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Radiofrequency echographic multi-spectrometry for early bone health: The REMS-bone study protocol (Trial acronym: REMS-bone)

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ABSTRACT

Background: Bone health begins with maternal health and nutrition, which influences skeletal mass and bone mineral density (BMD) already in utero. Maximization of bone mass during skeletal growth has become the goal of primary prevention of osteopenia and osteoporosis. The amount of bone gained during growth and its subsequent rate of loss are closely linked to the final skeletal mass in adulthood. Radiofrequency Echographic Multi Spectrometry (REMS) technology has proven to be useful in the assessment of BMD in pregnant women. However, the feasibility of REMS for the assessment of bone status in newborns remains unknown. This multicenter longitudinal study aims at using REMS to evaluate skeletal status in fetuses, newborns and children until 12 months of age.

Methods: Two hundred mother-newborn dyads, with infants born at term of gestation, will be consecutively recruited during the prenatal period and followed up until 12 months of life. BMD will be assessed with REMS technology in mothers, fetuses, newborns and infants at 1, 3 6 and 12 months of life. In all enrolled patients, blood will be collected at specific time points and oxidative stress biomarkers, specific microRNAs, and several bone metabolites will be measured in blood, whereas endocrine disruptors will be measured in urine.

Discussion: This study is designed to provide robust data on the best method to identify and evaluate bone status starting from intrauterine life. The associations among BMD, maternal nutrition, early exposure to endocrine disruptors, and other investigated molecules will also be investigated in relationship with subsequent body composition and bone health.

Trial registration: The protocol was retrospectively registered on ClinicalTrials.gov on December 3rd, 2024, with the ID number NCT06750523.

Introduction

Maximization of bone mass during skeletal growth has become the goal of primary prevention of osteopenia and osteoporosis. The amount of bone attained during growth and its consequent rate of loss is strictly related to the skeletal mass acquired at the beginning of adulthood.[1]

Any factors that might influence peak bone mass is important in determining the individual's risk of developing osteoporosis later in life.

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Bone health begins with maternal health and nutrition, which influences skeletal mass and bone density already in utero.[2]

Peak bone mass acquisition depends also on the genetic influence from both parents[3]. To date, some evidence suggests that the risk of osteoporosis later in life may be determined by environmental factors acting during intrauterine or early postnatal life. The mechanisms underlying the long-term effect of the intrauterine environment on bone health are currently unknown but definitely include 'fetal programming' of oxidative stress and endocrine systems. The risk of osteoporosis later in life is likely strongly determined by environmental influences during intrauterine or early postnatal life.[4; 5]

Change in bone mass density/composition involves mechanisms and pathways that aren't currently well defined. Among these exposure to endocrine disruptors (EDCs) plays a role[6]. EDCs are chemicals that interfere with the normal functioning of the endocrine system; they can mimic, block, or alter hormone signaling, potentially leading to adverse effects on growth, reproduction, metabolism, and other physiological processes.[7] EDCs are found in several natural and synthetic sources, including pesticides, plastics, personal care products, and industrial.[8] Changes in BMD or bone composition involve mechanisms and pathways that can be influenced by early exposure to EDCs. The action of EDCs during fetal and neonatal life is mainly exercised via epigenetic regulation of metabolic and endocrine pathways[6]. Among these, oxidative stress and regulatory mechanisms of gene expression, and the miRNA network, are of upmost importance[4]. The molecular mechanisms by which miRNAs exert their regulatory role in longitudinal bone growth are involved with the regulation of cell growth, particularly of chondrocytes.[5] In addition, exposure to EDCs at critical ages negatively impacts trabecular microarchitecture and cortical geometry in adulthood..[9, 10]

MicroRNAs (miRNAs) are small, non-coding RNA molecules that regulate gene expression post-transcriptionally by binding to messenger RNA (mRNA) and preventing its translation or promoting its degradation[11]. Multiple changes in miRNA abundance can occur, where simultaneously up- and down-regulated miRNAs can target the same gene with a range of predicted effects and, vice versa, a single miRNA can regulate several target genes.[12]

