Title: Defining reference intervals for a serum Growth Differentiation Factor-15 (GDF-15) assay in a Caucasian population and its potential utility in Diabetic Kidney Disease (DKD).

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Running Head: Reference Intervals & Potential Utility of GDF-15 in DKD.

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Abstract

Background:

Growth differentiation factor-15(GDF-15), a stress responsive cytokine, is a promising biomarker of renal functional decline in Diabetic Kidney Disease (DKD).

Aim:

This study aimed primarily to establish normative data and secondarily to evaluate the potential utility of GDF-15 in DKD using Roche Diagnostics electrochemiluminescence immunoassay (ECLIA) in an Irish Caucasian population.

Method:

Following informed consent, 188 healthy volunteers and 128 participants with diabetes (72 with and 56 without DKD) were recruited to a cross-sectional study. Baseline demographics, anthropometric measurements and laboratory measurements were recorded. Blood for GDF-15 measurement was collected into plain specimen tubes kept at room temperature and processed (centrifugation, separation of serum, freezing at -80°C) within 1 hour of phlebotomy pending batch analyses. Reference intervals were determined using the 2.5^{th} and 97.5^{th} percentiles for serum GDF-15 concentration.

Results:

Of 188 healthy participants, 63 failed to meet study inclusion criteria. The reference interval for serum GDF-15 was 399ng/L (90% CI: 399-399)- 1335ng/L (90% CI: 1152-1445). ROC curve analysis for DKD determined the AUC to be 0.931 (95% CI: 0.893-0.959; P<0.001). The optimum GDF-15 cut-off for predicting DKD was >1136ng/L providing a diagnostic sensitivity and specificity of 94.4% and 79% respectively and positive likelihood ratio of 4.5:1 (95% CI: 3.4-6.0).

Conclusions:

The reference interval for serum GDF-15 in a healthy Irish Caucasian population using Roche Diagnostics ECLIA was established and a preliminary determination of the potential of GDF-15 as a screening test for DKD was made. Further prospective validation with a

larger DKD cohort will be required before the cut-off presented here is recommended for clinical use.

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Introduction:

Growth Differentiation-15 (GDF-15) is a stress responsive cytokine and a divergent member of the transforming growth factor-β (TGF-β) superfamily [1]. Under physiological conditions, only the placenta expresses large amounts of GDF-15 [2]; peaking in the third trimester of pregnancy [3]. GDF-15 is found in a variety of other organs such as heart, brain, liver and adipose tissue and can be released from a variety of cells including macrophages [4], vascular smooth muscle cells [5], adipocytes [6], cardiomyocytes, endothelial cells [7] and fibroblasts under stressful stimuli [8]. There is considerable interest in the quantification of GDF-15 in serum and other biological fluids as studies suggest that GDF-15 may be a useful biomarker in several diseases [9]. There are limited studies evaluating reference intervals for GDF-15 in a healthy cohort [10]. To date, no such study has taken place in an Irish Caucasian population.

Diabetes mellitus (DM) is characterized by hyperglycaemia due to reduced/ absent insulin secretion or insulin resistance or both. Up to 425 million people or 8.8% of adults aged 20-79 years have DM and this figure is expected to rise to 629 million by 2045 [11]. Diabetic kidney disease (DKD) is the term used to describe kidney disease attributable to DM [12] and manifests as increased urine albumin excretion and/or impaired glomerular filtration rate (GFR) [13, 14]. DKD is one of the commonest causes of chronic kidney disease (CKD) especially in the Western World [15].

There is a significant unmet clinical need for the identification of biomarkers that serve as predictors or early indicators of disease progression in DKD [15]. Several authors [16-18] have long noted the need for biomarkers in DM care that can improve patient outcomes, even at the individual level by identifying which patients will develop micro- or macro-vascular complications, respond to therapy or are eligible for inclusion in clinical trials [19]. These markers may facilitate cardiovascular risk stratification and the introduction of therapy at an earlier time point to reduce renal risk [20-22].

Recent studies have identified GDF-15 as one such biomarker. In patients with Type 1 DM (T1DM) and DKD and in patients with Type 2 DM (T2DM) and microalbuminuria, elevated plasma GDF-15 concentrations predicted all-cause mortality and a faster decline in renal function [23, 24]. A nested case-control study of subjects with T2DM selected from the PREVEND cohort found that plasma GDF-15 was a valuable marker in predicting transition in stage of albuminuria beyond traditional risk factors [25]. A post hoc analysis of the sulodexide-macroalbuminuria (Sun-MACRO) trial that included 861 patients with T2DM and macroalbuminuria found that serum GDF-15 is independently associated with risk of renal decline [26]. In murine models, renal GDF-15 is upregulated upon induction of DM [27]. Intrarenal GDF-15 expression in the tubulointerstitial compartment of patients with CKD is reflected by circulating plasma GDF-15 levels [28]. In GDF-15 knockout (KO) mice with T1DM and T2DM, renal tubular and interstitial damage was increased despite similar diabetic states to wild type (WT) mice while glomerular damage was similar in both KO and WT mice [27]. The GDF-15 KO mice with T2DM had impaired renal function as measured by plasma creatinine concentration [27]. These results suggest that GDF-15 has an important functional role in preventing tubular and interstitial damage while also acting as an early circulating biomarker of DKD [27].

The primary objective of this study was to establish normative data for serum GDF-15 in an Irish Caucasian population. The secondary objective was to perform a preliminary determination of the potential diagnostic utility of serum GDF-15 in DKD.

Materials and methods:

Ethical approval for this study was granted by the Research Ethics Committees, Galway University Hospitals (GUH) and National University of Ireland, Galway (NUIG). This study was conducted in accordance with the World Medical Association Declaration of Helsinki.

Study Design:

A single centre cross-sectional study was conducted between March 2016 and November 2017 at GUH/NUIG. Recruitment of healthy volunteers (HV) was achieved using posters displayed at GUH/NUIG. Healthy volunteers completed a detailed questionnaire to confirm that they had no known medical conditions. Participants with DM with and without DKD were recruited by convenience consecutive sampling at routine DM, DM renal and nephrology clinics [19].

Reference population:

The inclusion criteria were stringent and included signed informed consent and Irish Caucasian (Table 1). The exclusion criteria were: taking prescribed medications (not including contraceptives) or over the counter medications such as nonsteroidal anti-inflammatory drugs (NSAIDs) in the week preceding recruitment, previous or new diagnosis at time of enrolment of prediabetes or DM, known diagnosis of cardiac, thyroid, liver or metabolic bone disease or anaemia or unwell in the previous 2 weeks; non-Caucasian and clinical or laboratory parameters outside the inclusion criteria identified following enrolment.

