ARTICLE

The Common H202D Variant in GDF-15 Does Not Affect Its Bioactivity but Can Significantly Interfere with Measurement of Its Circulating Levels

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Background: There is growing interest in the measurement of growth differentiation factor 15 (GDF-15) in a range of disorders associated with cachexia. We undertook studies to determine whether a common histidine (H) to aspartate (D) variant at position 202 in the pro-peptide (position 6 in the mature peptide) interfered with its detection by 3 of the most commonly used immunoassays.

Methods: Three synthetic GDF-15-forms (HH homo-, HD hetero-, and DD-homodimers) were measured after serial dilution using Roche Elecsys®, R&D Quantikine™ ELISA, and MSD R&D DuoSet® immunoassays. GDF-15 concentrations were measured by the Roche and the MSD R&D immunoassays in 173 genotyped participants (61 HH homozygotes, 59 HD heterozygotes, and 53 DD homozygotes). For the comparative statistical analyses of the GDF-15 concentrations, we used non-parametric tests, in particular Bland–Altman difference (bias) plots and Passing–Bablok regression. The bioactivity of the 2 different homodimers was compared in a cell-based assay in HEK293S-SRF-RET/GFRAL cells.

Results: The Roche assay detected H- and D-containing peptides similarly but the R&D reagents (Quantikine and DuoSet) consistently underreported GDF-15 concentrations in the presence of the D variant. DD dimers had recoveries of approximately 45% while HD dimers recoveries were 62% to 78%. In human serum samples, the GDF-15 concentrations reported by the R&D assay were a median of 4% lower for HH, a median of 36% lower for HD, and a median of 61% lower for DD compared to the Roche assay. The bioactivities of the HH and DD peptides were indistinguishable.

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IMPACT STATEMENT

The measurement of growth-differentiation factor 15 (GDF-15) is expanding from the research to the clinical arena, with GDF-15 levels related to prognosis in a number of chronic diseases. There is a very common (15% to 30% allele frequency) histidine to aspartate variant at position 202 of the GDF-15 pro-peptide in human populations. We undertook studies to establish whether this variant interfered with the measurement of GDF-15.

The D variant of GDF-15 substantially affects its measurement by the R&D reagents. These findings have implications for interpreting studies, which have used R&D reagents to measure GDF-15, and its emerging clinical use as a diagnostic biomarker.

Conclusions: The D variant of GDF-15 substantially affects its measurement by a commonly used immunoassay, a finding that has clear implications for its interpretation in research and clinical settings.

INTRODUCTION

Growth-differentiation factor 15 (GDF-15) is a member of the transforming growth factor β (TGF-β) cytokine superfamily. Its circulating concentrations in humans are elevated in a wide range of diseases such as cachexia (1), chemotherapy-related nausea (2) and vomiting, and hyperemesis gravidarum (HG) (3). It is also elevated in mitochondrial disorders (4–6), where it may have some diagnostic utility, and its levels are being investigated for potential prognostic value in some cancers and in heart failure (7,8). GDF-15 has recently gained further scientific and translational prominence with the discovery that its receptor is a glial cell line-derived neurotrophic factor (GDNF) family receptor alpha like (GFRAL)-Ret proto-oncogene (RET) heterodimer, which is found solely in the hindbrain (9–12). Activation of this receptor results in a reduction of food intake and body weight in several animal models (13). These findings have prompted exploration of GFRAL-Ret agonists as potential therapy for obesity and antagonists of GDF-15 or GFRAL in various forms of cachexia. While studies in rodents and primates have consistently demonstrated the weight loss effects of GDF-15, a recent Mendelian randomization study did not support the idea that increased levels of GDF-15 (measured by immunoassay using the R&D antibodies (14)) reduced human body mass index (BMI). Conversely, it was suggested that genetically determined increases in circulating GDF-15 might actually predispose to obesity. As there is a common coding variant in GDF-15, which changes residue 202 from histidine (H) to aspartate (D) (15,16) and is present in the vast majority of human populations studied to date with a reported minor allele frequency ranging from 14% to 35% across major continental groups (17), we speculated that the discrepancy between pharmacological and Mendelian randomization studies might be due to systematic bias in the measurement of GDF-15 by certain immunoassays. To test that hypothesis, we first examined the ability of the antibody combinations from 2 widely used assays for human GDF-15 to detect the H and D isoforms, using the relevant synthetic homodimers and heterodimers. Secondly, we studied human serum samples from participants of known genotype. Finally, we undertook studies comparing the functional properties of the H and D forms of GDF-15 as activators of signaling through GFRAL-Ret.

