CHAPTER 34

Evaluation of Hormonal Status

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Introduction

This chapter reviews the assessment of hormonal status in the practice of reproductive endocrinology. It is acknowledged that the patient will often provide useful biologic information, such as changes induced by hypoestrogenism, skin changes with androgen excess, and galactorrhea in hyperprolactinemia. The clinically relevant symptoms and signs associated with various disorders are discussed in other chapters in this text. Here we will describe various types of hormonal assays and dynamic tests used in the evaluation of reproductive disorders, as well as diagnostic radiographic techniques. We also offer several algorithms for the diagnosis of common clinical disorders with the use of hormonal assessments where necessary.

Principles of Hormone Assays

- *An understanding of the principles of all immunoassays is necessary to understand their usefulness and limitations.*
- *There are several types of immunoassays as well as detection systems, some of which have been developed for rapidity and automation.*
- *The gold standard for the measurement of steroid hormones, which may be at very low circulating levels, is with the use of mass spectrometry (MS).*

Immunoassays

The predominant assays used to measure steroid, peptide, and protein hormones in serum, plasma, and urine samples for over 45 years have been immunoassays. These assays have been widely used in both clinical diagnostic and research settings.

As the term *immunoassay* implies, this method of measuring hormones involves an antigen-antibody reaction, where the antigen is the hormone to be measured and the antibody, which is prepared against the hormone, binds to the hormone. The hormone can be a steroid, protein, or peptide. There are two types of hormone immunoassay methods: one uses excess hormone and a limited amount of antibody, whereas the other uses excess antibody. For quantification purposes, immunoassay methods require a labeled marker, which can be the radioactive [usually iodine-125(^{125}I)] or nonradioactive (chemiluminescent, fluorescent, or enzymatic) form of the hormone being measured, or a suitable chemical derivative of the hormone.

General Principles of Immunoassays

Principle of Antigen-Excess Hormone Immunoassays

The principle of an antigen-excess type of hormone immunoassay involves competition between the hormone being measured and the labeled form of the hormone for a limited amount of the antibody prepared against the hormone. When all three components are combined in a test tube, the net result is a mixture of labeled and unlabeled hormone bound to the antibody, and unbound labeled and unlabeled hormone (Fig. 34.1). From the practical standpoint in the assay, the bound hormone is separated from unbound hormone and the activity of the bound fraction is quantified (e.g., by determining the counts per minute of radioactive iodine in a gamma counter if a ^{125}I-labeled hormone is used as the marker). Separation of hormone-bound and unbound antibody is achieved by one of a variety of different methods, including use of a second antibody (prepared against the first antibody) when an iodinated hormone is used as the labeled antigen, or by magnetic particles when a nonradioactive hormone is used. In this manner, by using different concentrations of a pure hormone (standard), one can first generate a standard curve of the hormone. As the concentration of the standard is increased, the antibody-bound labeled marker is displaced; the higher the amount of standard that is added, the lower the amount of marker will be obtained. Thus, in an antigen-excess immunoassay, the standard curve shows an inverse relationship between the different amounts of antibody-bound labeled antigen (hormone) and the different concentrations of the standard (Fig. 34.2). A standard curve is essential in any immunoassay method used to measure the levels of a hormone in a biological fluid.

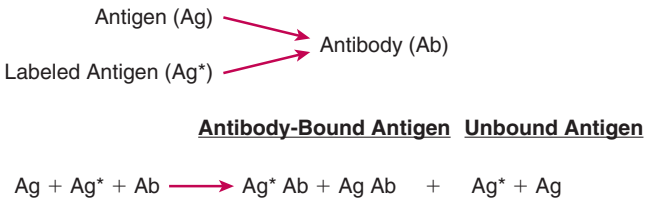
Measurement of a hormone in serum, plasma, or urine by an antigen-excess immunoassay is obtained by first determining the amount of the hormone that is antibody-bound

Abstract

The appropriate measurement of hormones, their interpretation, the judicious use of provocative tests, and targeted imaging are all important aspects of the discipline of reproductive endocrinology. In this chapter only clinically relevant tests have been included. Some algorithms have been provided suggesting a differential diagnosis for the evaluation of some disorders, but only to illustrate how testing plays an important role in making a correct diagnosis. Details of pathophysiology and treatment for specific disorders may be found in other chapters in this text.

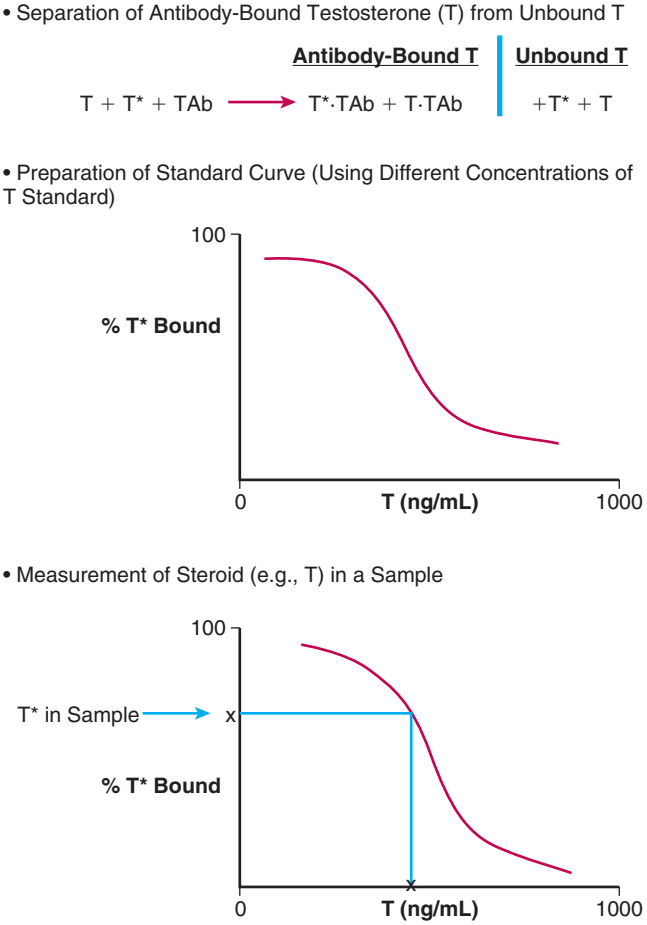
Keywords

Assays
LH
FSH
GnRH
prolactin
estrogens
progesterone
androgens
AMH
inhibins
ovarian reserve
HCG
insulin resistance
adipokines
GH
bone markers
vitamin D
thyroid hormones
CRH
ACTH
adrenal
CT
MRI
ultrasound



*Radioactive or nonradioactive (chemiluminescent, fluorescent, or enzymatic) tag.

FIGURE 34.1 Measurement of hormones by antigen excess radioimmunoassay: theoretical considerations. *(Modified from Stanczyk FZ, Glob. libr. women's med. [ISSN:1756-2228] 2009; DOI 10.343/GLOWM.10278.)*



*Radioactive or nonradioactive (chemiluminescent, fluorescent, or enzymatic) tag.

FIGURE 34.2 Measurement of hormones by antigen excess radioimmunoassay: practical considerations. *(Modified from Stanczyk FZ, Glob. libr. women's med. [ISSN: 1756-2228] 2009; DOI 10.343/GLOWM.10278.)*

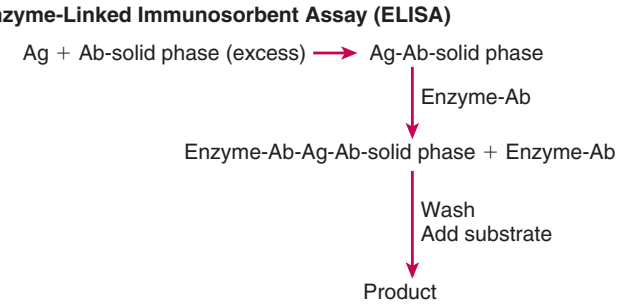


FIGURE 34.3 Principle of the antibody excess immunoassay: theoretical consideration shown for the ELISA. *(Modified from Stanczyk FZ, Glob. libr. women's med. [ISSN:1756-2228] 2009; DOI 10.343/GLOWM.10278.)*

in the sample. The concentration of the hormone is then extrapolated off the standard curve, as shown in Fig. 34.2. An appropriate computer program can be used to generate the data rapidly.

Principle of Antibody-Excess Hormone Immunoassays

In contrast to antigen-excess immunoassays, which use a limited amount of antibody resulting in competition between labeled and unlabeled antigens, there is no competition between these antigens in antibody-excess immunoassays (often referred to as immunometric assays [IMAs]). In general, two different antibodies that recognize two different parts of the antigen are used in antibody excess immunoassays, forming a "sandwich," with the antigen in the middle. Thus this assay method is useful for large molecules such as protein and peptide hormones. Quantitation is achieved by labeling one of the antibodies with a radioactive or nonradioactive marker. One of the most widely used antibody-excess immunoassays is the enzyme-linked immunosorbent assay (ELISA) in which quantitation is achieved by attaching an enzyme to one of the antibodies and measuring the optical density of a product formed by reaction of the enzyme with a specific substrate (Fig. 34.3). When one of the antibodies is labeled with a radioactive tracer, this type of assay is referred to as an immunoradiometric assay (IRMA), and when the labeling involves a chemiluminescent or fluorescent tag, it is referred to as an immunochemiluminometric assay (ICMA) or immunofluorometric assay (IFMA), respectively. In contrast to the inverse relationship between antibody-bound labeled antigen and concentration of antigen in the standard curve in an antigen-excess immunoassay, there is a direct relationship between the labeled antibody and antigen concentration in an antibody-excess immunoassay (Fig. 34.4).

Major Components of Immunoassay Systems

There are three major components of immunoassay methods: the antibody, unlabeled antigen (standard or substance to be measured in a biological fluid sample), and labeled antigen. These are essential in every immunoassay and are discussed below.

Antibody

The antibody is perhaps the most important component of an immunoassay method. If an antibody in an immunoassay

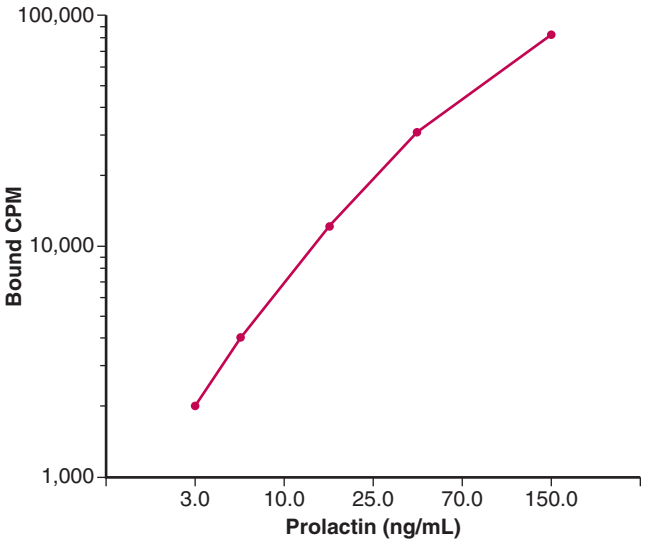


FIGURE 34.4 Typical standard curve obtained by abnormal antibody excess immunoassay. The example shown is for an immunoradiometric assay. *(From Nakamura RM, Stanczyk FZ: Immunoassays. In Lobo RA, Mishell DR Jr, Paulson RJ, Shoupe D, editors:* Mishell's Textbook of Infertility, Contraception and Reproductive Endocrinology*, ed 4, Oxford, 1997, Blackwell.)*

recognizes only the compound that it is intended to measure, then it is likely that the assay will be highly accurate.

Antibodies are commonly produced in animals (polyclonal) or via cell culture (monoclonal). The typical protocol for production of polyclonal antibodies is to disperse a small amount of antigen into an adjuvant (e.g., Freund adjuvant) and to inject it intradermally at multiple sites into an animal such as the rabbit. After approximately 3 months, a blood (serum) sample is obtained from the animal and the antibody titer is determined. This procedure involves addition of a fixed amount of labeled antigen to serially diluted aliquots of the serum obtained from the animal. The antibody dilutions typically start at 1:1000 and cover a range of at least two orders of magnitude. After the bound antigen is separated from the unbound antigen, the dilution at which the antibody binds between 30% and 70% (usually 40% to 50%) of the total activity of the labeled antigen is used for the assay.

To obtain a monoclonal antibody, it is first essential to inject the antigen into a mouse to induce an immunological reaction in the spleen. Each immunized spleen cell can produce an antibody with specific characteristics. The most important step in the production of monoclonal antibodies is screening the spleen cells to separate those capable of secreting a single antibody type. When a myeloma cell from the same species of mouse is fused with the selected spleen cell, a *hybridoma* is produced. This hybridoma continuously secretes antibodies with the same characteristics as the selected spleen cell. A hybridoma cell is capable of producing hundreds of specific antibody molecules per second. Thus a continuous clone line can be maintained in culture, becoming a source for the production of homogeneous monoclonal antibody molecules.

Antigen

Here the term *antigen* can refer to the substance that is injected into an animal to produce an antibody or to the standard that is used to generate a standard curve in an immunoassay. In addition, antigen can refer to the substance that is being measured in a biological fluid (e.g., serum).

Antigen Used to Prepare an Antibody

In general, any molecule larger than 10,000 Da can elicit an antibody response. With molecules in the 1000- to 10,000-Da range, eliciting an immune response becomes more difficult. Molecules smaller than 1000 Da are generally nonantigenic and are coupled to a large protein molecule (e.g., albumin); these small molecules are referred to as haptens. Thus steroids and some peptides, but not proteins, are prepared as hapten-proteins and injected into the animal to produce a polyclonal or monoclonal antibody.

The site of attachment of the protein carrier (e.g., albumin) is important because it determines the specificity of the antibody. In general, highly specific antibodies are formed when the attachment site does not involve any functional group on the steroid molecule. A commonly used approach is to prepare a carboxyl derivative at the carbon-6 position of the steroid molecule and to couple it to an amino group of the protein carrier.

Antigen Used as Standard

Steroids and peptides are available commercially and in most instances can now be obtained in a relatively pure state. However, it should never be assumed that these products are 100% pure when developing an immunoassay. If necessary, the compounds can be purified (e.g., by use of high-performance liquid chromatography [HPLC]). In contrast, proteins are generally considered to be more difficult to purify.

Labeled Antigen

Use of an optimum labeled antigen is also important in an immunoassay, as this determines how the compound being measured in a biological fluid, such as serum, is quantified in the assay. The labeled antigen must not only be identifiable by some physical or chemical method (e.g., a radioactivity counter or spectrophotometer) but also must bind to the antibody.

The classical labeled antigens for immunoassays have been radioactive antigens. For example, insulin, the first hormone measured for a radioimmunoassay (RIA), was labeled with radioactive iodine (^{131}I). This isotope had a very short half-life (8 days) and was soon replaced with iodine-125 (^{125}I), which has a half-life of 60 days. Peptides have also been labeled with ^{125}I. For steroids, tritium (^{3}H) was the initial isotope used in RIAs, but was subsequently replaced with ^{125}I. It is important to realize that, unlike proteins and peptides, the steroid molecule itself cannot be iodinated. Instead, the steroid molecule is attached chemically to an iodinated carrier molecule such as histamine or tyrosine methyl ester.

To eliminate the high cost of storage and disposal of radioactive material and to allow immunoassays to be carried out on an automated platform, nonradioactive labeled antigens began to be used in these assays. The predominant labels that were attached to antigens were chemiluminescent, fluorescent, and enzyme (which required a substrate); these labels were detected by use of a luminometer, fluorometer, and spectrophotometer, respectively. Presently, there is wide use of the chemiluminescent label in immunoassays on

automated platforms and the enzyme label in ELISAs carried out manually.

Automated Immunoassays

Over the past 30 years or so, highly sophisticated automated immunoassay systems have been developed. These systems consist of instruments that not only detect spectral properties of nonradioactive ligands (e.g., chemiluminescence), but also are able to analyze multiple analytes as well as to process multiple samples rapidly and efficiently. These immunoassay systems are capable of using both antigen-excess and antibody-excess methodology.

Currently a widely used immunoassay system is the Immulite analyzer (Siemens Healthcare Diagnostics, Deerfield, Illinois). The Immulite system employs enzyme-amplified chemiluminescent technology. The mechanism involves hydrolysis of a stable chemiluminescent substrate through the action of the enzyme alkaline phosphatase, resulting in an unstable anion that gives rise to sustained emission of light. The emitted light is quantified by use of a luminometer in the instrument.

As an example, the Immulite luteinizing hormone (LH) assay uses a solid-phase, two-site ICMA. The solid phase consists of a polystyrene bead coated with a monoclonal antibody against LH. The bead is sealed into an Immulite test unit, to which LH standard or serum sample, together with a polyclonal antibody conjugated to alkaline phosphatase, are added simultaneously. During an incubation period, LH is bound to the monoclonal antibody coating the bead and the polyclonal antibody-enzyme conjugate, forming a "sandwich complex." After unbound conjugated antibody is removed by a wash, the amount of complex, which is directly proportional to the LH standard or LH in the sample, is quantified by use of the chemiluminescent substrate described previously.

Steroid Hormone Immunoassays

The first RIA was developed in 1969 by Guy Abraham and that was for measurement of estradiol (E_2) in serum or plasma. The E_2 RIA method involved separation of E_2 from interfering metabolites by organic solvent extraction and Celite or Sephadex column chromatography, prior to its quantitation by RIA. The purification steps were added to remove most of the estrogen metabolites. E_2 is readily converted to estrone (E_1) and both E_2 and E_1 are converted to a total of approximately 90 metabolites. The extraction step removes the conjugated (water-soluble) steroids, whereas the chromatographic step separates E_2 from potential interfering unconjugated metabolites. The E_2 RIA included a specific antiserum against RIA in conjunction with tritiated E_2, and separation of the antibody-bound and unbound E_2 fractions using charcoal. This method, which is often referred to as a conventional RIA, was shown to be sensitive, specific, precise, and accurate. Soon afterward it was applied successfully to other sex steroid hormones, such as testosterone and progesterone.

During the decade of the 1970s, RIAs were developed for a variety of natural and synthetic steroid hormones. A notable change in the RIA method during those years was replacement of tritium with iodine in the labeled antigen to improve assay sensitivity. Because of their relatively low cost, RIAs became widely used in diagnostic and research laboratories.

Impact of the Conventional Steroid RIA Method

The immediate impact of the conventional steroid RIA method was that it allowed measurement of an immensely wide range of compounds of clinical and biological importance, and it opened new horizons in endocrinology. The long-term impact of the steroid RIA method was that its use in numerous studies enriched the field of endocrinology with new knowledge, and its use in diagnostic testing provided physicians with valuable information for diagnosing and treating countless patients. The steroid RIA methodology also allowed substantial research into the physiological and pathophysiological roles of steroid hormones in applications such as sexual differentiation, puberty, neuroendocrinology, the menstrual cycle, pregnancy, menopause, and male endocrinology. In addition, the RIA method opened the door for epidemiologic studies that permitted us to better understand the role of steroid hormones in the etiology of numerous diseases, notably the hormone-dependent breast and prostate cancers.

Advantages and Disadvantages of Conventional Steroid Radioimmunoassays

Steroid RIA methods with purification steps have the following advantages: First, steroid-binding proteins (BPs) (e.g., sex hormone-binding globulin [SHBG]) are denatured, thereby releasing the steroids (e.g., E_2 and testosterone) that they bind; second, the purification steps remove numerous potentially interfering metabolites prior to RIA; third, the RIAs are accurate and reliable when properly validated; finally, multiple steroids (usually up to five) can be measured in a single aliquot of serum.

