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Isolating mineralized bone and bone marrow mRNA from transiliac bone biopsies stored in a stabilizing solution: A comparative study

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ABSTRACT

The molecular mechanisms underlying metabolic bone diseases, including renal osteodystrophy, are poorly understood. Transcriptomics are increasingly used to characterize biological molecular networks and prove promising in identifying therapeutic targets and biomarkers. A reliable method for obtaining sufficient amounts of high quality RNA from human bone biopsies is a prerequisite for the implementation of molecular diagnostics in clinical research and practice.

The present study aimed to develop a simple and adequate method for isolating bone and bone marrow mRNA from transiliac bone biopsies. Several storage, separation, and extraction procedures were compared. The procedure was optimized in pig samples and subsequently validated in human samples. Appropriate amounts of mineralized bone and bone marrow mRNA of moderate to high quality were obtained from transiliac bone biopsies that were immersed in the stabilizing solution Allprotect Tissue Reagent at room temperature for up to 3 days prior to freezing. After thawing, bone marrow and mineralized bone were separated by a multistep centrifugation procedure and subsequently disrupted and homogenized by a bead crusher. Appropriate separation of mineralized bone and bone marrow was confirmed by discriminatory gene expression profiles.

1. Introduction

The skeleton is metabolically active throughout life with specific bone cells and paracrine/endocrine factors regulating its morphogenesis and remodeling. Aging and lifestyle factors (including diet and physical activity) have a significant impact on the skeleton. Systemic diseases, such as diabetes and chronic kidney disease may profoundly disturb bone homeostasis. The current diagnosis of metabolic bone disease relies on the measure of circulating biomarkers of bone and mineral metabolism, on analyses of bone density, volume, microarchitecture and remodeling using advanced imaging technologies and on bone histomorphometry. These diagnostic tools do not provide insights into the cellular and molecular mechanisms underlying the onset and progression of disease and thus are insufficient to understand the complexity of metabolic bone diseases, nor do they allow for precision medicine (Yang et al., 2020; Reppe et al., 2017).

Transcriptomics, representing the analysis of messenger ribonucleic acids (mRNAs) and small noncoding ribonucleic acids such as microribonucleic acids (miRNAs) by quantitative polymerase chain reaction (qPCR), microarray or RNA sequencing, from bone tissue or single bone cells may help to unravel pathophysiological mechanisms and to identify clinically relevant biomarkers (Martin and David, 2020).

The most commonly performed technique to obtain bone tissue in clinical practice is a transiliac bone biopsy. Preanalytical variables have a major impact on the integrity of biospecimens in biobanking (Ellervik and Vaught, 2015). This is the case for blood and urine, and probably even more so for bone. Thus, establishing a reliable method for collecting, storing, and processing transiliac bone biopsies for future molecular diagnostics is highly recommended. Immediate stabilization of RNA is of critical importance, because directly after harvesting the sample, changes in gene expression patterns may occur due to specific and nonspecific RNA degradation, or transcriptional induction. In the experimental setting, bone samples are most commonly snap frozen in liquid nitrogen and stored at −80 °C, pending further processing. In the

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clinical setting, however, safety regulations often preclude working with liquid nitrogen. A stabilization reagent does not require special safety precautions and, as a further benefit, allows bone to thaw to room temperature without loss of integrity (Pedersen et al., 2019). As such, additional steps such as the separation of bone and bone marrow may be scheduled at any convenient time point prior to isolation of RNA.

The present study aimed to investigate whether storage of a transiliac bone biopsy in a stabilizing solution is non-inferior to storage in liquid nitrogen with regard to preserving mRNA quality and to develop a simple and adequate method for separating bone and bone marrow mRNA.

2. Materials and methods

2.1. Animals

Three months old male Topigs-20 pigs were sacrificed in the frame of an ongoing study exploring the impact of warm ischemia time on pulmonary function. Pigs were premedicated with xylazine 2 mg/kg (VMD, Arendonk, Belgium) and tiletamine 8 mg/kg (Virbac, Barneveld, The Netherlands) allowing for oro-tracheal intubation and mechanical ventilation 10 min later. Anaesthesia was induced and maintained with isoflurane 1 % (Dechra Veterinary, Belgium) in a mixture of 50 % oxygen and room air. After cannulation of the marginal ear-vein, analgesia with fentanyl 8 μg/kg/h (Janssen-Cilag, Beerse, Belgium) was started. All experiments were performed in accordance with international guidelines regarding animal welfare and approved by the local ethical committee of the KU Leuven.

2.2. Bone biopsy

Pig bone tissue was harvested within 2 h after sacrifice in sterile conditions. A bone sample was retrieved from the iliac bone using a bone biopsy trephine with an internal diameter of 3.55 mm (Osteobell 7G, Mirandola, Italy).