MATERIALS AND METHODS

Study Participants

Circulating GDF-15 concentrations were measured in serum from 173 participants (126 women, 47 men with BMI range: 27.5 to 70 kg/m², age range: 21 to 74 years) from the Leipzig Obesity Biobank whose genotype at rs1058587 in the GDF-15 gene had previously been established from RNA sequencing data using adipose tissue obtained at surgery. Participants were randomly selected from the Biobank to represent an equal distribution of the genotype at rs1058587 (18). Serum samples were collected in the context of elective laparoscopic abdominal surgeries as described previously (19). After extraction, serum samples were immediately frozen in liquid nitrogen and stored at −80 °C. The study was approved by the Ethics Committee of the University of Leipzig (approval no: 159-12-21052012), and performed in accordance with the declaration of Helsinki. All subjects gave written informed consent before taking part in this study. Measurement of body composition and metabolic parameters was performed as described previously (18,19).

Protein Production

DNA encoding for mature GDF-15 was cloned into pBAT4 vector using *Nco*I and *Hind*III restriction sites, with the addition of a codon for an initiation methionine. The H202D mutation (histidine to aspartate variant present in position 6 of the mature GDF-15 protein) was introduced by PCR mutagenesis of the wild-type construct. The mature GDF-15 proteins were expressed in *E. coli* in 2x YT medium at 37 °C, with all the protein forming insoluble inclusion bodies. The bacterial cell cultures were pelleted by centrifugation. Pelleted cells were resuspended in 30 mL lysis buffer (50 mM Tris-HCl pH 8.0 and 5 mM EDTA with 10 mM dithiothreitol [DTT]) per 1 L of cell culture. Cell lysis was achieved under high pressure using an Emulsiflex C5 homogenizer, with 0.5% Triton-X added to the sample during lysis. The lysate was then incubated at room temperature with 0.2 mg DNase and 4 mM $MgCl_2$ for 30 min. The lysate was centrifuged at 15 000*g* for 20 min and the supernatant discarded. Centrifugation and removal of the supernatant was repeated after resuspension with the following wash buffers: buffer A (50 mM Tris-HCl pH 8.0, 5 mM EDTA, 10 mM DTT, and 0.5% v/v Triton-X 100), buffer A with 2 M NaCl, and finally buffer A without Triton-X 100. The purified inclusion body was pelleted by centrifugation and stored at −20 °C. Inclusion body pellets (from 1 L of bacterial cell culture) were re-suspended in 5 to 10 mL in 100 mM TCEP (tris-carboxy ethyl phosphine) pH 7.2, then solubilized with the addition of 15 to 30 mL 8 M guanidine hydrochloride, 50 mM Tris-HCl pH 8.0, and 10 mM EDTA. Samples were then incubated at room temperature for 20 min, clarified by centrifugation and buffer-exchanged into deionized 6 M urea with 20 mM HCl using a Sephadex G25 column (Cytiva). The protein concentration was adjusted to ≤ 1 mg/mL by rapid dilution in urea buffer (100 mM Tris-HCl pH 8.0, 1.2 M urea, 1 M PPS [pyridine propyl sulfonate], and 0.2 mM L-cystine). For refolding of the heterodimeric GDF-15 (HD), equal quantities of denatured H and D forms of GDF-15 were mixed before dilution in refolding buffer. This resulted in a "heterozygous mix" containing GDF-15 HD, HH, and DD as confirmed by mass spectrometry (MS) with an approximate 1:2:1 ratio of HH:HD:DD.

Refolded GDF-15 was captured by ion-exchange chromatography using a SOURCE 30S column (Cytiva) and fractions containing the disulfide-linked dimer polished using reversed-phase chromatography using an ACE5 C8-300, size 4.6 × 250 mm column (HiChrom). Eluted fractions were analyzed by non-reducing SDS-PAGE. Fractions with pure GDF-15 dimer were pooled together, with the concentration determined by UV absorption at 280 nM using a calculated absorption

coefficient. Aliquots of 250 µg were vacuum-dried and stored at −80 °C.

For subsequent assays, the proteins were suspended to 1 mg/mL in 10 mM HCl and diluted to the desired concentration in buffer containing bovine serum albumin.

Immunoassays

Roche Elecsys® GDF-15.

Samples were analyzed using the Roche Elecsys e411 GDF-15 Cobas electrochemiluminescent immunoassay (Roche Diagnostics). In the first incubation, GDF-15 in the sample, the biotinylated monoclonal GDF-15-specific antibody and a second monoclonal GDF-15-specific antibody conjugated to rheuthinium to form a sandwich complex. In the second incubation, after addition of streptavidin-coated microparticles, the complex becomes bound to the microparticles via interaction of biotin and streptavidin. The reaction mixture is aspirated into the measuring cell where the complex–microparticles are magnetically captured onto the surface of the electrode. Unbound substances are then removed with wash solution (ProCell II M). Application of a voltage to the electrode then induces electrochemiluminescent emission, which is measured by a photomultiplier. Results are determined via a calibration curve which is instrument-specifically generated by 2-point calibration and a master curve provided via the Cobas link. The limit of detection for the assay was 400 pg/mL, with an upper measurement limit of 20 000 pg/mL. Samples that were initially above the measuring range were diluted in Roche "Multiassay diluent." Assays were calibrated and quality controlled using the manufacturer's reagents. The coefficient of variation was 3.8% for the low control (at 1556 pg/mL) and 3.4% for the high control (at 7804 pg/mL).