Conventional steroid RIAs also have disadvantages. They are cumbersome, time-consuming, costly, and require relatively large sample volumes, especially when the steroid is present in low concentrations. Also, although multiple steroids can be measured in a single aliquot of serum, the measurements have to be done very carefully and are especially time-consuming. In addition, as with all antibody-based assays, since the measurement of the analyte is a surrogate approach (i.e., radioactivity is measured rather than the actual analyte itself), there is always the possibility of antibody cross-reactivity giving an erroneous result. Furthermore, the presence of autoantibodies within patients can further affect an assay, leading to falsely high or low values depending on the type of antibody interaction that occurs. Despite these concerns, a well-validated RIA preceded by organic solvent extraction and chromatography is typically an accurate and precise assay for the majority of applications.

Direct Steroid Immunoassays and Their Advantages and Disadvantages

Due to the time-consuming limitations of conventional RIAs, in the late 1970s the radio ligands in RIAs were replaced with nonradioactive ligands (chemiluminescent, fluorescent, or enzymatic) and the organic solvent extraction and chromatography steps used prior to RIA were eliminated, allowing direct immunoassays to be performed on an automated platform; this resulted in rapid measurements of steroid

hormones. Conventional RIAs continued to be used, but their use was overwhelmingly surpassed by direct immunoassays, particularly in clinical diagnostic laboratories.

The development of automated platforms gave direct assays, such as chemiluminescent immunoassays, the advantages of being convenient, simple, rapid, and relatively inexpensive, and requiring a lower sample volume (usually 0.1 mL). However, these assays also have serious disadvantages. They often overestimate the measurements due to lack of specificity of the antibody, especially in samples obtained from women treated with exogenous steroid hormones.[1] Also, matrix differences may exist between serum samples (particularly hemolyzed and lipemic samples) and solutions of the standard used to prepare the standard curve in the assay. In addition, steroids such as testosterone and E_2 may not be released efficiently from proteins such as SHBG to which they bind with high affinity in blood. Furthermore, direct immunoassays generally lack the sensitivity to measure accurately low levels of certain steroid hormones such as E_2 and testosterone. Limitations of direct immunoassays for quantifying E_2 in postmenopausal women and testosterone in both premenopausal and postmenopausal women are now well documented in the literature.[2]

Protein and Peptide Hormone Immunoassays

The first protein hormone RIA was developed for insulin and preceded the first steroid hormone RIA, which was for E_2, by about 10 years. The basis for the first protein RIA method was described in 1959 by Yalow and Berson, who showed that ^{131}I-insulin could be displaced by nonradioactive insulin from insulin-binding protein, and that ^{131}I-insulin was inversely and quantitatively related to the total amount of insulin present. This is the same principle that was described earlier for the steroid hormone RIA, and was subsequently used to develop RIAs for protein hormones other than insulin, as well as for peptide hormones. In 1967, Odell and coworkers reported the first RIAs for LH and follicle stimulating hormone (FSH). Subsequently, RIAs were developed for a variety of other protein hormones, such as human chorionic gonadotropin (hCG), prolactin and thyroid-stimulating hormone, and for peptide hormones such as gonadotropin-releasing hormone (GnRH) and adrenocorticotropic hormone (ACTH). The protein and peptide hormone RIAs used specific antisera in conjunction with the relevant ^{125}I-proteins and peptides. The change from the ^{131}I label to the ^{125}I label provided a longer-life for the labeled material (8 days vs. 60 days, respectively), so that the proteins and peptides did not require iodination as often for the RIAs.

Immunoradiometric Assay

In the late 1960s and early 1970s, the IRMA method was developed for measurement of proteins, which included insulin, FSH, and LH. Subsequently, the IRMA was also used to measure peptides such as GnRH and ACTH. The IRMA differed from the RIA in that the compound to be measured was assayed directly in combination with a specific labeled antibody rather than in competition with a labeled antigen for a limited amount of antibody.

Current two-site IMAs depend on the use of two antibodies directed to distinct antigenic epitopes on the protein or peptide being measured. This assay method is ideal for use of monoclonal antibodies, thereby increasing assay specificity. In addition, two-site IMAs offer greater sensitivity because they are much less dependent on the affinity of the antibody than RIAs. An appropriate choice of antibodies directed to different antigenic sites can further enhance the affinity and thereby the sensitivity of the assay. Thus highly sensitive IMAs opened up new clinical diagnostic opportunities (e.g., use of the sensitive thyroid stimulating hormone (TSH) assay to diagnose hypothyroidism).

Two-site IMAs also have certain limitations. They cannot be used to measure small ligand molecules such as steroids and very small peptides since they display only one antigenic site at a time. Also, IMAs are liable to high-dose "hook" effects. This results from very high levels of antigen that saturate the binding sites on the capture antibody and still leave antigen free to bind to the tracer antibody.

Enzyme-Linked Immunosorbent Assay

The ELISA differs from the IRMA in that the ELISA uses a solid-phase procedure and an enzyme-labeled instead of a radioactively labeled antibody. The most common format used for the ELISA is the multiwell plate made of polystyrene or polyvinyl. This is convenient to handle since centrifugation is not required and multiple wash steps are readily automated. Antibody is coated on the wall of each well and this antibody binds the corresponding antigen in the test sample. The antigen-antibody complex is quantified by addition of an enzyme-labeled specific antibody. Following addition of the appropriate substrate, the endpoint (product) can be "read" spectrophotometrically, using an automated reader.

Standards Used in Protein Immunoassays

Unlike steroids and small peptides (e.g., GnRH), proteins are considerably more difficult to purify. Although some proteins such as insulin are available in a highly purified state for use as standards in immunoassays, others such as FSH, LH, and hCG have not yet been prepared in sufficient amounts in a highly purified form. Although the latter proteins are available from international agencies, their purity, based on biological potency per unit weight, is often less than that of highly purified preparations reported by individual investigators. To be able to compare results obtained in different laboratories and at different times in the same laboratory, considerable effort has been made to use a single material as a standard.

Reference materials are available for FSH, LH, and hCG. They are provided by the World Health Organization (WHO), which obtains them from the National Institute for Biological Standards and Controls in Hertfordshire, England. Two types of reference materials are available: namely, an International Standard (IS) and an International Reference Preparation (IRP). The IS is a material that has a potency established in 10 to 20 expert laboratories throughout the world, whereas the potency of an IRP is established by only several laboratories.

With the advent of RIAs for gonadotropins, a partially purified extract of human pituitary glands (code name LER-907) was made available by the National Institutes of Health in the United States. This preparation was provided in large amounts to the WHO, which purified it further in 1976 and called it the First International Reference Preparation of

Pituitary FSH and LH (1st IRP-FSH and LH). Subsequently, another partially purified extract of LER-907 was prepared in 1980 to replace the 1st IRP-FSH and LH. It was called the Second International Reference Preparation of Pituitary FSH and LH (2nd IRP-FSH and LH) and was assigned the code number 78/549. Highly purified preparations of pituitary FSH and LH have also been prepared. The First International Reference Preparation for Human Pituitary LH (1st IRP-LH, code number 68/40) was available in 1974. Subsequently (in 1988), this material was replaced by the Second International Standard for Pituitary LH (2nd IS-LH; code number 80/552). In 1986, the First International Standard for Pituitary FSH (1st IS-FSH, code number 83/575) was established. Presently, both the 2nd IRP-FSH and LH and 2nd IS-LH are used as standards in gonadotropin immunoassays.

The initial standard used in hCG immunoassays was a partially purified hCG preparation obtained from urine of women in their first trimester of pregnancy and was called the Second IS for hCG. Subsequently, a highly purified preparation was developed and was referred to as the Third IS. This preparation is presently used as the standard in hCG immunoassays.

Validation of Immunoassays

Before an immunoassay can be used to measure any compound, it must first be validated with respect to sensitivity, specificity, accuracy, and precision. Procedures used to validate immunoassays are described later.

Sensitivity

The sensitivity of an assay is defined as the smallest amount of the substance being measured that can be distinguished from zero. In practice, assay sensitivity depends on the precision of the standard curve, which can be measured by assaying 10 replicates of each concentration of standard and the "zero" standard, which contains no standard. This allows calculation of the mean ± standard deviation (SD) amount of the antibody-bound marker, corresponding to each concentration of standard. The sensitivity of an assay is the lowest standard concentration that yields a mean amount (e.g., counts per minute) of antibody-bound marker differing by two SDs from the mean amount of antibody-bound marker associated with the zero standard.

Assay sensitivity expressed in that manner, in practical terms, refers to the sensitivity of the standard curve. However, it is important to know the assay sensitivity in terms of the lowest amount of analyte that can be measured per milliliter of sample. This can be determined by calculating the product of the lowest standard concentration (based on standard curve sensitivity) and, if applicable, dilution factors, as well as the factor accounting for procedural loss (in assays using an extraction and/or chromatographic step).

The sensitivity of an assay is especially important when very low serum levels of a compound are being analyzed (e.g., measurement E_2 in samples from postmenopausal women or from patients treated with an aromatase inhibitor).

Specificity

Assay specificity refers to the degree of interference from cross-reaction that is encountered from substances other than the target analyte. The specificity of an immunoassay is usually assessed in two different ways. First, the cross-reactivity (expressed as a percentage) of the antibody is determined by comparing the dose-response standard curve of the substance being measured with dose-response curves obtained for compounds that may be present in the same sample as the analyte, and that may bind to the antibody. The percent cross-reaction of an antiserum with a substance is calculated from the mass of standard that yields 50% inhibition of binding of the assay marker to the antibody, divided by the mass of the cross-reacting substance that gives the same percentage of inhibition (50%), and multiplied by 100%.

A second approach for defining immunoassay specificity is to compare the analyte values of a group of samples measured by an immunoassay method with those obtained by a classic method such as MS. If this is not possible and the analytes are being measured in an immunoassay without chromatography, the analyte values should be compared with the values obtained by an immunoassay that uses an extraction step followed by a chromatographic step (e.g., Celite column partition chromatography) to separate the analyte in question from interfering metabolites.

Accuracy

Assay accuracy defines the extent to which a given measurement agrees with the actual value. One commonly used method to establish the accuracy of an immunoassay is based on the finding of linearity (parallelism) between the assay standard curve and serial dilutions (using assay buffer) of several samples containing known high concentrations of the analyte. Another method is based on the recovery of added ("spiked") standard, at different levels, from patient samples. This method is often used for assays that require one or more purification steps (e.g., conventional steroid hormone assays). Both the parallelism and the "spiked" sample methods are limited by the precision of carrying out the dilutions or "spiking."

Precision

The precision of an assay refers to the variability that exists when multiple measurements of the compound are made on the same sample. In practice, both intraassay precision and interassay precision are determined and are usually expressed as the coefficient of variation of replicate measures. The coefficient of variation (expressed as a percentage) is calculated by dividing the SD by the mean of replicate determinations of an analyte, and then multiplying by 100. Intraassay precision is assessed by measuring the analyte in replicate samples (usually 7 to 10) within the same assay. Interassay precision is determined by measuring the analyte in replicate samples (at least five), with each sample included in a different assay. Both intraassay and interassay precision should be determined in three different concentrations of the analyte (high, midrange, and low).

Interferences in Immunoassays

Circulating human antibodies that react with animal proteins (anti-animal antibodies) are often an unrecognized and unsuspected source of interference in immunoassays. This is particularly true with two-site immunoassays. The most common human anti-animal antibody interference is caused

by human antimouse antibodies (HAMA). HAMA can cause either positive or negative interference in two-site mouse monoclonal antibody-based immunoassays. Strategies for preventing the development of anti-animal antibodies have been proposed. Awareness of laboratory personnel and clinicians of the problems associated with this type of interference in immunoassay methods is important.

Mass Spectrometry Assays

Measurements of steroid hormones by MS assays actually preceded their measurements by RIA. As early as 1966, the first comprehensive urinary steroid profile was produced by gas chromatography-mass spectrometry (GC-MS). This method combines the resolving power of gas chromatography with the high sensitivity and specificity of the mass spectrometer. Separation of steroids by gas chromatography requires that they be first derivatized to increase their volatility, selectivity, and detectability. The mass spectrometer functions as a unique detector that provides structural information on individual solutes as they elute from the gas chromatography column. The MS technique first involves ionization of the compound being measured at the ionization source, and is followed by separation and detection of the ions in the mass analyzer. A mass spectrum is produced in which the relative abundance of a particular ion is plotted as a function of the mass-to-charge (m/z) ratio, and the concentration of the compound is then obtained.

Due to the complexity of the methodology and associated costs of the instrumentation and reagents, as well as the need for a highly trained individual to carry out the assays, use of GC-MS assays was restricted to a limited number of laboratories, primarily at pharmaceutical companies. Thus, for a period of about 30 years after the development of the first RIA in 1969, conventional RIAs and direct immunoassays were the predominant methods used to quantify steroid hormones in clinical diagnostic and research laboratories due to their relative ease of performance and considerably lower overall costs. However, advances in liquid chromatography (LC) technology in the 1980s led to the development of a high performance liquid chromatography-MS (LC-MS) instrument in 1987. In addition, invention of an electrospray source by Nobel laureate, John B. Fenn, in 1990, and the subsequent development of chemical ionization greatly improved routine analysis of steroids. This technology facilitates ionization of compounds present in liquid droplets and sprays the molecules directly into the mass spectrometer from the HPLC unit. The advancements allowed for simple coupling of the LC eluent with the mass spectrometer and often negated the need for derivatizing the steroid, thereby reducing the complexity of the assay and shortening the assay run time dramatically. These factors greatly increased the throughput of patient samples, while still providing highly accurate and precise results.

In recent years, there has been a large increase in the use of assays that use either LC or gas chromatography with tandem MS (LC-MS/MS or GC-MS/MS). Tandem MS consists of two mass spectrometers in series connected by a chamber (collision cell). After chromatography, the sample is processed in the first mass spectrometer to obtain the precursor ion, which is then fragmented in the collision cell into product ions. The mass of the product ions is then determined in the detector of the second mass spectrometer. This method has high specificity, sensitivity, and throughput.

During the past decade there has been a substantial increase in the use of LC-MS/MS assays, particularly in major diagnostic clinical laboratories, and to a lesser extent in some research laboratories. However, there are still situations where a GC-MS assay provides higher chromatographic resolution and even sensitivity than LC-MS. A particular strength of GC-MS and GC-MS/MS assays is their high applicability to measurement of large numbers of structurally similar compounds. They remain the most powerful assay method for defining defects in steroid hormone metabolism.

Because of the high validity and throughput of steroid hormone MS assays, there is a rapidly growing use of this methodology in both clinical and research laboratories. In larger reference laboratories, these assays have replaced the conventional RIAs, which are cumbersome and time-consuming, and direct immunoassays, which lack specificity and/or sensitivity. The MS technology has been implemented successfully for routine analysis of steroid hormones in major clinical diagnostic laboratories. Although the high cost of MS instrumentation, related operating costs, and requirement for high technical expertise have prohibited smaller laboratories from using this instrumentation for high-throughput routine testing of steroid hormones, this situation is changing and MS assays are becoming much more widely used.

In addition to use of the MS methodology for routine analysis of steroid hormones, this methodology is now sufficiently rapid and robust for measuring these hormones in large epidemiological studies with high specificity and sensitivity. An important advantage of MS assays is their capability of measuring multiple steroids in a single aliquot of serum or urine. In contrast, generally only up to five different steroids can be measured in a single serum aliquot (usually 1 mL) by conventional RIA. Several years ago, at the National Cancer Institute, LC-MS/MS assays were developed for quantifying as many as 15 different estrogens in only 0.5 mL of serum or urine.[3] In addition LC-MS/MS assays have been established for simultaneous quantitation of 11 androgens, including principal adrenal and gonadal androgenic precursors and their 5α-reduced metabolites.[4]

Studies using MS have shown that the adrenal steroid, 11β-hydoxyandrostenedione, is a precursor to 11-ketotestosterone and 11-keto-5α-dihydrotestosterone, which are potent androgenic androgens. With LC-MS/MS several 11-oxygenated androgens (11β-hyroxyandrostenedione, 11-ketoandrostenedione, 11β-hydroxytestosterone, and 11-ketotestosterone) have been found to be elevated in women with polycystic ovary syndrome (PCOS) and cumulatively constitute a greater proportion of total circulating androgens than DHEA, androstenedione, and testosterone.[5] More work on the clinical significance of these MS-based 11-oxygenated androgens is clearly needed.

Measurement of metabolite profiles of a steroid hormone has the potential to provide highly valuable information in diagnosing patients and in a variety of studies, particularly epidemiological studies. Quantitative analysis of metabolite profiles of small-molecular-weight molecules such as steroid hormones is referred to as *metabolomics* and is a rapidly growing field of research.

Standardization of Steroid Hormone Assays

Although hormone assays are widely used by physicians, some of the assays lack accuracy. Significantly different results can be obtained by two laboratories measuring the same hormone in the same serum sample from a patient. Inaccurate test results can lead to missed diagnosis or incorrect treatment. In the research setting, inaccurate tests can make findings in a study uncertain and not repeatable. This makes it very difficult to apply meaningful evidence for clinical decisions and establishing normal ranges. Accurate hormone assays will lead to fewer medical errors, eliminating the need for costly test repeats, and reducing overall costs of health care.

Although there seems to be general agreement that MS assays will become the "gold standard" for steroid hormone measurements, there are many challenges to overcome before this occurs.[6] It is important to realize that the MS technology faces variability issues similar to those of conventional RIAs and direct immunoassays that need to be addressed. Differences in accuracy among MS assay methods appear to be attributable to variation in calibration of assay standards, whereas differences in assay precision may be explained, at least in part, by variations in sample preparation.

The Centers for Disease Control and Prevention (CDC), with broad input from various professional societies, particularly the Endocrine Society, is leading a Hormone Standardization Program with an initial focus on standardizing testosterone measurements.[7] The conceptual approach of this program is built on experiences gained from successful standardization programs that the CDC maintains or has supported (e.g., the cholesterol standardization program and national glycohemoglobin standardization program). The testosterone standardization program consists of three basic steps: calibrating individual assays, developing a reference system, and verifying end-user test performance. Although it is evident from previous efforts at the CDC that vast improvements in measurement performance can be achieved through assay standardization, based on their experience it could take years to accomplish such achievements.

Reference Intervals of Steroid, Protein, and Peptide Hormones

An important requirement in clinical diagnostic testing in which measurements of steroid, peptide, or protein hormones are determined by conventional and direct immunoassays or MS assays is reference intervals. These intervals should be derived from well-characterized, adequate-sized populations using standardized procedures such as those formulated by the Clinical and Laboratory Standards Institution. Frequently, only limited information is available about subjects used to establish reference intervals. Also, an often neglected aspect in establishing reference intervals for hormone measurements is biologically influencing factors that may affect these measurements. The major factors include gender, age, body mass index (BMI), pubertal stage, menopausal status, phase of menstrual cycle, pregnancy, and diurnal rhythm.

It is especially important to compare reference intervals for steroid hormones measured by MS assays to those obtained by conventional RIAs, since most of our knowledge about the role of steroid hormones in normal women and men, as well as in various endocrine diseases, is based on the data obtained by the latter methodology. Presently it appears that the reference intervals obtained by the two methods do not differ significantly.