2.3. Tissue stabilization

Next to snap freezing in liquid nitrogen, two different tissue stabilizers were tested on fresh biopsies i.e. Allprotect Tissue Reagent (Allprotect) and the less viscous RNAprotect Tissue Reagent (RNAlater) (both from Qiagen, Venlo, Netherlands). For both stabilizers, 1.5 ml was used in a RNase-free tube. After full immersion of the biopsy in the tissue stabilizer, samples were stored at −20 °C, either immediately (<1 h) or after standing on the benchtop for 3 days at room temperature, as per the manufacturer's recommendation, to guarantee optimal penetration of the bone tissue by the stabilizer. Snap frozen samples were stored at −20 °C or −80 °C. All pig samples were processed in quadruple.

2.4. Separation of bone and bone marrow

Flush and centrifugation techniques are commonly used to separate bone and bone marrow in long bones harvested from rodents (Dobson et al., 1999). Since these approaches are less convenient for transiliac bone biopsies, we developed a specific protocol. The transiliac bone biopsies were centrifuged in nested RNase free centrifugal tubes (Fig. 1). The inner tube (1,5 ml RNase free tube, Biosphere SafeSeal tube, Sarstedt, article number 1050299) was perforated thrice with a sterile 18G needle to allow marrow to elude into the outer tube (2 ml RNase free tube, Sample tubes RB, Qiagen, article number 1050299), containing 600 μl of lysis RLT buffer (Qiagen) with the addition of β-mercaptoethanol, according to the manufacturer's instructions. To reduce the migration time of the bone marrow during centrifugation, biopsies were split in 2 parts. Both parts were placed in the inner tube with the cortex

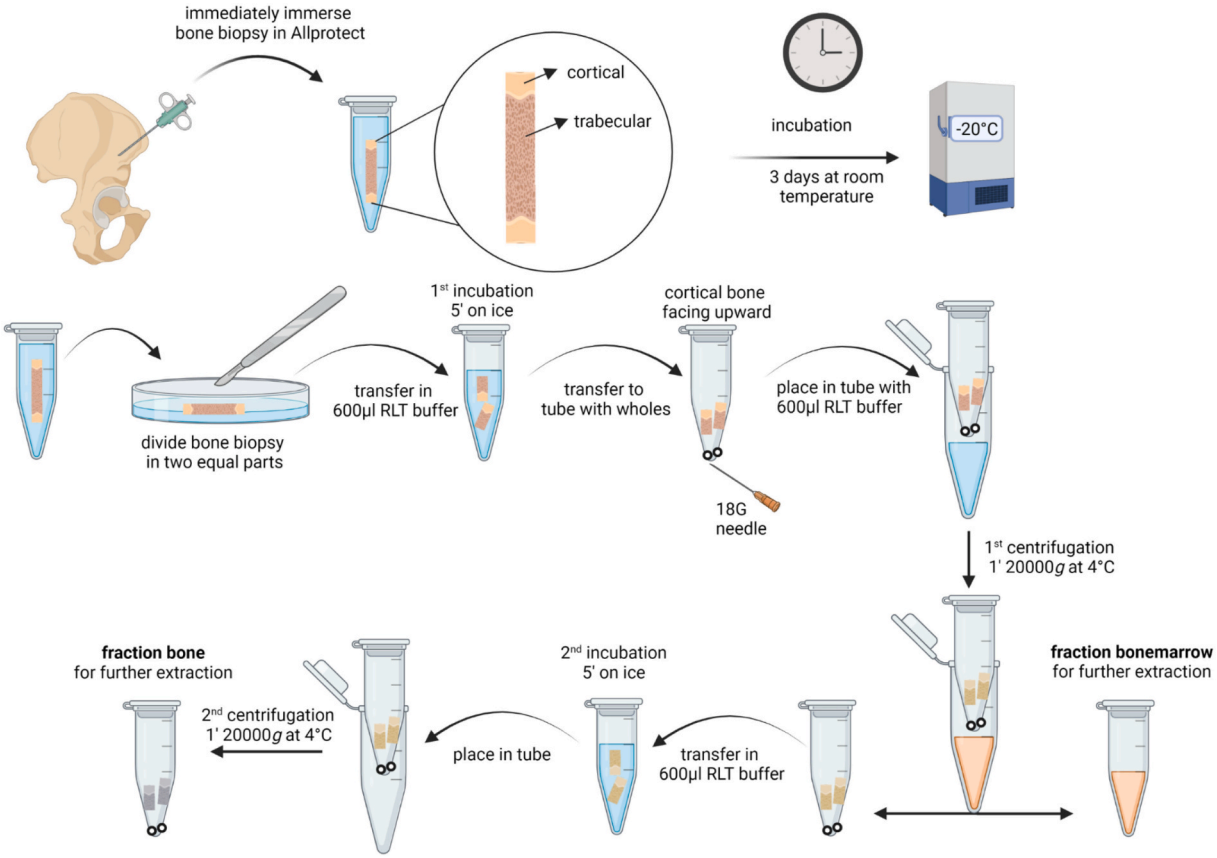

Fig. 1. Road map for isolating bone and bone marrow mRNA from transiliac bone biopsies.

pointing to the top of the tube. Three centrifugation protocols were tested: 1′ at 20,000*g*; 3′ at 20,000*g*, and 3′ at 5000*g*, all at 4 °C. Centrifugation cycles were repeated up to 3 times. All experiments were performed in triplicate (unless otherwise specified).