These molecules play a crucial role in regulating gene expression during pregnancy and are emerging as key players in fetal BMD development. MicroRNAs contribute to the tightly controlled regulation of pathways that govern skeletal development, such as osteoblast differentiation, extracellular matrix deposition, and mineralization.[13; 14; 15] Changes in miRNA expression due to maternal factors, such as poor nutrition, stress, or exposure to environmental toxins, can disrupt these processes, potentially impairing fetal bone formation. For example, aberrant miRNA activity can influence signaling pathways like Wnt/β-catenin, BMP, and RANKL/OPG, which are critical for bone development.[13]

A miRNA profiling study on micro-dissected individual growth plate zones in rats showed differential expression of 34 miRNAs between the proliferative and the hypertrophic zones suggesting that this expression pattern of miRNAs may be involved in the control of proliferative and differentiative mechanisms which regulate the cell fate of the specific growth plate zones[16].

We have recently proved that three specific miRNAs that regulate genes involved with bone growth are directly regulated by growth hormone, and of great interest with respect to longitudinal growth, namely miR-199a-5p, miR-335-5p, and miR-494-3p. With regard to the function of these specific miRNAs, miR-199a-5p and miR-335-5p, play a role in bone formation and osteoblast differentiation in vitro. In particular, miR-199a-5p is involved in osteoblast differentiation and its up-regulation increases alkaline phosphatase activity, calcification, and the expression of osteoblast differentiation markers such as Runx2, Osterix and Osteocalcin in human bone marrow stem cells.[17] The overexpression of miR-335-5p has been reported to promote bone formation and regeneration in a transgenic mouse model.[18] A previous study from this same group highlighted that miR-335-5p reduced the expression of DKK1, an inhibitor of Wnt signaling pathway, which is pivotal for bone development.[19] Moreover, miR-335-5p overexpression has been shown to promote chondrogenic differentiation of mesenchymal stem cells.[20]

MiR-494-3p has not been studied yet in the context of bone or growth plate development, however it has been reported to promote PI3K/AKT pathway hyperactivation in hepatocellular carcinoma by targeting PTEN.[21] PI3K/AKT pathway is known to control hypertrophic chondrocyte differentiation and to be involved in endochondral bone growth,[22] and promotes osteoblast differentiation.[23]

Interestingly, a miRNA profiling study on micro-dissected individual growth plate zones in rats showed differential expression of 34 miRNAs between the proliferative and the hypertrophic zones. The authors suggested that this expression pattern of miRNAs may be involved in the control of proliferative and differentiative mechanisms which regulate the cell fate of the specific growth plate zones. Moreover, the authors showed that these distinct patterns in growth plate were influenced by PTHrP concentration gradient across the zones. These findings definitely suggested that the mechanism of action of PTHrP in the control of the growth plate cell function was mediated, at least in part, by miRNAs, and the miRNAs of greater interest were mir-369-3p, mir-374-5p, mir-379-5p, and mir-503-5p.[16]

Other studies showed the histological cartilage sections from Dicer-null mice showing a reduction in proliferative chondrocytes and an expansion of the hypertrophic region being dicer is a key enzyme for miRNA biogenesis. In this study, specific miRNAs (miR-140 and let-7 family) were identified to play a key role in this process. Specifically, miR-140 null mice showed an acceleration of hypertrophic differentiation of chondrocytes with advancing endochondral bone formation as a consequence[24]. Conversely, the suppression of let-7 in another mouse model, reduced growth plate chondrocyte proliferation and increased cell death causing mild growth.[25] Finally, the mice with both miR-140 deficiency and let-7 downregulation presented severe growth deficiency, similarly to Dicer-null mice, suggesting a pivotal role for the above-mentioned miRNAs among others in growth plate development.[13; 25]

Understanding the role of miRNAs during pregnancy offers potential avenues for therapeutic intervention to enhance bone health, such as targeting specific miRNAs to optimize fetal skeletal development.

Studies performed by Basu and coworkers established a biochemical link between increased oxidative stress and reduced bone density and provide a rational for this project investigating the role of pro- and antioxidants in bone mineral density[26].

Oxidative stress in pregnancy impacts on fetal growth development and newborn infants are especially prone to oxidative stress harmful effects.[27]

Melatonin, a potent antioxidant, has been shown to prevent bone destruction in mice with retinoic acid-induced osteoporosis.[28]

Exposure to oxidative stress at critical ages has been related with the development of obesity and metabolic syndrome, that are related with changes in bone composition.[29] Osteopenia is recognized with increasing frequency in low-birth weight newborn infants.[30] Intrauterine growth restriction also increases the risk of obesity and metabolic syndrome which dramatically affect bone quality. Micro RNA and long non coding RNA have recently come into the spotlight due to their ability to control gene expression at the post-transcriptional level providing epigenetic modification and targeting the growth plate.