Measurement of Specific Reproductive Protein and Peptide Hormones and Steroid Hormones

- *Sensitive assays are now available for the measurement of gonadotropins that have in large part alleviated the need to depend on stimulation tests, which are still required on occasion; the greatest need is with amenorrhea and puberty alterations.*
- *Prolactin may circulate in several molecular forms including macromolecules. An understanding of this is important in the evaluation of prolactin secreting adenomas.*
- *Estradiol is the most important estrogen measured for clinical purposes and may require MS for the required sensitivity at low levels.*
- *Progesterone is primarily used for the detection of ovulation.*
- *Androgen measurements are used for evaluation of androgen excess in women and hypogonadism in men. Several derivatives of routine androgens are now detectable using MS. The work up of androgen excess is outlined.*

Measurement of Gonadotropins

Serum concentrations of LH and FSH are expressed in international units, using as a reference partially purified pituitary hormone preparations such as LER-907 or urinary gonadotropins (Second International Reference Preparation—human menopausal gonadotropin [2nd IRP-HMG]). The use of different hormone reference preparations complicates comparisons between different assays.

Classic RIAs of gonadotropins have low specificity and sensitivity and often cannot distinguish low levels from low-normal values. This is also compounded by the pulsatile nature of gonadotropin secretion. Immunometric assays are more sensitive and have improved the measurement of gonadotropins.[8] As previously described, IRMA (immunoradiometric), IFMA, or ICMA methods should be used when low levels of gonadotropins are expected but may not offer advantages when elevated or normal levels are anticipated. The ELISA for gonadotropins is considered to be less sensitive and exhibits higher nonspecific binding.

Additional problems that may arise in LH measurements include a significant cross-reactivity with hCG and the pulsatile secretion pattern (pulses approximately every 60 to 90 minutes). The requirement of precision in values would necessitate that samples, taken 15 to 20 minutes apart, be pooled for analysis. In the clinical setting, however, this is not necessary because low levels will usually remain low, and high values will not usually be in the normal range. The concern about pulsatility is more for research purposes and is far less pronounced for FSH samples, where pulses are less frequent, occurring approximately every 3 hours.

Apart from alterations in FSH and LH associated with various disorders and physiologic states, FSH levels are increased by levodopa and ketoconazole and decreased by the administration of estrogens and phenothiazines. LH is increased by ketoconazole and decreased by administration of sex steroids, phenothiazines, digoxin, and propranolol.

Blood Levels of Luteinizing Hormone and Follicle-Stimulating Hormone in Women

In adult women, using conventional RIA assays, during the follicular phase, serum levels of LH and FSH by RIA range between 4 and 15 mIU/mL (2nd IRP-HMG standard). Third generation commercial assays, using immunometric methods, report lower normal ranges of LH (1.0 to 8 mIU/mL).

During mid-cycle, serum LH levels increase four- to sixfold while serum FSH levels increase two- to threefold. The mid-cycle increase of gonadotropins lasts about 2 days.

During the luteal phase, serum LH and FSH levels are similar to or slightly lower than their respective levels during the follicular phase. Therefore, in healthy women the LH:FSH ratio is about 1.0 during the follicular phase but increases during ovulation.

Before puberty, gonadotropin levels are lower than in adult women, and the ratio of LH:FSH is less than 1.0 because of the relative predominance of FSH secretion.

During the perimenopausal period, serum FSH levels increase substantially while LH levels remain in the normal range. After menopause, both gonadotropins are high but serum FSH levels are higher than LH. In older women, LH levels decrease from their high levels in early menopause. In general a ratio of LH:FSH less than 1.0 reflects a hypoestrogenic state.

Gonadotropin Stimulation Tests (Gonadotropin-Releasing Hormone and the Gonadotropin Releasing Hormone Agonist)

In the past, synthetic GnRH, the hypothalamic decapeptide, has been widely used to stimulate LH and FSH secretion to uncover abnormalities that cannot be diagnosed with baseline determinations. However, in most instances, the test largely reflects baseline measurements.

In patients with PCOS and women with ovarian failure, GnRH stimulation results in exaggerated increases in LH (in the setting of PCOS) and in FSH elevations in women with ovarian failure. The GnRH test may be useful in the differential diagnosis of precocious puberty. However, because GnRH may not be commercially available for testing purposes in several countries, most stimulation tests are now carried out using a GnRH agonist (GnRHa), such as leuprolide acetate or naferelin. After administration of leuprolide acetate, LH levels are measured after 1 and 2 hours.

Measurement of Gonadotropins in the Diagnosis of Puberty Alterations

Using ultrasensitive LH assays, measurement of basal serum LH levels is often sufficient for making the diagnosis of central precocious puberty (CPP) while stimulation tests are reserved for those patients with inconclusive basal results. Some studies have reported that, using a third generation IMA, blood values of LH higher than 0.8 mIU/mL are sufficient for the diagnosis of gonadotropin-dependent precocious puberty. However, the sensitivity of the basal LH level for the diagnosis of CPP in girls is relatively low (about 65%) and about one third of patients with CPP have undetectable LH levels. Therefore while an increased basal LH level is highly suggestive of the diagnosis, a low value does not exclude a central pubertal activation.[9,10]

In patients with signs of precocious puberty but inconclusive basal LH levels, a GnRHa test is usually carried out. The test may be performed in many ways but most centers use leuprolide acetate at a dose of 20 μg/kg, up to a maximum of 500 μg. Peak LH of at least 5 IU/L indicates a pubertal response.

In most instances, gonadotropin measurements in the basal state and after the GnRHa test are not able to distinguish delayed puberty from hypogonadotropic hypogonadism.[11]

Measurement of Gonadotropins in Adult Female Reproductive Disorders

High values of LH with increased LH:FSH ratios are found in 50% of women with PCOS.[12] In the past, some clinicians based the diagnosis of PCOS on the finding of elevated LH and LH:FSH ratios higher than 2.0 or 3.0. While finding an increased serum LH or LH:FSH ratio may be useful for confirming the diagnosis of PCOS, many patients with PCOS have normal serum LH and LH:FSH ratios.[12,13]

High levels of serum LH and FSH are typical of premature ovarian failure/insufficiency and may be found in patients with gonadal dysgenesis.[14] Rarely, pituitary tumors may produce FSH or LH.[15] Measurement of α-subunits and determination of the gonadotropin responses to thyrotropin-releasing hormone (TRH) may be useful for the diagnosis of gonadotropin adenomas.

Measurement of Prolactin

Serum prolactin (PRL) is generally measured by RIA or IRMA. The upper limit of the normal range is generally reported to be 15 to 20 ng/mL in men and 20 to 25 ng/mL in women, although the true normal levels are usually lower (up to 18 ng/mL in women). Serum PRL values are influenced by estrogens, drugs (e.g., phenothiazines, metoclopramide), stress, food consumption, breast stimulation, and even venipuncture. Because of diurnal changes and transient increases after meals, routine samples should be obtained at mid-morning.

In normal women, blood immunoreactive prolactin results predominantly from monomeric prolactin (≥95%). Dimeric prolactin forms (so called big-prolactin), and big-big prolactin, a macroprolactin (due to a prolactin-antibody complex of molecular weight >100 kDa), are generally present in low concentrations (<1%). However, macroprolactinemia is present in 15% to 35% of subjects having increased immunoreactive prolactin, and in some patients hyperprolactinemia is the result of elevated macroprolactin levels (without a commensurate biological effect) and often leads to a misleading medical approach.[16,17]

The gold standard method for detecting macroprolactinemia is by gel filtration chromatography, a procedure that allows for quantification of all three variants of PRL. However, this method is labor intensive and most clinical laboratories prefer using precipitation with polyethylene glycol (PEG). A low PRL recovery after PEG treatment indicates the presence of macroprolactin.

Serum PRL levels greater than 150 ng/mL are diagnostic of the presence of a pituitary adenoma, but patients with microadenomas may have levels that are considerably lower.[16]

Mild elevations of serum PRL may occur with certain CNS tumors or granulomas compressing the pituitary stalk.

A mild elevation may also occur in hypothyroidism, with drugs stimulating PRL secretion, with high estrogen levels, and also during stress. If drugs and hypothyroidism are ruled out, magnetic resonance imaging (MRI) of the hypothalamic-pituitary region of the brain should be obtained. If no cause of hyperprolactinemia is found, macroprolactinemia should be ruled out, although this situation may be suspected when there are no symptoms or signs of elevated PRL, such as having normal menstrual function.

While it has been suggested to screen for macroprolactin in all hyperprolactinemic patients,[17] since the Pituitary Society has published guidelines indicating that values of blood prolactin ≥150 ng/mL are always suggestive of pituitary prolactinoma,[18] we suggest screening for macroprolactin only in patients having serum PRL values between 25 and 150 ng/mL. PRL recovery ≤30% following PEG precipitation indicates the presence of macroprolactinemia.[18] Because true hyperprolactinemia and macroprolactinemia may coexist, post PEG PRL values should not exceed 13 ng/mL.[18]

In pregnancy, serum PRL begins to increase by 6 weeks gestation and rises progressively to reach approximately 200 ng/mL at term, although the variability is large. In nonlactating women, PRL levels return to normal 2 to 3 weeks postpartum.[19] With menopause, serum PRL declines slightly as a result of the reduction of estrogen.[20]

Measurement of Estradiol and Other Estrogens

In most laboratories using commercial immunoassays, serum estradiol ranges between 20 and 80 pg/mL during the early to mid-follicular phases of the menstrual cycle and peaks at 200 to 500 pg/mL during the preovulatory LH surge. Refinements in assays, as discussed earlier and later, now show that these general "normal" ranges are higher than true values. However, most commercial assays and kits are not able to measure the low levels of estradiol that may be found in children, men, postmenopausal women, and women receiving aromatase inhibitors for the treatment of breast cancer.[21] Before puberty, serum estradiol levels are under 20 pg/mL, indistinguishable from cases of hypogonadism. After menopause, estradiol levels fall to prepubertal levels; mean serum estradiol is typically between 10 and 20 pg/mL, and levels are lower than 10 pg/mL in women who have undergone oophorectomy. Similar problems in the estradiol assay exist in men where serum estradiol should be less than 40 pg/mL.

The advent of radioimmunometric and other immunoassay methods has not improved the precision and accuracy of estradiol assays. For measuring low levels, samples should be extracted with organic solvents and subjected to chromatographic separation to remove interfering steroids, particularly estrogen conjugates. Recently, simpler analytical methods using LC coupled with MS have been developed and are increasingly used for measuring E2 samples.[21–23] These methods are particularly useful when a low estradiol level is expected and has a sensitivity, which is adequate to monitor patients with breast cancer treated with aromatase inhibitors. In these patients, methods need to be able to distinguish between suppressed levels of 1 pg/mL or less, from the pretreatment levels that are commonly 10 to 15 pg/mL.[24]

The measurement of serum estradiol in men may be useful in the clinical evaluation of gynecomastia. In pubertal gynecomastia, a normal estradiol level is generally found.[25] The finding of increased estradiol, testosterone, and LH but normal FSH in subjects with pubertal gynecomastia may indicate a syndrome of partial androgen insensitivity.[26]

Serum estrone is higher than values of estradiol in women after menopause[27] and in anovulatory women who do not produce adequate amounts of estradiol via the dominant follicle. Estrone levels are increased with obesity and are most closely linked to the increase in adipose tissue, where aromatization is increased. In women receiving any oral estrogen, estrone levels exceed those of estradiol; and its measurement is sometimes useful for the assessment of absorption and metabolism problems with oral estrogen therapy. Although not frequently measured, estrone sulfate reflects the circulating reservoir of estrogen and is quantitatively the highest circulating estrogen.[27]

Estriol may be measured by RIA in serum or by spectrophotometric methods or RIA in urine. Estriol, which is produced in the fetal-placental unit, may be analyzed for prenatal genetic testing to help detect aneuploidy, but is not useful today for the assessment of fetal well-being.

At present there is no clinical utility for the measurements of estetrol or catecholestrogens.

Measurement of Progesterone

Serum progesterone may be measured by a variety of immunoassays. The specificity of the assay is highly dependent on lack of cross reactivity with several pregnane metabolites.

Blood Levels of Progesterone and the Assessment of Ovulatory Function

Serum progesterone is low during the follicular phase, with levels being less than 1.5 ng/mL. Levels begin to increase just before the onset of the LH surge and then increase progressively to peak 6 to 8 days after ovulation. After menopause, serum progesterone of adrenal origin is under 0.5 ng/mL.

The measurement of serum progesterone during the mid-luteal phase (days 21 and 22) is most frequently used to assess ovulatory status. Although single samples are acceptable in clinical practice, it should be appreciated that progesterone levels exhibit a pulsatile pattern[28] as well as some diurnal variation.

During the mid-luteal phase, serum progesterone levels are usually higher than 7 ng/mL. Some physicians have proposed using three luteal determinations with a total serum value of 15 ng/mL or more to indicate normal luteal function.[29] For patients being monitored for fertility, other assessments may be used including basal body temperature charts, urinary LH kits, and timed endometrial biopsies. In conception cycles, properly timed mid-luteal progesterone levels are over 10 ng/mL.[30] Progesterone is also often used to assess ovulation after induction of ovulation. In clomiphene cycles, mid-luteal progesterone levels should be over 15 ng/mL. In assisted reproduction cycles such as in vitro fertilization (IVF) where gonadotropins are administered, a small rise in progesterone (>2 ng/mL) has been linked to decreased pregnancy rates.[31] This is related to luteinization rather than premature ovulation per se, but this level is detrimental to endometrial receptivity.

An alternative to the measurement of serum progesterone is urinary pregnandiol glucuronide. This provides a more

integrated assessment of luteal function and is usually normalized for urinary excretion by the determination of creatinine.

During pregnancy, serum progesterone may be useful to assess corpus luteum and placental function.[32] Maternal serum progesterone levels nadir in early pregnancy at 9 weeks with levels of approximately 10 ng/mL and then rise slowly to 40 ng/mL near the end of the first trimester and then increase progressively to reach 150 ng/mL at term. Low levels of progesterone (below 10 ng/mL) at 6 to 8 weeks signify an abnormal intrauterine pregnancy or an ectopic pregnancy.

Measurement of Androgens

Androgens in women arise from three different sources: the ovaries, the adrenal glands, and the peripheral compartment. Most androgens are produced or metabolized by more than one compartment and, in evaluating women with androgen excess, several androgens are usually measured.[33] In the past, the measurement of urinary 17-ketosteroids (17-KS) was the most common method used to evaluate androgen production in women. However, 17-KS measurements reflect adrenal androgen production, poorly reflect testosterone production,[34] and are nonspecific; therefore they have been abandoned for use in current practice.

Most commonly, serum testosterone and dehydroepiandrosterone sulfate (DHEAS) are measured, which largely reflect ovarian and adrenal contributions, respectively.[35] Serum testosterone reflects mostly ovarian androgen production, with two thirds of circulating levels resulting from the peripheral conversion of androstenedione; therefore, increases in either ovarian or adrenal androstenedione production results in elevated serum testosterone levels. On the other hand, while serum DHEAS reflects adrenal androgen secretion well, levels may be normal in certain cases of adrenal androgen hypersecretion (as in 21-hydroxylase deficiency).[36] The pathway of adrenal enzymatic blockade (affecting the Δ_4 pathway) explains this discrepancy. Conversely, serum DHEAS may be elevated in many patients with a prevalent ovarian source of hyperandrogenemia (as in PCOS)[37] and this is explained by the peripheral conversion of ovarian-derived DHEA to DHEAS in the circulation. Similarly, the increased levels of serum DHEAS also do not predict androgen responses to dexamethasone suppression,[38] which has been used as a test to determine adrenal responsivity to suppression.

Serum testosterone should be measured by RIA or ICMA, after extraction and chromatography. These assays are cumbersome, time-consuming, and relatively costly, and accordingly many laboratories use direct assays without purification. However, these methods have some major disadvantages including overestimation of the values and low specificity. It has been reported that these direct testosterone assays have poor or no validity in females.[39]

Many commercial diagnostic laboratories have switched to MS after liquid chromatography (LC/MS) assays.[39] It has been suggested that this method has high sensitivity and specificity, and permits a better differentiation between normal and increased levels in women. However, this method does not provide better results than those obtained with classic methods of testosterone evaluation that include extraction.[40]

Unbound testosterone may be measured as bioavailable testosterone[41] or as free testosterone.[42] The first method, which is also referred to as non-SHBG-bound testosterone, relies on SHBG to assess the percentage that is "free." In healthy women, up to 75% of testosterone is bound to SHBG; the percentage that is unbound includes testosterone that is entirely "free" and the moiety associated with albumin that is available to the target cells.

Free testosterone is measured by equilibrium dialysis and has remained the gold standard for assessing testosterone that is neither bound to SHBG nor associated with albumin. Variations in incubation temperature significantly affect results in the dialysis method,[43] which renders this method, which is tedious and expensive, rarely used clinically.

Commercial methods measure unbound testosterone by a direct method using a ^{125}I-labeled testosterone analogue as tracer. This analogue method gives 75% lower values than equilibrium dialysis assays and, although it is easy to perform, its clinical utility is questionable. It has been reported that direct assays of free testosterone result in unacceptably spurious values with high random variability and, therefore, should not be used in clinical practice.[44] It is most practical for clinical purposes, to calculate a free testosterone index (free androgen index, FAI) using levels of testosterone and SHBG and albumin. An average normal albumin concentration from the literature can be used in the equation, and the binding constants.[43] Calculated free testosterone levels in women have been found to be nearly identical with corresponding values determined by equilibrium dialysis.[45,46] The calculation is made using the ratio between testosterone and SHBG (in units of nmol/L): (T (ng/mL) × 3.467/SHBG × 100). However, FAI values have low validity in adult males[47] and are strongly dependent on the accuracy of testosterone and SHBG assays. In general, FAI should be used primarily for clinical rather than research purposes, when testosterone assays using chromatography and purification techniques are not available. It has also been thought that since saliva is a natural distillate, measurements of salivary testosterone may provide a measure of "unbound" testosterone. Lack of precision and reliability with salivary testosterone do not allow this measurement to be recommended for clinical use.

There are other androgens that may be useful to measure under different circumstances. These include androstenedione and 11β-hydroxyandrostenedione,[48] the latter of which reflects adrenal production of androstenedione, because 11β-hydroxylase activity is usually absent in the ovary.

Assessment of peripheral androgen production requires the measurement of products of 5α-reductase activity. While serum dihydrotestosterone (DHT) does not appropriately reflect increases in peripheral 5α-reductase activity, more distal metabolites may be helpful. Serum 3α-androstanediol glucuronide best reflects peripheral testosterone metabolism in hirsutism,[49] and androsterone glucuronide best reflects the state in androgenic acne.[50]

In assessing androgen excess in women, it is preferable to obtain blood samples in the morning because of diurnal variation from the adrenal. Values are often reduced during the afternoon or evening.

Blood Levels of Androgens in Women

In women of reproductive age, an accurate range for serum testosterone is between 20 and 50 ng/dL. With direct immunoassays, the upper end of the normal range is approximately 70 to 80 ng/dL, or even higher. Serum testosterone levels higher than 200 ng/dL are suggestive of ovarian neoplasms, but some tumors may present with lower levels.[51]

Because upper normal ranges vary, a rule of thumb is to be concerned about a tumor in women when values exceed 2.5 times the upper normal range of a particular assay. Patients with PCOS have mildly increased serum testosterone (usually to values <100 ng/dL), while markedly increased levels may be found in patients with hyperthecosis and in those who have classic congenital adrenal enzymatic deficiencies. Patients with nonclassical congenital adrenal hyperplasia (NCAH) have androgen values similar to those found in PCOS.[52]

The normal range of serum DHEAS is between 0.5 and 2.8 μg/mL. Values above 8 μg/mL are suggestive of an adrenal neoplasm; if the adrenal tumor produces testosterone, however, only modestly elevated values (3 to 4 μg/mL) may be found.[53]

Serum androstenedione levels range between 1.0 and 2.5 ng/mL, and the highest levels occur with a functioning tumor or in congenital adrenal hyperplasia.