2.5. mRNA isolation

Whole bone as well as the bone fraction were immersed in a lysis solution (1000 μl QIAzol Lysis buffer or 600 μl RLT buffer, both Qiagen), immediately followed by crushing and lysis on the TissueLyser LT (Qiagen). More specifically, the bone tissue was disrupted and homogenized by a 7 mm diameter stainless bead during two consecutive cycles at 50 Hz, each lasting 5′, with an in between 3′ cooling phase on crushed ice. The procedure was highly efficient as <10 % of the overall recovered RNA amount was isolated after the second crushing session of the remaining bone debris (data not shown).

The bone marrow fraction, collected in 600 μl RLT buffer (Qiagen) was disrupted and homogenized by a 5 mm bead at 50 Hz for 3′. The lysate was pipetted into a new sample tube and dissociation of nucleoprotein complexes was promoted by standing the homogenate 5′ on the benchtop.

mRNA isolation was performed using the semi-automatic system QiaCube (Qiagen).

Three commercial extraction kits of Qiagen were tested: RNeasy Lipid Tissue Mini (Lipid) kit, RNeasy Fibrous Tissue Mini (Fibrous) kit, and RNeasy Mini (RNeasy) kit, all followed by a DNase digest step (to make sure all genomic DNA was removed). All samples were eluted in 40 μl RNase-free water and stored at −80 °C until further analysis.

2.6. mRNA quantity, purity and integrity

The quantity (absorbance at 260 nm) and purity (ratio of the absorbance at 260 and 280 nm) of mRNA isolated from bone and bone marrow were measured using the NanoDrop ND-1000 spectrophotometer (Thermo Scientific, Life Technologies Europe B.V., Ghent, Belgium). A ratio 260/280 of ~2.0 is generally accepted as "pure" for RNA (Thermo Scientific T042 technical bulletin). RNA integrity was determined by RNA integrity number (RIN) using the Eukaryote Nano RNA Kit (Agilent Technologies Belgium NV, Diegem, Belgium) in the 2100 Bioanalyzer instrument (Agilent Technologies Belgium NV).

2.7. Validation in human samples

We next tested the protocol as optimized in pigs on human bone samples acquired by transiliac bone biopsy (Torres et al., 2014). All patients were enrolled in an ongoing prospective observational study investigating the impact of kidney transplantation on bone health (NCT01886950). The experimental protocol was in accordance with the Declaration of Helsinki and informed consent was obtained from all participants. We randomly selected 10 bone biopsies from 10 patients with chronic kidney disease (CKD), across stages of disease (men 8, mean age of 54.3 ± 5.9 years).

The optimized protocol was as follows: bone biopsies were immediately immersed in Allprotect for 3 days at room temperature after which they were stored at −20 °C. Bone and bone marrow were separated by centrifugation (2 cycles, 1′ at 20,000*g*). mRNA was isolated from bone and bone marrow using the Lipid and RNeasy kit, respectively (Fig. 1).

Complementary DNA (cDNA) was synthesized using 500 ng of mRNA of the extracted bone or bone marrow in 5 μl of total volume by SuperScript IV VILO master mix kit (Thermo Fisher Scientific, Waltham, MA, USA) according to the manufacturer's protocol.

The reaction was performed in a Veriti 96-well thermal cycler (Thermo Fisher Scientific, Waltham, MA, USA). For a better efficiency, the gradient was slightly adapted from the supplier protocol: 10′ at 25 °C, 10′ at 55 °C, 5′ at 85 °C and hold at 4 °C. The cDNA was stored at −20 °C or for longer period at −80 °C. The amount of cDNA produced was not quantified and it was assumed that mRNA was quantitatively converted into cDNA.

The qPCR was performed singleplex in a 96-well plate in a final volume of 20 μl containing 1.5 μl of cDNA, 18.5 μl of qPCR reaction mix (10 μl TaqMan Fast Advanced Master Mix (2×), 1 μl Taqman Assay (20×) with VIC/FAM fluorescent dye labeling and 7.5 μl nuclease-free water). The real-time PCR program was executed on a 7500 Fast Real-Time PCR System (Thermo Fisher Scientific, Waltham, MA, USA) in fast modus consisted of 2′ at 50 °C (UNG incubation), 20 s at 95 °C (polymerase activation) and followed by 50 cycles of denaturation at 95 °C for 3 s and annealing and extension at 60 °C for 30 s.