R&D Quantikine® ELISA assay.

This is a quantitative sandwich enzyme immunoassay with a monoclonal antibody specific for GDF-15 pre-coated onto a microplate. Standards and samples are pipetted, in duplicate, into the wells and any GDF-15 present is bound by the immobilized antibody. After washing, a horseradish peroxidase (HRP)-conjugated polyclonal antibody specific for GDF-15 is added to the wells. Following a wash to remove any unbound conjugate, a substrate solution is added to the wells and color develops in proportion to the amount of GDF-15 bound in the initial step. The color development is stopped with acid and the absorbance of the solution is measured on a Perkin Elmer Victor3 plate reader. The assay was controlled using in-house– generated controls based on anonymized serum and plasma pools as used for the MSD R&D DuoSet® in-house assay. The in-house assay is a MSD electrochemiluminescence assay format using antibodies and standards from the R&D DuoSet®.

The limit of detection of the assay is 17.6 pg/mL with an upper limit of the standard curve of 1500 pg/mL (manufacturer's data). The between-batch imprecision is 6.0% at 225 pg/mL, 4.7% at 442 pg/mL, and 5.6% at 900 pg/mL (manufacturer's data).

MesoScale Discovery (MSD) R&D DuoSet in-house assay.

The assay is an in-house, microtiter plate-based, two-site electrochemiluminescence immunoassay using the MesoScale Discovery assay platform (MSD). Antibodies and standards were purchased as a Human-GDF-15 DuoSet ELISA from R&D Systems (BioTechne). Standard-bind MSD microtiter plates were coated overnight with monoclonal anti-GDF-15 capture antibody diluted in phosphate-buffered normal saline (PBS), 1x solution. After coating, the plate was washed 3 times with PBS/Tween using a ThermoFisher automated plate washer and then blocked with MSD Blocker A. After blocking, 40 µL assay diluent plus 10 µL standards, QCs, and undiluted sample were added to the plate in duplicate. After 2 h incubation at room temperature on a plate shaker, the

plate was washed 3 times with PBS/Tween. Biotinylated polyclonal goat anti-human GDF-15 detection antibody was diluted in MSD Diluent 100 and added to the plate. After 1 h incubation at room temperature on a plate shaker and washing with PBS/Tween, MSD SULPHO-TAG™ Streptavidin labelled streptavidin (MSD) diluted in MSD Diluent 100 was added to the plate. The plate was the incubated on a plate shaker for a further 30 min.

After another wash step, MSD read buffer was added to all the wells and the plate was immediately read on the MSD s600 plate reader. Luminescence intensities for the standards were used to generate a standard curve using MSD's Workbench software package. The assay was controlled using in-house–generated controls based on anonymized serum and plasma pools.

The limit of detection of the assay is 3.0 pg/mL with an upper limit of the standard curve of 32 000 pg/mL (in-house data). The between-batch imprecision is 9.8% at 352 pg/mL, 7.8% at 1476 pg/mL, 8.0% at 6593 pg/mL, and 6.1% at 12 134 pg/mL (in-house data).

GDF-15 bioassay of recombinant GDF-15 protein constructs.

GDF-15 bioactivity assays were performed in HEK293S-SRF-RET/GFRAL cells. This stable cell line was modified to express full-length human RET and GFRAL receptors as well as serum response factor-responsive luciferase reporter gene by AstraZeneca. Cells were grown and seeded in T75 flasks in Dulbecco's Modified Eagle Medium (DMEM) GlutaMAX (Gibco) supplemented with 10% fetal bovine serum (FBS) (Gibco) and incubated for 30 h at 37 °C, with 5% carbon dioxide. Post incubation, the media was switched to serum-free DMEM and cells were then incubated overnight under the same conditions. The following day, cells were detached using accutase enzyme and then neutralized in DMEM with 1% FBS. Cells were then pelleted by centrifugation and resuspended in serum-free DMEM with an additional 25 mM HEPES pH 7.2 (GDF-15 bioassay media). Cells in suspension were counted using a Cedex automatic cell counter (Roche) and Trypan Blue (Sigma) staining as standard. After counting, cells were then diluted to 500 000 cells/mL in GDF-15 bioassay media before plating in 384-well plates (15 µL/well). After plating, 5 µL of recombinant GDF-15 in GDF-15 bioassay media was then added to the cells at 4× the final concentration. Cells with recombinant protein samples were then incubated for 5 h at 37 °C, with 5% carbon dioxide. After 5 h, the plate was then equilibrated to room temperature before the addition of Steady-Glo Luciferase Assay System solution (Promega) at 12 µL/well and incubated for a further 20 min. The luminescence was measured using an EnVision plate reader (Perkin Elmer) with crosstalk correction applied. Chinese hamster ovary cell-derived recombinant human GDF-15 (PeproTech) was used as a positive control. Negative controls of protein sample buffers only were also screened to confirm the absence of signaling activity. Triplicate samples were treated at each GDF-15 dose, and data was analyzed in GraphPad 7.0.

