Berger, P., and Lapthorn, A. J. (2016) The molecular relationship between antigenic domains and epitopes on hCG. Molecular Immunology, 76, pp. 134-145. (doi:10.1016/j.molimm.2016.06.015)

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Deposited on: 07 October 2016

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The molecular relationship between antigenic domains and epitopes on hCG

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Key Words

hCG, variants, antigenic domains, number of epitopes, harmonizing immunoassays, pregnancy testing

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Abstract

Antigenic domains are defined to contain a limited number of neighboring epitopes recognized by antibodies (Abs) but their molecular relationship remains rather elusive. We thoroughly analyzed the antigenic surface of the most important pregnancy and tumor marker human chorionic gonadotropin (hCG), a cystine knot (*ck*) growth factor, and set antigenic domains and epitopes in molecular relationships to each other. Antigenic domains on hCG, its free hCGα and hCGβ subunits are dependent on appropriate inherent molecular features such as molecular accessibility and protrusion indices that determine bulging structures accessible to Abs. The banana-shaped intact hCG comprises ~7,500 $Å^2$ of antigenic surface with minimally five antigenic domains that encompass a continuum of overlapping non-linear composite epitopes, not taking into account the C-terminal peptide extension of hCGβ (hCGβCTP). Epitopes within an antigenic domain are defined by specific Abs, that bury nearly 1,000 $Å^2$ of surface accessible area on the antigen and recognize a few up to 15 amino acid (aa) residues, whereby between 2 and 5 of these provide the essential binding energy. Variability in Ab binding modes to the contact aa residues are responsible for the variation in affinity and intra- and inter-species specificity, e.g. cross-reactions with luteinizing hormone (LH). Each genetically distinct fragment antigen binding (Fab) defines its own epitope. Consequently, recognition of the same epitope by different Abs is only possible in cases of genetically identical sequences of its binding sites. Due to combinatorial V(D)J gene segment variability of heavy and light chains, Abs defining numerous epitopes within an antigenic domain can be generated by different individuals and species. Far more than one hundred Abs against the immune-dominant antigenic domains of either subunit correspond to both ends of the hCG-molecule, the tips of peptide loops one and three (*Ł1+3*) protruding from the central *ck*, encompassing *hCGβŁ1+3* corresponding to aa 20-25+64+68-81 and *hCGαŁ1* aa 13-22 (*Pro16*, *Phe17*, *Phe18*) plus *hCGαŁ3* (*Met71, Phe74*), respectively, have been identified in two "ISOBM Tissue Differentiation-7 Workshops on hCG and Related Molecules" and by other studies. These Abs recognize distinct but overlapping epitopes with slightly different specificity profiles and affinities. Heterodimeric-specific epitopes involve neighboring *αŁ1* plus *βŁ2 (hCGβ44/45 and 47/48)*. Diagnostically important Abs recognize the middle of the molecule, the *ck* (aa *Arg10, Arg60 and possibly Gln89*) and the linear *hCGβCTP* "tail" (*aa 135-145; Asp139, Pro144, Gln145*), respectively. Identification of antigenic domains and of specific epitopes is essential for harmonization

of Abs in methods that are used for reliable and robust hCG measurements for the management of pregnancy, pregnancy-related disease and tumors.

1. hCG and variants are important clinical pregnancy and tumor markers

The dimeric glycoprotein hormone (GPH) human chorionic gonadotropin (hCG) is synthesized by the placental trophoblast and controls pregnancy by stimulating progesterone production in the *corpus luteum*. hCG and its variants are also secreted by hydaditiform mole, a benign gestational trophoblastic disease, as well as a variety of tumors in particular choriocarcinoma, testicular cancer and bladder cancer. Thus, they serve as reliable diagnostic markers for the detection of pregnancy, the management of pregnancy-related disease and trophoblastic and non-trophoblastic tumors [1-4].

In its strict sense hCG is defined as the biologically active heterodimeric hormone but hCG is also used as an umbrella term for a plethora of genomic, posttranslational and metabolized protein backbone variants that result from modifications like nicking, truncation, homo-dimerization, oxidation and variable glycosylation, thus does not denote a unique chemical entity. It occurs in highly varying concentrations and variant formulations in body fluids like serum, urine, seminal plasma, extra embryonic coelomic fluid and liquor (for reviews see [1-3, 5].

The "International Federation of Clinical Chemistry (IFCC) Working Group (WG) on Standardization of hCG Measurements" identified and defined six protein backbone variants of potential clinical interest: the bioactive αβ-heterodimer, hCG, its free subunits hCGα and hCGβ, hCGβ core fragment (hCGβcf) and peptidase nicked hCG (hCGn) and hCGβ (hCGβn). This WG established respective new 1st International Reference Reagents (IRR) and the 5th International Standard (IS) for hCG calibrated in SI units (molar units). These are meant to be used for the characterization of diagnostic antibodies (Abs) and methods for hCG measurement as well as its calibrations [6-10] (Figure 1, Table 1).

2. hCG, a cystine knot growth factor

hCG and the three pituitary-derived GPHs human (h) luteinizing hormone (hLH), follicle stimulating hormone (hFSH) and thyroid stimulating hormone (hTSH) are cystine knot (ck) growth factors composed of two non-covalently linked α- and β-subunits. The GPHs, in particular hCG and hLH that share approximately >85% structural homology in their β-subunits (hCGβ vs. hLHβ) and bind

to the same receptor, genetically, biochemically and in their antigenic appearance are closely related. Their prominent structural feature is a central ck in either subunit whereby two disulfide bonds link two anti-parallel β-strands forming a ring that is axially crossed by a third disulfide bond. From this knot three peptide loops protrude, *αŁ1 to αŁ3* and *βŁ1 and βŁ3*, respectively, whereby the two twisted neighboring loops *Ł1* and *Ł3* of each subunit are diametrically opposed by loop *Ł2* [11].

Symbol	**WHO code**	**Content/ampoule**	**Molecular definition**	**MW**[b]
hCG[c]	3rd IS 75/537	70µg or 650 IU		
hCG[c]	4th IS 75/589	70µg or 650 IU	intact αβ heterodimer, bioactive [a]	
hCG[d]	5th IS 07/364	0.39 nmol or 179 IU		35,336 – 37,338
hCG[d]	1st IRR 99/688	1.88 nmol		
hCGn	1st IRR 99/642	0.78 nmol	hCG nicked at *hCGβ44-48* [a]	
hCGβ	1st IRR 99/650	0.84 nmol	hCGβ subunit (*hCGβ1-145*) [a]	22,336 – 23,667
hCG□n	1st IRR 99/692	0.88 nmol	hCGβ nicked at *aa 44-48* [a]	
hCGβcf	1st IRR 99/708	0.33 nmol	hCGβ□core fragment; *hCGβ6-40* linked to *β55-92* [a]	
hCGα	1st IRR 99/720	1.02 nmol	hCGα subunit (*hCGα1-92*) [a]	13,000 – 13,671
hCGβ	1st IRP 75/551	70µg; 70 IU	hCGβ subunit (*hCGβ1-145*)	
hCGα	1st IRP 75/569	70µg; 70 IU	hCGα subunit (*hCGα1-92*)	
hCGβCTP	[e]		hCGβ C-terminal peptide, (aa *hCGβ109/114-145*)	
-CTPhCG	[e]		hCG missing most of the CTP (aa *β121-145*)	
-CTPhCG□	[e]		hCGβ missing most of the CTP (aa *hCGβ121-145*)	
hCGav	[e]		acidic variant of hCG, synonymic to hhCG [12]	
hhCG	[e]		hyperglycosylated hCG	

Table 1, hCG and hCG-related variants: Nomenclature, International Standards (IS), International Reference Reagents (IRR) and International Reference Preparations (IRP)

[a] Abbreviations and definitions for hCG and hCG-derived molecules as put forward by the IFCC Working Group for Standardization of hCG [6, 7].
[b] determined by RP HPLC-ESI-MS; non-trypsinized i.e. native frozen concentrate [13]
[c] bioactivity/mass = 9,200 IU/mg
[d] bioactivity/mass = 12,240 IU/mg determined by immunoassay
[e] no standard available
aa…amino acids

hCGβ carries a unique carboxyl-terminal peptide "tail" (hCGβCTP, aa *β113-145*) which structurally discerns hCGβ from hLHβ. It has evolved by a mutational frameshift caused by a base pair deletion at aa *β113* and loss of the first stop codon in the ancestral hLHβ gene leading to a read-through event and incorporation of a previously untranslated peptide sequence into the open reading frame [14]. It is not involved in receptor-binding and biological activity [15] but significantly prolongs metabolic half-life in serum [16, 17]. This hCGβ and hCG-specific sequence is of great interest for the design of highly

specific immunoassays, fertility regulating and anti-hCG cancer vaccines as well as long-acting erythropoietins [7, 15, 17, 18].

