Human Reproduction Update, Vol.15, No.1 pp. 69–95, 2009
Advanced Access publication on October 22, 2008 doi:10.1093/humupd/dmn036

human reproduction update

Human choriogonadotrophin protein core and sugar branches heterogeneity: basic and clinical insights

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TABLE OF CONTENTS

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 choriogonadotrophin / heterogeneity / structure / application

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 were 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

Table I Alpha and beta hCG composition

	hCGα	hCGβ
Number of amino acids	92	145
Molecular weight (Da)	14 900	23 000
Carbohydrate weight (Da)	4700	7000
Protein weight (Da)	10 200	16 000
Number of cysteine residues	10	12
Number of disulfide bonds	5	6
N-linked oligosaccharides	Asp 52, Asp 78	Asp 13, Asp 30
O-linked oligosaccharides	—	Ser 121, Ser 125, Ser 132, Ser 138
Complex-type *N*-oligosaccharides	SA α2,3 Gal β1,4 GlcNAcβ1,2 Manα1,6 ↘ Manβ1,4 GlcNAcβ1,4 GlcNAc - Asp ↗ SA α2,3 Gal β1,4 GlcNAcβ1,2 Manα1,6	SA α2,3 Gal β1,4 GlcNAcβ1,2 Manα1,6 ↘ Manβ1,4 GlcNAcβ1,4 GlcNAc - Asp ↗ SA α2,3 Gal β1,4 GlcNAcβ1,2 Manα1,6
Complex-type *O*-oligosaccharides	—	SA α2,3 Gal β1,3 GalNac-Ser

SA, sialic acid; gal, galactose; GlcNAc, *N*-acetylglucosamine; man, mannose.

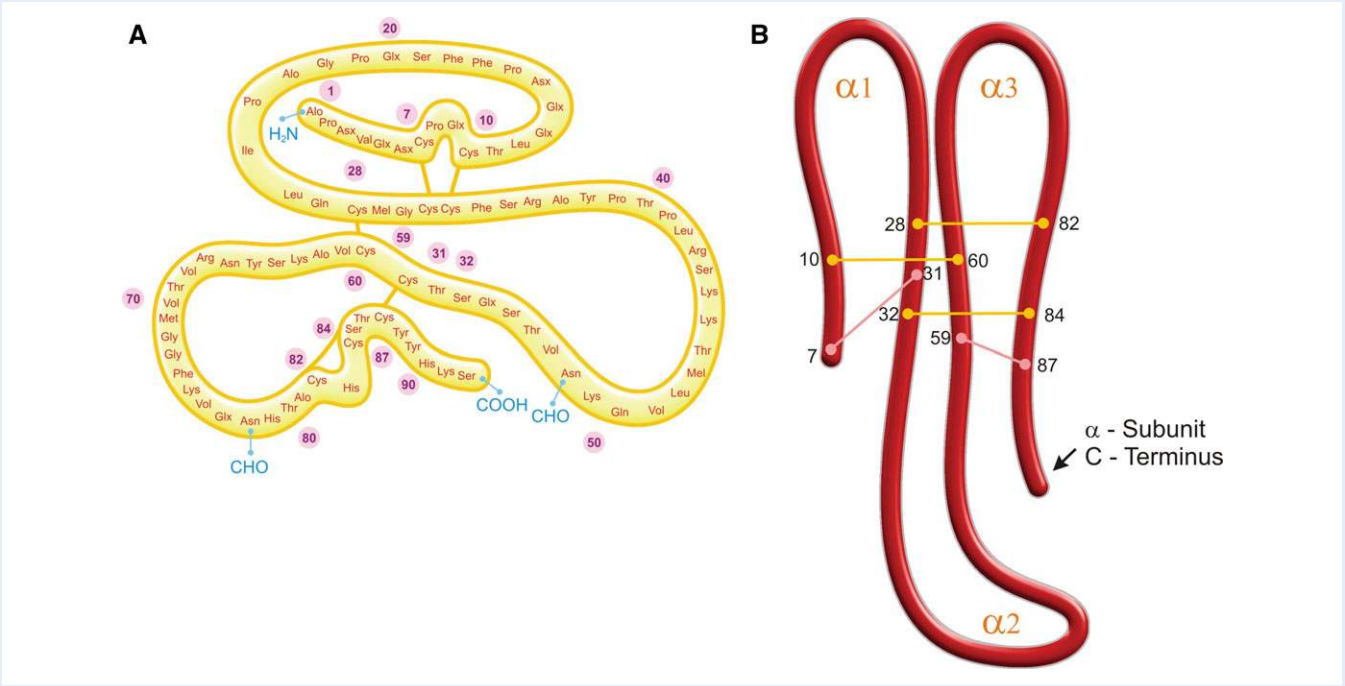


Figure 1 (**A**) Primary sequence of hCGα and locations of the disulfide bonds. From Mise and Bahl (1980), with permission.
(**B**) hCGα protein core and reassigned disulfide bonds forming three hairpin loops. From Xing *et al.* (2001), with permission.

