Peptide hormone analysis in diagnosis and treatment of differences of sex development: joint position paper of EU COST action 'DSDnet' and European reference network on rare endocrine conditions

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Abstract

Differences of Sex Development (DSD) comprise a variety of congenital conditions characterized by atypical chromosomal, gonadal, or anatomical sex. Diagnosis and monitoring of treatment of patients suspected of DSD conditions include clinical examination, measurement of peptide and steroid hormones, and genetic analysis. This position paper on peptide hormone analyses in the diagnosis and control of patients with DSD was jointly prepared by specialists in the field of DSD and/or peptide hormone analysis from the European Cooperation in Science and Technology (COST) Action DSDnet (BM1303) and the European Reference Network on rare Endocrine Conditions (Endo-ERN). The goal of this position paper on peptide hormone analysis was to establish laboratory guidelines that may contribute to improve optimal diagnosis and treatment control of DSD. The essential peptide hormones used in the management of patients with DSD conditions are follicle-stimulating hormone, luteinising hormone, anti-Müllerian hormone, and Inhibin B. In this context, the following position statements have been proposed: serum and plasma are the preferred matrices; the peptide hormones can all be measured by immunoassay, while use of LC-MS/MS technology has yet to be implemented in a diagnostic setting; sex- and age-related reference values are mandatory in the evaluation of these hormones; and except for Inhibin B, external quality assurance programs are widely available.

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Introduction

Differences of Sex Development (DSD) comprise a variety of congenital conditions characterized by atypical chromosomal, gonadal, or anatomical sex (1). DSD are medical conditions that are classified according to karyotype: (1.) 46,XX DSD, (2.) 46,XY DSD, and (3.) sex chromosome DSD, all with a wide phenotypic spectrum. Due to the complexity of these conditions, laboratory analyses with high sensitivity and specificity as well as age-adjusted sex-specific reference values are therefore crucial to optimize diagnosis and treatment monitoring.

Development of recommendations on laboratory assessment for DSD was one of the tasks of the European Cooperation in Science and Technology (COST) Action DSDnet (BM1303) (http://www.dsdnet.eu), which was active between 2013 and 2018. This task was performed in collaboration with the European Reference Network on rare endocrine conditions (Endo-ERN; http://www.endo-ern.eu), formed in 2017. DSDnet was a network of all people interested in DSD, from leading scientists to clinicians, as well as people with DSD, while Endo-ERN is a network composed of health care providers and patient organizations. A recent large, international survey reported that the diagnostics of a newborn suspected of having DSD is influenced by appropriate access to specialists, thereby resulting in a substantial variation in the initial evaluation of the child (2). This underlines the necessity of networks consisting of highly specialized laboratories with knowledge of DSD. The laboratory assessment of patients with known or suspected DSD requires biochemical and genetic assessment.

Biochemical hormone analysis comprises two distinct groups: peptide and non-peptide hormones, predominantly steroid hormones. DSDnet recently published two position papers, one on steroid hormone analysis in diagnosis and treatment of DSD (3) and one addressing diagnostic genetic approaches in DSD (4). There is currently no position paper addressing peptide hormones in relation to DSD. Working Group 3 (Harmonisation of Laboratory Assessment) in DSDnet and Work Package 5 in Endo-ERN (Diagnostics and Laboratory Analysis) therefore present a position paper on the quantification of peptide hormones in patients suspected of DSD conditions. In this paper, we discuss the aspects that underlie the quality assurance of peptide hormone assays used for the diagnosis and monitoring of patients with DSD. The importance of the quality of peptide hormone analyses in the diagnosis and management of these specific and rare endocrine conditions is illustrated herein.

Clinical application of peptide hormones

Analysis of peptide hormones is important in first-line testing and during monitoring of DSD conditions and include the gonadotropins follicle-stimulating hormone (FSH), luteinizing hormone (LH), anti-Müllerian hormone (AMH), and Inhibin B. FSH and LH are synthesized in the anterior pituitary gland and are glycoproteins consisting of a common α-subunit and a unique β-subunit binding to the FSH-receptor and LH-receptor, respectively. Postnatally, LH stimulates testosterone production in the testicular Leydig cells and in the ovarian theca interna cells. FSH stimulates AMH and Inhibin B production in both the testicular Sertoli cells and the ovarian granulosa cells.

During infancy, boys have higher concentrations of LH and lower concentrations of FSH than girls. This sex dimorphism in gonadotropin concentrations has been reported for children during the first hours of life (5), during the first month of life (6), and during the first years of life (7). Thus, especially in mini-puberty – the period in which a transient, postnatal activation of the hypothalamic-pituitary-gonadal (HPG) axis in infancy occurs – the ratio of LH divided by FSH is an excellent classifier in separating the sexes (8). At this point in time, the LH/FSH ratio is high in boys and low in girls (Fig. 1). Mini-puberty therefore reflects an early window of opportunity in which to study reproductive function. Additionally, gonadotropins are of diagnostic value both when their concentrations are high, as seen in primary hypogonadism (e.g. 45,X (9), 45,X/46,XY (10), 47,XXY (11)), and when low, as seen in congenital hypogonadotropic hypogonadism (CHH) (12). A newborn with a 46,XY karyotype and CHH may present with micropenis and bilateral cryptorchidism at birth, which resemble DSD conditions.

