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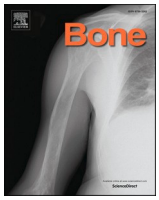

Review Article

Bone histomorphometry for the diagnosis of renal osteodystrophy – a European consensus statement

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ABSTRACT

Histomorphometric analysis of an iliac bone biopsy remains the gold standard for the diagnosis of renal osteodystrophy (ROD), which comprises various histological lesions induced by chronic kidney disease (CKD). ROD belongs to the framework of CKD-associated osteoporosis. The use of bone biopsy in the routine management of CKD-associated osteoporosis has decreased over the past decades for various reasons, including diminishing expertise in performing the procedure, and major variability in processing bone samples and reporting of results. In this context, the European Renal Osteodystrophy group, a part of the CKD-mineral and bone disorder working group of the European Renal Association launched an initiative to evaluate various issues related to bone histomorphometry in the context of ROD. To this effect, 28 experts from 14 European countries engaged in rounds of discussions to reach a consensus related to the bone biopsy procedure, sample handling, and reading and reporting findings. Key conclusions include a recommendation that all practitioners in this field move towards reporting diagnostic findings by the turnover, mineralization, and volume (TMV) classification and that external quality control is prioritized to ensure validity and reproducibility of results. The consensus group recognises that the lack of an accepted normative reference for bone histomorphometry is a barrier towards uniform diagnostic definitions and recommends further collaborative efforts in this area. Until these issues are solved, transparent reporting on the choice of reference and diagnostic definitions applied should be adhered to, both in clinical reports and research settings.

1. Introduction

Osteoporosis in chronic kidney disease (CKD) is responsible for a substantial increase in morbidity and mortality, particularly in the late stages of kidney failure [1–3]. The metabolic bone disease component of CKD-associated osteoporosis, which comprises the histological bone lesions collectively known as renal osteodystrophy (ROD), is associated with progressive disturbances in calcium and phosphate metabolism that form the underlying basis for bone fragility, of which fractures are the major complication [4,5]. In ROD, bone turnover may be increased or decreased, primary mineralization may be impaired, and bone volume may be high, normal or low.

Quantitative bone histomorphometry of an iliac crest biopsy is the gold standard for the precise diagnosis of ROD lesions. Although there have been significant improvements in the predictive value of serum biomarkers of bone remodeling [6], the diagnostic specificity and sensitivity of biomarkers remain below 80–85 % and the combination of non-kidney cleared markers such as TRAP5b and trimeric P1NP may be necessary to optimize prediction [7,8]. Further, it remains difficult to diagnose bone mineralization defects strictly based on serum markers [9,10], especially after a major fracture, due to the modeling-related increase in bone formation during the fracture healing process [11]. A degree of uncertainty thus remains in several patients in predicting the underlying ROD lesion without supportive evidence from the histological findings of a bone biopsy, particularly when a mineralization defect is suspected. This diagnostic uncertainty is particularly relevant when, for instance, fracture preventive treatment is considered.

The performance of bone biopsies has significantly declined in routine management of CKD-associated osteoporosis over the last twenty years [12], despite the recommendations of consensus working groups to consider a bone biopsy if the results are likely to impact therapeutic decisions [13]. Reasons for this downward trend include perceived invasiveness of the sampling, the loss of expertise in the performance of the procedure, the gradual dismantling of laboratories performing bone histomorphometry, and the lack of consensus in the interpretation and reporting of results. Indeed, in a previous work of the European Renal Osteodystrophy (EUROD) initiative, Jørgensen et al. reported substantial disagreements in the categorization of ROD in two cohorts of kidney transplant recipients, depending on the diagnostic criteria applied [14]. This concerning situation led the EUROD initiative to set up a consensus-based approach to harmonize techniques and procedures for processing bone samples, reading sections, and interpreting results, which we report in the present work.

2. Methods

2.1. Participants

To identify participants of this consensus, a systematic review of the literature over the last 10 years was performed with various combinations of the following key words: “humans”, “CKD-MBD”, “bone biopsy”, “bone histomorphometry”, “bone formation rate”, “osteoclastic surface”, “renal osteodystrophy”, “quantitative bone histology”, or “osteomalacia”, “adynamic”, “osteitis fibrosa” in both PubMed and Google Scholar® search engines in order to detect European authors or laboratories using bone histomorphometry for research or clinical purposes. Similar keywords were used in Google® to detect physicians or medical institutions using bone histomorphometry for clinical purposes. When electronic addresses were available, e-mails including an initial survey (Supplementary Table S1) to map bone biopsy practices in Europe were sent. A total of 24 potential laboratories were contacted, and 12 practitioners from Belgium, Denmark, Finland, Italy, France, Netherlands, Portugal, Sweden, Switzerland and the United Kingdom, responded. The results of this first survey were reported via a first videoconference during which the goals and the methodology of the consensus work was planned. The objectives defined were to clearly state the questions related to ROD diagnosis that we needed to answer and to reach a consensus. Following this initial meeting, a second set of e-mails were sent to recruit physicians using bone histomorphometry and/or performing bone biopsies and/or reading bone biopsies for research or clinical care. A final group of 35 people were finally invited to participate in the consensus work, including 2 pathologists, 16 nephrologists, 5 endocrinologists, 3 rheumatologists and 6 from other disciplines (paediatricians, orthopedic surgeons, researchers).

[1] Contributed equally

2.2. Methodology of the consensus

An approach derived from the Delphi method [15] was adopted, in which a panel of experts is involved in several rounds of questions and discussions until a consensus is achieved. To facilitate this process, a second survey was conducted where the consensus group was asked to provide a level of agreement on a 5-point scale (completely disagree, disagree, neutral, agree, firmly agree) on 82 closed statements regarding the bone biopsy procedure, analysis and interpretation. Open-ended comments were also encouraged to refine, rephrase or add questions as necessary.

After this second survey, 28 participants from 14 European countries were split in four working groups which met 3 to 4 times in order to elaborate on, and refine, the consensus statements. *Group 1*: Methodology of the biopsy procedure and sample processing was led by S Mazzaferro and included J Cannata-Andia, A Ferreira, P D'Haese, P Chavassieux, J Bacchetta, H Kröger; *Group 2*: Trabecular lesions was led by MH Lafage-Proust, and included EF Eriksen, AC Ferreira, D Hansen, M Haarhaus, R de Jongh, P Chavassieux, and M Cohen-Solal; *Group 3*: Cortical lesions was led by N Bravenboer and included J Cannata-Andia, MH Lafage-Proust, A Trombetti, M Gerbaix, MJ Begin, A Fahrleitner-Pammer, M Cohen-Solal, H Kröger, and XY Tong and *Group 4*: Reference values was led by P Evenepoel, and included P D'Haese, G Behets, A Ferreira, A D Lalayiannis, S Salam, J Bacchetta, and A Fahrleitner-Pammer. Issues related to ROD in children were specifically addressed in each group thanks to the input of J Bacchetta, A Fahrleitner-Pammer, A Lalayiannis and R Shroff. HS Jørgensen participated in all the group meetings.

It was agreed that at least a 70 % level of consensus was required for each statement, failing which the recommendation would be adapted after further discussion and reviewed again. A consensus was reached after one to several rounds as the experts settled on a mutual agreement. In addition to several meetings within each workgroup, two plenary meetings were held gathering the whole panel for the final consensus.

3. Results

3.1. Biopsy procedure

3.1.1. Clinical practice points

- Iliac crest bone biopsy is a safe procedure that can be performed in an outpatient setting, with light sedation and local anesthesia.
- Double tetracycline labeling is recommended before the bone biopsy procedure.
- Both manual trephines and electric drill-trephines are suitable, depending on the experience of the physician performing the bone biopsy. Manual trephines are generally preferred.
- Both horizontal and vertical approaches are valid for iliac crest bone biopsy retrieval. The horizontal approach is generally preferred.
- While a regular-size trephine (7.5 mm) is considered optimal, a $\geq$3.5 mm diameter biopsy core is sufficient for a histomorphometric analysis of trabecular bone.

