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HIGHLIGHTS

- Renal transplantation has a major impact on both bone metabolism and bone turnover
- Bone biomarkers may be useful in guiding therapy, acknowledging limitations
- Further standardization and harmonization of bone biomarker assays is needed

ABSTRACT

Successful kidney transplantation (partly) corrects the physiologic and metabolic abnormalities driving chronic kidney disease – mineral and bone disorders. At the same time, renal transplant recipients are exposed to immunosuppressive agents that may affect bone metabolism. Bone biomarkers have been suggested as surrogates of or adjuncts to bone biopsy and imaging techniques to assess bone health and to classify risk of bone loss and fractures. Bone biomarkers may be classified as circulating factors that affect bone metabolism (commonly referred to as bone metabolism markers) or that reflect bone cell number and/or activity (commonly referred to as bone turnover markers). A growing body of evidence shows that successful renal transplantation has a major impact on both bone metabolism and bone turnover. Analytical issues, including the cross-reactivity with fragments, complicate the interpretation of bone biomarkers, especially in the setting of a rapid changing kidney function, as is the case after successful renal transplantation. Overall, bone turnover seems to decline following renal transplantation, but inter-individual variability is substantial. Preliminary evidence indicates that bone biomarkers may be useful in guiding mineral and bone therapy in renal transplant recipients.

INTRODUCTION

Disturbances in mineral and bone metabolism occur early in the course of chronic kidney disease (CKD) to become almost universal in patients with advanced stage disease. Disturbances in mineral and bone metabolism, with secondary hyperparathyroidism (SHPT) being a hallmark, trigger renal bone disease and extra-skeletal calcification, which are associated with decreased quality of life, fractures and increased (cardiovascular) morbidity and mortality. Currently, the term CKD-Mineral and Bone Disorder (CKD-MBD) is preferentially used to describe a clinical syndrome that develops as a systemic disorder of mineral and bone metabolism due to CKD, which is manifested by abnormalities in bone and mineral metabolism and/or extra-skeletal calcification. The term renal osteodystrophy is recommended to be used exclusively to define alterations in bone morphology associated with CKD, which can be further assessed by histomorphometry, and the results reported based on a unified classification system that includes parameters of turnover, mineralization, and volume[1].

Successful kidney transplantation corrects, at least partly, the physiologic and metabolic abnormalities responsible for SHPT. However rather than solving CKD-MBD, renal transplantation only changes its phenotype. Posttransplant CKD-MBD reflects the effect of immunosuppression, previous CKD-MBD persisting after transplantation and *de novo* CKD-MBD[2;3].

The histomorphometric analysis of the tetracycline double-labeled bone biopsy is considered the gold standard for evaluating bone health. A bone biopsy provides information on bone turnover, volume,

mineralization and cellular number/activity. Bone biopsies can only be obtained at the iliac crest, except for surgical samples taken at other sites under specific conditions. Given the heterogeneity of the skeleton, caution is warranted when extrapolating iliac crest bone biopsy results to other skeletal sites. Taking a bone biopsy is invasive and requires the necessary skills whilst its analysis is expensive and necessitates specific histopathological expertise which is not widely available. Therefore, implementing a bone biopsy in routine clinical care is not feasible [4;5].

Noninvasive imaging techniques may inform on bone mass/mineral density and bone quality (e.g. microarchitecture) but have intrinsic limitations such as limited spatial resolutions, limited availability, bias by skeletal deformities, are limited by skeletal heterogeneity, and do not inform on bone cell cellular number/activity and degree of mineralization[6].

Bone biomarkers have been suggested as surrogates of or adjuvants to bone biopsy and imaging techniques to assess bone health, to classify risk of bone loss and fractures, and to guide therapeutic decisions[7].

The exact role of bone biomarkers in the management of metabolic bone diseases remains a topic of controversy[7;8]. In this review we will present a state of the art of the impact of renal transplantation on bone biomarkers. After discussing general aspects we will briefly discuss established and emerging bone biomarkers.

