

## Reference intervals and biological variation for kallikrein 6: influence of age and renal failure

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### Abstract

**Background:** Kallikrein 6 (KLK6) is a serine protease involved in numerous cellular processes, up-regulated in many cancers and associated with some neurodegenerative disorders. The aim of this study was to establish a reference interval and estimate the biological variation of KLK6 in serum samples of adults. Furthermore, levels of this protein in patients with renal failure were also studied.

**Methods:** Serum samples from healthy volunteers (n=136) were collected. Between 15 and 18 additional samples from four of these subjects were obtained over a period of 2 months. Samples from individuals (n=1043) who visited the University Health Network for a routine check-up were collected to study the association between KLK6 with age and gender. Samples from patients with renal failure (n=106) were also obtained and KLK6 and creatinine concentrations were analyzed by ELISA and an automated enzymatic method, respectively.

**Results:** The reference interval was established to be 1.04–3.93 ng/mL. The index of individuality was 0.43 and the reference change value was 35%. Only two serum samples would be required to estimate the homeostatic setting point of an individual. There is a weak but highly significant positive correlation between KLK6 and age ( $p < 0.0001$ ). Furthermore, there is a significant positive correlation between serum concentrations of KLK6 and creatinine ( $p < 0.0001$ ), in patients with renal failure.

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**Conclusions:** The established reference interval for KLK6 and the estimation of its biological variation will further aid in the clinical use of this protein as a serum marker of malignancy and other diseases.

**Keywords:** biological variation; kallikrein 6; reference interval; serum.

### Introduction

Human kallikrein 6 (KLK6) is a secreted serine protease and a member of the human kallikrein gene family, located on chromosome 19q13.4 (1). The mature form of KLK6 is a 223-aminoacid protein with trypsin-like activity but unclear physiological role (2). This protein is expressed in many tissues, such as breast, kidney, lung and skin, with highest concentrations in brain and spinal cord (3). Bayani and Diamandis (4) have recently reviewed the physiology of KLK6 and its role in disease, linking this protein with numerous cellular processes and disorders, such as cancer and neurodegeneration.

KLK6 has been proposed as a biomarker for a variety of malignancies, such as breast, colorectal, gastric, lung, pancreatic, salivary, skin, bladder, uterine, ovarian carcinoma, and glioma (4). Thus, for instance, Kim et al. (5) have recently described an over-expression of KLK6 in colon cancer, including elevation in serum samples. An over-expression of this protein has also been described in ovarian cancer (4). Several studies have shown that the combination of KLK6 with CA-125 could enhance their diagnostic power for this disease (6, 7). However, KLK6 has also been linked to neurodegenerative diseases, such as Alzheimer's disease (8), multiple sclerosis (9) and spinal cord injury (10).

Therefore, serum levels of KLK6 could assist in diagnosis of diverse diseases, including cancer and neurological disorders. However, few studies have evaluated this protein in serum and the reference values of KLK6 in healthy individuals are unknown. The aim of this study was to establish a reference interval for KLK6 in serum samples of adults as well as to estimate its biological variation. The serum KLK6 concentration in patients with renal failure was also studied.

### Materials and methods

#### KLK6 antibodies and calibration

KLK6 was analyzed with an in-house sandwich-type spectrophotometric ELISA assay, based on two monoclonal antibodies from

mouse. One antibody for capture (clone 27–4) and other for detection (clone E24). The assay was calibrated with in-house-produced, purified and characterized recombinant KLK6, in a yeast expression vector, as previously described (11). KLK6 calibrators of 0, 0.2, 1, 2.5, 5 and 10 ng/mL were prepared by diluting recombinant purified KLK6 protein in a 50-mm Trizma-maleate buffer, pH 7.0, containing 0.5% of bovine serum albumin (BSA).

### KLK6 assay

Calibrators, controls or samples (10 µL) were pipetted into a pre-coated white polystyrene microtiter plate with 200 ng/100 µL anti-KLK6 (27–4) monoclonal antibody and 100 µL of assay buffer (50 mM Tris, 6% BSA) were added, incubated for 120 min and washed. After washing, 100 µL of biotinylated detection anti-KLK6 (E24) were added, incubated for 60 min and washed. Streptavidin-HRP solution (100 µL) was then added, incubated for another 15 min and washed. Then, 100 µL of TMB substrate (Neogen) were added and incubated for 10 min. All the incubations were at room temperature on a horizontal microplate shaker at 700 rpm and all washes were performed four times with washing buffer (5 mM Tris, 0.05% Tween-20). Finally, 100 µL of stop solution (0.2 M H<sub>2</sub>SO<sub>4</sub>) were added and the signal was read at 450/620 nm.

### Analytical features of assay

The detection limit of KLK6 assay is 0.03 ng/mL, with a dynamic range from 0.03 ng/mL to 10 ng/mL (Supplemental Data, Figure 1 which accompanies the article at <http://www.degruyter.com/view/j/cclm.2012.50.issue-5/issue-files/cclm.2012.50.issue-5.xml>). The analytical sensitivity was determined by calculating the mean +2 standard deviations for 10 replicates of the zero standard. The within-run coefficient of variation was 2.0%, 2.9% and 2.3%, at KLK6 levels of 6.35, 3.29 and 2.09 ng/mL, respectively, and the between-run coefficient of variation (CV<sub>A</sub>) was 4.2%, 4.9% and 6.7%, at levels of 5.54, 1.48 and 0.61 ng/mL, respectively. Overall recovery ranged from 89% to 102% (Supplemental Data, Table 1). Comparison with a previously established immunofluorometric method showed good correlation ( $y=0.995x-0.071$  ng/mL,  $r^2=0.98$ ).

### Creatinine assay

Creatinine was measured with a fully automated enzymatic assay on the Abbott Architect system.

### Blood sampling

Serum samples from healthy volunteers (n=136) were collected according to the recommendations of CLSI (12), to establish the reference interval for KLK6. Heparin-plasma samples from 42 of these individuals were also obtained. To study the biological variation of KLK6, between 15 and 18 additional serum samples from four of these subjects were collected over a period of about 2 months (between 57 and 63 days). Additionally, serum samples from individuals (n=1043) who visited the University Health Network (Toronto) for a routine check-up (family practice) were collected, to study the association between KLK6 with age and gender. Finally, serum samples from patients with renal failure (n=106) were also obtained and KLK6 and creatinine concentrations were analyzed. Samples were stored at –80°C until assayed. Our protocol was approved by the Institutional Review Board of University Health Network.

### Statistical analysis

Statistical analyses were performed with SPSS® 15.0 software package (SPSS Inc., Chicago, IL, USA). A p-value <0.05 was considered statistically significant. The distributions were analyzed using parametric or non-parametric tests. Data were expressed as mean±standard deviation. Outliers were identified with Reed's test and the normal distribution was evaluated using Shapiro-Wilk test and by inspection of Q-Q plots. The reference interval and associated confidence intervals (CI) were calculated using the parametric test (mean±1.96 standard deviations). Student's t-test or Mann-Whitney U-test were performed for comparison between independent samples and Wilcoxon signed-rank test was used for paired samples. Associations between variables were assessed by Spearman's rank correlation coefficient.

The biological variation of KLK6 was estimated according to the method of Fraser (13). The within subject biological variation (CV<sub>I</sub>) and the between-subject biological variation (CV<sub>G</sub>) were calculated as:

$$CV_I = 100/M \times (S_{A+I}^2 - S_A^2)^{1/2}$$

$$CV_G = 100/M \times (S_T^2 - S_{A+I}^2)^{1/2}$$

where  $S_{A+I}^2$  is the experimental variance obtained with the results of each individual,  $S_A^2$  is the analytical variance,  $S_T^2$  is the total variance obtained with the results of all individuals and M is the mean concentration.

