A Pilot Study of α-KLOTHO Serum Concentrations in CKD Patients using a Highly Sensitive Fluorescence Immunoassay based on Plasmonic Microtiter Plates

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Introduction

α-KLOTHO consists of a large extracellular domain, including 980 N-terminal residues followed by a 21-amino-acid transmembrane domain and a small domain of 11 residues corresponding to the intracellular C-terminus. The extracellular domain of membrane can be cleaved by the metalloproteinases ADAM-10/17 (1) and released into circulation as soluble KLOTHO. Although also expressed in placenta, small intestine and prostate, the main source for KLOTHO is the kidney.

Therefore it is not surprising that it has been well established that this protein, being an essential co-receptor for FGF23, plays an important role in chronic kidney disease and thus also in CKD related mineral bone disorders (CKD-MBD). The pathogenic mechanisms of the components of CKD-MBD include vascular calcification, loss of renal membrane bound KLOTHO, hyperphosphataemia, osteodystrophy, vitamin D deficiency, increased FGF23, and hyperparathyroidism which is the result of an imbalance in the bone–kidney–parathyroid endocrine axes mediated by FGF23 and KLOTHO (Fig. 1).
So studying KLOTHO as a biomarker for CKD and CKD-MBD has sparked a lot of interest and a large number of studies have been performed but data are very inconsistent and partly contradictory (2). Therefore we decided to use our recently developed highly sensitive and reproducible fluorescence immunoassay platform to provide a new reliable tool for α-KLOTHO serum measurements.

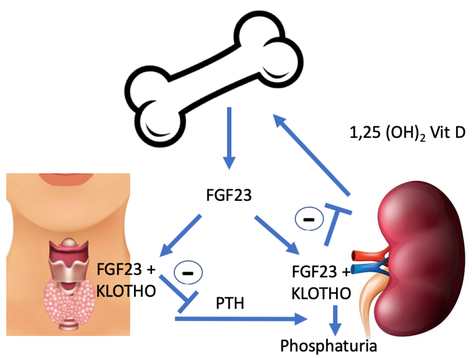


Fig. 1, Bone-Kidney-Parathyroid Axis maintaining vitamin D and phosphate homeostasis

FluoBolt™-Technology (FBT):

FBT is based on metal-enhanced fluorescence (MEF), an electromagnetic interaction of excitation light with nanometer-sized metal structures, which dramatically increases the quantum yield of fluorescent molecules in the immediate vicinity of the metal structures (Fig. 2). This increase in light yield can be used to generate **highly sensitive** biomarker tests. In addition the suppression of bulk fluorescence allows **immunoassays without any washing steps**. Since highest reproducibility of the required metal nanostructures is a key issue for diagnostic application of MEF, we are (in cooperation with our partner STRATEC Consumables) using structuring technologies originally developed for Blu-Ray and DVD manufacturing (Fig. 3) guaranteeing utmost homogeneity for manufacturing of the plasmonic microtiter plates (MEF-MTPs), which are used in our assays (3).

