requiring the presence of the hCG carboxy terminal extension. After evacuation, or chemotherapy, of gestational trophoblastic disease (GTD), an assay directed to the hCGn variant is preferred to following-up because both hCG and free hCGβ becomes nicked as their levels diminish.
Monitoring of invasive trophoblastic disease/choriocarcinoma requires hCGβn variant detection because the occurrence of nicking of the hCG dimer leads to rapid dissociation of αβ subunits. For screening of Down syndrome, assays able to detect total hCG and hCGb should be chosen, because in this trisomy, native hCG, free hCGβ and hCG are increased as a consequence of transcriptional hyperactivation of the hCGβ gene, increased half-life of the hyperglycosylated hCG form, lower utilization of circulating hCG or poor expression of full-length cognate hCG/LH receptors (LHCGR). To predict preeclampsia, both total hCG in serum and urinary hCGβc assays can be used. In this condition, there is abnormal trophoblastic secretion as a proliferative and compensatory response to trophoblastic damage. In non-trophoblastic tumors, the follow-up can be done by using total hCG assays because hCG is increased in nearly 50% of patients with non-seminomatous germ cell tumor and in 10–15% of patients with seminoma. As the free hCGβ subunit is increased in 30–70% of these patients, a specific hCGβ assay may also be used alone. Urinary hCGβc assay may have potential clinical utility to monitor many gynecological tumors in which this fragment is found over 70% of patients. Worldwide, there is a need for greater awareness and use of the more specific and sensitive new immunoassays recognizing different forms of CG.

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