

**Title:**

A rapid and sustained improvement of calcification propensity score after successful kidney transplantation: reanalysis of a randomized controlled trial of ibandronate.

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## Authorship Page

### *Specific contributions per author:*

K.T.S. participated in research design, performance of the research, data analysis and writing of the article. A.Å. participated in research design, performance of the research, data analysis and writing of the article. H.K. participated in data analysis and critical revision of the manuscript. A.P. participated in research design, performance of the research, measurement of calcification propensity and critical revision of the manuscript. D.O.D. participated in research design, performance of the research and critical revision of the manuscript. J.B. participated in research design, performance of the research and critical revision of the manuscript. K.G. participated in performance of the research and critical revision of the manuscript. A.H. participated in research design, performance of the research, data analysis and writing of the article.

### *Disclosures:*

A.P. is an inventor of the T<sub>50</sub> test, and is currently an employee and holds stock in Calciscon AG, Berne, Switzerland, a company which markets the T<sub>50</sub> test. All other authors declare no conflicts of interest.

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## **Abbreviations Page:**

**ANCOVA**, analysis of covariance

**BMD**, bone mineral density

**BMP-7**, bone morphogenic protein 7

**CPP**, calciprotein particle

**FGF23**, fibroblast growth factor 23

**iPTH**, intact parathyroid hormone

**OPG**, osteoprotegerin

**OPN**, osteopontin

**TNF $\alpha$** , tumor necrosis factor alpha

**VSMC**, vascular smooth muscle cells

## **Abstract.**

*Background.* A serum test called  $T_{50}$  assesses the overall propensity for calcification of blood and is associated with cardiovascular outcomes in kidney patients. We aimed to examine the evolution of  $T_{50}$  over time in kidney transplant recipients and also address any effects of ibandronate, an inhibitor of bone turnover.

*Methods.* Serum samples that had been frozen at minus 70°C, taken from kidney transplant patients included in a prospective, randomized placebo controlled study of ibandronate were analyzed in retrospect. Adequate analyses could be performed at baseline about 3 weeks after transplantation in 129 patients, at 10 weeks in 127 patients and at one year in 123 patients.

*Results.* Ibandronate caused no differences to placebo in  $T_{50}$  at 10 weeks ( $p=0.094$ ) or at 1 year ( $p=0.116$ ). Baseline  $T_{50}$  was a significant covariate ( $p < 0.0001$ ) for  $T_{50}$  scores at 10 weeks and 1 year. In the total cohort there was a highly significant ( $p < 0.0001$ ) increase in  $T_{50}$  of 26.6% from  $186.5 \pm 47.2$  min to  $236.1 \pm 58.6$  min after 10 weeks and  $T_{50}$  remained stable at  $232.8 \pm 62.9$  min after one year with an overall increase from baseline of 24.8% ( $p < 0.0001$ ). The only biochemical variable correlated with the change in  $T_{50}$  was an inverse correlation to phosphate of -0.515 ( $p < 0.0001$ ) and a smaller positive correlation with change in serum albumin ( $p < 0.03$ ).

*Conclusions.* We found that  $T_{50}$  increased from baseline to 10 weeks after transplantation with no further change after one year. Ibandronate had no effect on  $T_{50}$ .

## Introduction

Chronic kidney disease (CKD) is associated with increased risk of cardiovascular complications and premature death compared to the general population<sup>1,2</sup>. As recently reviewed<sup>3</sup>, such patients are prone to excessive vascular and tissue calcifications, and this is again highly correlated with cardiovascular morbidity and mortality<sup>4,5</sup>. These complications accelerate with increasing stages of kidney disease, are most pronounced in patients receiving renal replacement therapy with dialysis, and are associated with a multi-fold increased risk of morbidity and mortality<sup>6,7</sup>. Renal replacement therapy with kidney transplantations appears to yield lower rates of cardiovascular complications compared with persisting dialysis therapy, although still much higher than that of the general population<sup>8-10</sup>. The reasons for this discrepancy are not fully understood but the tendency to form accelerated tissue and vessel calcifications may have a role<sup>11-14</sup>.

Further, calcium metabolism is altered in several ways after successful kidney transplantation due to alterations in renal and endocrine functions. There are many links or similarities between vascular calcification and bone metabolism: decreased expression of calcium-sensing receptors located in the vascular smooth muscle cells (VSMCs) promotes osteoblastic transformation of VSMCs and thereby accelerates vessel wall calcification<sup>15</sup>; the activity of osteoprotegerin (OPG) and osteopontin (OPN), which are both secreted locally from the vascular wall as well as by osteoblasts<sup>16</sup>.