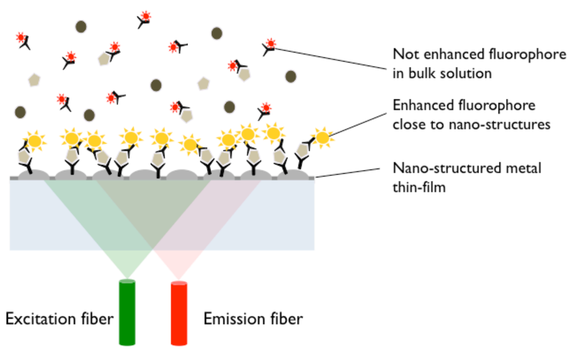


Fig. 2, Schematic representation of metal-enhanced fluorescence

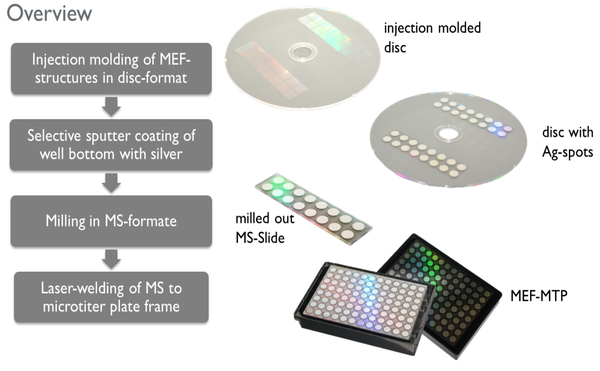


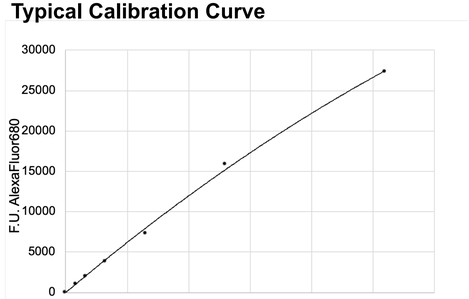
Fig. 3, Manufacturing process of MEF–MTPs

Assay Characteristics

Protocol

- Add 50 µl of fluorescence (FITC, Cy3, Cy5 or AlexaFluor680) labeled detection antibody to all wells required
- Add 10 µl of the human serum sample to be assayed, swirl gently to mix
- Incubate over night at room temperature
- Read the plate in bottom configuration (BOT) without any further processing at the Ex/Em wavelength fitting to the used detection antibody
- Alternatively, discard or aspirate the content of the wells, wash 3x with diluted wash buffer and read the plate in TOP configuration

Typical Calibration Curve



Specificity

The assay uses antibodies raised in rats and rabbits against amino acids 34-981 and 34-483 of human KLOTHO that shares 96-98% homology with primates, 90-91% with horse, dog and cow and 87-90% with rat and mouse.

Analytical Characteristics:

•Detection limit: (0 pM + 3xSD):	4,8 pmol/L
•Intra- / Inter-assay CVs (n=3):	6% and 12%
•Recovery in human serum (n=3):	105%
•Linearity of dilution (n=3):	113%

Pilot-Study with CKD-Samples

The assay described above was used to investigate a small set of serum samples from patients (Fig. 4) with mild to severe kidney disease (classified according to ICD-10-CM coding and purchased from Central BioHub GmbH, Germany):

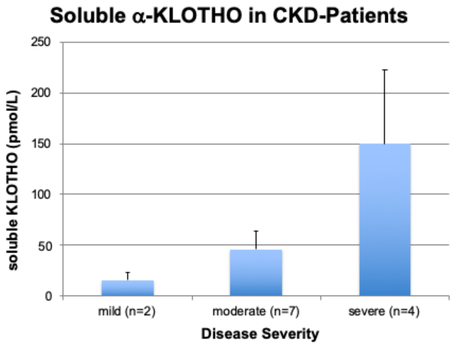


Fig. 4, Soluble KLOTHO in serum samples from patients with chronic kidney disease

Summary

Although we found a profound increase of KLOTHO serum levels with disease severity, the number of samples is much too low to draw final conclusions. However, the data indicate that the developed assay has highest sensitivity combined with excellent reproducibility & specificity to investigate patient collectives with diseases having relevance to this biomarker. Further studies are required to confirm this preliminary results and inter-assay comparisons will show if the developed assay indeed is an improvement over currently used methods.

Acknowledgements:
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Literature:

1. Insulin stimulates the cleavage and release of the extracellular domain of Klotho by ADAM10 and ADAM17. Chen CD et al., Proc Natl Acad Sci U S A. 2007;104(50):19796–801.
2. Correlation between Soluble α-Klotho and Renal Function in Patients with Chronic Kidney Disease: A Review and Meta-Analysis. Wang Q et al., Biomed Res Int. 2018 Aug 12; 2018:9481475.
3. Single step, direct fluorescence immunoassays based on metal enhanced fluorescence (MEF-FIA) applicable as micro plate-, array-, multiplexing- or point of care-format. Hawa G et al., Anal. Biochem. 2018 May; 594:39-44.