Human chorionic gonadotropin (hCG) isoforms and their epitopes: diagnostic utility in pregnancy and cancer

P Berger, Ph.D, Professor, Head Endocrinology Division[1], C Sturgeon, Ph.D.[2]

[1]Institute for Biomedical Aging Research, Austrian Academy of Sciences, Innsbruck, Austria, [2]Dept. of Clinical Biochemistry, Royal Infirmary, Edinburgh, U.K.

Key Words
diagnostics, epitopes, hCG, immunoassay standardization, international standards, monoclonal antibodies, variants

Correspondence
Peter Berger, Ph.D.
Head Endocrinology Department
Institute for Biomedical Aging Research
Austrian Academy of Sciences
Rennweg 10
A6020 Innsbruck
Austria

Tel.: ++43-512-583919-24
Fax.: ++43-512-583919-8
E-mail: peter.berger@oeaw.ac.at

List of Abbreviations

hCG	human chorionic gonadotropin
hCGn	nicked hCG
hCGα	hCG alpha subunit
hCGβ	hCG beta subunit
hCGβn	nicked hCG beta subunit
hCGβcf	hCG beta core fragment
hCGβCTP	carboxyl-terminal peptide of hCGβ
hCGαα	dimer of hCGα
hCGββ	dimer of hCGβ
hCGh	hyperglycosylated hCG variants
GPH	glycoprotein hormone
mAbs	monoclonal antibodies
LH	luteinising hormone
LHβ	luteinising hormone beta subunit
FSH	follicle stimulating hormone
TSH	thyroid stimulating hormone
CKGF	cystine knot growth factor
NGF	nerve growth factor
PDGF-B	platelet derived growth factor
TGFβ	transforming growth factor beta
LH/hCG-R	LH/hCG-receptor
CGβ1	hCGβ gene 1
IRR	International Reference Reagent
IFCC	International Federation of Clinical Chemistry
V	Valine, amino acids single letter code
Ser	Serine, amino acids triple letter code
avhCG	acidic variants of hCG
LC-ESI-MS	liquid chromatography-electrospray ionisation-tandem mass spectrometry
c-mAbs	mAbs recognizing conformationally intact holo-hCG
ISOBM	International Society of Oncodevelopmental Biology and Medicine
β_1	epitope 1 on hCGβ
INN	Innsbruck
IU	International Units
WG	Working Group
IS	International Standard
WHO	World Health Organisation
NIBSC	National Institute for Biological Standards and Control
trunc-hCG	truncated hCG

ABSTRACT

Background: Clinical management of pregnancy, pregnancy-related disorders and trophoblastic tumors is dependent on immunoassay measurements of the complex analyte human chorionic gonadotropin (hCG). Differences in hCG results using different methods affect clinical interpretation with potentially adverse consequences for patient care. *Objectives/Method:* To provide an overview of factors contributing to method-related differences in hCG measurements and how to overcome these drawbacks. *Results/conclusion:* Six recently established highly purified and molar unit calibrated WHO Reference Reagents for important hCG-variants provide means for indepth characterization of diagnostic immunoassays for hCG. For different clinical applications in pregnancy and cancer appropriate epitopes and specificities of pairs of monoclonal antibodies against hCG in immunoassays have been clearly defined. This lead to the conclusion that in routine clinical situations assays are preferred that measure all relevant hCG-variants. The adoption of new nomenclature unambiguously describing what is being measured and the anticipated introduction of a new highly pure International Standard (IS) for hCG represent significant progress towards improved analytical reliability and comparability of diagnostic hCG results.

1. INTRODUCTION

Human chorionic gonadotropin (hCG) is a heterodimeric glycoprotein hormone (GPH) structurally closely related to luteinising hormone (LH), follicle stimulating hormone (FSH) and thyroid stimulating hormone (TSH). These are members of the cystine knot growth factor (CKGF) superfamily that also includes nerve growth factor (NGF), platelet derived growth factor (PDGF-B), transforming growth factor beta (TGFβ) and other molecules. Three-dimensional X-ray crystallographic studies

demonstrate that the structural hallmark of each of the two subunits of hCG (hCGβ and hCGα) is a central cystine knot consisting of two disulfide bonds which connect two β-sheets to form a ring which a third disulfide bond penetrates. Two shorter hairpin-like loops of double stranded β-sheets protrude from one side of the cystine knot (loops 1 and 3) that are opposed by a single longer loop (loop 2) (Figure 1) [1,2]. The two subunits are associated in a head-to-tail fashion. This αβ-heterodimer is termed hCG, is biologically active and regulates sex steroid production through a 7 transmembrane G-protein-coupled chimeric receptor (LH/hCG-R), which contains a large leucine rich repeat extracellular binding-domain [3].

The α-subunit is encoded by a single gene on the human chromosome 12q21.1-23. It is 92 amino acids (aa) in length and common to all four human GPHs. Consequently measurement of the α-subunit is not specific for hCG or any other GPH. The β-subunits of GPHs confer receptor- as well as immunological specificity with the exception of hCGβ and hLHβ that share to a high degree genetic, biochemical, biological and immunological features. hCGβ- and related hLHβ-encoding genes (*CGβ1* to *CGβ9*) located on chromosome 19q13.3 are presumed to have arisen from a hLHβ precursor gene by gene duplications and a rare read-through event in which a previously untranslated region was incorporated into the coding sequence following a mutational frameshift due to a nucleotide deletion in codon 113 and subsequent loss of a stop codon (for review see [4]). This gave rise to a unique carboxyl-terminal peptide extension of hCGβ (hCGβCTP corresponding to amino acids *113-145*), which facilitates design of specific immunoassays for determination of hCG even in the presence of hLH, with which hCG shares over 85% structural homology in the first 114 amino acids of the β-subunit.

Measurement of intact heterodimeric hCG and to a lesser extent hCG-related analytes such as hCGβ have important diagnostic applications in both pregnancy and oncology [5,6,7,8]. The intact hormone hCG and its isoforms, subunits and variants are secreted by trophoblastic cells of the placenta and by a variety of tumor tissues as well as by various normal tissues [9,10]. Some metabolites of hCG-related molecules (e.g. beta core fragment, hCGβcf) are primarily generated in the kidney [11] but have also been observed in bladder cancer tissue [12]. These metabolites are found in relatively high concentrations in the urine of healthy pregnant women and some cancer patients and at low concentrations in urine from non-pregnant individuals [6].

In non-pregnant healthy individuals free and combined levels of hCGα in serum and urine are predominantly determined by the pulsatile secretion of the pituitary and to a minor extent by various other tissues [9,13,14,15,16]. In non-pituitary tissues hCGα secretion may be regulated by G-protein-coupled receptors other than those for gonadotropin releasing hormone [17].

Until recently, the diagnostic reliability of immunoassays for hCG isoforms has been limited by (i) the remarkable heterogeneity of the analyte, (ii) insufficient information on epitope distribution and sharing of these epitopes by hCG isoforms, (iii) lack of consensus about the most appropriate antibody specificities for use for clinical applications, (iv) lack of highly purified and well-characterized reagents with which to assess the specificities of diagnostic mAbs used in current immunoassays, and (v) difficulties in comparing and describing the extent of detection of molecular variants by different commercial immunoassays. Data from external quality assessment (EQA) and proficiency testing schemes consequently demonstrate significant method-related differences in results [18,19].

However, as a result of several international initiatives, considerable progress has been made in addressing these problems. Clear descriptive nomenclature for some of the most important hCG-related analytes has been developed [19](Table 1) and highly purified International Reference Reagents (IRRs) for six of these established [20,21]. Consensus has also been achieved regarding specificity and epitope requirements for mAbs suitable for use in different clinical applications [22]. These developments should facilitate progress towards improved comparability of immunoassays for hCG. The approach that has been taken for hCG should ultimately improve the comparability of diagnostic methods for this analyte while also providing a useful template for standardization of other complex analytes [18,19,22].

2. hCG variants

hCG is not a single well-defined molecular entity but comprises a family of closely related isoforms, displaying considerable molecular heterogeneity, both protein and carbohydrate in nature [4,7]. Its protein variants mainly result from post-transcriptional and metabolic modifications such as nicking, truncation, homo-dimerization and oxidization. It is not clear whether all modifications of the protein backbone are naturally occurring variants, and their biological function and clinical significance are not all known. The nomenclature developed for hCG and well-defined hCG-variants (Table 1) by the International Federation of Clinical Chemistry (IFCC) Working Group for Standardization of hCG enables clearer description of some of these variants according to the protein backbone structure [19,20].

2.1. Genes and protein diversity

hCGβ gene multiplicity and diversity does not appear to contribute significantly to protein diversity with the exception of hLHβ. Of nine genes in the hCGβ/hLHβ genes cluster (designated *CGβ1* to *CGβ9*) on chromosome 19q13.3, gene 4 encodes for LHβ, genes *CGβ1* and *2* probably are pseudogenes and theoretically should not encode hCGβ protein, and *CGβ6* and *7* are allelic as are *CGβ3* and *CGβ9*. Thus four genes and two alleles (*CGβ3/9, 5, 6/7* and *8*) remain that code for genuine hCGβ protein of 145 amino acids in length. This gene cluster can be further subdivided into Type I (*CGβ6/7*) and Type II (*CGβ3/9, 5 and 8*) genes. They differ in a silent mutation and more importantly in aa hCGβ117: Type I gene contains Ala, type II genes Asp [23].

The gene *CGβ5* accounts for approximately 50% of hCGβ transcripts in normal placentas [24]. An *hCGβV79M* transition polymorphism of gene *CGβ5* identified in 4.2% of the normal population causes assembly inefficiency *in vitro* [25].

2.2 Posttranslational protein backbone variants

The best investigated protein backbone variants are nick-free biologically active heterodimeric hCG (hCG), nicked hCG (hCGn), the non assembled dissociated alpha-subunit of hCG (hCGα), the non assembled dissociated beta-subunit of hCG (hCGβ), nicked hCG beta-subunit (hCGβn) and hCG beta-core fragment (hCGβcf). Nicking of hCG or hCGβ occurs after amino acids (aa) *45, 46, 48* and *49*, (most frequently after *45* and *49*), presumably after aa *Asn 77*, and to a minor extent after aa *76, 78, 79* and *80* [26,27,28]. It is a matter of debate whether some nicked and N- and C-terminally variants are true in vivo variants or purification artifacts.

hCGβcf is a di-peptide corresponding to amino acids *hCGβ6-40* and *55-92*. Predominantly generated in the kidney as a metabolite of hCGβ [11], it is a major hCG-variant in the urine of pregnancy and tumor patients but not at significant concentrations in serum [5]. Less well-defined degradation products of hCG potentially give rise to variable hCG results particularly in pregnancy- and tumor-derived urine [29,30].

2.3 Carbohydrate heterogeneity

Glycosylation of the protein backbone of both subunits accounts for approximately one third of the molecular weight of hCG and influences subunit assembly, intra-cellular trafficking, secretion, receptor activation and hCG half-life in serum [31]. hCGβ contains 2 putative N- (*Asn 13* and *30*) and 4 O- (*Ser 121*, *Ser 127*, *Ser 132*, *Ser 138*) glycosylation sites. hCGα carries 2 carbohydrate chains at *Asn 52* and *78*. These glycans are monoantennary (core-1), biantennary (core-2) or sometimes triantennary structures or in some cases even missing [26]. Terminal sialylation of the carbohydrate antennae varies considerably (8 – 15 sialic acids), which not only influences the above-mentioned biological features but also affects molecular charge heterogeneity as reflected in large variation in isoelectric points (pI) observed for highly purified preparations of e.g. pregnancy-derived hCG (pI 4 – 6.5 for hCG 1st IRR 99/688), hCGα (pI 6.5-8.5; 1st IRR 99/720) and hCGβ (pI 4 – 5.5; 1st IRR 99/650) [21].

Acidic variants of testicular cancer-derived hCG (avhCG) have lower pI's (3.6 – 3.9) [32,33] as do variants produced by other tumors and some very early pregnancy variants [34]. The expression avhCG [32,35], denoting hCG with complex carbohydrate antennae that are highly terminally sialylated - thus conferring a low pI

to hCG and its subunits - was later replaced by the term ”hyperglycosylated” [36], a term ultimately restricted to hCG variants carrying a biantennary core 2 O-glycan on *Ser 132* that seems to be preferentially recognized by assays employing a particular mAb termed B152 [37].

Most hCGβ N-glycans are of the biantennary type, with increasing triantennary glycans at *Asn 30* and fucosylation at *Asn 13* observed in malignancy-derived hCG. Of the O-linked carbohydrates on the carboxyl-terminal peptide of hCGβ, core-2 glycans are located on *Ser 121* and core 1 glycans on *Ser 138*. Core-1 glycans on *Ser 127* and *Ser 132* in pregnancy are frequently replaced by core-2 glycans in cancer-derived hCGβ [26].