3. Glycosylation and microheterogeneity

Glycosylation of hCG is required for correct intracellular folding and assembly of subunits, secretion, receptor activation and metabolic half-life in circulation [19]. Both subunits are heavily glycosylated so that approximately one third of hCG's molecular mass consists of carbohydrates. hCGα carries two branched N-linked carbohydrate antennae at *Asn52* and *78*, whereas hCGβ contains two at *Asn13* and *30* plus four branches linked by O-glycosylation to *Ser121,127,132* and *138*. Molecular microheterogeneity is caused by variability in carbohydrate branching (mono-, di- or tri-antennae) and terminal sialylation (Figure 1).

The composition of hCG protein backbone variants and glycosylation isoforms show stark absolute and relative dynamics in concentrations with progression of pregnancy and tumor type [2, 20-23]. While it seems that more intensely glycosylated hCG isoforms dominate at the beginning of pregnancy a change in median molecular charge reflecting less glycosylated hCG isoforms occurs around week 13 of gestation in urine and serum [24, 25]. The major urinary hCG variant after 3 weeks of gestation until parturation is hCGβcf (Figure 1) that is largely deglycosylated and only contains rudimentary N-linked mannose cores [26]. It is generated primarily in the kidney [27].

In addition there do exist other less well defined variants such as the acidic variant of hCG (avhCG), including hyperglycosylated hCG (hhCG), observed in serum and urine of patients presenting with trophoblastic disease [12, 24, 30], -CTPhCG and -CTPhCGβ both truncated after amino acid hCGβ117 [12, 31-33], the clipped carboxyl-terminal peptide of hCG (hCGβCTP) encompassing approx. amino acids *hCGβ117-145*, hCGββ homodimers [34] and hCGαα homodimers that are observed in sera of testicular cancer patients [12], seminal plasma [35] and choriocarcinoma cells [19].

Variability in glycosylation is mirrored by a broader range of molecular masses [36] that, as determined by ion pair reversed phase (RP) HPLC-ESI-MS, ranged between 13,000 and 13,671 for the non-trypsinized concentrate of the 1st IRR for hCGα (99/720) and 22,336 to 23,667 for the 1st IRR for hCGβ (99/650). As both subunits were derived by dissociation of the purified starting material of the 1st IRR 99/688 and the 5th IS 07/364 for hCG, the calculated mass for intact pregnancy hCG ranges from 35,336 to 37,338 [13, 37] (Table 1). Despite the high number of potential glycosylation

variants, greater than 3,000 glycosylation isoforms for hCGα and 13,800 for hCGβ, only 14 major variants for hCGα (1st IRR 99/720) and 12 for hCGβ (1st IRR 99/650) have been observed [13].

4. Why are hCG epitopes important?

For reliable patient management diagnostic “hCG” results of measurements in pregnancy and disease should be comparable between Ab-based sandwich methods of different manufacturers and laboratories. The most important parameters to achieve robust and reliable “hCG” results are:

i. adequate universally adopted commutable international standards (IS) and international reference reagents (IRR) calibrated in molar units
ii. an unambiguous nomenclature of and clinical consent on the hCG variants and isoforms being measured and finally
iii. the harmonization of the diagnostic Abs applied in sandwich assays regarding recognition of epitopes and consequently specificity profiles [4, 7, 38].

In particular harmonization of epitopes in commercial assays has generally been neglected until now, not only for hCG but also for other analytes, and variability in method design. Non-standardized Abs’ specificities still leads to differences in results either due to selective over-specificity in variant recognition, undesired cross-reactivity or interference of non-measured metabolic variants like hCGβcf that are only detected by one of the two Abs in sandwich assays (hook-effect) [4, 38-41]. Thus identification and harmonization of epitopes recognized by diagnostic Abs will in future improve method comparability and consequently patient management [38].

5. hCG epitope identification

Unfortunately a comprehensive array of anti-hCG-antibody:antigen complexes with accurate data on the molecular location of recognized epitopes is not available, due in no small part to the difficulty of crystallizing hCG and other glycoprotein hormones. Therefore a combined approach to assign hCG epitopes to diagnostically relevant antibodies was developed and applied by

i. determining Ab specificity profiles by quantitative intra-molecular, intra-species and inter-species specificity characterization by separately testing mAbs in question with hCG, hCG related variants (non-assembled hCG subunits, hCGβcf, deglycosylated hCG, asialo-hCG, avhCG, etc.), hLH, hLHβ, LH and CG of other species and free subunits thereof or synthetic hCG peptides (soluble and solid-phase), and consequently grouping of mAbs according to the main specificities (α-, β- and c-mAbs) and preliminarily to epitope recognition (for reviews see [41-43].
ii. testing for mutual exclusion or binding of hCG and/or hCG related molecules in a chessboard-like fashion by pairs of mAbs in sandwich type assays for discerning epitopes and the elucidation of the spatial relationship of epitopes [44, 45],
iii. defining antigenic domains by schematic grouping of mAbs according to their specific reaction profiles [44-46] and finally
iv. pin pointing molecular localization of epitopes recognized by representative mAbs from each group by mutational analysis of hCGβ [47, 48] based on the three-dimensional X-ray structure of hCG [11]. Single selected amino acids were mutated by PCR and tested for effects on mAb binding whereby special care was taken not to disturb the overall molecular configuration of hCGβ as evaluated by molecular modelling using the programs O [49] and Sculpt [50] with the crystallographic coordinates of hCG [11].

On the basis of data generated with this methodology epitope maps were designed for hCG, hCGβ, hCGβcf and hCGα (Figure 2) and characteristic reference Abs for each epitope defined [7, 42, 43]. In two “ISOBM Tissue Differentiation (TD)-7 Workshops (TD-7 WSs) on hCG and Related Molecules” 96 Abs of 17 manufacturers and laboratories were epitope typed by cross-comparison to the reference Abs with previously determined molecular epitope recognition and their specificity profiles determined [7, 41].

6. Epitopes and antigenic domains

Antigenic domains and epitopes contained therein are dependent on two features:

i. appropriate inherent molecular structures of the antigen that are characterized by molecular accessibility and protrusion indices [51], and

ii. complementary Abs recognizing these structures [41, 52]. Thus, epitopes are molecular structures that acquire identity by complementary Abs [41, 53]. An epitope is ultimately defined by the Ab's complementarity determining regions (CDRs), the antigen binding site, and its contact points within the inherent molecular structure of an antigenic domain providing a functional epitope [54].

Epitopes within antigenic domains are determined by the complementary Abs' paratopes binding to slightly different sets of aa residues but covering more or less the same molecular region by its Fabs. Classification of Abs according to their recognition of certain antigenic domains is possible but definitions of epitopes are just approximations as most monoclonal (m) Abs, due to combinatorial V(D)J gene segment variability of Abs' heavy and light chains as well as Ab maturation, will show differences in their CDRs' aa sequences, quaternary structure and consequently in antigen binding as well as epitope recognition.

6.1 Antigenic domains of hCG

There seems to be a finite number of antigenic domains on hCG and its subunits that are determined by its above mentioned structural properties. On the other hand the number of epitopes within antigenic domains can be large and ultimately corresponds to the theoretical Ab repertoire that can be generated [41]. The size of the antigen-specific repertoire varies with individual immune responses, haplotypes and species. Thus there will be significant variation in the numbers of epitopes corresponding to particular amino acid combinations within given antigenic domains recognized by Abs of different species and individuals [41].

Thus even when interacting with and spatially covering very similar antigenic surfaces different Abs will display different binding energy of their Ag binding site with certain aa within the set of the up to 15 amino acids constituting an epitope [55] and in particular with a small number of aa residues providing essential binding energy. As an example, the major antigenic domain on the tips of *βŁ1+3* encompassing aa *hCGβ20-25+64+68-81* [7, 42, 43, 56] corresponds very well to the sizes of discontinuous epitopes and is recognized by large numbers of Abs that show substantial variability in recognition of a few essential contact aa even though they cover more or less the same surface with its cylinder-shaped paratopes (Figure 3.) [41, 48].

