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The use of a "grey zone" considering measurement uncertainty in pharmacological tests. The serum growth hormone stimulation test as an example.

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Keywords:	Measurement Uncertainty, Pharmacological test, Stimulation serum growth hormone secretion, Cut-off, Grey zone.

Dear Editor in-chief

Thank you very much for the evaluation of our manuscript entitled " The use of a “grey zone” considering measurement uncertainty in pharmacological tests. The serum growth hormone stimulation test as an example"

We have tried to make the modifications according to the suggestions of the reviewers. The modified or added text were highlighted in bold.

We welcome your comments.

Below you will find the answers to the reviewers.

Sincerely,

Eduardo A. Chaler

Answer to Reviewer 2

a) The title was modified according to your suggestions. We agree that "around the cut-off" is part of the definition of "grey zone". The new title is “**The use of a “grey zone” considering measurement uncertainty in pharmacological tests. The serum growth hormone stimulation test as an example”.**

b) Cut-off limit was changed into Cut-off value.

c) We have modified the text pointed out by you to “The combination of these factors and the lack of standardization and other unmeasurable variables has led to a lack of confidence”

d) We agree with the reviewer’s comment and have changed in the text "accuracy" for **"measurement uncertainty"**.

e), f), g), h) and i) We have completely changed the text relating to the calculation of the expanded uncertainty, and we have adjusted the terms to NORDTEST, the resulting text is as follows: “**The application of ISO 15189:2012 (International Organization for Standardization) to clinical laboratories requires the knowledge of the measurement uncertainty for each measurement procedure in the analysis phase used to inform the measured quantitative values of patient samples.**

All measurements are affected by a certain error. The measurement uncertainty tells us what size the measurement error might be. Therefore, the measurement uncertainty is an important part of the reported result.

Measurement uncertainty (3) should normally be expressed as U, the combined expanded measurement uncertainty (u_c), using a coverage factor k = 2, providing a level of confidence of approximately 95 %.

$$U = 2.\ u_c$$

The different contributions to the u_c are the within-laboratory reproducibility (Rw) and the uncertainty component for bias (*u(bias)*)

$$u_c = (u(R_w)^2 + u(bias)^2)^{1/2}$$

$u(R_w)$ is calculated taken into account the intermediate precision (R_w) and is obtained from the internal quality control data measured for at least 6 months using different operators, reactive lots, calibrations, and storage conditions; and using suitable material.

***u(bias)* can be estimated by**

$$u(bias) = [(bias)^2 + (S_{bias}/(n)^{½})^2 + u(Cref)^2]^{½}$$

bias is the difference between mean measured value from a large series of test results and an accepted reference value. The most common ways of estimating the bias components are: the use of Certified Reference Material (CRM), recovery tests or participation in interlaboratory comparisons (External Quality Control).

S_{bias} is standard deviation obtained from measurements on the CRM and n is the number of measurement on the CRM.

u(Cref) Uncertainty component for the certified or nominal value."

j) Throughout the text and in the Figure, Uncertainty zone (UZ) was changed into **Grey Zone (GZ)**

k) We changed in the text and in the figure the formula for the calculus of GZ and we have expressed U in an absolute value, not percentage. **"GZ = cut-off ± U".**

l) We used the corrected mathematical model en we agree with you that U is 10.8%, the range is between 4.2 and 5.2 ng/ml, we added information about the peer group that we used. The resulting text is as follows:

"Due to the lack of laboratory or reference material **R_w** is traceable uniquely to the laboratory and bias is traceable uniquely to the group. We used Lyphochek Immunoassay Pluscontrol - US-Bio Rad Laboratories Irvine – CA, with a concentration at a level = 4.5 ng/ml

Over a period of 3 years, we measured **GH in ng/ml in Human serum** with a **U = 10.8%. (R_w = 5.13%, bias = 1.75%, number of result of sampling used for R_w and bias = 669, Sbias is unmeasurable and u(Cref) is unknown) .** If we apply the U formulation, our **GZ** is between 4.2 and 5.2 ng/ml."

m) We have added a brief explanation about the international reference material that the growth hormone assay uses as calibrator n) the keywords were changed.

n) The keywords were changed.

The use of a "grey zone" considering measurement uncertainty in pharmacological tests. The serum growth hormone stimulation test as an example.

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Short title: The use of a "grey zone" considering measurement uncertainty in PhTs. Growth hormone as an example.

Keywords: **Measurement Uncertainty**, Pharmacological test, Stimulation serum growth hormone secretion, **Cut-off**, Grey zone.

The responsibility of clinical laboratories includes adequate assay methods, measurement procedures, and the definition of the appropriate quality specifications for each mensurand as well as the identification of criteria required for obtaining the optimal interpretation and utilization of results, with reference intervals and adequate decision limits (1).