Primers for genes that have been established to be highly expressed in bone tissue were selected (Supplementary Table 1). Cycle of quantification (Cq) was determined for each gene and compared to 3 housekeeping genes: beta-2-microglobulin (*B2M*), actin beta (*ACTB*) and ribosomal protein L41 (*RPL41*) (Reppe et al., 2010; Fujita et al., 2014).

Relative quantification was calculated with the $2^{-\Delta\Delta Cq}$ method. Expression levels of the gene of interest were normalized to the expression of *ACTB* and with a maximum Cq value of 35.

3. Results

3.1. Tissue stabilization

In a first set of experiments on pig samples, the impact of (a) storage medium (Allprotect vs RNAlater), (b) bench time on room temperature (short [<1 h] vs long [3d], samples collected in stabilizing solutions only), and (c) long term cold storage conditions (−20 °C vs −80 °C, samples collected in liquid nitrogen only), on mRNA quantity, purity and integrity was studied (Table 1). No major differences were observed for mRNA yield (amount/weight) between the different conditions. mRNA purity was within target for all conditions. Integrity of mRNA, appeared to be strikingly lower in both bone and bone marrow fraction from snap frozen liquid nitrogen samples stored at −20 °C. In line, suboptimal mRNA quality, arbitrarily defined by a median RIN <6, was observed in samples of the bone fraction collected in RNAlater, kept on the bench for 3 days at room temperature and subsequently stored at −20 °C. Apart from bone biopsies immersed in liquid nitrogen and subsequently stored at −20 °C, all bone biopsies yielded excellent bone marrow mRNA quality.

Optimal mRNA quality was observed in bone biopsy samples processed without separation of bone and bone marrow (i.e. 'whole' bone), whether stored in liquid nitrogen or stabilizer solution (Table 1).

To assess the impact of storage time on mRNA quantity and quality, pig bone samples (n = 2) of similar size were stored up to 2 years (Supplementary Table 2). Both quality and integrity indices (of whole bone, mineralized bone, and bone marrow) did not show major changes over time.

Although samples collected in RNAlater numerically yielded the highest RIN value, we preferred in our final protocol to store the samples in Allprotect. The reasons are twofold; first, samples collected in Allprotect yielded similar RIN values, independent of bench time; and second, Allprotect also stabilizes DNA and proteins, which may be considered an additional asset.

3.2. Separation of bone and bone marrow

3.2.1. Role of pre-incubation

Initial attempts to obtain bone biopsy samples free of bone marrow by centrifugation only (1′ at 20,000*g*, 4 °C) were not successful as blood residues were clearly present in the bone fraction on visual inspection. Furthermore, the co-elution of Allprotect solution in the bone marrow fraction technically prevented the aspiration of the sample into the Qiacube. To overcome this problem, we pre-incubated the bone biopsies in RLT buffer for 5′ on crushed ice. The pre-incubation and

Table 1
Stabilization strategies.

Storage condition	Separated bone (storage 2,5 months, n = 6 pig biopsies at each condition)									Non-separated bone (storage 1 week, n = 3 pig biopsies at each condition)				
	Bone biopsy	Bone fraction (extracted with Lipid kit)				Bone marrow fraction (extracted with RNeasy kit)				Bone biopsy	Whole bone (extracted with Lipid kit)			
	Weight (mg)	Amount (μg)	Amount/ weight	RIN	260/ 280	Amount (μg)	Amount/ weight	RIN	260/ 280	Weight (mg)	Amount (μg)	Amount/ weight	RIN	260/ 280
Allprotect < 1 h RT → −20 °C	92 ± 17	26 ± 24	0.29	6.6 ± 0.5	2.05 ± 0.02	14 ± 9	0.15	8.7 ± 0.7	2.04 ± 0.02					
Allprotect 3 days RT → −20 °C	95 ± 21	29 ± 4	0.31	6.2 ± 0.4	2.06 ± 0.01	13 ± 1	0.14	8.8 ± 1.1	2.03 ± 0.02	101 ± 31	40 ± 17	0.39	7.4 ± 0.07	2.09 ± 0.007
RNAlater < 1 h RT → −20 °C	134 ± 36	38 ± 10	0.29	7.5 ± 0.3	2.06 ± 0.01	23 ± 6	0.17	9.2 ± 0.2	2.07 ± 0.01					
RNAlater 3 days RT → −20 °C	104 ± 43	33 ± 17	0.32	4.1 ± 0.6	2.05 ± 0.02	9 ± 4	0.08	8.9 ± 0.6	2.06 ± 0.005					
Liquid N_2 → −20 °C	118 ± 31	28 ± 10	0.24	3.0 ± 1.5	2.08 ± 0.02	11 ± 9	0.09	4.5 ± 0.4	2.09 ± 0.03					
Liquid N_2 → −80 °C	121 ± 27	24 ± 13	0.20	5.4 ± 0.7	2.09 ± 0.01	16 ± 12	0.13	7.9 ± 1.2	2.06 ± 0.02	107 ± 69	59 ± 20	0.62	6.8 ± 0.5	2.06 ± 0.007