These considerations highlight that the evaluation of skeletal status from perinatal period is essential for both the early detection of the conditions characterized by defects in bone mass or mineralization and for the monitoring of the bone development process.

It is still unknow if and how fetal BMD varies according to maternal nutritional factors, if and how it impacts on some outcomes at childbirth (e.g spontaneous clavicle or skull fractures during operative birth) and on the long term bone mineralization.

Radiofrequency Echographic Multi Spectrometry (REMS) technology has proven to be useful in the assessment of bone mineral density in pregnant women.[31] Very few studies have reported that transmission ultrasound can be used for the assessment of bone status in newborns. The attractiveness of REMS technology in fetus and newborns stands in the lack of ionizing radiation, ease of use and, above all, the possibility to perform longitudinal studies on REMS patterns from intrauterine to extrauterine life and during the first year of life. New challenge relies in - development of transducers and software specific for fetus and newborns. which will be part of this project.

Starting from the mother's exposome information, the project will unravel the effect of environmental contaminants, and in particular of endocrine disruptors, of oxidative stress and of miRNAs within the field of epigenetics on bone health.

Objectives

A) To determine the feasibility of the use of REMS, as assessed by a new measurement approach to evaluate skeletal status in fetuses, newborns and children as a precise and innovative technology.
B) To assess how mother's REMS patterns, and her anthropometric and gestational features influence REMS parameters in the fetus, newborn and during the first year of life.
C) To quantify miR-199a-5p, miR-335-5p, and miR-494-3p, mir-369-3p, mir-374-5p, mir-379-5p, and mir-503-5p, miR-140, and Lin28a (Let-7 family) and oxidative stress biomarkers in cord serum in order to assess relationships with fetal ad newborn bone status, and with changes in bone parameters established by using the REMS technique throughout the first year of life.
D) To asses exposure to endocrine disruptors, and its relationships with oxidative stress, and mother's nutritional habits, as part of the in utero exposome, and with the specific miRNAs, and subsequent body composition and bone health.

Methods: participants, interventions and outcome

This is a multicenter longitudinal prospective study as Trial registration ClinicalTrials.gov NCT06750523. Registered on December 3rd, 2024.

The Azienda Ospedaliera Universitaria di Parma and Azienda Ospedaliera Universitaria di Messina are involved in the recruitment of mother-child dyads.

Two hundred mother-newborn dyads, with infants born at 37 weeks ± 0 days – 41 weeks± 6 days of gestation, will be consecutively recruited during the prenatal period.

Elegibility criteria

Inclusion Criteria:

Full-term newborns from low-risk pregnancies

37–42 weeks of gestational age

Singleton pregnancy

Absence of current or previous maternal conditions that could potentially interfere with bone metabolism (kidney or liver diseases)

Absence of maternal motor disabilities

No previous history of bone fractures or recent traumatic fractures (in the mother)

Maternal age >18 years

Absence of a diagnosis of osteopenia or osteoporosis according to the criteria of the Italian Society for Osteoporosis, Mineral Metabolism, and Skeletal Diseases (SIOMMMS)

Exclusion Criteria:

Preterm newborns

Newborns hospitalized from birth for specific conditions

Newborns with metabolic disorders

Newborns with genetic syndromes

In all enrolled patients, biological samples will be collected at specific time points: maternal peripheral blood at delivery, cord blood, neonatal peripheral blood at 48 hours of life, maternal urine at delivery, and neonatal urine at 1 and 3 months of life. Blood samples will be analyzed for miRNA expression and biomarkers of oxidative stress, while urine samples will be used for endocrine disruptor assays.

Who will take informed consent?