Patients with DM:

The inclusion criteria for patients with DM were: known diagnosis of DM, signed informed consent, age ≥ 18years and non-pregnant. Patients with DM were then subdivided into those with and without DKD. Patients with DM and DKD had one or more of the following: CKD stages 3-5 as defined by the National Kidney Foundation, Kidney Disease Outcome Quality Initiative (NKF KDOQI) [29]; Renal hyperfiltration - estimated GFR (eGFR)

>150mL/min/1.73m^2; or urinary ACR of >2.5mg/mmol (males) or >3.5mg/mmol (females) on ≥2 of the last 3 occasions on which ACR was tested.

The exclusion criteria for patients with DM were: haemoglobin level <10g/dL within the past 3 months and under active management for infection, cancer, acute cardiovascular event or a haematological condition other than anaemia.

Data Collection:

Basic clinical information including age, gender, ethnicity, body mass index (BMI), current medication usage, smoking and pregnancy status was recorded.

Blood (20 mL) was collected from each participant in appropriate specimen tubes (Becton Dickinson plain plastic vacutainer): serum tubes for GDF-15, C-reactive protein (CRP), renal indices, liver indices, bone indices, parathyroid hormone (PTH), free thyroxine (FT4), thyroid stimulating hormone (TSH), N-terminal pro b-type natriuretic peptide (NT-proBNP) and high sensitivity Troponin T (hsTnT) measurement; ethylenediaminetetraacetic acid (EDTA) anticoagulant containing vacutainer for glycated haemoglobin (HbA_{1c}) and haematological analysis (haemoglobin, white cell and platelet count). A mid-stream urine sample for urine albumin:creatinine ratio (uACR) measurement was collected in a plain sterile container. The eGFR was calculated using the CKD-Epidemiology Collaboration (CKD-EPI) formula [30].

For GDF-15 measurement, serum samples were collected from each participant, inverted 3 times and allowed to clot for 1 hour at room temperature (RT; 20-25°C). The samples were then centrifuged at 800Xg for 15 minutes at RT, serum separated, aliquoted and stored at -80°C pending batch analyses.

Analytical methods:

The Elecsys® GDF-15 assay is a sandwich immunoassay that employs two monoclonal antibodies, a biotinylated anti-GDF-15 capture antibody and a ruthenium-labelled anti-GDF-15 tag antibody [31]. The concentration of GDF-15 in the sample is directly proportional to

the chemiluminescent emission measured by the system photomultiplier. All analyses were carried out at the GUH clinical biochemistry laboratory, which is accredited to ISO15189:2012 standards for medical testing laboratories. We recommend a minimum volume of 120µL of serum to assay GDF-15. From successful calibration and assay initiation, the first result is released within 43 minutes and test throughput confirmed as 76 reportable tests per hour.

Assessment of assay performance specifications:

Assay precision and bias were assessed in accordance with the CLSI EP15 A3: User Verification of Precision and Estimation of Bias; Approved Guideline [32] using Roche PreciControl Cardiac II control material (Lot No: 158396; Expiry date 2017-11) based on human serum at two concentration ranges. A second approach to estimate bias employed the use of recombinant GDF-15 materials of known concentration, 400 ng/L and 13,500ng/L respectively. The latter were assayed in duplicate. Linearity was evaluated by performing a dilution study using patient's serum with a mean GDF-15 concentration of 7435ng/L and the Roche MultiAssay diluent in accordance with the manufacturer's instructions for use. All dilutions were analysed in triplicate, with the mean observed GDF-15 concentration calculated, corrected for the dilution and % recoveries calculated.

The effect of haemolysis on the in vitro measurement of GDF-15:

Interference of haemolysis was assessed using whole blood from a single volunteer collected into two plain (serum) and two EDTA (plasma) specimen tubes. Vigorous vortexing to one of each specimen tube type was performed to ensure red cell rupture causing haemolysis. GDF-15 and haemolytic index (HI) measurement was then performed on all samples and a comparison of GDF-15 results in haemolysed serum/plasma samples made with values in the respective non-haemolysed samples.

The effect of sample stability on the in vitro measurement of GDF-15:

Stability of serum GDF-15 was investigated over 2-time periods spanning 6 and 12-months respectively. In February 2017, serum samples of sufficient volume were collected from 6 patients and divided into 2 aliquots and frozen at -80°C. GDF-15 concentration in these 6 patient samples ranged from 524ng/L to 7577ng/L. At 6 months after the initial analyses (July 2017) the second serum aliquot from 3 of these 6 patients was thawed, mixed thoroughly and reanalysed for GDF-15 and results compared with those determined at baseline. At 12 months (February 2018), the same procedure was followed for the remaining 3 patient's samples with the GDF-15 results obtained at this time point again compared to those achieved one year earlier at initial testing.

The effect of repeated freeze-thaw cycles on the in vitro measurement of GDF-15:

The effect of freeze-thaw cycles on serum GDF-15 measurement was evaluated using 3 patient samples with low (524.3ng/L), medium (1220ng/L) and high (7577 ng/L) serum GDF-15 concentrations. GDF-15 results at baseline were compared to GDF-15 measurement obtained following 2 subsequent freeze-thaw cycles of these patient samples.

Statistical analyses:

All data and statistical analyses was recorded and performed using Microsoft® Excel 2016, Analyse-it® (Version 17), GraphPad Prism Version 6.01 for Windows, MedCalc® Statistical Software (Version 18.2.1) and R® V3.2.0 (R Foundation for Statistical Computing, Austria; accessible at www.r-project.org).

Establishing reference intervals for GDF-15:

Reference values within the Roche GDF-15 ECLIA reportable range were used to establish the reference intervals. GDF-15 results below the assay reportable range or Limit of Quantification (LoQ) of 400 ng/L for statistical purposes were assigned the arbitrary figure of 399 ng/L. Analyse-it® (Version 17) statistical software was used to illustrate the data. The

frequency distribution for GDF-15 in a healthy Irish population was established. The Anderson-Darling test was used to evaluate normality. The data was visually examined for apparent outliers (results that do not fit within the majority of reference values). Potential outliers were assessed in accordance with the criteria of Dixon [33] and Reed [34]. In brief, outlier removal is based on the use of the D/R ratio, where D represents the difference between an extreme observation and the next observation, and R represents the range of all observations including the extremes. An outlier is excluded when D is equal to one-third or greater than the range R [33, 34]. This approach to statistically significant outliers is supported by the International Federation for Clinical Chemistry and Laboratory Medicine (IFCC) working group [35]. The IFCC method that does not assume Gaussian type distribution was employed to establish the reference interval. GDF-15 lower and upper reference limits were estimated at the 2.5^{th} and 97.5^{th} percentiles, respectively. In Table 1, the data are represented as medians (ranges) to show the spread of clinical and laboratory indices amongst the reference interval population.