Recently, a novel in vitro test of serum has been launched which assesses the overall propensity for calcification of blood<sup>17</sup>. This test is a physiological test that measures the time of transformation of soluble primary calciprotein particles (CPPs)

to secondary, crystalline (hydroxyapatite-containing) CPPs during stimulation of calcification in vitro. The time to half maximal transition to the insoluble crystalline form is called  $T_{50}$ , and a shortened  $T_{50}$  means an increased tendency for calcification, or accelerated transformation. It has recently been found that  $T_{50}$  is a strong and independent risk factor for adverse long-term outcomes including cardiovascular death in elderly patients with stage 3 or 4 CKD, as well as in kidney transplant recipients<sup>18-20</sup>.

Little is hitherto known about drug treatment and influence on calcification propensity in the clinical setting. Bisphosphonates are drugs that inhibit bone turnover and thus halt bone loss, and are commonly used for treating bone disease in kidney transplant patients with adequate kidney function. There are mechanisms offered by bisphosphonates that may protect from calcification, such as decreased expression of tumor necrosis factor alpha, down-regulation of the inflammatory process and decreased uptake of LDL-cholesterol by macrophages within the atherosclerotic plaque<sup>21</sup>. Furthermore, bisphosphonates directly interact with amorphous and crystalline calcium phosphate<sup>22</sup>. Thus, exploring whether bisphosphonates influence the  $T_{50}$  score would be a valuable contribution towards understanding the usefulness of this new score in a new clinical setting.

In a prospective, randomized, double blind and controlled study during the first year after kidney transplantation, ibandronate reduced bone turn-over, improved bone mineral density (BMD) in femur and in ultradistal radius, but had no significant effect on BMD in the lumbar spine compared with placebo<sup>23</sup>. Serum samples from this clinical trial has been stored in a biobank, and provided us with an ideal model for studying the calcium propensity score in more detail. In the present study, the aim was to conduct secondary and post hoc analyses from the above-mentioned study

on the evolution of  $T_{50}$  during one year after transplantation, with a particular focus on the possible effect of ibandronate and on any biochemical variables with potential importance for the  $T_{50}$  scores during this period.

## **Materials and methods**

The selection of patients and details of treatment of the original patient cohort examined has been described in detail previously<sup>23</sup>. In short, 129 kidney transplant patients were included in a one-year prospective, randomized, placebo-controlled trial, with study baseline when a clinically stable and adequate graft function with an estimated glomerular filtration rate (eGFR) of at least 30 ml/min/1.73m<sup>2</sup> had been obtained, provided this was within four weeks after transplantation. Eligible patients were treated with either intravenous (i.v.) ibandronate or i.v. isotonic saline each third month during one year. All patients received supplementation of calcium (1 g/day) and active vitamin D<sub>3</sub> (0.25 mcg/day). Blood samples used for the present analysis were taken at baseline, after 10 weeks and after one year and the plasma had been stored at minus 70<sup>0</sup>C until analysis. The  $T_{50}$  analysis in all samples were analyzed in one batch using laser detection of fully calcified particles as described in detail elsewhere<sup>17</sup>. Adequate samples and  $T_{50}$  results were obtained from all patients remaining in the trial, i.e. from 129 patients at baseline, 127 patients at 10 weeks and 123 patients at one year. The demographic data and relevant biochemical analyses for the patients included in the present study are shown in Table 1.

## *Statistics*

An ANCOVA model was used to check for differences between treatments in  $T_{50}$  score at 10 and 52 weeks, using  $T_{50}$  score at baseline as a covariate. Further analyses were done using paired sample t-test and multiple linear regression to model  $T_{50}$  score at 10 weeks. Two sample t-tests and Wilcoxon two sample tests were used to compare variables at baseline, 10 weeks, and 52 weeks (Chi-square test was used for categorical data). Repeated measures were not accounted for in any of the analyses.

## **Results**

The study population consisted mainly of Caucasians with a mean age of 51 years, about three out of four were males, and 88% participated in occasional or regular physical activity. Patients were included into the study after an average of 18.5 days post transplantation, and this time-point thus represents study baseline. Further demographic and baseline data are presented in Table 1, and formal statistical tests demonstrated that there were no significant differences between the groups in terms of any of the demographic, clinical or biochemical variables at baseline (results not shown).