The index of individuality (II), the reference change value (RCV) bi-directional at 5% probability level and the number of samples required to estimate the homeostatic setting point (NHSP), at 95% confidence and 20% accuracy, were calculated as (14–16):

$$II = CV_I / CV_G$$

$$RCV = 2^{1/2} \times 1.96 \times (CV_A^2 + CV_I^2)^{1/2}$$

$$NHSP = [1.96 \times (CV_A^2 + CV_I^2)^{1/2} / 20]^2$$

## Results

### Reference interval

Serum samples from healthy volunteers (n=136), 73 women and 63 men, 20–86 years old, were analyzed. After exclusion of one outlier, the KLK6 results ranged from 1.00 to 4.14 ng/mL, with a mean of 2.48±0.74 ng/mL. Data followed a normal distribution (Supplemental Data, Figures 2 and 3). The reference interval was established from 1.04 (90% CI 0.86–1.21) to 3.93 ng/mL (90% CI 3.75–4.11). There were no significant correlation between KLK6 and gender and no significant association with age (Supplemental Data, Figures 4 and 5).

The association between serum levels of KLK6 with age and gender was re-analyzed in a much larger number of individuals (n=1043) who visited the University Health Network (Toronto) for a routine check-up, after exclusion of patients with KLK6 concentration outside of the established reference interval. These were 555 women and 488 men, 18–93 years old. There were no significant differences between genders (Supplemental Data, Figure 6) but there was a weak but highly significant positive correlation with age (Spearman rho=0.205, bilateral significance p<0.0001; Figure 1).

## Biological variation

The biological variation of KLK6 was estimated using serum samples ( $n=72$ ) from four healthy volunteers, two men and two women, 30–38 years old. After exclusion of two outliers, the remaining values followed a normal distribution (Supplemental Data, Figure 7), with KLK6 concentrations from 0.97 ng/mL to 4.09 ng/mL (Figure 2). The components of biological variation for KLK6 are described in Table 1. The  $CV_A$  used was the value corresponding to the concentration of KLK6 (1.48 ng/mL) closest to mean concentration in these four individuals (2.47 ng/mL). The  $II$  obtained was 0.43 and the RCV bi-directional (95% confidence) was 35.3%. Interestingly, only two serum samples would be required to estimate the homeostatic setting point (within  $\pm 20\%$ , 95% confidence) of an individual.

## KLK6 in renal failure

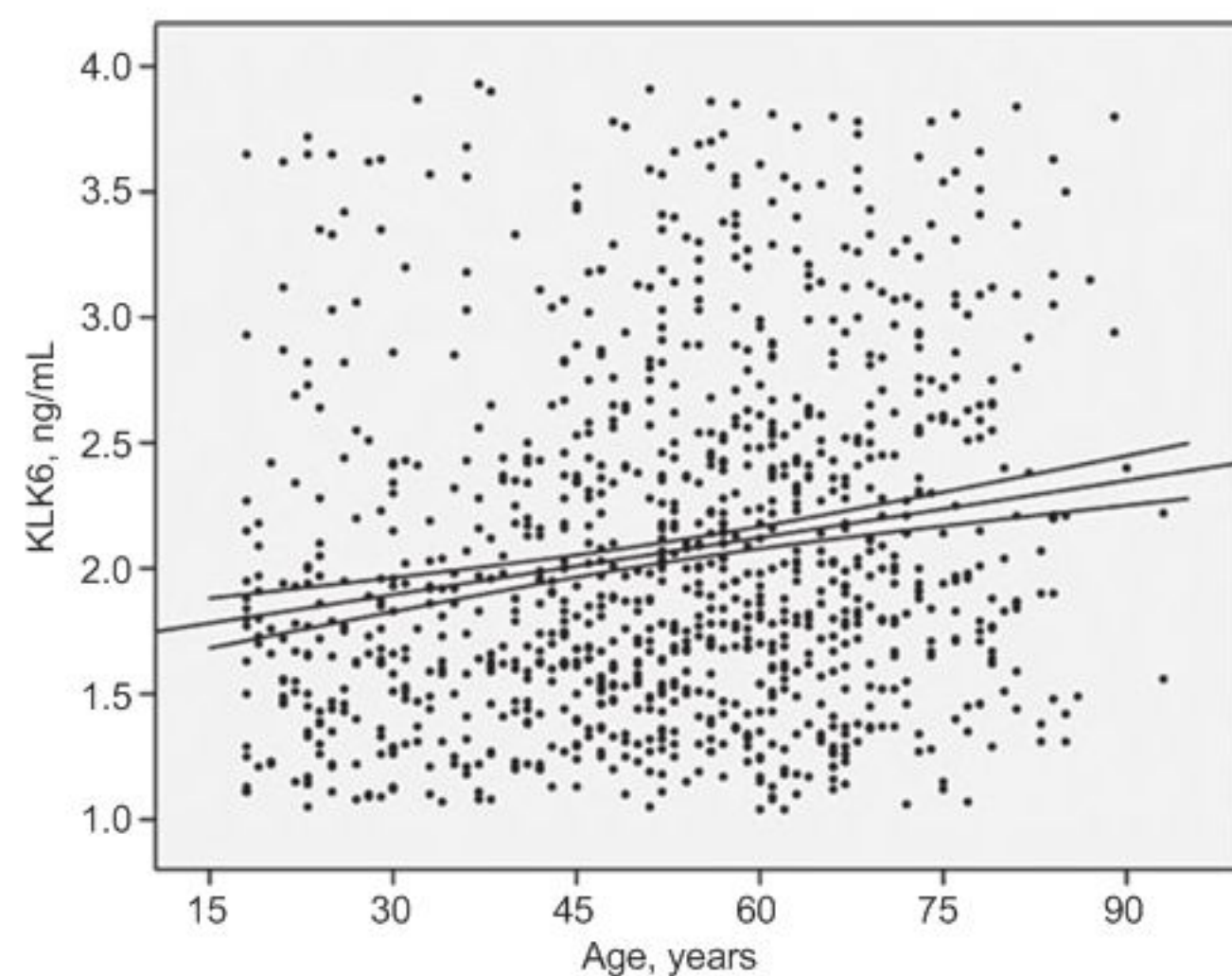
The KLK6 serum levels in individuals, between 21 and 95 years old, with renal failure ( $n=106$ ) were from 2.46 ng/mL to 22.8 ng/mL and their creatinine levels from 68  $\mu\text{mol/L}$  to 1263  $\mu\text{mol/L}$ . There was a significant positive correlation between these two biomarkers (Spearman  $\rho=0.772$ , bilateral significance  $p<0.0001$ ) (Figure 3). There was no significant association between KLK6 and age in this group of patients ( $p=0.585$ ).

## Comparison between serum and plasma

The KLK6 results of serum and heparin-plasma samples obtained from 42 individuals ranged from 1.78 ng/mL to 4.44 ng/mL. There were no significant differences between these two types of specimens ( $p=0.396$ ) (Supplemental Data, Figures 8 and 9).