3.1.2. Background and rationale

The anterior iliac crest is the preferred puncture site because it is easily accessible, has been proven safe and is associated with minimal morbidity [16]. Further, all published reference values pertain to the iliac bone. Bone tissue may also be retrieved from other sites during orthopedic surgery, e.g. for fracture repair, taking care to avoid the immediate fracture site, and being aware that different skeletal sites are not necessarily comparable with each other [17–19]. Bone biopsies at the anterior iliac crest can be obtained either in a vertical or horizontal direction (Fig. 1) [16]. The vertical approach allows sampling of a greater volume of trabecular bone. The use of a horizontal direction provides information on the outer and inner cortices, but sample size is limited by the thickness of the iliac bone. The horizontal transiliac technique is currently the most widely applied approach, with a 5 cm isolateral triangular area (Bordier's triangle) located behind the anterior superior iliac spine and below the iliac crest border being the most suitable biopsy site [20]. This site shows the closest relation to the lumbar bone mass [21]. Operator skills and the use of appropriate instruments determine the quality of bone sampling. Using disposable trephines obviates the need for sterilisation and sharpening of the teeth of the trephine in between bone biopsy procedures.

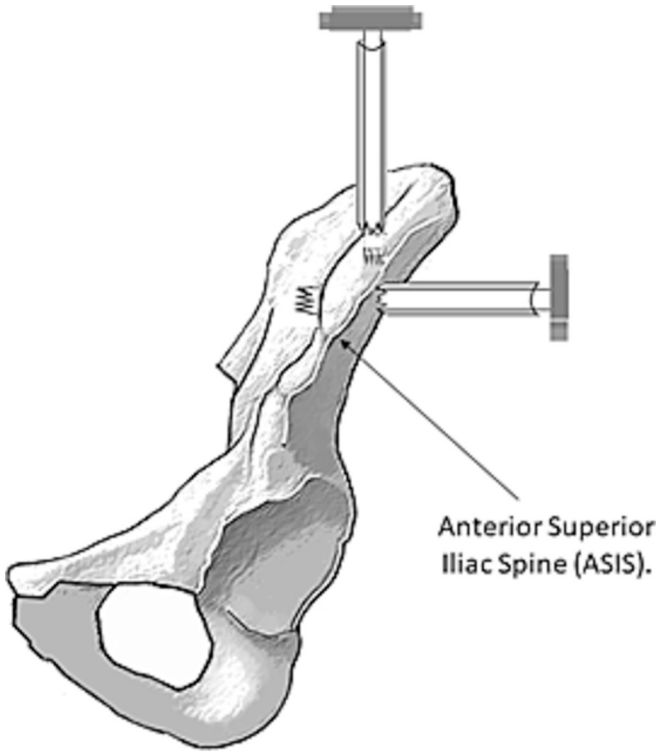

Fig. 1. Iliac crest bone biopsy for bone histomophometry by vertical versus horizontal approach.

While previously bone samples of 7.0–8.0 mm in diameter and 1.5–2.0 cm in length (for example from Landager, Paris, France) were considered appropriate, currently smaller (inner diameter 4–5 mm) Jamshidi-like trephines (for example from Biopsybell Medical, Mirandola, Italy) are gaining popularity [22]. A major asset of using smaller needles is decreased procedural complexity, e.g. no need to suture the skin incision and no need for blunt dissection through muscle and fascia. It may also be anticipated that the complication rate, already low with the larger needles (<1 %) [16,20], is further reduced with the smaller trephines [8]. The most common complication is pain, which can be countered by local anesthesia, particularly at the periosteum. Skin infections at the biopsy site can be avoided by adhering to aseptic techniques [16]. The potentially serious complication of bleeding and haematoma can be countered by applying a compressive dressing and positioning the patient on their side, so that their bodyweight presses down on the biopsy site, for 1 to 2 h after the procedure. The use of small needles obviates the need to interrupt antiplatelet agents or to modify the anticoagulation regimen including that of the dialysis session preceding and following the bone biopsy procedure (unless scheduled on the same day). With the use of smaller needles, the bone biopsy procedure is almost indistinguishable from the marrow biopsy performed in the work-up of a haematological disorder.

As a trade-off, bone cores harvested with the Jamshidi-like needles are more prone to crush artifacts. However, crush artifacts can be largely avoided by gentle pushing and rotating the needle, rather than applying fierce physical force.

Drill-assisted bone biopsies are at present not yet widely implemented in Europe (<10 %) but there are ongoing studies focusing on this approach [12,16,23]. Bone biopsies can also be performed under live-pulse fluoroscopy or incremental computed tomography guidance, allowing a precise localisation of the biopsy site and standardization of the procedure [24]. However, benefits such as secured specimen quality should be balanced against increased procedural complexity.

The transiliac biopsy can be repeated, preferably on the opposite side. A time interval of one year is advocated between two bone biopsies extracted at the same iliac side to avoid bias in interpretation of findings due to residual histologic changes resulting from the previous procedure and tetracycline labeling. The misconception held by patients and physicians that the bone biopsy procedure is highly invasive and painful is a major obstacle to its performance [12,23]. A bone biopsy may be performed in the setting of outpatient minor surgery facilities. The periosteum has a plentiful supply of sensory nerves and is exceedingly sensitive to pain, contrary to core bone. Pain can thus be largely avoided by adequate local anesthesia of the skin, subcutaneous tissue, and most importantly of the external (+/− internal) periosteum. If oxygen saturation and blood pressure can be monitored, light sedation (for example with a combination of benzodiazepine and opioid analgesic), can be considered to further enhance the procedural comfort of the patient.

To obtain information about dynamic parameters such as bone formation rate and mineralization state, double labeling of the bone surface needs to be performed using one of the fluorochrome tetracycline compounds such as demeclocycline, doxycycline or tetracycline hydrochloride given at a set schedule prior to the bone biopsy procedure, as these compounds are incorporated into newly mineralized bone [16,23]. The usual schedule consists of two dosing periods, 2–3 days on, 10 days off, and a further 2–3 days on (e.g. 500 mg tetracycline or 100 mg doxycycline twice daily), after which the biopsy is performed within the next 4 to 14 days. The labeling of bone can be shortened to a 1-day-on, 4–6-days-off, and 1-day-on schedule (e.g. 1000 mg tetracycline or 200 mg doxacycline). Although patients generally tolerate oral tetracyclines well, some side effects, such as gastrointestinal discomfort, allergic reaction or photosensitivity might be observed. Non-adherence and decreased bioavailability (related to ingestion with meals, particularly dairy products, antacids, phosphate binders) may explain the lack of fluorescent labels or the presence of only one label on bone slides. Patients ingesting phosphate binding agents should be advised to discontinue their phosphate binders during the days tetracyclines are taken. In some countries, demeclocycline or tetracycline are (increasingly) hard to obtain. In cases of longitudinal evaluation (i.e. treatment efficacy), two different tetracyclines that fluoresce in their distinct colors can be administered at the desired time points, allowing for a single biopsy to be used to assess dynamic indices of bone [25]. This “quadruple” bone biopsy labeling could represent a solution to the barrier of repeat bone biopsies, particularly in research, but potentially also in clinic when monitoring therapeutic response.

3.2. Sample handling and staining

3.2.1. Clinical practice points

- A bone biopsy can be stored in 70 % ethanol for up to 4 weeks before processing.
- UV microscopy of unstained sections is recommended
- A standard of 3–5 μm thick stained sections for light microscopy and 7–10 μm thick unstained sections for UV microscopy should be used.
- Goldner trichrome staining is recommended as a standard staining for bone histomorphometry.
- Toluidine blue, Pearl’s prussian blue and TRAP stainings are not mandatory, but provide valuable additional information.
- Auritricarboxilic acid or solochrome azurine staining for aluminium is recommended for laboratories receiving bone samples from regions of the world where the risk of aluminium exposure remains high.