Prominent antigenic domains of hCG are defined by structures bulging out from the molecule such as β*ck* residues *Arg10* and *Gln89* (epitope β_1), aa residues *α13-22* on *αȽ1* whereby three hydrophobic aa *Pro16*, *Phe17*, *Phe18* and and presumably nearby *αMet7*1 and *αPhe74* on *αȽ3* play pivotal roles for epitopes α_1, α_2, and α_4, and aa residues *β20-25+64+68-81* on *βȽ1+3*, encompass epitopes β_2 - β_6 and analogous epitopes [7, 42, 56]. The latter region is located on the two neighboring loops *βȽ1* and *βȽ3*, each between two reverse running β-sheets, with no particular secondary structure, but stabilized by an additional disulfide bridge between *Cys23* and *Cys72*. Such prominent bulges make good epitopes as observed in other proteins [51] and can act as antigenic domains independent of the immunized species [41, 45].

6.2 Spatial requirements of epitopes

hCG not considering its hCGβCTP is shaped like a "banana" (75 Å × 35 Å × 30 Å, diameters) [11, 57]. Its solvent accessible surface area is calculated by effectively rolling a probe sphere over the coordinates and amounts to 11,500 $Å^2$ for hCG without its CTP and its carbohydrates and around 16,000$Å^2$ including the CTP but without carbohydrates. Not all of its surface fulfills the criteria for Ab binding such as bulging structures and steric accessibility as e.g. the beginning of *hCGβȽ1+3* are blocked by bulky self-associated carbohydrate branches N-linked to *Asn13 and 30* which are close to the hCG N-linked *Asn52* antennae [11]. Thus N-linked carbohydrate antennae of hCG are not immunogenic *per se* but might shield some parts of the protein backbone from being recognized by Abs having an assumed footprint of the same size as an epitope of 1,000 $Å^2$ (Fig.3) [7, 36, 58]. Moreover some regions might be immunologically inert as no T-cell help is provided or due to gaps in the Ab specificity repertoire. Thus the surface theoretically suitable for binding of Abs might be in the order of 7,500 $Å^2$. Total binding surfaces of two cylinder-shaped anti-hCGα- and anti-hCGβ-Fabs were shown to be 734 $Å^2$ and 855 $Å^2$ in sizes. The corresponding antigenic solvent accessible surface areas covered by these Fabs were 780 $Å^2$ for an $\alpha_{2/4}$ epitope located in antigenic domain *αȽ1,3*, which is primarily constituted of aa *hCGα14-21+71+74*, and 930 $Å^2$ for a $\beta_{2\text{-}5}$ epitope in antigenic domain *βȽ1+3* (aa *hCGβ20-24* + *Arg68* + aa *hCGβ73-81*) [56].

The major antigenic domains of hCG are located at either end of the hCG molecule at the tops of the neighboring *Ƚ1+3* of either subunit (Fig. 2). As a consequence mAbs that interact with the

protruding loops exhibit canyon-like crevices in their binding sites as shown by co-crystallization of Fab(anti-α) – hCG - Fab(anti-β) ternary complexes [56].

hCG - neglecting its hCGβCTP - may harbor minimally five up to 7 antigenic domains [7, 42]. In fact Abs were shown to bind simultaneously to five epitopes $\beta_1 + \beta_3 + \alpha_2 + \alpha_3 + c_4$ [45] reflecting five spatial independent antigenic domains *ck* + *βŁ1+3* + *αŁ1* + *αŁ3* + c_4. Two additional independent domains (β_8 and β_9) are located on the *βctp*.

6.3 Assembled vs. linear epitopes

The majority of epitopes on hCG are arranged in structurally inherent antigenic domains that encompass continua of overlapping epitopes. It therefore is not possible to draw definite borders between them. Abs to hCG and related molecules preferentially bind to assembled i.e. composite, discontinuous epitopes determined by the tertiary and quaternary structure and rarely bind to linear continuous epitopes of the primary structure [59]. This is in agreement with findings for other antigens where more than 90% of Abs against native antigens recognize discontinuous epitopes [60].

Nevertheless, the boundary between discontinuous epitopes and stretches of aa residues is somewhat blurred since the former may contain short stretches of linear peptide segments of about five aa in length [51]. In hCG this e.g. is the case with overlapping epitopes α_2 (aa *hCGα13-18*) and α_4 (*hCGα17-22*) on *αŁ1*. As these sequences are only recognized with affinities that are orders of magnitude lower than those for the full length proteins hCG or hCGα, they seem to represent only part of the epitope with a limited degree of similarity [56, 61].

Only two epitope regions of hCG, epitope β_9 (aa *hCGβ113-116*) and β_8 (aa *hCGβ135-145*) on the hCGβCTP are unequivocally of linear appearance (for reviews see [7, 42, 43]). All other hCGβ and hCGβcf epitopes require conformational correct folding of the protein backbone as demonstrated with reduced and alcylated subunits and natural and synthetic peptides [59, 62] and with one exception (epitope β_{14} represented by a single mAb) map to the immunodominant region hCGβcf [7].

6.4 hCGβCTP epitopes

hCGβ-related epitopes not determined by hCGβcf, with one exception, are located on the hCGβCTP (aa *hCGβ113-145*) [59] (Fig. 2B). hCGβCTP is of low antigenicity as even in hyperimmunized animals few Abs of generally low affinity are elicited as compared to the remaining

part of the hCG molecule [42, 63]. The three-dimensional structure of hCGβCTP has not been resolved yet. hCGβCTP seems to be rather flexible “tail” with no defined secondary structures and is not constrained by any disulfide bridges [11]. As it has been incorporated into the coding sequence rather by chance it has no obvious function in receptor binding but hCG as compared to hLH has acquired prolonged metabolic half-life by its *Ser*-linked core-1 and 2 carbohydrate antennae [16, 17]. Nevertheless, hCGβCTP-mAbs by definition show high selectivity for hCG versus hLH and are used in various commercial sandwich type immunoassays where they show strong synergy in affinity with other β- and α-mAbs [64].

As mentioned above hCGβCTP located epitopes β_8 (*hCGβCTP135-145*) and β_9 (*hCGβCTP113-116*) are determined by the primary structure i.e. are composed of linear aa sequences [7, 42, 59]. Polyclonal antisera against the hCG or hCGβCTP recognize preferentially domain β_8 and to a much lesser extent β_9 [15]. The immunodominant region of hCGβCTP is located at its very carboxyl-terminal end encompassing aa residues *hCGβCTP135-145* (antigenic domain $\beta_{8.1\text{-}3}$).

The aa residues providing essential binding energy for this domain were determined in great detail. The full $\beta_{8.1}$ epitope consists of seven aa (*hCGβCTP139-145*: *Asp139-Thr140-Pro141-Ile142-Leu-143-Pro144-Gln145*) with *Asp139, Pro144* and *Gln145* essentially involved in binding and representing the functional epitope [54], whereby *Asp139* seems not to be directly recognized by Abs but rather contributes to correct spatial presentation of the target aa residues *hCGβCTP143-145*. Abs against this epitope bind with identical affinity to hCG, hCGβ, *hCGβCTP109-145*, *hCGβCTP135-145* and *hCGβ139-145*. Thus, this is a rare case where the native protein elicits Abs that recognizes a continuous peptide which represents the complete epitope (Figure 4).

6.5 hCGβ loops 1+3 epitopes

Antibodies against hCG recognize a limited number of dominant antigenic domains. Far more than hundred mAbs against the major antigenic domain *βŁ1+3* were identified by the two “ISOBM Tissue Differentiation (TD)-7 Workshops (TD-7 WSs) on hCG and Related Molecules” and by other studies. Within that domain Abs recognize epitopes with different affinities and slightly different specificity profiles [7, 41, 42, 44, 45, 56, 65, 66] (Fig. 2B and C). Variability in aa recognition of epitopes β_2 - β_5 in this particular antigenic domain causes differences in Abs’ specificity, e.g. hLH

cross-reactivity, and generally in affinity of Abs [41]. Abs against epitopes β_1, β_2/β_4 and β_3/β_5, respectively, display <0.1%, ≤1% and >10% cross-reactivity with hLH and hLHβ[7].