The fully trained specific researchers who are on-site will obtain informed prospective consent from participants. Written and oral information will, whenever possible, be offered to parents prior to birth when the mother is near term of gestation. Informed written consent will be signed by both parents, and sufficient time will be provided for consent. If parents do not speak the local language, consent will only be obtained if an independent interpreter is available. The informed consent will be obtained by the principal investigator of each participating center and their collaborators in charge. A senior investigator will be always available to discuss concerns raised by parents or clini- cians during the course of the trial. The subject's parents or legal representatives will be provided with comprehensive oral and written information regarding the nature, purpose, potential consequences, and possible risks of the clinical study. They will be informed of their right to withdraw the subject from the study at any time without incurring any disadvantages. Moreover, a specific questionnaire, administered to the mothers, will establish the possible exposure based on nutrition, clothing, use of cosmetics and detergents, upholstery, etc. Additionally, they will receive detailed information about how personal and health-related data of both the subject and themselves will be collected, processed, and used during the study, with assurances that the subject's identity and medical information will remain confidential

Interventions

Pre and postnatal assessment of the bone mineral density of the femur of mothers, fetuses, newborns and infants

As the novel application of REMS technology in the fetal and neonatal population proposed in this project requires the preliminary construction of a reference database (from which the corresponding reference curves will be in turn extracted), population-based data will be gathered (i.e., without adopting exclusion criteria based on possible bone-related disorders), which is the approach adopted in the National Health and Nutrition Examination Survey (NHANES) III database,[32] and is considered also as the best way to collect reference data in the field of bone densitometry.[33]

The first 100 enrolled fetuses will be included in the reference database. The same approach will be applied to the newborns, for which, obviously, the first 100 acquired cases will correspond to the first 100 enrolled fetuses.

The size of the sample to be included in the reference database (100 for the fetal database and 100 for the newborn one) was calculated taking into account the conclusions of the work by Hou et al. (2008), according to whom, a suitable reference database can be obtained from a population of 458 women of the same ethnicity aged 6-85 y, in which the most populated age interval includes 44 patients.[34] We considered the fetuses as corresponding to an age interval and each considered newborn/infant age (14 h, 1 month, 3 months, 6 months, 12 months) as corresponding to a different age interval. Then, according to the methodology adopted in the construction of the reference database for REMS technology application in adults[35] we rounded off this value (44) to 50 and multiplied it by a safety factor of 2.

The echographic acquisitions on fetuses and newborns will be performed at each established age by employing dedicated REMS devices provided in a customized research configuration and will take care to anonymize the corresponding datasets. For each considered age interval

a BMD value will be associated to each acquisition and from the first 100 values of each group mean value and standard deviation will be extracted, in order to obtain a 6-point reference curve of BMD as a function of age including fetuses (age 0) and newborns (age from birth to 12 months). The availability of such a curve will allow the expression of the densitometric result in terms of a statistically significant Z-score, which represents the most effective way to assess the bone growth. The whole process will be repeated separately for the male and female cases, in order to obtain a dedicated curve for each sex

Assessment of biomarkers involved in bone mineral density (MicroRNAs, oxidative stress profile, endocrine disruptors)

In cord serum at birth (artery) total RNA will be extracted, and retrotrascribed to cDNA using the cDNA TaqMan TM Advanced miRNA kit (ThermoFisher) following the manufacturer's instructions. The converted cDNA templates will be used for miRNA expression analysis using Real-Time PCR. The expression of the selected miRNAs will be quantified by Taqman Advanced miRNA assay kit (ThermoFisher) using QuantStudio 7 Flex (Applied Biosystem). Data will be normalized using using hsa-miR-16-5p (Assay ID: 477860_mir) as endogenous control. Using the $2-\Delta\Delta Ct$ approach, data will be analyzed and miRNAs with $Ct > 35$ will be classified as not expressed and removed from additional investigation.

