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Bone remodeling during pregnancy and post-partum assessed by metal lead levels and isotopic concentrations☆

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ABSTRACT

Bone remodeling is normally evaluated using bone turnover markers/indices as indicators of bone resorption and formation. However, during pregnancy and post-partum, there have been inconsistent results between and within biomarkers for bone formation and resorption. These differences may relate to pregnancy-related changes in metabolism and/or hemodilution altering measured marker levels. An alternative approach to evaluating bone remodeling is to use the metal lead (Pb) concentrations and Pb isotopic compositions in blood. These measurements can also provide information on the amount of Pb that is mobilized from the maternal skeleton. Despite some similarities with accepted bone turnover markers, the Pb data demonstrate increased bone resorption throughout pregnancy that further continues post-partum independent of length of breast-feeding, dietary intake and resumption of menses. Furthermore the isotopic measurements are not affected by hemodilution. These data confirm calcium balance studies that indicate increased bone resorption throughout pregnancy and lactation. They also indicate potentially major public health implications of the transfer of maternal Pb burden to the fetus and new born.

1. Introduction

The entire adult skeleton, comprised of about two-thirds mineral and one-third osteoid, is replaced about every 10 years with approximately 10% of the skeleton being involved in bone remodeling at any one time. The remodeling process begins when an area of bone is resorbed by osteoclasts, forming a discrete pit. Osteoblasts then deposit organic matrix, or osteoid, into the pit, and the osteoid then becomes mineralized or calcifies. The entire process occurs over about 3 months at any single site [1]. Bone resorption and formation are usually coupled with an increase in bone resorption followed by increased bone formation within approximately 6–8 weeks. This bone remodeling is commonly evaluated by biochemical markers and, because bone resorption and bone formation are tightly coupled, a marker from either group usually reflects bone turnover rate [1], [2,3], [4].

Pregnancy and lactation are characterized by major changes in maternal calcium homeostasis and bone metabolism in order to satisfy the needs of the fetus and the newborn infant for calcium during skeletal growth and mineralization [2,5]. The potential calcium sources are: increased intestinal absorption, decreased renal excretion, and increased resorption from the maternal skeleton. Calcium balance studies suggest that increased dietary intake and intestinal absorption are not sufficient to provide the calcium required by the fetus and the maternal skeleton is used as a source of calcium for the fetus [6] and particularly for the newborn infant during breast feeding. During pregnancy there is increased bone resorption despite high estradiol levels that could be expected to suppress bone resorption and even promote bone formation [7].

Biochemical markers used for analysis of bone formation and resorption have given divergent results and lead to alternative suggestions for markers (Table 1). The changes in Table 3 for post-partum are relative to values in trimester 3. Thus some studies observed increased bone resorption from early pregnancy [8,9] but others only during late pregnancy [10,11]. Likewise, bone formation markers in early pregnancy have been reported to be unchanged [11] or decreased [9] [12,13,10]. During lactation most studies have reported an increase in bone turnover [9–14]. As 1,25-dihydroxyvitamin D levels decrease following delivery resulting in normalization of the intestinal calcium absorption

☆ This paper is dedicated to Dr Paul Mushak who sadly did not wake up on February 3, 2016. Paul provided continual encouragement to the first author and without his perspicacity and encouragement it is highly unlikely that the use of lead isotopic tracing in environmental health would have reached its current status.

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Table 1
Summary of studies employing biochemical markers to evaluate bone remodeling.

Bone Formation Markers (measured in serum)

Marker	No. of subjects	Trimester 1	Trimester 2	Trimester 3	Postpartum	Reference	Changes over pregnancy & postpartum (PP) and comment
Osteocalcin (OC)							
	10	X (No change)	X	X	↑	Gallacher (1994)	Longitudinal study; abstract only available. Increase PP.
	40	↓	↓	↑	↑	Ardawi (1997)	Compared with non pregnant controls. Increase trimester 3 & PP.
	16	↓	X	↓	↑	Naylor (2000)	Compared with baseline. OC considered to be unreliable marker.
	22	X	↓	X	↑	Yoon (2000)	Compared with non↓pregnant controls. No change trimester 1 &3; decrease trimester 2.
	962	X	↓	X	-	Sowers (2001)	No background or controls. Decreased trimester 1 to 2, then unchanged.
	14	X	↓	X	↑	Umera (2002)	No controls. Low values. Decrease trimester 2, no change till PP.
	20	-	X	↑	↑	More (2003)	Compared with baseline. All indices increased during pregnancy but didn't reach baseline even after 12 months PP.
							Some results different to other studies.
	15	↓	X	↑	↑	Ulrich (2003)	Compared with baseline & non pregnant controls. Decrease baseline to trimester 2; baseline again trimester 3.
	95	X	↓	↓	-	Ainy (2006)	Compared with non pregnant controls. Higher trimester 1 compared with trimesters 2 & 3. Significant difference between trimesters 2 & 3.
	78+	↑	↓	X	-	Dorota (2012)	Cross-sectional study. Comparison with nonpregnant subjects. Highest values trimester 1, decreases in trimesters 2 & 3.
	92	↓	↓	X	↑	Möller (2013)	Compared with baseline & non pregnant controls. Decreases trimester 1 & 2.