GENERAL ASPECTS OF BONE BIOMARKERS

Bone biomarkers are commonly classified as circulating (systemic) factors that affect bone metabolism (further referred to as bone metabolism markers [BMMs], e.g. parathyroid hormone [PTH], sclerostin, osteoprotegerin [OPG] and factors that reflect bone cell number and/or activity (further referred to as bone turnover markers [BTMs]). The latter are generally subdivided into two categories: markers of bone formation and markers of bone resorption. Bone formation markers derive from the osteoblastic activity and include bone specific alkaline phosphatase (BsAP), osteocalcin, N-terminal propeptide (PINP), and C-terminal propeptide of type-I procollagen (PICP). The markers of bone resorption include degradation products of the type-I collagen such as the intermolecular crosslinks pyridinoline (PYD) and deoxypyridinoline (DPYD), the C-terminal telopeptide (CTX), the N-terminal telopeptide (NTX) and matrix-metalloproteases (MMP)-generated (CTX-MMP or ICTP) type I collagen fragments and osteoclasts enzymes, such as type 5b tartrate-resistant acid phosphatase (TRAP-5b) and cathepsin K. The deliberate use of bone biomarkers requires knowledge about their strengths and limitations[9].

Strengths

Undoubtedly the *easiness of sample collection* is an important asset of all bone biomarkers.

Biochemical BTMs reflect changes in bone turnover more *rapidly* than changes in other clinical tests such as bone mineral density and bone histomorphometry. Monitoring BTMs may thus be advocated to capture 'acute' effects of disease and therapy. The concentration of BTMs reflects the turnover rate of the *skeleton as a whole*, whereas histomorphometric indices reflect its activation at a definite site. This may be relevant as heterogeneity of the skeleton (site, endosteal vs periosteal envelope) exists, both with regard to the response to ageing, as to disease and therapy[10].

Limitations

BTMs originating from the degradation of type I collagen *lack tissue specificity*. Indeed, type I collagen is not only present in bone, but also in other connective tissues such as the skin and tendons. More importantly, most BTMs are characterized by a high *variability*. Sources of variability can be pre-analytical (related to patient characteristics and sampling/storage conditions), analytical (related to the assay)[11] and post-analytical (including biological variability). Many of circulating BTMs exhibit a circadian rhythm. The circadian rhythm is more pronounced for markers of bone resorption than those of bone formation and is partly related to food intake. Some BTMs are retained in renal failure, hampering their interpretation in the setting of CKD. This is of utmost relevance when monitoring bone turnover in conditions of (rapidly) changing kidney function, as is the case in (*de novo*) renal transplant recipients. Other pre-analytical confounding factors include age, gender, menopausal status, ethnicity, geographical location and therapy. Sample type (serum or plasma) and analyte stability in particular should be accounted for as well. BTMs are most commonly determined by immunoassays. Automated methods are not uniformly available and inter-method or inter-manufacturer variability may be high. The latter is related to problems of antibody specificity and standardization. Establishment of reliable reference ranges for BTMs is also challenging. Indeed, the reference population should be free of any condition that might lead to secondary hyperparathyroidism among which CKD and vitamin D deficiency, or low bone turn-over among which older age and diabetes. Unfortunately, most of manufacturers do not take these confounding factors into consideration, which may lead to reference ranges which are too wide with both a too high upper normal limit and a too low lower normal limit. Another post-analytical issue that should be accounted for is the biological variability (CVi)[12;13] (***Table 1***). The CVi is the random natural variation around an individual homeostatic set point. The CVi determines how much the concentration of an analyte must vary between two results before the change should be considered as clinically significant with 95% certainty. This change is commonly referred to as the critical difference or least significant change (LSC) and corresponds to approximately 3 times the CVi. In hemodialysis patients, the LSC for e.g. bone alkaline phosphatase is 36%, which is several fold higher than LSCs observed for other common biochemical parameters such as creatinine[12].