Decision limits in pharmacological tests (PhT) are used in the study of different hormonal axes (GnRH, TRH, serum growth hormone (GH) in PhT, etc) in which an inhibitory or stimulatory factor is administered to the patient and the effects are measured at different times. Subsequently, according to a defined cut-off **value**, it is considered whether stimulation/inhibition was effectively achieved. The problems behind an evidence-based approach to laboratory diagnostics can be clearly illustrated by the components of a PhT: 1) a variety of protocols, 2) secretagogues, 3) a variable biological response to stimulation, 4) a multiplicity of assays, and finally, 5) the variability in clinical interpretation. **The combination of these factors and the lack of standardization and other unmeasurable variables has led to a lack of confidence.**

Usually, the evaluation of the results obtained does not take into account that they have measurable magnitudes and are limited by the measurement system. Therefore, results have a certain **measurement uncertainty**, and the area around the cut-off value where the results are uncertain should be defined (2).

The application of ISO 15189:2012 (International Organization for Standardization) to clinical laboratories requires the knowledge of the measurement uncertainty

for each measurement procedure in the analysis phase used to inform the measured quantitative values of patient samples.

All measurements are affected by a certain error. The measurement uncertainty tells us what size the measurement error might be. Therefore, the measurement uncertainty is an important part of the reported result.

Measurement uncertainty (3) should normally be expressed as U, the combined expanded measurement uncertainty (u_c), using a coverage factor k = 2, providing a level of confidence of approximately 95 %.

$$U = 2.\ u_c$$

The different contributions to the u_c are the within-laboratory reproducibility (Rw) and the uncertainty component for bias (*u(bias)*)

$$u_c = (u(R_w)^2 + u(bias)^2)^{1/2}$$

$u(R_w)$ is calculated taken into account the intermediate precision (R_w) and is obtained from the internal quality control data measured for at least 6 months using different operators, reactive lots, calibrations, and storage conditions; and using suitable material.

u(bias) **can be estimated by**

$$u(bias) = [(bias)^2 + (S_{bias}/(n)^{½})^2 + u(Cref)^2]^{½}$$

bias is the difference between mean measured value from a large series of test results and an accepted reference value. The most common ways of estimating the bias components are: the use of Certified Reference Material (CRM), recovery tests or participation in interlaboratory comparisons (External Quality Control).

S_{bias} is standard deviation obtained from measurements on the CRM and n is the number of measurement on the CRM.

u(Cref) Uncertainty component for the certified or nominal value.

We propose to use a grey zone **(GZ)** based on U as it considers all sources of variation of a result attributed to the quantities in two terms: variation associated with precision and with trueness. This area consists of the **GZ = cut-off ± U.** Using the **GZ**, a **ternary classification is expected;** any individual result outside this zone - with its range of uncertainty included - is guaranteed to be above or below the cut-off. (Figure).

In this line, GH may be a paradigm. A diagnosis of growth hormone deficiency (GHD) implies expensive and prolonged treatment. A tremendous amount of scientific evidence regarding the physiology and physiopathology of synthesis mechanisms, secretion, and actions of GH has been published over the last years; however, in spite of these impressive advances and, deeply disappointing from a public health perspective, the

real picture of diagnosis is overshadowed by widespread diagnostic inaccuracies (underdiagnosis, overdiagnosis) as well as by treatment failures generated by under- or overtreatment. The scientific, medical, and patient communities as well as decision-makers worldwide are striving for the greatest possible health gains from available resources.

The diagnostic cut-off **value** of serum GH in PhT for the diagnosis of GHD has been an ongoing topic of discussion. For years there was no harmonization between assays (4, 5) until 2008, when a consensus (6) proposed the GH assays to measure the 22k form and use the second growth hormone-recombinant international standard IRP 98/574. Currently, there is no agreement on the cut-off point of serum GH in PhT below which we define GHD.

In patients the diagnosis of GHD is based mainly on auxological criteria. The diagnosis is biochemically confirmed by the maximum peak (maxp) reached during two PhTs; if one of them is above the diagnostic cut-off **value** the patient is considered to have adequate GH secretion.

In a recent publication (7), we defined a cut-off point for PhTs in GH of 4.7 ng/ml by chemiluminescent assay (Immulite 2000, Siemens Laboratories) using IRP 98/574 (international reference material prepared by genetic engineering).