Bone Formation Markers (measured in serum)

Marker	No. of subjects	Trimester 1	Trimester 2	Trimester 3	Postpartum	Reference	Changes over pregnancy & postpartum (PP) and comment
Alkaline phosphatase							
Bone Alkaline phosphatase	10	?	↓	↑	?	Gallacher (1994)	No data for trimester 1 & PP.
	14	X	X	↑	↑	Uemera (2002)	Increased with gestational age.
	20	-	↑	↑	↓	More (2003)	
	10	X	X	↑	↓	Black (2000)	Compared with baseline. No significant changes in formation markers before week 38.
	16	↓	↑	↑	↓	Naylor (2000)	
	17	X	X	↑	↓	Naylor (2003)	Compared with background.
	15	↓	X	↑	↑	Ulrich (2003)	Maximum trimester 3, decreased PPbut still above baseline.
	27	X	X	X	↑	Kaji (2007)	No controls; compared with bed rest subjects.
	92	↓	↓	X	↑	Möller (2013)	Decreased to baseline trimester 3.
	40	↓	↑	↑	↓	Ardawi (1997)	
Total Alkaline phosphatase	95	X	X	↑	-	Ainy (2006)	Significantly l ower values trimester 1 compared with trimester 3.
	10	X	X	↑	↓	Black (2000)	
	15	X	X	↑	↑	Ulrich (2003)	
Type I collagen propeptides							
Procollagen type I C propeptide	16	↓	X	↑	↓	Naylor (2000)	
	20	-	↑	↑	X	More (2003)	
	10	X	X	↑	↓	Black (2000)	
	17	↑	↑	↑	-	Anim-Nyame (2002)	
	266	-	X	↑	-	Puistola (1993)	Only measured markers PICP & PINP. Only abstract available.
P rocollagen type I N propeptide	16	↓	X	↑	X	Naylor (2000)	
		-	X	↑	-	Puistola (1993)	Increased 2 fold during Tr3; only measured TR2 & 3

Osteocalcin (OC) - a vitamin-K dependent protein that binds to hydroxyapatite in calcified bone; a marker of late stages of bone formation.
Alkaline phosphatase is measured as total ALP or bone specific ALP (boneALP).
PICP is procollagen type I carboxy-terminal (C) propeptide, derived from type I collagen during its post-translational modification into the tripeptide helical form of type I collagen.
PINP is procollagen type I N propeptide, also derived from type I collagen during its post-translational modification into the tripeptide helical form of type I collagen.
N denotes number of subjects; X denotes no change; - denotes not measured

Bone Resorption Markers

Marker	No. of subjects	Trimester 1	Trimester 2	Trimester 3	Postpartum	Reference	Changes over pregnancy & postpartum (PP) and comment
Pyridinoline (PYD) /Deoxypyridinoline(DPD)							
	10	?	?	?	?	Gallacher (1994)	PYD.
	10	↑	↑	↑	↓	Black (2000)	3 types of PYD; (data for Free PYD).
	10	↑	↑	↑	↓	Black (2000)	Total PYD data.
	16	↓	↑	↑	↑	Naylor (2000)	3 types of PYD & PYD(data for Total PYD).
	22	-	↑	↑	↑	Yoon (2000)	DPD.
	20	-	X	↑	↓	More (2006)	DPD.
	230+	X	↑	X	↑	Yamaga (1997)	PYD (Cross sectional study; comparison with non pregnant subjects).
	230+	X	X	↑	↑	Yamaga (1997)	DPD.
Type I collagen telopeptides							
C-terminal crosslinking telopeptide (CTX-I)	230+	X	X	↑	X	Yamaga (1997)	
	16	X	↑	↑	↓	Naylor (2000)	
	17	X	X	↑	↑	Naylor (2003)	Note difference between the 2 Naylor studies.
	27	X	↑	↑	↓	Kaji (2007)	No baseline or non pregnant controls.
	78+	X	↑	↓	-	Dorota (2012)	Trimesters 1 & 3 same. Similar results for OPG & RANKL (Cross-laps).
ICTP (CTX-MMP)	14	X	X	X	↑	Uemura (2002)	No change until PP.
	15	↑	X	↑	↑	Ulrich (2003)	
	17	↑	↑	↑	-	Anim-Nyame (2002)	
N-terminal crosslinking telopeptide (NTX)	230+	X	↑	↑	X	Yamaga (1997)	