Contact amino acids were identified by mutational analyses of hCGβ, whereby special care was taken not to disturb the overall spatial conformation of the molecule: *Pro24, Val25, Arg68, Gly71* and *Gly75* were identified to be involved in epitope β_3, residues *Lys20, Glu21, Gln22, Gly75* and *Asn77* in free hCGβ subunit epitope β_6, and *Arg68* in epitopes β_2, β_4 and β_5 thereby forming functional epitopes [54]. By different techniques other laboratories confirmed *βŁ1+3* as major antigenic domain with essential contact amino acids *Lys20, Glu21, Gln22, Pro24* plus *Arg68* and *hCGβ73-81* [56, 65].

By definition, identical epitopes recognized by Abs within an antigenic domain only exist in the rare cases where genetically identical Abs are used. For reasons of simplicity, Abs with highly related but not identical features that recognize and cover very similar molecular surfaces, are defined to recognize the same epitope [7, 41].

6.6 hCGβ cystine knot epitopes

The second important antigenic domain of hCGβ is far less immunogenic, but diagnostically highly useful as it is distinct and not shared by hLH [7]. It is the only hCG/hCGβ-specific domain that is not located on the unique hCGβCTP, but centered around the ck and comprises epitopes β_1 and β_7 with *Arg10, Arg60, Asp61 and possibly Gln89* as contact aa residues [47, 48].

6.7 hCGα and αβ epitopes

As in assembled and free hCGβ, and hCGβ-derived variants where most Abs recognize domain *βŁ1+3*, the majority of hCGα-mAbs is directed against neighbouring beta-strand loops *αŁ1 and αŁ3* [43]. The antigenic surface of hCGα is composed of four antigenic domains [7, 61]:

i. The immunodominant region on N-terminal loop *αŁ1* encompasses amino acids *hCGα13-22* containing parts of epitopes α_1, α_2 (*hCGα13-18*) and α_4 (*hCGα17-22*) [61]. This is in agreement with the results of other laboratories [56, 65, 67] and with the fact that there is an unusual bend at aa hCGα *Asn15* resulting in *Pro16*, *Phe17* and *Phe18* bulging away from the antiparallel strand [11]. This structure together with nearby *Met71 and Phe74* of loop *αŁ3* provides a prominent immunogenic surface [56],

ii. the region on *αŁ3* around *Tyr65/Arg67* that encompasses a large part of the accessible surface area contains epitopes $\alpha_{3/5}$ [43],

iii. the sequence *hCGα33-42* on the single opposing loop *αŁ2* is important for subunit association [11] and thus specific for non-combined i.e. free hCGα (epitope α_6) [61, 68] and

iv. the very carboxyl-terminal end of hCGα contains epitope α_7 (*hCGα87-92*) [61, 67, 69].

Heterodimer specific epitopes are only present on hCG±hCGn. Abs against such epitopes are sterically incompatible with Abs recognizing hCGα epitopes on loop *αŁ1* (α_1, α_2 and α_4) [44, 45] and sensitive to nicking of between aa hCGβ44/45 and 47/48 in assembled *βŁ2* [29, 70]. The X-ray structure show both regions to be in close proximity as the two subunits are associated in a head-to-toe fashion [11].

6.8 Epitopes and carbohydrates

Some molecular regions are immunologically inert and no Abs have been identified against them, due to lack of T-cell help, shielding by glycans or inaccessibility due to protein folding [41, 53]. All hCG epitopes, except for epitope $\beta_{8.3}$ on hCGβCTP that consists of a core-2 type glycan attached to *Ser132* and surrounding peptide structure [71, 72] (Figs.1 and 2B), are determined by the protein backbone and consequently are present on asialo- and deglycosylated hCG [36, 58]. Moreover neither affinity of mAbs nor numbers of epitopes or spatial relationships of epitopes nor receptor or biological activity were severely compromised even in highly acidic and heavily glycosylated pregnancy and tumor isoforms [12, 36].

The amino acids *Asn13* and *Asn30* of hCGβ carrying N-linked carbohydrate branches spatially are in proximity, are mutually associated and form a prominent bulky structure that is hydrophilic, thus surface exposed and therefore bulges away from the stem of β-sheet *βŁ1*. As no Abs bind to that molecular region these bi- or tri-antennary N-linked carbohydrates obviously render this region immunologically inert [7, 47, 48].

7. hCG methods

Harmonization of antigenic domains and epitopes based on epitope maps and Ab specificity profiles is essential for reliable hCG measurements. The important features for reliable pregnancy

detection in urine seem to be adequate clinical and analytical sensitivity, specificity versus hLH/hLHβ (<1%) and, most importantly, it has to be assured that non-measured hLH and hCG variants such as hCGβcf do not interfere with signal. Detection has to reliably work irrespective of the high dynamics of absolute levels and relative hCG variant proportions, be it at the first few days of missed menses with an over-the-counter test or from few days to weeks of pregnancy with point-of-care tests.

Pregnancy urine may contain as much as 2.7 $\times 10^6$ pmol hCGβcf/L and 1 × 105 pmol hCG/L, whereby approximately three weeks from ovulation to term hCGβcf levels exceed those of hCG by up to ten fold [2, 75]; Upper reference limits for hCG, hCGβ and hCGβcf are ≥12 pmol/L in non-pregnant women [2]. In pregnancy serum hCGβcf is virtually absent and hCGβ levels are lower than 1% of hCG levels; This percentage increases in choriocarcinoma.

Testicular cancer patients' serum levels of hCG may even rise up to 1.4 × 107 pmol/L as compared to a normal range of <0.5–5 pmol/L in sera of normal men [81]. Non-trophoblastic cancers may predominantly produce hCGβ and 20–50% of these hCGβ only [2, 23, 82].

7.1 hCG detection by three categories of methods

Qualitative hCG detection in serum and particular in urine is achieved by three categories of methods that reliably and robustly measure either hCG±hCGn only, preferably via mAbs directed against epitopes in domains *αŁ1 - βŁ1+3* or $c_{2/3}$ - *βŁ1+3* (category I assays), or hCG+hCGβ+variants excluding hCGβcf by using epitopes β_8 and β_2 in antigenic domains *βctp* and *βŁ1+3*, respectively, (category ii assays with restricted pan-hCG/β recognition) or including hCGβcf by using epitopes β_1 – β_2 or β_4 in antigenic domains *βck* - *βŁ1*+3, (category iii assays) providing broad pan-hCG/β and variants measurement [4, 73] (Fig. 5).

Numerous other epitope combinations are theoretically feasible for hCG±hCGn detection in category (I) methods, each of which has its own advantages and drawbacks; thus ultimately no method can be advocated to be superior. Diagnostic first line category (iii) assays are constructed with pairs of mAbs directed against a *βck* related epitope (β_1) combined with *βŁ1+3* mAbs against epitopes β_2 or β_4 as used in the hCG+hCGβ+hCGβcf wide spectrum assay [41].

For the qualitative detection of pregnancy in urine it generally makes no difference which of the three principle assay designs are used addressing either hCG-only or a restricted or broad range of pan hCG/β variants [73, 74]. All of these hCG-related surrogate markers rise in parallel at the

beginning of pregnancy [75] and regardless of the assay scheme used (category i-iii) pregnancies seem to be reliably detected. However in some extreme situations, presumably at the end of the 1st trimester of pregnancy when hCG and variant levels are highest, some difficulties may arise for hCG-only (category i) and restricted pan hCG/β concepts (category ii) [39, 40, 76]. To avoid such reliability issues category (iii) *βck – βȽ1+3* assay designs are preferable [4].

When hCG is quantified in urine antigenic determinants and specificity profiles of Abs have to be harmonized as large differences in results arise when other assay concepts than pan hCG/β/βcf via *βck – βȽ1+3* antigenic determinants are used [4].

7.2 Method specificity and high-dose hook effects

Possible impairment of signals in sandwich methods by non-measured variants like hCGβcf thereby causing high-dose hook effects is due to differences in the specificity profiles of the two Abs used, or generally due to an overload of the binding capacity of either Ab. When in a pregnancy test e.g. an anti-alpha Ab (epitope α_4, antigenic domain *αȽ1*) is combined with an anti-hCGβ-mAb (e.g. epitope β_1; antigenic domain *βck*) [39] it will specifically provide the desired signal for hCG plus hCGn but will be susceptible to signal inhibiting interferences with all hGPHs and its free α-subunits, due to the specificity profile of the α-mAb, and with all hCGβ variants, in particular hCGβcf, due to the mAb directed against epitope β_1 (Fig.3).