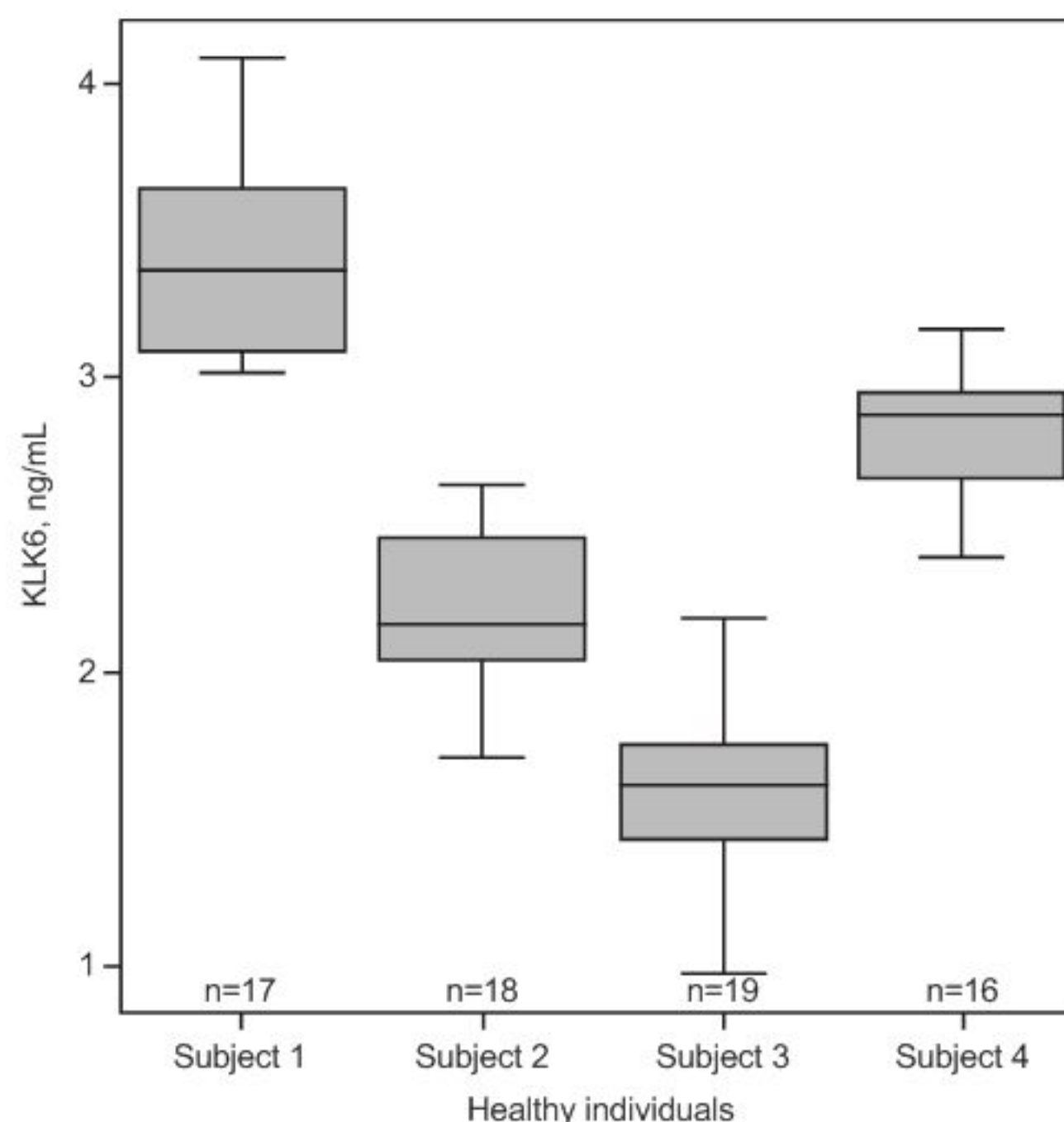
## Discussion

To our knowledge, this is the first study describing a well-established reference interval for serum concentrations of



**Figure 1** Correlation between serum concentration of kallikrein 6 and age ( $n=1043$ ).

The solid lines represent the mean and confidence interval at 95%.



**Figure 2** Box and Whisker diagram of serum kallikrein 6 levels (ng/mL) from four healthy individuals.

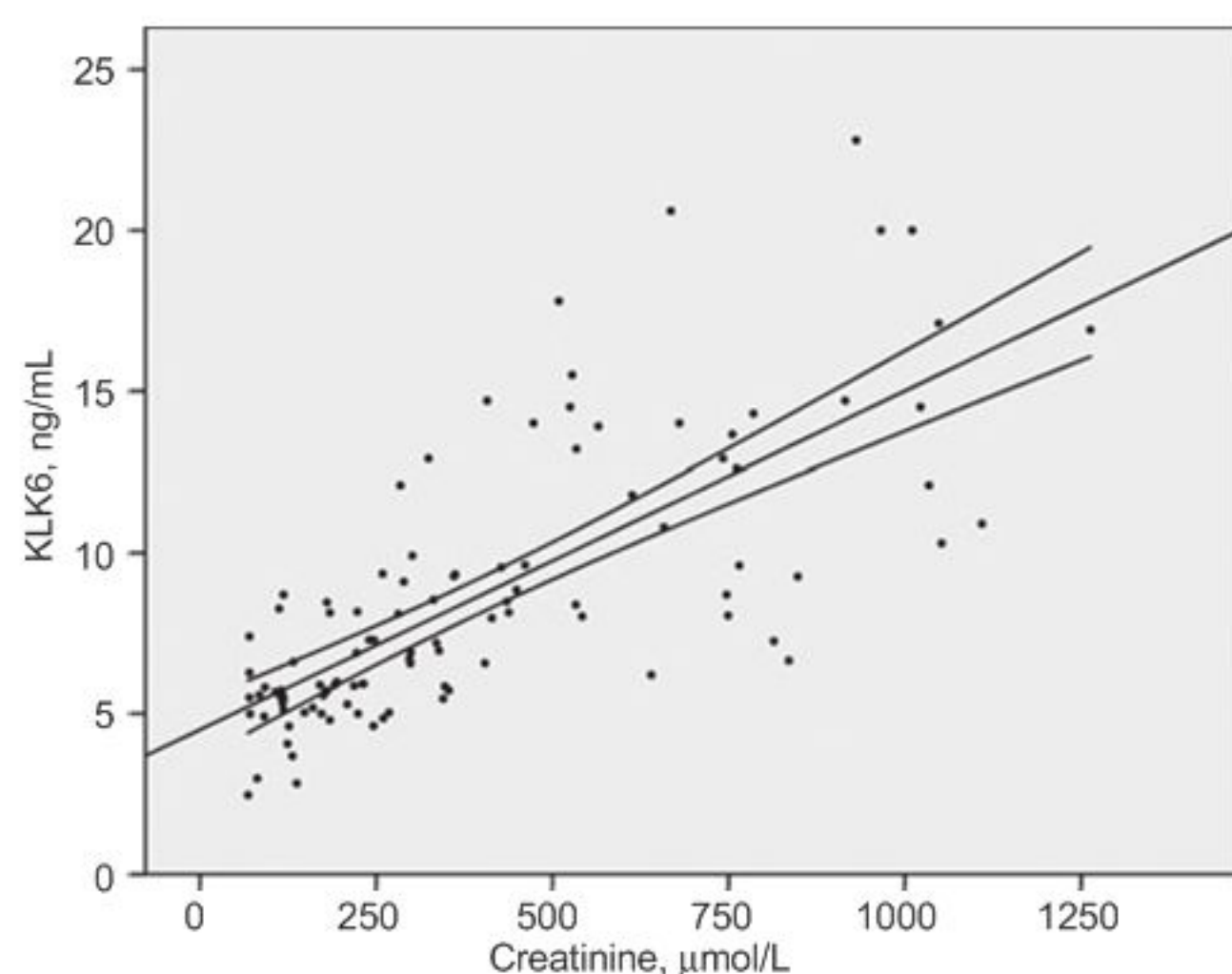
KLK6. Analysis of a large number of samples from patients with KLK6 levels within the reference interval showed that there were no differences in KLK6 concentrations between women and men and that there is a highly significant positive correlation with age. Others described the same association, both in cerebrospinal fluid (17) and plasma (8) (although in a smaller number of subjects) and speculated that KLK6 may be related to aging and Alzheimer's disease.