The reference ranges for free and bioavailable testosterone can vary considerably depending on the assay method used to determine these testosterone fractions. Again, it is important to know the reference ranges in the specific laboratory where the samples are being analyzed. Reference ranges of 1.1 to 14.3 ng/dL and 1.1 to 6.3 ng/mL for bioavailable and free testosterone, respectively, are used in one large clinical diagnostic laboratory. The reference range for SHBG in women is generally approximately 30 to 90 nmol/L.

Serum androgens are low before puberty, with adrenal androgen levels beginning to increase 1 or 2 years before the onset of the puberty. Serum DHEAS values higher than 0.8 μg/mL indicate the presence of adrenarche.[54]

In women, adrenal androgens begin to decline in the third decade of life and the greatest reduction in values occurs between age 20 and the fifth and sixth decades of life.[55] Ovarian androgens begin to decline a little later, generally in the third decade[56] and this reduction continues after menopause.[57] Similar declines of ovarian and adrenal androgens are observed in PCOS and may explain the improvement in menstrual function observed in many patients with PCOS after age 40.[58]

Measurement of serum DHEA-S levels may be helpful in that very low levels for age may suggest adrenal deficiency as occurs in young women with autoimmune ovarian failure.[59]

Measurement of Circulating Androgen Precursors in Women

Measurements of intermediates in steroid metabolism are useful for the diagnosis of adrenal enzymatic deficiencies. Serum 17α-OHP is used in the diagnosis of 21-hydroxylase deficiency.[52] Because 17α-OHP is secreted by the corpus luteum, the measurement should be carried out in the morning during the follicular phase. In healthy women, serum 17α-OHP is generally lower than 1 ng/mL, but hyperandrogenic patients (mostly those with PCOS) generally have slightly higher levels.

Patients with classic neonatal forms of 21-hydroxylase deficiency have very high levels of 17α-OHP (50 to 200 ng/mL), while the diagnosis of NCAH relies mostly on the finding of serum 17-OHP concentrations above 10 ng/mL (30 nmol/L).[52] An increased basal 17-OHP result between 2 and 10 ng/mL (30 nmol/L) must be confirmed by the finding of 17-OHP above 10 ng/mL (30 nmol/L) after stimulation of adrenal function by the administration of synthetic ACTH (cosyntropin), which is usually given as an intravenous bolus but can also be given intramuscularly.[52]

From a practical standpoint, the *CYP21A2* locus responsible for 21-hydroxylase deficiency is complex, precluding its molecular genetic analysis as the first line diagnostic test for NCAH. However, it is essential for genetic counseling since many patients with NCAH carry a severe allele, which may result in findings associated with CAH in the progeny.[52]

The measurement of serum 11-deoxycortisol may be used to diagnose 11β-hydroxylase deficiency when 17α-OHP is elevated. This is a rarer condition that may be associated with hypertension.

The measurement of serum 17α-hydroxypregnenolone (and the ratio of 17α-OHP to 17α-hydroxypregnenolone), as well as the ratio for DHEA to androstenedione is useful in the diagnosis of 3β-hydroxysteroid dehydrogenase deficiency.[60] In adults, no genetic mutations in the 3βHSD gene have been uncovered and most patients previously diagnosed with this enzymatic deficiency probably have PCOS with an enhanced adrenal component.[61]

Adrenocorticotropic Hormone Stimulation Test

Because serum androgens may arise from different sources in women, several tests have been established to distinguish adrenal from ovarian hyperandrogenism. However, most tests are not specific and their use is generally limited. Only the ACTH stimulation test is currently used.

The ACTH stimulation test is generally used for the diagnosis of cortisol deficiency or to uncover mild adrenal enzymatic deficiencies such as CAH. Also, in the past it has been used to distinguish between adrenal and ovarian sources of hyperandrogenism, but is not generally used for this purpose because of its lack of specificity.

The test is best performed between 8 and 9 a.m., with 0.25 mg of cosyntropin injected intravenously. While the test may also be accomplished by intramuscular injection, more consistent results have been obtained with intravenous administration. Blood samples are generally obtained at 30 and 60 minutes, but the 60-minute value suffices as a single time point for practical purposes. For the diagnosis of nonclassic 21-hydroxylase deficiency, serum cortisol and 17α-OHP are measured. Serum 11-deoxycortisol may be evaluated if the rare 11-hydroxylase defect is suspected. Nonclassic 21-hydroxylase deficiency may be diagnosed if peak 17α-OHP is higher than 10 ng/mL[2] (Fig. 34.5).

Hormonal Evaluation of Hirsutism

Hirsutism is excessive hair growth in women where it is not normally found, usually with a central body distribution. When this biologic signal is manifest, laboratory tests may be indicated to pinpoint the abnormality. Most commonly, total testosterone, DHEAS, and SHBG are measured (as well as 17α-OHP to exclude nonclassic congenital adrenal hyperplasia). In about 10% to 15% of hirsute women, all of these hormone levels are in the normal range and with the occurrence of normal menstrual cycles, the diagnosis of "idiopathic hirsutism" is made.[62] While it is not our intent to discuss details of the differential diagnosis of hirsutism, a simple algorithm is provided in Fig. 34.6 that describes the possible diagnoses. There are several other algorithms for this evaluation, namely that of the Androgen Excess and PCOS Society[63] and a recent updated guideline by the

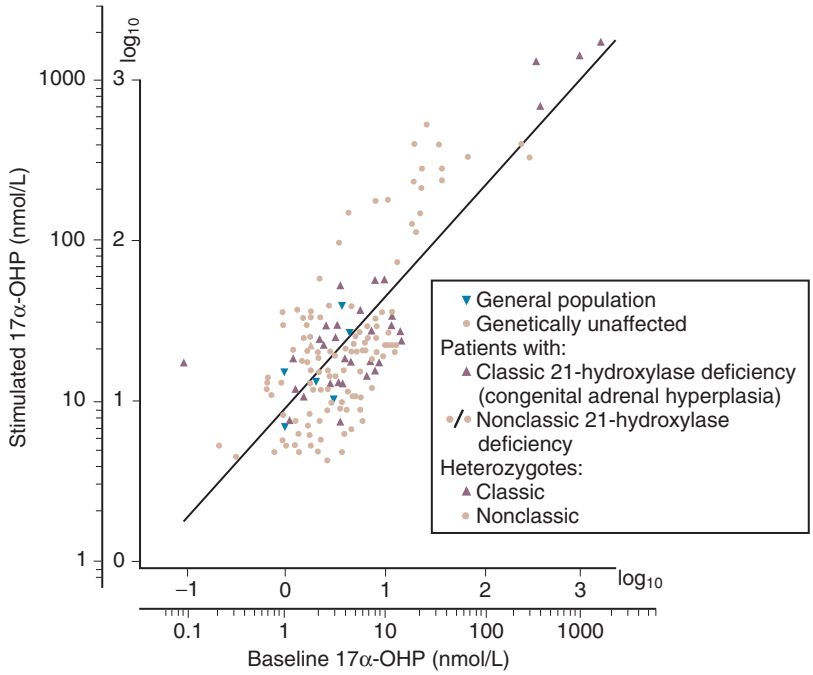


FIGURE 34.5 Nomogram of 17α-hydroxyprogesterone *(17α-OHP)* in different types of patients with congenital adrenal hyperplasia. *(Modified from New MI, Lorenzen F, Lamer AJ, et al: Genotyping steroid 21-hydroxylase deficiency: hormonal reference data.* J Clin Endocrinol Metab *57:320–326, 1983.)*

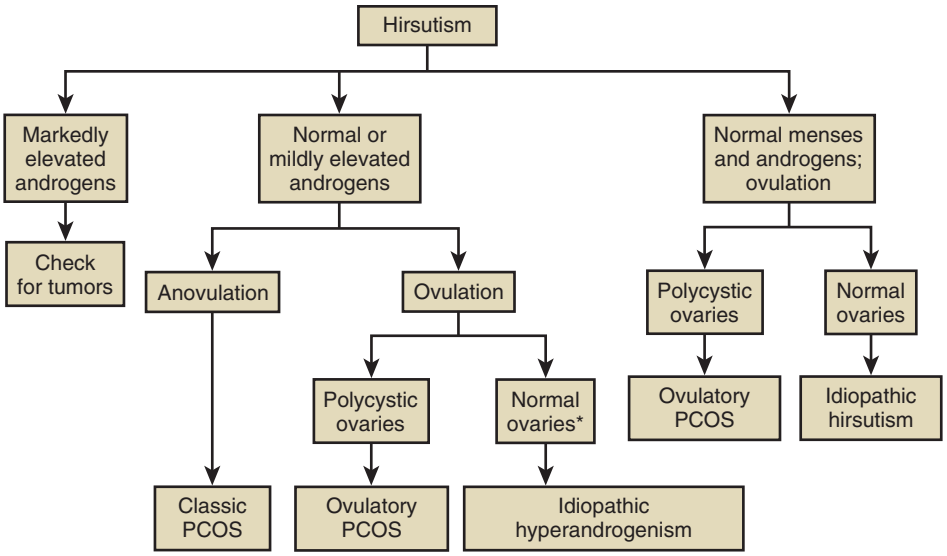


FIGURE 34.6 Diagnosis of hirsutism. *Note that the ultrasound diagnosis of polycystic ovaries is somewhat subjective. Therefore the diagnostic distinction between ovulatory polycystic ovary syndrome *(PCOS)* and idiopathic hyperandrogenism is fluid.

Endocrine Society.[64] Specifically the latter guideline stresses that only the measurement of testosterone is needed initially, with other measurements being reserved for when there is a suspicion for other disorders such as PCOS or NCAH.

Blood Levels of Androgens in Men

In adult men, total and unbound testosterone are the only androgens measured in the evaluation of testicular function. Serum testosterone levels range between 300 and 1000 ng/dL (a slightly lower range with more sensitive assays). Serum testosterone decreases during the day and in the evening the levels are approximately 15% lower.

Similar to women, the reference ranges for bioavailable and free testosterone depend on the assay methods used. The reference range for bioavailable testosterone in one large clinical diagnostic laboratory is 60 to 430 ng/dL; for free testosterone, it is 52 to 280 ng/mL. These assays may be useful in patients with low testosterone values. Serum DHT values range between 30 and 86 ng/dL, but this assay is not recommended on a routine basis.

In evaluating children and neonates for disorders of sexual differentiation, serum DHT and testosterone are helpful for ruling out 5α-reductase deficiency, where the ratio of testosterone to DHT is increased, exceeding 10.

Measurement of Anti-Müllerian Hormone

- *Anti-müllerian hormone (AMH) is a valuable marker of granulosa and Sertoli cell function and in women has become a valuable marker in the assessment of ovarian reserve and the trajectory to menopause.*
- *The measurement of AMH has not been consistent with several assays being available and the lack of an international standard.*

AMH is a homodimeric glycoprotein linked by disulfide bonds with a molecular weight of 140 kDa that belongs to the transforming growth factor-β (TGF-β) superfamily. AMH is generally measured by ELISA assays; this measurement requires close scrutiny of values depending on how the samples have been analyzed.[65] Lack of an international standard for AMH is the major reason for this inconsistency. Initially, two different commercial ELISA kits were available to measure AMH: one produced by Immunotech (IDT) and the other by Diagnostic Systems Laboratory (DSL). However, the two companies were acquired by Beckman Coulter, and both kits continued to be available until 2010 when Beckman Coulter developed a second-generation (Gen II) AMH ELISA kit. The antibodies from the DSL kit were used in the Gen II assay, which was standardized to the IDT assay.[66] Inconsistencies in performance of this assay were reported,[67] which were apparently due to complement interference in the assay with fresh samples; a modified protocol had to be developed. Recently, two automated AMH assay were developed using the modified Gen II assay (Roche Elecsys and Beckman Coulter Access) and seem to be more reliable than the original Gen II assay.[68] Two additional commercial AMH ELISA kits have been developed by Ansh Labs. These two kits use the same monoclonal antibody pair directed against specific linear epitopes in the stable pro and mature regions of the associated form of human recombinant AMH, and have been shown to be accurate and reliable.[69] The ultrasensitive AMH assay (Ansh Lab) gives higher normal values than the Gen II method but has a lower detection limit (0.34 pmol/L). In almost 70% of patients with undetectable values, the pico AMH ELISA kit yielded a measurable result.[69]

AMH plays an important role in male sex differentiation as its production by the embryonic Sertoli cells induces the regression of müllerian ducts. Prepubertal boys have high circulating levels of AMH. The levels of AMH diminish markedly in boys as they approach puberty, with values in adults being about 10% of the levels found in infancy.[70]

In women, AMH is produced by the granulosa cells of preantral and small antral follicles and values are virtually undetectable at the birth but increase gradually until puberty and then remain relatively stable during the early reproductive period.[65] By age 30, AMH levels start declining and become very low during the perimenopausal period.[71] Fig. 34.7 depicts prospective data on the decline in AMH levels with aging in individual women (panel A) and also the decline to undetectable levels close to menopause (panel B).

The measurement of AMH in blood represents an important tool in differentiating a number of alterations in both males and females. At birth, the AMH assay may be used in the evaluation of disorders of sexual differentiation, with ambiguous genitalia (neonates with testes have much higher levels than normal female neonates).[70,72] In males, the measurement of AMH may be useful in cryptorchidism and distinguishing between hypogonadotropic hypogonadism and delayed puberty.[72]

In women, AMH has been used to assess ovarian reserve, the risk of hyperstimulation with gonadotropin therapy, and has been suggested to be useful in the diagnosis of PCOS.[71] Serum AMH reflects the number of small preantral follicles, which are increased in women with PCOS.[73,74] While different cutoff values have been reported on AMH levels that suggest the diagnosis of PCOS, a meta-analysis of the literature and our own data show that a cutoff of 4.7 ng/mL (using the

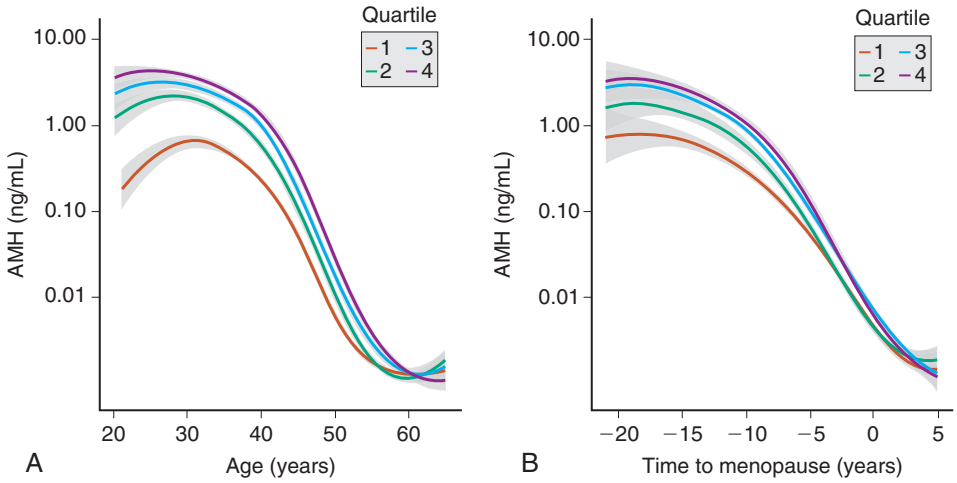


FIGURE 34.7 Anti-müllerian hormone *(AMH)* decline with age and time to menopause. *White lines* represent individual trajectories based on observed AMH levels. *Colored lines* represent the group trajectories of women based on baseline age-specific AMH quartiles and *gray areas* indicate the standard error of a group trajectory. (A) The trajectories of women in the baseline age-specific AMH quartiles are distinguishable until they overlap between ages 55 and 60. The difference between women in low and high age-specific quartiles is largest at age 20. (B) The trajectories of women in the baseline age-specific AMH quartiles are distinguishable until they overlap around 5 years before the final menstrual period. The difference between women in low and high age-specific quartiles is largest at 20 years before the final menstrual period. *(Data from de Kat AC, van der Schouw YT, Eijkemans MJ: Back to the basics of ovarian aging: a population-based study on longitudinal anti-Müllerian hormone decline.* BMC Med *14:151, 2016.)*

GEN II assay) is useful in differentiating between normal and those with PCOS.[75,76] However, it is mainly useful in women with "classic" PCOS—Rotterdam phenotype A.[76] AMH is less sensitive in mild (ovulatory and normoandrogenic, phenotypes C and D) forms of PCOS.[76]

AMH values have been found to be increased in about 30% to 40% of women with functional hypothalamic amenorrhea,[77] where some women with this diagnosis have an increased number of small preantral follicles. AMH may also be useful in some forms of hypogonadism[71] where low levels of AMH are found.

In PCOS, as in normal women, serum AMH begins to decline around age 30, and the decline becomes more marked after the fourth decade (see Fig. 34.7). Depicted in this figure are individual declines over time in a normal population of women (panel A). We have observed in a longitudinal follow-up of women with PCOS that those women who have lower AMH values with aging have a higher probability of becoming ovulatory with aging (Fig. 34.8).[78]

The trajectory of declining AMH has been used to help determine the age of menopause,[79] although the clinical utility of this is questionable. In general, once AMH levels are undetectable, menopause occurs in 4 to 5 years (Fig. 34.9; see also Fig. 34.7, panel B).

Measurement of Inhibins

- *Inhibin B is highest in the follicular phase, while inhibin A peaks in the luteal phase, reflecting production by different populations of granulosa cells.*
- *Inhibin A is useful as a marker in obstetrics, and inhibin B is useful as a tumor marker and when low in the follicular phase reflects poor ovarian reserve.*

Serum inhibin A and B may be measured by specific immunoassays and reflect the secretion from various populations of granulosa cells. Serum values vary in adult women during the menstrual cycle, with inhibin A being lowest during the follicular phase and peaking during the midluteal phase; inhibin B exhibits an opposite pattern (Fig. 34.10).[80] During pregnancy, inhibin A decreases during the second trimester while inhibin B rises from the third trimester to peak at term.[81] After menopause, both inhibins levels decline markedly.[82]

The measurement of inhibin may be useful in several conditions.[83] Inhibin A increases in gestational diseases such as preeclampsia and fetal Down syndrome, and this increase in inhibin A improves the early diagnosis of both conditions. Inhibin B increases markedly in women with granulosa cell tumors, along with AMH, and it is particularly useful for postoperative surveillance, where it can signal tumor recurrence.[84] Inhibin B decreases in women with declining ovarian function and correlates with a poor response to ovulation induction. A low level of inhibin B on day 3 suggests declining ovarian reserve.

