Clinical Assays for Human Chorionic Gonadotropin: What Should we Measure and How?

Within the last year, human chorionic gonadotropin (hCG) has been a topic of great discussion in both the scientific and popular press. Recent reports have demonstrated that because of hCG’s heterogeneity, assay standardization is an important problem, with different immunoassays giving different results for the same specimens. In addition there have been reports of false-negative results due to high concentrations of certain hCG variants. Finally, the popular press has reported on illicit use of hCG by athletes for performance enhancement and by dieters for weight loss. In all, these publications raise questions about what variants of hCG are currently being measured, what should be measured, and how best to do it. In this Q&A article, 5 leaders in the field have been asked to comment on current clinical assays for hCG and what the future might hold.

There has been a lot in the literature lately about the lack of standardization of hCG immunoassays. The data suggest that in most cases we don’t know what hCG variants our immunoassays are measuring. What are the most immediate changes that you feel need to be made to hCG immunoassays regarding standardization?

Catharine Sturgeon: I think it is really important that laboratories know the specificity of the method used as the specificity needs to be different for pregnancy and cancer applications. It’s important to know the extent to which different hCG variants are recognized in different methods. The highly purified WHO International Reference Reagents (IRR) for 6 hCG-related isoforms, which were produced by the IFCC Working Group for hCG, now enable manufacturers to characterize the extent to which their immunoassays measure those important isoforms. Reaching consensus about how best to assess this, as well as how to present the data so as to allow easy comparison of methods, will represent a major step forward. Studies to assess the influence of calibrator purity on results are also in progress. Thankfully, we can now describe the major variants using the systematic and user-friendly nomenclature developed by the IFCC Working Group. We all need to work hard to encourage its universal adoption.

Since the IRR are calibrated in molar units, we can begin to understand the method-related differences in hCG results that often cause interpretative confusion if patients are monitored in different hospitals. Achieving equimolar recognition of the different isoforms will be a major target when designing the next generation of hCG immunoassays. Preparing IRR for some of the other variants, including hyperglycosylated hCG (hCGh), would also be desirable.

Thanks to round-robin antibody workshops carried out under the auspices of the International Society for Oncology and Biomarkers, we already know which broad antibody combinations are likely to be most appropriate for different clinical applications. Pulling all these strands together should lead to much better comparability of hCG immunoassay results as assessed by proficiency-testing schemes. The impact of improved comparability on clinical practice will also require careful audit.

Ulf-Håkan Stenman: I agree that the most important change would be to start using the IRR for standardization, and to report results for these in moles per liter in addition to International Units per liter according to the 3rd/4th IS and IRR 99/688. Due to the presence of some nicked hCG in the 3rd/4th IS, the relationship...
will to some degree be method dependent, but the differences for measurement of hCG should not exceed 10%. However, measurement of samples containing subunits and fragments may still pose problems. Some assays are designed to measure only intact hCG while others measure both hCG and the free β subunit (hCGβ), and in some cases the core fragment of hCGβ (hCGβcf). It is difficult to design assays that measure all of these equally on a molar basis. Therefore, between-method differences will occur when the samples contain these hCG variants. Furthermore, the concentrations of hCGβ and hCGβcf can be expressed correctly only in moles per liter.

Do you feel that, along with hCG results, laboratories should report which hCG variants their assay measures?

Ulfr-Håkan Stenman: Yes, this should be mandatory and it is readily possible by use of the IRRs. To facilitate comparison of the recognition of different variants, the results should be expressed in substance concentrations (mol/L).

Catharine Sturgeon: Laboratories certainly need to know which hCG variants their assays measure and should be able to provide this information if asked. I strongly believe that it is the responsibility of laboratories to ensure that the test results they provide are fit for the clinical purpose for which they are being used, and I suspect that most clinicians take it for granted that this is the case. I doubt many clinicians would find detailed information about recognition of hCG variants helpful on the printed reports.

The NACB Guidelines on Quality Requirements for Tumor Markers recommend that the name of the assay method be stated on the laboratory report. I think that when reporting hCG results it would be very helpful if the report indicated—perhaps in brackets after the method or alternatively in an appended comment—whether the method should be restricted to pregnancy applications because it measures only intact hCG, or whether it has broad specificity and is suitable for oncology applications, appreciating that this may be an off-label use. Whether such reporting is feasible will depend very much on the flexibility of the laboratory information system and the layout of the report.