AMH is a homodimeric disulphide-linked glycoprotein and a member of the transforming growth factor b family β (TGFβ). In male fetuses, AMH is synthesized soon after testicular differentiation and is essential for regression of the Müllerian ducts, whereas AMH production in female fetuses first is initiated in the 36th week of gestation (13). In females, in the absence of AMH, the Fallopian tubes, uterus, and the upper part of the vagina are developed. Inhibin B is a dimeric disulphide-linked glycoprotein consisting of two subunits (α and β) and is, like AMH, a part of the TGFβ protein family. The main role of Inhibin B is the downregulation of FSH synthesis. There is a well-known difference in concentrations of AMH and Inhibin B between sexes (Figs 1, 2 and 3). Thus, plasma concentrations of AMH and Inhibin B in boys

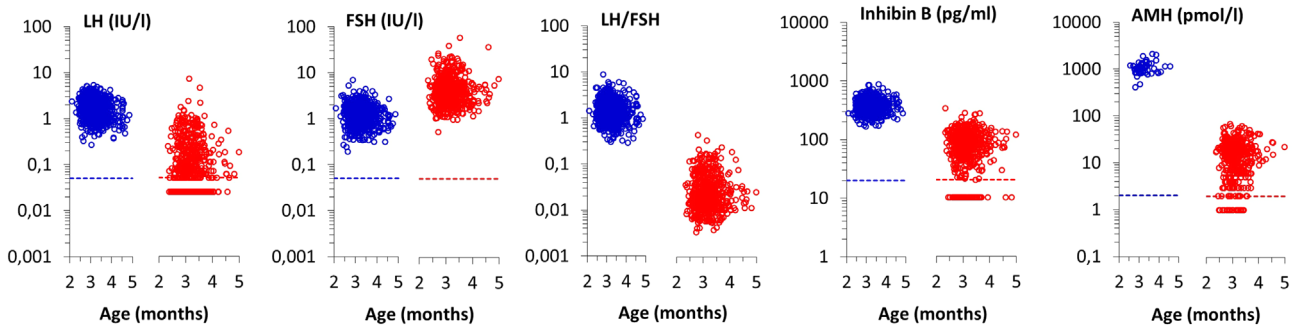

Figure 1

Serum concentrations of luteinizing hormone (LH), follicle-stimulating hormone (FSH), the LH/FSH-ratio, Inhibin B, and anti-Müllerian hormone (AMH) in 1041 healthy males (blue) and 799 healthy females (red) during mini-puberty. The concentrations are shown on a log10-transformed y-axis (dotted lines: limit of detection). Modified from (8).

have been reported to be, respectively, up to 100 times (14) and 10 times higher (15) than in girls. During mini-puberty, the Inhibin B concentrations in boys are higher than the concentrations seen in men (15). AMH and Inhibin B therefore represent important biomarkers in the evaluation of gonadal type and function in DSD conditions, as low concentrations are markers for the absence of functioning testicular tissue (16, 17). AMH and Inhibin B concentrations are viable biomarkers reflecting ovarian function and follicle reserve in girls and may, together with the gonadotropins, be used in the diagnostics of, for example, primary or premature ovarian failure. Additionally, as AMH production reflects Sertoli cell function, while testosterone production reflects Leydig cell function, a human choriogonadotropin (hCG) test can offer further information on gonadal function. As hCG stimulates Leydig cells directly to produce testosterone, the test is useful to test the HPG axis after infancy and until puberty, where the gonadotropin concentrations are low. The test evaluates the testosterone response to hCG administration, but the interpretation of the testosterone response should be done cautiously as described in detail by Ahmed *et al.* (18). In a recent, large study of hCG testing, a normal AMH concentration before hCG administration

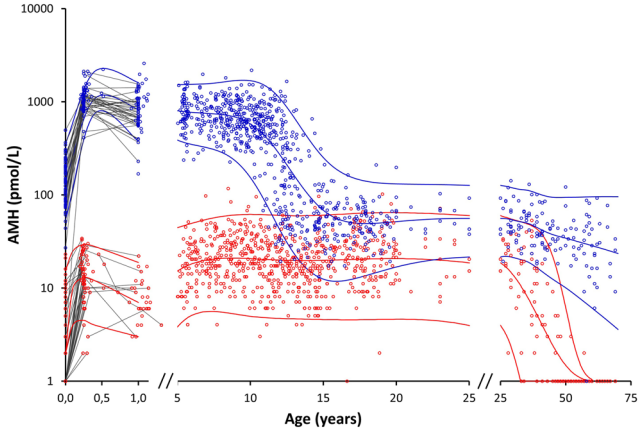

Figure 2

Serum concentrations of AMH in 1027 healthy males (blue) and 926 healthy females (red) throughout life. Longitudinal values during infancy are connected via black lines. Blue and red lines mark male and female reference ranges (mean, ±2S.D.). The concentrations are shown on a log10-transformed y-axis. AMH was measured using a double antibody enzyme-immunometric assay (Immunotech, Beckman Coulter Ltd., Marseilles, France). Modified from (24) and (25).

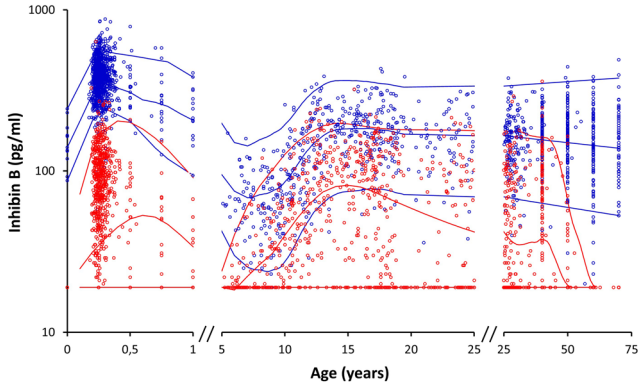

Figure 3

Serum concentrations of Inhibin B in 1161 healthy males (blue) and 1344 healthy females (red) throughout life. Blue and red lines mark male and female reference ranges (mean, ±2S.D.). The concentrations are shown on a log10-transformed y-axis. Inhibin B was measured using a double antibody enzyme-immunometric assay (Oxford Bio-Innovation, Oxfordshire, UK; later named Serotec, Oxford, UK (73)).

was correlated to a normal hCG response. However, a subnormal AMH concentration did not always predict a subnormal testosterone response (19). Thus, the AMH concentration provides important additional information on the testicular function, although it cannot replace the hCG test (18).