RT: room temperature.

centrifugation procedure (see below) were repeated several times. The yield of bone marrow mRNA in the eluent rapidly decreased over subsequent centrifugation cycles: 43 %, 36 %, 14 %, and 5 % (all median, n = 6 pig biopsies) of the total mRNA was obtained after the first, second, third, and fourth cycle, respectively. mRNA purity and integrity were monitored along the procedure, and did not show contamination nor progressive disintegration (data not shown). In parallel, experiments were also performed with PBS as pre-incubation solution. As shown in Table 2, pre-incubation with PBS resulted in lower amounts of bone marrow mRNA of inferior quality.

A quicker separation was achieved in human samples (n = 6); 84 %, 13 %, and 3 % of the total bone marrow mRNA was obtained after the first, second, and third centrifugation cycle, respectively (data not shown).

3.2.2. Role of centrifugation force and time

We next studied the impact of centrifugation force and time by comparing 3 centrifugation programs. Post-procedural visual inspection of the bone biopsies showed blood residues in bone samples spun for 1′ at 5000*g*. In line with the hypothesis of incomplete separation, we also observed a higher RNA amount in bone fraction samples spun for 1′ at 5000*g* (39 ± 9 μg) vs samples spun at 20,000*g* (15 ± 3 μg). Extending the centrifugation time to 3′ did not make any difference. Based on these observations and that we visually notice that after a second incubation in RLT bone fraction looked cleaner, we defined the standard procedure as 2 subsequent centrifugation cycles, 20,000*g* for 1′ at 4 °C, following a 5′ pre-incubation with RLT buffer on crushed ice. The eluent obtained after the first centrifugation cycle was used for bone marrow processing.

Table 2
impact of pre-incubation condition (n = 6 pig biopsies for each condition).

	Bone (extracted with Lipid kit)			Bone marrow (extracted with RNeasy kit)		
	Amount (μg)	RIN	260/ 280	Amount (μg)	RIN	260/ 280
RLT buffer	21 ± 12	6.0 ± 0.8	2.04 ± 0.06	19 ± 15	6.9 ± 0.8	2.04 ± 0.02
PBS buffer	27 ± 15	3.1 ± 0.9	2.06 ± 0.10	6.7 ± 5.6	5.4 ± 0.2	1.99 ± 0.02

The bone 'remnant' after the second centrifugation cycle was used for mineralized bone processing.

3.3. RNA isolation

RNA was isolated from 18 pig bone samples. Nine samples were analyzed as such ('whole bone'), while for the remaining bone samples, mineralized bone and bone marrow were first separated (as described earlier). All samples were disrupted and homogenized as described in the Materials and methods section. The performance of 3.

3 commercial mRNA extraction kits (RNeasy, Lipid and Fibrous kits, all Qiagen) were studied (Table 3).

With regard to the whole bone and bone fraction, mRNA yield with the RNeasy kit was very low, precluding the assessment of purity and quality. The other kits yielded comparable mRNA purity and integrity, but the yield was superior with the Lipid kit.

With regard to the bone marrow fraction, the lipid kit could not be used for the extraction of mRNA, as bone marrow was collected in RLT buffer. The RNeasy and Fibrous kit yielded high amounts (14 ± 6 and 18 ± 8 μg, respectively) of moderate-to-high quality (purity 260/280: 2.06 ± 0.02 and 2.07 ± 0.03; RIN: 7.06 ± 0.3 and 6.97 ± 0.2, respectively, n = 7). The QiaCube processing time was shorter with the RNeasy kit.

Therefore, the Lipid kit and RNeasy kit were selected as extraction kits for mineralized bone and bone marrow, respectively.