Comparative Statistical Analyses

Note that for 3 participants, the GDF-15 concentrations measured by the Roche Elecsys assay were below the detection limit of 400 pg/mL. These individuals were excluded from the comparative analyses. All data analysis steps were run using R 4.1.1 (20).

Normality of the GDF-15 concentrations was assessed using the Shapiro–Wilk test. To determine method differences, we constructed Bland–Altman relative difference plots. As the data were non-normally distributed (see below), we estimated the confidence intervals for the relative differences empirically as the 2.5th percentile and 97.5th percentile (21). To formally test the impact

of the genotype of the H202D variant on the GDF-15 concentrations measured by the MSD R&D DuoSet in-house and Roche Elecsys immunoassays, a repeated measures ANOVA of the aligned and rank-transformed data was computed (22,23) using the R package ARTool (v.0.11.1) (24). Spearman rank correlation coefficients were determined, stratified by genotype.

Passing–Bablok (PB) regressions (25) were run using the R package mcr (v.1.2.1) (26) for n = 170 patients to obtain potential adjustment equations between the 2 immunoassays, stratified by genotype.

RESULTS

Recovery of the D Variant in Synthetic Peptides

We synthesized 3 forms of GDF-15 (HH homodimers, DD homodimers, and HD heterodimers, the result of a mixture of HH:HD:DD in the ratio 1:2:1). Equal amounts of each dimer were serially diluted and assayed using the Roche Elecsys and the R&D Quantikine ELISA and MSD R&D DuoSet immunoassays (see Methods). Table 1 shows the data for the sample with a theoretical concentration of 667 pg/mL. This is the only concentration that was within the working ranges of all 3 assays. The full data set is included in online Supplemental Table 1. Recoveries of HH and HD with the Roche Elecsys assay were very similar, with a slight reduction (8% to 15%) in recovery when assaying the D dimer (Table 1 and Supplemental Table 1). With the Roche Elecsys assay, reported results were proportional to dilution from 2000 to 667 pg/mL. The Roche Elecsys assay has a lower limit of detection of 400 pg/mL and therefore further dilutions were reported as <400 pg/mL, as expected (Supplemental Table 1). With the MSD R&D DuoSet immunoassay, which has a lower limit of detection of 3.0 pg/mL, the measurements were also proportionate to the dilutions and the percentage recovery was similar across all dilutions. However, there was a marked difference in measured levels between the types of dimer (Table 1 and Supplemental Table 1).

The R&D Quantikine ELISA and MSD R&D DuoSet immunoassays gave comparable results. Recovery of DD dimers with both of these assays was approximately 45% of that seen with HH dimers, and between 62% and 78% with the HD dimers (Table 1 and Supplemental Table 1).

Recovery of the D Variant in Human Serum Samples

We compared the circulating concentrations of GDF-15 as measured by the Roche Elecsys and MSD R&D DuoSet immunoassays in serum samples from 170 participants (124 women and 46 men) from the Leipzig Obesity Biobank whose genotype at rs1058587 in the *GDF-15* gene had previously been established from RNA sequencing data using adipose tissue obtained at surgery (18). We selected samples from 59 HH homozygotes, 58 HD heterozygotes, and 53 DD homozygotes.

Based on the evidence from the synthetic peptides, we hypothesized that the genotype at H202D had a significant impact on the degree of concordance between the 2 immunoassays. The 6 variables relevant for this analysis (measurements by 2 immunoassays used for each individual stratified by 3 genotypes) were not normally distributed (P values $< 10^{-6}$ for all Shapiro–Wilk tests). Therefore, we applied a non-parametric framework.