When comparing hCGβ from different sources using liquid chromatography-electrospray ionisation-tandem mass spectrometry (LC-ESI-MS), characteristic glycosylation patterns are observed. The major carbohydrate structures of hCGβ of both pregnancy- and malignancy-derived hCG are depicted schematically in Figure 2 [26].

When variability of glycosylation is taken into account, molecular microheterogeneity increases dramatically. On theoretical grounds more than 3000 carbohydrate variants for hCGα and >13,800 for hCGβ can be predicted but only 14 major variants are observed for the native non-trypsinized starting material of the 1st IRR for hCGα (99/720) based on a maximum ±5.0 Da mass tolerance [38]. The measured glycoprotein masses after ion-pair reversed phase (RP) HPLC-ESI-MS were between 13,000 and 13,671 for hCGα and 22,336 to 23,667 for hCGβ (1st IRR 99/650). These are, however, results for free subunits prepared by dissociation of intact pregnancy hCG for which the calculated glycoprotein masses range from 35,336 to 37,338. Results may be slightly different for naturally occurring free subunits which have

higher molecular weights in serum [39], seminal plasma [16] and choriocarcinoma cells [31,40,41].

3. Epitopes on hCG and hCG variants

3.1 Three classes of main specificities of ”hCG“-directed mAbs

Deciding which variants are diagnostically and clinically relevant, whether they should be measured, and if so, how, represents a major challenge. In order to clarify this, mAbs directed against hCG and hCG-related variants have been produced by our own [42,43,44,45] and other laboratories [46,47,48,49], extensively tested to determine their specificities and molecular epitope recognition (for reviews see [50,51]) and then used to design immunoassays of various specificities for hCG and/or hCG-related variants.

Three major classes of mAbs recognizing epitopes on hCG are defined by protein backbone recognition, i.e. epitopes located (i) on assembled and/or free hCGα (hCGα-mAbs), (ii) on assembled and/or free hCGβ or hCGβ metabolites (hCGβ-mAbs) or (iii) exclusively on the assembled intact or modified hCG$\alpha\beta$ heterodimer but on neither uncombined subunit or metabolites thereof (c-mAbs) [42,43]. These three classes can be further subdivided into mAbs recognizing epitopes on a single hCG-variant (e.g. mAbs recognizing hCGβcf only) or some hCG-variants (e.g. hCGβ and hCGβcf). Most epitopes are primarily determined by the protein backbone [32,33], with the exception of a few rare mAbs located on hCGβ_{CTP} that are influenced by O-linked carbohydrates attached to *Ser 132* or *Ser 138* [22,52]. The core region of hCGβ corresponding to hCGβcf carries the immunodominant epitopes of hCGβ. The tips of the adjacent loops 1 and 3 in either subunit form the immunodominant regions of hCG. [22].

3.2 Molecular epitope localization

Detailed knowledge of the three-dimensional molecular structure of hCG [1], together with extensive epitope mapping studies using mutational analyses, synthetic peptides, hCG metabolites, homologous and heterologous hormones and subunits, and biochemically modified hormones and fragments have yielded precise information on the molecular localization and sharing of epitopes between hCG, its variants and other related hormones. These studies have helped to define the immunologically, biologically and diagnostically relevant domains on hCG and hCG-variant molecules (for review see [22,50,51]). Epitope maps for hCG, hCGβ, hCGα and hCGβcf (Figure 3) show the topographical distribution of epitopes on the protein backbone of hCG and hCG-variants [22].

The epitope maps of hCG and related molecules defining the molecular localization of 24 epitopes were taken as a scientific basis for an hCG Antibody Epitope Mapping Workshop held under the auspices of the International Society of Oncodevelopmental Biology and Medicine (ISOBM) (Table 2) (for review see [22,51]). Mapping of 27 mAbs directed against hCG and hCG-variants provided by a number of major diagnostic companies has facilitated development of practical recommendations regarding the most appropriate mAb specificities and immunoassays designs for hCG methods for clinical use. These results should influence the choice of antibodies for the next generation of hCG immunoassays, thereby leading to improved between laboratory comparability of assays for hCG and hCG variants and ultimately improved patient management [22].

3.2.1 hCGβ (β_1 – β_9) and hCGβcf-only (β_{10} – β_{13}) epitopes

hCG epitope presence differs from variant to variant. Whereas epitopes β_1 – β_5 are present on a broad spectrum of hCG and hCG-variants (hCG, hCGn, hCGβ, hCGβn, hCGβcf) other epitopes are expressed on selected variants only: (i) epitopes β_6 and β_7 on hCGβ, hCGβn and hCGβcf but not αβ-heterodimers (ii) epitopes β_8 and β_9 on hCG, hCGβ, hCGβn and hCGβ$_{CTP}$ but not hCGβcf and (iii) epitopes β_{10} – β_{13} exclusively on hCGβcf but not present on hCG or any other hCG variant, including hCGβ [22,51,53].

The hCG-specific epitope β_1 is located around the cystine knot in the core region of hCGβ that corresponds to hCGβcf and is partially determined by *Arg 10 and Arg 60* (reference-mAb INN-hCG-2) [54,55]. The β_1 epitope not present on hLH or hLHβ but is shared by all relevant hCG/hCGβ-derived variants - including hCGβcf - and is therefore an excellent candidate epitope for specific and comprehensive hCG/hCGβ-variant detection [22].

Most antibodies of hCG-immunized animals are directed against epitopes β_2 to β_5 on the top of hCGβ β-strand loops 1 and 3. Antibodies directed against this immunodominant cluster of epitopes are of significantly higher affinity compared to antibodies directed against other regions of the hCGβ molecule [22,50,56]. Epitopes β_2 to β_5 encompass aa regions *hCGβ20-25* and *hCGβ68-77*. These can be subdivided as follows: *Pro 24, Val 25, Arg 68, Gly 71* and *Gly 75* contribute to epitope β_3; residues *Lys 20, Glu 21, Gln 22, Gly 75* and *Asn 77* to the free subunit epitope β_6; and *Arg 68* to epitopes β_2 (mAb INN-hCG-22), β_4 (mAb INN-hCG-24) and β_5. Epitopes β_2 and β_4 are highly specific for hCG/hCGβ derivatives, having <1% cross-reactivity with hLH/hLHβ, and are therefore highly suitable for hCG and hCGβ-variant measurement. Antibodies to epitopes β_3 and β_5 recognize all relevant hCG and

hCGβ-derived variants but also recognize hLH/hLHβ equally well and consequently are of limited use for specific hCG measurement [51,54].

In evolutionary terms *hCGβCTP* has developed relatively recently, as the nucleotide deletion causing the genetic read-through event resulting in incorporation of *hCGβCTP* into the reading frame is only be observed in primates and not in other species like mice, rats or sheep. This might be one of the reasons why this extension does not possess a significant secondary let alone tertiary 3-dimensional peptide structure. Moreover it does not contain any cystines which could participate in disulfide-bridges. Thus, *hCGβCTP* seems to be essentially linear in structure and consequently *hCGβCTP* epitopes are the only hCG epitopes that are truly defined by linear peptide sequences. Antibodies preferentially recognize epitopes determined by protein structures of higher orders rather than linear peptide sequences. Thus epitopes on *hCGβCTP* are generally of low antigenicity and antibodies recognizing *hCGβCTP* of low affinity. Even in hCG-hyperimmunized animals fewer mAbs are directed against the two linear sequence epitopes on hCGβCTP designated β_8 (*hCGβ135-145*) and β_9 (*hCGβ111-116*) as compared to the core region of hCGβ. The aa sequence *hCGβ135-145* encompassing epitope β_8 is the immunodominant region of the *hCGβCTP*. All other hCGβ-epitope are defined by the tertiary (mAbs recognizing the hCGβ core region) or quaternary (i.e. c-mAbs recognizing only heterodimeric hCG isoforms) structure of hCG as reactivity with synthetic linear peptides is not observed or negligible [53].

hCGβCTP antibodies show low affinity but by definition high hCG specificity [50,53] and are used in several commercial hCG immunoassays. Such sandwich assays, although specifically recognizing hCG and - depending on the specificity of the second antibody - hCGβ and hCGβn, may be over-specific and fail to detect

important hCGβ-derivatives such as hCGβcf, particularly when measurements are being made in urine.

Additional epitopes giving rise to rare and unique mAb specificities (e.g. hCGβ only, no hCGβcf recognition) were not considered in the ISOBM mapping study [22].

3.2.2 Epitopes on hCGα (α_1 - α_7)

hCGα epitopes on loops 1 and 3 are determined by aa *hCGα13-22* (α_1, α_2, α_4) and Tyr 65 (α_3, α_5), respectively [57]. As is the case of hCGβ, where immunodominant regions are located on neighboring β-strand loops 1 and 3 protruding from the cystine knot, the immunodominant regions of hCGα include epitopes α_1, α_2, α_4 (*hCGα13-22*) on β-strand loop 1, and spatially separated epitopes α_3 and α_5 on the adjacent loop 3. Antibodies against epitopes α_2, α_3, α_4 and α_5 essentially recognize all hGPH and their α-subunits equally well, whereas those against epitopes α_1 bind uncombined α-subunit preferentially. The subunit interaction site is represented by epitope α_6, which is only accessible on free and non-assembled hCGα (aa *hCGα33-42*) [57]. Thus mAbs against epitope α_6 bind uncombined hCGα exclusively and specific assays for hCGα generally employ anti-α_6 antibodies (reference-mAbs INN-hCG-72 and –80). Epitope α_7 is defined by a single low affinity anti-peptide mAb against the extreme carboxyl-terminal end of hCGα (aa *hCGα87-92*) [58].

Again, analogously to mAbs against hCGβ core epitopes, epitopes of mAbs which recognize hCGα are predominantly not determined by the primary linear sequence. However a few hCGα-mAbs do react with synthetic peptides with very low affinities (e.g. α-mAbs against imunodominant antigenic domain on β-strand loop 1 or the subunit interaction site or the extreme C-terminal end of hCGα).

3.2.3 Epitopes exclusively or predominantly present on the hCG αβ-heterodimer (c_1 - c_4)

The four epitopes on αβ-heterodimeric hCGare not expressed in adequate conformations on free subunits or metabolites thereof [42,43]. Epitopes c_1 and c_2 are specific for hCG whereas c_3 and c_4 are shared between hCG and hCGn and their truncated forms lacking the hCGβ-carboxyl-terminus [30]. The assumed molecular locations of epitopes c_1 and c_2 are based on circumstantial evidence: c_1 and c_2 mAbs (i) are not compatible with mAbs against epitope β_1 which is located around the cystine knot, (ii) do not recognize hCGn (nicks around aa *hCGβ44-48*, large single β-strand loop 2), and (iii) are mutually exclusive with α-mAbs in sandwich assays (epitopes α_1, α_2, α_4 on the β-strand loop 1 of hCGα; *hCGα17-22*), thus c_1 and c_2 epitopes seem to be located near the cystine knot and loop 2 of hCGβ and close to loop 1 on hCGα (Figure 3).

MAbs against heterodimeric epitope c_3 (reference mAb INN-hCG-45) are highly hCG-specific (<<1% cross-reactivity with hLH), are not influenced by nicking (i.e. recognize hCG and hCGn equally well) and thus are suitable for use in specific hCG assays designed not to cross-react with hCGβ, hCGβ derived variants, hCGα and hLH and derivatives.

Epitope c_4 is remote from all other known epitopes, is not destroyed by nicking and is partially shared by hCGβ. However the affinity for hCGβ is several orders of magnitude lower than that for hCG. Furthermore, epitope c_4 is shared by hLH and is thus not suitable for specific hCG measurement [22,42,59].

3.2.4 Carbohydrate isoforms and immunoreactivity

Although almost all hCG antibodies recognize the protein backbone of hCG and its derivatives [32], their affinity may be influenced by carbohydrate content in general [33] and in particular by the biantennary core 2 O-glycan on the aa *Ser 132* of hCGβ [37] or on *Ser 138*. The latter might be reflected by a cross-reactivity of a group of mAbs directed against the extreme carboxyl-terminal end of hCGβ that is 3 to 4 orders of magnitude higher when glycosylated hCGβ is tested as compared to non-glycosylated synthetic peptide [22]. The clinical utility of assays for glycosylation variants of hCGβ is still a matter of debate [5].