Diagnostic Utility of GDF-15 in DKD:

In Tables 2 and 3, continuous data were represented using means (standard deviations) where data was normally distributed and medians (minimum to maximum) for non-normally distributed data. Comparisons of means between healthy volunteers, participants with DM without DKD and participants with DM with DKD were performed using analysis of variance (ANOVA) with Tukey's multiple comparison test for normally distributed data. Non-parametric data was compared using Kruskal-Wallis multiple comparison test with Dunn's post hoc multiple comparison test. Multiplicity adjusted P values are reported for each comparison. Categorical data was summarized with frequencies (percentages). Comparisons of proportions were performed using a chi-square test. The relationship between GDF-15 and different clinical and biochemical parameters in HVs was explored using correlation coefficients.

The diagnostic utility of serum GDF-15 to identify participants with known DKD, in a cohort comprised of the reference population and DM patients with and without DKD, was evaluated using Receiver Operator Characteristics (ROC) curve analysis [36]. The ROC curve was generated by plotting sensitivity (true positive rate) against 1-specificity (false positive rate). Test accuracy is measured by the area under the ROC curve (AUC). An AUC value of >0.9 is classified as a highly accurate test. Additionally, if the p-value is <0.05, then it can be concluded that the AUC is significantly different from 0.5 (null hypothesis: area=0.5) and that there is evidence that serum GDF-15 is capable of distinguishing between those with and without DKD. ROC analysis was also used to evaluate the accuracy of multiple logistic regression models aimed at calculating the probability of a given individual as affected/unaffected by DKD [37]. The potentially relevant clinical and biochemical indices assessed were age, gender, BMI, pulse rate, SBP, DBP, CRP, GGT, hsTnT and NT-proBNP.

The sample size required to identify an AUC of >0.75 with a null hypothesis AUC value of 0.5, a ratio of sample size in no disease: disease group of 2.5, α of 0.05 and β of 0.1 (power 90%) is 19 cases with disease and 48 cases without disease.

Results:

In total 188 HVs and 128 patients with DM were recruited (Figure 1). Of the HVs recruited for the normative data study, 63 were excluded as they failed to meet our strict study inclusion criteria. This left a total of 125 Caucasian HV who formed the reference population (Table 1). Of note, 27 of 125 control subjects (21.6%) had GDF-15 values that were below the assay reportable range of 400ng/L. There was a significant weak correlation between GDF-15 and potassium (r=0.193), GGT (r=0.195) and haemoglobin (r=-0.196). There was a significant mild correlation between GDF-15 and age (r=0.274), CRP (r=0.272) and eGFR (r=-0.303) (Table 1). While GDF-15 levels were higher in smokers (726±366ng/L) than non-smokers (602±225ng/L) this was not statistically significant (P = 0.255).

Analytical Performance of the Roche Diagnostics GDF-15 Assay:

The intra-assay precision at a mean GDF-15 concentration of 1370ng/L and 7302ng/L was 2.6%, inter-assay precision at these concentrations was almost identical at 6.1% and 5.9% respectively. The control materials used in the assessment of imprecision (5 replicate analyses of each material on 5 separate days) had a mean assay value of 1370ng/L ± 83.3ng/L for level 1 and 7302ng/L ± 438.1ng/L for level 2. This is in agreement with the manufacturer's assigned GDF-15 values of 1400ng/L ± 84ng/L (Range: 1148-1652) and 7300 ng/L ± 438ng/L (Range: 5986-8614) for level 1 and 2 respectively.

At a known GDF-15 concentration of 13,500ng/L bias was estimated at 3.6%. In the sample containing recombinant GDF-15 at a concentration of 400ng/L repeated analyses gave GDF-15 results below the assay's limit of quantification (LoQ: CV_A of ≤ 20%). Linearity was verified to a mean GDF-15 concentration of 7435ng/L using a patient sample with recoveries of 100+/-10% demonstrated in dilutions up to 1:4.

Increasing the degree of haemolysis in either serum/plasma was associated with decreasing GDF-15 levels. A serum sample with a baseline GDF-15 concentration of 662.4ng/L and a HI of 7 demonstrated a decrease in GDF-15 concentration of 35% to 428ng/L when the HI increased to 499. Studies using EDTA plasma demonstrated similar findings.

Serum GDF-15 stability was assessed at 2-time intervals of 6 and 12 months using 6 patient samples with GDF-15 concentrations that ranged from low (524ng/L) to high (7577ng/L) values. Serum GDF-15 at the concentrations assessed was determined to be stable when stored at -80°C for a period of at least 12 months with all results within 100±10% of the baseline result.

A single freeze-thaw cycle post initial analysis minimally affected GDF-15 measurement with results within 100±10% of the baseline result (GDF-15 concentration range 524-7577ng/L). Further freeze-thaw cycles (n=3) showed poor GDF-15 recovery with results deviating by ±15% of the initial value.

Establishing the reference interval for GDF-15:

The frequency distribution of GDF-15 in the reference population was shown to be non-Gaussian (Figure 2). The Anderson-Darling test rejects normality, A^2 statistic = 6.96, P <0.001. No outliers were found using the Dixon and Reed approach to outlier detection [33, 34]. The non-parametric method defined the GDF-15 lower and upper reference limits at the 2.5^{th} and 97.5^{th} percentiles: 399ng/L (90% CI: 399-399) - 1335ng/L (90% CI: 1152-1445) respectively.

Diagnostic Utility of GDF-15 in DKD:

Of the 128 participants with DM recruited to this study, 56 had DM without DKD and 72 had DM with DKD as outlined in Tables 2 and 3. These tables report multiplicity adjusted P values indicating the differences in clinical characteristics and biochemical/haematological indices between HVs, participants with DM without DKD and participants with DM and DKD. Median GDF-15 was higher in participants with DM and DKD (3022 (759-7577) ng/L) compared to participants with DM without DKD (1265 (399-5729) ng/L) and HV (540 (399-1452) ng/L) (P<0.001) (Figure 3).

ROC curve analysis was carried out in the following sample: HVs (n=125) and patients with DM without DKD (n=56) compared to those with DKD (n=72) provided for an AUC of 0.931

(95% CI: 0.893-0.959; P<0.001) (Figure 4). In this study, the optimum GDF-15 cut-off for predicting DKD was >1136ng/L providing a diagnostic sensitivity and specificity of 94.4% and 79% respectively and positive likelihood ratio of 4.5:1 (95% CI: 3.4-6.0). Multivariate ROC analysis determined the AUC to be minimally influenced by age, gender, BMI, pulse rate, SBP, DBP, CRP, GGT, hsTnT and NT-proBNP (supplementary Table 1).

Discussion:

Prior to introduction of an assay into routine clinical use, reference intervals, the decision support tools used for the interpretation of quantitative pathology reports, should be established in the local population [35]. In this study, we established normal reference intervals for a well-defined healthy Irish Caucasian population using the newly developed electrochemiluminescence immunoassay (ECLIA) for the *in vitro* quantification of GDF-15 on the Cobas® series of immunoassay analysers. Furthermore, we demonstrated the discriminatory potential of GDF-15 to distinguish patients with DM and DKD from those without DKD and HVs. Although further studies in patient cohorts are required, the results add to a growing body of literature that this biomarker has the potential to inform clinical decision-making.