In summary, the combination of measuring gonadotropins, AMH, and/or Inhibin B therefore provides a rapid and powerful tool to differentiate between primary and secondary failure in the HPG axis in the initial evaluation of the patient with DSD. The analyses of peptide hormones mostly need to be supplemented by measurements of steroid hormones and genetic tests.

Position 1: Peptide hormones of interest for the diagnosis of patients with DSD include FSH, LH, AMH, and Inhibin B. The request for these peptide hormones should be based on clinical examination and supplemented with relevant steroid hormones and genetic analyses.

Impact of matrices

Blood (serum and plasma) is the most commonly used matrix for peptide hormone analyses, while urinary gonadotropins have proven valuable in preliminary research settings. It is recommended that clinicians be aware of the laboratories' recommendations regarding correct patient preparation and pre-analytical handling of the samples. In addition to an appropriate sampling technique (including knowledge of type of collection device), the necessary knowledge of the timely processing from collection to freezing or measuring is important for sample integrity. Such knowledge includes storage and durability of the sample as well as the correct way to transport. The package insert and/or the analytical method standard operating procedure therefore should be consulted for the preferred matrix and stability for each of these hormones. Plasma and serum are the matrices of choice for AMH and Inhibin B. The use of plasma facilitates both stability and the speed of processing (i.e. eliminating the waiting time for clotting prior to centrifugation). FSH and LH can also be measured in plasma, but as these gonadotropins are relatively stable and frequently analyzed by laboratories, serum is acceptable. Additionally, in first morning voided urine, an increase in LH concentrations occurs before the clinical signs of puberty (20) and is correlated with basal and gonadotropin releasing hormone (GnRH)-stimulated LH concentrations in serum. Thus, urinary LH concentration has recently been suggested by several groups as a novel non-invasive method to evaluate pubertal progress (21, 22). Furthermore, the use of umbilical blood has proved useful for peptide hormone measurements, for example, in reference ranges for gonadotropins in extreme prematurity (23) and for AMH in healthy newborns (24, 25). The use of saliva for FSH and LH determination has been reported (26), but there is little validation evidence available in this case.

Position 2: Serum or plasma is the preferred matrix for the determination of FSH, LH, AMH, and Inhibin B. Furthermore, LH can be measured in urine in experimental settings.

Analytical methods

The Endo-ERN recently conducted a questionnaire-based survey among its associated 71 Reference Centers on their use of biochemical analyses. In development of this position statement, the 33 replies from 13 countries reporting a platform/method within the topic *Sex Development & Maturation* were included. FSH and LH resulted in 29 replies, AMH in 26 replies, and Inhibin B in 14 replies (Fig. 4 and Supplementary Table 1, see section on supplementary materials given at the end of this article). All laboratories reported the use of immunoassays.

In immunoassays, the analytes are typically measured using, for example, chemiluminescent, enzymatic, or fluorescent-labelled ligands. The benefits immunoassays

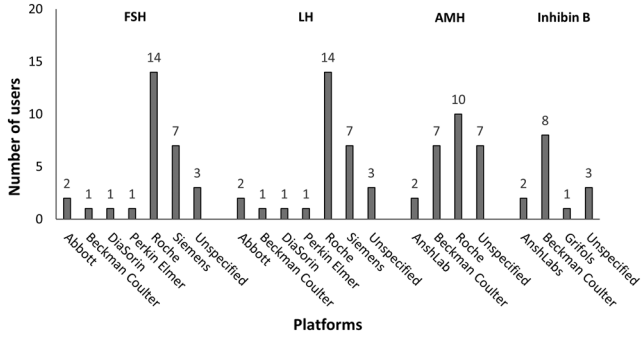

Figure 4
Reported analytical platforms for follicle-stimulating hormone (FSH, *n* = 29), luteinizing hormone (LH, *n* = 29), anti-Müllerian hormone (AMH, *n* = 26), and Inhibin B (*n* = 14) within the theme *Sex Development & Maturation* of the European Reference Network on Rare Endocrine Conditions (Endo-ERN). The replies are from a questionnaire survey among Reference Centers within Endo-ERN. A full description of methods and laboratories is provided in Supplementary Table 1.

offer include the extensive experience that has been gained with them and their availability to all laboratories. For some of the analytes, for example, gonadotropins (27), AMH (28), and Inhibin B (29), highly sensitive methods exist. However, immunoassays may be subject to cross-reactivity and/or matrix effects and, thus, low specificity (or higher detection limit). Additionally, in immunoassays only one analyte is measured at a time.

Peptide hormones can exist in many molecular forms, so metrological traceability to a 'true' value is an ongoing challenge. Within this limitation, the World Health Organization (WHO) provides an invaluable service of developing peptide hormone reference preparations (RPs), which are endorsed through the WHO Expert Committee on Biological Standardization. These preparations provide the basis for manufacturers' peptide hormone assay kit calibration, and their package inserts routinely provide the reference information (e.g. WHO International Standard Follicle Stimulating Hormone, Pituitary NIBSC code: 83/575, https://www.nibsc.org/documents/ifu/83-575.pdf). Examples have arisen in which change to a peptide hormone RP can cause a dramatic change in the calibration slope and therefore the reference values and decision points will need adjusting (30). Hence, as part of characterizing reference values and decision points in guidelines and publications, it is important to include the relative RP to which this applies to.