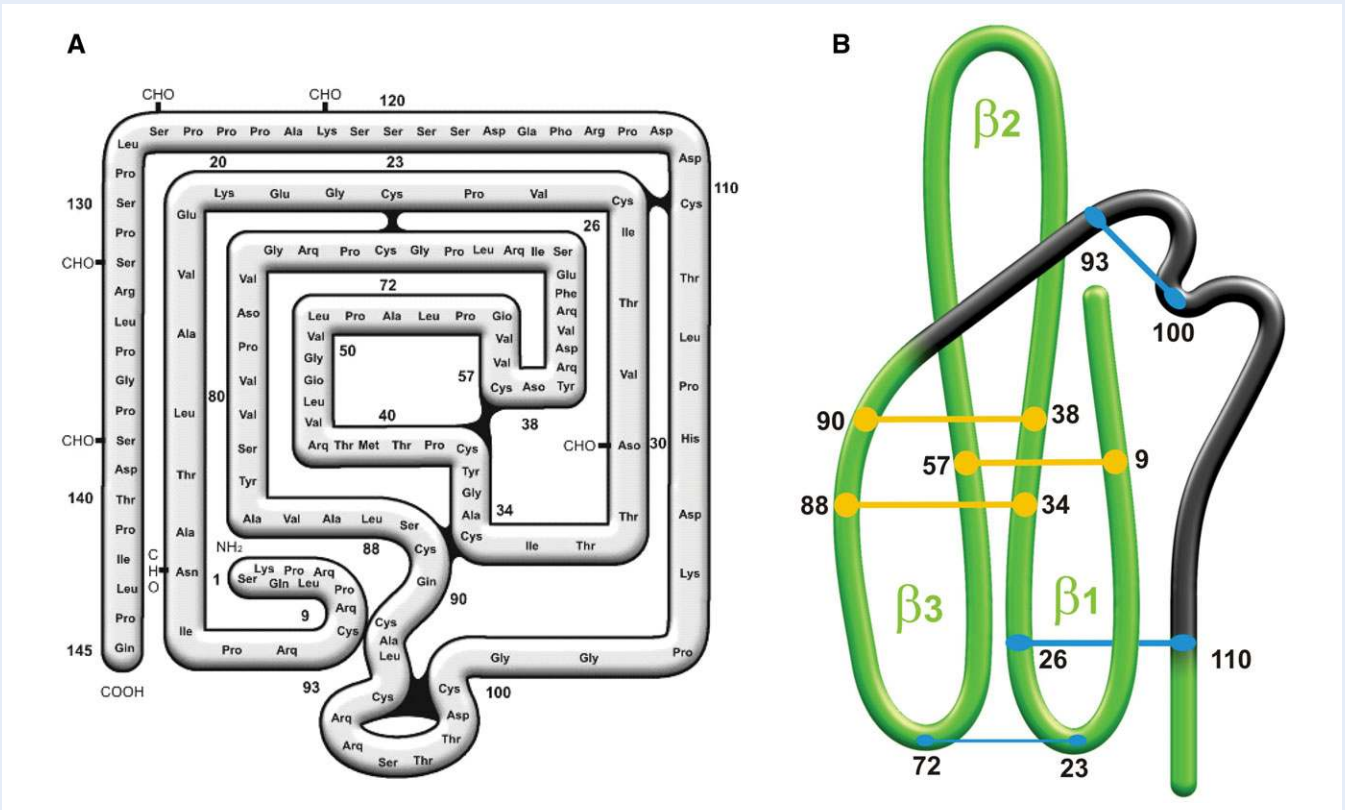


Figure 2 (**A**) The amino acid sequence of hCGβ showing the locations of disulfide bonds and carbohydrate moieties (CHO). From Mise and Bahl (1981), with permission.
(**B**) hCGβ subunit polypeptide chain (thick green line) with its seatbelt region (thick black line) and reassigned disulfide bonds. Numbers, amino acid numbers; yellow bars, cystine knots; thick blue lines, seatbelt disulfide bonds; thin blue line, small loop disulfide bond; B_1, B_2, B_3, loops of hCGβ. From Xing *et al.* (2001), with permission.

β38–90 bridged by disulfide bonds) through which the β9–57 disulfide bridge penetrates. In its carbohydrate branches, hCG has asparagine units on both α and β subunits and unique O-glycosydically 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 rugous endoplasmatic 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 are 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 transcriptor, 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 peptidases 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 β93–110) 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