3.4. Human gene expression

To validate the procedure for clinical samples we next assessed gene expression in 10 human transiliac bone biopsies that were processed according to the protocol as optimized in pigs (Fig. 1). Briefly, upon harvesting, bone biopsies were immediately immersed in Allprotect for 3 days at room temperature after which they were stored at −20 °C. Bone and bone marrow were separated by centrifugation (2 cycles, 1′ at 20,000*g*). mRNA was isolated from bone and bone marrow using the Lipid and RNeasy kit, respectively. Storage time was 1.76 ± 0.99 years (mean ± SD). RNA amount, RIN, and purity amounted to 4.6 ± 3.7 μg, 4.4 ± 1.2, 1.95 ± 0.06 for the bone fraction and 9.4 ± 7.7 μg, 5.1 ± 0.8, 2.04 ± 0.02 for the bone marrow fraction. We were able to obtain acceptable expression profiling data, even in samples with suboptimal RINs (defined as RIN < 6.0). Not surprisingly, the expression of bone

Table 3
Impact of RNA extraction kit on whole bone and bone fraction (n = 3 pig samples at each condition).

Homogenisation	Extraction kit Qiagen	Whole bone			Bone fraction		
		Amount (μg)	RIN	260/280	Amount (μg)	RIN	260/280
1 ml QIAzol, 5′ 50 Hz, 3′ on ice, 5′ 50 Hz	Lipid kit	47 ± 4	6.8 ± 1.4	2.06 ± 0.0	21 ± 12	6.3 ± 0.1	2.04 ± 0.02
600 μl RLT, 5′ 50HZ, 3′ on ice, 5′ 50 Hz	Fibrous kit	18 ± 9	8.8 ± 0.5	2.07 ± 0.02	17 ± 3	5.6 ± 0.1	2.05 ± 0.01
600 μl RLT, 5′ 50HZ, 3′ on ice, 5′ 50 Hz	RNeasy kit	1 ± 0.4	NA	1.85 ± 0.0	1 ± 0.2	NA	1.88 ± 0.007

NA: not available.

genes was several-fold higher in the bone as compared to the bone marrow fraction ($p < 0.05$, all) (Table 4 and Fig. 2). Of note, *SOST* expression was not observed in bone marrow.

4. Discussion

The main finding of the present study is that both bone and bone marrow mRNA of sufficient quality for qPCR analysis can be obtained from transiliac bone biopsies using a simple and pragmatic protocol.

Molecular diagnostics may prove useful in unravelling the complex pathophysiology of metabolic bone diseases including renal osteodystrophy (ROD) (Fig. 3). In clinical research, progress in molecular diagnostics has been limited, mainly due to difficulties in obtaining bone tissue of sufficient quality. In humans, bone samples may be obtained during orthopedic surgery or by trephine needles, most commonly from the iliac crest (Torres et al., 2014). Small (inner diameter < 5 mm) and disposable trephine needles are gaining popularity at the expense of the large, non-disposable Bordier and Bedford trephine needles (Evenepoel et al., 2017). Immediate stabilization of RNA in bone samples is necessary because, directly after harvesting the samples, changes in the gene expression pattern occur due to specific and nonspecific RNA degradation as well as to transcriptional induction. In an experimental setting, bones are commonly flash-frozen in liquid nitrogen and stored at −80 °C before RNA extraction. Due to safety concerns, use of liquid nitrogen is more problematic if not prohibited in clinical practice. We demonstrate that immersion of the bone biopsy in a **stabilization reagent** may be a valid alternative. The quality of bone and bone marrow mRNA extracted from transiliac bone biopsies immersed in Allprotect and RNAlater upon harvesting was at least as good as mRNA extracted from bone biopsies stored in liquid nitrogen. Allprotect not only stabilizes RNA but also provides immediate and convenient preservation of DNA and proteins in tissues, enabling reliable results in gene expression analysis as well as protein and DNA analyses. A stabilization reagent, furthermore, allows bone to thaw at room temperature without complete loss of RNA integrity. As such, separation of bone and bone marrow may be scheduled at a convenient time point prior to isolation of RNA. Failure to elute the bone marrow, comprising hematopoietic and stromal stem cells and their respective lineages, fibroblasts and endothelial cells may results in extensive 'contamination' of bone RNA (Evenepoel et al., 2017). In addition, separation may prove useful to explore the cross talk between these two compartments. Centrifugation has been reported to remove bone marrow more efficiently than flushing, as least in long bones of rodents (Pedersen et al., 2019; Evenepoel et al., 2017). The present study shows that spinning bone biopsies at 20,000*g* for 1′ at 4 °C following a 5′ pre-incubation in RLT buffer also yields good **separation**