Even if the expected concentrations of interfering hGPHs were low, hCGβ variants like hCGβcf in urinary samples and hCGn and truncated hCG in standards [7] could still pose problems. Up to twenty-fold excess of hCGβcf may occur in second trimester pregnancy urine when hCG levels peak. hCGn recognition in itself will give rise to calibration problems if the 3rd or 4th IS for hCG were used that contain approximately 10% of it. To overcome hCG variant interference it is necessary to choose hCGβ-Ab pairs of identical variant recognition and thereby avoiding over-specificity of one of the two Abs and consequently potential interference in signal of important analytes like hCGβcf in urine.

7.3 hCG variant selective methods

For qualitative and quantitative “hCG” pregnancy tests no selective detection of hCG variants is necessary. Separate hCG and hCGβ measurements can be helpful to discern normal pregnancy from choriocarcinoma, for the diagnosis of testicular cancer and non-trophoblastic cancers when the either

the ratio of hCGβ to hCG or hCGβ itself is selectively increased. Specific assays for hCGβ in serum are also used for Down's syndrome screening in the first trimester of pregnancy. Such assays again can be constructed with a mAb directed against a *βck* related epitope (β_7) combined with the same anti-*βŁ1+3* mAbs against epitopes β_2 or β_4 as in the hCG+hCGβ+variant wide spectrum assay (Table 2.) [41]. All assays for selective measurement of hCG, hCGβ, hCGβcf, hCG- and hCGβ-derived molecules involve a mAb directed against *βŁ1+3* epitope β_2 or β_4 combined with the respective variant-specific mAb.

In addition to the epitopes on holo-hCG, two epitopes are located on free hCGβ and hCGβcf (β_6 and β_7) but not on hCG. One additional epitope (β_{14}) is present on hCGβ only, four epitopes are specific for hCGβcf (β_{10} - β_{13}), thus are not shared with hCGβ, hCG or hLH/hLHβ, and two (α_6 and α_7) are present on free hCGα only. MAbs against such epitopes are highly useful for variant-selective immunoassays designed to measure free hCGβ, hCGβcf or hCGα, respectively, in the presence of holo-hCG, other GPHs and hCG protein backbone variants (Table 2).

8. Conclusion

hCG and its molecular variants carry a limited number of antigenic domains that are structurally determined by bulging surfaces and protrusion indices. These antigenic domains provide the structural bases for overlapping epitopes that only acquire identity by complementary recognition through CDRs in Abs' Fabs. Thus each genetically different Ab defines its own epitope. The elucidation of the molecular relationship of antigenic domains, epitopes and Ab specificity profiles are a crucial first step to harmonize Abs in diagnostic methods and thereby improve patient management.

	MAb and method specificities				
Analytical targets	**Code Epitopes**	**Antigenic domain**	**Molecular localization**	**Specificity mAbs & assay**	**Appropriate clinical use**
hCG+hCGβ	β_1	*βck*	*β10+60+89*	pan hCG/β	Oncology, Down's
+hCGβcf	β_2[a)]	*βŁ1+3*	*β20-25+68-77*	pan hCG/β	Early pregnancy
Category iii	β_1 - β_2[b)]	*βck - βŁ1+3*		pan hCG/β	OTC, POC, lab assay
hCG+hCGβ	$\beta_{8.1}$	*βctp*	*β141-44*	hCGβCTP	Oncology
no hCGβcf!	β_2[b)]	*βŁ1+3*	*β20-25+68-77*	pan hCG/β	Early pregancy
Category ii	β_8 - β_2[b)]	*βctp–βŁ1+3*		restr.panhCG/β	Prenatal Screen
	c_3[c)]	*βŁ2+αŁ1*	*β45-48+α13-22*	hCG + hCGn	Early pregnancy
hCG	β_2[b)]	*βŁ1+3*	*β20-25+68-77*	pan hCG/β	Oncology, Down's
Category i	c_3 - β_2[a)]	*βŁ2+αŁ1-βŁ1+3*		hCG + hCGn	(plus β detection)
	β_7	*βck*	β*61+89*	free hCGβ	Oncology
hCGβ	β_2[b)]	*βŁ1+3*	*β20-25+68-77*	pan hCGβ	Prenatal Screen
	β_7 - β_2[c)]	*βck - βŁ1+3*		hCGβ + βcf	(plus hCG detection)
	β_{11}	hCGβcf	*hCGβcf*	hCGβcf	Pregnancy, Down's
hCGβcf	β_2[b)]	*βŁ1+3*	*β20-25+68-77*	pan hCG/β	Oncology
	β_{11} - β_2[c)]	*βcf - βŁ1+3*		hCGβcf	Clinical utility?
	α_6	*αŁ2*	*α33-42*	free GPHα	Oncology
hCGα	α_5[d)]	*αŁ3*	*αTyr65+Met67*	pan GPHα	(pituitary, testis)
	α_6 - α_5[c)]	*αŁ2 - αŁ3*		free GPHα	Clinical utility?

Table 2

Key points of sandwich assays for hCG and/or hCG variants as recommended by the two ISOBM WS on measurement of hCG Modified with permission. [7, 41]: Over-the-counter, point-of-care and laboratory based hCG tests are constructed according to three epitope and specificity profile concepts: category (i) hCG-only measurements, category (ii) restricted pan hCG/β recognition (no hCGβcf) and category (iii) wide pan hCG/β specificity including hCGβcf. For first line hCG tests a pan hCG/β specificity profile was suggested that is best achieved by the use of pairs of mAbs against antigenic domains β*ck - βŁ1+3* whereby epitopes β_1 – $\beta_{2/4}$ are superior candidates (category iii tests) [4]. The popular category (ii) tests using the hCGβCTP as a target (combination of antigenic domains *βctp - βŁ1+3*; epitopes β_8 – $\beta_{2/4}$) are of restricted pan hCG/β + variant recognition recognition; it does not measure hCGβcf, that at high concentrations might induce a high-dose hook effect. The category (i) hCG-only method does not detect free subunits or hCG βcf but is prone to hook effects caused by both due to non-congruent specificity profiles of the two mAbs [39, 40, 76]. Many more epitope combinations are possible for the measurement of hCG-only [4] but no clear recommendation be given. The method for hCGβ (+hCGβcf + hCGβ-variants) is highly discriminative versus hCG and hCGn. hCGα and hCGβcf methods are highly specific for their target analytes but clinical utility of such assays is not established.

[a)]… alternatively a mAb directed against epitope β_4 (domain *βŁ1+3*) can be used
[b)]… Sandwich method with mAbs directed against given epitopes
[c]… alternatively a mAb directed against epitope c_2 (domain *βŁ2+αŁ1*) can be used
[d)] … alternatively a mAb directed against epitope α_4 (domain *αŁ1*) can be used
GPHα… glycoprotein hormone alpha subunit; hCGβ cystine knot … *βck*; loop … *Ł*

Acknowledgements

The authors thank the IFCC (International Federation of Clinical Chemistry) Working Group for Standardization of hCG, the National Institute for Biological Standards and Control (NIBSC), South Mimms, U.K., and the ISOBM TD-7 (International Society of Oncology and BioMarkers Tissue Differentiation 7) Workshop on hCG and Related Molecules.