	193	X	↑	↑	-	Tellez-Rojo (2004)	No comparison with baseline or non pregnant controls.
	92	↑	↑	↑	↓	Möller (2013)	Peak NTX in trimester 3.
	10	↑	↑	↑	↓	Black (2000)	All resorption markers show similar patterns.
	17	↑	↑	↑	↓	Naylor (2003)	
	16	↓	↑	↑	↓	Naylor (2000)	
	15	X	↑	↑	↓	Ulrich (2003)	Peak NTX in trimester 3.
	27	X	↑	↑	↓	Kaji (2007)	

ICTP Serum Cross-linked telopeptide type I collagen
CTX Serum C-terminal telopeptide type I collagen CTX and ICTP recognize different segmental domains of the C-terminal telopeptideregion of type I collagen and respond differently to bone metabolic processes[18]
NTX cross-linked N-telopeptide of type I collagen (measured in urine) -based on an antibody against the N-terminal of collagen, including the crosslinking region1
Pyridinium cross links -deoxypyridinoline (Dpy), pyridinoline (Pyr) stabilise the collagen triple helix in mature bone, cannot be metabolised and areexcreted in urine

Other Bone Mineral MetabolismParameters

Parameter	No. of subjects	Trimester 1	Trimester 2	Trimester 3	Postpartum	Reference	Changes over pregnancy & postpartum (PP) and comment
Insulin-like growth factor I(IGF-I)							
	92	↓	↓	↑	↓	Möller (2013)	Peak in trimester 3.
	16	↓	↑	↓	↓	Naylor (2000)	Peak in trimester 1 compared with baseline.
	10	X	X	↑	↓	Black (2000)	
	962	X	X	↑	-	Sowers (2001)	
Parathyroid hormone (PTH)							
Intact	14	X	X	X	X	Uemera (2002)	No signifi cant change.
	20	-	↑	X	X	More (2003)	No significantincrease during pregnancy.
	92	↓	↓	↓	↑	Möller (2013)	Decreased.
	16	↓	↓	X	↑	Naylor (2000)	Decreased trimester 1 compared with baseline.
	10	X	↓	↑	X	Black (2000)	Trimester 1 & 2 bone remodeling uncoupled, bone resorption greater. Bone formation not until trimester 3.
PTHrP	10	X	X	X	X	Black (2000)	No change.
	95	X	X	X	-	Ainy (2006)	No change.
Intact PTH	40	X	↑	↑	↓	Ardawi (1997)	Intact PTH
	10	X	X	X	↑	Gallacher (1994)	
Osteoprotegrin (OPG)							
	14	X	↑	↑	↓	Uemera (2002)	Increased with gestational age.
	78+	X	↑	↓	-	Dorota (2012)	RANKL Tr1&3 same
	393	X	X	↑	-	Hong (2005)	Cross sectional.
	17	↑	↑	↑	↓	Naylor (2003)	
p-estradiol (E2)							
	92	↑	↑	↑	↓	Möller (2013)	Increased during pregnancy, maximum at delivery, back to baseline PP.
	17	↑	↑	↑	↓	Naylor (2003)	
	15	↑	↑	↑	↓	Ulrich (2003)	