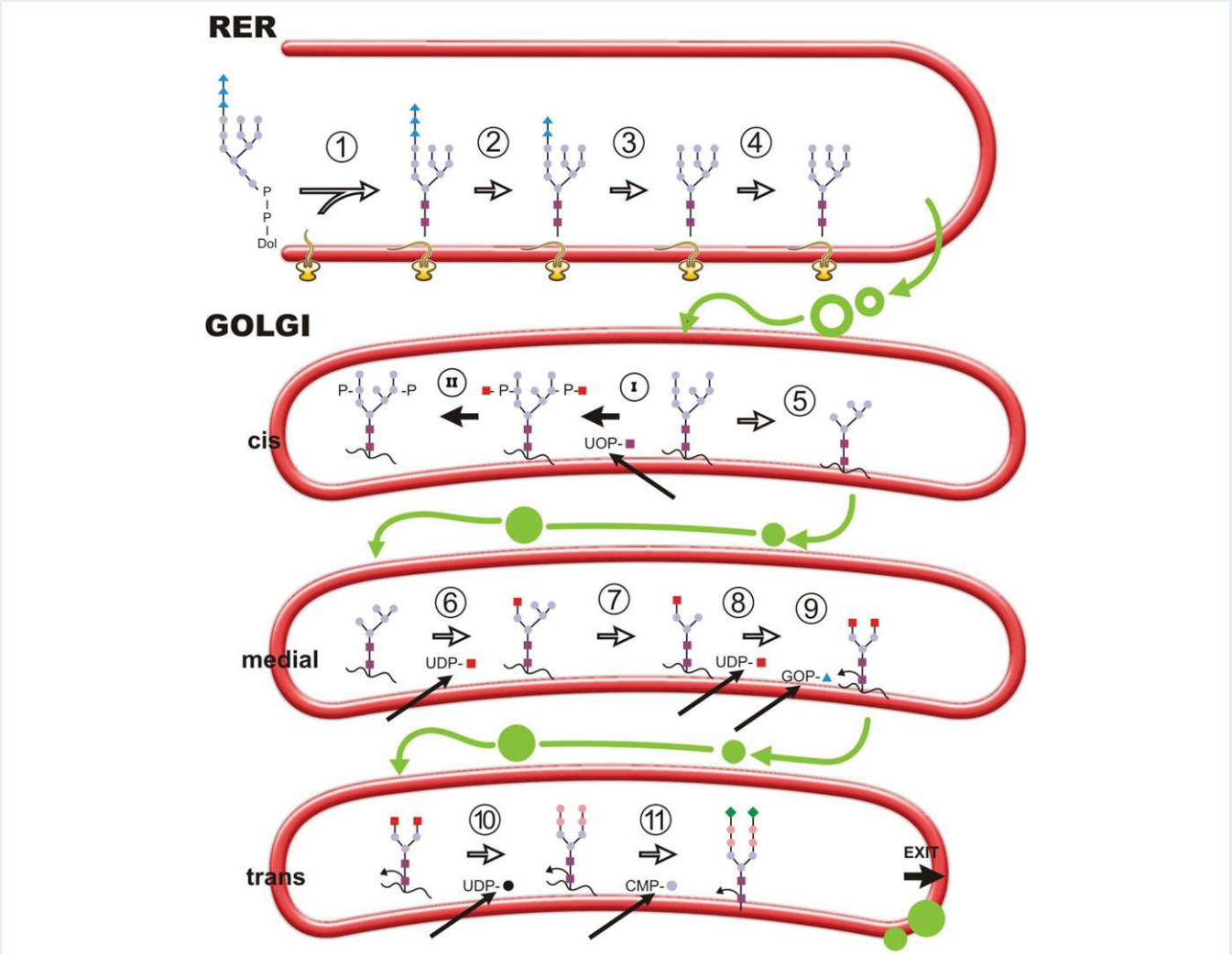


Figure 3 Schematic pathway of oligosaccharide processing on hCG molecule.

Arabian numbers represent: 1, oligosaccharyltransferase; 2, α-glucosidase I; 3, α-glucosidase II; 4, RER α1,2-mannosidase I; 5, golgi α-mannosidade I; 6, *N*-acetylglucosaminyltransferase I; 7, golgi α-mannosidade II; 8, *N*-acetylglucosaminyl transferase II; 9, fucosyltransferase; 10, galactosyltransferase; 11, sialyltransferase. Roman numbers represent: I, *N*-acetylglucosaminylphosphotransferase; II, *N*-acetylglucosaminylphosphotransferase-1-phosphodiester α-*N*-acetylglucosaminidase. The symbols represent: ■ *N*-acetylglucosamine, ▲ glucose, • galactose, ○ mannose, Δ Fucose and ◆ sialic acid. From Kornfeld and Kornfeld (1985), with permission.

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 hCGα and its removal reduces αβ correct dimerization (Matzuk and Boime, 1988; Feng *et al*., 1995). The Asn-30 oligosaccharide on the hCGβ is important for secretion but not assembly, and the Asn-13 branch influences mainly the assembly (Fares, 2006).

The polypeptide sequences α27–40 and α38–42 have an important role in the dimer formation. Whereas αTyr-37 is a critical residue for proper combination of αβ, the αTyr-65 is involved in holding both subunits in native conformation. In addition, the single substitution of αThr 39 with Phe or Ala eliminates or reduces αβ dimerization (Xia *et al*., 1994). The residues β90–111 wrap around a helical loop of the hCGα and play an important role in subunits assembly, but the whole hCGβ-CTP (β115–145) extension is not required for subunit association. Besides the sugar branches and the polypeptide composition of α and β 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 α7–α31, α59–87 and α10–32 are not essential for the hCGα combination with the hCGβ, but the hCGβ disulfide bonds β9–57, β34–88 and β38–90 are essential for heterodimer formation (Mishra *et al*., 2003). The disulfide bond β26–110 is formed only after αβ assembly (Huth *et al*., 1992). The αβ subunits are aligned 'head-to-toe' such that α_2 loop is adjacent to β_1, β_3 and β_2 is adjacent to α_1,

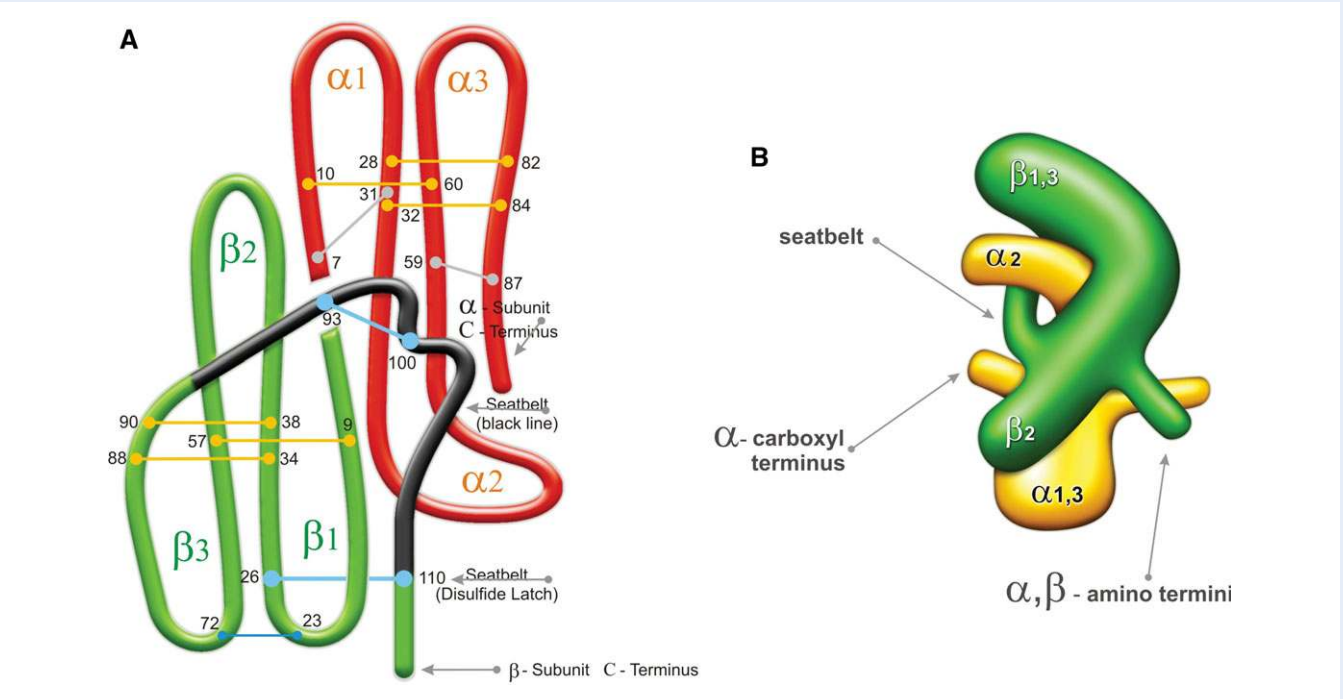


Figure 4 (**A**) Assembly of α and β subunits to form hCG dimer.