This was confirmed by a repeated measures ANOVA of the aligned rank-transformed data that revealed a significant interaction effect of the genotype at H202D and the type of immunoassay used ($F_{2,\ 167} = 102.10$, $P < 2 \times 10^{-16}$). Post hoc tests showed significant group differences between the GDF-15 concentrations measured by the 2 assays for HD heterozygotes ($P < 0.001$)

Table 1. Percentage recovery of synthetic GDF-15 dimers HH, HD, and DD using 3 immunoassays.[a]

GDF-15 dimer type	Theoretical, pg/mL	Roche Elecsys assay			MSD R&D DuoSet in-house assay			R&D Quantikine ELISA		
		Measured, pg/mL	Recovery to theoretical, %	Recovery to HH, %	Measured, pg/mL	Recovery to theoretical, %	Recovery to HH, %	Measured, pg/mL	Recovery to theoretical, %	Recovery to HH, %
HH	667	882	132	NA	1027	154	NA	1154	173	NA
HD	667	877	132	99	795	119	77	716	107	62
DD	667	774	116	88	446	67	43	523	78.	45

[a]Abbreviations: HH, homozygote for wild type; HD, heterozygote; DD, homozygous mutation; NA, not applicable.

and DD homozygotes ($P < 0.001$) but not for HH homozygotes ($P = 0.069$).

In quantitative terms, the concentrations of GDF-15 reported using the MSD R&D DuoSet immunoassay were a median 4% lower (95% CI, 35 lower to 13 higher) in HH, a median 36% lower (95% CI, 16–55) in HD, and a median 61% lower (95% CI, 54–73) in DD than those measured by the Roche Elecsys assay (Fig. 1 and Supplemental Fig. 1). The Bland–Altman plots suggest that for the HD genotype, the relative difference between the methods is consistent across concentrations, whereas for the HH and DD genotypes, the underreporting by the MSD R&D DuoSet immunoassay appears to moderately increase for high GDF-15 concentrations (online Supplemental Fig. 1). However, these patterns need to be interpreted with caution due to the low sample sizes in the higher concentration range.

To further compare the 2 immunoassays, we assessed their relationship stratified by genotype at H202D using Spearman rank correlation and found that they were highly correlated. For the HH genotype Spearman's rho is 0.93 ($P < 2 \times 10^{-16}$, 95% CI: 0.84–0.96), for the HD genotype 0.96 ($P < 2 \times 10^{-16}$, 95% CI: 0.90–0.98), and for the DD genotype 0.89 ($P < 2 \times 10^{-16}$, 95% CI: 0.75–0.96).

Figure 1 shows individual data points showing distribution (median, interquartile range [IQR], and 1.5-fold IQR above/below the first/third quartiles respectively). In human serum samples, the GDF-15 concentrations reported by the R&D DuoSet assay were a median of 4% lower in HH, a median of 36% lower in HD, and a median of 61% lower in DD compared to the Roche assay. The bioactivities of the HH and DD peptides were indistinguishable.

From the data produced using the synthetic peptide, it appears that the Roche Elecsys assay is able to detect the 3 forms of GDF-15 approximately equally. From this, we can then attempt to establish a potential adjustment equation. It