Critical to defining the reference intervals is the appropriate selection of reference individuals, as the quality of the reference intervals can play as large a role in result interpretation as the quality of the result itself [38]. We defined reference intervals for serum GDF-15 in an Irish Caucasian population with strict inclusion and exclusion criteria. Serum GDF-15 concentration ranged from 399 ng/L-1452 ng/L in the reference population and the reference interval established was 399ng/L-1335ng/L. A recent German reference interval study defined the reference range for serum GDF-15 as 400ng/L-3976ng/L [10]. The difference in the reference intervals observed is likely due to the stringency of the inclusion criteria for our study. Of note, the authors of the German reference interval study acknowledged that some of their apparently healthy population may have had subclinical disease [10].

It is important to understand what factors contribute to the variability of GDF-15 levels amongst healthy individuals. While we acknowledge that only 34% of the study participants were male, Wollert *et al* in their reference range study (739 participants; 364 (49.3%) male) found that partitioning for gender was not required [10]. This is in accord with other published works that found no association between gender and plasma/serum GDF-15 [39, 40]. As

age increases serum/plasma GDF-15 increases [10, 39, 40]. In obese individuals, the strongest predictor of plasma GDF-15 is age [41]. GDF-15 maybe upregulated as part of the normal physiological process. However, in the Rancho Bernado study, plasma GDF-15 remains predictive of mortality in age-adjusted analysis [42]. While we observed a mild correlation between serum GDF-15 and age, the size of our study prevented partitioning for age. Similar to Ho *et al* we noted a mild correlation between renal function and GDF-15 in our healthy cohort without renal impairment [39]. Thus, serum GDF-15 does not appear to be influenced by gender but the effects of age and renal function in healthy individuals need to be considered.

In our cohort, serum GDF-15 had a high diagnostic accuracy for identifying patients with DKD with an AUC of 0.931 (95% CI: 0.893-0.959; P<0.001). The optimum GDF-15 cut-off for predicting DKD in the population assessed was >1136ng/L providing a diagnostic sensitivity and specificity of 94.4% and 79% respectively. Moreover, multivariate logistical regression modelling determined that the AUC was minimally influenced by age, gender BMI, pulse rate, SBP, DBP, CRP, GGT, hsTnT and NT-proBNP, suggesting that the observed relationship between serum GDF-15 levels and DKD is not explained by co-association with the selected biomarkers and demographic factors.

Li *et al* in a cohort of patient with T2DM and different degrees of DKD found an AUC for plasma GDF-15 of 0.801 compared to an AUC of 0.717 for urinary albumin in identifying those patients with an eGFR <90mL/min/1.73m^2. With a cut off of 733.78ng/L, plasma GDF-15 had a sensitivity of 88.1% and a specificity of 58.1% for a diagnosis of renal dysfunction [43]. This coupled with the ability of GDF-15 to predict decline in renal function in patients with T1DM and DKD and patients with T2DM and microalbuminuria or macroalbuminuria [23, 24, 26] as well as transition in stage of albuminuria in patients with DM [25] suggests that GDF-15 has the potential to provide additional relevant clinical information beyond that currently provided by standard renal indices.

While concerns exist over the stability of GDF-15 following long periods of serum/plasma storage (>15 years) [23], a strength of our study is the shorter sample storage period and the stability of GDF-15 shown over a 12-month period. Furthermore, we used strict inclusion and exclusion criteria for our volunteers to ensure that the HVs were well defined. While we recognise that GDF-15 concentrations below 400ng/L are rarely observed in patients with acute coronary syndrome or heart failure [44], a limitation of the Roche Diagnostics GDF-15 ECLIA is its relatively high LoQ. This is illustrated by the finding that 21.6% of HVs were found to have a GDF-15 result that was less than the assay's reportable range (<400ng/L). This is almost double the rate (11.8%) seen in the recent paper by Wollert *et al* [10], which had far less stringent inclusion criteria. This finding together with the correlation between renal function and GDF-15 in adults with no renal disease, suggests that an assay with higher analytical sensitivity is required to measure the full range of concentrations of GDF-15 expected in health. Moreover, this finding makes the provision of interpretative guidance on pathology reports challenging. In the routine clinical setting, we advocate using the medically important limit, the URL (97.5^{th} percentile) or GDF-15 <1335 ng/L.

We acknowledge that the reference intervals defined in our study apply only to a healthy Irish Caucasian population and that further studies will need to be undertaken to determine the effect of ethnicity on GDF-15 levels. Furthermore, while we acknowledge that histological assessment of renal biopsy is considered the "gold standard" to diagnose DKD [45], it is not routinely performed due to the risk: benefit of the procedure. In our study, the clinical diagnosis of DKD is defined based on eGFR and ACR - a "tarnished gold standard". Consequently, the diagnostic accuracy of GDF-15 may be subject to imperfect gold standard bias. This type of bias has the potential to make GDF-15 look better (if it makes the same errors as the tarnished gold standard) or worse than it is (if it outperforms the tarnished gold standard). We have not compared the potential diagnostic utility of GDF-15 to that of

standard renal indices (eGFR or ACR) due to incorporation bias – eGFR and ACR are integral to our tarnished gold standard.

While the results of the diagnostic utility study for DKD using the Roche GDF-15 ECLIA on the Cobas® are encouraging, validation is required with a larger DKD cohort before the diagnostic threshold presented here could be recommended for routine clinical use. Longitudinal studies to demonstrate the predictive value of GDF-15 are warranted.

Conclusions:

We defined robust reference intervals for serum GDF-15 in a healthy Irish Caucasian population using the Roche Diagnostics ECLIA. We performed a preliminary determination of the potential diagnostic utility of serum GDF-15 in discriminating adults with DKD from those without. The current and growing literature suggests that assays of blood for GDF-15 concentration have potential to stratify patients at risk of progression of DKD and, therefore, to inform clinical decision-making. Further prospective validation with a larger DKD cohort is required before the cut-off presented here is recommended for clinical use.