In men, inhibin B is a good marker of Sertoli cell function and its measurement is useful in the diagnosis of the different forms of hypogonadism, particularly in boys.[85]

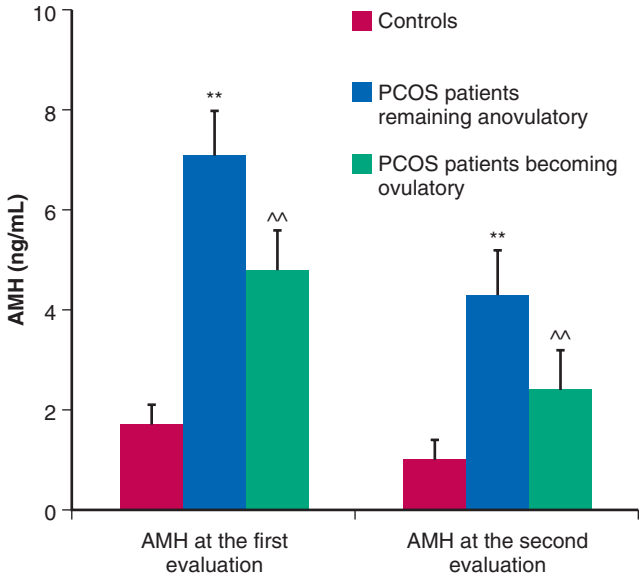


FIGURE 34.8 Decline in anti-müllerian hormone *(AMH)* in women with polycystic ovary syndrome *(PCOS)* and controls in a 20 year follow-up study where AMH declines with age but women with lower AMH levels have more ovulatory cycles. $P < .01$ versus controls and PCOS becoming ovulatory, $P < .01$ versus controls. *(Modified from Carmina E, Campagne AM, Mansueto P, Vitale G, Kort D, Lobo R: Does the level of serum antimüllerian hormone predict ovulatory function in women with PCOS with aging?* Fertil Steril *98[4]:1043–1046, 2012.)*

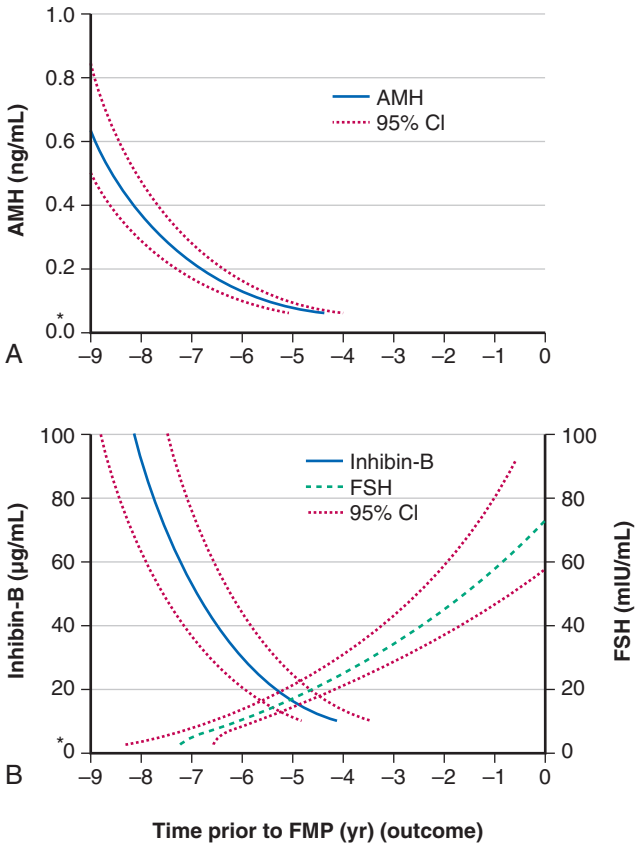


FIGURE 34.9 Anti-müllerian hormone *(AMH)* and inhibin-B and follicle-stimulating hormone *(FSH)* concentrations in relation to the last menstrual period. (A) Decline in AMH to undetectable levels in a prospective cohort from Study of Women Across the Nation. "0" denotes menopause (final menstrual period *[FMP]*). (B) Decline in inhibin B and rise in FSH to FMP. *CI*, Confidence interval. *(Modified from Eyvazzadeh AD, McConnell D, Sowers MR, et al: Anti-mullerian hormone and inhibin B in the definition of ovarian aging and the menopause transition.* J Clin Endocrinol Metab *93[9]:L34768–L34783, 2008.)*

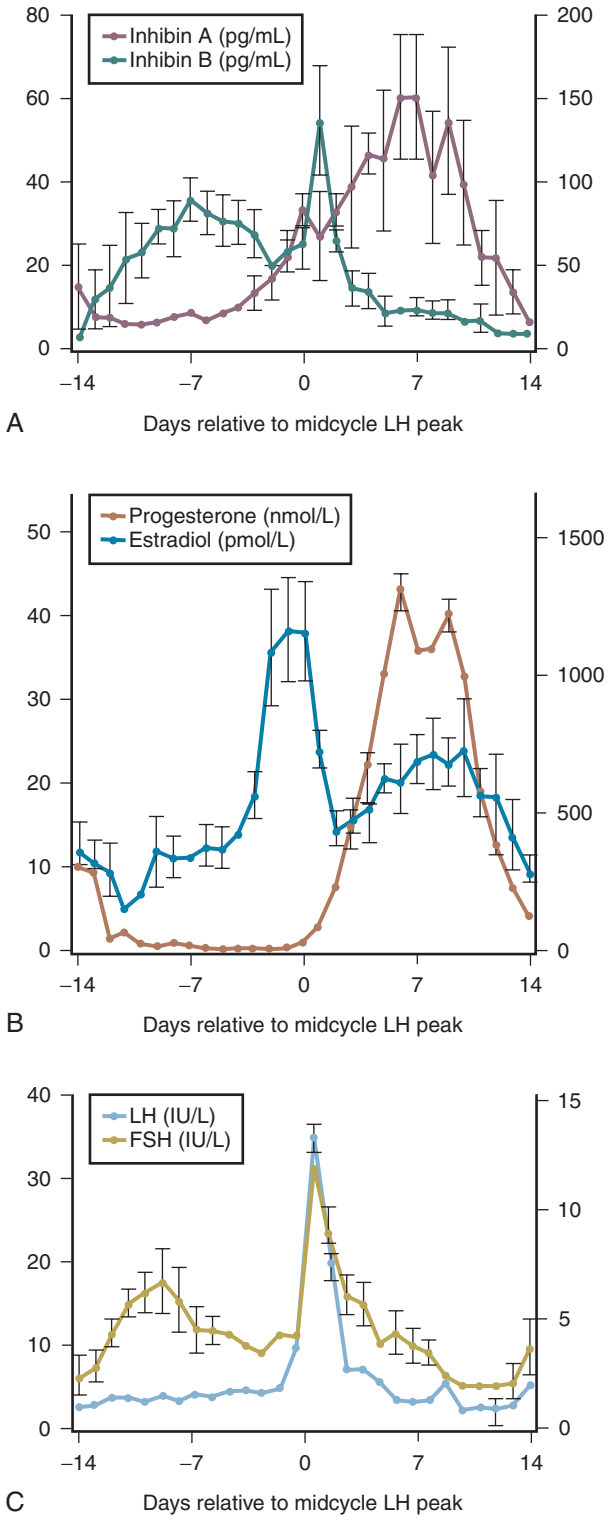


FIGURE 34.10 Plasma concentrations of inhibin A and inhibin B. (A) Estradiol and progesterone (B), and luteinizing hormone *(LH)* and follicle-stimulating hormone *(FSH)* (C), during the female menstrual cycle. Data displayed with respect to the day of midcycle LH peak. Mean concentrations are shown ± standard error of the mean. *(Modified from Groome NP, Illingworth PJ, O'Brien M, et al: Measurement of dimeric inhibin B throughout the human menstrual cycle.* J Clin Endocrinol Metab *81:1401–1405, 1996.)*

Assessment of Ovarian Reserve

◆ *While several methods are available to assess ovarian reserve, the most valuable markers are the antral follicle count (AFC) and AMH levels, which have been found to have equal predictive value.*

Assessing ovarian reserve has become an extremely important part of the management of women with reproductive disorders. While ovarian aging becomes accelerated in the late 30s, even younger women, particularly those who have undergone cancer treatments, may have diminished ovarian reserve.[86] In the past, a day 2 to 3 serum FSH level along with an estradiol level had been the mainstay of assessment.[87] This was followed by the clomiphene stimulation test, which is still carried out occasionally, but has been shown by meta-analysis to be no more valuable than the day 3 FSH assessment.[88] Day 3 FSH values (with estradiol levels <75 pg/mL) should be less than 10 mIU/mL, and values above 15 mIU/mL suggest a poor prognosis for ovarian stimulation, although due to cycle variation, repeated FSH values may be lower in subsequent cycles.

The clomiphene challenge test is carried out by baseline determinations of estradiol and FSH on day 3 and then the administration of clomiphene 100 mg on days 5 to 9. On Day 10 FSH is measured again. FSH levels above the normal value for the lab (usually 10 to 12 mIU/mL) on either day 3 or 10 is considered an abnormal test and is indicative of an abnormal ovarian reserve.

Day 2 to 3 inhibin B measurements have also been studied but these measurements exhibit wide variability and are not reliable as a sole assessment tool.[89,90] Also, the assessment of ovarian response has been assessed with short-term gonadotropin or GnRH agonist stimulation. However, these assessments are not frequently used.

Currently, obtaining an early follicular phase AFC by ultrasound (described later) and the measurement of AMH are considered the better indicators of ovarian reserve and are equally valuable by ROC analysis (Fig. 34.11).[91] Measurements of AMH are not as cycle dependent as the fluctuations which occur during the menstrual cycle; however, use of contraceptive steroids will lower AMH values by as much as 20%.[92]

AMH and AFCs decrease with age in all women and values are usually interpreted on an age-specific basis. However, critical values suggesting the potential of a diminished ovarian response at any age are values of 1.0 ng/mL for AMH and/or an AFC value of less than 8.[91]

Measurement of Human Chorionic Gonadotropin

◆ *There are several circulating forms of hCG. hCG is most useful for pregnancy diagnosis, the assessment of a viable intrauterine pregnancy, molar pregnancies, and as part of a panel for the noninvasive detection of aneuploidy.*

Many tissues, including the pituitary, produce hCG (at very low levels) while the placenta produces the largest quantities of hCG. Because of the heterogeneity in the hCG

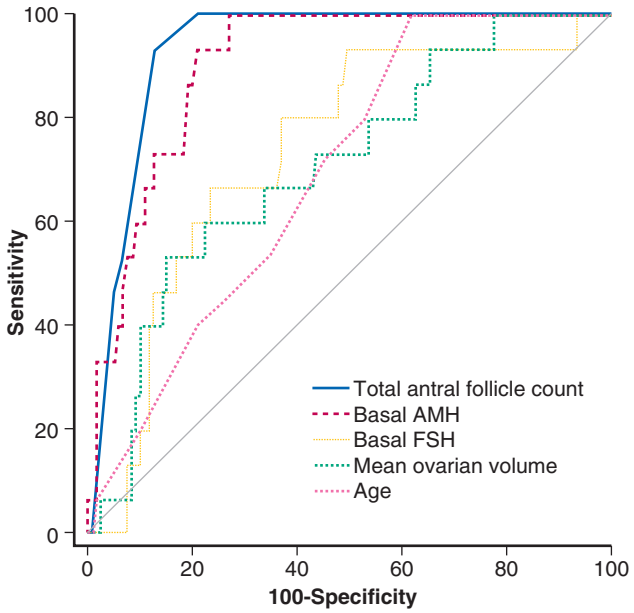


FIGURE 34.11 Prediction of poor response to controlled ovarian hyperstimulation. Antral follicle count *(AFC)* and anti-müllerian hormone *(AMH)* are best; the combination is not better. *FSH,* Follicle-stimulating hormone. *(Modified from Jayaprakasan K, Deb S, Batcha M, et al: The cohort of antral follicles measuring 2-6 mm reflects the quantitative status of ovarian reserve as assessed by serum levels of anti-müllerian hormone and response to controlled stimulation.* Fertil Steril *94[5]:1775–1781, 2010.)*

molecule and the various forms such as "nicked" and "β core fragment," choosing the assay method is key in determining what is occurring in a clinical situation, and possibly repeating the measurement in a different assay system if there is an unexpected result. For example, low levels of hCG that are falsely positive may occur due to cross-reacting heterophile antibodies and can be correctly determined by switching from a "sandwich" based assay to an RIA or measurement in urine. Among the automated assays available, the Siemens Immulite system has been shown to be able to detect 8 out of 9 variants of hCG, while some other commercial assays are not able to detect many of the varied forms.[93]

Apart from certain assays incorrectly detecting positive hCG levels, there are situations where measurements of hCG are real when the individual is neither pregnant nor has a tumor such as choriocarcinoma. Familial hCG syndrome occurs when low levels of hCG are present and is due to biologically inactive variants of hCG which are picked up in various assays but do not have biological significance; this occurs as a genetic variation in metabolism.[94] The second source of authentic hCG secretion occurs when the pituitary source is increased, as occurs in postmenopausal women. Here the average hCG concentration has been found to be 11 mIU/mL, but is suppressible by pituitary suppression as with high doses of estrogens and progestogens.[95]

During pregnancy, hCG levels increase rapidly, with levels doubling approximately every 2 days until 12 weeks. After the third month of pregnancy, hCG levels decrease and plateau at a constant level until term. These assays are used for diagnosis and follow-up of normal intrauterine pregnancy, with abnormally rising values suggesting an abnormal pregnancy (either an ectopic pregnancy or one destined for early pregnancy loss).[96] It has been determined that the minimal rise for a normal pregnancy over 2 days based on the first percentile is a rise of 53%.[97] Pregnancies with values over 2 days exceeding a 53% rise can be handled expectantly, while values below this threshold signify an abnormal pregnancy. Because the exact timing of when blood is obtained for this assessment is a variable, it has been suggested that the number of 53% might be even lower for a normal pregnancy. However, this has been recently studied with the conclusion suggesting that there should be no change in the minimal threshold for viability.[98]

Measurements of β-hCG during pregnancy, when combined with other tests (pregnancy associated plasma-protein A and inhibin A), as well as nuchal translucency by ultrasound, may be useful for screening for aneuploidy such as in Down syndrome.[99]

Assays designed for detection of the free hCGβ subunit are particularly useful for the assessment and follow-up of trophoblastic tumors.[100] Measurement of the hyperglycosylated form of hCG also is most useful in the diagnosis and follow-up of trophoblastic tumors.[101] Having an increased proportion of hyperglycosylated hCG over total hCG (reflecting the invasive biological activity of this moiety) correlates well with the finding of an early successful intrauterine pregnancy.[102]

Assessment of Glucose Metabolism, Insulin Activity, and Adipose Mass

- *There are several methods used for the clinical assessment of insulin sensitivity that are merely surrogates for insulin clamp studies, which remains the gold standard.*
- *There are several adipokines which may be valuable to assess, and of these, leptin and adiponectin are the most useful at present.*
- *Adipose mass may be measured in several ways. Dual x-ray absorptiometry (DEXA) scans have been the most used.*

Normal fasting blood glucose levels are lower than 100 mg/dL. Any of the following criteria are diagnostic for diabetes[103]:

1. Fasting plasma glucose (FPG) ≥126 mg/dL (7.0 mmol/L).
2. Two-hour plasma glucose ≥200 mg/dL (11.1 mmol/L) during an oral glucose tolerance test (OGTT).
3. Glycated hemoglobin A1C (HbA1C) ≥6.5%.

Prediabetes represents an elevation of plasma glucose above the normal range but below that of clinical diabetes. Prediabetes is diagnosed as either impaired fasting glucose (IFG) (fasting blood glucose levels between 100 and 125 mg/dL) or impaired glucose tolerance (IGT). The latter is detected by oral glucose tolerance testing (see later discussion) with blood glucose levels greater than 140 mg/dL 2 hours after glucose administration indicating IGT. Both IFG and IGT are risk factors for type 2 diabetes, and risk is even greater when IFG and IGT occur together.

Glycated hemoglobin A1c (HbA1c) represents a useful tool for predicting and diagnosing diabetes mellitus. HbA1c has slightly lower sensitivity than FPG in detecting diabetes, but slightly higher specificity and a cutoff value of greater than 6.1% may be used for the diagnosis of diabetes mellitus. However, because optimum cutoff values vary by ethnic

group, age, gender, and population prevalence of diabetes, a slightly higher value of 6.5% may be used.

Different strategies exist for diagnosing gestational diabetes mellitus (GDM).[104,105] The most used approach involves screening with a 1-hour 50-g glucose challenge test with a cutoff point at 140 mg/dL followed by a 3-hour 100-g glucose tolerance test (a two-step approach). Other diagnostic strategies rely on the administration of a 2-hour glucose tolerance test (a one-step approach) with plasma glucose evaluated fasting, and after a 75-g glucose load. Using the one-step approach, the diagnosis of GDM requires at least two of the following four venous plasma glucose levels:

1. FPG ≥105 mg/dL (5.8 mmol/L).
2. One hour plasma glucose after OGTT ≥190 mg/dL (10.6 mmol/L).
3. Two hour plasma glucose after OGTT ≥165 mg/dL (9.2 mmol/L).
4. Three hour plasma glucose after OGTT ≥145 mg/dL (8.1 mmol/L).

Measurement of Serum Insulin

Serum insulin may be measured by different immunoassays. Use of heparin causes falsely elevated values, while hemolysis of the blood may result in falsely low values. Insulin antibodies in blood result in low levels in some assays and high levels in others.

Using commercial assays, normal fasting insulin levels range between 5 and 15 μU/mL but with more sensitive assays normal fasting insulin should be lower than 12 μU/mL. Obese subjects have increased values, while very high circulating levels are found in patients with severe insulin resistance. Forty to 50% of women with normal BMI who have classic PCOS have elevations in fasting insulin levels,[106,107] with levels in obesity being significantly higher in women with PCOS, reflecting more insulin resistance.[107]

Evaluation of Insulin Resistance

Insulin resistance is extremely important in health and disease and has an important role in diabetes, metabolic syndrome, and has also been implicated in the pathophysiology of PCOS. Insulin resistance is generally considered to be operative in peripheral tissues (muscle and fat). There are several ways to assess peripheral insulin sensitivity.[108] The most precise methods involve the use of glycemic clamps. However, these methods are very cumbersome and should be reserved for research purposes. Use of the minimal model of Bergman applied to a frequently sampled intravenous glucose tolerance (FSIGT) test is considered to be the "gold standard" for assessing insulin resistance. This method is as sensitive as the euglycemic insulin clamp.[109] An insulin tolerance test (ITT) is a good alternative for use in clinical practice.[110] Fig. 34.12 provides data on the kinetic decline in glucose after insulin KITT in various states.

Methods based on fasting blood concentrations of glucose and insulin at one time point, with the use of various calculations (glucose/insulin ratio, HOMA-IR and QUICKI and others—see later), are highly dependent on the quality of insulin assay and several other clinical variables. These methods should not be used when β-cell function is impaired (as in diabetes mellitus or in the elderly). In addition, these methods measure mostly hepatic, and not peripheral, insulin sensitivity. With all these limitations, these methods may be useful in the clinical setting, and have been used to follow the effect of various interventions in clinical trials, for example in patients with PCOS who do not have diabetes.[111] Methods based on the OGTT have similar limitations as the fasting measurements of glucose and insulin but have the advantage of providing information on glucose tolerance.[112]

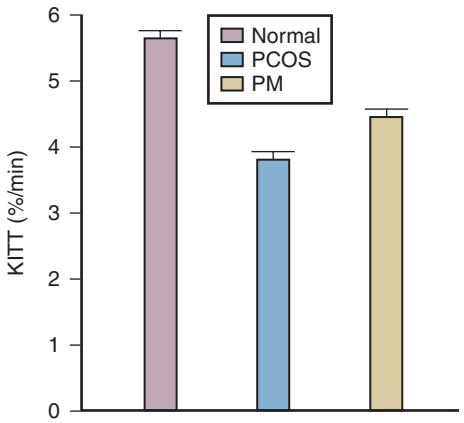


FIGURE 34.12 Insulin tolerance test to evaluate insulin sensitivity. *PCOS*, Polycystic ovary syndrome; *PM*, postmenopausal women. *(Modified from Carmina E, Lobo RA: Dynamic tests for hormone evaluation. In Lobo RA, Mishell DR, Paulson Rj, Shoupe D, editors:* Mishell's Textbook of Infertility, Contraception, and Reproductive Endocrinology, *ed 4, Oxford, 1997, Blackwell Science.)*

Oral Glucose Tolerance Test

The OGTT is generally used to evaluate glucose tolerance, but may be also used to obtain an estimate of insulin resistance. The test is generally performed administering 75 g of glucose orally after an overnight fasting. Blood samples are taken before the glucose administration and each 30 minutes for 2 to 3 hours. For evaluating glucose tolerance, a blood glucose measurement at 120′ after glucose administration is sufficient, with values ≥140 mg/dL indicating IGT and values ≥200 mg/dL showing type II diabetes. For assessing insulin resistance, serum insulin also should be measured and blood samples at 30′, 60′, and 120′ are needed. At least nine indices have been proposed to measure insulin sensitivity by the OGTT but few show a significant correlation with the "gold standard" clamp techniques. The Matsuda index [(fasting glucose × fasting insulin) × (mean glucose × mean insulin during OGTT)] and the log sum insulin give the best estimates of insulin sensitivity.