4. Applications of assays for hCG variants in pregnancy and oncology

Measurement of hCG and hCG-derivatives is important for monitoring of pregnancy, in prenatal screening for Down's syndrome and in the diagnosis of hCG-secreting tumors [4,5,60,61]. Numerous variants of hCG have been described (Table 1), including hyperglycosylated or acidic hCG [35] [32] and hCGn [62], truncated hCG and hCGβ (truncated after aa *hCGβ117*) [22,30], the free carboxyl-terminal extension of hCGβ (hCGβCTP; aa approx. *hCGβ117-145*), N-terminally truncated hCGα, and hCGββ [63]. hCGαα homodimers are found in sera of testicular cancer patients [32], in seminal plasma [16] and in choriocarcinoma cells [31].

The extent to which measurement of hCG, hCG-variants and metabolites are clinically relevant depend both on the clinical condition being investigated and on the type of specimen (most often serum or urine). In serum, hCG/hCG-derived molecules are reasonably homogeneous with respect to their protein backbone but they differ substantially in their glycosylation and/or terminal sialylation patterns. hCG-protein backbone variants in pregnancy serum consist predominantly of hCG (>95%),

together with minor percentages of uncombined hCGα and hCGβ. Early pregnancy serum contains a high proportion of hyperglycosylated/acidic hCG. Sera of patients with gestational trophoblastic disease and germ cell tumors may have increased proportions or even selective elevations of hCGα or hCGβ, particularly hCGβ [6,64] [65] [6,66,67]. Various non-trophoblastic tumors may show selective increases of either non-combined subunit [6,8,12,15,68,69]. Moreover as pregnancy proceeds or hCG-secreting tumors develop the relative proportions of hCG-variants in serum or urine can change dramatically. It is essential that in parallel the specificities of diagnostic immunoassays can fulfil these changing requirements.

For early detection of pregnancy, qualitative hCG determinations in serum or urine may be adequate, but quantitative serum measurements are essential for the diagnosis and management of miscarriage and ectopic pregnancy and for Down's syndrome screening. Serial measurements of hCG in serum contribute to the management of benign gestational trophoblastic disease (hydatidiform mole), molar pregnancy, ectopic pregnancy and induced or spontaneous abortion. hCG and hCGβ are also highly reliable tumor markers in testicular cancer [30], choriocarcinoma and ovarian germ cell tumors, and hCGβ is a diagnostic and prognostic marker for some non-trophoblastic tumors [6,8]. hCGα is of little diagnostic value.

Genomically determined type I hCGβ *Ala117* (allelic genes *CGβ6* and *CGβ7*) is reportedly associated with nontrophoblastic tissues, renal cell carcinoma and type II hCGβ Asp117 with normal trophoblastic cells and many other tumors [23,70]. The diagnostic relevance of the *V79M* transition polymorphism in gene *CGβ5* has not yet been determined.

4.1 Diagnosis of pregnancy

In humans hCG is indispensable for the maintenance of pregnancy as it stimulates the production of progesterone in the corpus luteum. Thus assay of hCG and hCG-related molecules in urine is most commonly used for home pregnancy testing. Serum or plasma is also used in professional laboratories. Ideally semi-quantitative pregnancy tests including those sold over-the-counter should have clinical sensitivities of around 25 IU hCG/L as hCG and hCG-like molecules may be present in non-pregnant individuals at concentrations up to 10 – 15 IU/L [5,71].

The composition of hCG immunoreactivity differs between serum and urine [72,73] and it also changes during the course of pregnancy. hCG and hCGβ are both present in pregnancy serum and urine [73,74], and urine also contains nicked forms, metabolic degradation products (e.g. hCGn and hCGβcf) and highly acidic i.e "hyperglycosylated" variants of hCG and hCGβ [5]. In urine the proportions of hCG and of its highly acidic "hyperglycosylated" variant decrease with time while those of the metabolite hCGβcf increase [5,73,75]. Measurement of hCG in pregnancy sera reflects bioactivity as the vast majority of hCG-like molecules are intact αβ-heterodimers that act via LH/hCG-receptors in the corpus luteum to maintain pregnancy. hCG and hCG-like molecules in pregnancy urine are surrogate markers, which only indirectly reflect levels of bioactive hCG in circulation.

Pregnancy tests are used in the first few days and weeks before and after the missed menses. Ovulation (day 0) and conception is followed by implantation on day 9 (6 –12) and a concomitant rise in hCG [73,76]. Day 14 is the approximate day of the expected menses. Few prospective longitudinal data on proportions and urinary levels of hCG and hCG-variants are available [5,73] but it seems that hCG (most often its hyperglycosylated acidic variants) is the predominant form [5,75] up to day

21 [34,73]. From day 21 from ovulation to term hCGβcf urinary concentrations might exceed those of hCG [73]. In the second trimester most ”hCG” immunoreactivity is due to hCGβcf [5,77]. For the measurement of “hCG” immunoreactivity this means that only between day 14 (missed mensis) and day 21 after ovulation hCG is the most important marker which is then gradually replaced by hCGβcf [5,73]. The pattern of varying hCGβ proportions in urine is not entirely clear but it seems to be of minor importance [5,77]. Although present in both serum and urine, hCGα is of little diagnostic value during pregnancy as its concentration varies widely and is superimposed by pituitary secretion.

Within individuals, fluctuations in hCG concentrations may limit the clinical utility of quantitative urine hCG assays but these are largely eliminated by normalizing against urinary creatinine or urine density [73]. Highly selective measurement of individual hCG-variants is generally not feasible so semiquantitave detection of pregnancy in urine requires an assay that detects all relevant hCG-variants, including hCG, hCGn, hCGβ, hCGβn and hCGβcf [22,73]. However relatively few commercially available assays achieve this [72,78]. Hyperglycosylated hCG/hCGβ-variants must be detected and any non-detected fragments or other hormones/hormone fragments should not interfere (i.e. diminish the signal) (e.g. free hCGβCTP) or cross-react (e.g. hLH and hLH-variants). It is essential that these requirements are taken into account when designing immunoassays for early pregnancy applications. [22,79].

4.2 Quantitative monitoring of hCG in pregnancy

It is well accepted that immunoassays measuring the protein backbone do not necessarily reflect hCG bioactivity in serum. The in vivo bioactivity of hCG is influenced by differences in glycosylation patterns. More acidic variants in early

pregnancy show increased bioactivity presumably not due to increased receptor activity as shown in vitro [33,80] but rather due to secondary effects like prolonged half life. Quantitative serum measurements of hCG are useful for the diagnosis and management of early miscarriage and ectopic pregnancy. Serum hCG levels are about twice as high as those in urine and highly correlate to urine concentrations [5]. Intact heterodimeric hCG is the most prominent protein backbone variant in serum, whereas hCGn and hCGβcf seem to be present only in negligible concentrations [81,82]. Free hCGβ can be measured separately but is normally present at serum concentrations <1% those of hCG, except during the first days of pregnancy when proportions are significantly higher [5,74,75]. Its major measurement application in pregnancy is in Down's syndrome screening [6]. Whether nicked or hyperglycosylated variants pose problems for hCG detection is still a controversial issue but is rather unlikely for most antibody sandwich combinations [30,32,33]. The change to less acidic forms in serum occurs around the 13th week of gestation. These forms have shorter serum half-lives and this may contribute to lower measurable serum levels of hCG after the first trimester [83].

5. Improving the comparability of immunoassays for hCG

5.1 Nomenclature for hCG variants and immunoassays

Early quantitative hCG measurements depended on radio-immunoassays (RIAs) using polyclonal antisera. By careful selection of polyclonal antibodies it was possible to ensure that RIAs were specific for hCG and did not recognize LH [84]. As the preferred rabbit antibody (SB6) for such RIAs was raised against hCGβ, the widely used assays based on this and similar antibodies were of broad specificity, recognizing αβ-heretodimeric hCG and uncombined hCGβ as well as other variants

[84] and used to measure mostly hCG and coincidentally but conveniently hCGβ and other hCG and hCGβ-related variants and metabolites. However the assays, somewhat unhelpfully, were called “hCGβ”- or “βhCG”- assays to indicate that the antibodies were raised against hCGβ. Use of this terminology unfortunately persists and still causes considerable confusion, as it is not always clear whether what is being described is an assay specific for hCGβ or an assay detecting a broad and that have a broad and spectrum of hCG-related molecules (e.g. hCG ± hCGn ± hCGβ ± hCGβn ± hCGβcf ± truncated hCG variants).

With this background, the International Federation of Clinical Chemistry and Laboratory Medicine (IFCC) established a Working Group (WG) for the Standardisation of hCG measurement, giving it the broad remit of exploring how best to improve comparability of results for a complex analyte, taking hCG as a prototype.

Development of clear nomenclature for hCG and related molecules (Table 1) was addressed as a priority by the IFCC WG [19,20], which extended these recommendations to include nomenclature permitting clear description of exactly what hCG assays measure [22]. The IFCC WG recommends that “hCG”-assays should be described with respect to their specificities. An assay described as an hCGβ-assay therefore measures exclusively hCGβ but not hCG or any of its variants [5,19].

5.2 International Standards and Reference Reagents for hCG variants

While existing International Standards (IS) for hCG have served the scientific and community very well, some limitations were identified by the IFCC WG, particularly with respect to the purity of these IS and the units in which they were established. The current and previous IS for hCG (4th IS 75/589 and the essentially identical 3rd IS

75/537) were purified in 1972 from pregnancy urine and were established as WHO International Standards some years later [85]. The International Reference Preparations for hCGα (IRP 75/569) and hCGβ (IS 75/551) were prepared by dissociating portions of the same purified preparation.

Although current hCG immunoassays measure protein concentrations and not bioactivity, the IS for hCG were assigned values in arbitrary international units (IU), as for earlier hCG International Standards, according to their biological activity as assessed by bioassay. As the free subunits have no measurable bioactivity, their unitage was arbitrarily defined as 1 IU/µg of hCG subunit. Trying to relate numerical results in terms of these different standards is therefore complex [5] [21], and a major source of confusion, as 1 IU of the 3rd IS for hCG corresponds to roughly 0.1µg, but 1 IU of hCGα (IRP 75/569) or of 1 IU hCGβ (IRP 75/551) to 1µg of hCGα and hCGβ respectively. By assuming molecular weights of 36.7 kDa for hCG and 22.2 kDa for hCGβ, and then calculating the number of picomoles that are approximately equivalent to 1 IU of hCG (~2.9 pmoles) and hCGβ (~45 pmoles), it is possible to conclude that in an assay recognizing both species on an equimolar basis, the response expected from 1 U of hCGβ will be approximately 15.5 times greater than that from 1 IU of hCG [61]. However this approach provides only an estimate and is not scientifically rigorous.

In order to address this problem, and permit improved understanding of which hCG variants different methods recognize, the Working Group therefore prepared and characterized highly purified preparations for six important hCG variants (Table 1) [21]. Portions of each were ampouled at the National Institute for Biological Standards and Control (NIBSC) and were subsequently established by the World

Health Organization (WHO) as the 1st International Reference Reagents for hCG, hCGn, hCGβ, hCGβn, hCGβcf and hCGα (Table 1) [20].

Crucially, ampoule unitage was assigned not in arbitrary units as for the previous International Standards for hCG (e.g. IS 75/589) but – for the first time for such a complex molecule and exploiting progress in protein purification and characterization – in molar units based on amino acid analysis. This represents a major advance as molar unitage permits ready comparison of the extent to which different methods recognize each of the International Reference Reagents. As described above, previous calculations were both complex and unsatisfactory and were also limited to hCG, hCGβ, and hCGα, the only International Standards then available [61]. The new Reference Reagents are now being used as recommended by the WHO to characterize the extent to which currently available hCG immunoassays recognize each variant. The same preparation of hCG used to prepare IRR 99/688 (Table 1) is currently one of two candidate preparations (the other being a recombinant hCG) in an International Collaborative Study which will determine the replacement for the 4th International Standard for hCG (IS 75/589), stocks of which are rapidly diminishing.

The preparation from which IRR 99/688 was ampouled is a worthy candidate for the next i.e. 5th IS for hCG, as it is well-characterized and of higher purity than IS 75/589, as reflected by its higher bioactivity [21]. Four specific immunoassays from the author's laboratory have been used to assess percentages cross-reactivities (mol/mol) of the six 1st IRRs for hCG and hCG-related molecules (Table 5). The diagnostically most important 1st IRR for hCG (99/688) shows significantly less cross-contaminations than the existing 4th IS for hCG (75/589) and is devoid of hCGn [21] which has reportedly given rise to inconsistencies in hCG measurements [5]. hCG and hCG-like cross-contaminations in the hCGβ (99/650), hCGβcf (99/708) and

hCGα (99/720) preparations are much less than 1%. These observed values are due to cross-contaminations and not to cross-reactions of the immunofluorometric assays (IFMA) as assay specificities with respect to discrimination of hCG-variants are far better. “True” cross-reactivities of the four hCG-variant selective IFMAs were evaluated with immunaffinity-purified standard preparations of potentially cross-reacting variants. The assays generally showed extremely low values for cross-reactivities, e.g. < 0.001% for hCGβ in the assays for (i) hCG + hCGn, (ii) hCGβcf and (iii) hCGα (Table 5) [45,79,86].