Other Bone Mineral MetabolismParameters

Parameter	No. of subjects	Trimester 1	Trimester 2	Trimester 3	Postpartum	Reference	Changes over pregnancy & postpartum (PP) and comment
Vitamin D parameters							
Calcitriol	14	↑	↑	↑	↓	Uemera (2002)	$1,25(OH)_2D_3$; increasedwith pregnancy.
	92	↑	↑	↑	↓	Möller (2013)	$1,25(OH)_2D_3$, increased with pregnancy.
	40	X	↑	↑	↓	Ardawi (1997)	$1,25(OH)_2D_3$
Calcidiol	95	X	X	X	-	Ainy (2006)	$25(OH)D_3$
	40	X	X	↓	X	Ardawi (1997)	$25(OH)D_3$
	20	-	X	X	X	More (2003)	$25(OH)D_3$No significantincrease in pregnancy.
Calcitonin	40	X	↑	↓	X	Ardawi (1997)	
	92	↓	X	X	X	Möller (2103)	
Calcium							
	10	X	X	X	X	Gallacher (1994)	
	10	X	↓	X	X	Black (2000)	
	14	X	X	X	X	Uemera (2002)	
	20	X	X	X	X	More (2003)	
	15	↓	X	X	↑	Ulrich (2003)	
	92	X	X	↑	X	Möller (2013)	
	16	↑	X	X	↓	Naylor (2000)	
Phosphorous							
	14	X	X	X	↑	Uemera (2002)	
	20	X	X	X	↑	More (2003)	
	92	↑	X	X	↑	Möller (2013)	

[13], increased mobilization of skeletal calcium would appear to be the only source for sufficient calcium for breast-milk secretion [14,15,16,17]. One flaw that does exist is that many of the studies did not have preconception measurements for comparison in each individual subject studied.

Bone formation markers may be enzymes or other proteins, measurable in serum/plasma and associated with osteoblast function, or may reflect the formation of type I collagen. During bone formation, procollagen is cleaved at the N-and C-terminal ends and thus, for example, procollagen type I N-terminal propeptide (PINP) reflects the rate of new bone formation [1,18].

Osteocalcin, although one of the 'pioneer' biomarkers of bone formation has fallen out of favor in recent years due to the problems with standardization and the need for prompt and special handling of the specimen due to its instability [1,8,[18]. The most consistent results suggest decreases in osteocalcin in the 2nd trimester compared with 1st trimester in most [2,6] [19,20,4,21,22] but not all studies [10,8,11].

Alkaline phosphatase or bone specific alkaline phosphatase, as with osteocalcin, exhibits considerable variability during pregnancy and also post-partum with no consistency across studies (Table 1).

Procollagen type I carboxy-terminal propeptide, (PICP) is another formation marker derived from type I collagen during its post-translational modification into the tripeptide helical form of type I collagen. Most results for this marker indicate increases for the 3rd trimester compared with the 2nd trimester. Procollagen type I N-terminal propeptide (PINP) is another formation marker derived from type I collagen during its post-translational modification into the tripeptide helical form of type I collagen. A two-fold increase between the 2nd and 3rd trimesters was reported in two studies [8] [23] and no change at 2 weeks post-partum compared with the end of the third trimester.

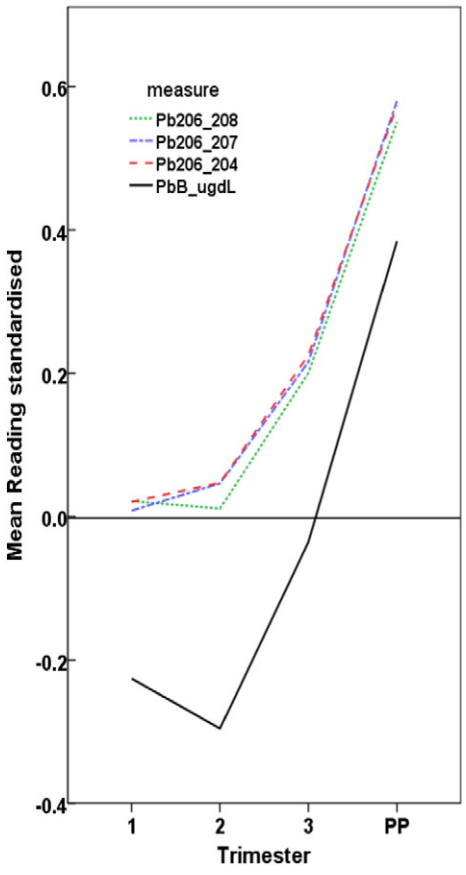

Fig. 1. T=Standardized values of the four Pb measurements by trimester for the Low Ca cohort showing minimal skeletal Pb mobilization between the 1st and 2nd trimesters and rapid increase after the 2nd trimester. PP denotes post-partum.