Glenn D. Braunstein5: It is important for the clinician to know what the hCG assay they are using actually measures. During pregnancy, the predominant form of hCG in the circulation is intact hCG with some variation in carbohydrate due to varying degrees of glycosylation. However, during the first several weeks of pregnancy, the predominant form of hCG is hyperglycosylated, which appears to be detected by most assays that measure intact, normally glycosylated hCG. In pregnancy urine, the predominant form is hCGβcf. Therefore, an assay that measures only intact hCG would be excellent for quantifying hCG in the serum, but would miss the major hCG-related molecular variant in the urine. Patients with gestational trophoblastic disease secrete intact hCG, hCGβ, nicked hCG and hCGβ, and in some cases hCG devoid of the carboxyterminal portion of the hCGβ subunit and hCGβ. Individuals harboring nongestational trophoblastic neoplasms such as germ cell tumors of the testes and ovaries frequently secrete predominately hCGβ with lesser amounts of intact hCG, while a third to two-thirds of patients with nontrophoblastic neoplasms secrete only hCGβ and not intact hCG. In these cases, an assay designed to serve as an hCG tumor marker assay should detect all of these variants, ideally in equimolar quantities. Based upon these considerations, it is essential that laboratories carefully calibrate their assays with the new IRR preparations and report the limit of quantification and specificity of their assay in molar units, as stated above. This will help the clinician to interpret both positive and negative assay results in a patient with a known or suspected condition associated with hCG production.

hCG has been in the popular press lately because of illicit use by athletes, and as adjunct to fad diets. Have you seen this type of hCG use and do you feel that the hCG assays we have available today have the limits of quantification and specificity to detect illicit hCG use?

Anthony W. Butch6: Our Olympic drug-testing laboratory at UCLA has observed isolated cases in ath-

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6 Anthony W. Butch, Professor, Department of Pathology and Laboratory Medicine, UCLA Medical Center, and Director of the Olympic Analytical Laboratory at UCLA, Los Angeles, CA.
letes of increased hCG concentrations measured in urine by immunoassay; such results are consistent with the use of hCG. Current hCG immunoassays have adequate limits of quantification for doping purposes and many can measure urine hCG concentrations as low as 1 IU/L. This is below the minimum required performance concentration of 5 IU/L required by the World Anti-Doping Agency. The major problem with hCG immunoassays is variable recognition of the different forms of hCG. For example, many of the assays fail to detect hCG/β, the predominant form in urine. This results in urinary hCG concentrations that can be widely divergent depending on assay-related differences. This issue is especially problematic in athlete testing programs since a second immunoassay recognizing a different antigenic epitope on the hCG molecule is required for confirmation of an initial positive test.

David A. Cowan: hCG has been banned in human sport since the 1980s after it was discovered that weightlifters and road-race cyclists were misusing this hormone. Although mass spectrometry was used by sports drug-testing laboratories, it was recognized that immunoassays were needed for hCG. Fortunately, relatively specific assays were available that could distinguish luteinizing hormone from hCG to a sufficient certainty for use in sport. Obviously, the sole finding of an increased hCG concentration in a single untimed urine specimen cannot distinguish a pregnant female from an hCG-secreting tumor or from an hCG user. Sport has a strict liability principle in which the mere presence is prima facie evidence that an offense has been committed. To avoid conviction, the athlete must provide convincing evidence that he or she has a physiological or pathological abnormality (or is pregnant, but since the test is performed only on males, this is a defense that is unlikely to be successful).

The testing kits used by sports drug-testing laboratories are usually the types that detect down to 1 IU/L of hCG, sometimes as total but more often as intact hCG. The threshold used by sport is usually 5 IU/L, which most would consider sufficient. We use a confirmation process that includes ultrafiltration of the sample and a second hCG assay that recognizes different epitopes before confirming an initial finding.

Fortunately, hCG does not rank highly amongst the substances found in samples from top level sports competitors who are most drug tested. For example, in 2007, 15 samples were reported to contain hCG out of 223 898 samples collected worldwide, i.e., about 67 in a million.

*What do you think the future holds for hCG methods? Do you think that immunoassay methods will give way to mass spectrometry–based methods?*

**Anthony W. Butch:** Although immunoassays are exclusively used to measure urinary hCG for doping control purposes, liquid chromatography–tandem mass spectrometry (LC-MS/MS)-based methods are preferable because they have better discriminating power. Previously developed LC-MS/MS based methods lacked adequate detection capabilities, but recent improvements in mass spectrometers will allow methods to be developed with lower limits of quantification. Because immunoassays for hCG are fast and do not require sample cleanup, it is likely that they will continue to be used to screen for hCG misuse. Screen-positive urine samples can then be confirmed by improved LC-MS/MS methods as they become available.