3.2.2. Background and rationale

The histologic evaluation of the bone biopsy sample should include a standard histologic identification of the bone surfaces and cells (for which small samples are sufficient), in addition to the two-dimensional histomorphometric quantification of bone elements and observation under a UV light microscope (for which tetracycline administration is necessary). For research purposes, immunohistochemistry and in situ hybridization are possible additional procedures. These histologic analyses require specific processing which may differ between laboratories. As a first step, the fresh bone samples are immediately immersed in a fixative, which could be ethanol or formaldehyde. Ethanol (70 %), the most widely used, dehydrates the tissue, and penetrates deeply into the fatty marrow up to the bone surfaces without inducing decalcification since it does not contain hydrogen ions. Alternatively, formaldehyde fixatives, which may produce some decalcification, preserve macromolecular epitopes and enzymes better than ethanol, thus improving the quality of the sample for immunohistochemistry or in situ hybridization if desired for research purposes. Therefore, if the bone sample is large enough, it can be longitudinally halved and fixed with both methods [26].

Following fixation, which with ethanol 70 % allows up to 4 weeks storage when kept refrigerated at 4 °C, the next processing step is embedding. Evaluation of bone structures and cells requires that thin slices of integral and undecalcified tissue are obtained. A polymerized resin like methylmethacrylate is the most frequently used because of its rapid penetration into the tissue, a low rate of artifacts (e.g. bubbles), a final hardness similar to bone (which allows homogeneous cutting), and the availability of a solvent for eventual dissolution [23,27]. The process is applied to undecalcified bone samples and allows good quality histology, mineralization and enzyme staining evaluation. Alternatives include glycol methacrylate, which is not ideal for mineralized tissues but useful for thin sections of soft tissues and for preserving enzyme activities, and paraffin embedding which is suitable for soft tissues and decalcified bone samples to be stained with hematoxylin and eosin. After embedding, thin slices of different thicknesses are obtained according to the histologic procedure, e.g. 3–5 μm for light microscopy and 7–10 μm for UV microscopy. In addition to a good-quality microtome, a skilled technician is recommended for this step [26].

Several staining techniques are available for bone tissue (Table 1), but to discriminate calcified bone from uncalcified matrix, the most frequently used are the modified Masson-Goldner trichrome stain [28], the solochrome cyanine stain [29], and the von Kossa stain [30]. Of note, the often-used Goldner stain may overestimate osteoid. To specifically identify cells, proteins or minerals, special stains are necessary. For example, osteoclasts are identified by histochemical staining of tartrate resistant acid phosphatase [31] and osteoblasts by histochemical staining of alkaline phosphatase [32]. Among the several minerals that can accumulate in bone, those of potential interest for the nephrologist are aluminium and iron. The Aluminon staining method has been used in the past to identify accumulation of aluminium and resulting bone disease in CKD patients [33], while the Pearl’s prussian blue technique can be employed to reveal iron [34]. Although not routinely performed, these stainings could be helpful to check for possible bone disease in specific clinical settings, like prolonged aluminium-based phosphate binder therapy or chronic high dose intravenous iron administration. During discussions in the expert panel, it was highlighted that although aluminium-induced bone disease is becoming rare, it is still an issue in some parts of the world and thus a relevant staining particularly for centers receiving referral biopsies from such regions.

Despite efforts to simplify the biopsy procedure [35], the panelists felt that tetracycline labeling was necessary for the diagnosis of ROD, to measure histodynamic parameters, and thus to be recommended

Table 1
List of the many possible stains available for bone tissue evaluation.

Method	Purpose
Alcian blue	Revealing acid mucins in tissues
Alizarin red/Alcian blue	Distinguishing mineralized vs. unmineralized bone in whole tissues
Alkaline phosphatase	Histochemical localization of the enzyme in osteoblasts, diminished in formalin fixed tissues
EVG (Elastin/Van Gieson)	Revealing elastic fibers
Goldner's Trichrome	Distinguishing mineralized and non-mineralized areas
Gomori's trichrome	Visualizing collagen
Hematoxylin/Eosin (H&E)	Cellular details best on paraffin sections
Fluorescent bone labeling In vivo	Calcein, xylenol orange and alizarin red labeling in vivo for bone mineralization front
Lee's Methylene Blue	H&E "lookalike" stain used for glycolmethacrylate sections
Oil Red O (frozen sections only)	Demonstrating lipid droplets in tissues sectioned frozen and not processed through any solvents
Osmium Staining	Quantifying infused lipids using μCT. Requires decalcification
Prussian Blue	Revealing iron
Safranin O	Staining proteoglycans
Tartrate Resistant Acid Phosphatase (TRAP)	Revealing the enzyme specific of osteoclasts
Toluidine Blue O	Demonstrating mineralized bone, osteoid seams, osteoblasts, osteoclasts, and cells of the bone marrow
Von Kossa	Demonstrating mineralization in bone, tissues and cell cultures

Modified from https://medicine.yale.edu/ortho/research/histo/services/special/.

whenever feasible [36]. This entails the quantification of localization and extent of fluorescence under UV light microscope after administering two courses of tetracycline to the patient prior to the bone biopsy. For the clinician and the pathologist, it may be useful to remember that tetracycline hydrochloride and doxycycline has a yellow fluorescent label, while demeclocycline hydrochloride has a yellow-orange label, which allows to better distinguish between the two-time elapsed administrations of the drugs and to better evaluate mineralization rate.

The information gained from several different staining procedures that are favored by different centers is similar. Therefore, while ideally a single protocol could be adopted worldwide for ROD, it is mainly crucial that each laboratory uphold a specific protocol to ensure quality and comparability of the results.

3.3. Evaluation of trabecular bone

3.3.1. Clinical practice points

- 2D reporting of bone histomorphometric parameters is recommended as standard
- The following static parameters are recommended for diagnosing ROD: Osteoid width or thickness (O.Wi or O.Th, μm), Osteoid perimeter or surface (O.Pm/B.Pm or OS/BS, %), Osteoid area or volume (O.Ar/B.Ar or OV/BV, %), and Osteoclast perimeter or surface (Oc.Pm/B.Pm or Oc/BS, %).
- Other measurements, such as Osteoblast perimeter or surface (Ob.Pm/B.Pm or Ob/BS, %) and Eroded perimeter or surface (E.Pm/B.Pm or ES/BS, %) provide additional information about bone turnover.
- The following dynamic parameters are recommended for diagnosing ROD: Mineral apposition rate (MAR, μm/d), Tetracycline labelled mineralizing area or surface (Md.Ar/B.Ar or MS/BS, %), and Bone formation rate (BFR). Bone formation rate should be expressed as BFR per bone perimeter or surface, BFR/BPm (BFR/BS, $\mu m^3/\mu m^2$ per day or yr)
- Calculation of adjusted apposition rate (Aj.AR, μm/d) and Mineralization lag time (Mlt, d) are recommended.
- Bone turnover is primarily evaluated by bone formation rate (BFR/BS, $\mu m^3/\mu m^2$ per day or yr).
- Bone mineralization is evaluated by MAR or Mlt, in combination with osteoid parameters.
- Bone volume is evaluated by bone volume/tissue volume (B.Ar/T.Ar or BV/TV, %).
- In the absence of successful tetracycline labeling, bone turnover is evaluated by static parameters of remodeling, and mineralization is evaluated by osteoid amounts.
- Other assessments such as woven bone, marrow fibrosis, and metal stainings should be included in the qualitative assessment, but not necessarily as quantitative measurements.
- Expression of parameters should be standardized across laboratories.