Four markers commonly used to assess bone resorption: pyridinoline, N-terminal crosslinking telopeptide (NTX), C-terminal crosslinking telopeptide of type I collagen (CTX-I) and C-terminal crosslinking telopeptide of type I collagen generated by matrix metalloproteases (CTX-MMP or ICTP) are relatively specific for bone [1] [18]. During bone resorption, post-translationally modified fragments of type I collagen that cannot be reduced to their native amino acids and 'recycled', enter the circulation, and are excreted in urine in the case of NTX and CTX measurements. Similarly, pyridinium crosslinking molecules stabilize the collagen triple helix in mature bone, cannot be metabolized and are excreted in urine [1]. As with the bone formation markers, there is considerable variability in outcomes for the bone resorption markers during pregnancy (Table 1). Three of the four CTX studies showed increases in the 3rd trimester compared with earlier trimesters but variability post-partum. Two of three ICTP studies found increases for the 3rd trimester and post-partum. NTX studies reported increases in the 2nd and 3rd trimesters but decreases post-partum (Table 1). All of the *pyridinium crosslinks* studies reported decreases [3,11] or increases [8,21,24] post-partum compared with the 3rd trimester. Further discussion of bone markers is given in the Supplementary Notes.

1.1. Lead and lead isotope tracking of bone turnover

Given these conflicting findings, there is a need for another approach that would not be affected by differences in metabolism or hemodilution. The use of Pb concentrations and Pb isotopic tracing is one such alternative assessment. Being similar chemically to calcium, the main repository for Pb in the human body is in the skeleton and for an adult, 90% of the body burden of Pb resides in the skeleton [25]. Although the skeleton has been considered to be a 'safe' repository for Pb, it has been demonstrated from several studies (see following), that Pb can be mobilized from the skeleton during pregnancy and lactation as well as menopause, extended bed rest and weightlessness. The critical evidence for mobilization of skeletal Pb comes from studies employing the stable Pb isotopic tracing method.

2. Material and methods

2.1. Lead and Pb isotopic composition

Lead in the environment is dominated by industrial sources that in turn have been derived from relatively few mineral deposits, most of which have distinctive isotopic patterns (signatures or fingerprints). People growing up in a particular environment will take Pb into their bodies with a signature characteristic of that environment. A proportion of this Pb will be incorporated into their skeletons that are then effectively labeled with this signature. If an individual moves to an environment with a different Pb signature, the original signature can be detected in the individual's blood for many years because Pb is released from the skeleton as part of normal bone remodeling. What is measured in the blood (and urine) is a mixture of the signatures from the new and old environment. Any process that affects bone turnover rate will affect the proportions of the two signatures. In addition to changes arising from different environments and the levels of Pb in blood (Blood Pb), the Pb isotopic measurements incorporate an "in-built" control as there are 4 isotopes of Pb which are typically measured and expressed as $^{208}Pb/^{206}Pb$, $^{207}Pb/^{206}Pb$ (or their inverse) and $^{206}Pb/^{204}Pb$ ratios. High precision isotopic measurements as reported in this paper are measured by thermal ionization mass spectrometry (TIMS) but can now also be measured by multi-collector inductively coupled plasma mass spectrometry (MC-ICP-MS). Details of the analytical methods are described previously [27,28].

The prevalence of geologically old Pb (~1700 million years old) in the Australian environment has resulted in a unique isotopic fingerprint or signature, generally not available on other continents. We have

established from blood analyses of almost 300 subjects that the Pb isotopic signature in subjects from other countries is significantly different from those of multi-generational Australian residents [27]. By monitoring the Blood Pb isotopes of immigrant subjects as soon as possible after arrival in Australia, it is possible to detect changes in isotopic composition and Blood Pb concentration related to mobilization of skeletal Pb during pregnancy and lactation [26,27] and aging [28]. It is also possible to quantify the extra amount of Pb released (flux) from the skeleton during pregnancy and lactation [29]. We studied 31 female subjects from mostly European countries whose Pb isotopic signature in their skeleton and their blood was imprinted with Pb exposure from different sources to that prevailing in the Australian subjects. Estimates of their skeletal Pb isotopic signature can also be obtained from analyses of tooth Pb. In earlier isotopic investigations we demonstrated that from 42 to 70% of the Pb in blood in female adults under equilibrium conditions was derived from their skeleton [30]. Although lead in blood may change because of inputs from the environment and diet, these inputs were monitored at the same time as blood and urine sampling by collection of ambient air, long-term dust accumulation (3-monthly) in the residence, soil and dietary intake by a 6-day duplicate diet and tap water.