Abovementioned characteristics of BTMs may explain why the correlation between histomorphometric parameters obtained from the iliac bone and the integrated mean of the overall skeletal turnover represented by serum BTM concentration is at best only modest [14] [7].

Tissue specificity may also be an issue for some BMMs. Recent evidence suggest that sclerostin is not only expressed by the skeleton (osteocytes), but also by calcifying vasculature. BMMs, furthermore, are characterized by an even higher pre-analytical, analytical and post-analytical variability. For some analytes, inter-assay variability has a clear explanation. For instance, some assays not only detect the intact BMMs, but also some degradation products. This is best acknowledged for second-generation PTH assays. These PTH assays, which are currently the most widely implemented in clinical practice, use a capture antibody that binds near the N-terminus and a second solid phase-coupled antibody that binds to the C-terminus. Differences in antibody specificities and affinities of the assays translate in different inter-assay recoveries of (1-84)PTH and cross-reactivities with (7-84)PTH and other fragments[15]. The third generation assays use the same capture antibody but the detection antibody is more specific for the first 4 amino acids of PTH, thereby avoiding cross-reactivity with the N-terminal truncated PTH fragments. Renal retention of PTH fragments may (falsely) inflate the severity of secondary hyperparathyroidism along the progression of CKD, when monitored by second generation assays.[16;17]. For other analytes (e.g. sclerostin), we can only speculate about the causes of the inter-assay variability[18].

BMMs at best provide a poor proxy of bone metabolism[19;20]. Besides abovementioned analytical issues, extra-skeletal generation (e.g. OPG, sRANKL, sclerostin, DKK-1) and skeletal hyporesponsiveness (PTH, FGF23) represent important and hard to quantify confounders. The pathophysiology of CKD-related hyporesponsiveness to the action of hormones involved in mineral metabolism, including, PTH, FGF23, calcitriol, sex steroids …, is complex and multifaceted[21]. PTH hyporesponsiveness, for example, involves competing downstream signals, inhibitory local factors and PTH receptor downregulation[16].

BONE METABOLISM MARKERS

PTH

Because PTH is a key regulator of bone remodeling and because of the widely availability of rapid and automated PTH immunoassays, regular monitoring of PTH levels has become the *lingua franca* of renal bone disease management. PTH levels show a biphasic decline after successful renal transplantation: a rapid drop (by approximately 50%) during the first 3 to 6 months, attributed to a reduction of the parathyroid functional mass (and clearance of fragments), followed by a more gradual decline[22;23]. The long lifespan of parathyroid cells (approximately 20 years) contributes to the very slow involution of the hyperplastic parathyroid glands after renal transplantation[24]. As a result, elevated intact PTH

levels persist in a substantial proportion of renal transplant recipients, even on long-term [23;25]. While the involution of hyperplastic parathyroid glands is slow, PTH hyporesponsiveness rapidly wanes after renal transplantation parallel to the recovery of renal function. PTH levels thus may become inappropriately high in a substantial proportion of *de novo* renal transplant recipients, a condition commonly referred to as tertiary hyperparathyroidism (HPT)[2]. Since renal function does not recover completely following transplantation, hyperparathyroidism (HPT) in the post-transplant setting is always a combination of tertiary (inappropriate) and (*de novo*) secondary (appropriate) HPT[2]. The correct estimation of the contribution of each is challenging, but important to define the optimal treatment strategy. When presenting together with hypercalcemia, hypophosphatemia or elevated BTMs, a high PTH points to tertiary HPT as dominant pathology. Ultimate proof can be obtained by bone histomorphometry [26;27]. PTH at the time of transplantation does not associate with fracture risk[28] [29]. Post-transplant HPT, conversely, associates with BMD loss[30], progressive cortical porosity [31;32] and high fracture risk[29]. Intervention studies (with calcimimetics, VitD (analogs) or parathyroidectomy) assessing bone endpoints, so far also yield inconsistent findings [33-35], partly related to case-mix and differences in the magnitude of PTH suppression. Hard end-point (fracture) studies, finally, are lacking at all.