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Table 1: Baseline characteristics of the reference interval population and correlation with GDF-15

Parameter	Healthy Volunteers (n=125)			
	Inclusion Criteria	**Median (Range)**	**Correlation Coefficient***	**P-Value**
Age (years)	≥18	30.4 (18.1-62.2)	0.274	**0.002**
BMI (kg/m^2)	≤32.5	24.2 (16.7-32.4)	0.051	0.573
Pulse (beats per min)	N/A	69 (42-108)	0.038	0.67
SBP (mmHg)	<146	122 (93-145)	-0.112	0.212
DBP (mmHg)	<89	75 (49-88)	0.013	0.882
HbA_{1c} (mmol/mol)	20-42	32 (21-39)	-0.028	0.759
CRP (mg/L)	<10	0.7 (0.5-8.9)	0.272	**0.002**
Sodium (mmol/L)	134-145	140 (134-145)	-0.071	0.428
Potassium (mmol/L)	3.5-5.2	4.1 (3.6-5.0)	0.193	**0.031**
Chloride (mmol/L)	N/A	101 (94-105)	0.011	0.905
Urea (mmol/L)	N/A	5.0 (2.5-9.3)	0.01	0.911
Creatinine (µmol/L)	45-110	75 (47-109)	-0.135	0.133
eGFR (mL/min/1.73m^2)	≥60	98 (65-133)	-0.303	**<0.001**
Adj. Calcium (mmol/L)	2.15-2.51	2.31 (2.16-2.45)	0.054	0.546
Phosphate (mmol/L)	0.7-1.5	1.14 (0.71-1.46)	-0.047	0.6
Total Bilirubin (µmol/L)	≤23	8 (2-23)	-0.229	**0.01**
ALP (U/L)	<130	59 (29-104)	-0.103	0.251
ALT (U/L)	<1.5x URL (40) or <60	18 (7-48)	-0.074	0.41
GGT (U/L)	<3x URL (35) or <105	16 (6-83)	0.195	**0.029**
Cholesterol (mmol/L)	N/A	4.6 (2.9 -7.0)	0.122	0.175
Triglycerides (mmol/L)	N/A	0.9 (0.3-4.7)	0.078	0.385
HDL (mmol/L)	N/A	1.7 (1.0 -3.1)	-0.041	0.653
LDL (mmol/L)	N/A	2.3 (1.1-4.2)	0.044	0.631
Free T4 (pmol/L)	10.5-24	15.9 (11.3 -24.0)	-0.082	0.364
TSH (mIU/L)	0.27-4.78	1.82 (0.28 - 4.71)	0.036	0.691
iPTH (ng/L)	<65	31.2 (8.1-64.2)	0.132	0.144
hsTnT (ng/L)	<14	4 (2-10)	0.117	0.193
NT-proBNP (ng/L)	≤150	24.4 (5.0-150.0)	0.171	0.056
uACR (mg/mmol)	N/A	0.8 (0.1-19.5)	0.082	0.374
WCC (10^9/L)	3-12	6.3 (3.1-11.6)	0.174	0.052
Haemoglobin (g/dL)	M >13; F >11	13.6 (11.3-16.1)	-0.196	**0.028**
Platelet Count (10^9/L)	128-450	247 (129-390)	0.129	0.15

BMI = body mass index, SBP = systolic blood pressure, DBP = diastolic blood pressure, HbA_{1c} = glycated haemoglobin, CRP = C-reactive protein, eGFR = estimated glomerular filtration rate, Adj. Calcium = adjusted calcium, ALP = alkaline phosphatase, ALT = alanine aminotransaminase, GGT = gamma-glutamyl transferase, LDL = low density lipoprotein, HDL = high density lipoprotein, T4 = thyroxine, TSH = thyroid-stimulating hormone, iPTH = intact parathyroid hormone. hsTnT = high sensitivity troponin T, NT-proBNP = N-terminal pro b-type natriuretic peptide, uACR= urine albumin:creatinine ratio, WCC = white cell count.
*URL: Upper Reference Limit; M: Male; F: Female; *Pearson's correlation.*

Table 2: Comparison of biochemical and haematological indices of the reference population to participants with diabetes with and without DKD

Parameter	HV	No DKD	DKD	P-Value			
	n=125	n=56	n=72	HV *vs* No DKD[≠]	HV *vs* DKD[≠]	No DKD *vs* DKD[≠]	Overall[¥]
GDF-15 (ng/L)^	540 (399-1452)	1265 (399-5729)	3022 (759-7577)	<0.001	<0.001	<0.001	<0.001
HbA_{1c} (mmol/mol)*	32 (3)	62 (14)	61 (15)	<0.001	<0.001	0.915	<0.001
CRP (mg/L)^	0.7 (0.5 - 8.9)	1.6 (0.5 - 46.6)	2.6 (0.5-47.3)	<0.001	<0.001	0.81	<0.001
Sodium (mmol/L)*	140 (2)	139 (2)	140 (3)	0.03	0.04	0.957	0.01
Potassium (mmol/L)*	4.3 (0.3)	4.3 (0.4)	4.7 (0.5)	0.709	<0.001	<0.001	<0.001
Chloride (mmol/L)*	101 (2)	99 (2)	100 (3)	0.001	0.334	0.096	0.002
Urea (mmol/L)*	5.0 (1.3)	5.6 (1.8)	11.3 (5.3)	0.526	<0.001	<0.001	<0.001
Creatinine (µmol/L)*	76 (13)	72 (15)	145 (61)	0.84	<0.001	<0.001	<0.001
eGFR (ml/min/1.73m^2)*	99 (14)	96 (20)	48 (26)	0.563	<0.001	<0.001	<0.001
Adj. Calcium (mmol/L)*	2.31 (0.06)	2.33 (0.07)	2.35 (0.07)	0.129	<0.001	0.282	<0.001
Phosphate (mmol/L)*	1.12 (0.18)	0.99 (0.18)	1.07 (0.22)	0.132	<0.001	0.044	<0.001
Total Bilirubin (µmol/L)*	9 (4)	9 (6)	7 (3)	0.952	0.007	0.015	0.004
ALP (U/L)*	60 (15)	82 (22)	84 (26)	<0.001	<0.001	0.9	<0.001
ALT (U/L)*	19 (8)	23 (10)	23 (12)	0.033	0.023	0.997	0.007
GGT (U/L)*	16 (6-83)	24 (8-279)	26 (9-782)	<0.001	<0.001	0.812	<0.001
Cholesterol (mmol/L)*	4.6 (0.8)	4.1 (1.0)	4.1 (1.2)	0.008	0.003	0.999	0.001
Triglycerides (mmol/L)^	0.9 (0.3-4.7)	1.4 (0.3-4.4)	1.8 (0.6-8.2)	<0.001	<0.001	0.03	<0.001
HDL (mmol/L)*	1.7 (0.4)	1.5 (0.5)	1.2 (0.4)	0.018	<0.001	0.812	<0.001
LDL (mmol/L)*	2.4 (0.7)	1.9 (0.7)	1.9 (0.9)	<0.001	<0.001	0.974	<0.001
FT4 (pmol/L)*	16.2 (2.2)	17.0 (2.7)	16.0 (3.4)	0.172	0.889	0.113	0.107
TSH (mIU/L)*	2.04 (0.99)	1.74 (0.89)	2.59 (1.50)	0.25	0.003	<0.001	<0.001
iPTH (ng/L)^	31.2 (8.1-64.2)	27.2 (10.2-90.8)	47.4 (6.3-311.1)	0.158	<0.001	<0.001	<0.001
hsTnT (ng/L)^	4 (2-10)	5 (2-55)	16 (2-132)	0.005	<0.001	<0.001	<0.001
NT-proBNP (ng/L)^	24.4 (5.0-150.0)	33.9 (5.0-239.4)	157.4 (5.8-13509)	0.142	<0.001	<0.001	<0.001
uACR (mg/mmol)^	0.8 (0.1-19.5)	0.8 (0.2-15.3)	10.2 (0.4-484.7)	0.999	<0.001	<0.001	<0.001
WCC (10^9/L)*	6.4 (1.6)	7.1 (2.0)	7.6 (1.8)	0.063	<0.001	0.258	<0.001
Haemoglobin (g/dL)*	13.8 (1.0)	14.0 (1.2)	12.8 (1.8)	0.411	<0.001	<0.001	<0.001
Platelet Count (10^9/L)*	251 (50)	241 (63)	242 (93)	0.559	0.603	0.992	0.476