The estimation of components of biological variation for KLK6 could help in a better interpretation of this biomarker. The  $II$  was defined by Harris (18) as the value that describes the distributions of measured values of samples from healthy individuals in relation to a population-based reference interval. When this index is higher than 1.4, the population-based reference intervals are relevant. However, when it is lower than 0.6, the reference interval does not have enough sensitivity to detect individual sample changes and the comparison of serial results

**Table 1** Components of biological variation for kallikrein 6.

Component	Results
Mean, ng/mL	2.47
$CV_A$ , %	4.9
$CV_I$ , %	11.8
$CV_G$ , %	27.6
Index of individuality	0.43
RCV, %	35.3
NHSP	2

$CV_A$ , between-run analytical variation at 1.48 ng/mL;  $CV_I$ , within-subject biological variation;  $CV_G$ , between-subject biological variation; NHSP, number of samples required to estimate the homeostatic setting point, at 95% confidence and 20% accuracy; RCV, reference change value bi-directional at 95% confidence.



**Figure 3** Correlation between serum concentrations of kallikrein 6 and creatinine (n=106).

The solid lines represent the mean and confidence interval at 95%.

from the same individual is more informative (19). Iglesias et al. went further, stating that the II should be lower than 0.48 to have a 90% probability of detecting a change in a monitoring situation (20). The II obtained for KLK6 was 0.43, indicating that the use of the RCV could be very informative. The RCV allows assessing the significance of changes in serial results from an individual. For KLK6, this value was 35% and, therefore, changes in KLK6 concentration higher than 35% between two serial samples, could be attributed to disease. Interestingly, only two samples from a subject are necessary to establish the homeostatic setting point of KLK6, with 20% accuracy.

The KLK6 concentration in individuals with renal failure correlates significantly with creatinine, with levels >5 ng/mL in most patients studied (Figure 3). Hence, renal function should be checked in subjects with high levels of KLK6, to avoid misinterpretations.

Finally, comparison of serum and heparin-plasma results in 42 individuals revealed very similar KLK6 concentrations, suggesting that these two types of specimen are equivalent for the analysis of KLK6.

### Conflict of interest statement

**Authors' conflict of interest disclosure:** The authors stated that there are no conflicts of interest regarding the publication of this article.

**Research funding:** None declared.

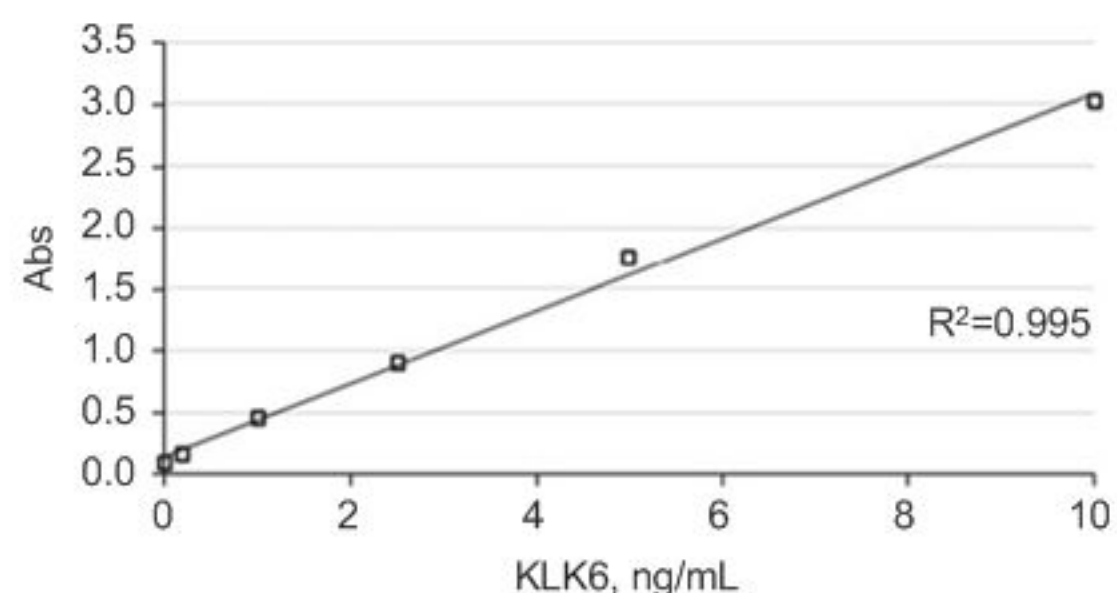
**Employment or leadership:** None declared.