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 α:β assembly in which the hCGα carboxyl terminal extension penetrates the hCGβ and is locked by the β-seatbealt portion. Adapted from Moyle *et al.* (1998).

α_3 (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).

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 serial 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 progestational 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.

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 down-regulation. 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 apposition of the α loop 2 and β loops 1 and 3 (Moyle *et al.*, 1995) (Fig. 5). The conformational

Table II Endocrine and paracrine/autocrine regulators of trophoblast hCG secretion *in vitro*, according to the pregnancy time

Factor	Trimester	Effect on hCG biosynthesis	Reference
hCG	Third	Stimulates	Licht *et al.* (1993)
GnRH	First, Third	Stimulates	Barnea and Kaplan (1989)
GnRH-antagonist	First	Inhibits	Sidler-Khodr *et al.* (1983)
cAMP	Third	Stimulates	Fenstermaker *et al.* (1989)
β adrenergic	First	Stimulates	Oike *et al.* (1990)
Dexamethasone	Third	Stimulates	Ringler *et al.* (1989)
Activin	Third	Potentiates GnRH	Petraglia *et al.* (1989)
EGF	First, Third	Stimulates	Cao *et al.* (1994)
Interleukin-1	First	Stimulates	Masuhiro *et al.* (1991)
Interleukin-6	First	Stimulates	Masuhiro *et al.* (1991)
Inhibin	Third	Inhibits	Petraglia *et al.* (1989)
Progesterone	First	Inhibits	Szilágyi *et al.* (1992)
Prolactin	Third	Inhibits	Yuen *et al.* (1980)
LIF	Third	Inhibits	Sawai *et al.* (1995)
TNF-α	First	Stimulates	Li *et al.* (1992)
M-CSF	First	Stimulates	Saito *et al.* (1991)
Leptin	Third	Stimulates	Cameo *et al.* (2004)
TGF	First	Inhibits	Morrish *et al.* (1991)
Mifepristone	First	Inhibits	Das and Catt (1987)
Glycodelin A	First	Stimulates	Jeschke *et al.* (2005)
17β-estradiol	First	Inhibits	Sharma *et al.* (1993)
GABA	First	Stimulates	Licht *et al.* (1992)
PTH	First	Stimulates	Dodeur *et al.* (1991)
Opioids	First	Stimulates	Cemerikic *et al.* (1988)
Insulin	First	Inhibits	Barnea *et al.* (1993)
HGH	Third	Stimulates	Di Simone *et al.* (1995)
H_2O_2	Third	Stimulates	Aris *et al.* (2007)
Triiodothyronine	First	Stimulates	Maruo *et al.* (1991)

EGF, epidermal growth factor; LIF, leukemia inhibiting factor; TNF, tumor necrosing factor; M-CSF, macrophage-colony-stimulating factor; TGF, transforming growth factor; GABA, gamma amino butyric acid; PTH, parathyroid hormone; HGH, human growth hormone.

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 hCGβ, the amino acid residues Tyr-Tyr-His-Ly-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