5.3 Improved characterization of current immunoassays for hCG

hCG measurements contribute to critical clinical decisions. It is therefore highly desirable that different immunoassays for hCG provide results that lead to the correct clinical interpretation, to ensure consistency of treatment in different clinical centres. It would have been expected that with the advent of mAb technology and replacement of RIAs with sandwich immunoassays, more selective determination of hCG and related molecules became possible.

However these advances did not lead to improved comparability of analytical results, with between-method geometric coefficients of variation (which indicate the scatter of results) >20% still observed in EQA schemes [18], and little awareness of what methods measure. It was not until the early 1990s that it was widely appreciated that when considering analytical performance and clinical utility, hCG assays which do not recognize the free beta-subunit should be considered separately from those which do, with use of the latter mandatory for applications in oncology [19].

Providing the possibility of interference from heterophilic antibodies that are present in some patient sera can be excluded [18], between-method discrepancies are most

likely to reflect non-equimolar recognition of hCG-variants in different assays. While most frequently observed for macroheterogeneic variants (e.g. hCG, hCGβ and hCGβcf), discrepancies may also reflect differences in recognition or interference of other variants (e.g. nicked forms of hCG and hCGβ, glycosylation variants and variants potentially carrying mutations or allelic transition polymorphisms).

Highly purified and calibrated in molar units, availability of the six IRRs for hCG variants enables elucidation of what is being measured in current hCG immunoassays, e.g. whether hCG and hCGβ are recognized on an equimolar basis or whether either is recognized preferentially. The difficulties inherent in attempting to measure mixtures remain [87], but these reference reagents provide a sound basis for improved assay design and calibration.

5.5 Clinically relevant antibody combinations

Available sandwich assays measure various combinations of hCG and related molecules, usually hCG±hCGn or hCG+hCGn + various hCGβ-variants + degradation products (e.g. hCGβ, hCGβn, hCGβcf and truncated forms of hCGβ) [72]. Complementing the IFCC work on new standards described above, an ISOBM Workshop carried out an “hCG”-mAb epitope mapping study. The fine specificities and the recognized epitopes of 27 mAbs from seven major international diagnostic companies were determined, with experimental work undertaken in four laboratories. Combining these results with current understanding of clinical requirements for hCG immunoassays, recommendations about appropriate epitope combinations for specific clinical applications were then successfully formulated [22].

Given a pair of compatible mAbs (i.e. that form a sandwich in appropriate antigen recognition studies) hCG-variant detection, unwanted cross-reactivities (e.g. of hCG

or hLH in assays measuring hCGβ), and potentially interfering ligands can be predicted [22,88]. Highly variant-selective, specific and sensitive assays have been constructed for hCG and the common protein backbone variants by combining pairs of mAbs of known specificity. Measurement of defined protein backbone variants (e.g. hCG, hCGβ, hCGβcf, hCGα, respectively) or combinations thereof (e.g. hCG + hCGn + hCGβ + hCGβn + hCGβcf + trunc-hCG + trunc-hCGβ) can then be achieved using different epitope pairs that either represent optimal combinations or, where unavoidable, compromises e.g. with respect to interferences with non-measured variants recognized by one but not the other mAb in sandwich formats.

5.6 Sandwich assay construction principles

Principles for the construction of sandwich immunoassays are illustrated in Figures 4a and tabulated in Table 3a, with some less suitable alternatives shown in Figures 4b and Table 3b. Numerous other epitope combinations are of course possible and have been used for clinical and research purposes. Such assays either miss the detection of desired variants (over-specificity) or show undesired cross-reactivities of hCG-variants. Immunoassays using one mAb against hCGβ and one against hCGα will fail to recognize hCGβcf and uncombined hCGβ and some metabolic variants (e.g. hCGβn), while assays using a mAb against free and assembled hCGβ combined with a second mAb against hCGβCTP may be sensitive to glycosylation differences at the extreme carboxyl-terminal end of hCGβ, will miss truncated hCG/hCGβ-variants, hCGβcf and may be prone to interference with clipped i.e. free hCGβCTP.

Final signals of ”hCG” sandwich immunoassays are composed of signals from variants recognized by both mAbs. Some assay formats are prone to signal

interference with excess hCG-variants that are recognized by one of the two mAbs only leading to lower signals of desired analytes due to competition [79].

For routine diagnostic purposes, where a single assay is required for reasons either of cost or logistics, methods that recognize a broad spectrum of hCG + hCGβ and variants (e.g. hCG + hCGn + hCGβ + hCGβn + hCGβcf + -CTPhCG + -CTPhCGβ), ideally on an equimolar basis, are the best choice [5,22,30].

These rather stringent requirements exclude all mAbs recognizing selectively free subunits, hCGβcf or the holo hormone hCG. Significant differences in reactivity towards glycosylation and genetic variants should also be avoided. For example although mAbs directed against hCGβCTP will be highly specific for hCG + hCGn + hCGβ+ hCGβn, they may be too specific as they do not recognize truncated hCG/hCGβ-variants or most importantly hCGβcf or some of them only recognize core 2 *Ser132* and *Ser 138* "hyperglycosylation" hCGβ variants and miss out most frequent core 1 *Ser 138* or non-glycosylated *Ser 132* variants [22,37]. Specificity versus other glycoprotein hormones and their variants particularly hLH, hLHβ and hLHβcf as well as hCGα is of course mandatory.

Specificity requirements can be fulfilled using pairs of antibodies directed against the two most important antigenic domains of the core region of hCGβ, i.e. mAbs against the cystine knot associated epitope β_1 (reference antibody INN-hCG-2 or alternatively INN-hCG-32), and epitopes located on the top of β-sheet loops 1+3 (β_2, reference mAb INN-hCG-22 or alternatively β_4, reference mAb INN-hCG-24) (Figure 4a, Table 3a) [22]. These represent the only hCG-specific epitopes that are located in the core region of hCGβ and not on hCGβCTP. Such mAbs show near equimolar recognition of hCG + hCGn + hCGβ + hCGβn + hCGβcf, do not react with other human glycoprotein hormones or subunits or with hCGβCTP and do recognize

hyperglycosylated, deglycosylated and/or asialo hCG/hCGβ isoforms [32,33]. Immunoassays constructed in this way are close to ideal in terms of antibody recognition of diagnostically relevant hCG and hCG variants. With these assays, measurements in serum reflect biologicial hCG activity much better than measurements in urine. As discussed above, assays providing selective measurement of single variants (e.g. hCGβ or hCGβcf) might give additional useful clinical information as well as being much easier to standardize [67], but they are not always practicable.

6. Conclusion

State of the art measurements of hCG and other high molecular protein- or glycoprotein analytes still rely on sandwich immunoassay formats based on mAbs. Progress in standardizing immunoassays for complex analytes is limited by their extreme heterogeneity and variability and the differences in ”hCG“-variant recognition of mAbs, but in recent years considerable progress has been made towards improving the comparabilility of hCG and hCG-variant measurements. Six highly purified and well-characterized hormone standards, prepared under the auspices of the IFCC and calibrated in molar units, are now available and appropriate epitopes and specificities of pairs of mAbs for important clinical applications have been clearly defined as the result of the 1st ISOBM Workshop on hCG.

Together, the IFCC and ISOBM projects provide, for the first time for any analyte, a means of elucidating and clearly describing what is being measured in hCG immunoassays, of calibrating hCG immunoassays more accurately, and of improving future hCG immunoassays by more informed choice of antibody combinations appropriate for particular clinical applications.

7. Expert Opinion

7.1 Replacement for hCG IS 75/589

Supplies of the current IS for hCG (IS 75/589) are expected to be exhausted within the next few years. The new 1st IRR for heterodimeric intact hCG (IRR 99/688) is therefore to be included, together with a recombinant hCG preparation, in an International Collaborative Study being coordinated by the National Institute of Biological Standards and Control (NIBSC) to identify the next International Standard.

7.2 Harmonization of mAb specificities and reference methods for hCG

Differences in the extent to which hCG, hCG-variants, hLH and hLH-variants are recognized in different assays may give rise to discrepant results in some patient samples, reflecting differences in mAb specificities as well as assay design. To overcome these problems the successful 1st ISOBM Workshop on hCG formulated criteria for mAb specificities and epitope recognition as well as assay design. In routine clinical situations an assay measuring all relevant hCG and hCG-variants (i.e. hCG + hCGβ + hCGβcf + hCGn + hCGβn + -CTPhCG + -CTPhCGβ) is preferred. Achieving universal agreement about the most clinically appropriate specificities of antibodies required to produce hCG assays for pregnancy and oncology applications both in serum and in urine, and then working to encourage use of such antibodies in all commercially available hCG methods would represent a major step forward. This may be facilitated by results of the Second ISOBM Workshop on hCG antibodies which was launched in Prague in September 2007. It would be highly desirable to define more precisely which hCG and related molecules are to be detected in early pregnancy tests, particularly in urine.

7.3 Reference measurement procedure for hCG

Techniques have not yet advanced sufficiently to allow development of a mass spectrometric reference method for hCG. In the interim, further characterization of the hCG IRRs using mass spectrometric techniques is being undertaken by the IFCC Working Group. Development of an immunological reference method remains a possibility.

7.4 Improving the comparability of hCG immunoassyas

Work is in progress to determine the effect of using a more highly purified hCG standards on assay performance and results. A direct comparison of the use of IS 75/589, IRR 99/688 and a recombinant hCG preparation is currently being undertaken and includes measurement of a panel of more than specimens previously issued by the United Kingdom National External Quality Assessment Service (UK NEQAS) for hCG.

7.5 Clinical applications of hCG immunoassays and future clinical studies

Selection of the most appropriate hCG immunoassay (e.g. broad spectrum or specific for a single variant) depends on the clinical condition being investigated i.e. pregnancy detection, pregnancy-related disorders, hCG-producing tumors and risk of Down's syndrome.

Quantitative and qualitative requirements for hCG measurement in early pregnancy should be investigated further. Building on increasing knowledge of time-dependent dynamics of changes in variant composition in early pregnancy, consideration of appropriate choice of antibody specificity and assay design, the requirement for

additional standards (e.g. for hyperglycosylated hCG) and cut-off levels in early pregnancy tests should all be carefully considered.

An important issue requiring detailed evaluation is the frequency of false negative and false positive results in hCG assays (e.g. from heterophilic antibody interference) and the clinical consequences of these. Whether physiologically and pathophysiologically occurring genetic polymorphisms and variants like hCGβ *Ala117Asp* and *hCGβ V79M* are relevant has yet to be established. The former probably is not of importance as aa *hCGβ117* is not involved in any diagnostically relevant epitope but aa *hCGβ79* is situated close to the major epitope domain of hCGβ and its diagnostic importance should be clarified.

Such studies are a pre-requisite for achieving a clearer understanding of the effects of disease on hCG-related variants and may ultimately help to identify those variants that provide the best diagnostic discrimination in given clinical circumstances and hence lead to more appropriate immunoassays.

Encouraging manufacturers to use the new 1st IRRs, to adopt the IFCC nomenclature when describing hCG analytes and immunoassays, and to follow recommendations about assay construction should in the future lead to improved and analytically more comparable hCG immunoassays that better fulfil clinical requirements.

Acknowledgements

The authors thank all the members of the IFCC (International Federation of Clinical Chemistry) Working Group for Standardization of hCG and those of the International Society for Oncodevelopmental Biology and Medicine (ISOBM) Tissue Differentiation (TD)-7 Workshop on hCG Measurement. Special thanks to Dr. Ulf-Håkan Stenman [Helsinki, Finland] for advice and Dr.Wolfgang Merz [Heidelberg, Germany] for continuous collaboration and critically reading the manuscript. Hormones and hormone variants were kindly supplied by the IFCC, the National Institute for Biological Standards and Control (NIBSC) [South Mimms, U.K.] and by Drs. Klaus Mann and Rudy Hörmann [Essen, Germany]. Dr. Natalie Sampson [Innsbruck, Austria] provided editorial help. The skilled technical assistance of Regine Gerth is highly appreciated. The Austrian Science Fund (NFN S 9307-B05) in part supported the work of P.B.