The lower limit of quantitation or functional sensitivity is usually a function of the lowest calibrator or the point at which the level of imprecision is found to reach 20%. However, guidelines to calculate the lower limit of an assay differ. In Table 1 and Supplementary Table 1, the listed 'lower values' may correspond to the analytical sensitivity, the limit of detection, the limit of quantification, or something else, and thus they may not be entirely comparable. Nevertheless, they give an indication of the sensitivity of these methods. In the Endo-ERN survey, the 'lower value' that was reported varied between laboratories, even when the same manufacturer's kit was used. The 'lower value' reported from the survey ranged from 0.02 to 0.5 IU/L for FSH, from 0.05 to 0.5 IU/L for LH, from 0.07 to 2.5 pmol/L for AMH, and from 1 to 10 pg/mL for Inhibin B (Supplementary Table 1). Furthermore, guidelines differ on how to calculate the lower limit of an assay, so this may differ between manufacturers as well.

The specificity of immunoassays creates challenges for interpretation, which have led to the move by some laboratories toward liquid chromatography-tandem mass-spectrometry (LC-MS/MS) methods to allow for improvements in specificity and the simultaneous determination of multiple analytes (31). MS-based techniques have now been successfully introduced for the quantification of small, well-defined molecules such as steroids. However, within the field of protein analysis, while there is an increasing interest in the use of LC-MS/MS, clinical applications are still lacking. Considerations for peptide analysis include defining the peptide molecular weight(s) to include in quantitation, whether relevant standards and internal standards are available, and the choice of using a bottom-up or a top-down approach for analysis. In addition, the large dynamic concentration range of proteins in plasma may hinder the detection of less abundant components by LC-MS/MS analysis (32), thereby resulting in detection limits that are above the needed range for the diagnostic peptides. With the LC-MS/MS techniques for peptide quantification, the future challenges lie within the validation for the clinical application.

Position 3: As LC-MS/MS technology for the measurement of FSH, LH, AMH, and Inhibin B has yet to be implementated in a diagnostic setting, measurement by immunoassay is the current and recommended method.

Reference values

An important basis in the clinical evaluation of the measured concentrations of any biomarker is the availability of reference data from healthy volunteers. Sex- and age-related reference values are of paramount importance, especially for reproductive hormones due to the sex dimorphism and physiological fluctuations in concentrations according to age and developmental stage. This is illustrated with AMH and Inhibin B in Figs 2 and 3, respectively. Reference ranges for FSH, LH, AMH, and Inhibin B are previously reported (e.g. (24, 25, 33, 34)). In addition to the reference data for hormones individually, reference data with cut-off values for ratios of hormones may add in the evaluation of DSD; for example, in healthy infants, the LH/FSH-ratio has been shown to separate healthy boys and girls, and in infants with complete AIS, the LH/FSH-ratio has been reported to lie within male range (8). However, the recruitment of healthy volunteers for the establishment of sex- and age-related reference values is often a challenge, especially in young children and adolescents. Furthermore, the aim of getting the value of a compound within the corresponding reference limits may not in all cases serve as optimal disease monitoring, as exemplified in acceptably treated CAH, in which 17-hydroxyprogesterone and androstenedione

Table 1 Lowest and highest measurement ranges without dilution of follicle-stimulating hormone (FSH), luteinizing hormone (LH), anti-Müllerian hormone (AMH), and Inhibin B and numbers of instruments according to analytical platforms.

	Measurement range		Number of instruments (%)	
	Low	High	Bio-Rad	Labquality
FSH, IU/L				
n			1600	96
Roche Elecsys & Cobas e411*	0.10	200	269 (17)	5 (5)
Roche Modular E & Cobas e601-e801	0.1 (e801: 0.3)	200	19 (1)	35 (36)
Roche Cobas 6000 & Cobas 8000	0.1	200	291 (18)	NA
Siemens Advia Centaur	0.3	200	300 (19)	17 (18)
Siemens Immulite	0.1	170	53 (3)	11 (11)
Siemens Dimension Vista	0.2	200	5 (0.3)	7 (7)
Abbott Architect	0.05	150	305 (19)	15 (16)
Beckman Coulter Access & Unicel DxI	0.2	200	168 (11)	1 (1)
bioMérieux Vidas Group	0.1	110	80 (5)	2 (2)
Vitros Systems	0.66	200	49 (3)	1 (1)
Tosoh	0.1	250	27 (2)	NA
DiaSorin Liaison	0.25	400	15 (1)	1 (1)
Perkin Elmer AutoDelfia	0.05	256	1 (0.1)	1 (1)
LH, IU/L				
n			1601	94
Roche Elecsys & Cobas e411*	0.10	200	260 (16)	5 (5)
Roche Modular E & Cobas e601-e801	0.1 (e801: 0.3)	200	18 (1)	35 (37)
Roche Cobas 6000 & Cobas 8000	0.10	200	301 (19)	NA
Siemens Advia Centaur	0.1	200	304 (19)	16 (17)
Siemens Immulite	0.05	200	52 (3)	12 (13)
Siemens Dimension & Vista	0.2	150	6 (0.4)	7 (7)
Abbott Architect	0.09	250	299 (19)	14 (15)
Beckman Coulter Access & Unicel DxI	0.2	250	167 (10)	1 (1)
bioMérieux Vidas Group	0.1	100	81 (5)	1 (1)
Vitros Systems	0.216	200	50 (3)	1 (1)
Tosoh	0.1	250	26 (2)	NA
DiaSorin Liaison	0.2	250	14 (1)	1 (1)
Perkin Elmer AutoDelfia	0.05	250	1 (0.1)	1 (1)
AMH, pmol/L				
n			99†	18
Roche Elecsys/Cobas	0.071	164.2	44 (44)	16 (89)
Beckman Coulter Access	0.14	171	28 (28)	NA
Beckman Coulter AMH Gen II	0.57	160.7	24 (24)	2 (11)
INHIBIN B, pg/mL				
Beckman Coulter Inhibin Gen II ELISA	7	1000		
DSL**	7	1000		
Serotec**	15	1000		
Oxford Bio-Innovation Ltd.**	15	1000		
ANSH LABS Inhibin B ELISA AL-107 (RUO)	1.6	1390		