**Ulf-Håkan Stenman:** Most hCG assays are very reliable for detection and monitoring of pregnancy, but there is a need for better methods for diagnosis and monitoring of cancer. These needs can be met with immunoassays, and presently I am not aware of any clinical condition for which mass spectrometry would provide important new information if specific assays for hCG and hCG/β are available. However, mass spectrometry is a very useful method for characterization of hCG variants and this may eventually lead to new clinical applications. I think that immunoassays will still be used during the next decade.

**Catharine Sturgeon:** The IFCC Working Group for hCG is investigating the possibility of developing a reference method for hCG that will almost certainly require use of mass spectrometry. The Working Group recently agreed that the rapidity of technological advances in mass spectrometry may make this feasible within the next 5 years. It will be interesting to hear whether specialists in the field agree.

However, for routine applications it seems unlikely that mass spectrometry will replace immunoas-

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7 David A. Cowan, Professor, Head of the Department of Forensic Science and Drug Monitoring and Director, Drug Control Centre, King’s College London, London, UK.
say measurements in the near future. The heterogeneity of the glycan variations of hCG, its subunits, and fragments would make use of mass spectrometry challenging, and in addition, preparification steps using antibodies would probably be necessary.

David A. Cowan: I agree. Immunoassays benefit from operating with a batch size of dozens through to hundreds, whereas mass spectrometry is essentially a sequential process and is unlikely in the foreseeable future to be as rapid as immunoassay.

What are some of the limitations to measuring hCG by mass spectrometry?
David A. Cowan: Mass spectrometry, unlike immunoassays, has the benefit of providing a vast amount of information content. Thus mass spectrometry has the potential discrimination power that can be tailored to provide specificity. It can also be used to determine the multiplicity of components in a complex matrix. Mass spectrometry provides very low detection limits, but often immunoassay has the edge. Since analysis is performed on just a few milliliters of urine, the limit of quantification is an issue; 5 mIU of hCG equates to about 500 pg or about 15 fmol. Furthermore, glycoproteins like hCG often exist as multiple isoforms and a decision needs to be made as to which are important or detection could be further compromised.

Ulf-Håkan Stenman: There are several problems, e.g., the highly heterogeneous carbohydrate structure and the low concentrations that need to be measured in cancer patients. The problem with heterogeneity can be solved by producing homogeneous fragments of hCG by tryptic digestion. However, at the same time, information about variants is lost because many fragments from various forms are identical. The detection limit can be improved by using an antibody to capture hCG before analysis by mass spectrometry, but at the moment immunoassays facilitate detection of much lower concentrations.

Anthony W. Butch: The major limitations are limit of quantification and extensive sample preparation. Sample preparation involves immunoextraction of hCG isoforms from urine, denaturation to separate the subunits, reduction of disulfide bonds, derivatization and tryptic digestion, followed by separation and analysis by LC-MS/MS. Major advantages include separate measurement of hCGβ core fragment and free hCGβ chain concentrations based on tryptic peptide analysis and improved specificity provided by structural information.

Qualitative urine hCG immunoassays also suffer from lack of standardization. In addition, they are subject to false-negative results when certain hCG variants are present in excess. Given their limitations, do you think that qualitative assays are still clinically useful?

Ulf-Håkan Stenman: Most of the qualitative urine hCG immunoassays presently available are reliable when used for detection of early pregnancy, but some of these assays may give false-negative results when used later in pregnancy, when the urine concentrations of hCGβcf are very high. This is a problem if these tests are used to exclude pregnancy before initiation of cancer therapy. Serum tests are not affected by this problem.

Catharine Sturgeon: Data from the National External Quality Assessment Service for Pregnancy Testing, which regularly assesses performance of qualitative tests at hCG concentrations close to the claimed detection limits, suggest they are generally reliable and meet manufacturers’ claims. However, I certainly agree that clear information should be provided about which variants are recognized by qualitative tests.

If exclusion of pregnancy is essential, for example before having an abdominal x-ray or starting chemotherapy, the consequences of a false-negative result may be severe. If the date of the last menstrual period is known, qualitative testing should be fine as a confirmatory check, but if there’s any doubt, a quantitative test would be desirable. Qualitative results that do not seem in accord with the clinical picture should always be rechecked and also confirmed in a quantitative hCG immunoassay.