3.3.2. Background and rationale

Quantitative analysis of histomorphometric parameters should be performed according to the standardized nomenclature of the American Society of Bone and Mineral Research (ASBMR) [37]. Primary measurement of histological sections result in two-dimensional (2D) terms (length, area, distance, number). Adapting these to the three-dimensional (3D) character of bone requires extrapolation based on assumptions, although methods for 3D histomorphometry have been suggested and may become more accessible, using artificial intelligence and modern imaging techniques [38,39]. While the ASBMR nomenclature accepts both 2D and 3D terms, most of the expert panel recommended reporting directly measured 2D terms. In agreement with the nomenclature, the panel recommended strict consistency regarding dimensionality with the only exception being the 2D term number, which should also be used when reporting 3D terms.

Commonly, cancellous bone is used for quantitative measures of bone histomorphometric parameters. In ROD, bone turnover and mineralization are important aspects that should be described by the histomorphometric parameters determined in a bone biopsy. The third aspect suggested by KDIGO for the description of ROD is bone volume [13], which contrasts the aforementioned parameters by its 3D character that cannot be directly measured by histomorphometry. It is estimated, based on determination of bone area relative to tissue area (B.Ar/T.Ar, or bone volume by tissue volume, BV/TV) and represents the proportion of the marrow cavity which is occupied by trabecular bone.

Bone volume is only one of several quantifiable structural aspects of bone, and others, e.g. trabecular number, thickness, spacing, and connectivity may also have clinical relevance [40,41].

Mineralization and turnover are best determined after labelling of bone with a tetracycline before retrieving the biopsy. Among the expert panel, there was strong agreement on parameters that should be included in the evaluation (Table 2), with minor disagreement regarding the inclusion of osteoblast surface (Ob.S/BS or Ob.Pm/B.Pm) and eroded surface (ES/BS or E.Pm/B.Pm). Bone turnover should primarily be determined by the bone formation rate (BFR) per bone surface, most often expressed as $\mu m^3/\mu m^2$ per day or yr, although other variations (such as %/year) are also used (see formula Table 2) [37]. Another parameter that can be used is activation frequency (Ac.f) [42]. Estimation of bone turnover in unlabelled biopsies is challenging, as demonstrated by low agreement of static parameters of turnover (i.e. osteoblast surface (Ob.S/BS), osteoclast surface (Oc.S/BS), and eroded surface (ES/BS) with BFR/BS) [43], though diagnostic performance of combinations of static parameters may be acceptable [44]. However, the panel agreed that a rough estimate can be performed, excluding very high or low bone turnover, which may be of benefit in the clinical setting. Assessment of woven bone and marrow fibrosis is strongly recommended as part of the qualitative evaluation of bone turnover. Mineralization of newly formed bone matrix is driven by substrate availability and the balance of mineralization promotors and inhibitors [45], but in contrast to bone volume and turnover none of the clinically available non-invasive indicators of bone mineralization have demonstrated sufficient accuracy for clinical applicability. There was strong agreement among panelists that bone mineralization is evaluated

by static osteoid parameters (osteoid thickness (O.Th or O.Wi), osteoid surface (OS/BS or O.Pm/B.Pm), and osteoid volume (OV/BV or O.Ar/B.Ar)) in combination with the dynamic parameters mineralization lag time (Mlt) or mineral apposition rate (MAR). A narrow majority agreed that in absence of dynamic parameters, either due to lack of labeling, or very low bone turnover or severe mineralization defect leading to absence of labels in the biopsy, mineralization can be estimated by static osteoid parameters alone. However, it should be recognized that increased osteoid thickness can be observed both in full-blown osteomalacia and in high bone turnover with high osteoblastic appositional rates with normal or even accelerated mineralization – the discrimination between these two states being challenging without labeling. Histomorphometric findings characteristic of ROD are shown in Fig. 2.

3.4. Evaluation of cortical bone

3.4.1. Clinical practice points

- For an analysis of cortical bone, a full-size trephine (7.5 mm) is optimal, and the horizontal approach is preferred, as it ensures two cortices
- A qualitative evaluation of the cortex is recommended.
- This assessment should include cortical thickness, cortical porosity and subperiosteal resorption
- Qualitative evaluation of trabecularization of cortical bone provides additional information.

3.4.2. Research recommendations

- A reference set for cortical bone should be established to enable future quantitative assessment.
- An imaging library of qualitative features of cortical bone, normal and pathological, should be established.

3.4.3. Background and rationale

Cortical bone is regarded as a significant component in biopsy assessment due to its potential role in predicting fracture risk [46]. This may be even more relevant in ROD due to the effects of hyperparathyroidism specifically in cortical bone [47–49]. The majority of laboratories represented in the consensus reported routinely evaluating cortical bone with the agreement that this assessment is important in the context of ROD, due to the specific deleterious effects of hyperparathyroidism on cortical bone. In most cases, this evaluation is performed qualitatively – typically through visual inspection with or without measurements of key variables such as cortical thickness and porosity. For quantitative reporting, several methodological issues remain to be solved, such as a uniform delineation of the trabecular/cortical boundary. Further, there is a lack of normative reference values for cortical bone. While microcomputed tomography (micro-CT; Fig. 3) is considered a useful adjuvant to bone histomorphometry, the consensus agreed that this was mainly relevant for research purposes at present and not recommend as part of routine clinical practice.

Regarding relevant parameters, most centers conduct a qualitative evaluation of cortical bone, encompassing assessments of cortical thickness, cortical porosity and subperiosteal resorption, with cortical thickness being the only quantitative parameter. Subperiosteal resorption is a common feature in adults with hyperparathyroidism-related high turnover. None of the centers taking part in the consensus incorporated quantitative cortical histomorphometry into clinical cases, neither bone structural nor remodeling indices. However, for research purposes, a more comprehensive analysis of the cortex is often undertaken, involving quantitative assessments of cortical area, thickness, porosity, and osteon parameters such as numbers and density, typically measured via micro-CT [50,51].

Several challenges arise in the quantitative analysis of the cortex. For instance, in CKD-associated osteoporosis, very thin cortices are common [52], rendering the measurement of cortical porosity unreliable due to the overlap of a significant proportion of pores with the endosteal surface. Another challenge stems from the trabecularization process of cortical bone [53,54], particularly prevalent in the context of kidney failure [47], necessitating a form of reconstruction of the microscopic image. Consequently, only an indication of this process's occurrence can be provided in the report. Additionally, defining the endocortical surface presents difficulties in such cases, wherein qualitative reporting may offer more meaningful insights compared to quantitative measurements. Finally, the lack of established histomorphometric reference values challenges quantitative analysis of the cortex.

In contributing to the broader discourse on biopsy methodology, the cortical working group deliberated on the merits of horizontal versus vertical bone biopsies and the trend towards smaller needles [12]. Smaller needles restrict the number of measurable fields, while a horizontal biopsy enables measurement of both inner and outer cortices. Moreover, given the possibility of biopsy damage or crushing, which may leave only a portion of the cortex analyzable, the preferred method for cortical assessment is a transiliac bone biopsy using a Bordier trephine drill. In general, there was a consensus that quantitative

Table 2
Recommended parameters for the diagnosis of renal osteodystrophy according to the TMV classification.