For FSH, LH and AMH, the platform groups that constituted at least 1% of the total number of platforms signed up to at least one of the external quality assurance programs (Bio-Rad, Labquality, and UK-NEQAS) are listed with numbers of participants (percentages in brackets). No widely available EQA-programs currently exist for Inhibin B. For Inhibin B is shown the most commonly used Inhibin B-assays found by an internet search at PubMed. †Instruments for AMH signed up to UK NEQAS; **No longer commercially available; ‡measurement range is provided in pg/mL; *Range for Roche Elecsys, but data on number of Roche Elecsys and Roche Cobas e411 are added from LabQuality; Permission to publish this information has been given from the EQA organizers

Bio-Rad: Immunoassay (Monthly) Program (Distribution no.: Cycle 14. Dec 2016–Dec 2017, Sample No. 8); High, Highest reportable value without dilution (exact value may depend on the kit), whatever present; Labquality (AMH): Tumour Markers (Distribution no.: 2017/02); Labquality (FSH and LH): Hormone Determinations B (Distribution no.: 2017/03); Low, Lowest reportable value; i.e. ananlytical sensitivity, limit of detection, limit of quantification, lowest calibrator value (except the 0-calibrator), whatever present; RUO, Research use-only analysis; UK NEQAS, United Kingdom National External Quality Assessment Service for Peptide Hormones (Distribution no.: 445, February 21, 2017).

concentrations generally are high-normal to elevated despite adequate clinical control (35). Thus, instead of using absolute concentration values, the transformation of absolute values into S.D.-scores may improve patient management in the future. For example, in the long-term monitoring of Turner syndrome patients, for which

measurements of gonadotropins and AMH are suggested as predictive tools for ovarian function (9, 36) as well as for deciding the time and dose of estrogen replacement therapy using gonadotropins (9), the use of S.D.-scores would simplify and advance clinical management.

Evaluation of central precocious puberty includes a standard GnRH test with gonadotropin measurements before and after GnRH. A basal LH of at least 0.3 IU/L (37) or a maximal GnRH-stimulated LH above 5 IU/L (37, 38) is considered a sign of central HPG activation. However, caution should be considered when evaluating GnRH test results in girls aged 1 to 3 years in whom LH may increase to 10 IU/L in healthy prepubertal girls (39). A GnRH test additionally may have some diagnostic value in separating constitutional delay of puberty from permanent hypogonadotropic hypogonadism, although controversy surrounding this issue exists. Furthermore, it may be useful even in the evaluation of patients presenting with AIS in mid-childhood, as an exaggerated LH response up to six times the basal levels has been reported (40). With the alignment of laboratory tests, systematic differences between laboratories can be minimized; that is, common reference values can be developed and used across laboratories. This will present the opportunity for comparable evaluation of measurements from different laboratories (41).

Position 4: Due to sex dimorphism and physiological fluctuations in concentrations with age and developmental stage, sex- and age-related reference values are mandatory in the evaluation of the peptide hormones FSH, LH, AMH, and Inhibin B. Publications containing reference values and decision limits should include information related to the analytical method.

Harmonization of laboratory tests

The comparability of results across laboratories can be attained by establishing metrological traceability; that is, the process by which it is ensured that measurement procedures measure the same quantity and that the calibration of these procedures are traceable to a shared reference system comprising reference methods and reference materials (42). The two approaches to reach metrological traceability are (1.) standardization, in which traceability is ensured to the International System of Units (SI) and 'trueness' is reached by a top-down approach (e.g. as applied to serum testosterone) and (2.) harmonization, in which traceability is ensured to a reference system that is not traceable to SI, but instead decided by agreement via a bottom-up approach (e.g. inter-method comparison among routine measurement procedures).

With both harmonization and standardization, external quality assurance programs play a central role in supporting agreement (43). DSD are rare conditions, so international collaboration is necessary to improve diagnostics and treatment follow-up of these conditions, and therefore agreement in laboratory medicine is crucial. The individual laboratory must use analytical validation, for which at least parameters like accuracy, including determination of both systematic (bias) and random (imprecision) errors, and sex- and age-specific reference values have been established.

For many peptides (including FSH, LH, AMH, and Inhibin B), true absolute values cannot be determined, as reference methods are not defined and no reference laboratories perform reference measurements for these peptides. Instead, laboratory comparisons are achieved using reference materials (e.g. WHO International Standards) that are untraceable to SI. A prerequisite for a reference material is commutability, which is two measurement procedures' closeness of agreement between the relation observed for a reference material and the relation observed for clinical samples (42). However, due to, for example, matrix effects in the reference material, non-commutability (i.e. that the reference material does not mimic the clinical samples) may challenge method comparison also for these peptide hormones. International standards exist for FSH, LH, and Inhibin B, while no international standard currently exists for the measurement of AMH. Thus, the introduction of routine measurements of AMH has been complicated and confusing due to the implementation of different assays based on various antibody combinations and various calibrations (44). However, recently it was shown that two widely available automated assays (Roche Elecsys and Beckman Coulter Access 2) yielded high degrees of consistency over a wide range of concentrations (44). This was because Roche standardized against the Beckman Coulter AMH Gen II ELISA assay.

Participation in external quality assurance (EQA) programs should be a prerequisite to achieve comparability in the results across laboratories. Table 1 depicts an example of four representative EQA programs (used in Copenhagen, DK), for FSH, LH, and AMH: the Bio-Rad Immunoassay (Monthly) Program (FSH and LH) (Bio-Rad Laboratories, CA, USA), the Labquality programs Hormone Determinations B (FSH and LH) and Tumour Markers (AMH) (Labquality, Helsinki, Finland),

respectively, and the United Kingdom National External Quality Assessment Service for Peptide Hormones (AMH; UK NEQAS, Edinburgh, UK). Table 1 lists the platform groups that constituted at least 1% of the total number of platforms participating in the Bio-Rad, the Labquality, or the UK-NEQAS program. Importantly, other EQA programs exist for peptide hormones, for example, Stichting Kwaliteitsbewaking Medische Laboratoria (SKML, the Netherlands) for FSH and LH. To the best of our knowledge, only two EQA programs for Inhibin B exist currently (ProBioQual, France, and Qualimedlab, Italy), and there is no program for urine LH (Table 1).