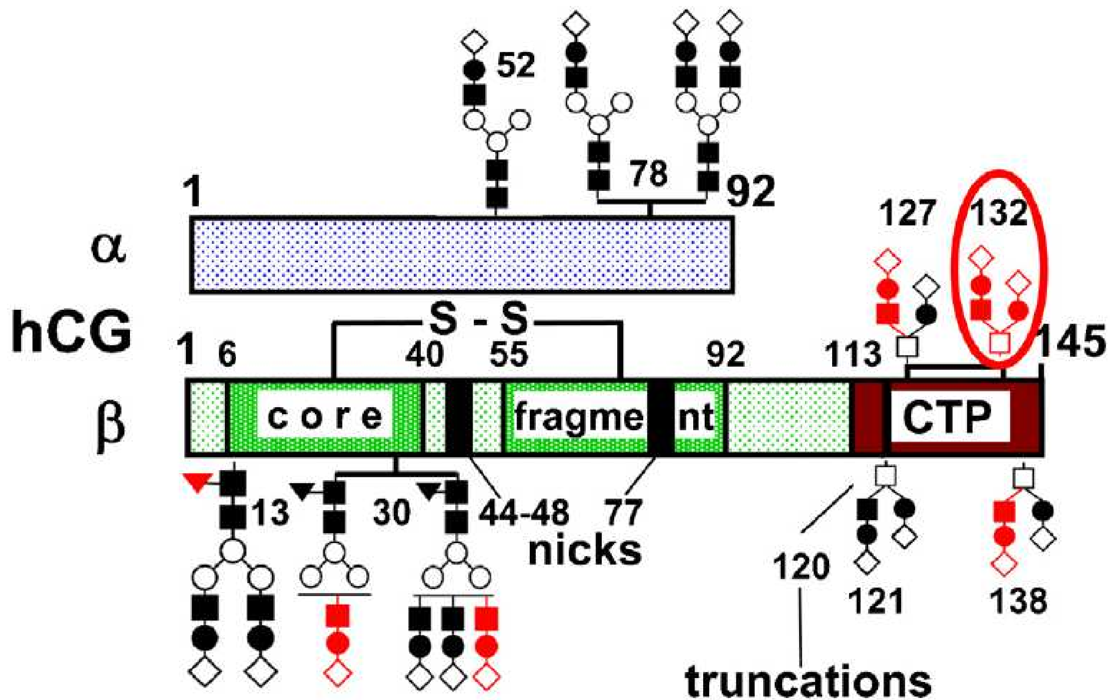

Figure 1

hCG protein backbone variants and isoforms

A clear nomenclature describing protein backbone hCG variants has been put forward [6] and adopted by the "IFCC WG on Standardization of hCG Measurements" [8]: hCG is the bioactive intact αβ-heterodimer (MW 36.3; 5th IS 75/364 and 1st IRR 99/688), nicked hCG (hCGn) is clipped between aa *hCGα44/45 and 47/48*, the hCGα-subunit (92 amino acids, MW 13.3; 1st IRR 99/720) is N-glycosylated at *Asn52* and *Asn78*, hCGβ (aa *hCGα1-145*, MW 23.0; 1st IRR 99/650) contains two N-linked (*Asn13* and *Asn30*) and up to four O-linked (*Ser121,127,132* and *138*) carbohydrate units, nicked hCGβ (hCGβn) is clipped around aa *hCGβ45-48* and hCGβ core fragment (hCGβcf, aa *hCGβ6-40+β55-92*, MW 9.8, isoelectric point 9.8!) retains the cystine knot and both adjacent loops 1+3 but has lost loop 2, the N-terminus and the hCGβCTP. Its N-linked carbohydrate branches are truncated to its Mannose cores [26]. Isoforms of hCG are differently terminally sialylated. Pregnancy hCGα and hCGβ-linked carbohydrates are of the bi-antennary type shown in black, whereas highly glycosylated malignancy associated hCGβ isoforms that show great variability are depicted in red. Large glycans seem to inhibit subunit association between free hCGβ and hCGα subunits. In pregnancy hCGβ *Ser121* always contains a bi-antennary core-2 and *Ser138* a core-1 carbohydrate structure with one or two sialic acids. Malignancy-derived hCGβ contains an increased proportion of O-linked core-2 type glycans [77]. The variant that is solely defined and detected by a single mAb (B152) directed against

hCGβCTP-located epitope $\beta_{8.3}$ is recognized independently of additional glycosylation modifications in the remaining part of hCGβ and itself is subject to variability (encircled in red). Numbers represent positions of amino acid residues in the peptide chains. The disulfide bridges are depicted by S – S.

■ … GlcNAc, … Fuc, ☐ … GalNAc, ○ … Man, ● … Gal, ◇ … NeuAc

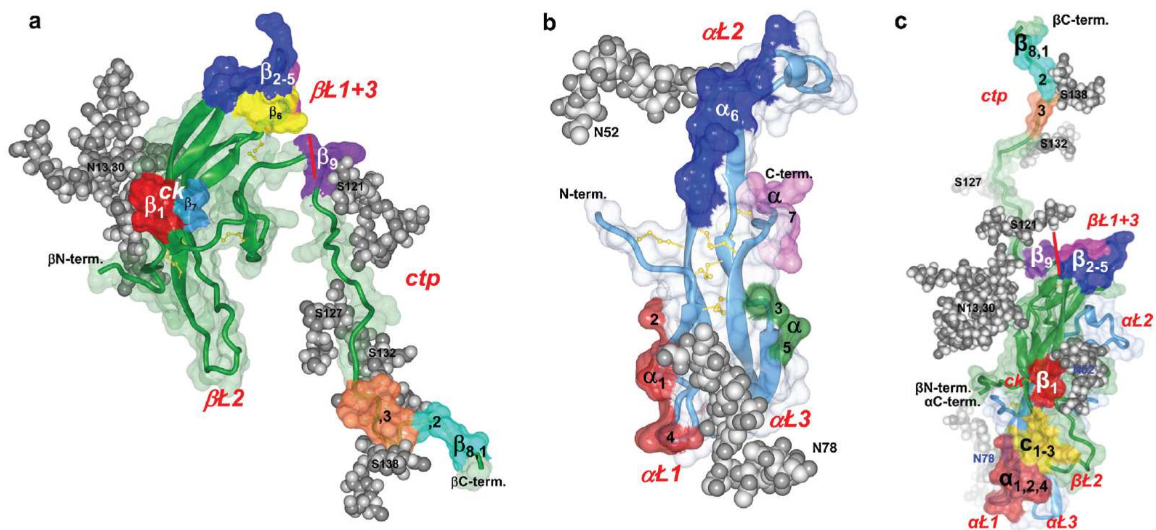

Figure 2

Epitope maps for hCGβ, hCGα and hCG.

Models are based on the X-ray structures of hCG (PDB codes 1HRPand 1QFW). The carboxyl-terminal peptide aa residues (hCGβ 111-145) have been modeled in an extended unstructured conformation with O-linked carbohydrates and the complete N-linked carbohydrates as extensions of the core carbohydrates visible within the crystal structures. The protein backbones are shown in a schematic form with β strands represented as arrows, hCGβ depicted in green and hCGα in blue, the disulphide bridges are shown in ball and stick coloured yellow. The extent of hCGβ-subunit carbohydrates are illustrated in space filling representation coloured in grey scale (black and blue fonts), and the protein solvent accessible surface as a transparent surface. Individual antigenic domains/epitopes are represented in discrete solid surface colours although there is likely to be significant overlap in adjacent epitopes. This structural diagram and others were produced using the CCP4MG program [78].

As previously shown the majority of epitopes is located on heterodimeric hCG as well as the non-assembled hCGα (epitopes α_1 – α_5) and hCGβ subunits (β_1 – β_5, $\beta_{8/1-3}$□□β_9) [59, 61]. Upon subunit dissociation free subunit epitopes become available (β_6, β_7, β_{14}, α_6, α_7) whereas □□□conformationally (c) determined epitopes (c_1 – c_4) defined by the quaternary intact structure disappear [11]. When hCGβ is metabolized to hCGβcf further epitopes emerge (β_{10} – β_{13}) [7, 43] (not shown).

(A) hCGβ antigenic domains *βck, βŁ1+3, βctp* and *βŁ2.*

hCGβ-associated epitopes are arranged in four antigenic domains (i) the *βck* domain (aa *hCGβ)* (ii) the tips of the two nearby loops *βŁ1+3* (*hCGβ20-25+68-75,* epitopes β_1 - β_6 plus *hCGβ64,77-81* [56]).

The major antigenic structures of hCG, hCGβ and of its metabolic variants are defined by the aa sequences corresponding to *hCGβcf* [7], (iii) the *βctp* with linear epitopes at the very carboxyl-terminal (*hCGβ135–145*, epitope β_8), the beginning of the CTP (*hCGβ113–116*, epitope β_9) and two minor antigenic determinants in between (not shown) [59, 79] and (iv) the *βŁ2* where αβ-heterodimer-specific c_1 and c_2 epitopes have a share on (*hCGβ44-48*) [8, 70]. The conclusions concerning molecular localization are just circumstantial as such c-type mAbs do not recognize uncombined hCGβ to a significant extent but are dependent on intact peptide bonds *hCGβ44/45* and *hCGβ47/48* [70].

In the hCGβ molecule the N-linked carbohydrate carrying amino acids *Asn13* and *Asn30* spatially are in proximity, mutually associated and form a prominent bulky structure that bulges from the stem of β-sheet *βŁ1*. As no Abs bind to that molecular region the N-linked carbohydrate branches obviously render this region immunologically inert [7, 47, 48].

