Clinical Biochemistry 46 (2013) 1079–1083

Contents lists available at SciVerse ScienceDirect

Clinical Biochemistry

journal homepage: www.elsevier.com/locate/clinbiochem

Soluble serum Klotho levels in healthy subjects. Comparison of two different immunoassays

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ARTICLE INFO

Article history:
Received 14 December 2012
Received in revised form 22 April 2013
Accepted 14 May 2013
Available online 23 May 2013

Keywords:
Klotho
Immunoassays
Reference ranges
FGF23
PTH
Ionized calcium
Phosphate
eGFR
BMI
Age

ABSTRACT

Objective: Soluble serum Klotho is a new biomarker linked to chronic kidney disease, cardiovascular disease and diabetes. This study describes the evaluation and comparison of two different immunoassays and establishment of assay specific reference intervals in adults.

Design and methods: Serum Klotho concentrations were determined in 120 healthy adults aged 19–66 years. Blood samples were collected, and stored sera were assayed for Klotho according to age and gender. In addition several other clinical and laboratory characteristics were determined in the cohort and compared to the levels of serum Klotho.

Results: Serum Klotho levels were significantly higher in time-resolved fluorescence immunoassay (TRF) compared to an ELISA (IBL) and no correlation was found between the assays. No signal was obtained in either assay when the standard curve was switched between the two different immunoassays. The median serum Klotho concentration using TRF was 61 ng/mL (2.5–97.5% reference limits; 11–181 ng/mL) for males and 99 ng/mL (2.5–97.5% reference limits; 19–316 ng/mL) for females while the ELISA gave a mean value of 472 pg/mL (2.5–97.5% reference limits; 204–741 pg/mL) with no difference between genders. Concentrations of serum Klotho were independently associated with estimated glomerular filtration rate (eGFR) and body weight using TRF whereas serum Klotho concentrations were associated with age using the ELISA.

Conclusion: Comparison of two different immunoassays for serum Klotho indicate, that the protein exists in human beings in different forms which may function as independent factors and whose role and potential value as biomarkers needs to be evaluated separately. Reference intervals specific for the different forms recognized by the different assays were calculated in this study.

Introduction

Klotho was originally identified as an anti-aging protein and is now known to interact with multiple target molecules in several different pathways and biological processes [1,2,6,8,11,12,17,20].

The Klotho-gene encodes a type I transmembrane protein (1014 amino acids, 130 kDa) with a large extracellular domain and a short intracellular portion (10 amino acids), predominantly expressed in the renal tubules [7] and in the parathyroid glands [10]. The extracellular domain of Klotho is found as a circulating soluble factor detectable in blood and in lesser extent in other biological fluids [9,13]. The expression of the secreted form of Klotho predominates over the transmembrane form of Klotho [13] but the main biological effects of Klotho are probably mediated through the transmembrane form of the protein. The functions of the circulating Klotho protein have not yet been identified.

So far studies addressing the use of soluble serum Klotho as a biomarker in relation to clinical disorders [4,14,18] and knowledge about the levels of serum Klotho in healthy subjects [4,18] have been few and limited due to the lack of available assays.

The aim of the present study was to evaluate and compare the analytical performance of two different immunoassays for measurement of soluble Klotho. By using samples from healthy adult subjects, reference values for each assay were determined. We furthermore aimed to increase the knowledge about the relationship between soluble serum Klotho levels and general parameters such as age, sex, height, and weight as well as several biomarkers of mineral metabolism and renal function in a normal population.