Bone	Recommended	Additional, optional
T Turnover	Osteoid perimeter by bone perimeter *(OPm/BPm or OS/BS, %)* Osteoid area by bone area *(OAr/BAr or OV/BV, %)* Osteoclast perimeter by bone perimeter *(OcPm/BPm or OcS/BS, %)* Mineral Apposition Rate *(MAR, μm/d)* Mineralizing surface / bone surface (double labelled Pm + 1/2 single labelled Pm)/ bone Pm × 100) *(MS/BS, %)* Bone Formation Rate / Bone Surface/ year (MAR × (MS/BS) × 365) *(BFR/Tar, BFR/BV, %/y or BFR/BS, $\mu m^3/\mu m^2/y$)*	Osteoblast perimeter by bone perimeter *(ObPm/BPm, Ob/BS, %)* Eroded perimeter by bone perimeter (EPm/BPm, ES/BS, %)
	Osteoid Thickness *(O.Th, μm)*	Adjusted Apposition Rate [MAR × (MS/OS) × 10^{-1}] *(Aj AR)*
M Mineralization	Mineral Apposition Rate *(MAR, μm/d)* Mineralizing surface / bone surface (double labelled Pm + 1/2 single labelled Pm)/ bone Pm × 100) *(MS/BS, %)*	Osteoid maturation time (O.Th / MAR) *(Omt, days)* Mineralization lag time (O.Th / Aj.AR) *(Mlt, days)*
V Volume	• **Trabecular** Bone area by tissue area or Bone volume per Tissue Volume *(B Ar/T Ar, BV/TV, %)* Trabecular thickness *(TbTh, μm)* Trabecular separation *(Tb Sp, μm)* Trabecular number *(TbN, /mm)*	
	• **Cortical** Cortical Thickness *(Ct Th, μm)* Cortical Porosity *(Ct Po, %)*	Trabecularization Subperiosteal resorption

Abbreviations in italic according to international nomenclature, formula is provided when parameter is calculated, Pm: perimeter. Note that MAR and MS/BS are used to evaluate both turnover and mineralization.

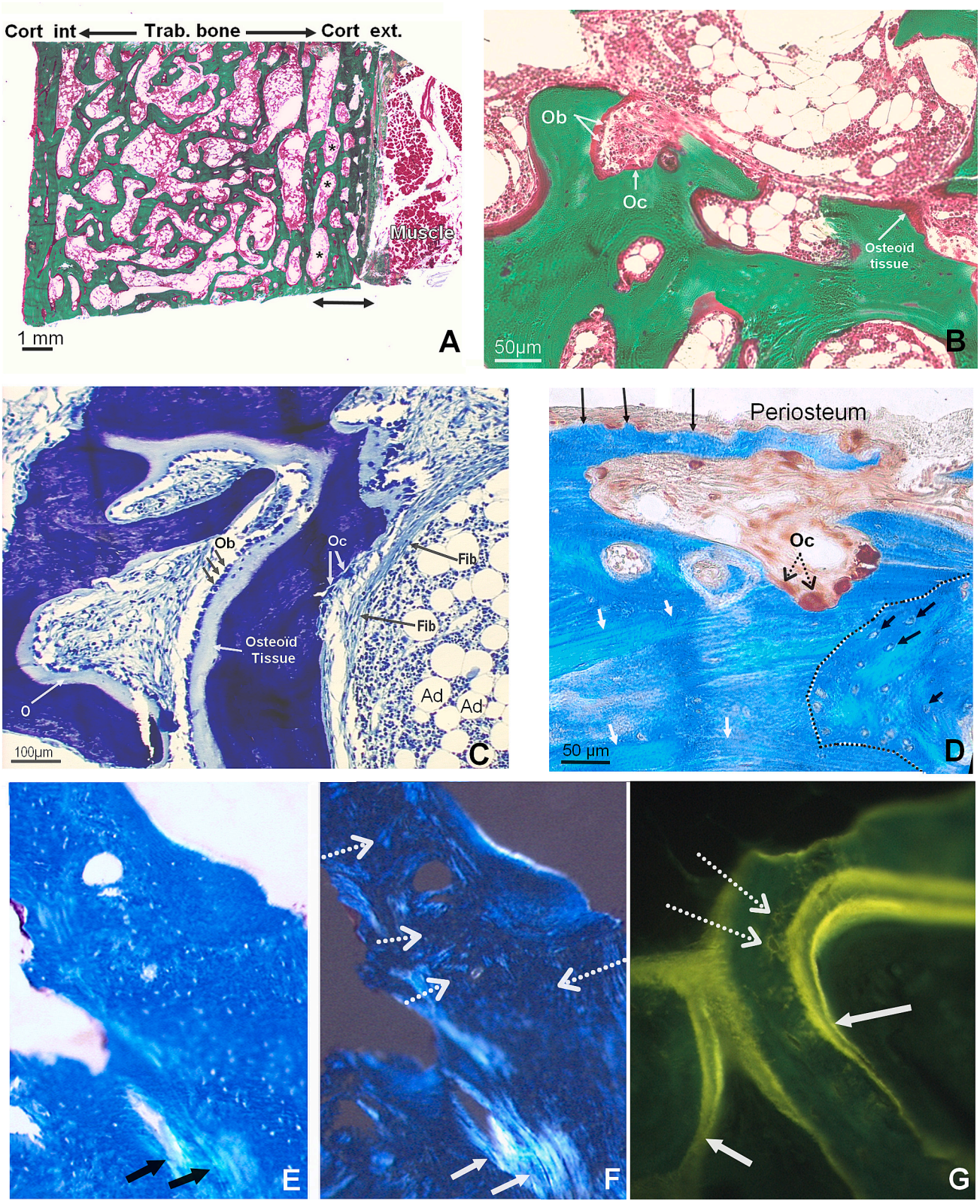

Fig. 2. Histomorphometric findings in renal osteodystrophy. A) Section of an iliac crest bone biopsy (×1.5)/Goldner staining with an 8 mm internal diameter trephine. Note the trabecularisation of the external cortex (*); the previous limits of the cortex is illustrated by ←→. B–G): High turnover related to secondary hyperparathyroidism. B: Goldner-stained section. (×20). Extended osteoid (pink) surfaces and high numbers of osteoclasts (Oc) and osteoblasts (Ob). C) High turnover related to secondary hyperparathyroidism. (×20) Toluidine blue stained section. (×20). Extended osteoid (light blue) surfaces and high numbers of osteoclasts (Oc) and osteoblasts (ob). Note the peri trabecular fibrosis (Fib), close to the resorption lacunae. D) TRAP histo-enzymology staining of osteoclasts with Aniline Blue counterstaining combined with polarized light. Subperiosteal and intracortical resorption. The area delineated with a black dotted line corresponds to woven bone (black arrows), with wide and numerous osteocyte lacunae, while the other mineralized bone areas are lamellar (white arrows). E–F: Same area under regular light (E) and polarized light (F), 10×, Bars 150 µm; plain arrows: lamellar bone, dotted arrows: woven bone. G: Tetracycline labeling under UV light:

Extended double labelings (plains arrows), Note the osteocyte lacunae and canaliculi closest to the bone surface enlighten by tetracycline. H–J) Low turnover. H: Goldner staining (thin osteoid seam, arrow, lack of osteoblasts), I: TRAP staining/aniline blue counterstaining (note the lack of osteoclasts). J: Tetracycline labeling under UV light: few single labels (arrow). K–L) Defect mineralization (Full blown osteomalacia) Goldner stained section. Note the extremely wide osteoid seams (red) (×20). H) Double tetracycline labeling associated high bone turnover and mineralization defect combining-surfaces where double labeling are clearly visible (dotted ar rows) and other surfaces exhibit blur labelled surfaces (solid arrows) where mineralization is impaired. Note that in this case cement lines might be highlighted by tetracycline labeling as well (thick arrows). (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