Due to a lack of laboratory or reference material **R_w** is traceable uniquely to the laboratory and bias is traceable uniquely to the group. We used Lyphochek Immunoassay Pluscontrol - US-Bio Rad Laboratories Irvine – CA, with a concentration at a level = 4.5 ng/ml

Over a period of 3 years, we measured **GH in ng/ml in Human serum** with a **U = 10.8%. (R_w = 5.13%, bias = 1.75%, number of result of sampling used for R_w and bias = 669, Sbias is unmeasurable and u(Cref) is unknown).** If we apply the U formulation, our **GZ** is between 4.2 and 5.2 ng/ml.

We analyzed the plotting of the results of two GH secretion PhTs (Clonidine and Arginine) in 338 patients. Using the GZ, 34.3% (n: 116) potentially had a secretory deficit (GHD group) and 57.7% (n: 195) had adequate secretion (AGH group). Finally, 8% (n: 27) was found in the GZ, in which GH secretion status cannot be appropriately determined (GZ group).

It is widely accepted to consider insulin-like growth factor type 1 (IGF-1) as a biomarker for GH action. There are publications that defined GHD in function of IGF-1 standard deviation score (SDS) (8). We found significant differences in IGF-1 SDS between the GHD group and the AGH group. The GZ group showed significant differences compared to the GHD group, but not compared to the AGH group. Moreover, if the GZ group is divided into the GZ group below the cut-off value (GZ low) and GZ group above

the cut-value value (GZ high), no significant differences were found, Remarkably, the GZ low group, which may be considered as having a deficit, presented with a significant difference compared to the GHD group but not compared to the AGH group (Table). Clearly, the GZ group has different characteristics that should be assessed differently, and a single cut-off value is not sufficient to define the diagnostic limits. Savage et al (9) defined the differences in status of secretion and sensitivity as a continuum and the use of GZ would be a practical approximation to this idea.

The concept of the "grey zone" is naturally used in other biochemical parameters such as those used in serologic diagnosis; however generally it is not considered in endocrine PhTs.

We strongly recommend to include an grey zone in the diagnostic cut-off **value** calculated for each analytical platform according to the U of the mensurand used.

We considered GH secretion in PhTs as a paradigm, as GHD implies expensive and prolonged treatment. If the maximum peak of patients is found to be within the GZ, the specialist should assess other features, such as family history, clinical and nutritional status, and diseases to decide whether to treat or not. If this grey zone is not taken into account, patients may misclassified leading to treatment errors.

As the sensitivity and specificity indicate diagnostic capacity of the tests in general, the GZ indicates the analytical limitation of the mensurand that should be taken into account for the biochemical counseling on the interpretation of the results.

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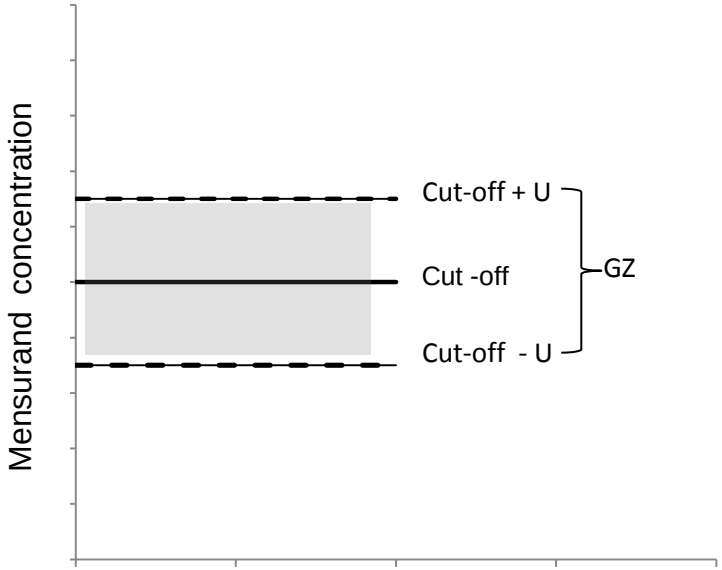
Mensurand concentration
Cut-off + U
Cut -off
Cut-off - U
GZ

Group	GH (ng/ml)	SDS IGF-1
GHD n: 116	1.89 ± 1.26	-1.63 ± 1.68[1]
AGH n: 195	12.13 ± 6.48	-0.58 ± 1.12[2]
GZ n: 27	4.85 ± 0.34	-0.47 ± 1.16[3]
GZ low n:11	4.51 ± 0.15	-0.38 ± 0.82[4]
GZ high n: 16	5.09 ± 0.18	-0.53 ± 1.37[5]

p< 0.0001 GHD vs AGH
p<0.001 GHD vs GZ
p<0.05 GHD vs GZlow; GHD vs GZhigh
ns GZ vs AGH; GZlow vs GZhigh; GZlow vs AGH; GZhigh vs AGH