With the aim of obtaining an overview of the most commonly used Inhibin B-assays, we performed a search on PubMed (https://www.ncbi.nlm.nih.gov/pubmed/) with the following criteria: (1.) Inhibin B *and* human *and* serum *and* 2016 and (2.) Inhibin B *and* human *and* serum *and* 2017. The most commonly used assays reported were the ones listed in Table 1. However, three of the listed assays are former versions of the Beckman Coulter Inhibin Gen II ELISA and therefore are no longer commercially available. Furthermore, several *research use-only* versions exist, of which the assay by Ansh Labs is listed. Thus, to achieve agreement across laboratories, an EQA program for Inhibin B is recommended.

Position 5: Except for Inhibin B, external quality assurance (EQA) programs are widely available for these relevant peptide hormones in blood. Laboratories should aim to participate in EQA programs, when available. When EQA programs are unavailable, laboratories should aim to participate in activities of peer comparison, such as sample exchange for Inhibin B.

Peptide hormone analysis in relation to DSD

The use of peptide hormones as markers for DSD can be viewed from the classification of DSD (1). The corresponding peptide hormone concentration levels within each condition are shown in Table 2.

Sex-chromosome DSD conditions

Turner syndrome

Turner syndrome is one of the most common chromosomal abnormalities, occurring in approximately 1 in 2000 live-born female infants (45). The classical X-monosomic Turner variant is mostly associated with a prenatal degeneration of ovarian follicles and development of streak gonads (46), while spontaneous pubertal development is more frequent in patients with mosaic (45,X/46,XX) Turner syndrome. In mini-puberty, gonadotropin levels have been reported to be above female reference range, but with LH/FSH ratios within normal female range (8). As in healthy children and adolescents, girls with Turner syndrome exhibit a biphasic pattern with increased gonadotropin secretion in early childhood and from puberty onward (9, 47). The gonadotropin concentrations overlap with reference values from childhood through adolescence (9), with the largest overlap in mid-childhood. However, as a group, Turner syndrome patients exhibit higher gonadotropin levels in early childhood and during puberty than observed in healthy controls, maybe as a consequence of gonadal failure. During mini-puberty, undetectable to low-level concentrations of AMH and Inhibin B have been reported (8). Furthermore, an AMH concentration of ≤3 pmol/L has been reported as a predictor of imminent premature ovarian failure (36).

45,X/46,XY mosaicism

While patients with a 45,X/46,XY constitution have a 'Turner'-cell line, these may present as phenotypically predominantly male or female, or with ambiguous genitalia. Females with Y-chromosome material are considered to have Turner syndrome (see previous section). Males with this karyotype vary greatly in terms of gonadal function and androgenization, which is reflected in the gonadotropins, AMH, and Inhibin B that all vary from normal to levels indicative of gonadal failure (i.e. high LH and FSH, low AMH and Inhibin B) (10, 48, 49).

Klinefelter syndrome

Klinefelter syndrome is the most common chromosomal abnormality, with an estimated frequency of 1:1000 to 1:1500 in male newborns (50). The classical form of Klinefelter syndrome, which is defined by the 47,XXY karyotype and results from the aneuploidy of the sex chromosomes, constitutes 80–90% of the cases, while 10–20% of Klinefelter cases are caused by higher-grade aneuploidies, structurally abnormal X chromosomes, or mosaicims (51). The cardinal stigmata include small testes, hypergonadotropic hypogonadism, gynecomastia, infertility, and variable degrees of learning difficulties.

Table 2 Concentration levels of luteinizing hormone (LH), follicle-stimulating hormone (FSH), anti-Müllerian hormone (AMH), and Inhibin B in Differences of Sex Development (DSD).

	LH	FSH	AMH	Inhibin B
Sex-chromosome DSD conditions				
Turner syndrome	Elevated for F	Elevated for F	Decreased for F	Decreased for F
45,X/46,XY mosaicism	Variable	Variable	Variable	Variable
Klinefelter syndrome	Elevated for M	Elevated for M	Decreased for M	Decreased for M
46,XX DSD conditions				
46,XX ovotesticular DSD	Variable	Variable	Variable	Variable
46,XX gonadal dysgenesis	Elevated for F	Elevated for F	Decreased for F	Decreased for F
21-hydroxylase deficiency	Normal for sex	Normal for sex	Normal for sex	Normal for sex
46,XY DSD conditions				
46,XY gonadal dysgenesis (complete)	Elevated for F	Elevated for F	Decreased for F	Decreased for F
46,XY gonadal dysgenesis (partial)	Variable	Variable	Variable	Variable
StAR, CYP11A1, CYP17A1, HSD3B2 and HSD17B3*	Elevated for sex	Elevated for sex	Normal for sex	Normal for sex
5α-reductase deficiency	Normal for M	Normal for M	Decreased to normal for M	Decreased to normal for M
Complete AIS	Elevated for F	Normal to Elevated for F	Elevated for F	Elevated for F
Partial AIS	Elevated for M	Normal for M	Normal for M	Normal for M
Leydig-cell hypoplasia	Elevated for M	Normal to Elevated for M	Normal for M	Normal for M
PMDS (impaired AMH production)	Normal for M	Elevated for M	Decreased for M	Normal for M
PMDS (AMH resistance)	Normal for M	Elevated for M	Elevated for M	Normal for M
PMDS (testicular dysgenesis)	Elevated for M	Elevated for M	Decreased for M	Decreased for M
Other				
Congenital hypogonadotropic hypogonadism	Decreased for sex	Decreased for sex	Decreased for sex	Decreased for sex