Sclerostin

Sclerostin, a protein produced by osteocytes, is a negative regulator of bone formation; it decreases bone formation through inhibition of the (canonical) Wnt–β-catenin pathway[36]. Clinical data show increasing circulating sclerostin levels with progression of CKD, to reach levels that are 2-4-fold higher in dialysis patients as compared to healthy controls [37]. It remains a matter of debate whether and if so, to what extent, renal retention (of fragments) or increased production accounts for the increasing circulating sclerostin levels along the progression of CKD [38;39].

Circulating sclerostin levels show a biphasic pattern with an early and profound drop, followed by a slight rebound towards levels observed in CKD counterparts [20;40-42]. Renal clearance (of fragments) most probably accounts for the early drop. Furthermore, HPT (suppression) [40] and glucocorticoids (upregulation) [43;44] may modulate sclerostin production. Bone biopsy data show increased expression of sclerostin in bone of renal transplant recipients as compared to CKD counterparts [20;45], fostering the hypothesis that the increase of circulating sclerostin in CKD is at least partly an analytical artifact.

Circulating sclerostin levels correlate negatively with circulating and histomorphometric parameters of bone turnover in dialysis patients [46;47] and renal transplant recipients [48]. Associations with other skeletal endpoints have not been reported in renal transplant recipients.

Fibroblast growth factor 23

Fibroblast growth factor 23 (FGF23) is a peptide hormone, secreted by osteoblasts and osteocytes, that regulates phosphate homeostasis and bone growth. Serum FGF23 levels rapidly increase in the course of CKD to reach levels in CKD 5D that are more than a 100-fold higher than in healthy controls. After successful renal transplantation, FGF23 rapidly decreases, parallel to the recovery of renal function. Inappropriately high levels of FGF23, however, may persist in a substantial proportion of transplanted patients [23;49;50]. Underlying pathophysiological mechanisms remain ill-defined. Both (hypercalcemic) HPT and glucocorticoids [51] may be involved in the pathogenesis of so-called "tertiary hyperphosphatoninism." As opposed to serum PTH levels, FGF23 levels return to normal by 1 year after transplantation in the majority of the patients [52]. High FGF23 levels associate with poor graft and patient survival[53], but associations with skeletal outcomes are limited. Patients with a high serum FGF-23 level at the time of transplantation were shown to be at risk for increased BMD loss during the first post-transplant year[54]. Both direct[55] and indirect (via hypophosphatemia) mechanisms may be involved.

Osteoprotegerin

Osteoprotegerin (OPG) is a member of the tumor necrosis factor receptor family, is produced by both bone and vascular cells and is an established key factor in bone remodeling. By binding to receptor activator of nuclear factor-B ligand (RANKL), OPG prevents the interaction of RANKL with RANK, and thereby inhibits osteoclast differentiation. Skeletal OPG is regulated by various calcitropic hormones (e.g. PTH ↓[56] and oestrogens↑[57]), and drugs (e.g. glucocorticoids↓ [58]). OPG levels increase along the progression of CKD [59], to reach levels in CKD5D patients that are about three times higher than those of healthy controls [60]. OPG levels decrease by approximately 50% shortly after successful renal transplantation, most probably due to increased renal clearance and suppressed production mediated by glucocorticoids [20;61;62]. OPG predicts survival and cardiac death in renal transplant recipients [63], probably through its association with vascular calcification. Data on associations of OPG with bone indices in renal transplant recipients are limited if not non-existing.

BONE TURNOVER MARKERS

Studies evaluating bone turnover in *de novo* renal transplant recipients are scarce and often hampered either by cross-sectional design, missing pre-transplant data or limitations inherent to the biomarker such as renal retention (e.g. osteocalcin, CTX, monomeric PINP, pyridinolines)[23;64-66]. Given the recovery of renal function following transplantation, we focus on BTMs that are not retained in kidney failure (***figure 2***).