**Honorarium:** None declared.

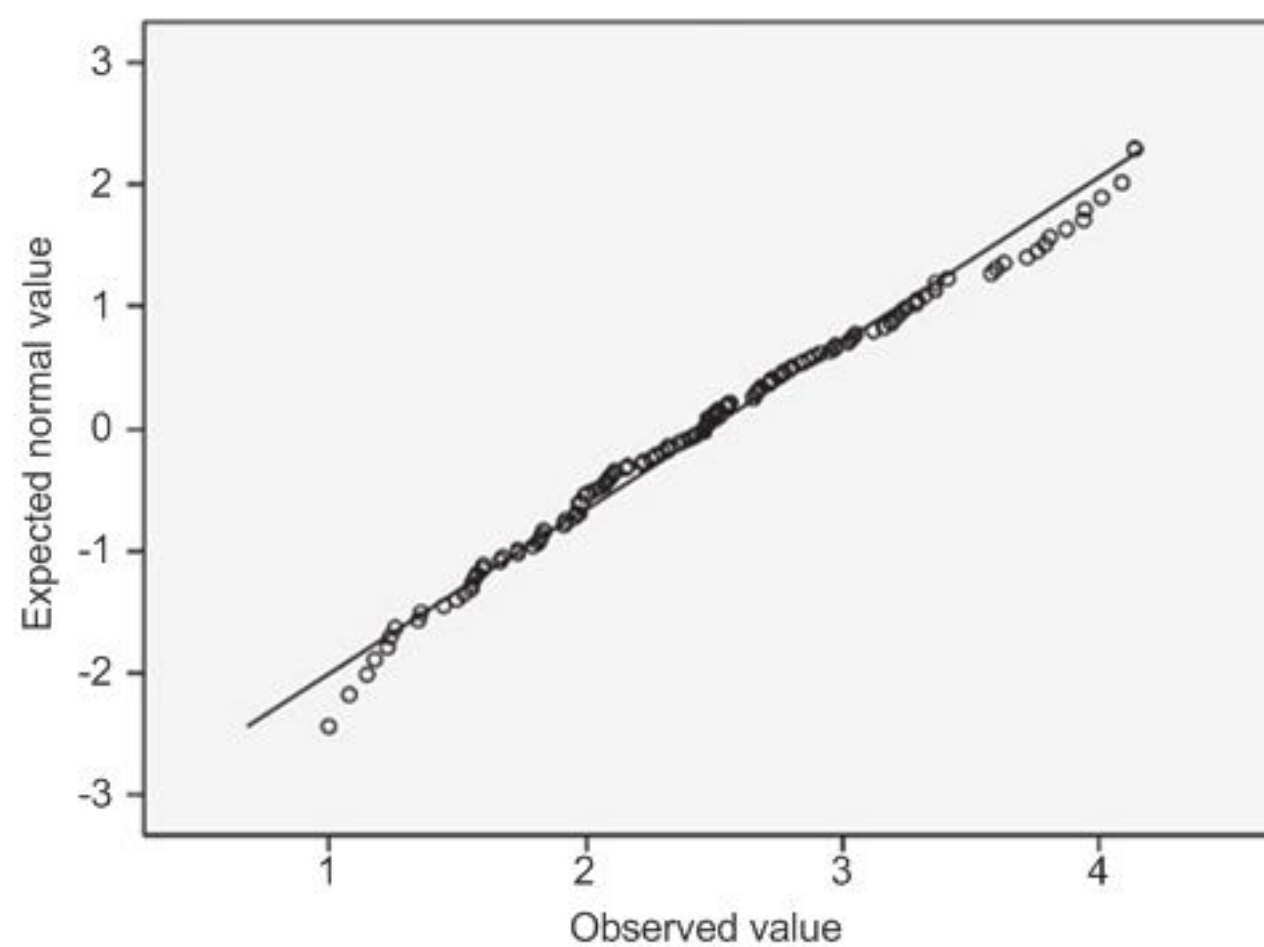
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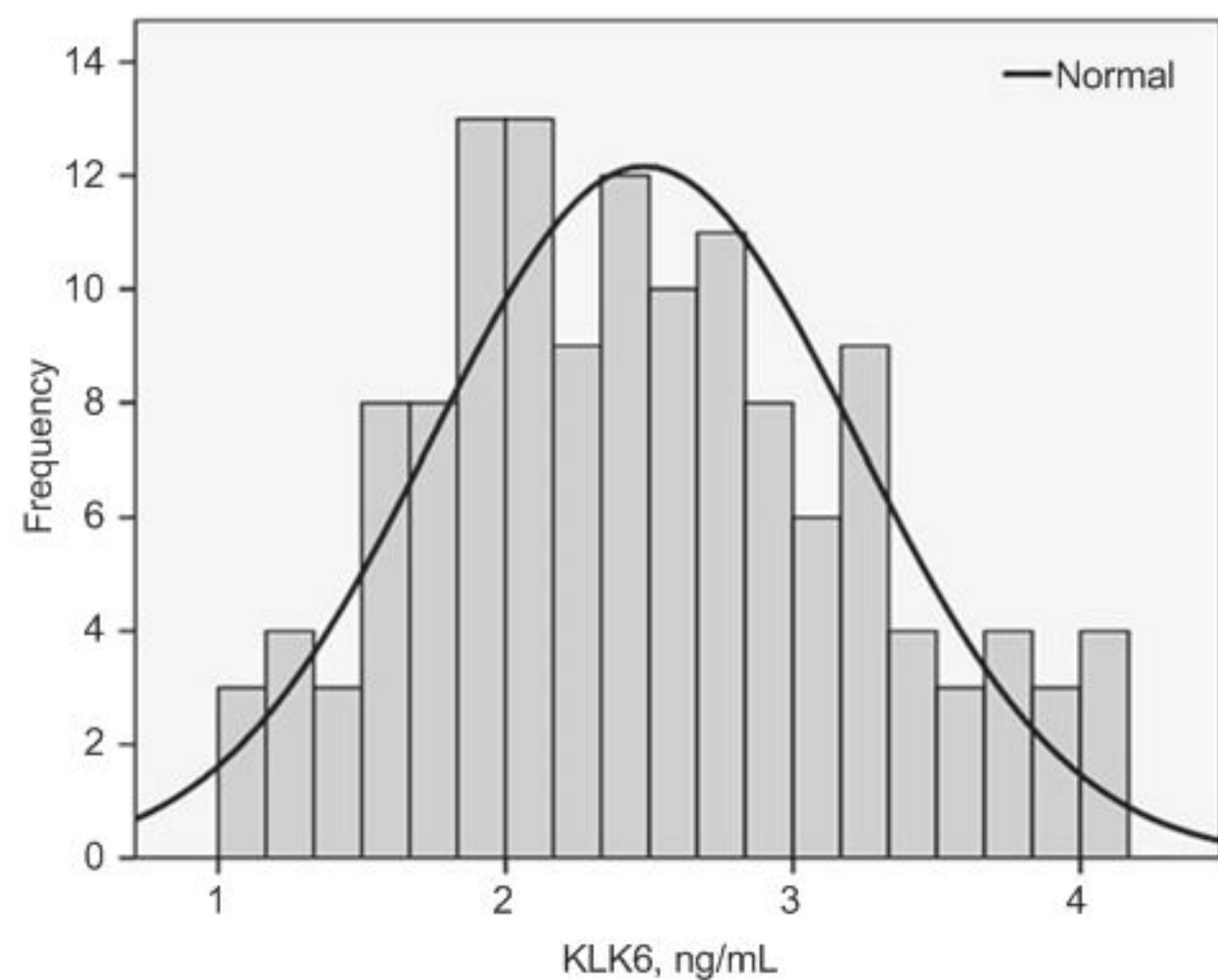
## Supplemental data



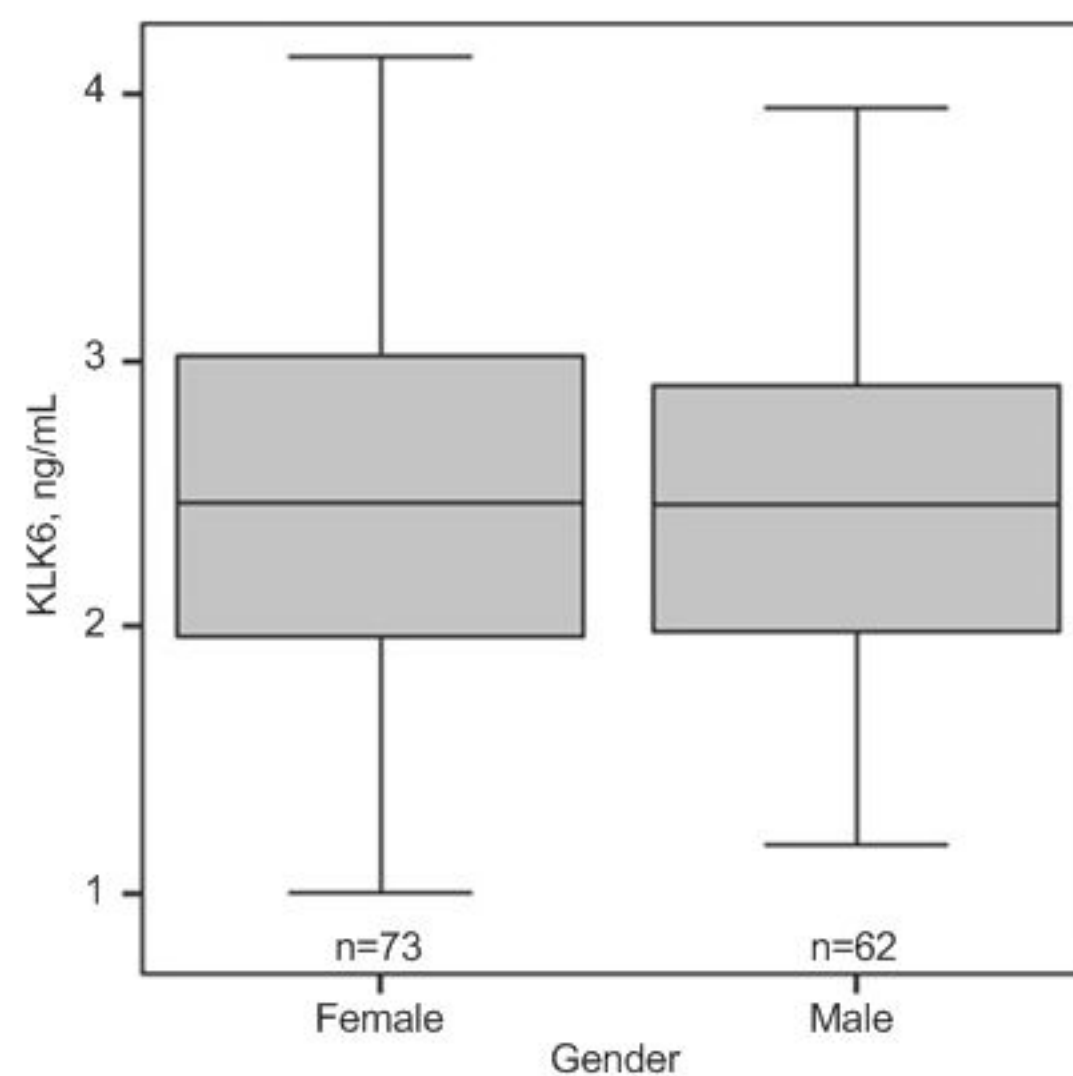
**Supplementary Figure 1** Calibration curve of KLK6 ELISA assay, using calibrators with 0, 0.2, 1, 2.5, 5 and 10 ng/mL of recombinant protein.