As DSD covers patients with a wide spectrum of phenotypes including ambiguous genitalia, patients may be evaluated based on the most predominant phenotypical features, regardless of chromosomal constitution.
AIS, androgen insensitivity syndrome; F, female; M, male; PMDS, persistent Müllerian duct syndrome. *genetic mutations resulting in the following androgen biosynthesis deficiencies, steroid acute regulatory protein deficiency (StAR), StAR, P450 side chain cleavage enzyme deficiency (CYP11A1), 17-hydroxylase deficiency (CYP17A1), 3β-hydroxysteroid dehydrogenase deficiency (HSD3B2), and 17β-hydroxysteroid dehydrogenase (HSD17B3);

Most often, the gonadotropins are increased due to an abnormal Leydig cell function, while AMH is decreased due to an abnormal Sertoli cell function (24). In mini-puberty, concentrations of gonadotropin, AMH, Inhibin B, and the LH/FSH-ratio have been reported within male reference ranges (8), although higher-than-normal gonadotropin concentrations are also described (11). However, the likelihood of abnormal biochemistry may depend on the extent of testicular abnormality, which again depends on the age of the boy.

46,XX DSD conditions

Disorders of gonadal (ovarian) development

46,XX ovotesticular DSD

In the very rare ovotesticular DSD, the gonads may contain both ovarian and testicular tissue (ovotestis) or there may be a combination of an ovary on one side and a testis or an ovotestis on the other side. In 46,XX DSD, the genital development depends on the extent of testosterone and AMH secretion and thus the stimulation and/or regression of Wolffian and Müllerian structures. Therefore, phenotypical presentation varies. Although AMH and Inhibin B measurements cannot contribute information on the gonads separately, the peptides may provide an overview of gonadal function.

46,XX gonadal dysgenesis

46,XX gonadal dysgenesis (46,XX GD) is a genetically heterogenous group of phenotypical females characterized by streak gonads that lead to primary ovarian failure. Different causative mutations in genes have been reported, for example, in *FSHR* (52). The phenotype includes delayed or absent puberty, primary amenorrhea, hypoplasia of the uterus, and hypergonadotropic hypogonadism. Thus, concentrations of gonadotropins are commonly elevated, while AMH and Inhibin B concentrations are commonly low.

Androgen excess

21-hydroxylase deficiency

Congenital adrenal hyperplasia (CAH) due to 21-hydroxylase deficiency is the most common cause of DSD in 46,XX individuals (53). Because of the overproduction of androgens, the majority of 46,XX individuals typically present with ambiguous genitalia without palpable gonads in infancy. Elevated 17-hydroxyprogesterone, 21-deoxycortisol, and Δ4-androstenedione are indicative of 21-hydroxylase deficiency in the first-line testing. Furthermore, although literature is scarce, gonadotropin levels in CAH females during infancy before and after onset of treatment have been reported to resemble those in healthy boys (high LH, low FSH), albeit both gonadotropins were lower prior to treatment (54). This resemblance supports the notion that the gonadotropin secretion pattern seen in CAH neonates is due to prenatal programming by the excessive androgen exposure *in utero*. Thus, before CAH is genetically confirmed, FSH LH, AMH, and Inhibin B provide important information when distinguishing between 46,XX and 46,XY individuals, thereby excluding other forms of DSD in newborns with ambiguous genitalia. However, patients may also present in childhood (precocious puberty, pseudo-, or central) or adulthood (menstrual disorders, benign testis tumor). Patients with pseudo-precocious puberty may not display an initial elevation of gonadotropins, while they may occasionally be high at presentation or upon the start of treatment in patients with central precocious puberty. AMH and Inhibin B are most often normal for sex and age.

46,XY DSD conditions

Disorders of gonadal (testicular) development

46,XY gonadal dysgenesis

46,XY gonadal dysgenesis (46,XY GD) is a genetically heterogeneous group characterized by deficient gonadal development (55). The primary etiology is unknown, but mutations in a list of genes (e.g. *SRY*, *WT1*, *SF1* and *SOX9*) are associated with 46,XY GD (56). In complete 46,XY GD, there is no testis development, while testis development, although abnormal, is present in partial 46,XY GD (57). The resulting phenotype depends on the timing and amount of sex hormones produced by the gonads. Thus, the hormonal pattern in complete 46,XY GD includes hypergonadotropic hypogonadism which has been reported from puberty onward (58) and undetectable levels of AMH is reported from infancy (59), while patients with partial 46,XY GD who may exhibit hypergonadotropic hypogonadism and decreased AMH levels have also been reported from puberty onwards (58).

Androgen biosynthesis defects

Steroid acute regulatory protein (StAR) deficiency, P450 side chain cleavage enzyme (CYP11A1) deficiency, 17-hydroxylase deficiency (CYP17A1), 3β-hydroxysteroid dehydrogenase deficiency (HSD3B2), and 17β-hydroxysteroid dehydrogenase (HSD17B3) deficiency

These are autosomal recessive conditions caused by enzymatic defects resulting in impaired androgen synthesis. 46,XY individuals have testes, thus no Müllerian structures are present because of AMH production, while Wolffian derivatives are present in varying degrees dependent on the testosterone synthesis, resulting in variable masculinization. Patients typically have hypergonadotropic hypogonadism and AMH and Inhibin B concentrations within the normal range for 46,XY individuals. However, in the first months of life, AMH is high within or above the normal male range (60). In androgen biosynthesis defects, the discrepancy between steroid hormones and peptide hormones may contribute to the diagnostics; that is, testosterone levels are low, whereas AMH levels are normal to high.