Legends to Figures

Figure 1

hCGβ is a member of the structural superfamily of cystine knot growth factors. The 3-dimensional structure and the primary amino acid (aa) sequence (shown as circles, single letter code) are schematically depicted. Its hallmark is the central cystine knot that consists of a ring formed by 2 strands of aa (aa *hCGβ34-38* and *hCGβ88-90*) connected by 2 disulfide bonds (*Cys 34–88* and *Cys 38–90*) and a 3rd disulfide bond (*Cys 9–57*) between 2 other aa strands axially running through it. Loops 1 (aa *hCGβ9-34*) and 3 (aa *hCGβ57-88*) are adjacent to each other and protrude from one side of the cystine knot opposed by loop 2 (aa *hCGβ38-57*). The core region corresponds to the metabolic break-down product hCGβcf (black circles, *hCGβ6-40* and *hCGβ55-92* connected by 4 disulfide bonds to each other, *Cys 9-57*, *Cys 23-72*, *Cys 34-88* and *Cys 38-90*). The highly O-glycosylated carboxyl-terminal extension (CTP; shaded gray area) of hCGβ (aa *hCGβ113-145*) has been incorporated into the reading frame of the hLHβ/hCGβ ancestral gene by a read-through event caused by a base pair deletion at aa *hCGβ113* and subsequent loss of the first stop codon. It is not involved in hLH/hCG-receptor binding and has no protein structure of higher order.

Figure 2

hCGβ glycosylation variants (according to [26])

(A) In pregnancy hCGβ N-glycans are of the biantennary type. Concerning O-glycosylation of hCGβ it appeared that *Ser 121* always contained a biantennary core-2 and *Ser 138* a core-1 structure with 1 or 2 sialic acids.

(B) Malignancy-derived hCG contained increased triantennary complex-type N-glycans attached to hCGβ *Asn 30* and fucosylated carbohydrates attached to *Asn 13* were increasingly enriched in malignancy and in early pregnancy (not shown). Major differences between pregnancy- and malignancy-derived-hCG are depicted in red.

The carbohydrate structures of free hCGβ resembled that of "hyperglycosylated" hCGβ (not shown) containing a high proportion of trintennary N-linked glycans (*Asn 30*), core-2 type O-glycans at *Ser 127*, *Ser 132* and *Ser 138* and fucosylated *Asn 13*-linked glycan. The large glycans have been implicated to inhibiting subunit association with hCGα. Some glycosylation sites were not glycosylated in some variants (*Ser 138*, *Ser 121* and *Asn 13*).

■ GlcNAc, ▲ Fuc, □ GalNAc, ○ Man, ● Gal, ◇ NeuAc

Figure 3

Three-dimensional epitope map of hCG

This model is based on the X-ray structure of hCG [1]. The αβ heterodimer hCG carries at least 16 epitopes that are depited as balls clustered mainly at both immunodominant ends of the molecule on adjacent loops 1 and 3 of either subunit (β_2-β_5 on hCGβ and α_1-α_5 on hCGα). Additional epitopes are found at the very beginning and the very end of the carboxyl-terminal peptide (hCGβ$_{CTP}$; 2 epitopes, β_8 and β_9) and centered around the cystine knot (1 epitope β1) flanked by 3 epitopes that are only present on the heterodimer but not on either free subunit (c_1-c_3). Two of the latter are influenced by nicks in the third loop of hCGβ (c_1 and c_2). One epitope (c_4) could not be assigned to the 3 D structure (not shown).

Uncombined hCGβ harbours 2 (β_6 and β_7) and hCGβcf additional 4 epitopes (hCGβ_{10}-β_{13}; not shown). Epitopes hCGβ_{10}-β_{13} are specific for hCGβcf and neither shared by free hCGβ nor by hCG. Their exact locations on hCGβcf are not resolved.

All epitopes on uncombined and assembled hCGβ (except for those on hCGβCTP) as well as on hCGβcf are located in the core region of the molecule (aa *hCGβ6-40 + β55-92)*, which is the immunodominant structure of the hCGβ-subunit [50,51,53].

The vast majority of mAbs against is directed against immunodominant epitopes on loops 1 and 3 of either subunit. This can also be observed in other species like rabbits (unpublished data). MAbs against epitopes on hCGβCTP and hCGαCTP are rare and of much lower affinity than epitopes dependent on the tertiary or quaternary protein structure as they are rather linear in their three-dimensional structure and thus not well recognized by the B-cell repertoire of the immune system of mammals (man, mouse, rabbit).

Epitopes have been identified with different approaches like mutational analyses of selected amino acids of hCGβ controlled by molecular modelling, the sythetic overlapping peptide approach for hCGβCTP and hCGα and probing mAbs with subunits and fragments of various hormones of different species. (for review see [51] [43,53,54,57].

Figure 4

(a) **Suggested epitopes for construction of hCG and hCG-variant sandwich assays**

Assay for **hCG + hCGβ +hCGβcf** + hCG-variants + hCGβ-variants; “wide spectrum”; clinically most important assay

MAb and assay specificities and interferences see Table 3a.

(b) **Sandwich assay for hCG and hCG variants of limited use**

Assay for **hCG + hCGβ** + hCG-variants + hCGβ-variants; no hCGβcf recognition! Overspecificity versus most abundant glycosylation variants of *hCGβSer132* and *Ser 138* is possible (some mAbs directed against epitope β_8 recognize core 2 variants preferentially [6,22,37];

MAb and assay specificities and interferences see Table 3b.

References

1 LAPTHORN AJ, HARRIS DC, LITTLEJOHN A *et al.*: Crystal structure of human chorionic gonadotropin. *Nature* (1994) **369**(6480):455-461.

2 WU H, LUSTBADER JW, LIU Y, CANFIELD RE, HENDRICKSON WA: Structure of human chorionic gonadotropin at 2.6 A resolution from MAD analysis of the selenomethionyl protein. *Structure* (1994) **2**(6):545-558.

3 MCFARLAND KC, SPRENGEL R, PHILLIPS HS *et al.*: Lutropin-choriogonadotropin receptor: an unusual member of the G protein-coupled receptor family. *Science* (1989) **245**(4917):494-499.

4 O'CONNOR JF, BIRKEN S, LUSTBADER JW *et al.*: Recent advances in the chemistry and immunochemistry of human chorionic gonadotropin: impact on clinical measurements. *Endocr Rev* (1994) **15**(5):650-683.

5 STENMAN UH, TIITINEN A, ALFTHAN H, VALMU L: The classification, functions and clinical use of different isoforms of HCG. *Hum Reprod Update* (2006) **12**(6):769-784.
- **A comprehensive review on hCG isoforms**

6 STENMAN UH, ALFTHAN H, HOTAKAINEN K: Human chorionic gonadotropin in cancer. *Clin Biochem* (2004) **37**(7):549-561.
- **A well-structured review on hCG and cancer**

7 MANN K, KARL HJ: Molecular heterogeneity of human chorionic gonadotropin and its subunits in testicular cancer. *Cancer* (1983) **52**(4):654-660.

8 MARCILLAC I, TROALEN F, BIDART JM *et al.*: Free human chorionic gonadotropin beta subunit in gonadal and nongonadal neoplasms. *Cancer Res* (1992) **52**(14):3901-3907.
- **Utility of hCG as a tumor marker**

9 BERGER P, KRANEWITTER W, MADERSBACHER S *et al.*: Eutopic production of human chorionic gonadotropin beta (hCG beta) and luteinizing hormone beta (hLH beta) in the human testis. *FEBS Lett* (1994) **343**(3):229-233.

10 DIRNHOFER S, HERMANN M, HITTMAIR A *et al.*: Expression of the human chorionic gonadotropin-beta gene cluster in human pituitaries and alternate use of exon 1. *J Clin Endocr Metab* (1996) **81**(12):4212-4217.

11 WEHMANN RE, AMR S, ROSA C, NISULA BC: Metabolism, distribution and excretion of purified human chorionic gonadotropin and its subunits in man. *Ann Endocrinol (Paris)* (1984) **45**(4-5):291-295.

12 DIRNHOFER S, KOESSLER P, ENSINGER C *et al.*: Production of trophoblastic hormones by transitional cell carcinoma of the bladder: association to tumor stage and grade. *Hum Pathol* (1998) **29**(4):377-382.

13 STENMAN UH, ALFTHAN H, RANTA T *et al.*: Serum levels of human chorionic gonadotropin in nonpregnant women and men are modulated by gonadotropin-releasing hormone and sex steroids. *J Clin Endocr Metab* (1987) **64**(4):730-736.

14 ODELL WD, GRIFFIN J: Pulsatile secretion of chorionic gonadotropin during the normal menstrual cycle. *J Clin Endocr Metab* (1989) **69**(3):528-532.

15 DIRNHOFER S, FREUND M, ROGATSCH H, KRABICHLER S, BERGER P: Selective expression of trophoblastic hormones by lung carcinoma: neurendocrine tumors exclusively produce human chorionic gonadotropin alpha-subunit (hCGalpha). *Hum Pathol* (2000) **31**(8):966-972.

16 BERGER P, GRUSCHWITZ M, SPOETTL G *et al.*: Human chorionic gonadotropin (hCG) in the male reproductive tract. *Mol Cell Endocrinol* (2007) **260-262**:190-196.
- **hCG variants are found at high concentrations in seminal plasma**

17 CASELLA I, LINDNER H, ZENZMAIER C *et al.*: Non GnRH-mediated transcription and secretion of large human glycoprotein hormone {alpha}-subunit in HEK293 cells. *Endocrinology* (2008) **149**(3):1144-1154

18 STURGEON CM, ELLIS AR: Standardization of FSH, LH and hCG--current position and future prospects. *Mol Cell Endocrinol* (2007) **260-262**:301-309.

19 STENMAN UH, BIDART JM, BIRKEN S *et al.*: Standardization of protein immunoprocedures. Choriogonadotropin (CG). *Scand J Clin Lab Invest Suppl* (1993) **216**:42-78.
- **Intellectual basis for the design of new IS for hCG**

20 BRISTOW A, BERGER P, BIDART JM *et al.*: Establishment, value assignment, and characterization of new WHO reference reagents for six molecular forms of human chorionic gonadotropin. *Clin Chem* (2005) **51**(1):177-182.
- **Six new hCG standards for immunoassay**

21 BIRKEN S, BERGER P, BIDART JM *et al.*: Preparation and characterization of new WHO reference reagents for human chorionic gonadotropin and metabolites. *Clin Chem* (2003) **49**(1):144-154.
- **Characterization of the six new WHO IRRs for hCG**

22 BERGER P, STURGEON C, BIDART JM *et al.*: The ISOBM TD-7 Workshop on hCG and related molecules. Towards user-oriented standardization of pregnancy and tumor diagnosis: assignment of epitopes to the three-dimensional structure of diagnostically and commercially relevant monoclonal antibodies directed against human chorionic gonadotropin and derivatives. *Tumor Biol* (2002) **23**(1):1-38.
- **International study on antibody requirements for hCG measurement**

23 BELLET D, LAZAR V, BIECHE I *et al.*: Malignant transformation of nontrophoblastic cells is associated with the expression of chorionic gonadotropin beta genes normally transcribed in trophoblastic cells. *Cancer Res* (1997) **57**(3):516-523.

24 MILLER-LINDHOLM AK, LABENZ CJ, RAMEY J, BEDOWS E, RUDDON RW: Human chorionic gonadotropin-beta gene expression in first trimester placenta. *Endocrinology* (1997) **138**(12):5459-5465.

25 MILLER-LINDHOLM AK, BEDOWS E, BARTELS CF *et al.*: A naturally occurring genetic variant in the human chorionic gonadotropin-beta gene 5 is assembly inefficient. *Endocrinology* (1999) **140**(8):3496-3506.

26 VALMU L, ALFTHAN H, HOTAKAINEN K, BIRKEN S, STENMAN UH: Site-specific glycan analysis of human chorionic gonadotropin beta-subunit from malignancies and pregnancy by liquid chromatography--electrospray mass spectrometry. *Glycobiology* (2006) **16**(12):1207-1218.

- **Detailed analyses of hCG glycosylation**

27 KARDANA A, ELLIOTT MM, GAWINOWICZ MA, BIRKEN S, COLE LA: The heterogeneity of human chorionic gonadotropin (hCG). I. Characterization of peptide heterogeneity in 13 individual preparations of hCG. *Endocrinology* (1991) **129**(3):1541-1550.

28 BIRKEN S, GAWINOWICZ MA, KARDANA A, COLE LA: The heterogeneity of human chorionic gonadotropin (hCG). II. Characteristics and origins of nicks in hCG reference standards. *Endocrinology* (1991) **129**(3):1551-1558.

29 COLE LA, SEIFER DB, KARDANA A, BRAUNSTEIN GD: Selecting human chorionic gonadotropin immunoassays: consideration of cross-reacting molecules in first-trimester pregnancy serum and urine. *Am J Obstet Gynecol* (1993) **168**(5):1580-1586.