Fasting Glucose/Insulin Calculations

These methods are based on mathematical calculations applied on simultaneously obtained fasting glucose and insulin values. The most frequently used methods are:

- Glucose: insulin ratio
- Homeostasis model assessment of insulin resistance (HOMA-IR) = (glucose × insulin) ÷ 22.5
- Logarithm of the HOMA-IR
- Quantitative insulin sensitivity check index (QUICKI) = 1 ÷ (log glucose + log insulin)
- HOMA-IR/Adiponectin index [(fasting glucose × insulin)/(22.5 × fasting adiponectin)].

As already noted, these methods are easy to perform and useful in a clinical setting but have several limitations.[108] HOMA-IR is the most commonly used method and values higher than 2.7 suggest insulin resistance. However, because the relationships between the insulin and the glucose level are hyperbolic rather than linear, methods using logarithmic transformation may be preferable. We generally prefer to use the QUICKI method because it is easy to perform and correlates reasonably well with the euglycemic clamp. Values lower than 0.335 suggest insulin resistance and has been found to be present in most women with PCOS.[102] Recently, an index including the measurement of adiponectin, to account for adipocyte function in the index, the HOMA-IR/Adiponectin index has been suggested as a reliable method for assessing insulin resistance. While valuable, it may not add substantially more information than HOMA-IR alone.[113]

Measurement of Adipose Hormones

Adipose tissue is a complex endocrine organ that secretes hormones, cytokines, free fatty acids, and various proteins that have profound effects on metabolism and the cardiovascular system.[114] In recent years, many of these factors have been characterized and measured in various endocrine disorders, including PCOS.[115] While most assays are useful only for research purposes, the measurement of two adipose hormones, leptin and adiponectin, also may be useful in clinical practice.

Leptin is a 146-amino acid protein that acts mostly as a signaling factor from adipose tissue to the central nervous system to serve as a metabolic indicator of energy sufficiency.[114] It may be measured by RIA or ELISA and, in our laboratory, in adult women with normal body weight, leptin values are between 10 and 30 pg/mL. Serum levels of leptin increase in obese subjects, probably because in obesity there is a condition of leptin resistance. However, some massively obese subjects have a genetically determined leptin deficiency and the finding of low leptin in severe obesity suggested the possibility of using leptin in the treatment of these subjects. In PCOS, the levels of leptin are strictly related to body weight,[115] and are not different when compared to weight-matched normal women. Finally, leptin levels are lower in children than in adults and increase progressively during puberty.[116]

Adiponectin is a large protein comprising 244 amino acids which has a major role in preventing or counteracting the development of diet-induced insulin resistance.[117] Moreover, adiponectin has been reported to have an important function in protecting endothelial cells from injury.[118] Adiponectin may be measured by RIA or ELISA and in our laboratory, in adult women with normal body weight, adiponectin values are between 8 and 18 μg/mL. Adiponectin is a heterogeneous molecule which has low-, intermediate-, and high-molecular-weight forms. While in the past, total adiponectin has been measured as noted previously, it is the high-molecular-weight form which is most biologically active, and specific ELISAs for this specific measurement are now available. The measurement of high-molecular-weight adiponectin has been shown to have a better predictive power for detecting insulin resistance and metabolic syndrome than total adiponectin.[119] Adiponectin is decreased in metabolically active obesity, probably because of the increased cytokine production in visceral and ectopic adipose tissue,[120] and this reduction has an important role in facilitating the effects of obesity on insulin resistance, endothelial disease, and cardiovascular risk.[121]

In PCOS, adiponectin is lower than that in weight-matched controls, and serum levels less than 8 μg/mL may suggest a particular risk for developing early endothelial disease.[121]

Evaluation of Fat Quantity and Distribution

Because excess fat and/or altered fat distribution represent very important factors in determining insulin resistance and metabolic and cardiovascular risk, many methods have been developed to assess these parameters.

The most simple way to assess fat quantity is to calculate the BMI by the formula: body weight (kg)/height^2 (cm). Values of 30 or higher indicate obesity, with values over 40 indicating severe obesity. Values between 25 or more and less than 30 suggest an overweight status while values between 19 and less than 25 indicate normal body mass. However, BMI does not take into account differences in lean muscle mass and bone mass; thus it is more valuable to measure fat quantity directly by total body DEXA.[122] This method permits the measurement of fat quantity in the total body and in several different areas. Particularly important is the measurement of fat quantity in the trunk because this represents a way to assess abdominal fat mass. An increase in abdominal fat, mainly visceral fat, has been suggested to be the main cause of cardiovascular and metabolic risks of obesity.

Because of the role of abdominal fat, it has been suggested that the measurement of waist circumference is more important than the evaluation of body weight in assessing the health problems linked to obesity. In women, values of 88 cm or higher indicate excessive abdominal fat accumulation and increased metabolic and cardiovascular risks. However, in young women, a normal waist circumference is less than 80 cm and values greater than 80 and less than 88 cm may indicate some abdominal fat increase.

To assess abdominal fat more accurately, other methods may be useful. Abdominal CT or MRI are very sensitive tests but cannot be used routinely in clinical practice for this purpose, and, therefore, abdominal ultrasound or DEXA are generally the preferred methods. Ultrasound is a simple method to assess abdominal fat but requires skill and is operator-dependent. With this method, omental thickness in centimeters is assessed by using the distance between the posterior surface of the aponeurosis of the rectus abdominal muscle and the anterior wall of the aorta, 5 cm above the umbilicus.

DEXA scans are simple and sensitive, but the software used usually also measures fat in the entire trunk, which includes the thorax. The sensitivity of this method may therefore be enhanced by measuring abdominal fat in an area of 50 cm^2 around the central point of the midline between the lateral iliac crests and the lowest rib margins at the end of a normal expiration.[107] This point generally corresponds to the umbilicus (but may not) and is located in the midline. Other authors have preferred to measure fat quantity between L1 and L4. Because DEXA measures total body fat and trunk (or central abdominal) fat, it is possible to calculate with the same examination the percentage of abdominal fat.

Table 34.1 Evaluation of Abdominal Fat by Dual X-Ray Absorptiometry*

	Mean	Upper Normal Limits	
Trunk fat (g)	5200	8000	High values indicate abdominal fat accumulation
Trunk fat (%)	31.4	38	High values indicate abnormal fat distribution (abdominal obesity)
Central abdominal fat (g)	300	520	High values indicate abdominal fat accumulation. More sensitive index than trunk fat

DEXA, Dual X-ray absorptiometry.
*Modified from Carmina E, Legro RS, Stamets K, Lowell J, Lobo RA: Difference in body weight between American and Italian women with polycystic ovary syndrome: influence of the diet. *Hum Reprod* 11:2289-2293, 2003.

We have recently determined normal values and the upper limits (Table 34.1) of fat parameters (by DEXA) in young normoweight women. In general, values of 40% or more of trunk fat indicate the presence of abdominal obesity.[107]

Depending on the population studied, 30% to 60% of women with PCOS may be considered obese and most of them will have abdominal obesity.[123] However, 60% of overweight women with PCOS and 30% of normoweight women with PCOS will be found to have an increase in abdominal fat.[107]

Measurement of Growth Hormone and Growth Factors

- *Circulating growth hormone (GH), measured by immunoassay, may not be sufficient to assess GH status and this is complemented by provocative stimulation tests and by measurements of IGF-1 and IGF-BP3.*

Circulating GH may be measured by chemiluminescent RIA or immunoassay. With any method, because of very low basal values and pulsatile secretion, measurement of an unstimulated random GH is usually not very meaningful.[124] Overnight urinary GH may be useful[125] but the urinary concentrations of GH are also very low and may not be detectable in some assays. There is also a high degree of variability in GH renal excretion.

Insulin-like growth factor 1 (IGF-1) is measured by specific immunoassays and, because these values reflect the integrated 24-hour concentrations of serum GH, IGF-1 may be used for screening for GH deficiency.[126] Finally, because IGF-1 is low in malnutrition, this assay also may be useful in assessing the general nutritional status of patients.

Diagnosis of Growth Hormone Deficiency

GH is low in basal conditions and its measurement is not useful in the diagnosis of growth hormone deficiency (GHD). A simple alternative to GH measurement is serum IGF-1 (measured by chemiluminescent or ELISA assays), which may be used for the screening of GHD. However, circulating IGF-1 levels are low in prepubertal children, making differentiation between healthy and GH-deficient children difficult; also in adults, IGF-1 measurements have a low accuracy for the diagnosis of GH deficiency.[126] Moreover, several conditions result in low IGF-1 concentrations (malnutrition, hypothyroidism, renal failure, and diabetes mellitus) and therefore the confirmation of the diagnosis requires a reduced response to a GH provocative test.

Many provocative or inhibitory tests to diagnose GH deficiency or excess have been described.[127,128] Although differences exist in expert recommendations and guidelines, the cutoff for a normal peak GH level in response to provocative tests is generally 7 ng/mL.

Significant problems exist with GH stimulation tests. False positive results are common in normal prepubertal children. In addition, peak GH is reduced in obesity and in adults, requiring the need for specific cutoff values. Because of this, the reduced GH response to at least two provocative tests is needed for making a firm diagnosis of GHD.

Insulin Tolerance Test

This test is the preferred first choice for the diagnosis of GH deficiency. Blood samples are obtained at 0, 15, 30, 60, and 90 minutes when glucose and GH are measured. The measurement of glucose is required for validation of hypoglycemia (<40 mg/dL), which is necessary to evoke a GH response.

Glucagon Stimulation Test

Intramuscular glucagon administration is a safe test that may be used for the diagnosis of GH deficiency. Glucagon 1 mg is administered after an overnight fast and blood samples for GH are obtained at 60, 120, 150, 180 minutes. The GH peak generally occurs 2 to 3 hours after glucagon administration.

Arginine Stimulation Test

The arginine stimulation test may be a safe alternative to insulin-induced hypoglycemia, particularly for those patients who have a predisposition to hypoglycemia such as patients with diabetes, or in patients with a history of seizures. Following an overnight fast, arginine is administered intravenously 0.5 g/kg (maximum dose 30 g) over 30 minutes.

Growth Hormone–Releasing Hormone and Arginine Test

This test is considered to be an alternative to the ITT for the diagnosis of adult growth hormone deficiency (AGHD) in patients for whom insulin hypoglycemia is contraindicated. Growth hormone–releasing hormone (GHRH; 1 μg/kg) and arginine (0.5 g/kg to a maximum of 30 g in 30 minutes) are combined in the same stimulation test.

Diagnosis of Growth Hormone Excessive Production

Serum IGF-1 is more useful in the diagnosis of GH hypersecretion as occurs in acromegaly, as IGF binding protein-3 (see later). Elevated serum IGF-1 can identify acromegaly

better than several random GH serum measurements and overnight urinary GH tests; however, some acromegalic patients may have normal IGF-1 levels, requiring other investigations to be done if acromegaly is suspected. Serum IGF-1 assessment is particularly useful in evaluating the effects of treatment in acromegalic subjects; finding an increased level of IGF-1 indicates that the disease is not well-controlled. The diagnosis of excessive GH secretion (acromegaly) requires the measurement of IGF-1 as a baseline screening test and the failure of GH to suppress after oral glucose.[129]

Oral Glucose Tolerance Test

The OGTT is the primary test used for the diagnosis of GH hypersecretion. After an overnight fast, 75 to 100 g of oral glucose will cause a suppression of serum GH. Blood samples are obtained every 30 minutes for 2 hours. Using an ultrasensitive assay, failure of GH to suppress to below 0.4 ng/mL is diagnostic of acromegaly. However, because many GH assays do not have adequate accuracy to measure low GH levels, a cutoff for nadir GH during OGTT below 1 μg/L is acceptable for excluding the diagnosis.[129]

Measurement of Insulin-Like Growth Factor-2 and Insulin-Like Growth Factor Binding Proteins

Insulin-like growth factor 2 (IGF-II) may be evaluated by specific IRMA or ELISA, but this level is less dependent on GH secretion than IGF-1. The measurement of IGF-II is useful only in islet-cell tumor hypoglycemia (NICTH), an uncommon syndrome caused by some tumors (generally mesenchymal tumors) and associated with low levels of insulin, IGF-I, and IGF-BP3.[130]

There are six IGF-BPs. Among these, circulating IGFBP-1, IGF-BP2, and IGFBP-3 have been the subject of evaluation for clinical purposes. IGFBP-3 is GH-dependent and reflects changes in GH levels. Its long half-life and nonpulsatile secretion make IGFBP-3 measurements an alternative to IGF-1 measurement for the screening of perturbations in GH.[128]

Levels of serum IGFBP-1 are significantly influenced by hyperinsulinemia, with low levels being found in cases of insulin resistance; for example, in PCOS, where IGF-1 levels are found to be normal, "free" IGF-1 is increased because of a reduced level of IGFBP-1.[131] Serum IGFBP-1 may be measured in diabetes to evaluate whether hyperglycemia is due to inadequate insulin dosage or to other factors.

Serum IGFBP-2 correlates inversely with GH secretion, and the ratio IGFBP-2/IGFBP-3 is a marker of GH action. Serum IGFBP-2 is also increased with some tumors (mostly gliomas and prostate cancers) and may be used to assess the growth of these tumors in conjunction with other markers, such as PSA in the management of certain patients with prostate cancer.[132]

Measurement of Calcium-Regulating Hormones and Bone Markers

- *Measurement of PTH, 25 (OH) vitamin D, and bone markers for resorption and formation complement the assessment of bone mass by DEXA scans.*

In humans, the main calcium-regulating hormones are parathyroid hormone (PTH) and 1,25-dihydroxyvitamin D.

The measurement of PTH is complicated by the complex metabolism of this hormone, which results in a multiplicity of circulating molecular forms with different bioactivities as well as the presence in blood of PTH fragments.

In the past, immunoassays using antibodies that recognize the mid- or carboxyl-terminal regions of PTH have been largely used, but the most used PTH immunoassays are the two site immunoradiometric or chemiluminescent assay for intact PTH. These assays use antibodies directed against the amino-terminal and mid-region (or carboxyl-terminal) fragments.[133] With these methods, normal PTH values range from 10 to 65 pg/mL and elevated levels are found in the majority of patients with primary hyperparathyroidism. In some patients the parathyroid hormone level is normal, but it tends to be in the upper range of normal and thus clearly "abnormal" when hypercalcemia is simultaneously present.

The finding of increased serum PTH and calcium is diagnostic of primary hyperparathyroidism, although some patients may have normocalcemic hyperparathyroidism that results in a form of osteoporosis that is often clinically indistinguishable from postmenopausal osteoporosis. This condition has to be differentiated from the more common mild deficiency of vitamin D, where PTH levels are moderately increased but calcium blood values are normal. In these patients, the administration of vitamin D normalizes serum PTH values.

The clinical assay techniques for circulating monohydroxylated (25-OH) and dihydroxylated (1,25-OH) have been used in the screening of osteoporotic women for underlying vitamin D deficiency.[134] Two assays that have been widely used to measure 25(OH)D include a manual RIA and an automated LIAISON chemiluminescent immunoassay (DiaSorin, Stillwater, Minnesota). Both assays use an antibody-based detection system with 100% recognition of both the D_2 and D_3 forms. However, in the last few years the state of the art assay method uses LC-MS/MS. This assay measures both forms of 25(OH)D with high accuracy and precision, as well as high throughput. In comparison with LC-MS/MS as the gold standard, several automated assays in clinical use were variable in terms of performance. However, the LIASON system performed well.

It has been suggested that a large percentage of the female population, particularly in the Northern hemisphere, is vitamin D deficient. Blood levels of 25-OH-D lower than 10 ng/mL indicate a condition of severe vitamin D deficiency.

Disagreement exists on 25-OH-D values indicating vitamin D deficiency. In fact, the American Institute of Medicine has suggested that serum 25-hydroxyvitamin D values lower than 20 ng/mL indicate vitamin D deficiency[135] while guidelines of Endocrine Society have indicated that all 25-OH-D values lower than 30 ng/mL are low.[136] The American Institute of Medicine indicated a threshold value of 20 ng/mL because it corresponds to 2 SD above the median calculated needs of vitamin D.[135] Putting the threshold value at 30 ng/mL was based on criteria linked to changes in circulating PTH.[136] While it is clear that increases in circulating PTH may reflect the bone effect of mild vitamin D deficiency, it may cause an over diagnosis of vitamin D deficiency in that small changes of PTH may not have a clinical impact on bone metabolism. More recently, based on changes in the femoral neck T-scores by DEXA, a 25-OH-vitamin D cutoff of 25 ng/mL has been suggested to be particularly useful for the diagnosis of mild vitamin deficiency.[137]

Box 34.1 Markers of Bone Turnover

FORMATION (SERUM)
Bone alkaline phosphatase
Osteocalcin
Propeptides of type I collagen:
- Carboxyterminal (PICP)
- Aminoterminal (PINP)

RESORPTION (SERUM)
Telopeptides of collagen cross-links:
- Carboxytelopeptides (CTX)
- Aminotelopeptides (NTX)
- Tartrate resistant acid phosphatase (TRAP)

RESORPTION (URINE)
Pyridinolines cross-links (PyD)
Deoxypiridinolines cross-links (DpD)
CTX
NTX
Hydroxyproline

Several biochemical markers of bone turnover are measurable and may be useful in assessing bone formation and resorption (Box 34.1).[138] Improved immunoassays have increased their role in studying bone metabolism and clinical consequences of osteoporosis. The most sensitive and specific markers of bone formation include serum bone alkaline phosphatase, total osteocalcin, and the procollagen type I N-terminal propeptide assay. Among the various markers of bone resorption, serum C-terminal cross-linked telopeptides are the most sensitive and specific. Markers of bone turnover can be used to predict the rate of bone loss in postmenopausal women, during treatment and can also be used to assess the risk of fractures. In osteoporosis-treatment studies, markers of bone turnover have been found to be even more strongly associated with fracture risk reduction than bone mineral density (BMD).[139] However bone turnover markers should not be used as an alternative to validated measurements of bone density in the clinical evaluation of patients.

Methods for Measurement of Bone Density

Measurement of bone density is essential for the diagnosis of osteopenia and osteoporosis. While many methods have been widely used, including ultrasound evaluation of the calcaneus, a precise determination of bone density requires DEXA. The lumbar spine, the hip, and the femoral/neck are the most important regions to be evaluated. The data are expressed as a T-score, which provides the number of SDs below the mean peak bone density of a normal adult. Values between –1.0 and –2.5 are indicative of osteopenia, and values less than –2.5 indicate osteoporosis. DEXA of peripheral bones (e.g., forearm) is possible and is sometimes used as an additional assessment, particularly in metabolic disease. Osteoarthritis and other degenerative bone diseases may result in artificially higher T scores; therefore this condition can be suspected when there is a discrepancy in T scores between different lumbar vertebrate in the spine.