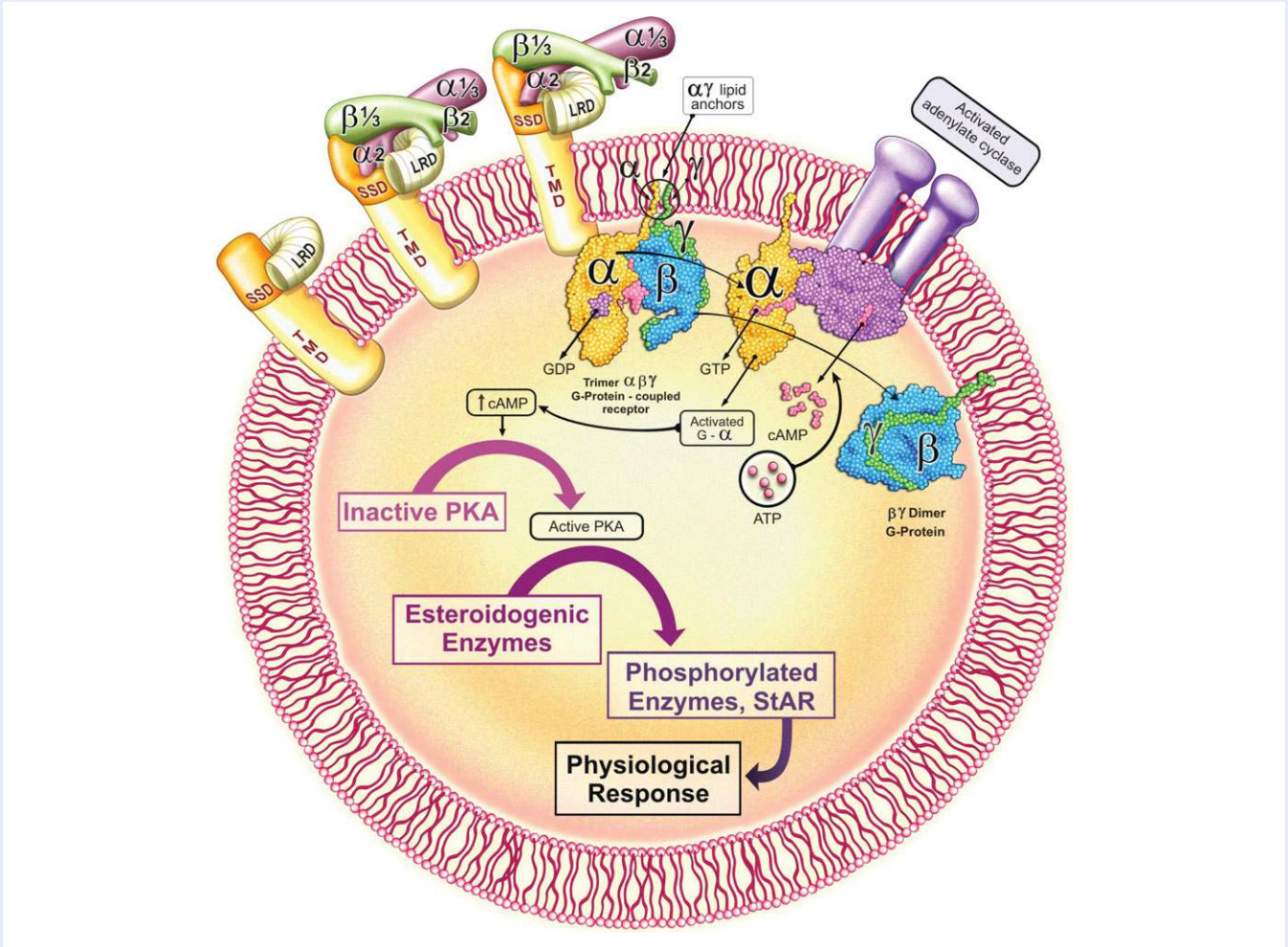


Figure 5 Model of hCG signal transduction showing the signaling specificity domain (SSD) on the extracellular surface of the transmembrane domain (TMD) and the leucine-rich domain (LRD) near the SSD–TMD complex.

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. Gα activates adenylate 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).

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

(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 βO-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-RII 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 glycogenolysis in human placental villi (Demers *et al*., 1973), stimulates prostaglandin synthesis by placental tissue and inhibits myometrium contractility (Ticconi *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 prolactin 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 B_2–B_5) (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, R525, 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 antigenic site and recognizes heterodimeric hCG, hCGβ and the hCGβ core fragment (hCGβcf). Three monoclonal antibodies (B_{101}, B_{107} and B_{109}) bind to site III (β38–56) on dimeric hCG and measure exclusively intact hCG. Site IIIa recognizes antibody A_{109} which has affinity to free hCGα and antibody A_{102} which bind almost exclusively hCGα on hCG. Site IV (β100–109), binding B_{201}, B_{202}, B_{204}, B_{205}, B_{208} and B_{210} antibodies, is present on hCGβcf and on free hCGβ. B_{201} antibody does not discriminate between free hCGβ and hCGβcf, but it does not bind to dimeric hCG. B_{204} and B_{205} antibodies bind the hCGβcf with an affinity 10 times higher than that for free hCGβ. B_{210} 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

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

HCGβcf, hCGβ core fragment.

Two monoclonal antibodies (A_{103} and A_{105}) 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 DeM_3) or monoclonal (B_{201}, B_{208} and B_{210}) 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 B_1–B_9 (Berger *et al*., 1996). The first hCGβ loop accommodates epitope B_6 (β20–25) and the third one the B_7 (β68–77) (Jackson *et al*., 1996). While epitopes β_6 and β_7 are specific for free hCGβ (Table III), epitopes B_1–B_5, B_8 and B_9 are accessible both on the free and assembled forms of hCGβ. Epitopes B_1, B_6, B_7, B_8 and B_9 are specific to the βCTP, epitopes B_2, B_3, B_4 and B_5 cross-react with LH and LHβ molecules, but their location has not been determined yet. In addition, there exist four epitopes specific for hCGβcf (B_{10}–B_{13}) not shared by free or assembled hCGβ (Dirnhofer *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 (A_1–A_7), arranged in four spatially distinct antigenic domains. One of these clusters to loop α1 and includes sequences α13–22 (A_1 and A_4). The second epitope (A_2), located on loop 3 α65–68, clusters on the intact hCG, is conformation sensitive. The third epitope (A_3), portion α32–41, specific for the free hCGα, is masked by the hCGβ in the native hormone (Dirnhofer *et al*., 1994b). The sequences α13–18, α17–22, α33–42 and α87–92 form the epitopes A_1, A_4, A_6 and A_7, respectively, and are dependent on the tertiary structure. The sequence α6–7 is specific for the free hCGα subunit. The free hCGα epitope A_6, 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 this 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 Kuppfer cells in liver tissue and distributed along the sinusoides (Nisula *et al*., 1989). In the ovary, variable amounts of hCG molecules are internalized by granulosa/theca lutein cells (Zimnisky *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,

Table IV Intact hCG and variant molecular forms

Molecular form	Source	References
hCG		
Intact, standard		
Hyperglycosylated	Pregnancy, tumors	Cole (2007)
Nicked	Pregnancy, tropoblastic neoplasia	Cole *et al.* (1991)
Hypoglycosylated	Pregnancy, tumors	Dufau *et al.* (1972)
Asialo-hCG	Choriocarcinoma, gestational thyrotoxicosis	Mizuochi *et al.* (1983)
Acidic hCG	Trophoblast tumor, testicular tumor	Cassels *et al.* (1989)
Pituitary hCG	Non-pregnant women	Odell and Griffin (1989), Braunstein (2002)
Deglycosylated hCG	?	Manjunath and Sairam (1982)
Large hCG	Placenta extracts	Maruo *et al.* (1980)
hCGβ		
Free hCGβ	Pregnancy, tumors	Cole (1998)
Hyperglycosylated	Pregnancy, tumors	Butler and Iles (2004)
Pré-beta form	Placenta	Hussa *et al.* (1986)
Nicked	Pregnancy, tumors	Birken *et al.* (2001)
hCGα		
Free hCGα	Pregnancy, tumors	Weintraub *et al.* (1977)
Hyperglycosylated	Pregnancy, tumors	Nemansky *et al.* (1998b)
Pre-alpha form	Placenta	Sakakibara *et al.* (1986)
Big hCGα	Pituitary, pregnancy, choriocarcinoma	Blithe and Nisula (1985)
Small hCGα	Pregnancy, non-tropoblastic tumors	Bielinska *et al.* (1989)
Other fragmented forms		
hCGβ missing CTP	Trophoblastic, non-trophoblastic tumors	Berger *et al.* (1993)
Asialo β-CTP	Choriocarcinoma, pregnancy	Amr *et al.* (1985)
hCGβ peptide 48–145	Pregnancy, tropoblastic disease	Nishimura *et al.* (1998)
hCGβ missing CTP	Trophoblastic neoplasia	Wang *et al.* (1989)

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 Nicopoulou, 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 neoplasm (GTN) (Norman *et al.*, 1990a).