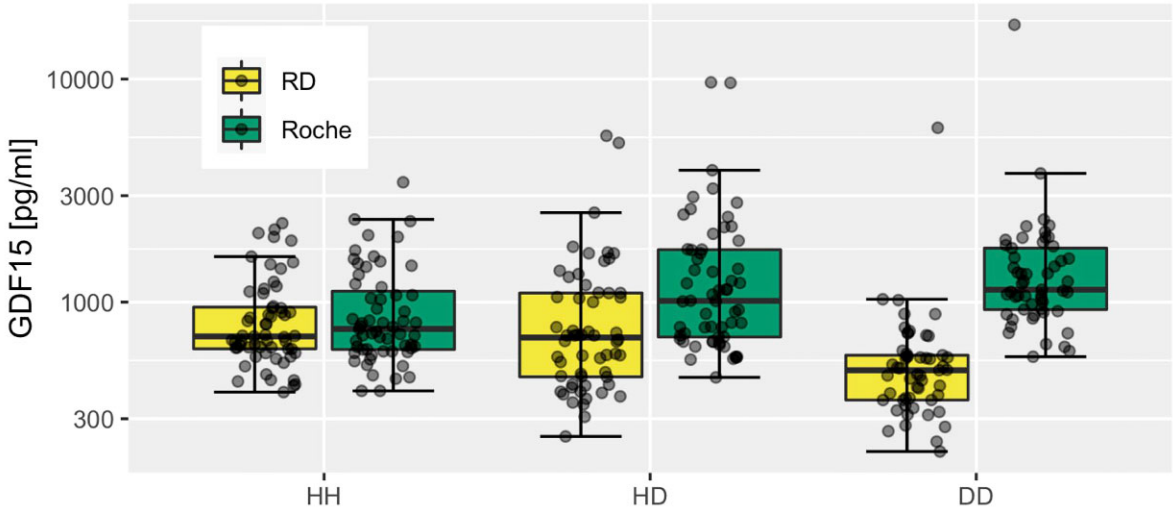

Fig. 1. Circulating levels of GDF-15 measured in genotyped human participants using MSD R&D DuoSet and Roche Elecsys immunoassays.
Box plots overlaid with individual data points showing distribution (median, interquartile range [IQR] and 1.5-fold IQR above/below the first/third quartiles respectively) of serum GDF-15 concentrations in genotyped 170 participants of the Leipzig Obesity Biobank measured by MSD R&D DuoSet immunoassay (RD) against serum GDF-15 measured by the Roche Elecsys immunoassay (Roche) in the same individuals. Abbreviations: Wild type (HH) n = 59; heterozygote (HD) n = 58; homozygote (DD) n = 53.

could be used in situations where genotype is known to convert MSD R&D DuoSet immunoassay results to an estimate of the Roche Elecsys assay for samples from individuals who are homozygous or heterozygous for the D mutation.

Figure 2 provides a graphical representation of the relationship between GDF-15 levels measured using the 2 assays in the same individuals. PB regression (25) was applied to obtain adjustment equations as it is more resistant to outliers than simple linear regression. The results of PB regression (Fig. 2) were consistent with those of ANOVA and the Bland–Altman plots. Equations 1 and 2 suggest adjustments for HD heterozygotes and DD homozygotes, respectively; confidence intervals for the regression parameters are given in Figure 2. The 95% confidence intervals for the slopes do not include 1 and do not overlap with each other. This is consistent with strong evidence against the null hypothesis that the slopes for all 3 observed genotypes at rs1058587 represent the same relationship between the 2 assays. For the HH homozygote genotype, evidence from multiple analyses (ANOVA, Bland-Altman plots, and PB regression) is not strong enough to suggest a consistent difference between the immunoassays.

$$\text{GDF} - 15\ \text{Roche Elecsys}^{®}\text{estimate [pg/ml]} = (GDF - 15\text{R\&D [pg/ml]}) \times 1.62 - 36.52\ \text{pg/ml} \quad (1)$$

$$\text{GDF} - 15\ \text{Roche Elecsys}^{®}\text{estimate [pg//ml]} = (GDF - 15\text{R\&D [pg/ml]}) \times 2.84 - 161.7\ \text{pg/ml} \quad (2)$$

H- and D-containing Forms of GDF-15 Have Equivalent Bioactivity on the GFRAL-Ret Receptor

Stably transformed HEK293S cells expressing both components of the GDF-15 receptor, GFRAL and Ret, and containing a serum response factor-responsive luciferase reporter gene, were exposed to varying concentrations of synthetic forms of