Bone formation markers

Type I collagen and other bone matrix components are produced by osteoblasts, which also produce enzymes essential for the mineralization of osteoid. Bone formation markers reflect different stages of bone formation.

Propeptides of type I procollagen

In the early phase of bone formation osteoid is formed. Around 90% of osteoid consists of type I collagen. The formation of type I collagen requires cleavage of the propeptides of type I procollagen, C-terminal (PICP) and N-terminal (PINP) which are released into the circulation. In human serum, PINP is present in two major forms, an intact trimeric form and a monomeric one which accumulates in patients with renal failure [67]. Immunoassays that only recognize the trimeric form are called "intact PINP" whereas those recognizing the trimer and the monomer are named "total PINP" assays. PINP is cleared by the liver. The serum concentration of PINP shows little diurnal or seasonal variation and does not differ between men and women. Data on the natural history of PINP in renal transplant recipients are scarce. A more than 50% decline of total PINP was observed 6 months after transplantation in a cohort of 70 renal transplant recipients [66]. As monomeric PINP accumulates in CKD, these data are hard to interpret (if measured with the "total" assays). In the placebo arm of a Swiss open-label, prospective, randomized trial to assess the efficacy and safety of RANKL inhibition with denosumab to prevent the loss of BMD in *de novo* renal transplant recipients, PINP levels (assay not specified) showed an increase during first post-transplant year[68]. We recently assessed intact PINP in a cohort of 69 *de novo* renal transplant recipients, and showed a non-significant decline (-36%) during the first post-transplant year, which thereafter continued at a slower pace [69].

Bone Specific Alkaline phosphatase

Approximately 2 weeks after formation of osteoid, mineralization of the matrix occurs. Essential to this process is bone specific alkaline phosphatase (BsAP) activity, an ectoenzyme of the osteoblast which hydrolyzes pyrophosphate to inorganic phosphate. The bone isoform of alkaline phosphatase accounts for approximately half of circulating total alkaline phosphatase activity, the remaining representing mainly the liver isoform. With regard to its diagnostic potential, BsAP presents very interesting features, like liver clearance (thus concentrations not influenced by decreased glomerular filtration rate), relative high half-life in serum, storage stability and relatively low intra-individual (biological) variability [12;13;70]. In recent years, rapid, robust, and reproducible immunoassays have been developed for the quantification of circulating BsAP, often on fully automated platforms, but unfortunately results obtained by different assays are not interchangeable[71]. In abovementioned

prospective cohort study[69], BsAP, showed a significant 27% decrease during the first post-transplant year (23 vs 18 µg/L, time of transplantation [Tx] vs year 1, n=69). This observation is consistent with bone biopsy data showing impaired osteoblastogenesis and osteoblast apoptosis in the early posttransplant period. Both post-transplant hypophosphatemia and use of glucocorticoids may contribute to osteoblast dysfunction[72]. In another study, glucocorticoids were shown to decrease levels of bone formation markers dose-dependently within 2 days of onset of therapy[11]. Keronen *et al.,* conversely, failed to observe a decline of BsAP in a small cohort (n=27) of *de novo* renal transplant recipients (12 vs 14 µg/L, Tx vs year 2, despite histomorphometric data showing a decrease in bone turnover[73]. Also in the Swiss study[68], BsAP levels, overall remained stable during the first post-transplant year.

Bone resorption markers

Telopeptides of type I collagen

Peptide fragments of collagen are released into the circulation when bone is resorbed. Amino-terminal (NTX-I) and carboxy-terminal (CTX-I) telopeptides type I are non-helical fragments of type I collagen. Both NTX-I and CTX-I assays have been adapted for measurement on automated analyzers. Telopeptides show an important circadian rhythm, which, at least partly, is mediated by food intake[74]. Although the International Osteoporosis Foundation recommended serum CTX-I to be used as the reference marker for bone resorption, its utility is the setting of CKD is highly questionable due to important renal retention. Several studies showed marked decreases of CTX following renal transplantation, but whether this is the consequence of increased renal clearance or decreased bone resorption remains an open question[66;68;75].