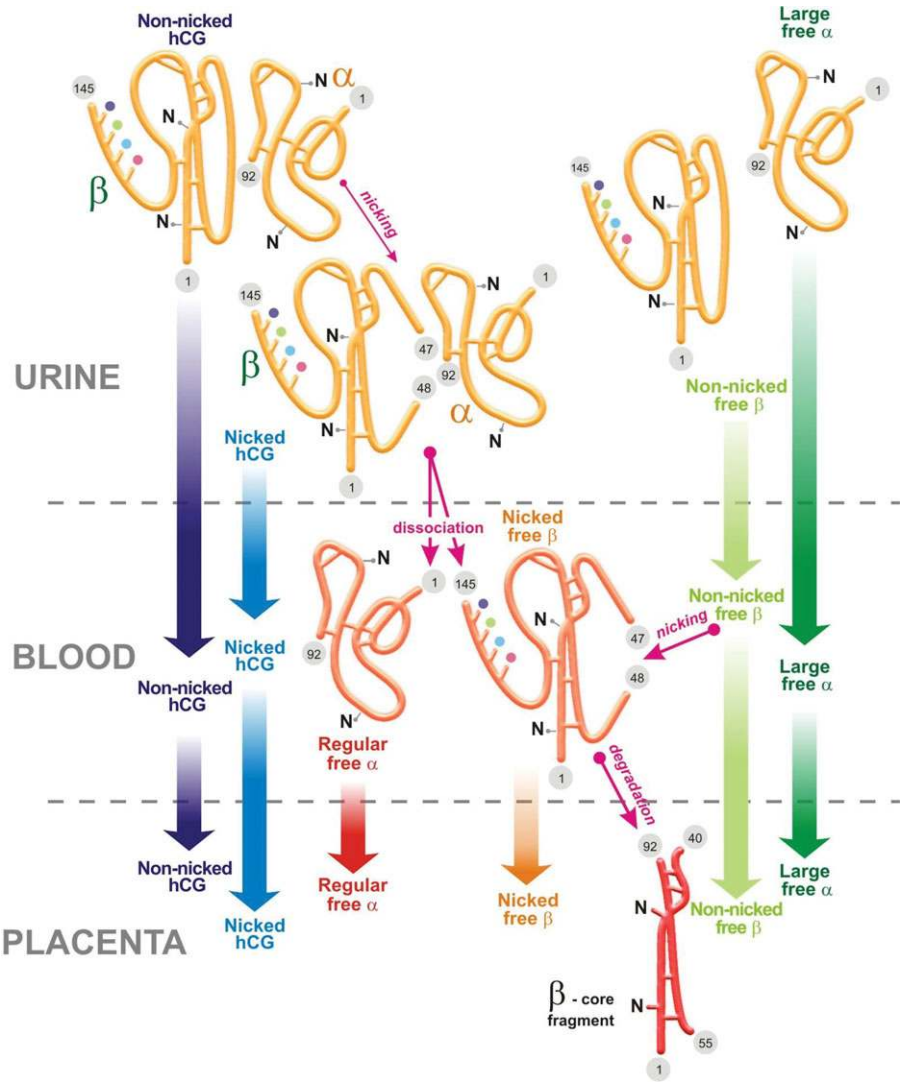
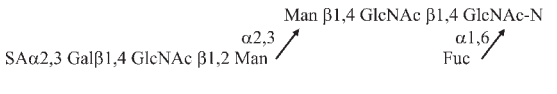
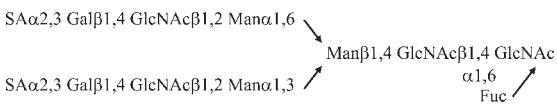
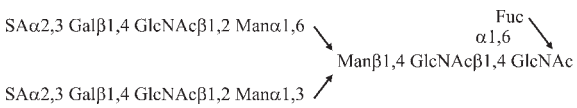
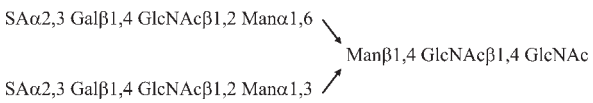
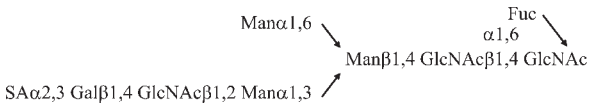
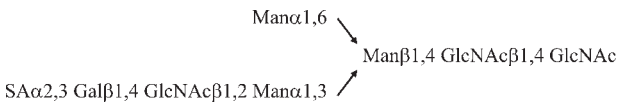
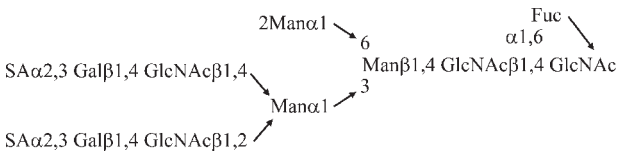
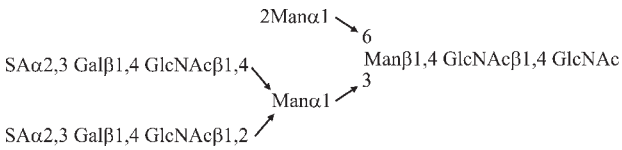
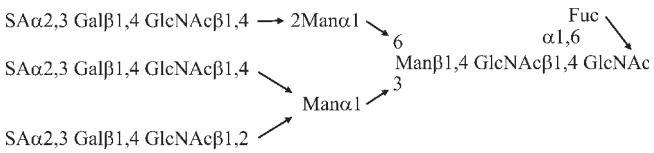
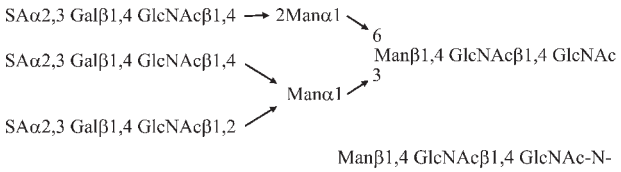
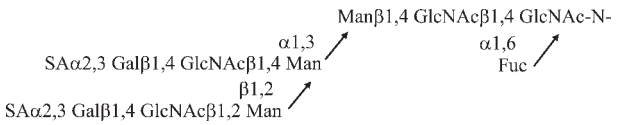
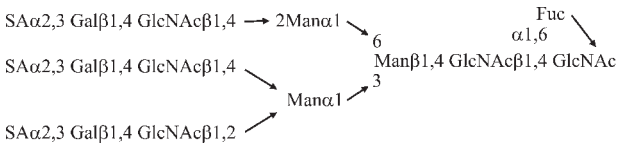