HV = Healthy volunteer, No DKD = DM with no DKD, DKD = DM with DKD, GDF-15 = growth differentiation factor -15, HbA_{1c} = glycated haemoglobin, CRP = C-reactive protein, eGFR = estimated glomerular filtration rate, Adj. Calcium = adjusted calcium, ALP = alkaline phosphatase, ALT = alanine aminotransaminase, GGT = gamma-glutamyl transferase*, LDL = low density lipoprotein, HDL = high density lipoprotein, T4 = thyroxine, TSH = thyroid-*

stimulating hormone, iPTH = intact parathyroid hormone. hsTNT = high sensitivity troponin T, NT-proBNP = N-terminal pro b-type natriuretic peptide; uACR= urine albumin:creatinine ratio, WCC = white cell count.

^Median (min – max), * Mean (SD), ¥ P values are the significance levels of multiple comparisons between the three groups as determined by Kruskal-Wallis test for non-parametric data and ANOVA for parametric data. ≠Multiplicity adjusted P values are reported for non-parametric data (Dunn's multiple comparisons test) and parametric data (Tukey's multiple comparisons test).

Table 3: Comparison of baseline demographics of the reference population to participants with diabetes with and without DKD

	HV	No DKD	DKD	P-Value			
Parameter	**n=125**	**n=56**	**n=72**	**HV *vs* No DKD[≠]**	**HV *vs* DKD[≠]**	**No DKD *vs* DKD[≠]**	**Overall[¥]**
Age (years)*	34.6 (12.2)	54.5 (16.7)	67.9 (14.1)	<0.001	<0.001	<0.001	<0.001
Male no. (%)[∞]	42 (34)	38 (68)	47 (65)	<0.001	<0.001	0.759	<0.001
BMI (kg/m^2)*	24.3 (3.6)	28.8 (6.0)	31.4 (5.8)	<0.001	<0.001	0.006	<0.001
Pulse (beats per min)*	71 (12)	79 (13)	78 (15)	<0.001	<0.001	0.943	<0.001
SBP (mmHg)*	122 (11)	130 (12)	134 (14)	<0.001	<0.001	0.104	<0.001
DBP (mmHg)*	74 (7)	74 (8)	71 (11)	0.994	0.061	0.174	0.061
Smoker no. (%)[∞]	13 (10)	9 (16)	7 (10)	0.469	0.922	0.473	0.71
Duration of DM (years)[Ω]	0	10 (0.2-59)	15 (2-49)	<0.001	<0.001	0.458	<0.001
Type of Diabetes Mellitus[∂,∞]							
Type 1 DM no. (%)	0 (0)	22 (39)	10 (14)	N/A	N/A	<0.001	N/A
Type 2 DM no. (%)	0 (0)	30 (54)	61 (85)	N/A	N/A		N/A
Secondary DM no. (%)	0 (0)	4 (7)	1 (1)	N/A	N/A		N/A

HV = Healthy volunteer, DM = Diabetes Mellitus, No DKD = DM with no DKD, DKD = DM with DKD, BMI = body mass index, SBP = systolic blood pressure, DBP = diastolic blood pressure.

*Mean (SD). [∞] Number (%). [Ω] Median (minimum – maximum). [¥]P values are the significance levels of multiple comparisons between the three groups as determined by ANOVA for parametric data, Kruskal-Wallis test for non-parametric data and Chi-squared for proportions. [≠]Multiplicity adjusted P values are reported for parametric data (Tukey's multiple comparisons test) and non-parametric data (Dunn's multiple comparisons test). [∂]For Type of Diabetes Mellitus, Chi-squared analysis was performed to determine if participants with Type 1 DM, Type 2 DM and Secondary DM are distributed differently between No DKD and DKD groups.

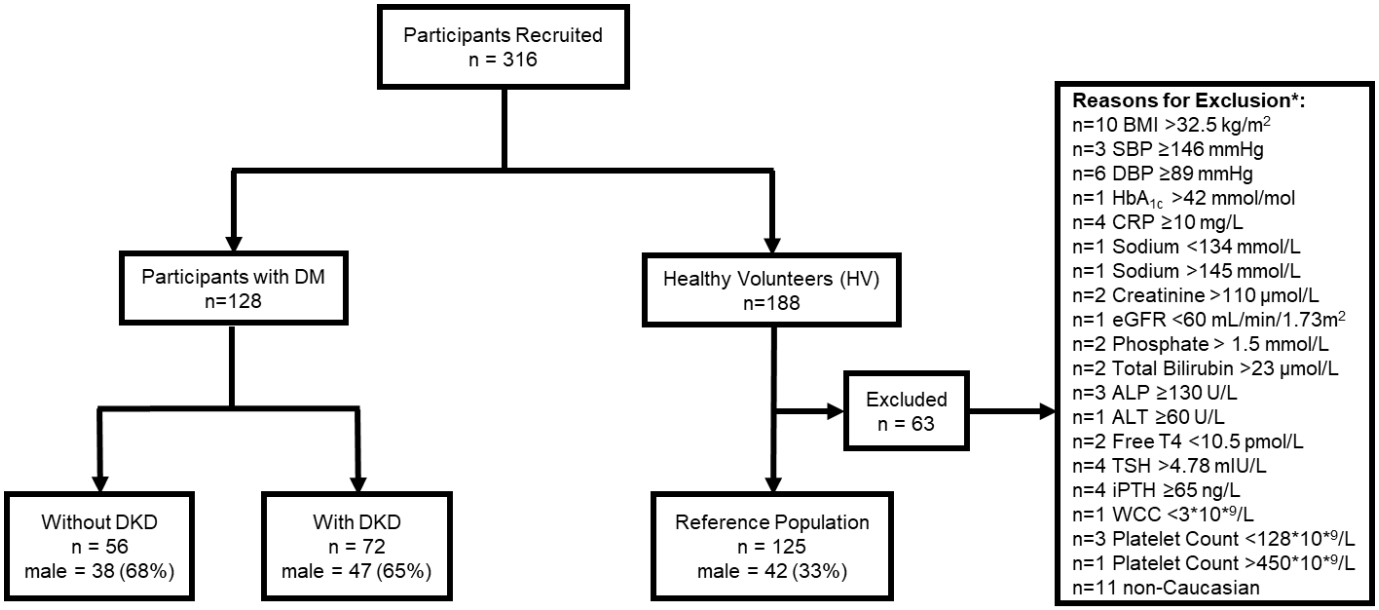

Figure 1: Recruitment schematic to establish normative data for GDF-15 in an Irish population and to assess the diagnostic utility of GDF-15 in patients with DKD.