Endocrine disruptors (EDCs): A specific mix of endocrine disrupting chemicals will be assayed. Specifically, Bisphenols (4 Chemical compounds) including Bisphenol A (BPA), bisphenol S (BPS), bisphenol F (BPF), and bisphenol F diglycidyl ether (BPFDGE)]; Parabens (7 Chemical compounds): Methylparaben (MePB), Ethylparaben (EtPB), n-Propylparaben (PrPB), iso-Propylparaben (iPrPB), n-Butylparaben (BuPB), iso-Butylparaben (iBuPB)]; Di-esters phthalates with its metabolites, mono-ester phthalates (14 Chemical compounds): Dimethyl phthalate (DMF), Diethyl phthalate (DEP), Dibutyl phthalate (DBP), Butylbenzyl phthalate (BBP), Di(2-ethylexyl) phthalate (DEHP), Di-n-octyl phthalate (DNOP), Monomethyl phthalate (MMP), monoethyl phthalate (MEP), Mono-n-butyl phthalate (MBP), Monobenzyl phthalate (MBzP), Mono(2-ethylexyl) phthalate (MEHP), Mono(2-ethyl-5-hydroxyhexyl) phthalate (MEHHP), Mono(2-ethyl-5-oxohexyl) phthalate (MEOHP), Monooctyl phthalate (MnOP)]; Perfluoroalkyl substances - PFAS (27 Chemical compounds): Perfluoro-n-butanoic acid (PFBA), Perfluoro-n-pentanoic acid (PFPeA), Perfluo-ro-n-hexanoic acid (PFHxA), Perfluoro-n-heptanoic acid (PFPHpA), Perfluoro-n-octanoic acid (PFOA), Perfluoro-n-nonanoic acid (PFNA), Perfluoro-n-decanoic acid (PFDA), Per-fluoro-n-undecanoic acid (PFUnDA), Perfluoro-n-dodecanoic acid (PFDoDa),Perfluoro-n-tridecanoic acid (PFTrDA), Perfluoro-n-tetradecanoic acid (PFTrDA), Perfluoro-1 butanesulphonamide (PFBS), Perfluoropentanesulphonic acid (PFPeS), Perfluorohexanesulphonic acid (PFHxS), Perfluoroheptanesulphonic acid (PFHpS), Perfluo-rooctanesulphonic acid (PFOS), Perfluorononanesulfonic acid (PFNS), Perfluorododec-anenesulfonic acid (PFDS), 2,3,3,3-tetrafluoro-2-(heptafluoropropoxy)propanoic acid (GenX), 4,8-Dioxa-3H-perfluorononanoic acid (ADONA), Perfluo-ro(2-((6-chlorohexyl)oxy)ethanesulfonic acid) (9Cl-PF3ONS), 11-chloroeicosafluoro-3-oxaundecane-1-sulfonic acid (11Cl-PF3OUdS), 3,3,4,4,5,5,6,6,6-nonafluorohexane-1-sulfonic acid (4:2 FTS), 3,3,4,4,5,5,6,6,7,7,8,8,8-tridecafluorooctane-1-sulfonic acid (6:2 FTS), 3,3,4,4,5,5,6,6,7,7,8,8,9,9,10,10,10-heptadecafluorodecane-1-sulfonic acid (8:2 FTS), Per-fluorobutane Sulfonamide (FBSA), Perfluorooctanesulfonamide (FOSA)]. The analysis will be performed by Waters® ACQUITY UPLC System coupled with XEVO TQ-S Detector (UPLC-MS/MS). MassLynx software will be used for data acquisition and analysis of peaks.

Oxidative stress (OS) profile and lipid mediators involved in oxidative stress: will be evaluated both by biomarkers of oxidative protein damage (Advanced Oxidation Protein Products, AOPP) and lipid peroxidation (Isoprostanes, IsoPs, malondialdehyde, MDA). A further evaluation of oxidative stress profile will be performed by measuring non-enzymatic antioxidant molecules (glutathione, GSH) and enzymatic antioxidant molecules (superoxide dismutase, SOD, catalase, CAT, and glutathione peroxidase, GPx). To this end, highly specific and sensitive methods, such as high resolution liquid chromatography (HPLC), and gas chromatography interfaced mass spectrometry (GC-MS) will be employed: HPLC to detect MDA, vitamin E, glutathione, and GSH. The involvement of fatty acid and lipid metabolism will be also investigated by evaluating specialized pro-resolving lipid mediators (Resolvin D1, by ELISA test).

Long-term outcome of enrolled babies

A long-term outcome of all enrolled babies will be performed to unravel the relationships among early exposure to endocrine disruptors, miRNA expression, oxidative stress, and mother's nutrition as part of the in utero exposome, and subsequent body composition and bone health.

At 1, 3-6-12 months of age, length of newborns and infants will be taken on a horizontal harpenden stadiometer, and weight on an electronic scale. Body mass index (BMI) will be calculated accordingly (Kg/m2) and evaluated according to WHO standards (WHO website -https://www.who.int/news-room/fact-sheets/detail/obesity-and-overweight). Head circumference, arm span, diameters of fontanels and pubertal stages will be recorded.