Figure 6 hCG and variant molecules in the placenta, blood and urine.Thick lines, polypeptide chains; numbers, amino acid numbers; thin lines, disulfide bonds; N, asparagines-linked oligosaccharides; O, serine-linked oligosaccharides; solid arrows, nicking, dissociation and degradation pathways. [Adapted from Cole (1997) with permission.]

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 fluoroimmunoassay 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, intrauterine 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, Shenhav *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,

Table V N-linked oligosaccharide types found in standard hCG and/or hCG-variant forms

N-linked oligosaccharide	Clinical condition	Prevalent hCG form	Mechanism
	Normal pregnancy	hCG, free hCGα	Glucosidases normal expression
			
	Normal pregnancy	hCG, hCGβ (Asp13), hCGα (Asn78)	Normal expression of glucosidases and sialyl fucosyltransferases
	Normal pregnancy	hCG, hCGβ (Asp 30)	Normal expression of glucosidases and sialyl fucosyltransferases
	Normal pregnancy Hydatiform mole Invasive mole	hCG	↑Expression α1-6 fucosyltransferase
	Normal pregnancy Invasive mole	hCG, hCGα (Asp52)	↑*N*-acetylglucosaminyl transferase IV
	Invasive mole	hCG	↑α1–6 fucosyl IV and α1–4 *N*-acetylglucosaminyl transferases
	Gestational diabetes Invasive mole	hCG	↑*N*-acetylglucosaminyl transferase IV
	Early pregnancy Invasive mole ↓Choriocarcinoma	hCG, hCGh	↑Expression *N*-acetylglucosaminyl transferase IV ↑(1→6/1→6 branches) β1→4 galactosyltransferase
	Choriocarcinoma	hCG	↑*N*-acetylglucosaminyl transferase V (1→4/1→3 branches)
	Choriocarcinoma	hCG	↑α1–6 fucosyltransferase IV β1–4 galactosyltransferase (1→6/1→6 branches)
	Choriocarcinoma Testicular germ cell cancer	hCG	↑*N*-acetylglucosaminyl transferases IV and V

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

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

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

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

Clinical condition	Prevalent molecule	Preferred assay	Available assays/source
Normal pregnancy, early detection (Weeks 1–5)	hCGh	Intact hCG, hCGh, hCGhn	• Baxter—Dade-Stratus II, IFMA, American Dade Delfia hCG Kit, Pharmacia Wallac hCGh assay, Nichols Isntitute Diagnostics
Normal single pregnancy detection, diagnosis and follow-up of ectopic pregnancy or early pregnancy loss	Dimer hCG	Total hCG (hCG + hCGβ), hCGn	• Elecsys hCG + hCGβ, ECLIA, Roche Diagnostics Tanden R hCG, IRMA, Hybritech, Inc. Immulite 2000, IRMA DPC, Inc.
Hydatiform mole	hCG, hCGβ, CTP-hCG	hCGβ, CTP-hCG	• hCGβ Delfia assay, CLIA, Pharmacia Wallac Immulite 2500 free βhCG, IRMA, DPC, Inc. UBI MAGIWEL free hCGβ, ELISA United Biotech, Inc. Tosoh AIA600, EFMA, Somagen Diagnostic, USA
Placental site trophoblastic tumor	hCGβ	Free hCGβ	• Immulite 2500 free βhCG, IRMA, DPC, Inc.
Choriocarcinoma	hCG, hCGh, hCGβ	Total hCG, hCGh, hCGβ	• Immulite 2000, IRMA DPC, Inc.
GTD after evacuation/chemotherapy	hCGn	hCGn, hCGβn	hCGn, IRMA, not commercially available/Dr J.C. O'Connor; Irving Center for Clinical Research, Columbia University Beckman Coulter Access/Access2, CLIA, Beckman Coulter Inc., USA
Down's syndrome screening Pre-eclampsia prediction	hCG, hCGβ, hCGh	Total hCG, hCGh, hCGβcf (urine)	• Baxter-Dade-Stratus II, IFMA, hCGh Nichols Assay
Germ cell tumors	hCG, hCGβ	Total hCG, hCGβ, hCGβcf (urine)	• UFG-EIA Toa-EIA, Toagosli Co. Ltd, Tokyo, Japan
Gynecologic tumors	hCGβ	hCGβ, hCGβcf (urine)	Immulite 2500 free βhCG, IRMA, UFG-EIA Toa-EIA

GTD, gestational trophoblast disease; hCGh, hyperglycosylated hCG; hCGn, nicked hCG.

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

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 mUI/ml in these tumors indicate greater risk and worse prognosis (Mead and Stenning, 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 Week 4 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. hCGh 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 hCGh 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 (Elliott *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 isoform (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).