30 HOERMANN R, BERGER P, SPOETTL G *et al.*: Immunological recognition and clinical significance of nicked human chorionic gonadotropin in testicular cancer. *Clin Chem* (1994) **40**(12):2306-2312.

- **hCG + hCGβ is best for monitoring testicular cancer patients**

31 MERZ WE, KRAUSE JM, ROIG J, SINGH V, BERGER P: Nonassembled Human Chorionic Gonadotropin Subunits and {alpha}{alpha}-Homodimers Use Fast-Track Processing in the Secretory Pathway in Contrast to {alpha}{beta}-Heterodimers. *Endocrinology* (2007) **148**(12):5831-5841.

32 BERGER P, SCHWARZ S, SPOTTL G, WICK G, MANN K: Variants of human chorionic gonadotropin from pregnant women and tumor patients recognized by monoclonal antibodies. *J Clin Endocr Metab* (1993) **77**(2):347-351.

33 LOTTERSBERGER C, HOERMANN R, MANN K, SCHWARZ S, BERGER P: Tumor- and pregnancy-derived isoforms of human chorionic gonadotropin: biological and diagnostic relevance. *Hormone Res* (2003) **59**(3):125-134.

34 KOVALEVSKAYA G, BIRKEN S, KAKUMA T *et al.*: Differential expression of human chorionic gonadotropin (hCG) glycosylation isoforms in failing and continuing pregnancies: preliminary characterization of the hyperglycosylated hCG epitope. *J Endocrinol* (2002) **172**(3):497-506.

35 MANN K, SCHNEIDER N, HOERMANN R: Thyrotropic activity of acidic isoelectric variants of human chorionic gonadotropin from trophoblastic tumors. *Endocrinology* (1986) **118**(4):1558-1566.

36 ELLIOTT MM, KARDANA A, LUSTBADER JW, COLE LA: Carbohydrate and peptide structure of the alpha- and beta-subunits of human chorionic gonadotropin from normal and aberrant pregnancy and choriocarcinoma. *Endocrine* (1997) **7**(1):15-32.

37 BIRKEN S: Specific measurement of o-linked core 2 sugar-containing isoforms of hyperglycosylated human chorionic gonadotropin by antibody b152. *Tumor Biol* (2005) **26**(3):131-141.

38 TOLL H, BERGER P, HOFMANN A *et al.*: Glycosylation patterns of human chorionic gonadotropin revealed by liquid chromatography-mass spectrometry and bioinformatics. *Electrophoresis* (2006) **27**(13):2734-2746.

39 BLITHE DL: Carbohydrate composition of the alpha-subunit of human choriogonadotropin (hCG alpha) and the free alpha molecules produced in pregnancy: most free alpha and some combined hCG alpha molecules are fucosylated. *Endocrinology* (1990) **126**(6):2788-2799.

40 KRAUSE JM, BERGER P, ROIG J, SINGH V, MERZ WE: Rapid maturation of glycoprotein hormone free alpha-subunit (GPHalpha) and GPHalpha alpha homodimers. *Mol Endocrinol* (2007) **21**(10):2551-2564.

41 ROIG J, KRAUSE JM, BERGER P, MERZ WE: Time-dependent folding of immunological epitopes of the human chorionic gonadotropin beta-subunit. *Mol Cell Endocrinol* (2007) **260-262**:12-22.

42 KOFLER R, BERGER P, WICK G: Monoclonal antibodies against human chorionic gonadotropin (hCG): I. production, specificity, and intramolecular binding sites. *Am J Reprod Immunol* (1982) **2**(4):212-216.

43 BERGER P: [Molecular morphology of placental and pituitary hormones: epitope mapping with monoclonal antibodies]. *Wien Klin Wochenschr* (1985) **97**(14):573-581.

44 BERGER P, PANMOUNG W, KHASCHABI D, MAYREGGER B, WICK G: Antigenic features of human follicle stimulating hormone delineated by monoclonal antibodies and construction of an immunoradiomometric assay. *Endocrinology* (1988) **123**(5):2351-2359.

45 BERGER P, KLIEBER R, PANMOUNG W *et al.*: Monoclonal antibodies against the free subunits of human chorionic gonadotrophin. *J Endocrinol* (1990) **125**(2):301-309.

46 KHAZAELI MB, ENGLAND BG, DIETERLE RC *et al.*: Development and characterization of a monoclonal antibody which distinguishes the beta-subunit of human chorionic gonadotropin (beta hCG) in the presence of the hCG. *Endocrinology* (1981) **109**(4):1290-1292.

47 BELLET D, BIDART JM, JOLIVET M *et al.*: A monoclonal antibody against a synthetic peptide is specific for the free native human chorionic gonadotropin beta-subunit. *Endocrinology* (1984) **115**(1):330-336.

48 ALFTHAN H, SCHRODER J, FRASER R *et al.*: Choriogonadotropin and its beta subunit separated by hydrophobic-interaction chromatography and quantified in serum during pregnancy by time-resolved immunofluorometric assays. *Clin Chem* (1988) **34**(9):1758-1762.

49 ARMSTRONG EG, EHRLICH PH, BIRKEN S *et al.*: Use of a highly sensitive and specific immunoradiometric assay for detection of human chorionic gonadotropin in urine of normal, nonpregnant, and pregnant individuals. *J Clin Endocr Metab* (1984) **59**(5):867-874.

50 BIDART JM, BIRKEN S, BERGER P, KRICHEVSKY A: Immunochemical mapping of hCG and hCG-related molecules. *Scand J Clin Lab Invest Suppl* (1993) **216**:118-136.

51 BERGER P, BIDART JM, DELVES PS *et al.*: Immunochemical mapping of gonadotropins. *Mol Cell Endocrinol* (1996) **125**(1-2):33-43.

52 KOVALEVSKAYA G, BIRKEN S, KAKUMA T, O'CONNOR JF: Early pregnancy human chorionic gonadotropin (hCG) isoforms measured by an immunometric assay for choriocarcinoma-like hCG. *J Endocrinol* (1999) **161**(1):99-106.

53 DIRNHOFER S, MADERSBACHER S, BIDART JM *et al.*: The molecular basis for epitopes on the free beta-subunit of human chorionic gonadotrophin (hCG), its carboxyl-terminal peptide and the hCG beta-core fragment. *J Endocrinol* (1994) **141**(1):153-162.

54 JACKSON AM, KLONISCH T, LAPTHORN AJ *et al.*: Identification and selective destruction of shared epitopes in human chorionic gonadotropin beta subunit. *J Reprod Immunol* (1996) **31**(1-2):21-36.

55 JACKSON AM. Towards an epitope-specific vaccine: Epitope-loss mutants of human chorionic gonadotropin beta subunit. In. London: University of London; 1996.

56 BERGER P, KOFLER R, WICK G: Monoclonal antibodies against human chorionic gonadotropin (hCG): II. Affinity and ability to neutralize the biological activity of hCG. *Am J Reprod Immunol* (1984) **5**(4):157-160.

57 DIRNHOFER S, LECHNER O, MADERSBACHER S *et al.*: Free alpha subunit of human chorionic gonadotrophin: molecular basis of immunologically and biologically active domains. *J Endocrinol* (1994) **140**(1):145-154.

58 TROALEN F, BELLET DH, GHILLANI P *et al.*: Antigenic determinants on human choriogonadotropin alpha-subunit. II. Immunochemical mapping by a monoclonal antipeptide antibody. *J Biol Chem* (1988) **263**(21):10370-10376.

59 SCHWARZ S, BERGER P, WICK G: The antigenic surface of human chorionic gonadotropin as mapped by murine monoclonal antibodies. *Endocrinology* (1986) **118**(1):189-197.

60 MANN K, SALLER B, HOERMANN R: Clinical use of HCG and hCG beta determinations. *Scand J Clin Lab Invest Suppl* (1993) **216**:97-104.

61 STURGEON CM, MCALLISTER EJ: Analysis of hCG: clinical applications and assay requirements. *Ann Clin Biochem* (1998) **35 (Pt 4)**:460-491.

62 COLE LA, KARDANA A, ANDRADE-GORDON P *et al.*: The heterogeneity of human chorionic gonadotropin (hCG). III. The occurrence and biological and immunological activities of nicked hCG. *Endocrinology* (1991) **129**(3):1559-1567.

63 BUTLER SA, LAIDLER P, PORTER JR *et al.*: The beta subunit of human chorionic gonadotropin exists as a homodimer. *J Mol Endocrinol* (1999) **22**(2):185-192.

64 KHAZAELI MB, BUCHINA ES, PATTILLO RA, SOONG SJ, HATCH KD: Radioimmunoassay of free beta-subunit of human chorionic gonadotropin in diagnosis of high-risk and low-risk gestational trophoblastic disease. *Am J Obstet Gynecol* (1989) **160**(2):444-449.

65 MADERSBACHER S, KLIEBER R, MANN K *et al.*: Free alpha-subunit, free beta-subunit of human chorionic gonadotropin (hCG), and intact hCG in sera of healthy individuals and testicular cancer patients. *Clin Chem* (1992) **38**(3):370-376.

66 MADERSBACHER S, KRATZIK C, GERTH R, DIRNHOFER S, BERGER P: Human chorionic gonadotropin (hCG) and its free subunits in hydrocele fluids and neoplastic tissue of testicular cancer patients: insights into the in vivo hCG-secretion pattern. *Cancer Res* (1994) **54**(19):5096-5100.

67 ALFTHAN H, HAGLUND C, ROBERTS P, STENMAN UH: Elevation of free beta subunit of human choriogonadotropin and core beta fragment of human choriogonadotropin in the serum and urine of patients with malignant pancreatic and biliary disease. *Cancer Resd* (1992) **52**(17):4628-4633.

68 ILES RK, CHARD T: Human chorionic gonadotropin expression by bladder cancers: biology and clinical potential. *J Urol* (1991) **145**(3):453-458.

69 GROSSMANN M, TRAUTMANN ME, POERTL S *et al.*: Alpha-subunit and human chorionic gonadotropin-beta immunoreactivity in patients with malignant endocrine gastroenteropancreatic tumours. *Eur J Clin Invest* (1994) **24**(2):131-136.

70 HOTAKAINEN K, LINTULA S, LJUNGBERG B *et al.*: Expression of human chorionic gonadotropin beta-subunit type I genes predicts adverse outcome in renal cell carcinoma. *J Mol Diagn* (2006) **8**(5):598-603.

71 CHARD T: Pregnancy tests: a review. *Human reproduction (Oxford, England)* (1992) **7**(5):701-710.

72 COLE LA: Immunoassay of human chorionic gonadotropin, its free subunits, and metabolites. *Clin Chem* (1997) **43**(12):2233-2243.

73 MCCHESNEY R, WILCOX AJ, O'CONNOR JF *et al.*: Intact HCG, free HCG beta subunit and HCG beta core fragment: longitudinal patterns in urine during early pregnancy. *Human reproduction (Oxford, England)* (2005) **20**(4):928-935.

- **Secretion patterns of hCG variants during early pregnancy**

74 HAY DL: Discordant and variable production of human chorionic gonadotropin and its free alpha- and beta-subunits in early pregnancy. *J Clin Endocr Metab* (1985) **61**(6):1195-1200.

75 COLE LA, SUTTON-RILEY JM, KHANLIAN SA *et al.*: Sensitivity of over-the-counter pregnancy tests: comparison of utility and marketing messages. *J Am Pharm Assoc (2003)* (2005) **45**(5):608-615.

76 WILCOX AJ, BAIRD DD, WEINBERG CR: Time of implantation of the conceptus and loss of pregnancy. *N Engl J Med* (1999) **340**(23):1796-1799.

77 NORMAN RJ, MENABAWEY M, LOWINGS C, BUCK RH, CHARD T: Relationship between blood and urine concentrations of intact human chorionic gonadotropin and its free subunits in early pregnancy. *Obstet Gynecol* (1987) **69**(4):590-593.

78 COLE LA, SUTTON JM, HIGGINS TN, CEMBROWSKI GS: Between-method variation in human chorionic gonadotropin test results. *Clin Chem* (2004) **50**(5):874-882.

79 MADERSBACHER S, BERGER P: Antibodies and immunoassays. *Methods (San Diego, Calif* (2000) **21**(1):41-50.

80 MOCK P, KOVALEVSKAYA G, O'CONNOR JF, CAMPANA A: Choriocarcinoma-like human chorionic gonadotrophin (HCG) and HCG bioactivity during the first trimester of pregnancy. *Hum Reprod (Oxford, England)* (2000) **15**(10):2209-2214.

81 KOVALEVSKAYA G, BIRKEN S, KAKUMA T, SCHLATTERER J, O'CONNOR JF: Evaluation of nicked human chorionic gonadotropin content in clinical specimens by a specific immunometric assay. *Clin Chem* (1999) **45**(1):68-77.