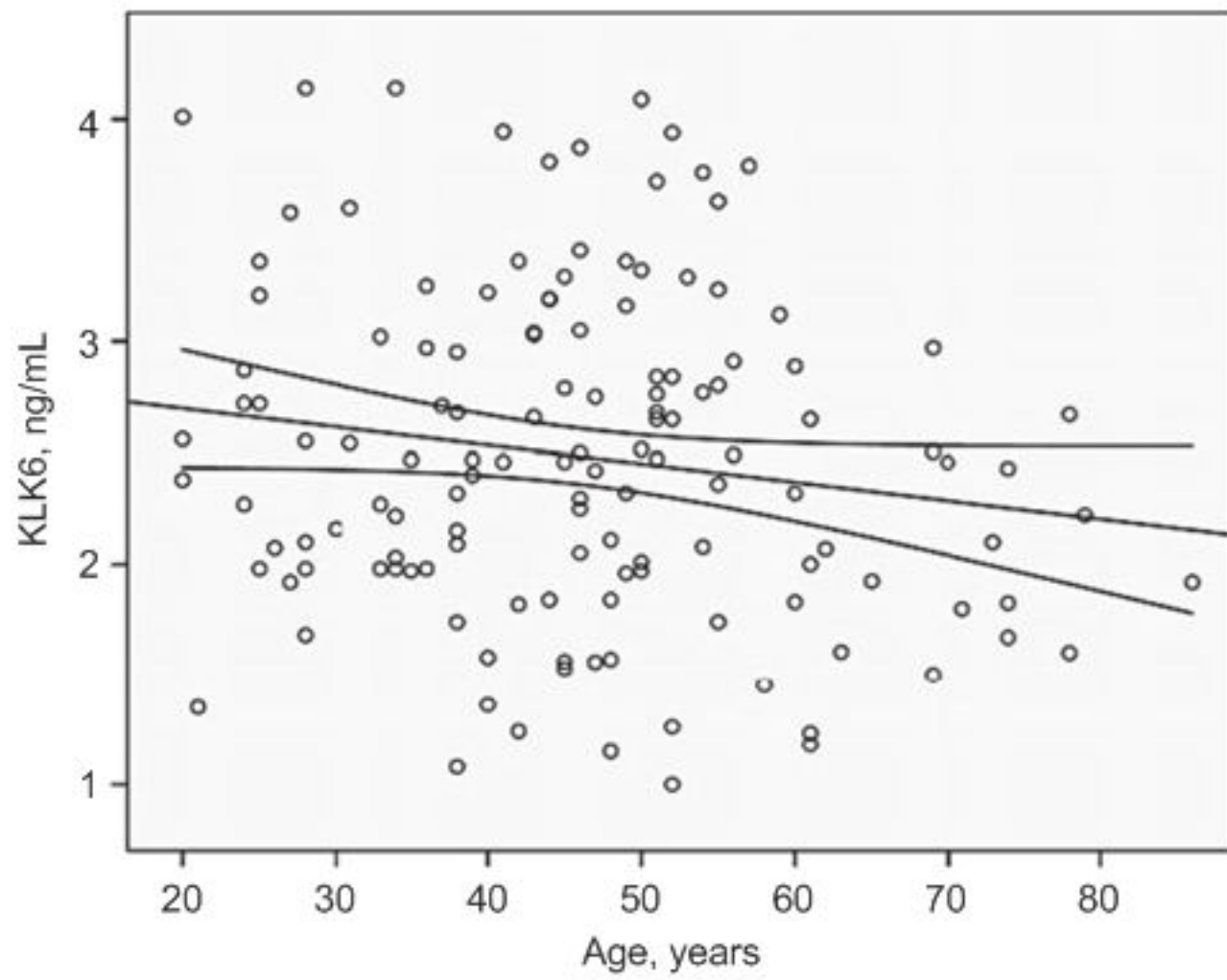
**Supplementary Figure 3** Q-Q plot of kallikrein 6 results (n=135) in serum samples from healthy individuals.