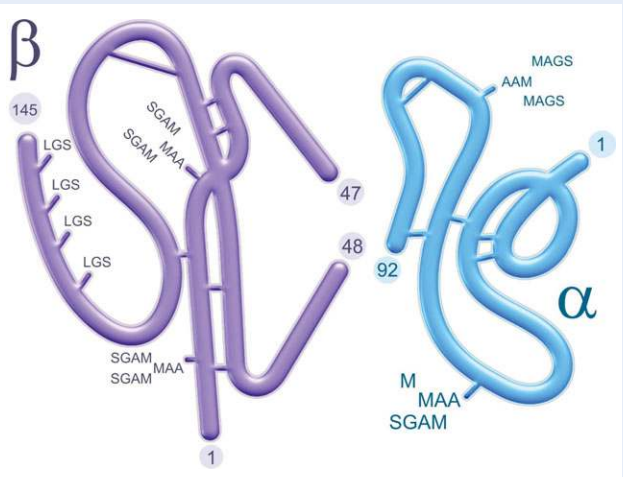


Figure 7 Folded structure of nhCG.

Numbers indicate amino acid sequence. A, *N*-acetylglucosamine; L, *N*-acetylgalactosamine; M, mannose; G, galactose; S, sialic acid; F, fucose. From Cole (1998).

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 mUI/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 (hCGα) 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., 1998b). 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 hCGα can itself be heterogeneous; about 70% of the molecules produced in normal placenta have their amino acid chains with the sequence Ala-Pro-Asp-NH2, 20% initiate with Val-NH2 and 10% with Asp-Va-NH2. hCGα 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 hCGα subunits as part of the dimer is nicked between residues α70 and α71 (Birken *et al*., 1978).

The free mature form of hCGα has been detected in hCG commercial preparations, urine and serum from pregnant women, placenta explants, pituitary and tumors. The free hCGα 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 (Benveniste and Scommegna, 1981). A hyperglycosylated hCGα form unable to combine with hCGβ was found in normal pregnancy and individuals with tumors. A big mature molecular form of hCGα (hCGαb) 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 hCGα (hCGαs), 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 hCGα 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, hCGα subunit is produced by the pituitary and reflects the pooled production of hCGα, FSHα, LHα and TSHα. In post-menopause women, the levels of hCGα increase about five times (Norman *et al*., 1987). About 10–20% of the patients with testicular cancer have elevated serum levels of hCGα and individuals with tumor carcinoids show even a much higher hCGα concentration (Alfthan and Stenman, 1996). Because it is produced in high levels, the measurement of hCGα can be useful for early detection of tumors in pituitary, lung, testes, insulinomas and gastric or pancreatic carcinoid (Braunstein *et al*., 1979). At least in a lung tumor cell line, hCGα seems to exert a paracrine growth-stimulating function (Rivera *et al*., 1989). Even though assays to quantify only the hCGα 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 hCGβ 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*., 2006) or 41 000 mUI/ml (Vaillant *et al*., 1996).

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; Piura *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 mUI/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 digynic origin (Spencer *et al*., 2002). When the free hCGβ 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 hCGβ 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 hCGβ 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 hCGβ 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 hCGβ intermediate which exposes hCGβcf 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 hCGβ molecule that is cleaved more easily, resulting in high levels of hCGβn form (Roig *et al*., 2007). In cases of trisomy 18, the levels of hCGβ are low in the first trimester and diminish yet more in the second trimester (Krantz *et al*., 2004). In trisomy 13, the levels of hCGβ 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 (hCGβcf) results from the removal of most of loop 2 of the hCGβ 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 hCGβ, 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 hCGβ, and contain only 5–11 residues of sugar. In addition, 30% of the hCGβcf molecules are completely deglycosylated (de Medeiros *et al*., 1993). The fragment has no proved function yet. The availability of highly purified preparations of hCGβcf allowed the development of specific assays for its quantification without cross-reaction with other related hCG molecules (de Medeiros *et al*., 1992c; Krichevsky *et al*., 1994). A new WHO standard for hCGβcf measurement was recently purified hCGβcf 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 hCGβcf (Cole *et al*., 1999).

The hCGβcf 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, choriocarcinoma and ovarian cancer cells secrete hCGβcf (de Medeiros *et al*., 1992a; Okamoto *et al*., 2001). The hCGβcf 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 hCGβcf was not seen in patients with gynecological neoplasmas, however (Neven, *et al*., 1994). In the second half of pregnancy, the hCGβcf concentration represents 15–75% of the total hCG immunoreactivity, either when estimated by assay using antibodies that also recognize free hCGβ 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 hCGβcf 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 hCGβcf in pregnancy is always in excess of about 1.6–9.6-fold between Week 6 and Week 41. The secretion hCGβcf emerge as the predominant form in 5 weeks after-conception.

In the first trimester, hCGβcf 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 hCGβ (Cole *et al*., 1994b). In MoM normalized for creatinine, the values of hCGβcf 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 hCGβcf 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 hCGβcf show positive correlation with gestational age between Week 4 and Week 6 in pregnancies with normal evolution, the measurement of hCGβcf 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 hCGβcf 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 hCGβcf increase. The decrease in trophoblast oxygen pressure which occurs in pre-eclampsia increases the hCG production and hCGβcf form (Roiz-Hernandez *et al*., 2006), probably as a consequence of increased H_2O_2 signaling (Aris *et al*., 2007). The relative risk of pre-eclampsia is 2.1 times higher in gestations with levels of hCGβcf $\geq$2.0 $\leq$4.0 MoM and 5.2 times in cases which the hCGβcf level is above 4.0 MoM, when compared with normal gestation.

The measurement of hCGβcf 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 hCGβcf 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 identify fetuses with trisomy 21 when its levels are $\geq$97th percentile; sensitivity of 61% and false-positive rate of 3.2% (Bahado-Singh *et al*., 1998). The studies examining the effectiveness of hCGβcf measurement in the detection of Down syndrome show sensitivity between 41% and 93% and specificity between 90%

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 its 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 32 mUI/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, preeclampsia 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 hCGh 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 α:β subunits. For screening of Down syndrome, assays able to detect total hCG and hCGh should be chosen, because in this trisomy, native hCG, free hCGβ and hCGh 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 pre-eclampsia, both total hCG in serum and urinary hCGβcf 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 hCGβcf 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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Submitted on May 7, 2007; resubmitted on April 18, 2008; accepted on July 14, 2008