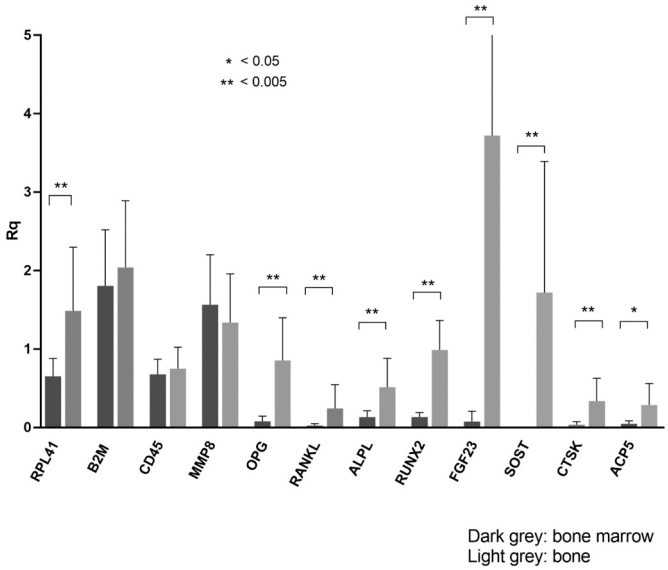

Fig. 2. Overview of the mean Cq values and stdev of the selected markers in bone fraction (light grey bars) and bone marrow fraction (dark grey bars).

Table 4
The relative quantification of representative bone marrow and mineralized bone genes in transiliac bone biopsies from 10 patients with CKD.

	Category	Bone Rq[a] median (IQ 25–75)	Bone marrow Rq[a] median (IQ 25–75)	p (Wilcoxon signed-ranks test)[b]
ACTB	Housekeeping gene	1	1	
RPL41	Housekeeping gene	1.290 (0.917–2.072)	0.656 (0.423–0.803)	***0.002***
B2M	Housekeeping gene	1.776 (1.418–2.775)	1.908 (1.090–2.343)	0.4316
CD45 (PTPRC)	Bone marrow marker	0.686 (0.528–0.973)	0.612 (0.576–0.845)	0.4316
MMP8	Bone marrow marker	1.237 (0.940–1.763)	1.406 (1.061–1.989)	0.1309
OPG (TNFRSF11B)	Cell signaling marker	0.812 (0.355–1.279)	0.056 (0.026–0.135)	***0.002***
RANKL (TNFSF11)	Cell signaling marker	0.105 (0.060–0.316)	0.014 (0.006–0.031)	***0.002***
ALPL	Osteoblast marker	0.476 (0.135–0.736)	0.112 (0.072–0.155)	***0.002***
RUNX2	Osteoblast marker	0.967 (0.785–1.095)	0.111 (0.097–0.159)	***0.002***
FGF23	Osteocyte marker/osteoblast marker	2.180 (0.974–4.997)	0.022 (0.000–0.101)	***0.002***
SOST	Osteocyte marker	1.454 (0.482–1.997)	0 (0.000–0.000)	***0.002***
CTSK	Osteoclast marker	0.260 (0.148–0.469)	0.023 (0.007–0.044)	***0.002***
ACP5 (TRAP5b)	Osteoclast marker	0.231 (0.121–0.319)	0.036 (0.018–0.056)	***0.039***

ΔΔCq = ΔCqsample − ΔCq reference sample.
ΔCq = Cqsample − Cq housekeeping gene.
Reference gene: bone sample.
[a] Rq (relative quantification) = 2^−ΔΔCq.
[b] Correlations with p<0.05 have been highlighted in bold.

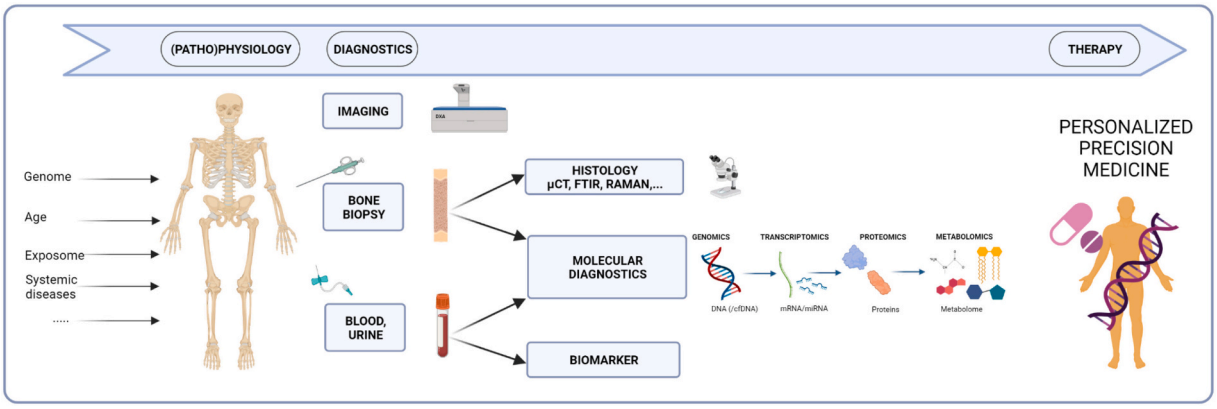

Fig. 3. On the road to precision medicine in metabolic bone disease.

of bone and bone marrow in transiliac bone biopsies, without loss of mRNA quality.