Materials and methods

Subjects

Serum and plasma samples were obtained from a cohort of healthy adult Danish blood donors in august and September 2011 at Odense University Hospital. A total of 120 donors were included targeting 30 in each

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0009-9120/$ – see front matter
http://dx.doi.org/10.1016/j.clinbiochem.2013.05.046

subgroup stratified by gender and above as well as below 40 years of age (obtained: 62 females, 58 males, age range 19–66 years). Informed consent was obtained and the study was performed according to the Declaration of Helsinki. The donors completed a questionnaire with information on age, sex, height, weight, night shift work, tobacco and alcohol consumption, cola consumption, physical activity, calcium and vitamin D supplements, and use of medical products containing estrogen. Use of medication in blood donors is restricted but estrogen usage is allowed. Inclusion of cola consumption was included because it has been suggested that cola due to the contents of phosphoric acid, induces reduction in bone mineral density [5].

Immediately after sampling ionized calcium was analyzed whereas all other samples were aliquoted and stored at −80 °C until analysis.

Measurements of serum parameters

Klotho

Serum Klotho levels were measured in duplicate using two different immunoassays.

Measurements of serum Klotho using an ELISA from Immuno-Biological laboratories (IBL), Japan were performed following the instructions provided by the manufacturer. The intra-assay and inter-assay coefficients of variations (CVs) were <3.6% and <11.5% (Klotho level 170 pg/mL), respectively. The detection limit of the assay was 6.15 pg/mL. The data for intra-assay and inter-assay CVs were confirmed in our laboratory by measurements of a serum control at level 435 pg/mL in 4 repeats on each ELISA plate (inter-assay CV <7.1%). The standard curve was found to be linear up to 3000 pg/mL. All readings were performed on a Victor X5 Multiplate Reader (PerkinElmer) at 450 nm.

Secreted soluble serum Klotho levels were also measured using a time-resolved fluorescence immunoassay (TRF) based on the antibodies provided in the ELISA kit from Cusabio, China (catalog number CSB-E13235h) and using recombinant human Klotho as standard (aa 34-981, R&D, catalog number 5334-KL-025). The only components used from the Cusabio Elisa kit were the antibody coated plates and the detection antibody. The detection method of the original ELISA was modified by replacing the Avidin conjugated to Horseradish Peroxidase (HRP) with Streptavidin conjugated to Europium (DELFIA Eu-N1-Streptavidin, Perkin Elmer, catalog number 1244-360) in the final incubation step, thereby changing the detection method from colorimetric detection to fluorescence detection. Changing the method from ELISA to TRF also included changing the Cusabio buffers to AutoDELFIA assay buffers. All samples and recombinant Klotho were diluted in AutoDELFIA diluent II (catalog number B132-100, PerkinElmer) while detection antibody and Streptavidin conjugated to Europium were diluted in AutoDELFIA assay buffer (catalog number 1244-111, Perkin Elmer). All wash steps were performed using AutoDELFIA wash buffer (catalog number, B117-100, PerkinElmer). All steps and measurements of the TRF assay were performed on a Perkin Elmer AutoDELFIA 1235 automated immunoassay system (PerkinElmer) with excitation of the Europium lanthanide at 340 nm and emission at 615 nm.

For the TRF assay a standard curve, that was linear up to 500 pg/mL, was constructed. Since the level of serum Klotho in all the samples was above the highest standard value, all samples were diluted 1:300 prior to analysis. The detection limit of the TRF assay was 3.1 pg/mL defined as the value which is 3 standard deviation (SD) above the mean value of the zero standard ($n = 7$).

The performance of the Klotho TRF assay was compared to the original colorimetric Klotho ELISA (Cusabio) measuring 46 patient samples and the 2 assays showed a good agreement (Fig. 1).