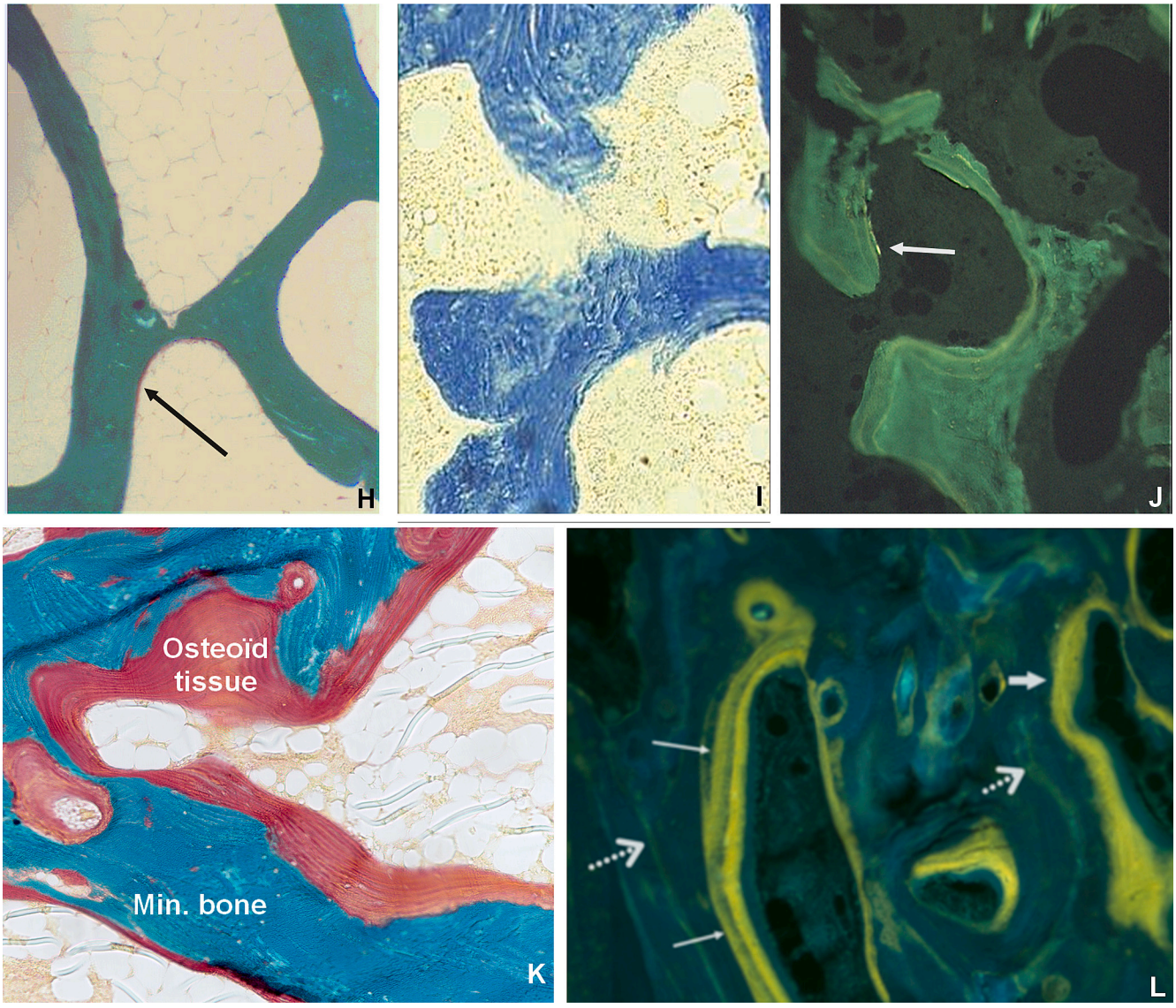

Fig. 2. (*continued*).

measurements of cortical bone should not be mandatory in the clinical report at this moment, as there is still doubt about threshold settings, normative values, and clinical utility.

3.5. Reference values

3.5.1. Clinical practice points

- Reference values should be separate for age categories.
- Reference values should be separate for men and women.
- Reference values should be separate for children and adolescents <25 years
- Histomorphometric results should be reported as numerical values and, once normative reference values have been established, as Z-scores

3.5.2. Research recommendations

- A normative reference set for all trabecular parameters, static and dynamic, should be expanded and harmonized.
- A normative reference set for cortical parameters should be established
- More research is needed to establish whether there are relevant differences in reference values based on ethnic groups, and if so, suitable reference values should be established.

3.5.3. Background and rationale

The lack of consensus on the diagnostic cut-offs likely contributes to the substantial variations reported in the spectrum of ROD. In the first and second surveys which preceded the consensus meetings, 30 % of the participants declared using in-house reference values for one to several parameters. This issue was recently emphasized by Jørgensen et al [14]

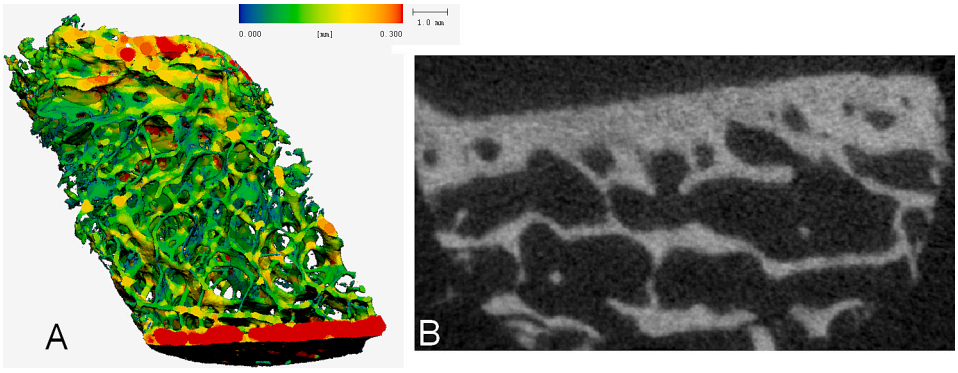

Fig. 3. High-resolution image findings in renal osteodystrophy. A) μCT 3D rendering of bone biopsy (μCT Scanco 40, 50 μm spatial resolution), Color scale related to thickness of structures (the redder the thicker). B) 2D section of external cortex. Note the thin cortex and open pores. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

who applied four different sets of references values (Recker et al. [55,56], Malluche et al. [57,58] and Vedi et al. [59]) on two cohorts of bone biopsies from kidney transplant recipients. The percentage of patients with high bone turnover varied from 24 to 73 %. This discrepancy was even greater for low turnover, the percentage of which varied from 10 % to 65 %. Similar differences were observed for the mineralization parameters depending on the cut-offs of osteoid amounts or Mlt chosen [14].

The working group gathered a considerable list of literature on reference values totaling ~1250 subjects, including cohorts of adults [56,60–65] and children [66–70]. Few studies reported on cortical parameters [36,70]. These studies demonstrate variation in histomorphometric parameters based on sex [56,63,65], age [63,64,71,72] or menopausal status [65,72,73], and ethnicity [36,72,74] indicating that normative reference values may need to be separate for these categories. These conclusions are also supported by data from a recent meta-analysis of histomorphometric data in healthy individuals. This study compiled data from 37 (out of 447 evaluated) manuscripts published until 2020 in an attempt to provide more comprehensive guidance for a normal reference range [75]. All regions of the world were represented, and categorizations were made based on sex, age, and ethnicity (white vs black). Pooled mean estimates with 95 % confidence intervals were established for 16 histomorphometric parameters. Sex and ethnicity had a significant effect on several histomorphometry parameters, and an age-by-sex trend was observed for most parameters as well. Although the format of these datasets does not allow for true reference values/ranges for diagnostic use, the authors provided suggestions for reference standards that may be used to evaluate individual bone biopsy results, by comparing the patients' histomorphometric values with a suitable reference group regarding age, and sex, and ethnicity [75].