Tartrate resistant acid phosphatase isoform 5b

Tartrate resistant acid phosphatase isoform 5b (TRAP-5b) is produced by osteoclasts during bone resorption and may spill over to the circulation. Similar to BsAP and trimeric PINP circulating TRAP-5b levels are not influenced by kidney or liver function and do not show a circadian variation[76]. The immunoassay for the measurement of TRAP-5b has been optimized over years to increase specificity and to decrease analytical variability[77]. Very recently, an automated method for determination of TRAP-5b was developed, which will facilitate its use in clinical practice. The intra-individual coefficient of variation of TRAP-5b in hemodialysis patients is low, translating in a reasonable LSC of 24%[12]. In abovementioned prospective cohort study [69], TRAP-5b showed a significant 38% decrease during the first post-transplant year. In another prospective bone biopsy study in *de novo* renal transplant recipients, changes in osteoclast number were paralleled by changes in circulating TRAP-5b levels[26]. In aggregate, current evidence indicates that renal transplantation causes a (further) decline of bone

turnover[26;73;78;79]. Reviewing the kinetics of the changes, it seems that the decline in bone resorption precedes the decline in bone formation and that the changes are more pronounced in patients with high bone turnover at baseline. Inter-individual variability however, is high and remains to be clarified. Of interest, changes in bone remodeling are inversely associated with areal BMD changes[80]. Thus monitoring bone turnover markers may inform on BMD changes and thus help identifying patients that may benefit most from bone sparing interventions.

CONCLUSIONS AND FUTURE DIRECTIONS

Bone biomarkers may be useful adjunct to bone histomorphometry and bone imaging. At present, none of the bone biomarkers fulfills all of the criteria of an 'ideal' biomarker (***Figure 3***); an ideal biomarker (a) undergoes little degradation, shows minimal variability diurnally and longitudinally, and does neither accumulate with GFR loss, nor is it cleared by dialysis; (b) can be analyzed by a high-throughput methodology that at the same time is accurate, reproducible and affordable; and (c) provides information that adds to, or improves upon existing tests, aids risk assessment or enhances patient management. The deliberate use of bone biomarkers requires knowledge about their strengths and limitations. Analytical limitations, including the cross-reactivity with fragments, often hamper interpretation of bone biomarkers especially in the setting of a rapid changing kidney function, as is the case after successful renal transplantation[20].

Moreover, inter-method variation of most, if not all bone biomarkers is high. This compromises widespread clinical implementation and calls for further standardization and harmonization. External reference material should be considered for quality control and quality assurance.

Current evidence indicates that bone turnover, overall, declines following renal transplantation [26;73;78]. Additional studies are required to identify determinants of bone turnover (changes) in de novo renal transplant recipients and how they associate with (hard) clinical outcomes. Undoubtedly, the panel of bone turnover markers will further expand in the near future to include molecular signatures, e.g. miRNAs. The challenge will be how to best integrate these novel diagnostics in clinical practice in order to decrease the burden of bone disease and related morbidity in renal transplant recipients.

Table 1: Tissue specificity, analytical variability and renal retention of common bone biomarkers

	Origin Tissue specificity	LSC in dialysis	Renal retention
PTH	Parathyroid gland	From 39% (Cavalier, AJKD 2013) to 72% (Gardham, CJASN 2010)	Yes (fragments)
FGF23	Osteocyte/osteoblasts	48% (C-terminal, according to Cavalier et al AJKD 2013), 43.6% intact, healthy subjects according to Jabor et al Ann Clin Biochem 2019)	No
Sclerostin	Osteocyte	Not known	Probable
OPG	Osteoblasts/stromal cells	No data in HD patients.	Yes
(Bs)AP	Osteoblast	From 23% (Cavalier AJKD 2013) to 36% (Sardiwal, KI 2012)	No
PINP	Osteoblast	32% (Cavalier AJKD 2013)	Yes (only monomeric)
Osteocalcin	Osteoblast	No data in HD patients.	Yes
TRAP5B	Osteoclast	24% (Cavalier AJKD 2013)	No
CTX	Osteoclast	No data in HD patients.	Yes