Intervention with ibandronate caused no statistically significant differences to placebo in  $T_{50}$  at 10 weeks ( $p=0.0937$ ) or at 1 year ( $p=0.1163$ ) as per ANCOVA analyses. Baseline  $T_{50}$  was a significant covariate ( $p < 0.0001$ ) in both ANCOVA models, for  $T_{50}$  scores at 10 weeks and 1 year.



Looking at the study cohort as a whole, a paired sample t-test showed that there was a highly significant ( $p < 0.0001$ ) early increase in  $T_{50}$  of 26.6% from  $186.5 \pm 47.2$  min at baseline to  $236.1 \pm 58.6$  min after 10 weeks (Figure 1). From week 10 to 1 year,  $T_{50}$  remained stable (-1.4% from week 10) at a high level ( $232.8 \pm 62.9$  min) maintaining a significant ( $p < 0.0001$ ) mean change of 24.8% compared with baseline.

To further understand the  $T_{50}$  scores in the first year after transplantation, and particularly the influence by other variables, multiple regression analyses were performed. Baseline  $T_{50}$  score was included in all regression models with a significant effect both for  $T_{50}$  at 10 weeks ( $p < 0.0001$ ) and for  $T_{50}$  at 1 year ( $p < 0.0001$ ). For all biochemical variables tested (albumin, hemoglobin, phosphate, magnesium and intact parathyroid hormone (iPTH)) only baseline calcium ( $p = 0.026$ ) had a predictive value for  $T_{50}$  score at week 10.

There were similarly significant changes ( $p < 0.0001$  for each variable, data not shown) in albumin, calcium, creatinine, hemoglobin, phosphate and iPTH from baseline to week 10. Table 2 shows the results of the correlation analysis of the change in  $T_{50}$  score against change in the biochemical variables from baseline to week 10. Change in phosphate was significantly correlated with the change in  $T_{50}$ , with a Pearson correlation coefficient of -0.515 ( $p < 0.0001$ ) so that a decrease in phosphate over those 10 weeks was correlated with an increased  $T_{50}$ . Also, change in albumin was significantly positively correlated with change in  $T_{50}$ .

## Discussion

The present post-hoc analyses share both insights into the evolution of calcification propensity over the first year after kidney transplantation and the modifying effects of bisphosphonate treatment over the same time period. The calcification propensity score showed a highly significant increase from baseline to 10 weeks with no further change up to one year after kidney transplantation, but there was no effect of ibandronate compared to placebo in any of the time-points. A prolonged accelerated level of this score is potentially beneficial since it has recently been shown that higher  $T_{50}$  at week 10 is associated with improved long-term cardiovascular outcomes in renal transplant patients<sup>19,20</sup>. Our study is the first one in kidney transplant recipients which provides longitudinal data on  $T_{50}$ . The improvement of  $T_{50}$  post transplantation is consistent with the lower risk for cardiac, cardiovascular and all-cause mortality post transplantation seen after half a year, despite the risk of the transplantation surgical procedure per se<sup>24</sup>.

A reduction of calcification propensity appears to precede the improvement and may thus represent an early marker for improved prognosis. Actually the improvement in calcium propensity score may be even higher than revealed in the present study. The baseline measures were done after stabilization of kidney function and normalization of plasma calcium, in this study about three weeks after transplantation. At this time the electrolytes and also metabolic acidosis were grossly restored. Still, there were significant changes in such electrolyte and other parameters from baseline to 10 weeks. As shown in our correlation analysis only phosphate and albumin changes were correlated with the increase of  $T_{50}$ , but the improvement of  $T_{50}$  could not be accounted for by these metabolic variables only. Potentially, the changes in  $T_{50}$  would be even more pronounced when compared to

the pre-transplant stage of the patients as perhaps there are more factors in the inflammatory milieu influencing the T<sub>50</sub> score that are reversed or improved with restoration of kidney function after successful kidney transplantation.

This study was originally designed to study the effect of ibandronate, a bisphosphonate, for prophylaxis against bone loss<sup>23</sup>. One could hypothesize that ibandronate and associated effects on bone turn-over, and its direct interactions with amorphous and crystalline calcium phosphate<sup>22</sup> might also have some impact on plasma calcification propensity but this was apparently not the case, at best such an effect was marginal and short-lasting.