2.2. Study group

In a long-term investigation on the biokinetics of lead in human pregnancy, changes in Blood Pb concentrations and Pb isotopic ($^{206}Pb/^{204}Pb$) ratio were monitored on a monthly basis during pregnancy and for 6 months post-partum in two immigrant cohorts and these changes were compared with an Australian control group (n = 6) who were sampled quarterly [27] [26]. One cohort of immigrant subjects to Australia was provided with a daily 1000 mg calcium supplementation (Calcium Supplement group; n = 10) and the other immigrant cohort, whose daily calcium intake was about 500 mg (Low Calcium group; n = 15), was not supplemented. In all except 3 cases, blood, urine and environmental sampling was possible preconception.

2.3. Statistical analyses

The changes in the four types of measurement ($^{206}Pb/^{204}Pb$, $^{206}Pb/^{208}Pb$ and $^{206}Pb/^{207}Pb$ and Blood Pb (in μg/dL) over trimesters 1, 2 and 3 and post-partum were investigated using a mixed model (SPSS v.22).

The measurements were standardized so that the values for each of the four types of measurement had a mean of zero and standard deviation of one for each subject. This meant that there was no overall difference between the means of the different measurements or the means for the groups but that overall changes over time could be assessed, along with the interaction contrasts for the two-way (measure by trimester) and three-way (group by measure by trimester) interactions. As the standardization resulted in zero intra-class correlation with respect to the participants, no random factor was necessary in the mixed analysis. Only the Low Calcium and Calcium Supplement groups (see below) were included in the analyses with results for the native-born Australian women shown for comparative purposes (see Fig. 3).

Informed consent was obtained for these studies.

3. Results

3.1. Lead as an indicator of bone remodeling

Changes in Blood Pb concentration (PbB_μgdL) and patterns for the 3 sets of isotopic composition (Figs. 1 to 3) are similar. Small perturbations to this uniformity, such as in Fig. 2, is usually in the $^{206}Pb/^{204}Pb$ ratio which is almost 2 orders of magnitude larger than the

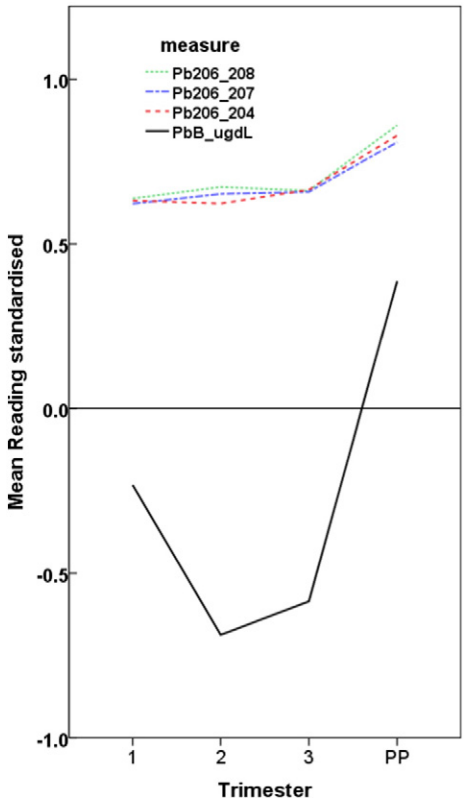

Fig. 2. Standardized values of the four Pb measurements by trimester for the Calcium Supplemented cohort showing minimal skeletal Pb mobilization during pregnancy and the increase during post-partum. PP denotes post-partum.

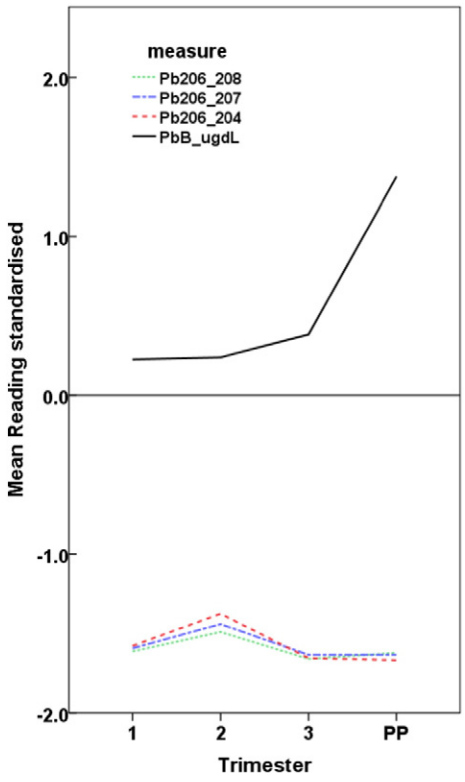

Fig. 3. Standardized values of the four Pb measurements by trimester for the Australian pregnant cohort. The increases in the isotopic values for the 2nd trimester are interpreted as intergenerational skeletal Pb mobilization in two subjects whose parents came from Europe. PP denotes post-partum.