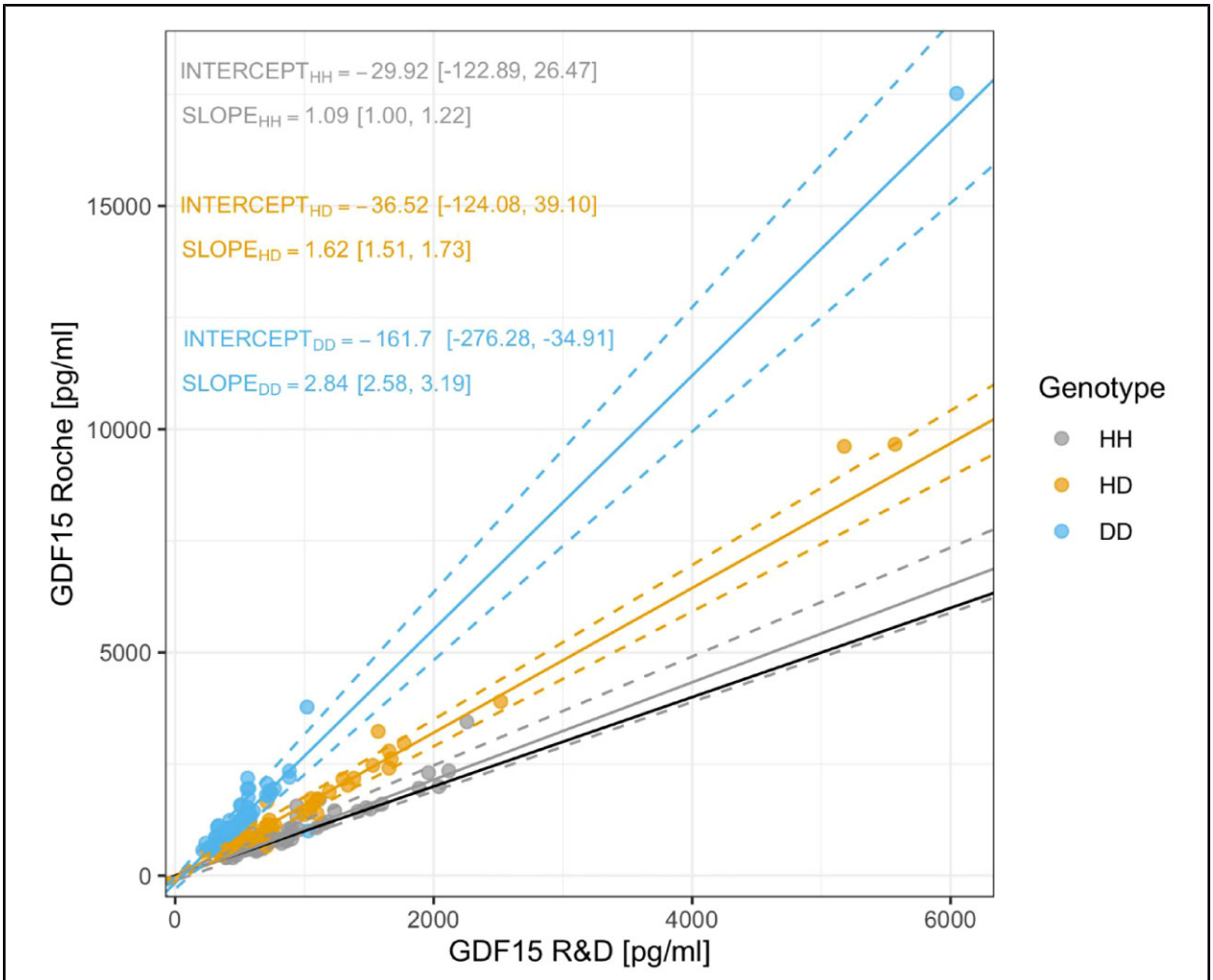

Fig. 2. Serum GDF-15 concentrations in genotyped persons (n = 173) measured by MSD R&D DuoSet immunoassay plotted against serum GDF-15 measured by Roche Elecsys immunoassay in the same individual.
PB regression lines are given; line colors represent the genotype of the respective person at rs1058587 and dotted lines the 95% CIs. Numerical values for intercepts and slopes with 95% CIs in square brackets are given. The solid black line denotes the diagonal. Abbreviations: HH, homozygote for wild type; HD, heterozygote; DD, homozygous mutation.

GDF-15 to establish their bioactivity. Both HH and DD homodimers of GDF-15 showed indistinguishable activity in their dose–response characteristics with EC_{50} (effective concentration at half maximal response) values of 0.39 nM (95% CI, 0.44–0.45 nM) for HH variant and 0.45 nM (95% CI, 0.38–0.52 nM) for DD variant (Figure 3).

DISCUSSION

We have found that the reagents used in one frequently used immunoassay for GDF-15, from R&D Systems, systematically and seriously underestimate circulating concentrations of GDF-15 in people who carry 1 or 2 copies of the D allele at