Recent reviews have expressed concern that the use of bisphosphonates may be contraindicated in patients with low GFR, and in those with low-turnover bone disease<sup>25</sup>. Our study of bisphosphonates administered for a year to such at-risk patients would possibly contribute to reducing this fear of worsening effects of bisphosphonates. Even if we were not able to detect any lasting additional effects of ibandronate over calcium and calcitriol, nothing in our findings suggest that ibandronate would worsen the calcification propensity. Furthermore, all our patients received additional calcium supplementation, and even if our study design did not allow us to separate any calcium effects from active vitamin D<sub>3</sub> or ibandronate, at least this additional and theoretically unwanted calcium did not seem to promote a tendency towards calcification as measured by serum calcification propensity.

Our study was, however, not planned to detect meaningful differences between ibandronate and placebo in terms of T<sub>50</sub> scores. The secondary analyses of T<sub>50</sub> were further of post-hoc nature and exploratory in terms of T<sub>50</sub>. We did not collect any imaging data and furthermore we did not analyse any relevant biomarkers

known to influence vascular calcification, such as fibroblast growth factor 23 (FGF23), bone morphogenic protein 7 (BMP-7), OPG, OPN, fetuin-A or matrix gla protein. The observation period in the current study is limited to 1 year post transplantation, only. On the other hand, the strengths of our study include the prospective, randomized and placebo-controlled design. The single-center approach has further assured a uniform approach to underlying treatment and procedures.

In conclusion, we found that  $T_{50}$  increased from baseline to 10 weeks after transplantation with no further change after one year. Ibandronate had no effect on  $T_{50}$  compared with placebo. The significantly improved calcification propensity measured with the  $T_{50}$  score, is encouraging in terms of utilizing this variable as a potential early biomarker for improved prognosis of cardiovascular mortality in the kidney transplant setting. Further confirmation of its clinical usefulness, over longer term and of its ability to predict therapeutic effectiveness of any interventions, would be reasonable follow-up work following this initial study.

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## Tables

Variable	Statistic	Ibandronate (N=66)	Placebo (N=63)	Total (N=129)
Gender (N, (%))	Males	48 (72.7)	51 (81.0)	99 (76.7)
Age (years)	Mean ± SD	50.2 ± 13.5	52.6 ± 14.0	51.4±13.8
	Range	21.4 – 71.3	18.0 – 77.6	18.0 – 77.6
Diabetes (%)	N	10 (15.2)	12 (20.6)	22 (17.1)
Smoking status (N (%))	Never	17 (25.8)	26 (41.3)	43 (33.3)
	Present	13 (19.7)	9 (14.3)	22 (17.1)
	Previous	35 (53.0)	28 (44.4)	63 (48.8)
Mean systolic blood pressure (mmHg)	Mean ± SD	134.6 ± 18.5	138.7 ± 17.9	136.6±18.2
eGFR (mL/min/1.73m <sup>2</sup> )	Mean ± SD	64.5 ±19.9	64.2 ±18.2	64.3±19.0
Creatinine (µmol/L)	Mean ± SD	111.8±27.2	112.3±24.0	112.1±25.6
Albumin (g/L)	Mean ± SD	40.3±2.7	39.2±3.2	39.8±3.0
Hemoglobin* (g/dL)	Mean ± SD	12.2±1.2	11.0±1.2	12.0±1.2
Phosphate (mg/dL))	Mean ± SD	0.73±0.21	0.67±0.18	0.70±0.20
Magnesium (mmol/L)	Mean ± SD	0.66±0.09	0.66±0.08	0.66±0.08
Calcium (mmol/L)	Mean ± SD	2.36±0.12	2.31±0.13	2.33±0.12
iPTH* (pmol/L)	Mean ± SD	15.1±11.6	16.5±11.1	15.9±11.3
T50 (min)	Mean ± SD	184.2 ± 43.7	189.0 ± 50.8	186.5±47.2

**Table 1. Demographics and baseline variables.**

\* This variable is not normally distributed. Wilcoxon two-sample test was used to check for differences between sample means. The confidence intervals assume normal distribution.



Biochemical variable	N	Pearson correlation coefficient	p
Creatinine	127	-0.122	0.173
Albumin	127	0.192	0.031
Hemoglobin	127	-0.064	0.477
Phosphate	127	-0.515	< 0.0001
Magnesium	17	0.369	0.145
Calcium	127	-0.119	0.184
iPTH	106	-0.049	0.615

**Table 2. Correlation analysis of change in serum T<sub>50</sub> versus change in covariates.**

## Figure legend

**Figure 1. Serum  $T_{50}$  scores (in min) following kidney transplantation per treatment group.**