Assay linearity of the TRF assay was determined using a sample with a Klotho concentration of 405 ng/mL which was serially diluted with diluent II to create 10 samples that were tested in duplicate. Results were linear over a concentration range of 2.7–405 ng/mL ($y = 1.07x + 0.14$, $R^2 = 0.997$). The intra-assay and inter-assay CVs were <10% for a serum control (Klotho level 80 ng/mL), respectively. Recovery of Klotho was evaluated by combining two serum samples with Klotho concentrations of 277 ng/mL and 44 ng/mL in ratios 1:3, 3:1 (high:low). Measured concentrations were 100 ng/mL for the 1:3 ratio and 221 ng/mL for the 3:1 ratio representing 97% and 101% of the expected concentrations, respectively. The stability of Klotho in serum during storage was determined in both serum and plasma samples under different conditions and compared to the level of a sample analyzed within 1 hour after centrifugation. Following storage of serum for 24 hours at 4 °C, 14 days at −20 °C, and 60 days at −80 °C, the recovery of serum Klotho was 96.7%, 97.5% and 101%, respectively. When plasma was stored 24 hours at 4 °C, the recovery of Klotho was 84.5%. Based on these data storage of serum at −80 °C before analysis of Klotho was chosen.

To further characterize the two different assays, the standard curve from the IBL ELISA where analyzed in the TRF assay and *vice versa*.

FGF23

Serum FGF-23 concentrations were measured in duplicate by a commercial FGF-23 ELISA kit (Kainos Laboratories, Tokyo, Japan) which measures the biologically active intact FGF23 using a combination of two monoclonal antibodies directed towards epitopes on either side of the cleavage site of FGF23 [19]. The analytical range was 3–800 pg/mL. The intra-assay and inter-assay CVs as reported by the manufacturer were 2.1–3.8% (FGF23 levels 19.5–119 pg/mL) and 2.0–3.0% (FGF23 levels 14.2–33.6 pg/mL), respectively.

Routine parameters

Inorganic phosphate and creatinine were determined by colorimetric end-point assays (Abbott Laboratories, Illinois, USA). Ionized calcium was determined by potentiometric measurements using ion-selective electrodes (ABL, Radiometer, Denmark). Intact parathyroid hormone (PTH) was determined by a chemiluminescence immunoassay (Immulite 2000, Siemens Diagnostics, Deerfeld, USA). 25-Hydroxy vitamin D was determined by TurboFlow liquid chromatography–tandem mass spectrometry (Thermo Scientific, CA, USA). Estimated glomerular filtration rate (eGFR) was calculated using the MDRD Study Equation (www.mdrd.com).

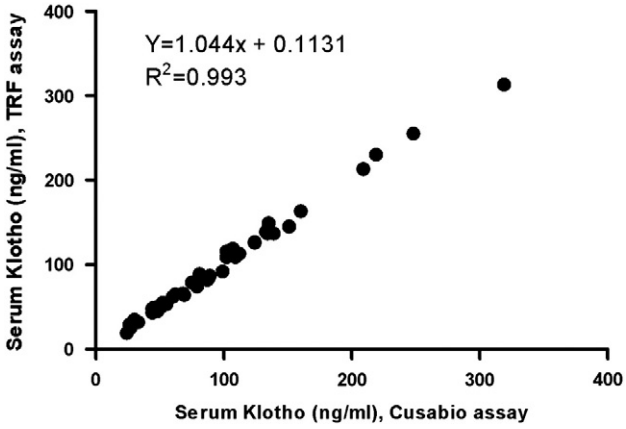

Fig. 1. XY-plot of serum Klotho levels in 46 samples measured with Cusabio ELISA and Klotho TRF immunoassay. Recombinant Klotho from R&D was used to calibrate both types of assays.[1]

[1] According to the product description leaflet the concentrations of Cusabio standards included in the kit cover a range from 0.78 to 50 ng/mL. However, optical density (OD) measurements at 450 nm of calibrators made from recombinant R&D Klotho (range 31.2–500 pg/mL) matched the OD's of Cusabio standards. Furthermore, the necessary 300 fold dilution of serum samples in order to reach measuring range, independently of standards, indicates that the nominal concentration of Cusabio standards was either mistyped in the leaflet, or the recombinant Klotho from Cusabio is impure ensuring a 100 fold higher avidity of the R&D antigen compared to the Cusabio standard.