82 ALFTHAN H, STENMAN UH: Pregnancy serum contains the beta-core fragment of human choriogonadotropin. *J Clin Endocr Metab* (1990) **70**(3):783-787.

83 WIDE L, LEE JY, RASMUSSEN C: A change in the isoforms of human chorionic gonadotropin occurs around the 13th week of gestation. *J Clin Endocr Metab* (1994) **78**(6):1419-1423.

84 VAITUKAITIS JL, BRAUNSTEIN GD, ROSS GT: A radioimmunoassay which specifically measures human chorionic gonadotropin in the presence of human luteinizing hormone. *Am J Obstet Gynecol* (1972) **113**(6):751-758.

85 STORRING PL, GAINES-DAS RE, BANGHAM DR: International Reference Preparation of Human Chorionic Gonadotrophin for Immunoassay: potency estimates in various bioassay and protein binding assay systems; and International Reference Preparations of the alpha and beta subunits of human chorionic gonadotrophin for immunoassay. *J Endocrinol* (1980) **84**(2):295-310.

86 MADERSBACHER S, STULNIG T, HUBER LA *et al.*: Serum glycoprotein hormones and their free alpha-subunit in a healthy elderly population selected

according to the SENIEUR protocol. Analyses with ultrasensitive time resolved fluoroimmunoassays. *Mech Ageing Dev* (1993) **71**(3):223-233.

87 EKINS R: Measurement of free hormones in blood. *Endocr Rev* (1990) **11**(1):5-46.

88 SCHWARZ S, BERGER P, WICK G: Epitope-selective, monoclonal-antibody-based immunoradiometric assays of predictable specificity for differential measurement of choriogonadotropin and its subunits. *Clin Chem* (1985) **31**(8):1322-1328.

Table 1

Nomenclature of hCG and hCG-variants, the new WHO-adopted 1st International Reference Reagents (IRR)* for hCG immunoassays and its starting materials (frozen concentrates…FC) as established by the International Federation of Clinical Chemistry (IFCC)

Symbol	Definition	WHO 1st IRR	FC	nmol/amp
hCG	human chorionic gonadotropin, bioactive αβ heterodimer	hCG 99/688	853	1.88
hCGβ	non assembled free β-subunit	hCGβ 99/650	863	0.78
hCGα	non assembled free α-subunit	hCGα, 99/720	862	0.84
hCGβcf	hCGβ core fragment; dipeptide, aa hCGβ6-40 linked to hCGβ55-92	hCGβcf 99/708	855	0.88
hCGn	hCG nicked around aa hCGβ45-49	hCGn 99/642	865	0.33
hCGβn	hCGβn nicked around aa hCGβ45-49	hCGβn 99/692	-	1.02
Nomenclature of less well defined hCG variants				
hCGβCTP	carboxyl-terminal peptide of hCGβ; aa hCGβ109/114-145	n.a.	n.a.	n.a.
hCGav	acidic variant of hCG	n.a.	n.a.	n.a.
hCGh	hyperglycosylated hCG	n.a.	n.a.	n.a.
-CTPhCG	truncated hCG missing hCGβCTP	n.a.	n.a.	n.a.
-CTPhCGβ	truncated hCGβ missing hCGβCTP	n.a.	n.a.	n.a.

*The IRRs for hCG and hCG variants are available from the National Institute for Biological Standards and Control (NIBSC), Potters Bar, Herts, U.K.
n.a., not available

Table 2: hCG-MAbs: Epitopes and Specificity

Epitopes		MAb Specificities											Reference mAbs[1)]	
Code	Molecular Localization	hCG	hCGβ	hCGβcf	hCGn	hCGβn	-CTP hCG	-CTP hCGβ	hLH	hLHβ	hFSH hTSH	GPHα	mAb-Code	Characteristics
β_1	Cystine knot hCGβ 10+60	■	■	■	■	■	■	■					INN-hCG-2	highly specific
β_2	hCGβ loops 1+3 hCGβ 20-25 + 68-77	■	■	■	■	■	■	■	▒	▒			INN-hCG-22	pan hCGβ
β_3		■	■	■	■	■	■	■	■	■			INN-bLH-1	
β_4		■	■	■	■	■	■	■	▒	▒			INN-hCG-24	
β_5		■	■	■	■	■	■	■	■	■			INN-hCG-58	
β_6	hCGβ core		■	■		?		?					INN-hCG-64	free hCGβ
β_7			■	■		?		?					INN-hCG-68	
β_8	hCGβ135-145	■	■		■	■							h54	hCGβCTP
β_9	hCGβ111-116	■	■		■	■	■	■					FB-12	
β_{10}	hCGβcf			■									INN-hCG-103	hCGβcf highly specific
β_{11}				■									INN-hCG-104 INN-hCG-106	
β_{12}				■									INN-hCG-105	
β_{13}				■									INN-hCG-112	
α_1	hCGα loop 1 hCGα13-22	■			■		■		■		■	■	INN-hFSH-73	all human GPH and GPHα
α_2		■			■		■		■		■	■	INN-hFSH-98 INN-hFSH-100	
α_4		■			■		■		■		■	■	INN-hFSH-132	
α_3	hCGα loop 3?	■			■		■		■		■	■	INN-hFSH-179	
α_5		■			■		■		■		■	■	INN-hFSH-158	
α_6	hCGα loop 2 hCGα 33-42											■	INN-hCG-72,-80	free GPHα sbt. interaction
α_7	hCGα 87-92		?	?	?	?	?	?		?	?	■	FA36	free GPHα?
c_1	hCGβ loop 2	■					?		▒				INN-hCG-10	hCG no XR hCGn minor XR hLH
c_2		■					?		▒				INN-hCG-40,-53	
c_3		■			■		n.t.						INN-hCG-45	hCG + hCGn
c_4	hCGβ?	■			■		?		■				INN-hCG-26	hCG + hLH

[1)] "INN-Reference mAbs" can be obtained from the author (P.B.); Filled squares…strong reactivity; Open squares…no reactivity; Grey squares…minor reactivity (<1%); GPHα…glycoprotein hormone alpha subunit; n.t.…not tested

Table 3

Cross-contaminations (% mol/mol) of hCG and hCG-related molecules in the frozen concentrates of the starting material for the 1st WHO RR hCG, hCGn, hCGβ, hCGβn, hCGβcf and hCGα and the 3rd I.S. for hCG (75/537).

	Percentage cross-contamination (mol/mol)[2)]			
Assay specificity[1)] / **Preparation[3)]**	**hCG/n** + -CTPhCG	**hCGβ/βn+** -CTPhCGβ̃βn+ hCGβcf	**hCGβcf**	**hCGα**
hCG 3rd I.S. (75/537)	**100.000**	**3.866**	**0.812**	**1.415**
hCG (99/688)	**100.000**	**1.076**	**0.208**	**1.001**
hCGn (99/642)	100.000	1.314	0.523	1.324
hCGβ (99/650)	0.312	100.000	0.178	0.013
hCGβn 99/692)	0.168	100.000	0.982	0.269
hCGβcf (99/708)	0.004	n.a.	100.000	<0.001
hCGα (99/720)	0.031	0.015	0.043	100.000
Assay design/epitopes	**c_3 - $\beta_2^{Eu4)}$**	**β_7 - $\beta_2^{Eu\ 5)}$**	**β_{11} - $\beta_2^{Eu\ 6)}$**	**α_6- $\alpha_5^{Eu\ 7)}$**

[1)] Recognition of hCG variants truncated at the CTP of hCGβ is likely but not proven

[2)] Percentages correlated to the frozen concentrates of the new 1st WHO IRRs and <u>not</u> to existing I.S. and I.R.P.s

[3)] Frozen concentrates of the starting material for the 1st WHO IRRs except for the 3rd I.S. for hCG (75/537)

[4)] Assay cross-reactivity: hCG 100% hCGβ <0.001% hCGβcf <0.1% hCGα 0.01%

[5)] Assay cross-reactivity: hCG <0.001% hCGβ 100% hCGβcf n.d., hCGα <0.001%

[6)] Assay cross-reactivity: hCG <<0.1% hCGβ <0.001% hCGβcf 100% hCGα <0.001%

[7)] Assay cross-reactivity: hCG 0.001% hCGβ <0.001, hCGβcf<0.001% hCGα 100%

n.a. ... not applicable; Eu ... Europium-labeled mAb

Table 4
Suggestions for "hCG"-sandwich assay specificities, mAb combinations and appropriate clinical use

Assay specificity	Recommended mAb combinations	Appropriate clinical use
hCG+hCGβ+hCGβcf (+hCGn+βn …)	β_1 mAb combined with β_2 or β_4 mAbs	Wide spectrum: Oncology, Early pregnancy
hCGβ (+ hCGβn+βcf)	β_6 mAbs combined with β_2	Prenatal (Downs) Oncology
hCGβcf	β_{11} mAb combined with β_2 or β_4 mAbs	In urine only Clinical utility to be established
hCGα	α_6 mAb combined with α_5 or α_4 mAbs	Oncology (pituitary/testis) Clinical utility to be established