Figure 1: Natural history of bone metabolism markers after transplantation

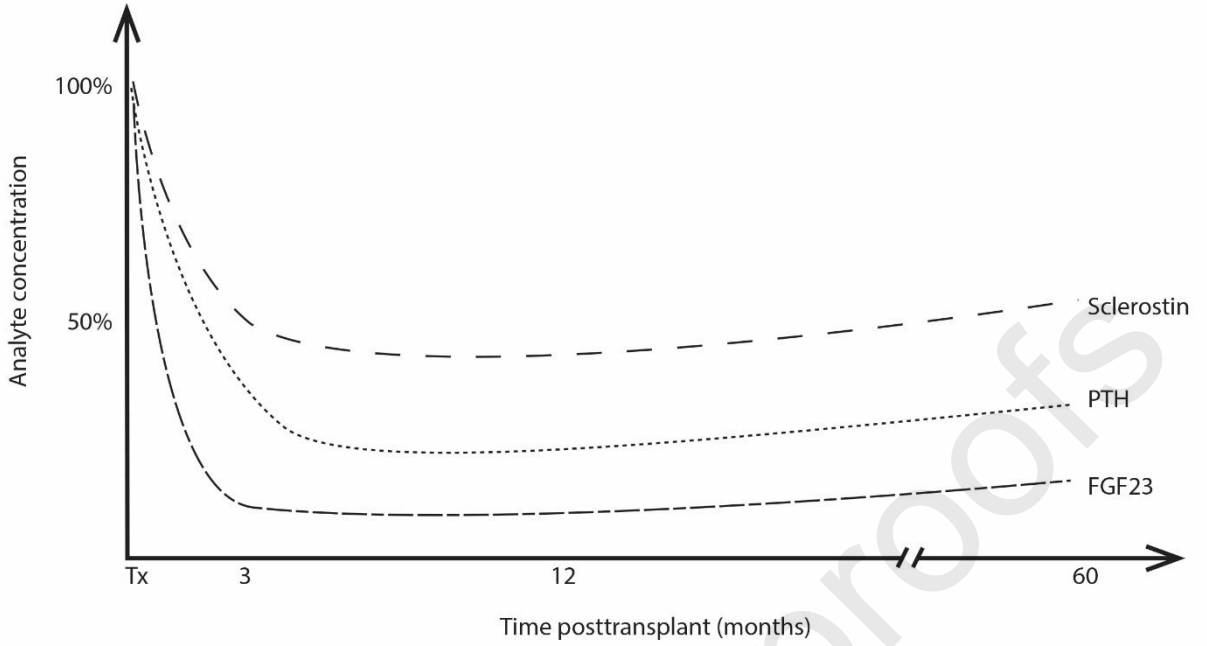


Figure 2: Natural history of bone turnover markers after transplantation

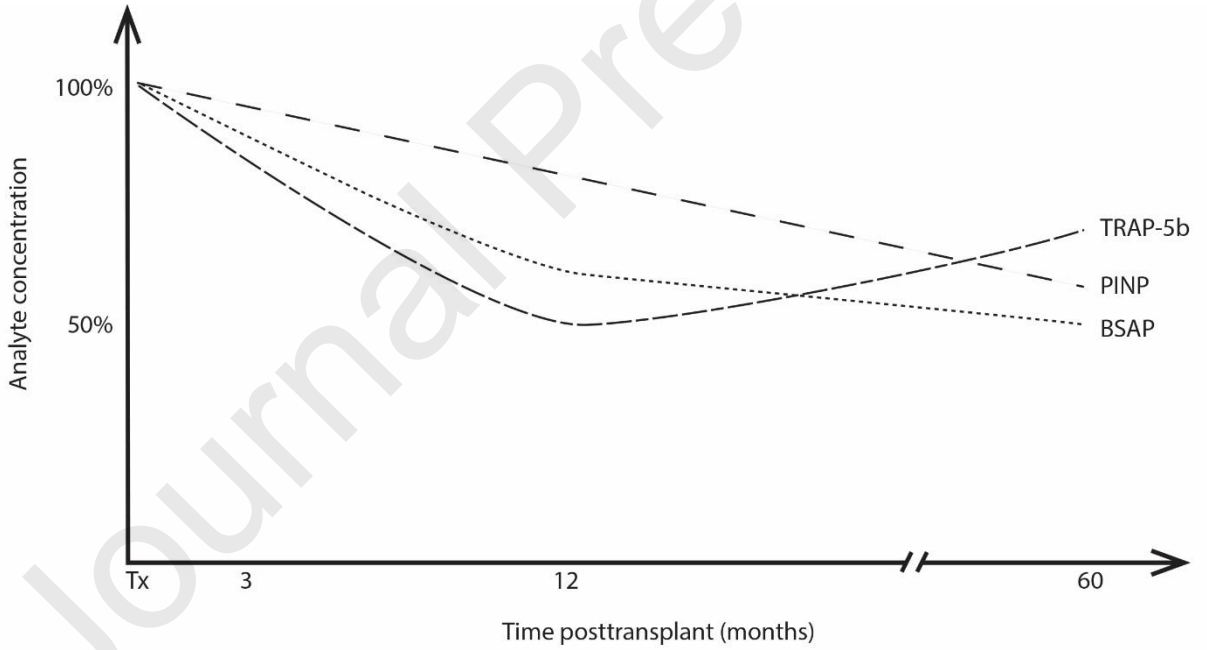
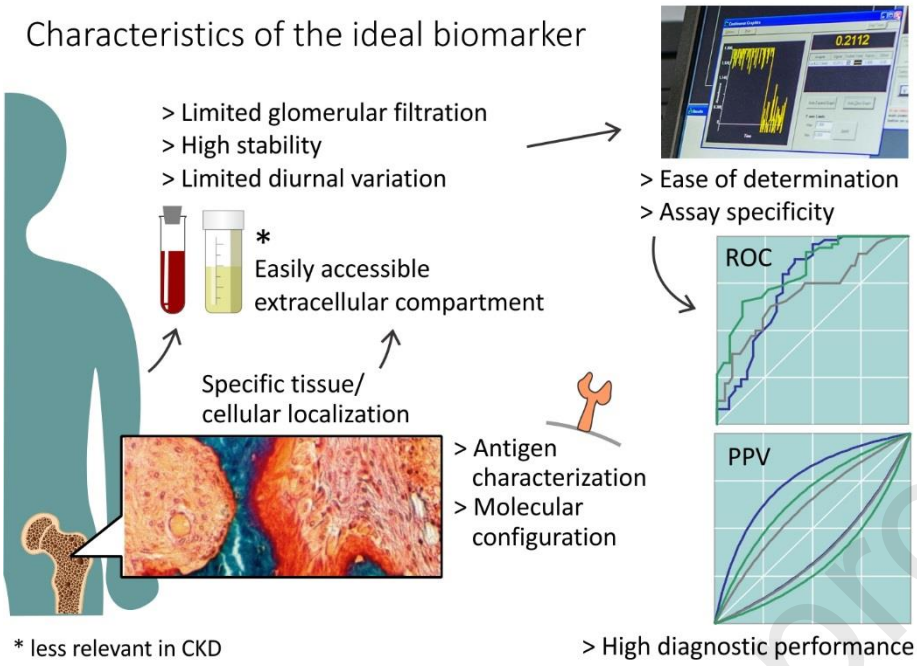


Figure 3: Characteristics of the ideal biomarker



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HIGHLIGHTS

- Renal transplantation has a major impact on both bone metabolism and bone turnover
- Bone biomarkers may be useful in guiding therapy, acknowledging limitations
- Further standardization and harmonization of bone biomarker assays is needed