The working group concluded that further work would be needed to define reference values for diagnostic use. Such an initiative could include the compilation of data from bone biopsies of non-CKD patients (convenience sample) or establishment of a contemporary reference dataset through common research protocols between collaborating laboratories. An example of an approved and ongoing research protocol is procurement of bone biopsies from living kidney transplant donors taken at the time of kidney transplantation – the planned and scheduled nature of the procedure allows for prior tetracycline labelling, and the procedure can be performed under general anesthesia, minimizing discomfort for the donor. A separate initiative could involve retrieving and reanalyzing individual-level data from laboratories that previously published reference ranges, in order to generate more robust and representative values. This would require a global effort. In the meantime, we may refer to the recent meta-analysis of bone biopsy data in healthy individuals [75], which provides a useful overview of reference values available. At the present moment, meticulous reporting of the reference values utilized should be adopted, e.g. by including details of the reference material used both on clinical reports and in research. The expert panel recognized that the lack of a normative reference standard constitutes a barrier for a consensus on the diagnostic definitions of ROD lesions.

3.6. *Diagnosis of renal osteodystrophy*

3.6.1. *Clinical practice points*

- The TMV classification should be given when reporting results of the bone histomorphometric analysis and should replace the previous definitions of ROD lesions (osteitis fibrosa, osteomalacia, adynamic bone, mixed uremic lesions).

3.6.2. *Research recommendations*

- Further efforts are needed to define common diagnostic definitions to ensure a uniform classification of ROD.

3.6.3. *Background and rationale*

The recommendation to use the TMV system, consisting of the three key histologic parameters of bone turnover, mineralization and volume to describe bone biopsy findings, was already proposed by the 2004 KDIGO controversies conference 'Definition and Classification of Chronic Kidney Disease' [5], with reference to a previous report published by the American Society for Bone and Mineral Research in 1987 [76]. The main argument in favor of this classification was having a unified evaluation and classification of bone histology that was clinically relevant and easily applicable. However, many practitioners have continued to report findings based on the older, categorical classification of 'hyperparathyroid bone disease', 'mild hyperparathyroidism', 'mixed renal osteodystrophy', 'adynamic bone', and 'osteomalacia'. In the initial survey of this consensus, which was performed in order to map current practices, 1/10 of respondents replied that they reported the TMV classification exclusively, 3/10 replied that they were still reporting by the previous classification, while 5/10 provided both (1 did not reply to this question). During the consensus discussions, participants expressed a familiarity with the old system as their main reason for continuing this practice. Others informed that clinicians would specifically request whether or not a patient fell into a category of, for example, 'adynamic bone' or 'hyperparathyroid bone disease'. Several disadvantages of the older categorization were acknowledged, such as ambiguity or partial overlap between categories, different definitions of mixed lesions, and frank disagreement on what should constitute "adynamic bone disease" versus other (for instance age-related) conditions of low bone turnover – all of which would presumably lead to relevant differences in the clinical diagnosis.

Ultimately, there was broad agreement that implementation of the TMV system into clinical practice was desirable. The TMV system offers a flexible diagnostic classification, that gives a concise and clinically relevant overview of pathology, which can be followed by a more detailed and descriptive report including both qualitative features and quantitative parameters.

As detailed in the previous paragraph (3.5), the lack of uniform reference values constitutes a barrier to agreement on common diagnostic definitions. Current controversies in this area include whether or not to use age- and sex-specific references for bone turnover and volume, and how to define abnormal mineralization. The currently proposed categorization of mineralization into 'Normal' or 'Abnormal' was felt to be quite restrictive, considering that mineralization defects occur on a spectrum [77]. As an example, a recent publication [14] demonstrated that a mineralization defect was only diagnosed in 2–3 % of contemporary kidney transplant recipients using traditional, stringent definitions (osteoid thickness >11–20 μm and Mlt>50–100 days [58,78]), but an additional 8–10 % could be described as having delayed mineralization, based on previously suggested definitions [77]. The consensus recommended that further efforts should be spent on defining common diagnostic definitions to ensure a uniform classification of ROD.

3.7. Biopsy report

3.7.1. Clinical practice points

- Relevant data on medical history, evolution of biochemistry, drug therapy and imaging should be included in the bone biopsy referral form.
- The report should provide an overall interpretation of histological findings, to aid clinical decision making.
- A TMV classification of trabecular bone is mandatory, followed by a full quantitative report of trabecular bone
- If a full bone histomorphometric analysis is not possible, a qualitative histological evaluation should be provided.
- A qualitative report of cortical bone should be provided if available.
- Presentation of all individual available parameters is recommended, including resorption parameters, with specification of reference data used.

3.7.2. Research recommendations

- The development and clinical validation of a standard for referrals and for reports shared between clinical referral centers and the histomorphometric laboratory would be beneficial.
- AI-based tools could be developed to aid in clinical decision-making, integrating the interpretation of histomorphometric findings with clinical parameters, resulting in a diagnosis and treatment recommendations.

3.7.3. Background and rationale

Considering the time and cost invested in a bone biopsy, the consensus participants found it important to stress that sufficient clinical information should be included in the referral. This would entail a thorough description of the current clinical problem, a medical history including the progression and current state of CKD, relevant medical therapy, current and previous trends in biochemical variables, and any information gained from additional investigations such as bone imaging.

For the report, presenting the results according to the current semi-quantitative reporting standard of TMV classification with bone turnover (low, normal high), bone mineralization (normal, abnormal) and volume (low, normal, high) is considered mandatory, and should be followed by a qualitative description of the histological findings, including a description of both trabecular and cortical bone. The quantitative histomorphometric variables should be given together with their normal reference ranges, with a specification of the reference material used.

It was generally felt that the information that can be gained from a rapid qualitative assessment of the bone biopsy should be highlighted. An initial report of findings from an 'eyes on slide' qualitative evaluation of the biopsy enables a preliminary diagnosis to be provided to the referring clinician, reducing diagnostic delay caused by the long processing time of a full histomorphometric analysis. For centers without the ability to perform bone histomorphometry, collaboration with a laboratory where this expertize is available is encouraged. This could be facilitated by the establishment of reference laboratories with the necessary expertize and willingness to receive bone biopsy samples from their region. If the expertize to perform and prepare (fixing, embedding, cutting procedures) the biopsy is available, a qualitative, histological diagnosis of the bone biopsy could be performed for an initial, clinical diagnosis, which could then be followed by a more comprehensive analysis from a reference center, if the clinical question cannot be sufficiently answered by the qualitative analysis.

An actual standard for referrals and for reports shared between clinical referral centers and the histomorphometric laboratory would be beneficial. The focus of the report should be to provide an interpretation of findings and a conclusion to aid in clinical decision-making. Multidisciplinary teams, either local or regional, integrating different clinical specialties, may be utilized to improve the clinical decision process.

3.8. Pediatric considerations

3.8.1. Clinical practice points

- Iliac crest bone biopsy is safe and can be performed in an outpatient setting in children.
- A 2-day course of tetracycline can be given for bone turnover/mineralization evaluation in children.
- Reference values for children should be age-adjusted.
- The horizontal approach is preferred in pediatric patients as otherwise the iliac growth plate may be a confounding factor.

3.8.2. Background and rationale

Bone biopsy is not routinely recommended in children with CKD in the European pediatric bone evaluation guidelines [79], but can be considered if the clinical and biochemical findings do not explain underlying bone disease, e.g. severe bone deformity or pain, low energy fracture, persistent hypercalcemia or hypophosphatemia despite optimized treatment [79]. An iliac crest bone biopsy is safe and well tolerated in children [16]. It can be performed in an outpatient setting [16], or during a general anesthesia for another procedure, for example the replacement of a dialysis catheter, a G-tube placement or an orthopedic surgery. Before the procedure, bone is labelled with tetracyclines in order to evaluate bone turnover. A two-day course of tetracycline is administered at 15 mg/kg/day (divided in twice or thrice daily doses that should be given without dairy products). In children younger than 8 years, tetracycline dosage is usually kept below 10 mg/kg/day to avoid toxicity. The two-day course is repeated fourteen days later [16]. It is important to keep in mind that children and teenagers have growing skeletons with high bone turnover and elevated new bone formation: as such, adult reference values should not be used to interpret a bone biopsy in pediatrics. Glorieux et al. provided reference data for white children and adolescents aged 1.5 to 22 years in 3- to 4-year age brackets [67]. Interestingly, neither Aj AR, Mlt or resorption parameters were dependent on age. Thus, it could be proposed to analyse structural parameters such cortical thickness and BV/TV, that increase with age, by age groups, and to keep the analysis of Mlt and other parameters of bone remodeling as a global pediatric group. Since children and teenagers with CKD usually display pubertal delay [80], it could even be discussed whether the the age-group analysis should be based on skeletal (and not chronological) age; but to date there is no evidence to support such an approach.