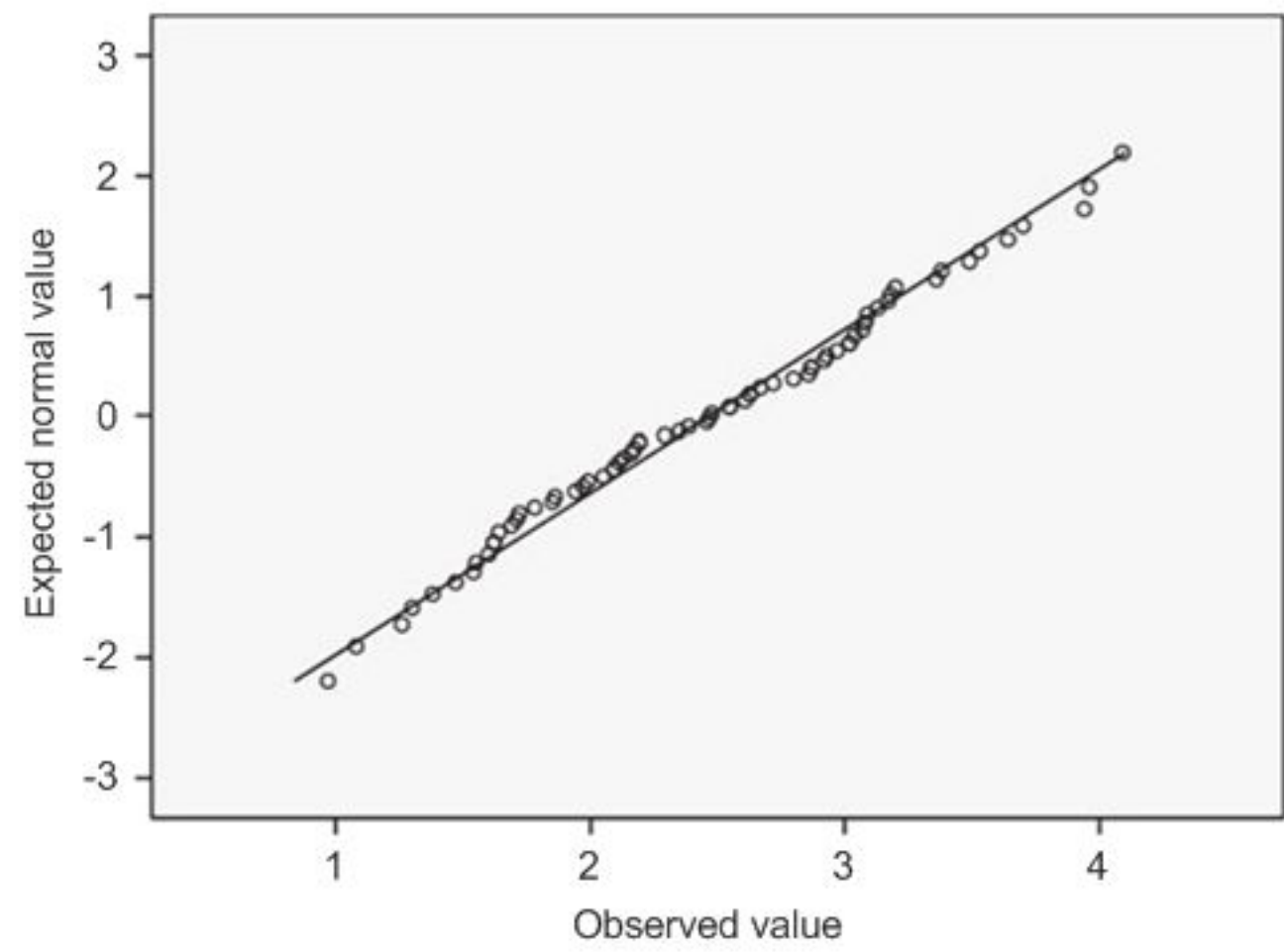
**Supplementary Figure 2** Histogram of kallikrein 6 results (n=135) in serum samples from healthy individuals. The solid line represents the normal distribution.



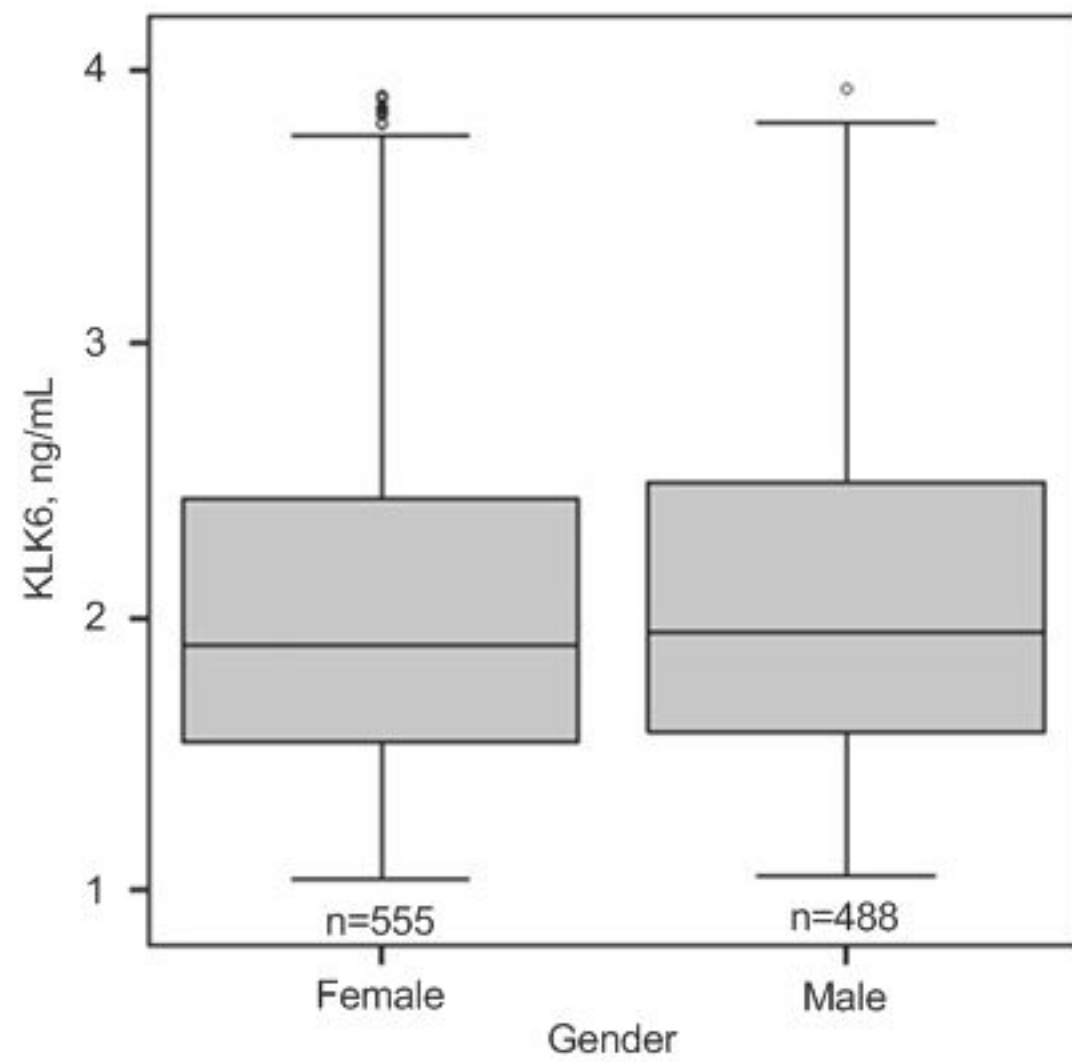
**Supplementary Figure 4** Box and Whisker diagram of serum kallikrein 6 levels (ng/mL) in healthy individuals, women (n=73) and men (n=62).