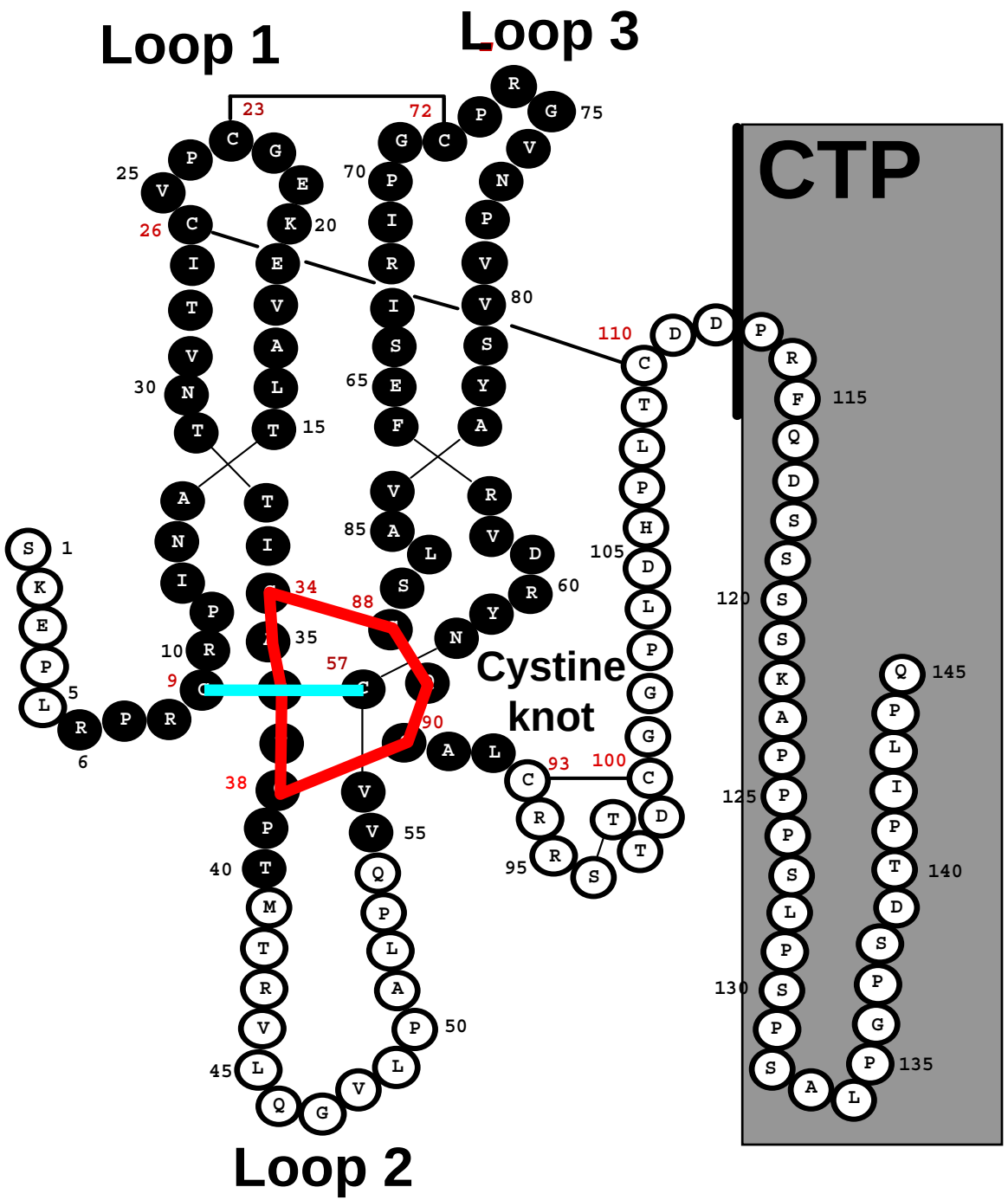

Figure 1

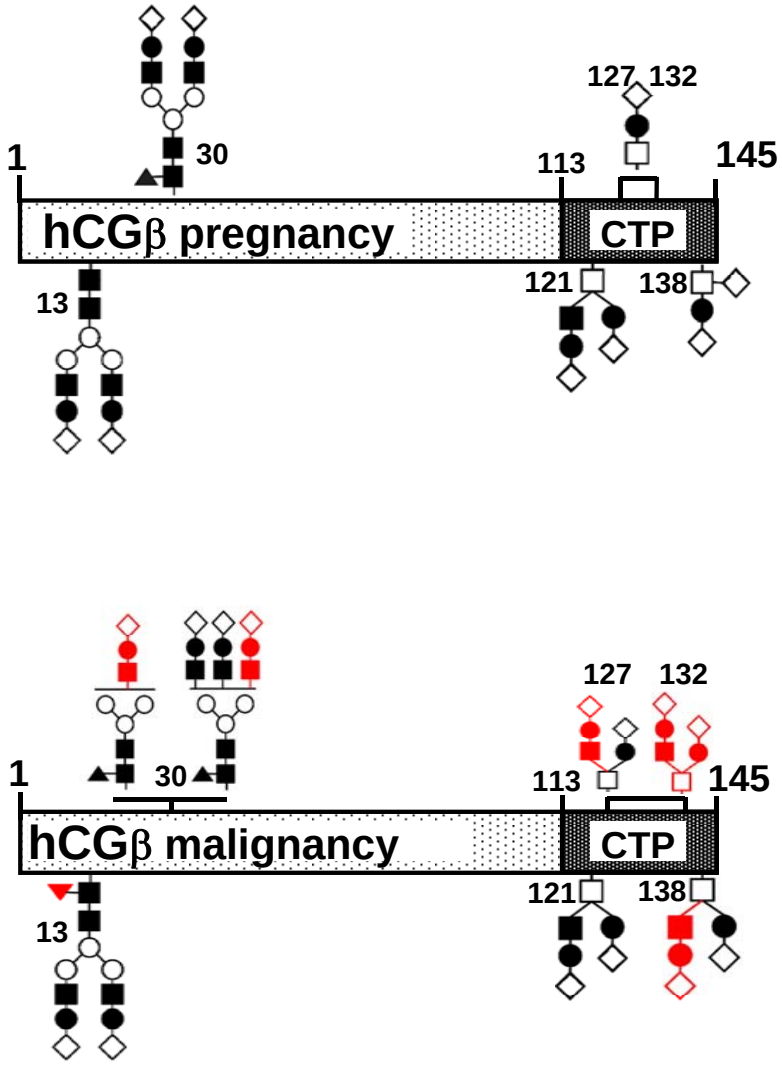
1
30
127
132
113
145
hCGβ pregnancy
CTP
13
121
138
1
30
127
132
113
145
hCGβ malignancy
CTP
13
121
138

Figure 2

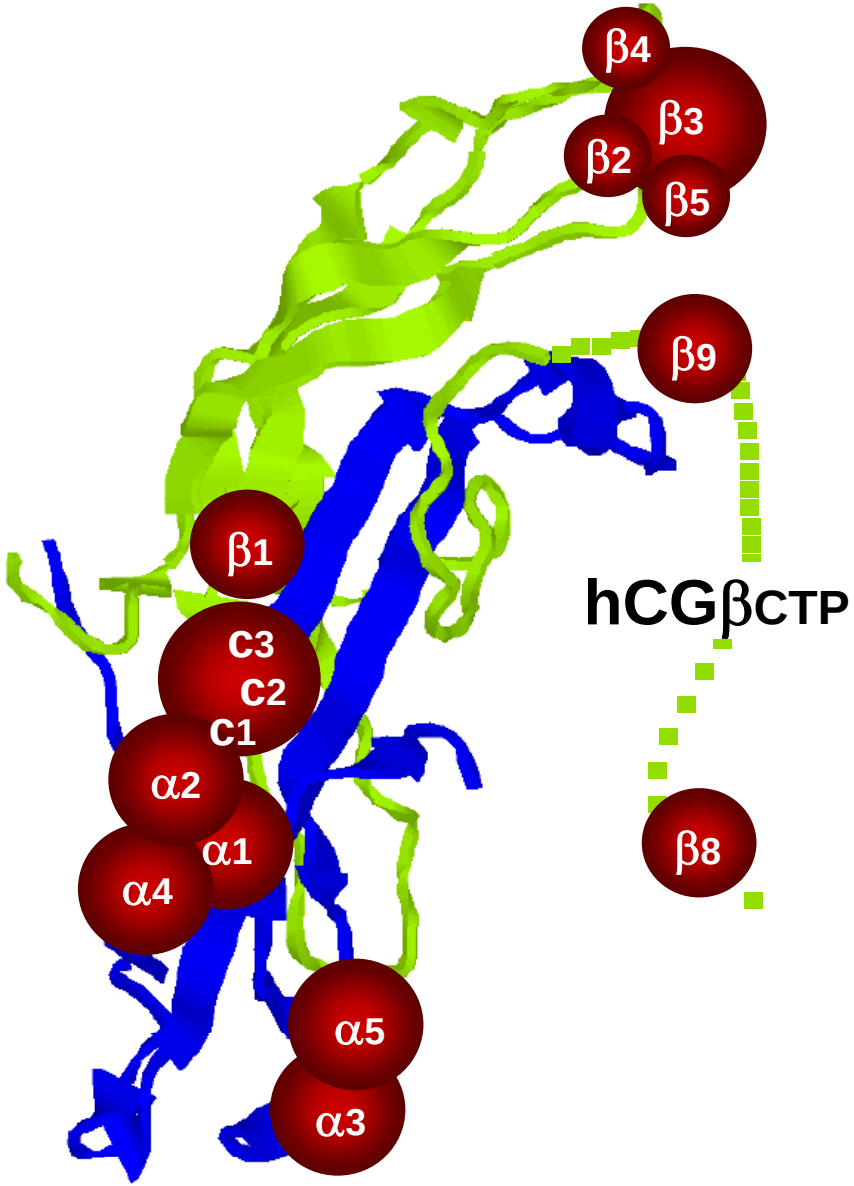

Figure 3

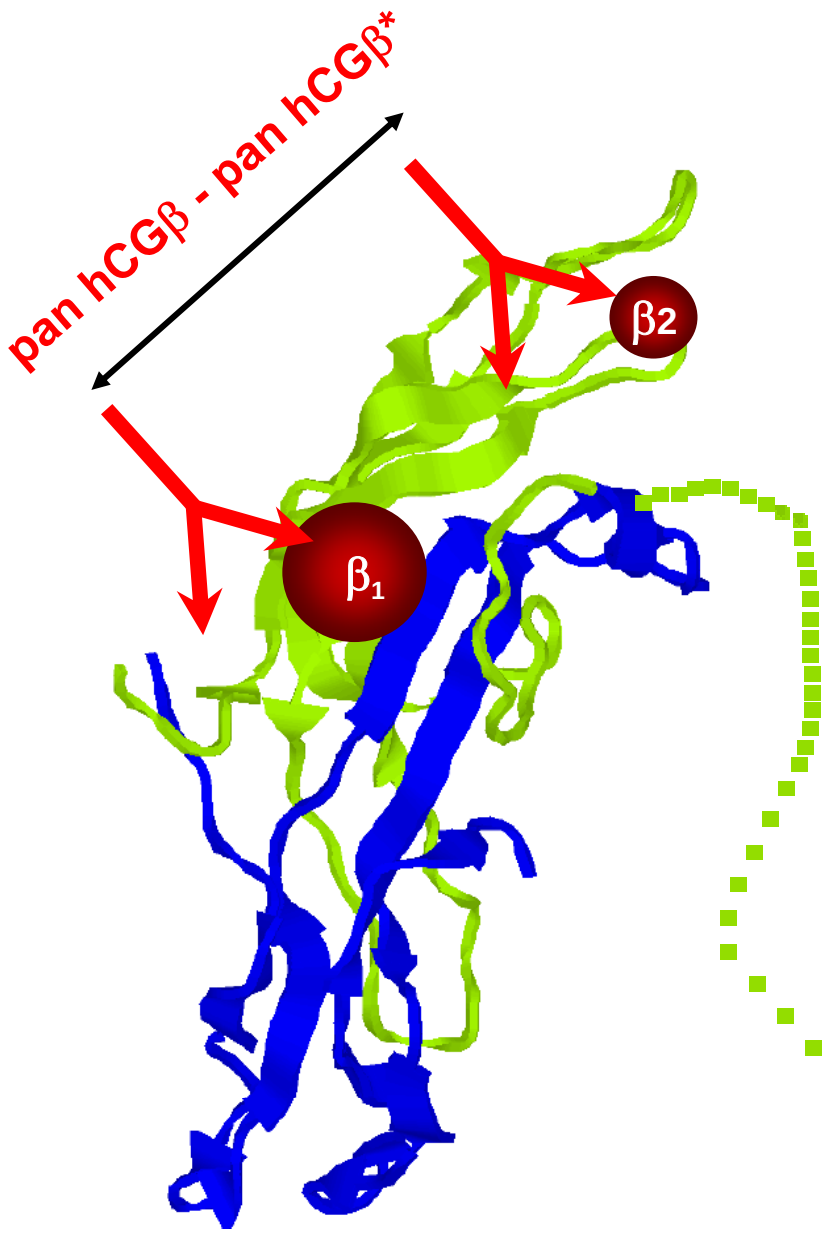

Figure 4a

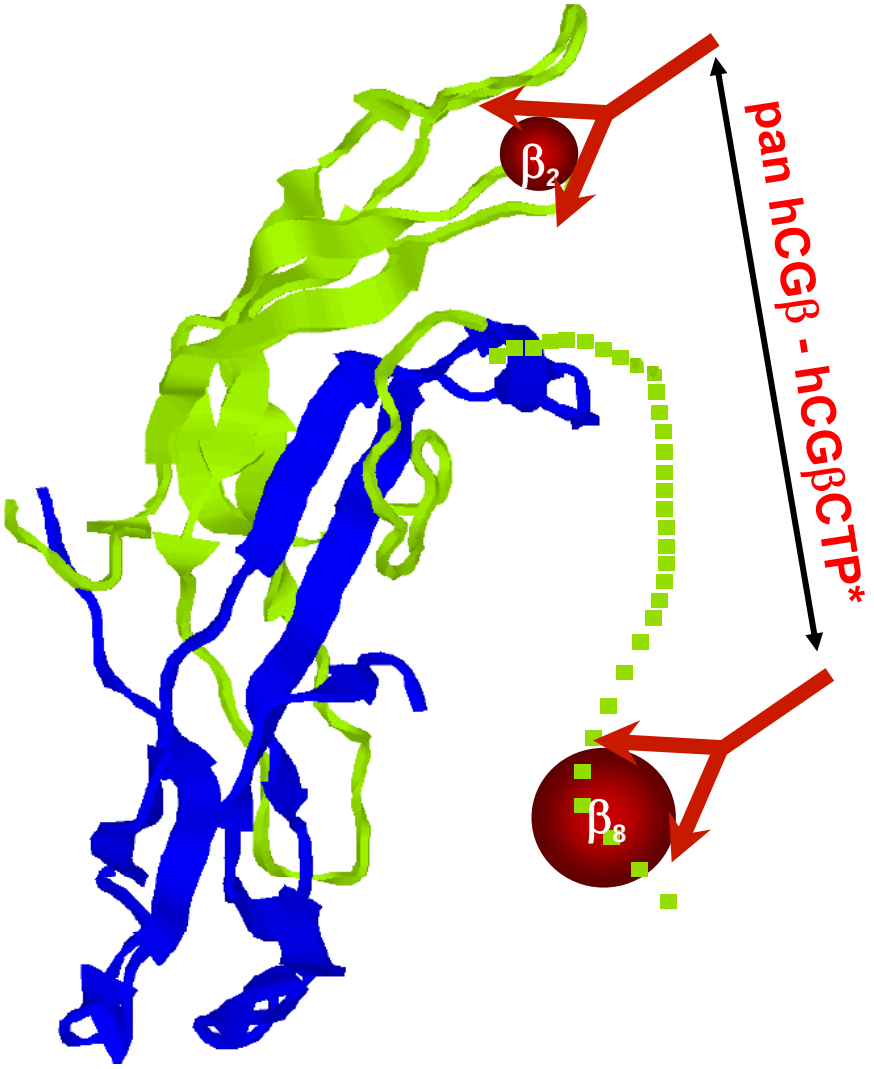

Figure 4b