The technical performance of commercially available **extraction** kits showed marked heterogeneity across mineralized bone and bone marrow fractions. For mineralized bone, the mRNA yield was inadequate, appropriate, and superior with the RNeasy, Fibrous and Lipid kits, respectively. mRNA extracted with the Lipid and Fibrous kits showed good and similar RmNA quality. For the bone marrow faction, excellent mRNA yield and quality were obtained with the RNeasy and Fibrous kits.

Thus, appropriate amounts of mineralized bone and bone marrow mRNA of moderate to high quality (RIN 7.2 and 9.0, respectively) were obtained from pig transiliac bone biopsies that were harvested on stabilization solution Allprotect, rested on the bench at room temperature for up to 3 days, and finally stored at −20 °C until further processing. Despite identical pre-analytical and analytical sample processing, human samples yielded lower bone and bone marrow mRNA quality than pig samples (RIN 4.4 and 5.1, respectively). The reasons for this discrepancy remain obscure. Storage duration appears not to be an issue as repeated analyses up to 2 years failed to show a decay of mRNA quality. Of note, pigs were sacrificed at a young age (3 months), and pig bone appeared much softer on retrieval. It may be speculated that a higher proportion of trabecular bone accounts for the higher RIN. Indeed, a previous experimental study showed that RNA quality may differ across bone regions, being higher in trabecular bone (RIN 6.3 vs 4.4, trabecular vs cortical bone) (Kelly et al., 2014). The trabecular bone is also more easily accessible to the stabilizer solution. The same line of reasoning may also explain the higher mRNA quality in bone marrow as compared to bone. Studies reporting RNA quality in human bone biopsy samples are very limited. Picard et al. reported a RIN varying between 7.6 and 8.5 in transiliac bone biopsy samples stored in liquid nitrogen (Picard et al., 2020). Importantly, and at variance with our approach, Picard et al. extracted RNA from whole bone biopsies only. This is important to emphasize since our data indicate that separation of mineralized bone and bone marrow confers a risk of loss of RNA quality in bone samples collected in liquid nitrogen. This observation aligns with studies describing the fragility of snap frozen samples, being critical the moment just before RNA extraction (Esteva-Socias et al., 2020).

The validity of RINs as a key measure of quality is challenged by some researchers (Sonntag et al., 2016). Analysis of electropherograms showed that RINs were not consistently correlated with RNA or cDNA profiles and appeared to be poor predictors of overall cDNA quality. In another study, differences introduced by RNA degradation were largely outweighed by biological differences (Opitz et al., 2010). Thus, RINs provide an incomplete measure of tissue quality. However, the 'minimum information for publication of quantitative real-time PCR experiments (MIQE) guidelines' demand transparency of the pre-PCR steps like documentation of sample RNA quality (Huggett et al., 2013).

Transcriptomic studies attempt to catalog and quantify the RNA content of a cell, tissue, or organism. In some cases, the goal is to target all transcripts, regardless of their structure or function. Other studies, though, home in on one specific subset of the transcriptome, such as mRNAs, miRNAs or long noncoding RNAs. Essentially, there are three techniques for tackling the transcriptome: qPCR, microarrays, and Next Generation RNA sequencing. The qPCR technique is highly quantitative and sensitive, but generally best for investigating a relatively small number of transcripts in a large set of samples. In the present study, we used qPCR to examine the expression of a panel of bone and bone marrow genes. We were able to obtain acceptable expression profiling data, even from samples with suboptimal RINs. As expected, the expression of bone genes was several-fold higher in the bone as compared to the bone marrow fraction. The bone marrow fraction did not express *SOST*, a marker of osteocytes, which are the most predominant bone cells. This finding confirms that the bone marrow fraction mRNA was not contaminated by bone cells.

The present protocol for collecting, storing, and preanalytically processing transiliac bone biopsies has major strengths, most importantly user-friendliness and thus broad clinical applicability. Some limitations should be acknowledged. Further refinements of the protocol will be needed to obtain mRNA with sufficient quality to allow for RNA-Seq, and for analyses separating the specific bone compartments (e.g. trabecular vs cortical (Varanasi et al., 2010)), and ultimately, to single cell analyses.

Supplementary data to this article can be found online at https://doi.org/10.1016/j.bonr.2022.101624.

CRediT authorship contribution statement

Study design: HdL and PE
Study conduct and data collection: HdL, CM, AVC and PE
Data analysis: HdL, AVC and PE
Data interpretation: all authors.
Drafting manuscript: HdL and PE.
Revising manuscript content: all authors.
Approving final version of manuscript: all authors.
PE takes responsibility for the integrity of the data analysis.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data availability

The data that support the findings of this study are available from the

corresponding author upon reasonable request.

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