5α-reductase deficiency

5α-reductase type 2 deficiency is an autosomal recessive condition due to mutations in *SRD5A2*. In 5α-reductase type 2 deficiency, there is an inability to convert testosterone to the more potent dihydrotestosterone that is required to masculinize external genitalia in genetic males during embryogenesis. The phenotype therefore ranges from varying degrees of under-virilization to complete feminization. The hormonal pattern is characterized by gonadotropin concentrations within the reference range, while AMH and Inhibin concentrations are low to normal (60, 61).

Defects in androgen action

Androgen insensitivity syndrome

Androgen insensitivity syndrome (AIS) is characterized by normal androgen production and metabolism in 46,XY individuals with complete (CAIS) or partial (PAIS) resistance of the androgen receptor to circulating

androgens. Thus, despite a male chromosomal constitution, the phenotype varies from male to female. AIS is reported to have a prevalence of 4.1 per 100 000 females (62). In CAIS, the phenotype is female due to the lack of masculinization of the Wolffian ducts and external genitalia, but internal female genitalia fail to develop due to regression of the Müllerian ducts. Caused by some residual binding affinity of the androgen receptor in PAIS, this phenotype ranges from a predominantly female phenotype with mild clitoromegaly and sparse labial fusion to a predominantly male phenotype with cryptorchidism, micropenis, hypospadias, and gynecomastia (63). During the first months of life, LH levels are low in CAIS infants, but high in PAIS infants (64). From puberty onward, AIS is characterized by elevated LH concentrations due to androgen resistance at the hypothalamic-pituitary level (65), and an LHRH stimulation test can further serve to highlight these elevated LH concentrations (40). Adults with testes *in situ* have normal to high concentrations of FSH (66). As the Sertoli cell function is intact, AMH and Inhibin B concentrations in general are within the male range (60, 67). In the first year of life, AMH levels are within or above male range in CAIS infants and above male range in PAIS infants (60).

LH-receptor defects

Leydig-cell hypoplasia

Leydig cell hypoplasia can be the result of inactivating mutations in the gene encoding the Leydig cell receptor, that is, the *l*uteinizing hormone-chorionic gonadotropin receptor *(LHCGR)* gene. In 46,XY DSD due to Leydig cell hypoplasia, there is a failure in pre- and post-natal virilization due to the scarcity of Leydig cells and consequently a lack of testosterone (68). The resulting phenotype depends on the degree of inactivation and varies therefore from male hypogonadism over genital ambiguity to female external genitalia. LH levels are typically increased, FSH levels are within or above male range, and AMH and Inhibin B levels are within male range. Particularly, in the first year of life, AMH is within or above male reference range (59).

Disorders of AMH and AMH receptor

Persistent Müllerian duct syndrome

Persistent Müllerian duct syndrome (PMDS) is the result of mutations in the *AMH* gene or the AMH receptor type 2 gene, resulting in AMH deficiency or AMH-receptor resistance, respectively, or the result of testicular dysgenesis. The genetic abnormalities only interfere with the regression of the Müllerian structures, so the Fallopian tubes and uterus are present, while external virilization is normal. Patients with PMDS due to mutations thus have normal male external phenotypes. In testicular dysgenesis, typically both Sertoli and Leydig cells are affected, resulting in external genital ambiguity (69). In PMDS, the normal concentration of Inhibin B indicates AMH deficiency when accompanied by low AMH concentrations and AMH-receptor resistance when accompanied by high AMH concentrations. As testosterone production is not affected, LH concentrations are normal. In testicular dysgenesis, the AMH- and Inhibin B concentrations are both lower than normal and dependent on the degree of testicular impairment.

Other

Congenital hypogonadotropic hypogonadism

In some cases of congenital hypogonadotropic hypogonadism (CHH), the external genitalia may be under-virilized and thereby qualify as a DSD phenotype. In CHH, the postnatal gonadotropic surge provides a useful window of opportunity to explore the gonadotropic axis, as gonadotropin concentrations are low in CHH. Due to the lack of stimulation of the testis, low concentrations of AMH and Inhibin B have been reported at this time of life (12).

Future directions

In the near future, it is expected that also peptide hormones may be analyzed using LC-MS/MS-technique because of its potentially higher specificity and lower detection limit than obtained by immunoassays. As this technique, as mentioned, also allows for simultaneous determination of multiple analytes, the challenges lie within the requirements for clinical application. An example of a LC-MS/MS analysis that recently has been developed for clinical application is quantification of the peptide hormone Insulin-like factor 3 (Insl-3) (70). This peptide is mainly produced in gonadal tissues in males and females and is thus expected to be a candidate within routine DSD diagnostics. Insl-3 is the major factor controlling the gubernacular growth and differentiation, it mediates the intra-abdominal testicular descent, and it is

involved in the transinguinal and inguinoscrotal descent (71). In animals, mutations in the gene encoding Insl-3 may lead to cryptorchidism, but such mutations are rarely the cause of cryptorchidism in humans. Insl-3 is thought to be a marker of functional Leydig cells and will, thus, be undetectable with the absence of Leydig cells.

Furthermore, targeted proteomics seems attractive with respect to future peptide analyses, as it allows for simultaneous analysis of several peptides at one time and as this method is compatible with existing mass-spectrometry platforms (72).

Conclusions

One of the aims of Endo-ERN is to establish a network of highly specialized endocrine reference laboratories with expertise in DSD. Differences of Sex Development constitute a heterogenous group of rare conditions. In a diagnostic setting, both a clinical and a biochemical approach is mandatory and knowledge of peptide hormone physiology and structure is essential. Five position statements have been developed to support the alignment of peptide hormone analysis in patients suspected of DSD conditions. This paper will support the establishment of common sex- and age-related references for normative data to optimize diagnostics and treatment. Comparability between laboratories requires participation in EQA.

Supplementary materials
This is linked to the online version of the paper at https://doi.org/10.1530/EJE-19-0831.

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