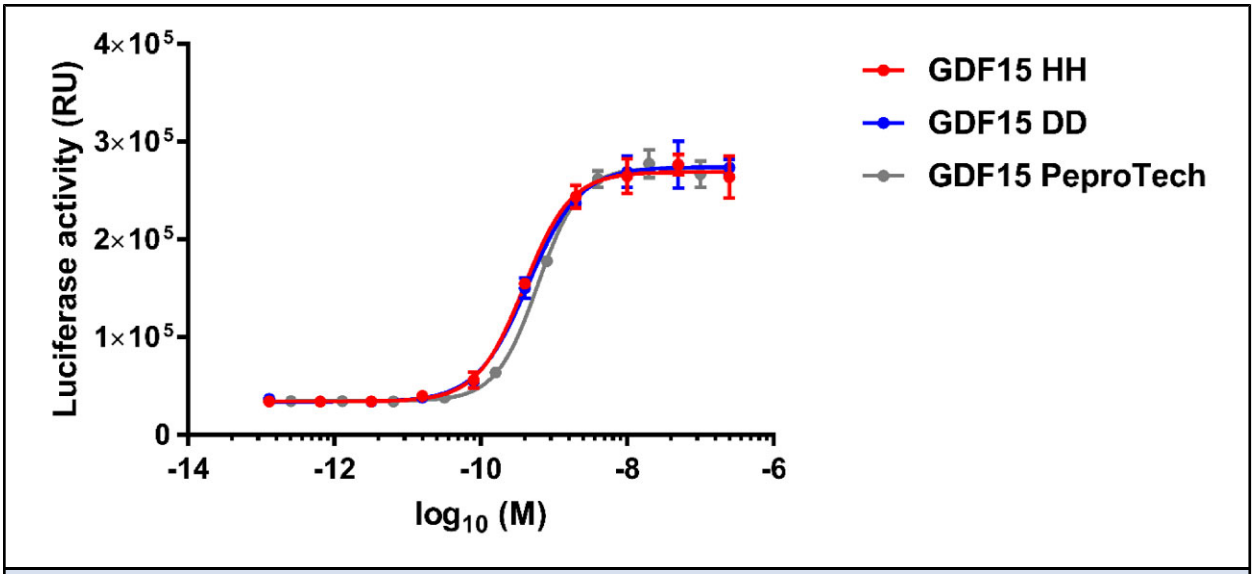

Fig. 3. Bioactivity of H- and D-containing GDF-15 dimers on GFRAL-Ret signaling. Bioactivity of HH and DD variant mature GDF-15 in HEK293S-SRF-RET/GFRAL cells with luciferase reporter under serum responsive factor control. CHO-derived recombinant human GDF-15/MIC-1 (PeproTech) was used as a positive control.

position 6 of the mature peptide (position 202 of the pro-peptide). In contrast, the Roche Elecsys assay appears to be far less affected, though studies with in vitro peptides suggest that homozygote DD dimers may be under-recovered by around 10%. As the epitopes targeted by commercial immunoassays are proprietary information, we are not in a position to report whether this difference in assay performance reflects the selection of the antibodies used. However, residue 6 of the mature peptide is in a flexible and accessible region of the GDF-15 molecule and is likely to be a site against which antibodies are readily generated. This common amino acid polymorphism might also affect the accurate measurement of GDF-15 by other types of assay components, such as aptamers (27). A substantial proportion of the published literature regarding circulating levels of GDF-15, including work from our own laboratory, has used methodology based on the R&D antibodies and the conclusions of such studies will need to be re-evaluated on the basis of this new information. In general, the assay appears to perform well within a given individual, so findings that measure levels in research participants before and after any experimental manipulations or serially over time should retain their validity. However, any cross-sectional studies comparing individuals will need to be viewed with caution as there is a serious risk that an inadvertent and unrecognized imbalance of genotypes between groups would be a critical source of inaccuracy.

Furthermore, clinical trials of GDF-15 antagonism are currently being undertaken in states of cachexia (28) with entry criteria for the trials including elevated concentrations of GDF-15. The GDF-15 concentrations may be significantly underestimated in individuals with the D genotype if a genotype-affected assay is used, resulting in the unnecessary exclusion of patients from the trial.

Genetic variants in and around the GDF-15 gene, one of which is the rs1058587 encoding

the H202D variant, are associated with the pregnancy-specific condition HG at genome-wide levels of significance (29). As GDF-15 administration in animals such as shrews and primates results in vomiting, it might have been expected that the Single Nucleotide Polymorphisms (SNPs) would affect HG risk through increasing circulating levels. However, studies of circulating levels in relation to genotype have not always been directionally consistent with such an effect. The knowledge that the D variant has such a significant effect on measurement by certain assay formats provides a possible explanation for the puzzling findings reported to date. Similarly, when Karhunen et al. (14) performed Mendelian randomization studies asking whether genetic variants increasing GDF-15 levels were associated with BMI, their surprising conclusion that GDF-15 might actually increase body weight could again be explained by the fact that the assay used to measure GDF-15 used R&D reagents. Although we cannot definitely report that these assay inconsistencies explain inconsistencies in the published epidemiological data, we suggest adjustment factors which we propose as a potential tool to re-analyze data from genotyped individuals in such Mendelian randomization studies. However, we acknowledge that independent replication will be important to test how well these adjustment equations apply in other contexts. It seems plausible that the H202D variant under-recovery will also occur using other assay methodologies that have been employed in large-scale proteomic studies (26).

A further level of complexity would be provided if there were differences in bioactivity between the H and D variants. The known structure of the GDF-15-GFRAL complex indicates that position 6 in the mature growth factor is not critically involved in this interaction as the data presented here support the idea that they are bioequivalent. It is at least theoretically possible that the H and D variants could act in subtly different ways in vivo, such as through differential interaction with components of the extracellular matrix.

CONCLUSION

The D genetic variant of GDF-15, present in 15% to 30% of people worldwide, substantially affects its measurement by one or more widely used assays with implications for the interpretation of studies employing such assays.

SUPPLEMENTAL MATERIAL

Supplemental material is available at *The Journal of Applied Laboratory Medicine* online.

Nonstandard Abbreviations: GDF-15, growth differentiation factor 15; H, histidine: D, aspartic acid; GFRAL-RET, glial cell line-derived neurotrophic factor (GDNF) family receptor alpha like (GFRAL)-Ret proto-oncogene (RET) ; DMEM, Dulbecco's Modified Eagle Medium; PB, Passing–Bablok.

Human Genes: GDF15

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