*BMI, body mass index; SBP, systolic blood pressure; DBP, diastolic blood pressure; HbA_{1c}, glycated haemoglobin; CRP, C-reactive protein; eGFR, estimated glomerular filtration rate; ALP, alkaline phosphatase; ALT, alanine aminotransaminase; T4, thyroxine; TSH, thyroid-stimulating hormone; iPTH, intact parathyroid hormone; hsTNT, high sensitivity troponin T; NT-proBNP, N-terminal pro b-type natriuretic peptide; WCC, white cell count. *Participants were excluded sequentially based on a single criterion.*

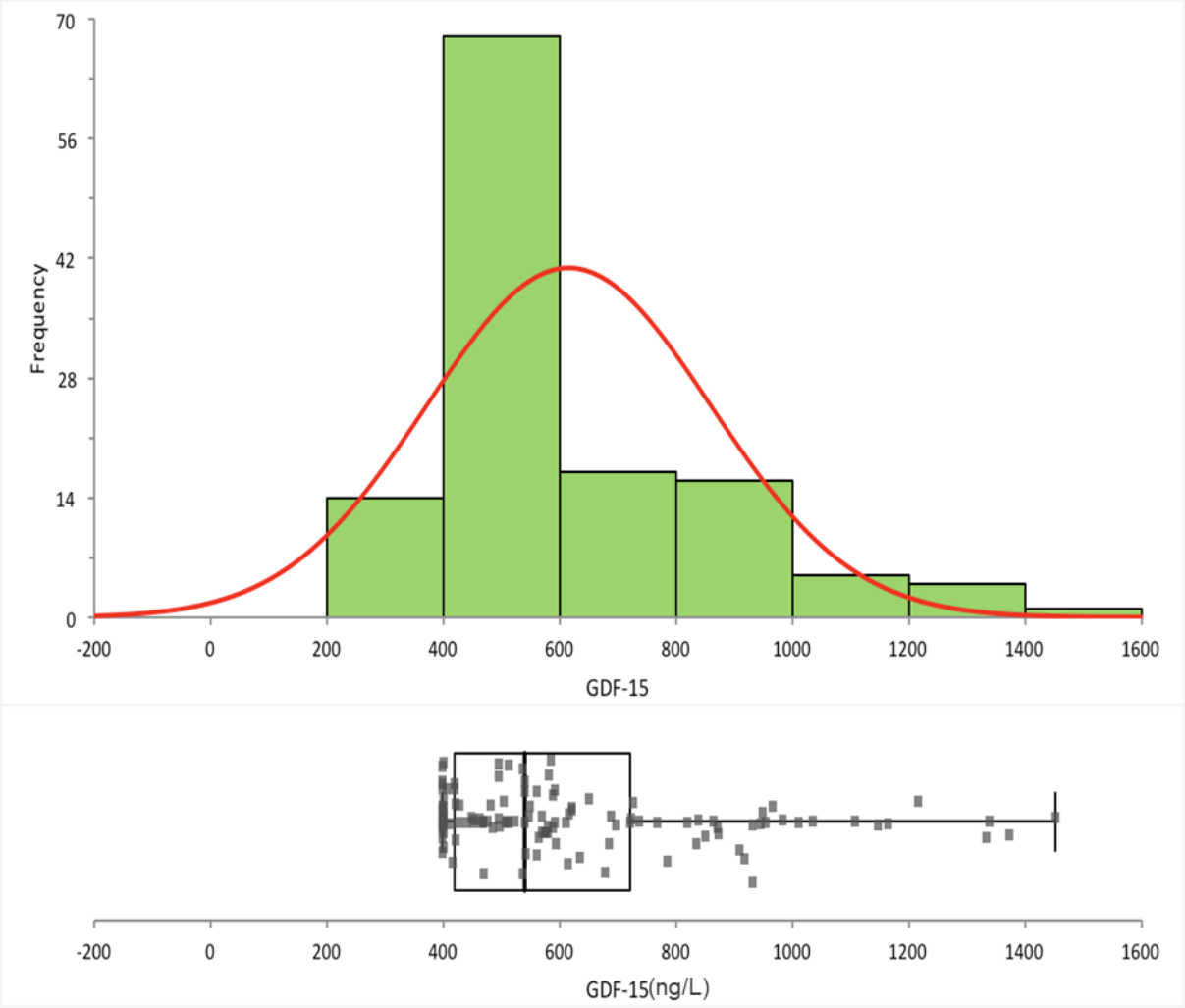

Figure 2: GDF-15 reference population histogram and box and whiskers plot.

The distribution of the reference population histogram visually demonstrates a non-Gaussian curve. The box portion of the box & whisker plot includes 50% of the data, the lower, median (represented by a line) and upper quartile. The whiskers extend to the minimum and maximum values. Disconnected points are potential outliers.

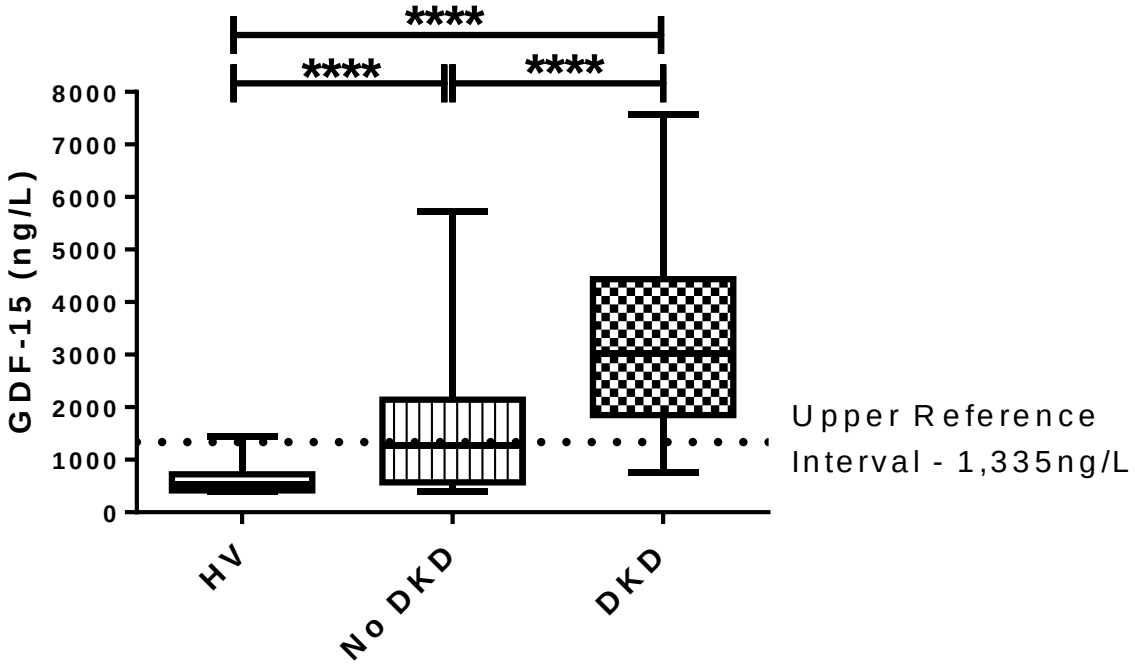

Figure 3: Schematic showing GDF-15 concentrations in healthy volunteers and participants with DM (without DKD (no DKD) and with DKD). **** P<0.001