The expression of hCGβ epitopes differs between hCG and variants which has impact on the specificity profiles of Abs. Abs against epitopes β_1 - β_5 are pan-hCG/β reagents, that recognize hCG, hCGn, hCGβ, hCGβn, hCGβcf and to various degrees hLH and hLHβ whereby in liquid phase titration and competitive immunoassays LH cross-reactivity of <0.1% is characteristic for mAbs against epitope β_1, <1% for β_2 and β_4 and >>1% for β_3 and β_5. Other epitopes are present on particular variants only: (i) epitopes β_6 and β_7 on hCGβ, hCGβn and hCGβcf but not on αβ-heterodimers, (ii) *βCTP*-epitopes β_8 and β_9 on hCG, hCGn, hCGβ, hCGβn and hCGβCTP but not on hCGβcf, (iii) epitopes β_{10} to β_3 are specific for hCGβcf and neither shared by hCG nor any other hCG-related variant and (v) epitope β_{14} is specific for hCGβ (not shown) [7, 41].

(B) hCGα antigenic domains *αŁ1* and *3, αŁ2* and *αctp*

The antigenic surface of hCGα comprises 2 major and 2 minor antigenic domains [7, 61]. As in assembled and free hCGβ, most hCGα-mAbs are directed against the adjacent beta-strand loops 1 and 3 [43]. Loop 1 is characterized by a protruding molecular structure around aa *hCGα16-18* [11] that is part of the most important antigenic domain comprising amino acids *hCGα13-22* [61] hosting part of epitopes α_1, α_2 (*hCGα13-18*) and α_4 (*hCGα17-22*) [56, 61, 65, 67, 68] that is connected to *hCGα71 & 74* on loop 3 [56]. C-type epitopes on heterodimeric hCG and also on hFSH seem to be associated with these loop 1 sequences [45, 46]. The second immunodominant region of hCGαon loop 3 around *Tyr65/Arg67* determines epitopes α_3 and α_5 [43]. The sequence *hCGα33-41* on the single opposing loop 2 is important for subunit assembly, covered in the heterodimer and thus specific for free hCGα. It

is rarely recognized by mAbs (epitope α_6) [61, 68] as is the very carboxyl-terminal end of hCGα (*hCGα87-92*, epitope α_7) [67, 69].

(C) Epitope map of hCG

In the hCG αβ-heterodimer the free subunit specific epitopes β_6, β_7, α_6 and α_7 as well as hCGβcf-specific epitopes β_{10} to β_{13} are cryptic and not accessible to the respective mAbs whereas c-type epitopes determined by the quaternary structure appear (epitopes c_1 – c_4). Some epitopes flow together to large antigenic superdomains in the αβ-heterodimer, e.g. epitopes $\alpha_1/\alpha_2/\alpha_4$ - $c_2/c_1/c_3$ - β_1 corresponding to molecular regions *αŁ1 – βŁ2 – βck*. This is structurally conceivable as *hCGαPhe17+18+74* are in close hydrophobic contact with *hCGβVal44* and *Leu45* [11], the peptide bond between which has to be intact for c_{1+2} epitopes.

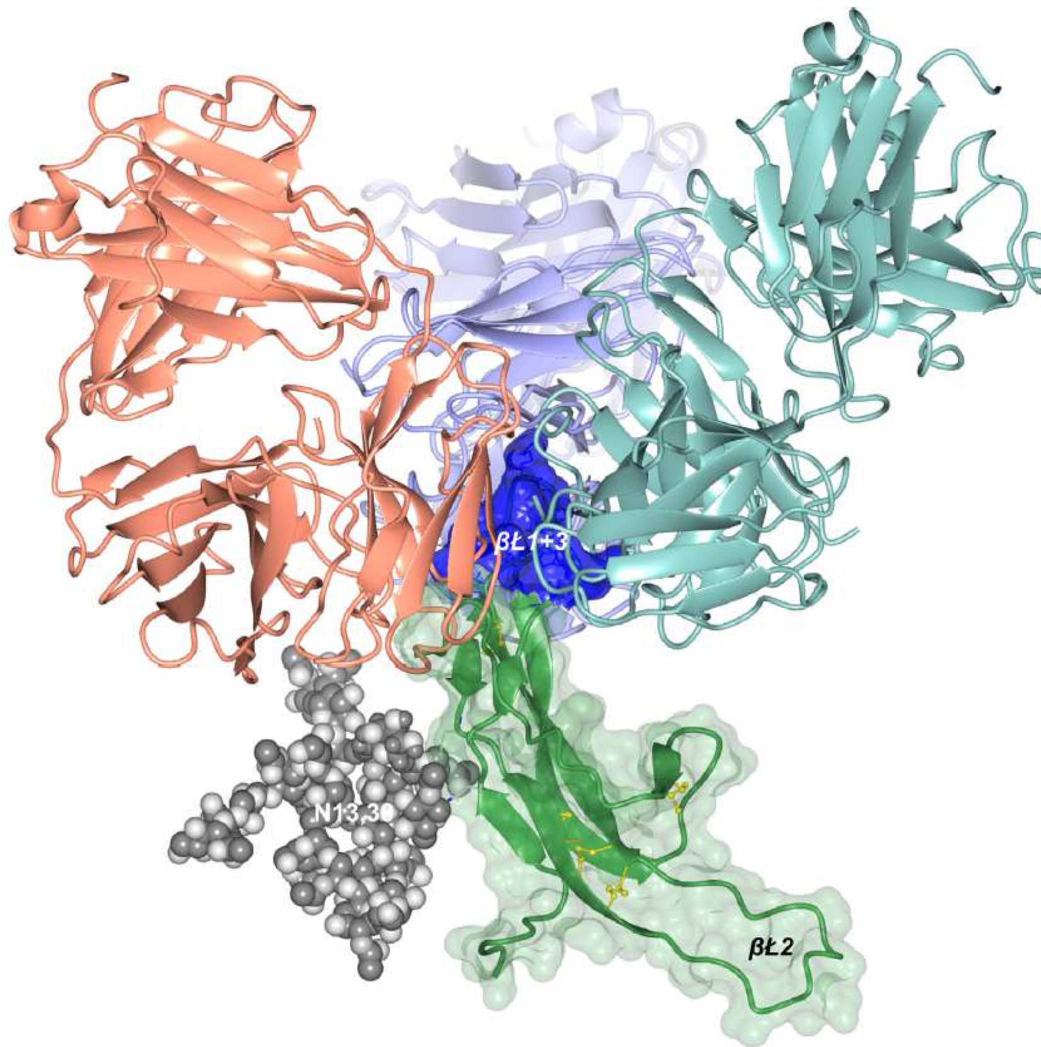

Figure 3

The major antigenic domain of hCGβ

The most immunogenic domain of hCGβ is located at the top of *beta loops 1+3* (*βŁ1+3*) corresponding to aa residues *hCGβ20-25+64+68-81*. It is striking that when molecular complexes of Fab and hCG are modeled true to scale it appears that the Fab's binding site substantially covers the top of *βŁ1+3* like an umbrella. Thus the antigenic domain of *βŁ1+3* encompassing the β_2 to β_6 epitope cluster plus epitopes defined by other laboratories looks small in comparison to the Fabs bound to it [43]. Moreover the two self-associated bulky N-linked hCGβ carbohydrates that cover the peptidic stem of *βŁ1* prevent recognition of the respective amino acid structures by Abs [7, 47, 48]. Thus not much spatial range is available for the recognition of *βŁ1+3* by the Fabs' binding sites and spatial differences in location between epitopes within this antigenic domain are only subtle and are mostly based on variability of preferential recognition of certain aa within that region thereby providing a functional epitope even when the different Abs' Fabs cover more or less the same molecular region.