Bone strength is the most important factor influencing the risk of fracture and is not totally reflected in measurements of bone density. Bone strength is a reflection of bone density and bone turnover as assessed by biochemical indices. A specialized quantified peripheral bone CT scan (qCT) has been suggested to provide such information, akin to a virtual bone biopsy, and has been useful in research studies (see Chapter 14).

Measurement and Blood Levels of Thyroid Hormones

◆ *With the availability of third generation assays for TSH, the diagnosis of thyroid disorders has become more straightforward; the normal range of TSH is lower in pregnancy, and the measurement of anti-thyroid antibodies is important in several clinical states.*

Circulating TSH is measured by two-site noncompetitive IMAs using enzymes, luminescent compounds, or ^{125}I as labels.[140] These assays have completely replaced the original RIAs because of the need for greater sensitivity to distinguish the low levels in hyperthyroid individuals from suppressed levels due to other effects.

Normal serum TSH ranges between 0.5 and 4.0 μU/mL. Increased TSH levels suggest the presence of primary hypothyroidism while suppressed TSH levels (0 to 0.1 μU/mL) are characteristic of Graves disease. However, for a more complete evaluation of thyroid function the measurement of thyroid hormones is necessary.

Circulating total thyroxine (T_4) and triiodothyronine (T_3) and their free fractions may be measured. Many laboratories prefer to measure only total T_4, but in some conditions (such as pregnancy), free thyroid hormones have to be evaluated. Any condition that increases circulating thyroxine-binding globulin (TBG)—as occurs in pregnancy and with estrogen treatment—also increases total thyroid hormone. During pregnancy, TBG increases approximately 2.5 times, with peak levels at 15 to 20 weeks' gestation and normal T_4 levels are higher than 7.8 μg/dL.[141] Free thyroid hormones remain in the normal range, the only exception occurring late in the first trimester, when a transient increase may occur.

The most precise determination of free T_4 is obtained by equilibrium dialysis, while some commercial RIA kits—which use a T_4 analogue—may give inaccurate results.[142]

Disagreement exists on the need for treating patients having mildly elevated TSH values (between 4 and 10 μU/mL) but with normal thyroxine levels (subclinical hypothyroidism). Patients with subclinical hypothyroidism who have normal levels of anti-thyroid antibodies, and those with old age (≥70 years) do not generally need any specific treatment.[143,144]

During pregnancy, the circulating TSH levels are lower and the normal range is 0.1 to 2.5 μU/mL during the first trimester, 0.2 to 3 μU/mL during second trimester, and 0.3 to 3 μU/mL during third trimester. It is particularly important to maintain serum levels of TSH in the normal range (<2.5 μU/mL during first trimester and <3 μU/mL during second and third trimester). Subclinical maternal hypothyroidism is associated with complications of pregnancy and adverse effects on the fetus.[145,146] In these women, l-thyroxine at low doses is started immediately at the beginning of pregnancy and serum TSH is monitored each month, increasing the treatment if TSH levels increase. In general, women who are receiving thyroxine treatment before pregnancy will need

to have their dose increased by 50% at the onset of pregnancy.[145,146]

Because of the need to maintain TSH levels under 2.5 μU/mL in the first trimester, it has been suggested that this level should be achieved by replacement in women attempting pregnancy who have values above 2.5 but less than 4 μU/mL (upper normal range). However, recent guidelines have suggested there is no benefit in doing this in terms of achieving pregnancy and preventing pregnancy wastage.[147]

Assessment of Thyroid Autoantibodies

Because the most common causes of thyroid disorders are autoimmune disorders, the assay of thyroid autoantibodies is often useful.[148,149] Several autoantibodies may be measured but the most used in clinical practice are the measurements of thyroid peroxidase antibody (TPO), thyroglobulin antibody (TgAb), and TSH receptor binding antibodies (TRAb).

TPO is useful for diagnosing autoimmune chronic thyroiditis but may be increased also in Graves disease. The finding of positive TPO in patients with mild elevations of TSH but normal thyroid hormone levels (subclinical hypothyroidism) may be important in establishing the need of treatment.[145,146]

In pregnant women, the finding of increased TPO in the presence of a normal TSH and thyroid hormone levels has been linked to increased miscarriage but disagreement exists on the need of treating these women.[145,146]

If TPO is negative and there is a strong clinical suspicion of autoimmune thyroid disease, TgAb may be measured but has a low sensitivity and it is not recommended. TgAb measurement is needed in treated patients with thyroid cancer to help interpret thyroglobulin levels.[149]

TRAb assays are useful for confirming the diagnosis of Graves disease. TRAb assays can be divided into two categories: assays that detect antibodies binding to the TSH receptor and assays that measure cAMP production in cells incubated with patients' sera (TSI). The first group of assays cannot differentiate between stimulating TRAb or nonstimulating (inhibiting or neutral) TRAb, while the second group of assays identify only stimulating TRAb. In clinical practice, the need for a test differentiating TSI from nonstimulating antibodies (TSHR-inhibiting antibodies or neutral antibodies) is very limited because in most cases TRAb is measured in patients with thyrotoxicosis and serves to differentiate Graves disease from other causes of thyrotoxicosis (e.g., subacute thyroiditis). Therefore, in the appropriate clinical setting (a patient with hyperthyroidism), the TRab assay is preferred because it is much easier and economical to perform.[150] With improved immunoassays with high sensitivity, the TRab assay has become a hallmark in the diagnosis of Graves disease and is reported to reach a 99% specificity and sensitivity.[151]

The TRAb assay is very useful in distinguishing postpartum thyroiditis from de novo or relapsing Graves disease in lactating women. The TRAb assay is also an important predictor of fetal or neonatal thyrotoxicosis in pregnant women with Graves disease. American Thyroid Association guidelines for the management of thyroid disease in pregnancy recommend the measurement of TRAb at 24 to 28 weeks' gestation, and if the value is over 3 times the upper limit of normal, close follow-up of the fetus is recommended.[151]

Thyrotropin-Releasing Hormone Stimulation Test

With the improvement and sensitivity of TSH assays, the TRH stimulation test is not required in the evaluation of primary hypothyroidism or hyperthyroidism with low TSH levels. However, the test is still useful in the evaluation of central hypothyroidism, in rare patients with TSH-dependent hyperthyroidism, and in patients with pituitary tumors.

After an overnight fast, a 200-μg dose of TRH is administered intravenously. Blood samples for TSH are then taken at 0, 30, and 60 minutes. The mean peak TSH response is a value 8 to 9.5 times the basal values; however, the range in euthyroid subjects is large (3 to 23 times baseline).

Evaluation of Glucocorticoid Adrenal Function

- *Sensitive assays are now available for urinary free cortisol and salivary cortisol as well as ACTH.*
- *Low- and high-dose dexamethasone suppression tests and corticotropin-releasing hormone (CRH) and ACTH stimulation tests are sometimes required to assess adrenal states.*
- *A proposed algorithm of the assessment of Cushing is offered.*

Cortisol, the main glucocorticoid produced by the adrenal, is conveniently measured by chemiluminescent immunoassay. Several corticosteroids, including prednisone, prednisolone, and cortisone, may cross-react in the routine assay.[152] Because cortisol secretion is highly pulsatile and exhibits circadian variations, with the highest values occurring in the morning and the lowest levels in the late evening, a single random serum cortisol determination is not diagnostic for adrenal disorders. It is preferable to assess cortisol status by an integrated 24-hour urinary collection for "free" cortisol and/or by salivary cortisol, which also provides a measure of free cortisol.

Plasma ACTH is also measured by chemiluminescent immunoassay. Plasma ACTH levels greater than twofold the upper limit of the reference range in the presence of low serum cortisol (<5 μg/mL) are suggestive of primary adrenal insufficiency (Addison syndrome).[153] In patients with Cushing syndrome, a plasma ACTH level above 20 pg/mL at 9 a.m. is highly indicative of ACTH-dependent Cushing syndrome. A level below 10 pg/mL suggests ACTH-independence and primary adrenal pathology.[154] However, in many individuals, the evaluation of blood ACTH and cortisol are not sufficient to diagnose adrenal disorders; therefore specific diagnostic procedures have to be performed.

Diagnosis of Pathologic Hypercortisolism (Cushing Syndrome)

The evaluation for the diagnosis of Cushing syndrome may be complex.[154] Because the clinical characteristics of Cushing syndrome may be found in many other common pathologic disorders, the first step in the evaluation is to validate the presence of hypercortisolism. Three first-line diagnostic tests are currently used to screen for Cushing syndrome: measurement of free cortisol in 24-hour urine (UFC), cortisol suppression by low doses of dexamethasone, and assessment

of the level of cortisol using its circadian rhythmicity by measuring late-night salivary cortisol.

Late Night Salivary Cortisol Measurement

Loss of circadian rhythmicity is the most sensitive indicator of pathologic hypercortisolism. Ideally, midnight sleeping cortisol should be a very sensitive indicator of Cushing syndrome but it is impractical for screening purposes. Assessment of late-night salivary cortisol is increasingly being considered as the simplest and more convenient outpatient procedure.[154,155] This method is also useful for the diagnosis of early or mild Cushing syndrome.

Saliva samples are collected late at night (23:00 h) using a commercially available device and assayed for cortisol by radioimmunoassay. A cutoff level of 1.5 μg/mL provides high sensitivity and specificity for both subclinical and overt Cushing syndrome. However, the cutoff value varies between studies because of different assays and populations studied. Normal values also differ between adults and children and may be affected by other comorbidities such as diabetes, and the method by which the saliva is collected.

Urinary Free Cortisol

Twenty-four-hour urinary free cortisol provides an integrated index of cortisol secretion throughout the day. In fact, unbound cortisol is filtered by the kidney, with the majority being reabsorbed in the tubules, and the remainder excreted. When hypercortisolism is present, the binding capacity of CBG is exceeded and large quantities of free cortisol are excreted by the kidney. While the upper normal range of urinary cortisol should be 50 to 60 μg/day, some commercial assays report normal ranges as high as 100 to 130 μg/day. One of the drawbacks of 24-hour urinary collection is the completeness of the collection, which therefore requires a simultaneous measurement of creatinine. In some studies of patients with Cushing syndrome, urinary-free cortisol measurement showed a sensitivity of 95% for the diagnosis. However, because many patients have sporadically normal values of urinary-free cortisol, 3 to 4 completely normal 24-hour UFC values may be needed to exclude the diagnosis.

The use of LC or tandem MS measurement of UFC has solved the issues of cross-reactivity of some exogenous glucocorticoids and other structurally similar steroids that were present when using conventional radioimmunoassays. However, drugs such as carbamazepine, digoxin, and fenofibrate may coelute with cortisol during HPLC and cause falsely elevated results. In summary, UFC measurements have a high sensitivity if collected correctly, and several completely normal values of UFC make the diagnosis of Cushing syndrome very unlikely. Values greater than fourfold above the normal range are rare except in Cushing syndrome. Patients with marginally elevated levels require further investigation. Because of all these caveats, most centers now prefer using other screening tests for hypercortisolism.[154]

Overnight and Classic Low-Dose Dexamethasone Suppression Test

As a simple screening test for hypercortisolism, overnight dexamethasone suppression has been frequently used. Dexamethasone (1 mg) is ingested at 11 p.m. and serum cortisol is measured the morning after. Using the criteria of a postdexamethasone serum cortisol of less than 50 nmol/L (<1.8 mg/dL), the *specificity* of this test for excluding Cushing syndrome is relatively good (88%), but false positives remain significant.

The classic low-dose dexamethasone suppression test has greater diagnostic accuracy and may be used in screening for Cushing syndrome, often together with late night salivary cortisol measurement.[154] The test is performed reliably in an outpatient setting and involves the administration of dexamethasone, 0.5 mg every 6 hours for 2 days, then collection of blood for cortisol measurement on the morning of the third day. Serum cortisol greater than 1.8 μg/dL is a sensitive indicator of Cushing syndrome. Some drugs that increase hepatic clearance of dexamethasone, such as carbamazepine, phenytoin, nifedipine, rifampicin, and phenobarbital, may interfere with the dexamethasone test and these drugs should be stopped prior to investigation. Estrogens may give falsely elevated cortisol levels (increasing CBG) and should be stopped 4 to 6 weeks before testing.

Differential Diagnosis of Cushing Syndrome

If the diagnosis of hypercortisolism is confirmed, the next step is to differentiate between ACTH-dependent and ACTH-independent causes, and this is initially carried out by the measurement of plasma ACTH (Fig. 34.13). Two-site ACTH IRMAs have replaced radioimmunoassays and are able to detect low levels of the hormone. In terms of the measurement, because ACTH is susceptible to degradation by circulating peptidases, the blood sample must be kept cold and centrifuged, aliquoted, and frozen within a few hours to avoid falsely low results.

Baseline plasma ACTH 10 pg/mL indicates an adrenal source of the hypercortisolism, while the distinction between adrenal causes: unilateral adrenal tumor, primary pigmented nodular adrenal dysplasia associated with Carney complex, and ACTH-independent macronodular adrenocortical hyperplasia will require a careful imaging study by CT.

Increased or normal levels of plasma ACTH (>20 pg/mL) associated with hypercortisolism suggests an ACTH-dependent form of Cushing syndrome. Distinction between pituitary Cushing disease or the ectopic ACTH syndrome requires detailed pituitary imaging. Finding of a pituitary adenoma (generally a microadenoma) confirms the diagnosis of pituitary Cushing disease. When no pituitary adenoma is found, further dynamic tests (high-dose dexamethasone suppression test, CRH test) may be needed. Often the results of these tests are equivocal, and/it may be difficult to distinguish between pituitary and ectopic forms of ACTH-dependent Cushing. In this situation full-body CT scanning and sampling plasma ACTH from the inferior petrosal sinus may be necessary.

Diagnosis of Adrenal Insufficiency

The clinical suspicion of adrenal insufficiency can be confirmed by demonstrating low serum cortisol concentrations.[153] Morning serum cortisol lower than 3 μg/dL is strongly suggestive of adrenal insufficiency. The simultaneous measurement of plasma ACTH helps to confirm the diagnosis of primary adrenal insufficiency.[153] In primary adrenal insufficiency, the 08:00 plasma ACTH concentration is increased

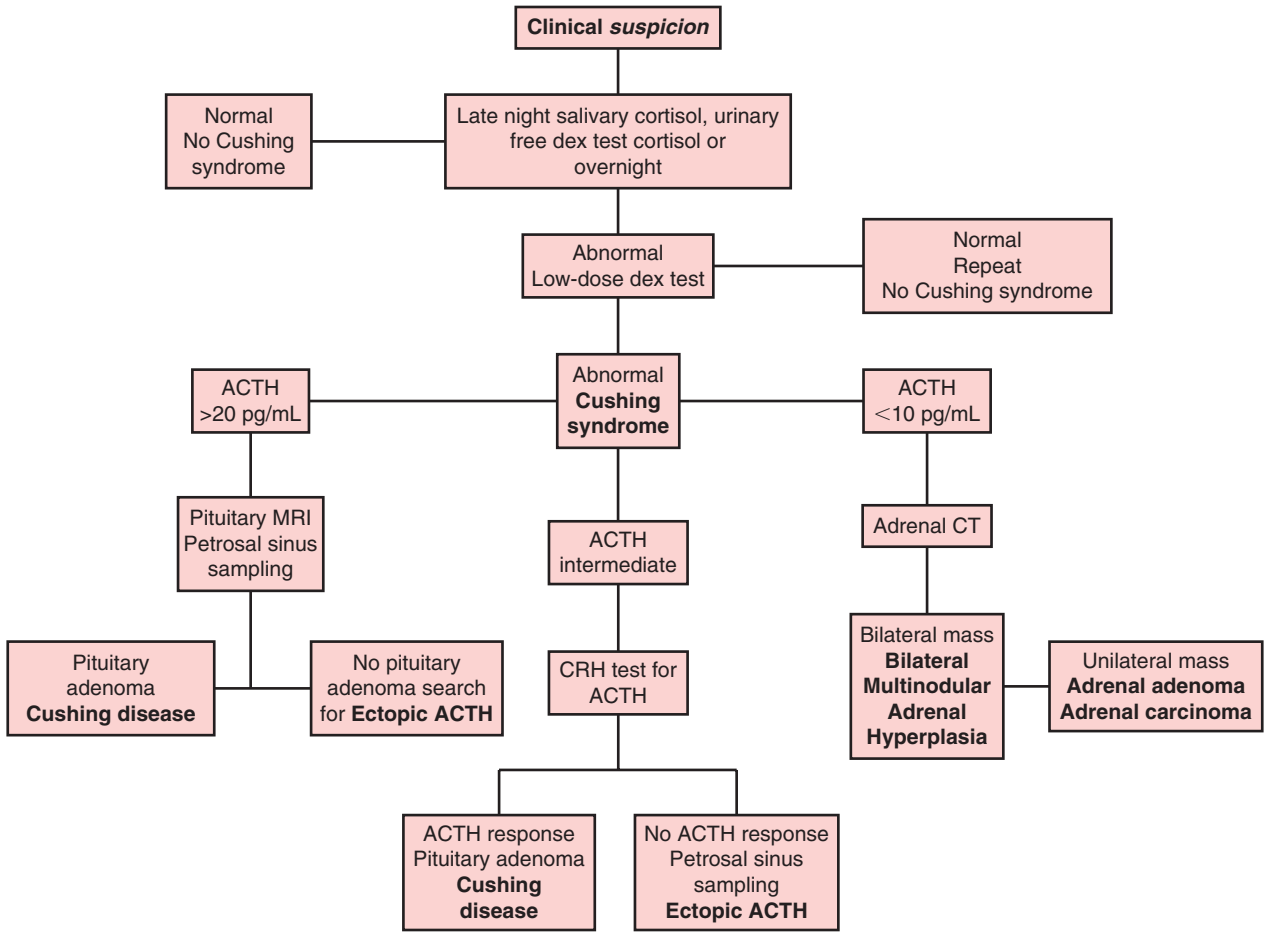


FIGURE 34.13 Diagnostic algorithm for Cushing syndrome. *ACTH*, Adrenocorticotropin hormone; *CRH*, corticotropin-releasing hormone; *CT*, computed tomography; *MRI*, magnetic resonance imaging.

and often is very elevated. However, when plasma ACTH levels are only mildly elevated or in cases of secondary or tertiary adrenal insufficiency, an ACTH test has to be performed to confirm the diagnosis. Additional clues to adrenal insufficiency may be found with low (for age) levels of DHEAS.

Adrenocorticotropic Hormone Test

Stimulation with ACTH (250 μg), administered intravenously with measurements of cortisol at 0, 30, and 60 minutes, is an excellent test for primary adrenocortical insufficiency.[154] Serum cortisol levels lower than 13 μg/dL after ACTH ensure the diagnosis of adrenal glucocorticoid insufficiency. Levels between 13 and 18 μg/dL are intermediate and require additional diagnostic evaluation. Serum cortisol levels higher than 18 μg/dL after ACTH stimulation excludes the diagnosis. If secondary adrenal insufficiency is of recent onset, the adrenal glands may still be capable of responding normally to a standard-dose ACTH test. In these cases, low-dose ACTH (1 μg intravenously) given at 2:00 p.m. is necessary.[148] Serum cortisol values higher than 18 μg/dL (500 nmol/L) or more are indicative of normal adrenal function. The low-dose test is preferred in patients with a suspect of secondary or tertiary adrenal insufficiency.

CRH test is used to differentiate between secondary and tertiary adrenal insufficiency. It consists of intravenous administration of CRH (1 μg/kg) and determination of serum cortisol and plasma ACTH concentrations at 0, 15, 30, 60, 90, and 120 minutes following stimulation (Fig. 34.14). Patients with secondary adrenal insufficiency demonstrate little or no ACTH response. Patients with tertiary adrenal insufficiency present an exaggerated and prolonged response of ACTH to CRH stimulation, which is not followed by a cortisol response.