$^{206}Pb/^{207}Pb$ and $^{206}Pb/^{208}Pb$ ratios and has less precision. The similar isotopic patterns in the figures reinforce the reliability of the isotopic approach. A lack of change or decrease in slope indicates there is negligible or limited skeletal mobilization of Pb whereas an increasing slope indicates skeletal Pb mobilization.

The 3 groups showed significant increases in Blood Pb during post-partum compared with during pregnancy, consistent with increased skeletal resorption. During pregnancy there is a small decrease in Blood Pb from the 1st to 2nd trimester for the Low Ca group (Fig. 1) but a much larger decrease for the Ca supplemented group (Fig. 2), consistent with other studies that only measured Blood Pb concentrations [31–44] [45]. Of interest, there was no change in Blood Pb for the Ca supplemented group from the 2nd to 3rd trimester compared with a small increase for the Low Ca group. Similarly, increases in Blood Pb occurred between the 2nd and 3rd trimesters at about 6 to 8 months in all of the Calcium Supplemented subjects. In contrast, subjects from the Low-Calcium cohort showed an increase in Blood Pb from the minimum value that was observed at 3–4 months. Hemodilution has been considered an issue in measurements of Blood Pb concentrations and can be corrected using measurements of haematocrit. In the Calcium Supplemented cohort, the changes in PbB during pregnancy were obvious whether the PbB values were corrected or uncorrected for haematocrit [46].On the other hand, the Pb isotopic ratios are unaffected by hemodilution.

As with Blood Pb, there was a significant difference between the results of Pb isotopic ratios at post-partum and each of the other three times reflecting the increased skeletal mobilization of Pb during post-partum. The mixed model analyses showed that the main effect of trimester was significant ($p < 0.001$) and there were also significant interaction contrasts between trimester and isotopic ratios reflecting the isotopic changes between the 1st and 2nd and 1st and 3rd trimesters. For the Low Calcium cohort the average change in the isotopic ratios from the 1st trimester to the 2nd trimester is small to insignificant (Fig. 1) and is interpreted to indicate minimal skeletal remodeling. In contrast, the significant increases in isotopic ratios from the 2nd to 3rd trimester and the 3rd trimester to post-partum are attributed to skeletal remodeling. As pregnancy progressed, the contribution of skeletal Pb as indicated by the isotopic ratios and Blood Pb showed a linear increase for subjects in the Low-Calcium cohort (Fig. 1). In contrast to the Low Calcium cohort, the isotopic ratios for the Calcium Supplemented cohort increase in the 1st to 2nd trimester, then level off or show small increases and during post-partum show consistent increases associated with skeletal mobilization.

In the immigrant cohorts, changes in Blood Pb and isotopic ratio from late pregnancy through the 6 months post-partum did not appear to be related to length of breast-feeding, calcium intakes or resumption of menses [47,48,49]. When an equilibrium was re-established post-pregnancy, the contribution of skeletal Pb to Blood Pb ranged from 60 to 80% with the remainder deriving from other sources such as air, dust, and diet.

3.2. Bone type and Pb during pregnancy and lactation

Bone mineral density and biochemical markers of bone turnover and especially resorption indicators during pregnancy and lactation are influenced by bone type with much slower exchange from cortical than trabecular bone (see Supplementary notes).

3.3. Epigenetics and skeletal mobilization of lead during pregnancy and post-partum

The term epigenetics refers to heritable changes in gene expression that does not involve changes to the underlying DNA sequence (What is Epigenetics 2015). DNA methylation, whereby a methyl (CH_3) group from S-adenosylmethionine is added to a cytosine nucleotide or lysine or arginine residue, is one process that is thought to initiate and sustain epigenetic change. An individual's global DNA methylation profile can be affected by coming into contact with metal toxicants such as lead (Pb) [50] and arsenic [51,52]. Wright and colleagues [50] reported an "inverse correlation between patella bone Pb levels and global DNA methylation of LINE1 repeat elements in umbilical cord blood (UCB), suggesting that methylation might serve as a marker for past Pb exposure." Global expression patterns and their correlation with DNA methylation in a mouse model of prenatal exposure to Pb revealed significant association between an increase in DNA methylation and transcriptional repression of genes associated with immune response, metal binding, metabolism and transcription/transduction coupling [53]. In recent studies Pb-exposure caused locus-specific changes in DNA methylation detectable in dried blood spots [54] and in human embryonic stem cells [55].