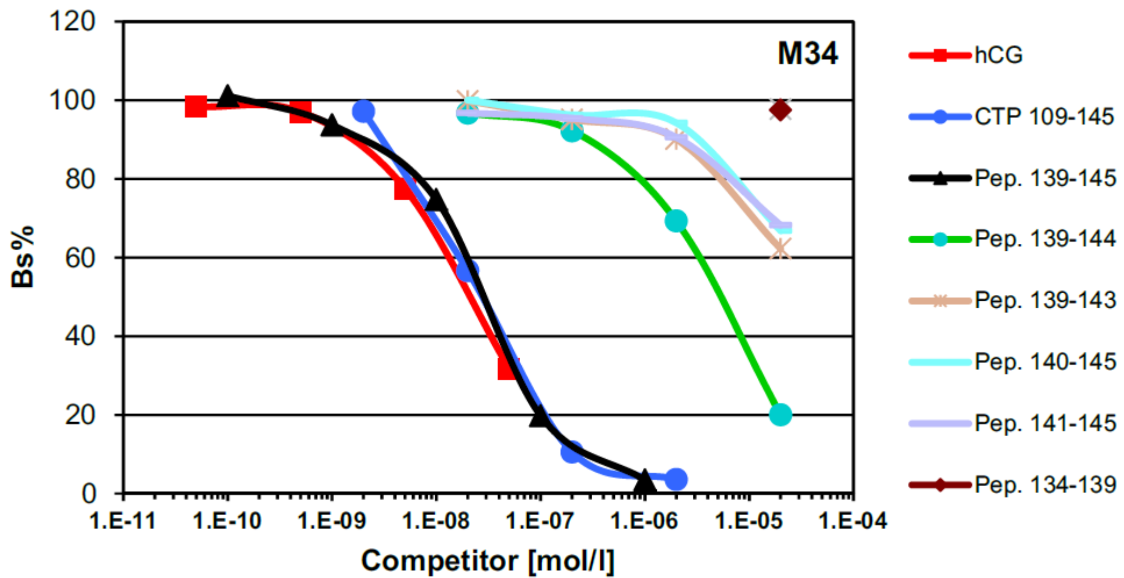

Figure 4

***hCGβ139-145*, the truly linear epitope $\beta_{8.1}$ on natural hCG**

This is the rare case where a truly linear epitope exist that is recognized by a number of monoclonal antibodies (mAbs) generated against the native full-length protein. In the two ISOBM WSs four mAbs [7, 41] plus mAb M34III (kindly provided by Roche, Penzberg, Germany) were directed against epitope $\beta_{8.1}$. hCG, hCGβ, *hCGβCTP109-145* and *hCGβ139-145* (Asp-Thr-Pro-Ile-Leu-Pro-Gln) all are equally well recognized when compared on a molar basis in competitive RIA [7], whereas peptides shortened by only one amino acid on either end (*hCGβ140-145* and *hCGβ139*-144, respectively) each lose affinities by two to three orders, thus indicating pivotal roles for amino acids *Asp139* and *Glu145*. No involvement of carbohydrate antenna *Ser138* in epitope $\beta_{8.1}$ was observed! Five mAbs directed against the facet $\beta_{8.2}$ of this antigenic domain recognize naturally glycosylated hCG 3 to 4 orders of magnitude better than synthetic *hCGβ135-145* [7] indicating an involvement of carbohydrate antennae *Ser132* and/or *Ser138* as seems to be the case for epitope $\beta_{8.3}$ as shown by mAb B152 [25].

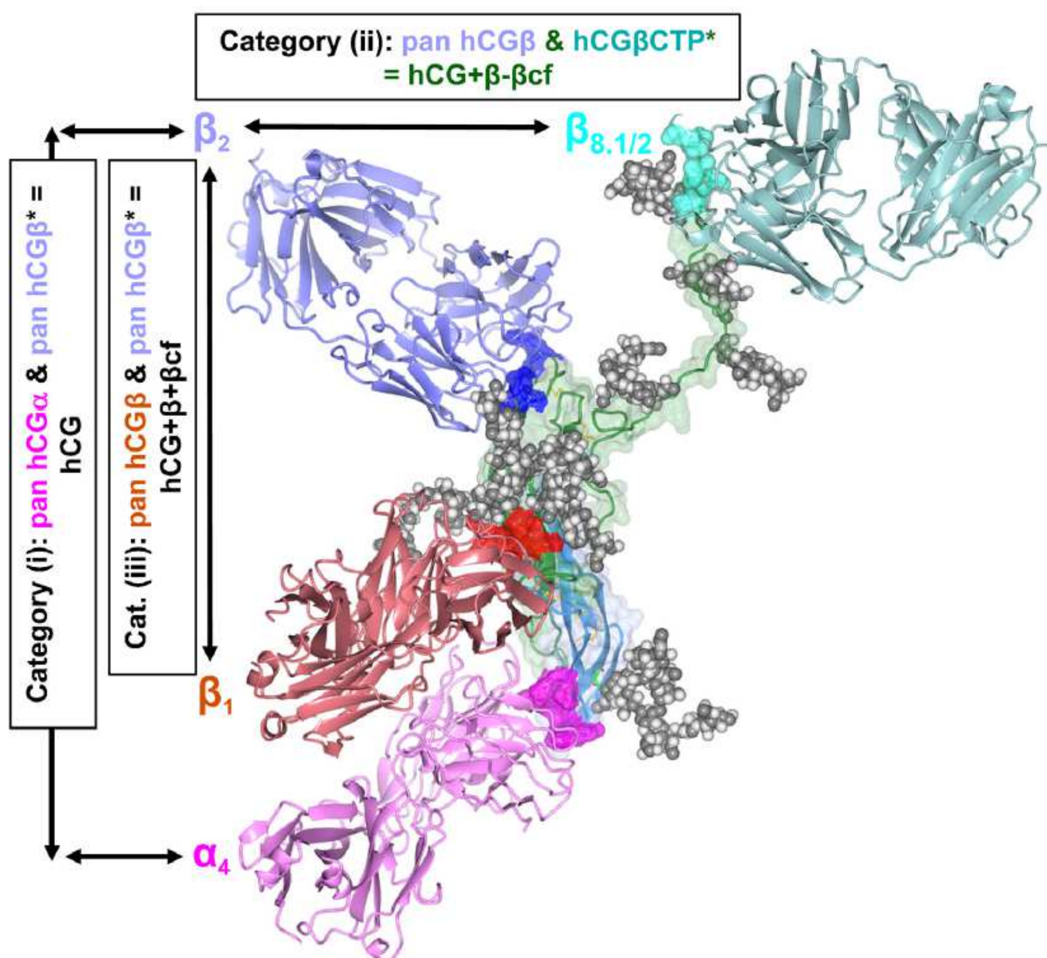

Figure 5

Measurement of hCG and variants

Models of Fab interactions with hCG, hCGβ and hCGα based on the X-ray structures of hCG and the hCG Fv complex (PDB codes 1HRP and 1QFW respectively) and also the CTP specific Fab [80] (PDB code 1SBS). The hCGα and βprotein backbones are shown in a schematic form with β strands represented as arrows (colored green and blue, respectively), the disulphide bridges are shown in ball and stick colored yellow. The antigenic domains involved in assay construction are depicted in red font. The extent of carbohydrates on the α and β subunits are illustrated in space filling representation colored in grey scale, and the protein solvent accessible surface as a transparent surface. Individual antigenic domains/epitopes are represented in discrete solid surface colors. The Fab structures were modeled from the Fv fragments in structure 1QFW [56] are represented in a schematic form with β strands represented as arrows and the individual immunoglobulin folded domains as transparent light yellow ellipsoids. This structural diagram and others were produced using the CCP4MG program [78].

Over-the-counter, point-of-care and laboratory based pregnancy tests are constructed according to three epitope and specificity profile concepts: (a) Category (i) assays for hCG-only measurement: epitope pairing α_4 - β_2, the α_4 epitope highlighted in red, the β_2 epitope in dark blue. (b) Category (ii) assays for restricted panhCG/β measurement (no recognition of hCGβcf and CTP-truncated hCG and hCGβ variants): $\beta_{8.1}$ - β_2, the β_2 epitope highlighted in dark blue, the $\beta_{8.1}$ epitope in cyan. (c) Category (iii) assays for panhCG/β measurement: epitope combinations β_1 - β_2, the β_1 epitope highlighted in red, the β_2 epitope in dark blue. From all combinations shown, exclusively the category (iii) pairing of epitopes β_1 – $\beta_{2/4}$ will fulfill the criteria of a multipurpose method measuring a wide range of hCG and hCGβ and variants including hCGβcf, showing no hLH cross-reactivity and not even a theoretical possibility of interferences in signal such as high-dose hooking of non-measured metabolic variants like hCGβcf.

Many more epitope combinations for hCG and hCG variant measurement are possible but only such combinations have been selected that *per se* will not cross-react with hLH or hLH derivatives. All other epitope combinations due to non-congruence of specificity profiles of the respective Ab pairs might be susceptible to such interferences [39, 40].

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