Either the right or left iliac crest can be biopsied. In pediatrics, bone specimens for histomorphometric evaluation should be horizontal, full-thickness biopsies of the ilium from a site 2 cm posterior from the anterior superior iliac spine [81]. This should yield a sample containing two cortices that are separated by a trabecular compartment. Vertical samples (from the iliac crest downwards) are of questionable value because of the presence of the growth plate at the top of the iliac crest that may be a confounding factor [16,81]. In either approach, a manual trochar or electric drill can be used [16]. Even though most papers report the use of modified Bordier trephine with a trocar core diameter of 5 to 7 mm [16], recent data of the smaller Jamshidi needles for quantitative histomorphometry in a CKD-MBD setting seem especially promising in children [78]. Special consideration during the processing of the bone biopsy may be required with specific diagnoses, for example in children with primary hyperoxaluria, where care should be undertaken to avoid washout of oxalate crystals [82–84].

In principle, histomorphometric analysis could be performed in any bone. In clinical pediatrics, the usefulness of samples from nonstandard sites is, however, limited because detailed reference data are only available for the ilium [67]. It can nevertheless be considered in a research context to better understand the underlying mechanisms of rare genetic diseases [85,86].

3.9. Quality control

3.9.1. Clinical practice points

- Bone histomorphometry reporting laboratories should perform regular assessment of their methodology precision.
- At least 2 non-sequential sections should be assessed per biopsy specimen.

3.9.2. Research recommendations

- Novel digital solutions, such as high-resolution imaging of bone biopsy slides, could be explored for quality assurance initiatives between collaborating laboratories.

3.9.3. Background and rationale

In addition to a current lack of standardization of the bone biopsy procedure, sample handling, reading, and reporting, lack of external cross-validation represents a barrier towards collaborative research and data synthesis in the field of ROD. There is to date no available common standard of bone histomorphometry for the assessment of ROD to ensure quality control. This lack of common standard had already been identified at the 2004 KDIGO controversies conference [5], at which the development of a "*quality control and assurance protocol with ongoing inter-laboratory exchange of bone biopsy material*" was recommended to promote standardization. There are certain difficulties in the exchange of biopsy material, of both technical (fading tetracycline labels) and legal character (patient informed consent, material transfer agreements), which may hamper efforts of quality control. However, several laboratories reported the incorporation of conversion of bone biopsy sections to high-resolution images into their workflow. Several image analysis software solutions used for bone histomorphometry support the analysis of these images. This technological advance represents a promising opportunity for quality assurance protocols to be applied without the need for physical transport of biopsy material and may also aid in training and education of young histopathologists.

4. Comments on the results of the Delphi survey

Following the second survey and subsequent group-discussions, a final survey round was performed to assess agreement on the statements proposed by the work groups. The results of this survey were presented and discussed at a final meeting with all consensus participants present. Statements with agreement of >70 % ('Firmly agree' or 'Agree') were accepted, statements with agreement of 50–70 % were discussed and either accepted or rejected, and statements with agreement <50 % were discarded. The results of the second survey are available as Supplementary Table S2.

5. Concluding remarks

Bone histomorphometry is an orphan diagnostic procedure with several strengths, weaknesses, opportunities and threats (Fig. 4). One of its major weaknesses is heterogeneity of practices, which dilutes knowledge and expertise, and furthermore represents a considerable barrier to research synthesis and collaboration. The present initiative addresses this weakness and provides recommendations to harmonize and standardize the procedure. By promoting consensus between bone histomorphometrists, we aim to safeguard this diagnostic procedure as a valuable diagnostic tool in patients presenting with complex CKD-associated osteoporosis. These cases may benefit from a multidisciplinary team (MDT) approach [87]. MDTs consist of nephrologists with expertise in bone disease, osteoporosis specialists, and optimally pathologists whenever histopathological expertise is available, radiologists, and other health care professionals working with CKD-associated osteoporosis. Ideally, this team should have access to advanced bone diagnostics including high-resolution peripheral quantitative computed tomography (HR-pQCT) and bone biopsy/histomorphometry. MDTs can act as referral units for nephrologists from surrounding primary and secondary healthcare facilities. Standardization and harmonization of quantitative bone histomorphometry are likely to represent future prerequisites to enable artificial intelligence and deep learning [88] to lift bone histomorphometry to the next level.

CRediT authorship contribution statement

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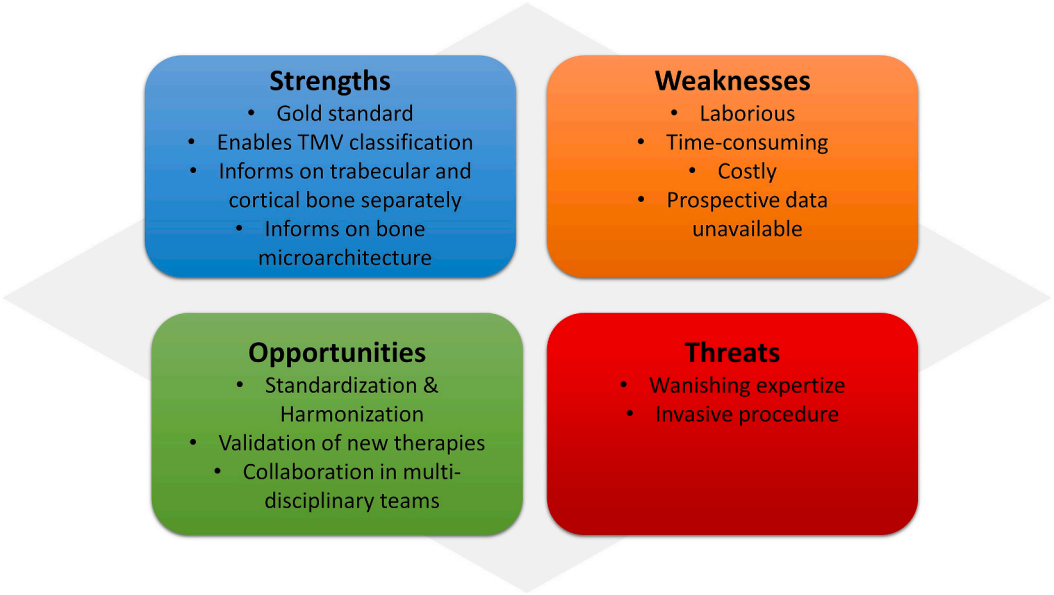

Fig. 4. Bone biopsy for the diagnosis of renal osteodystrophy; an analysis of strengths, weaknesses, opportunities and threats (SWOT).

original draft, Investigation. **Pablo Ureña:** Writing – review & editing, Writing – original draft, Investigation. **Justine Bacchetta:** Writing – review & editing, Writing – original draft, Investigation. **Sandro Mazzaferro:** Writing – review & editing, Writing – original draft, Investigation. **Mathias Haarhaus:** Writing – review & editing, Writing – original draft, Investigation. **Pieter Evenepoel:** Writing – review & editing, Writing – original draft, Visualization, Supervision, Project administration, Methodology, Investigation, Formal analysis, Conceptualization.

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Declaration of competing interest

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.bone.2025.117544.

Data availability

The data underlying this article is available in the article and in its online supplementary material.

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