Which hCG variants do you think quantitative immunoassays should be able to detect? Do you think that assays should be designed and marketed for specific uses such as to detect pregnancy vs as a tumor marker vs as a marker of gestational trophoblastic disease?

Glenn D. Braunstein: In this field there are “lumpers” and “splitters.” Lumpers want a single test that measures all of the hCG-related molecular variants, ideally in equimolar quantities. This would be the only test they would use to diagnose and monitor pregnancy, gestational and nongestational trophoblastic disease, and hCG (or variant)-producing nontrophoblastic tumors. As the tumor grows, the markers rise, as it shrinks, the markers fall. The splitters want to have separate assays that are highly specific for the different variants because the type of variant or the ratios of some the variants to each other may provide some additional diagnostic information, such as the differentiation of benign or malignant trophoblastic disease. I fall into the lumper camp because performing a single assay is less expensive than performing multiple assays. I acknowledge that multiple assays can occasionally provide important additional in-
formation, but I would need to see a prospective study comparing the diagnostic outcome of the 2 approaches that shows the superiority of multiple assays vs an all-inclusive single assay, and the cost-effectiveness of this approach before I would advocate multiple separate assays.

**Ulf-Håkan Stenman:** This is a complicated question. According to Braunstein’s terminology I would be a splitter. We prefer determining hCG and hCGβ separately, but if an hCGβ assay with low limit of quantification and high specificity is not available, an assay measuring hCG and hCGβ together has advantages for monitoring of patients with testicular and nontrophoblastic cancers that express hCGβ. However, the diagnostic sensitivity of a specific assay for hCGβ is superior to that of assays measuring hCG and hCGβ together. This is because pituitary secretion causes a background concentration of hCG that limits the clinical utility of assays measuring both forms together. Assays measuring hCGβ only have a theoretical advantage for measurement of hCG immunoreactivity in urine, but I am not aware of any clinical condition for which this is of clinical value.

**Catharine Sturgeon:** For most clinical applications in pregnancy, measurement of intact hCG alone is adequate as it is present in much higher concentrations than any of the other variants. However, when using hCG as a tumor marker it is essential that the free β-subunit is also measured as some tumors may produce only the free subunit. In gestational trophoblastic disease tumors are highly heterogeneous and other variants may be produced.

The patterns of variants most likely to be encountered in practice are not yet well defined clinically or biochemically, so it is prudent to use immunoassays with broad specificity that will recognize most hCG variants. This can be achieved by using an hCG monoclonal antibody with β₁ specificity as the capture antibody with a β₂ or β₃ monoclonal antibody for the signal.

Whether it would be helpful to design and market immunoassays for specific uses seems debatable. Availability of assays specific for individual variants is critical if we are to improve our understanding of which isoforms are produced by different tumors, knowledge which is likely to improve our understanding of tumor development. Specific assays can also be standardized much more readily. It would certainly be attractive to be able to report the molar concentrations of individual hCG variants, which is only likely to be feasible in routine clinical use if microarray assays are widely adopted. Such an approach could be advantageous in the management of cancer patients but would probably not add much to management of most pregnancies. In the immediate future I think that in the routine laboratory we are likely to continue to use broad specificity assays (i.e., “lumpers” according to Braunstein’s terminology), ultimately designed to recognize variants on an equimolar basis, for both pregnancy and oncology applications, but the specific assays will be important for increasing our understanding of disease processes.

Do you have any additional comments?

**Ulf-Håkan Stenman:** It would be valuable if assay manufacturers could provide information on epitope specificity of the antibodies used. Also, it has been claimed that hCGh is poorly recognized by some hCG assays, but a standard for hCGh is not yet available. Establishment of a standard for hCGh would be useful, but is demanding because its structure is not well defined and there is considerable variation in the structure of hCGh from different sources, e.g., patients with trophoblastic tumors and choriocarcinoma cell lines.

**Catharine Sturgeon:** It is also critical to remember other aspects of laboratory provision when providing hCG results! It is important to ensure correct specimen identity and be aware of potential assay interferences, including antireagent antibodies and the potential for high-dose hooking. Failures to recognize these have led to serious clinical incidents. hCG results that are not in accord with the clinical picture should always be called to the attention of the laboratory and actively investigated.

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