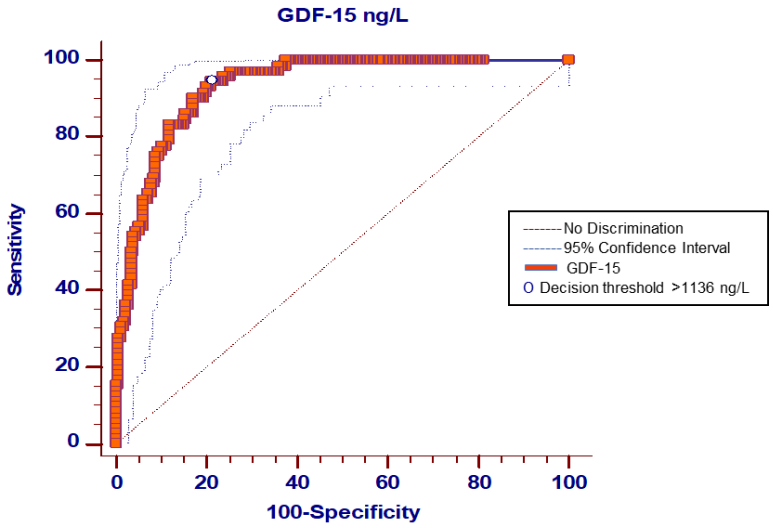

Figure 4: Receiver Operating Characteristics (ROC) curve for GDF-15 in the diagnosis of DKD.
ROC curve analysis performed using HV's (n=125), patients with DM without DKD (n=56) and patients with DM with DKD (n=72). The area under the ROC curve was 0.931 (95% CI: 0.893-0.959; P<0.001). The optimum GDF-15 cut-off for predicting DKD was >1136ng/L providing a diagnostic sensitivity and specificity of 94.4% and 79% respectively and positive likelihood ratio of 4.5:1(95% CI: 3.4-6.0).

	Univariate Model			Multivariate Model #1			Multivariate Model #2			Multivariate Model #3		
	Coeff	**P-value**	**AUC**	**Coeff**	**P-value**	**AUC**	**Coeff**	**P-value**	**AUC**	**Coeff**	**p-value**	**AUC**
Constant	3.357	<0.001	0.85	5.559	<0.001	0.87	5.617	<0.001	0.88	17.342	<0.001	0.88
Serum GDF-15 (ng/L)	-0.001	<0.001		-0.001	<0.001		-0.001	0.013		-0.001	<0.001	
Age (years)				-0.054	<0.001		-0.036	0.047		-0.060	0.004	
CRP (mg/L)							-0.063	0.036				
GGT (U/L)							-0.002	0.659				
hsTnT (ng/L)							-0.085	0.022				
NT-proBNP (ng/L)							-0.007	0.039				
Gender (m)										-0.837	0.078	
BMI (kg/m^2)										-0.118	0.003	
Pulse (beats per min)										-0.02	0.249	
SBP (mmHg)										-0.035	0.06	
DBP (mmHg)										-0.02	0.474	

Supplementary Table 1: ROC curve analysis of univariate and multivariate logistic regression models

Coeff= regression coefficient, AUC = area under the curve, GDF-15 = growth differentiation factor-15, CRP = C-reactive protein, GGT = gamma-glutamyl transferase, hsTNT = high sensitivity troponin T, NT-proBNP = N-terminal pro b-type natriuretic peptide, BMI = body mass index, SBP = systolic blood pressure, DBP = diastolic blood pressure.

In the above statistical analysis, R® V3.2.0 (R Foundation for Statistical Computing, Austria; accessible at www.r-project.org) was used.

The univariate AUC for GDF-15 presented in the main manuscript was calculated using MedCalc®. Here the ROC curve is calculated by plotting the true positive rate (sensitivity) against the false positive rate (100-specificity) for different cut off-points of serum GDF-15. Each point on the ROC curve represents a sensitivity/specificity pair corresponding to a decision threshold [1].

To take multiple co-variates into account, logistic regression analyses were performed using R® V3.2.0. Logistic regression analysis illustrates the relationship between an independent variable (such as serum GDF-15) or variables (such as serum GDF-15 and age) and a dichotomous variable (in this case the presence or absence of DKD) [2]. By using multiple logistic regression models that take multiple covariates into account it is possible to calculate each individual's probability of having a disease [2]. Using the univariate regression coefficient or the multivariate regression coefficients and the constant of the associated model, the log odds for each individual is calculated. The log odds can then be transformed to give the probability of each individual having DKD.

The univariate model presented above is a logistic model including only serum GDF-15. This gives an AUC of 0.85. This is different to the AUC calculated using MedCalc® because of the different methodology used. Multivariate model #1, #2 and #3 are logistic models which include serum GDF-15 in addition to other relevant clinical variables. For multivariate model #1, age was selected as a covariate as we found a mild correlation between GDF-15 and age which corresponds to the published literature. For multivariate model #2 in addition to age, CRP, GGT, hsTNT and NT-proBNP were selected as it was the authors opinion from reviewing the published literature and the correlation coefficients of serum GDF-15 with different clinical parameters in the reference population that these variables had the potential to lead to the greatest improvement in the AUC. For multivariate model #3 in addition to age, gender (male), BMI, pulse, SBP and DBP were included in the model to determine the impact of relevant baseline clinical demographics on the model.

The addition of multiple variables to the models did not have a clinically relevant impact on the AUC while increasing the complexity of the model. Consequently, we chose to use the MedCalc® ROC curve analysis – as the statistical methodology to generate the AUC is more easily accessible to clinicians who are using serum GDF-15.

References:

1. MedCalc Statistical Software version 18.2.1 (MedCalc Software bvba, Ostend, Belgium; https://www.medcalc.org; 2018)

ROC curve analysis. Available at: https://www.medcalc.org/manual/roc-curves.php. Accesed: 04/07/2018

2. Tripepi G, Jager KJ, Dekker FW, Zoccali C. Diagnostic methods 2: receiver operating characteristic (ROC) curves. Kidney Int 2009;76:252-6.