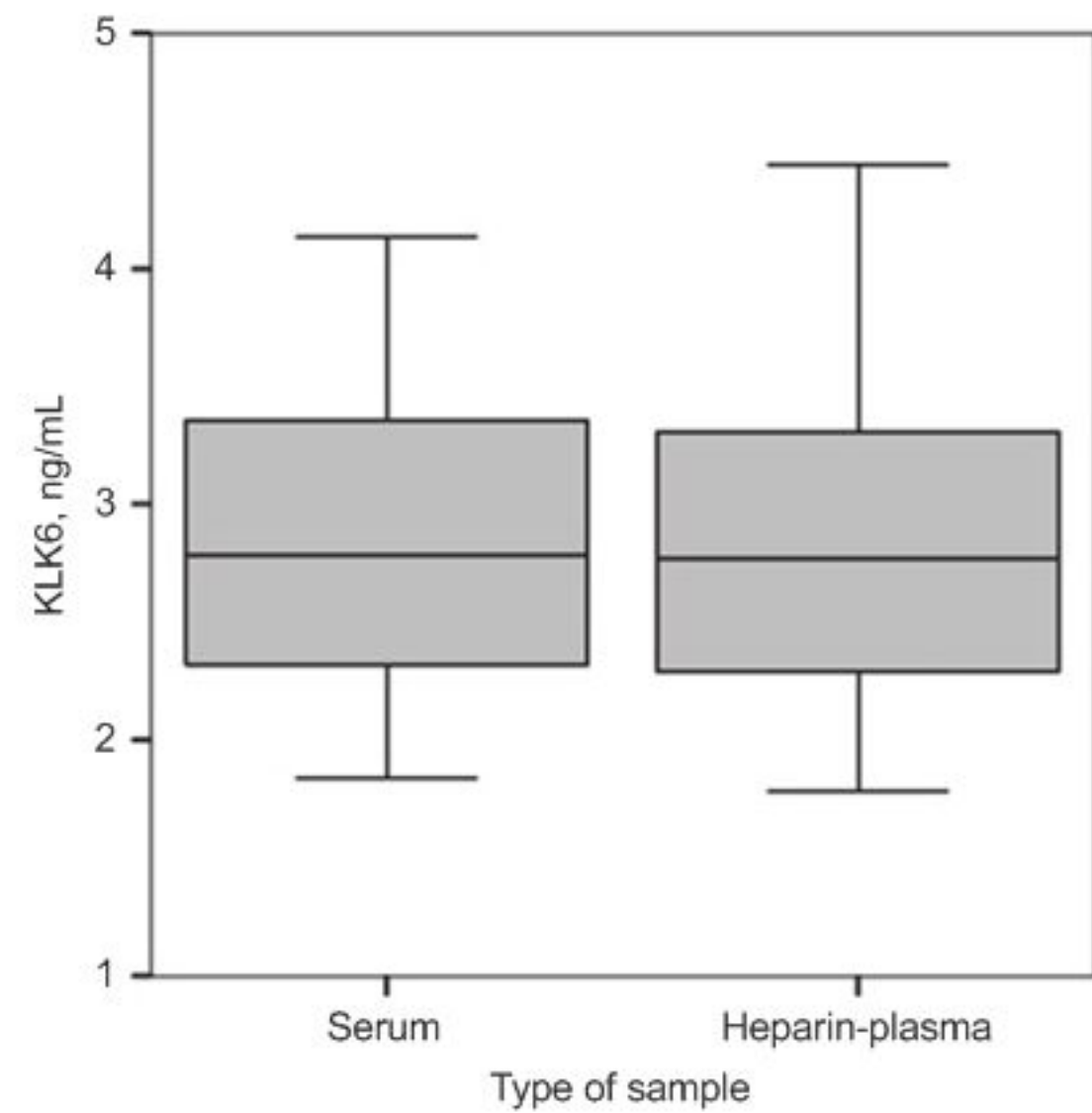
**Supplementary Figure 5** Correlation between serum concentration of kallikrein 6 and age in healthy individuals (n=135). The solid lines represent the mean and confidence interval at 95%.



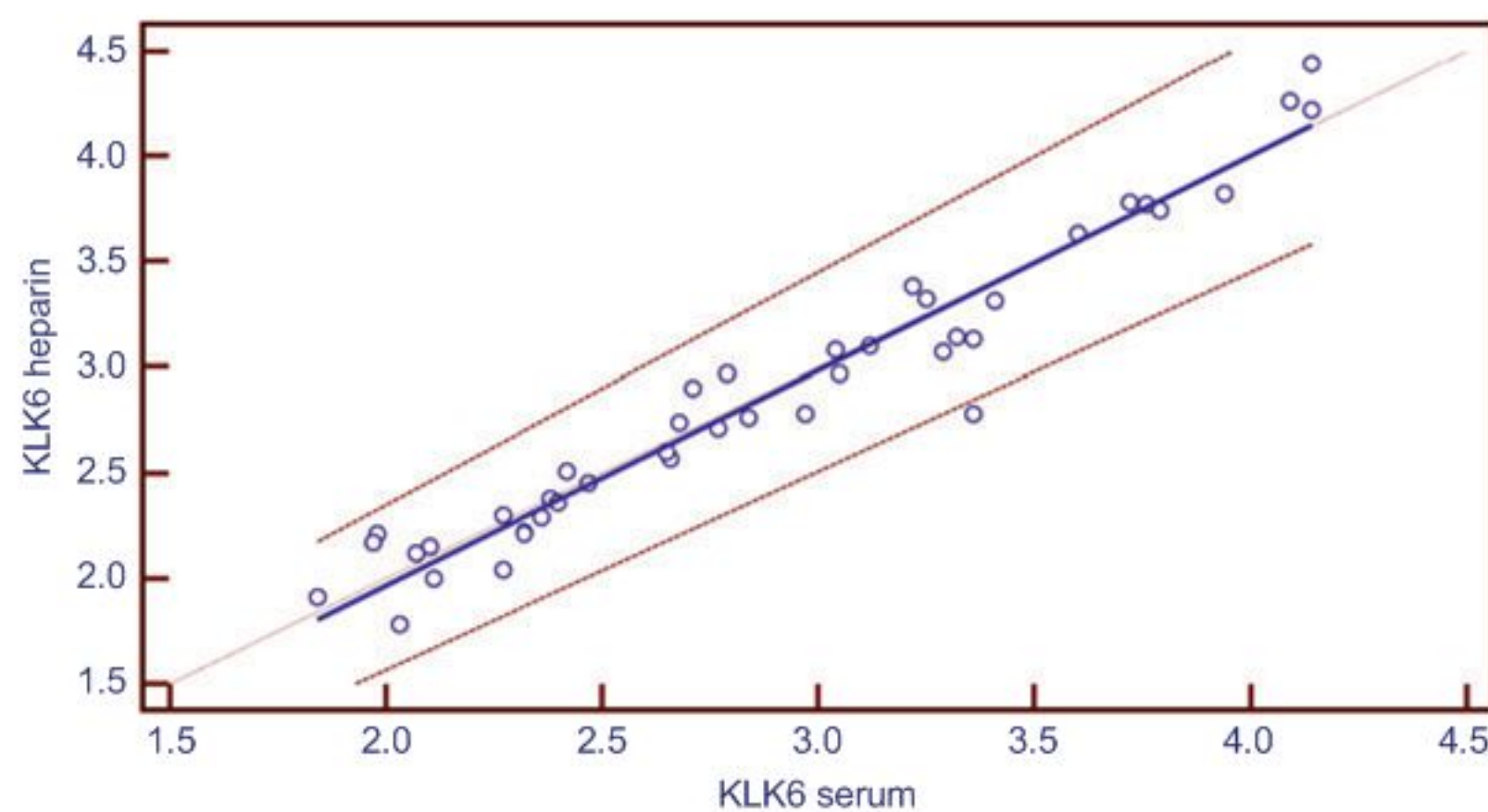
**Supplementary Figure 7** Q-Q plot of kallikrein 6 results in four healthy individuals (n=70).



**Supplementary Figure 6** Box and Whisker diagram of serum kallikrein 6 levels (ng/mL) in women (n=555) and men (n=488).



**Supplementary Figure 8** Box and Whisker diagram of serum kallikrein 6 levels (ng/mL) in serum and heparin-plasma samples (n=42).



**Supplementary Figure 9** Passing-Bablok regression for serum kallikrein 6 levels (ng/mL) in serum and heparin-plasma samples (n=42).

**Supplementary Table 1** Recovery of recombinant KLK6 added in three serum samples, at three different concentrations (1.45, 2.50 and 3.46 ng/mL).

Sample	Endogenous, ng/mL	Added, ng/mL	Observed, ng/mL	Recovered, ng/mL	Recovery, %
1	1.45	1.45	2.75	1.30	90
		2.50	3.68	2.23	89
		3.46	4.71	3.26	94
2	1.46	1.45	2.81	1.35	93
		2.50	3.75	2.29	92
		3.46	4.68	3.22	93
3	1.84	1.45	3.16	1.32	91
		2.50	4.20	2.36	94
		3.46	5.37	3.53	102