Previously the impact of various environmental exposures on DNA methylation had not been demonstrated in humans beyond one generation. However in their study of 35 mother-infant pairs, Sen et al. [56] found that elevated Pb in the mothers' blood resulted in changes in DNA methylation of her children and grandchildren, as assessed in dried blood samples from the Michigan Neonatal Bank.

As shown above the evidence for skeletal Pb mobilization during pregnancy and post-partum was definitive for both immigrant cohorts in that skeletal Pb from other countries was detected in their blood and urine. On the other hand, it was predicted that the data for long-term Australian subjects should have shown little or no change in their isotopic signature although the blood Pb would show increases during the third or possibly second trimester as has been demonstrated in several studiesas seen in as seen in Figs. 1 and 2. However, in three subjects there were unexpected changes in isotopic ratios which were indicative of European Pb (Fig. 1). Similar changes were detected in 24 h urine samples which better define body burden of Pb compared with 'spot' urine samples. In tracing the history of the mothers of the three subjects it was found that they came from Europe, one each from Holland, Poland, and Russia consistent with multi-generational passage of lead associated with pregnancy as found by Sen et al. [56].

These data are consistent with multi-generational passage of lead and potential effects on DNA methylation with its known effects on biology and biological versus chronological age. Further evidence for these processes could be obtained from the children of the cohorts.

4. Discussion

The variability in outcomes for the bone formation and resorption markers (osteocalcin, BAP, PICP, PINP) for the 1st and 2nd trimester preclude any definitive statement about bone remodeling during pregnancy and past-partum using different markers. While most studies suggest increased bone resorption during the 3rd trimester, some do not [4,6]. The overall conclusion from the biochemical markers is that during pregnancy bone turnover is increased with the increase in bone resorption preceding that of formation [8].

The 2nd trimester results for Blood Pb show decreases suggesting that there is little skeletal resorption. However, all Pb parameters show significant and consistent increases in the 3rd trimester compared with the 2nd trimester indicative of increased mobilization of Pb from the skeleton. If there is bone formation taking place, as suggested by the bone formation markers, then this is subservient to resorption. There are increased amounts of Blood Pb and increases in the isotopic ratios during post-partum compared with the 3rd trimester indicative of increased mobilization of Pb from the skeleton during this period. The timing of increase in Blood Pb is important for fetal exposure. Even though there is an increase of similar magnitude in Blood Pb from a minimum value during pregnancy in both the migrant cohorts, the fetus in the group receiving calcium supplements would be exposed to considerably lower Pb flux (mean 145 μg Pb) than those whose mothers had a low calcium intake (mean 330 μg Pb) [29]. A reduction in Pb flux was noted in cynomolgous monkeys [57] and in one human subject in another study [58]. In this regard, increases in isotopic ratios during the 2nd trimester were observed for three Australian-born mothers (Fig. 3) whose own mothers were migrants from other

countries, viz. Holland, Poland and Russia. These changes demonstrate the sensitivity of the Pb isotopic tracing method and indicate multi-generational transfer (epigenetic transfer) during pregnancy and/or lactation with obvious public health significance.

The agreement between the isotopic ratios shows that it is not necessary to measure all 3 ratios. Where there are large changes in isotopic ratios, the measurements, for example of only the $^{206}Pb/^{207}Pb$ ratio, could be assessed more rapidly using inductively coupled plasma mass spectrometry.

These Pb studies are consistent in showing an increase in bone resorption at least from the 2nd trimester onwards and during lactation. The Pb approach offers a powerful tool in studies of bone remodeling during aging and osteoporosis as shown by an earlier pilot study [28]. Interestingly, the increase during lactation appears to be relatively insensitive to dietary calcium intake. The mobilization of maternal skeletal calcium has public health implications. The data from the Australian-born mothers of migrant mothers provide evidence of transgenerational transfer of the skeletal Pb burden with obvious public health implications. The significance of these findings in mothers with high skeletal Pb burdens in relation to fetal and neonatal Pb exposure needs further consideration and study.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at http://dx.doi.org/10.1016/j.bone.2016.05.005.

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