Diagnostic Procedure in Suspected Pituitary Tumors

◆ *CT and MRI scan with and without contrast now provide great precision in the diagnosis of pituitary lesions.*

Patients with pituitary tumors seek evaluation because of the symptoms (e.g., headache, diabetes insipidus, visual field alterations) or because of the finding of increased hormone secretion (e.g., acromegaly, Cushing syndrome, amenorrhea with or without galactorrhea). Once a pituitary lesion is suspected, radiographic investigation is required.

MRI with gadolinium enhancement is the preferred technique to evaluate hypothalamic-pituitary lesions[156,157]; however, CT scanning with intravenous contrast and thin coronal sections may be also used. Examples of MRI and

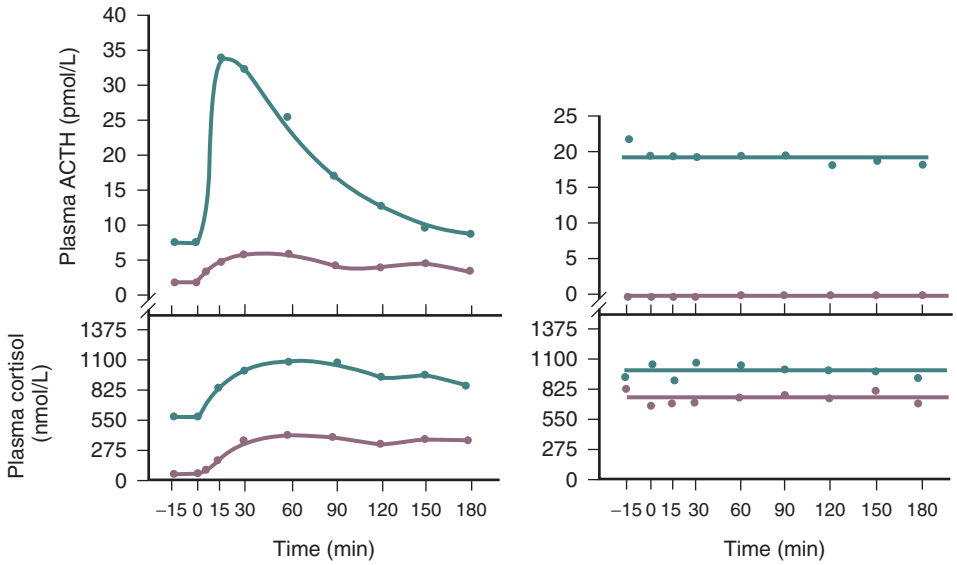


FIGURE 34.14 Adrenocorticotropin hormone *(ACTH)* response to corticotropin-releasing hormone. *Colored lines* represent different patients. *(Modified from Kaye TB, Crapo L: Cushing's syndrome: an update on diagnostic tests.* Ann Intern Med *112:435–444, 1990.)*

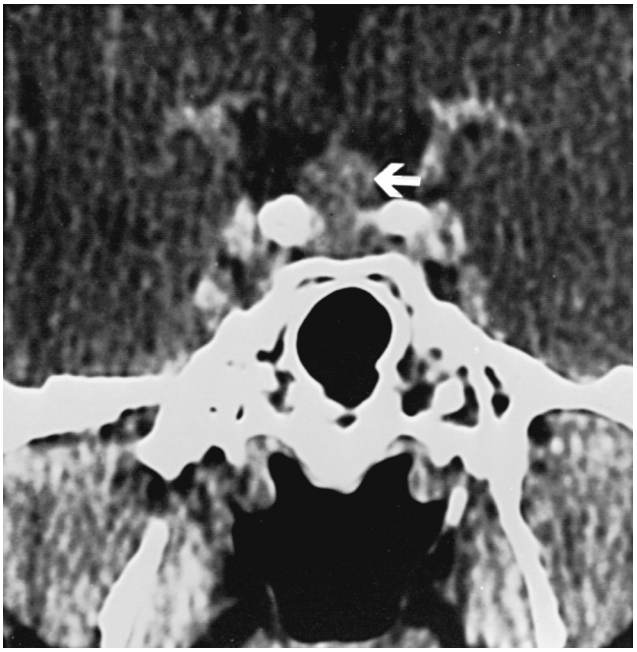


FIGURE 34.15 Typical close-up coronal view of a pituitary microadenoma by computed tomography *(CT)*. The neoplasm cannot be distinguished from normal pituitary. Thus the *arrow* indicates an enlarged abnormal pituitary bulging superiorly. The stalk is seen superior to the gland in the midline. The bone appears white on CT scan. Magnetic resonance imaging may allow better resolution of neoplasms from normal pituitary. *(Modified from Rebar RW: Practical evaluation of hormonal status. In Yen SC, Jaffe RB, Barbieri RL, editors:* Reproductive endocrinology, physiology, pathophysiology, and clinical management*, ed 4, Philadelphia, 1999, WB Saunders, p 709.)*

CT techniques may be found in Figs. 34.15 and 34.16. Table 34.2 lists the major MRI characteristics of the more common hypothalamic-pituitary lesions. Dynamic pituitary MRI may increase the number of microadenomas that may be visualized.

If hypothalamic or pituitary lesions are found, detailed evaluation of all pituitary hormones should be performed.[158] Regardless of the size of the lesion, patients should initially be evaluated for hormone hypersecretion. Serum PRL, GH with IGF-1, and 24-hour urinary free cortisol could be used for the initial screening. In selected patients, basal and TRH-stimulated gonadotropins and α-subunits should be measured for the evaluation of possible gonadotroph adenomas. Hyperthyroid patients should be screened for rare TSH adenomas. In a large series of 1043 pituitary tumors,[159] the most common pituitary hormone secreting adenomas were prolactinomas (27.2%), followed by GH-secreting adenomas (14%), GH- and PRL-secreting adenomas (8.4%), and ACTH-secreting adenomas (8%).

Some pituitary adenomas produce only alpha-subunit, a component of glycoprotein pituitary hormones (LH, FSH, TSH). Therefore, in patients with pituitary tumors and no increase of pituitary hormones, the assay of alpha-subunit may be used. The subunit is measured by a RIA method and in young adults the circulating values are less than 1 ng/mL. Some gonadotropin secreting pituitary tumors produce high quantities of alpha-subunit and this assay may be useful in postmenopausal women with pituitary adenomas. However, it has to be remembered that normal values of alpha-subunit are higher in postmenopausal women (<3.6 ng/mL) than in young women.

Pituitary microadenomas do not generally cause disruption of pituitary function; therefore, pituitary hypofunction should be assessed only in those patients with large sellar masses that do not have increased secretion. GnRH, TRH, GH-releasing hormone, and CRH tests (all four releasing factors) may be performed together for contemporaneous evaluation of gonadotropins TSH, PRL, GH, and ACTH.[160]

Pelvic Evaluation

- *Ultrasound provides a great deal of information with either 2D or 3D technology and particularly with a vaginal probe.*
- *MRI and specialized scans with contrast are sometimes required for special conditions such as with steroid producing tumors.*

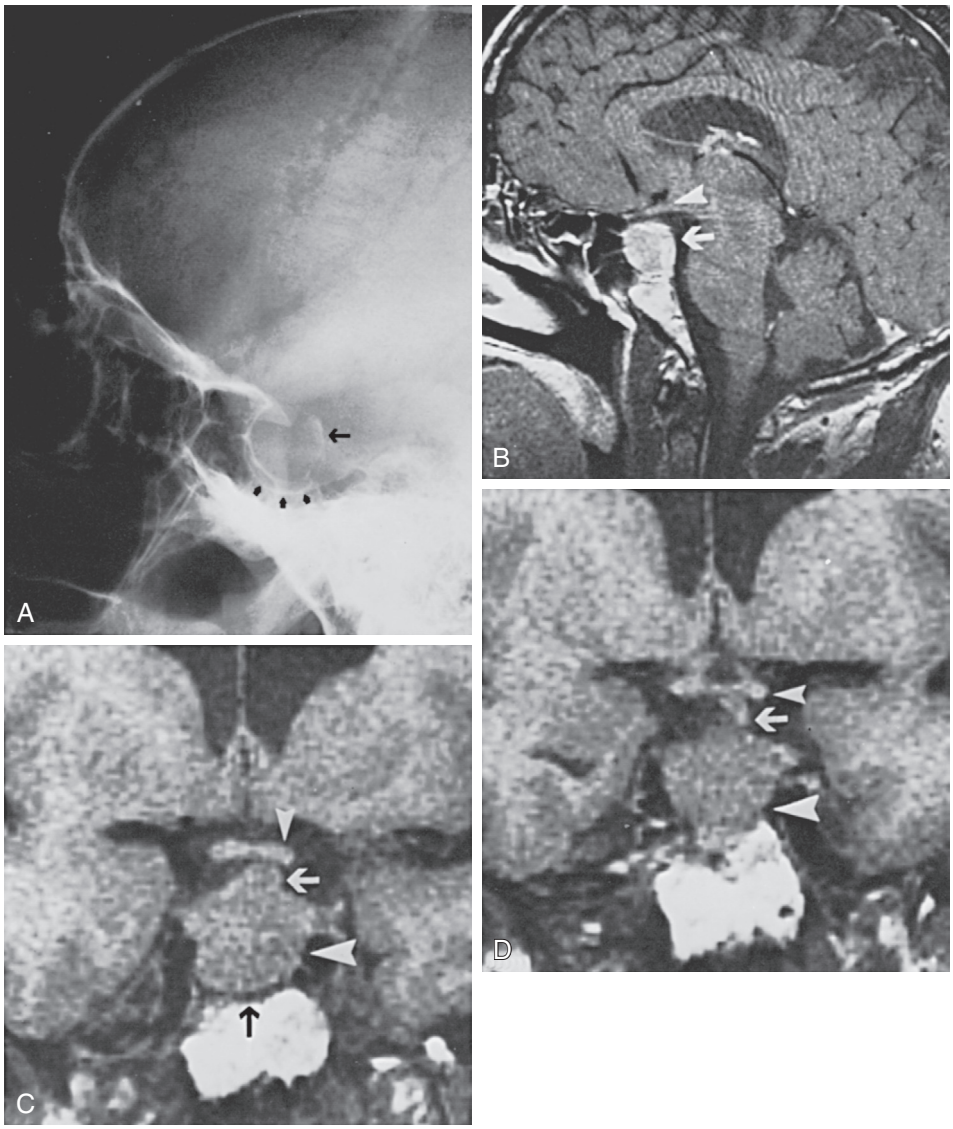


FIGURE 34.16 Selected views in a 39-year-old woman with a probable nonsecreting neoplasm who has amenorrhea, galactorrhea, and prolactin levels of approximately 50 ng/mL. The radiographs suggest that the mild hyperprolactinemia is due to stalk compression. (A) Lateral skull film showing ballooning of the sella turcica with a thin double floor *(small arrows)* and erosion of the clinoid processes posteriorly *(large arrow)*. (B) Sagittal view of magnetic resonance imaging with contrast material showing the large pituitary tumor *(arrow)* and normally positioned optic chiasm *(arrowhead)*. (C) Coronal view showing the large neoplasm *(large arrowhead)* bulging superiorly *(white arrow)* toward the optic chiasm *(small arrowhead)*. The *black arrow* shows the diaphragm sella. (D) Another view of the neoplasm shows displacement of the optic chiasm *(arrow)* below the optic nerves and chiasm *(small arrowhead)*. The *large arrowhead* indicates the lesion. The diaphragm is indistinct and there is the suggestion of bony erosion. *(Modified from Rebar RW: Practical evaluation of hormonal status. In Yen SC, Jaffe RB, Barbieri RL, editors:* Reproductive endocrinology, physiology, pathophysiology, and clinical management*, ed 4, Philadelphia, 1999, WB Saunders, p 709.)*

Table 34.2 Magnetic Resonance Imaging Characteristics of Some Hypothalamic-Pituitary Lesions

Type of Mass	MRI Images	MRI With Contrast Images	Particular Features
Pituitary adenoma	Hypointense	Hypointense	Hyperintense with hemorrhage
Pituitary cyst	Hyperintense	No change	Cystic; may be hypointense if CSF is in the cyst
Craniopharyngioma	Hyperintense	No change	Cystic with iso-hypointense solid portions
Meningioma	Isointense	Hyperintense	Hyperostosis of adjacent bone

CSF, Cerebrospinal fluid; *MRI*, magnetic resonance imaging.

Pelvic ultrasound has become an extremely important tool for the reproductive endocrinologist (see Chapter 35). Assessment of ovarian function and monitoring for endometrial development are useful for clinical management and have been discussed elsewhere (see Chapters 8 and 9).

Assessment of the AFC has become a valuable parameter for the assessment of ovarian reserve, and is equal in prediction value to measurements of AMH by ROC analysis as noted earlier. To have consistency in this subjective measurement, it is best to perform the count on days 2 to 4 of the menstrual cycle, including all follicles in both ovaries between 2 and 10 mm. Vaginal ultrasound with a 7 MHz transducer should be used and a systematic method of counting should be carried out, with the elimination of all cystic structures over 10 mm.[161] AFC assessment has been carried out at various ages (Fig. 34.17) and values less than 8 signify a diminished ovarian reserve.

The finding of polycystic ovaries (PCO) on ultrasound has been included as a criterion for the diagnosis of PCOS[162] and therefore use of ultrasound has become a valuable asset in patients being evaluated for a possible diagnosis of PCOS, showing the typical follicular appearance and increased ovarian blood flow (Fig. 34.18). At the same time, the ultrasound exam provides information on endometrial thickness (and the concern of endometrial hyperplasia or cancer) in women with irregular cycles or amenorrhea.

According to the original Rotterdam criteria, a number of 12 or small follicles for ovary and/or a volume of at least 10 cc was considered sufficient for making diagnosis of polycystic ovaries (PCO).[163] However, with new ultrasound technology, 50% of normal ovulatory women have follicle counts of 12.[164] Using vaginal probes of at least 8 MHz, a new cutoff value for the total follicular number (FNPO) of 25 or greater has been suggested as being able to establish the diagnosis of PCO.[165] In our experience, the FNPO cutoff is slightly lower (using ROC curves we calculated a FNPO cutoff of 22 for diagnosing PCO morphology).[76] We suggest that this is a very sensitive criterion for the diagnosis of PCOS and is useful in all phenotypes of the disorder, including mild phenotypes (ovulatory and normoandrogenic phenotypes, or phenotypes C and D).[76] Increased ovarian size may be also used for determining the existence of a PCO but its sensitivity is much lower than increased FNPO. Most centers use an ovarian size cutoff of 10 cc, while in our population the cutoff has been recently reviewed and set at 8.8 cc or greater.[76]

In the past, increased thecal/stromal volume within each ovary has been used for making the diagnosis of polycystic ovarian morphology and ovarian stromal/total surface area (S/F) ratio has been suggested as a sensitive criterion for determining PCO morphology with a cutoff value of 0.32 (Fig. 34.19).[166] It is logical that this increased thecal/stromal volume correlates well with the androgen level. However, with new ultrasound machines, the image of the theca has completely changed, with many PCO being so full of follicles that no theca tissue may be easily measured. This ratio has a lower sensitivity and specificity that FNPO.[165]

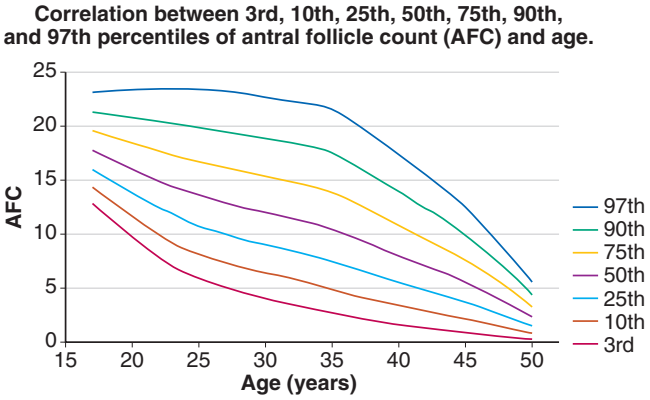


FIGURE 34.17 McGill nomogram for antral follicle count *(AFC)* with declining age. *(From Almog B, Shehata F, Shalom-Paz E, Tan SL, Tulandi T: Age-related normogram for antral follicle count: McGill reference guide.* Fertil Steril *95[2] 663–666, 2011.)*

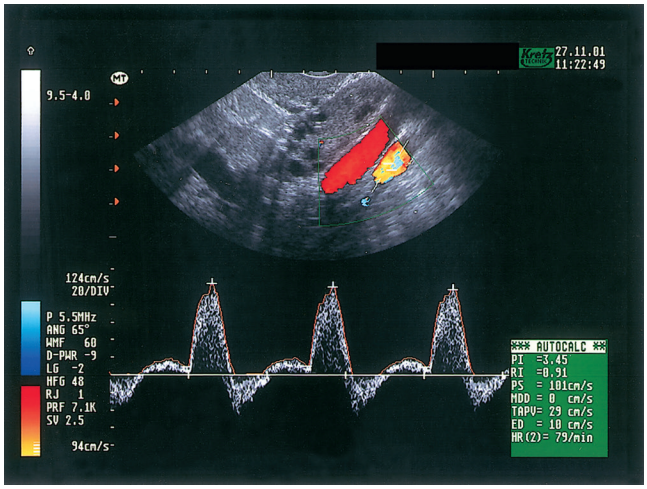


FIGURE 34.18 Typical color Doppler ultrasound of a polycystic ovary showing increased blood flow.

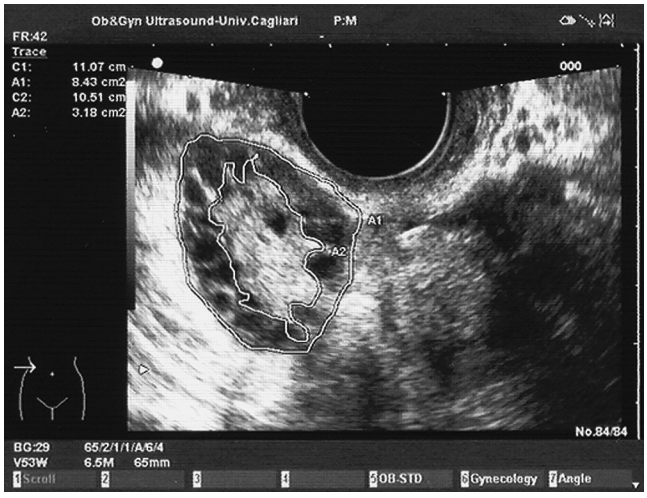


FIGURE 34.19 Example of median ovarian section with outlined ovarian and stroma areas. A1 is the total area and A2 is the stroma area. *(From Fulghesu AM, Angioni S, Frau E, et al: Ultrasound in polycystic ovary syndrome—the measuring of ovarian stroma and relationship with circulating androgens: results of a multicentric study.* Hum Reprod *22:2501–2508, 2007.)*

Using 3-D US, which has also been found to be useful for the diagnosis of PCOS, müllerian anomalies can be detected. While routine 2-D ultrasound is not able to distinguish between various müllerian anomalies, 3-D US is as sensitive as MRI in this regard. Nevertheless, MRI may still have an advantage over US when differentiating between fibroids and adenomyosis (see Chapter 35).

Various imaging technologies have advanced to the point that older techniques such as selective venous catheterization for localization of tumors are rare. This includes the traditional CT and MRI scans and the use of specific tracers for steroid producing tumors such as ^{131}I-6β-iodomethyl norcholesterol (NP-59) using SPECT/CT or various derivatives of glucose uptake using PET/CT.

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