Skinfold thickness will be measured using a Holtain skinfold caliper at the triceps, biceps, subscapular and suprailiac sites. Specific validated algorithms for age will be used to describe subcutaneous body fat distribution.

Specific questionnaires to mothers will be administered at the time of enrollment and the clinical evaluation of the newborn infants at follow-up, to establish any possible exposure to endocrine disrupting chemicals related with nutrition, clothing and use of cosmetics and detergents, upholstery, etc in their houses.

Questionnaires 1 and 2 (mother lifestyle) and 3 (mother-to-child) are an adaptation of validated questionnaires (mother- child) within the previous project "Phthalates and bisphenol A biomonitoring in Italian mother-child pairs: link between exposure and juvenile diseases (LIFE PERSUADED) LIFE13 ENV / IT / 000482, coordinated by the Istituto Superiore di Sanità. They have therefore been reworked on the basis of the recent report commissioned by the European Parliament (https://www.europarl.europa.eu/RegData/etudes/STUD/2019/608866/IPOL_STU(2019)608866_EN.pdf) and current scientific literature, in order to identify possible sources of EDC release (foreseen in the project analyzes) in the usual environment of the mother-child couples recruited for the study. When possible, an e-survey with partially assisted administration will be used for the administration of the Mother and Child questionnaires. The questionnaires will be built as a Google Form (Google Form): this structure allows to build a questionnaire made available through the URL of the page, without the need to install it on a single server.

Any possible relationships among BMD established using the REMS technique, biochemical markers of bone metabolism measured in the mothers just before birth, and in the infants at 6 months of age, and anthropometric measurements will be studied.

All measurements will be related with the miRNAs in cord serum, with the EDCs in mothers urine and in cord serum, and with the biomarkers of oxidative stress in serum. BMD during extrauterine life will be related with fetal measurements. Data in adequate for gestational age infants (AGA), small for gestational age infants (SGA) and large for gestational age infants (LGA) will be compared.

Data management

A customized electronic case report form (eCRF) will be created for the study. Data processing will take place in compliance with current Italian and European legislation regarding the General Data Protection

Regulation. Data sharing with non-European Union centers will be performed according to Standard Contract Clauses. Each participating center will be identified by a three-digit code, and within each center, patients will be identified with a progressive number. Pseudo-anonymized study data will be collected and managed using REDCap electronic data capture tools hosted at Azienda-Ospedaliero Universitaria di Parma.

REDCap is a secure, web-based application designed to support data capture for research studies, providing (1) an intuitive interface for validated data entry, (2) audit trails for tracking data manipulation and export procedures, (3) automated export procedures for seamless data downloads to common statistical packages, and (4) procedures for importing data from external sources[36, 37]. To prevent possible data entry mistakes and to improve data quality, the eCRF will be implemented by design according to validation, branching, and skipping logic quality criteria.

Statistical analysis

Sample size was determined by performing a simulation using a one sided (alpha= 0.025) confidence intervals for Linear Regression Slope test, in which 300 newborns will allow to test if the regression coefficient, which represent the association degree between baseline and the 1 month BMD value, will be at least equal to 1.1 with a lower limit precision distance equal to 0.094. Higher thresholds of coefficient regression were evaluated showing a relative increase of the precision interval. This result was obtained by assuming a correlation value for the covariance matrix of the residual equal to 0.8. Alpha was set to 0.025 to allow a secondary key evaluation with an intermediate time point at 14 hours after birth.

Main descriptive statistics will be adopted to summarize the sample size characteristics in terms of mean and standard deviation for continuous variables and absolute and relative (percentage) frequencies for qualitative variable.

The main hypothesis will be firstly evaluated by means of linear regression model in which the dependent variable is the BMD value at 1 month and the independent regressor is the BMD value at baseline. More complex models comprehensive of mixed effects and repeated measures (MMRM) analysis will be implemented to evaluate the within and between variability components and the evolution of BMD values over time. Multivariable models will be applied to identify independent factors associated (MicroRNAs, Endocrine disruptors and Oxidative stress biomarkers, questionnaire items on nutrition, clothing and use of cosmetics) with the outcome.

To take care of the multicollinearity issue generated in the multivariable model, a LASSO regression will be performed in order to shrink the regression coefficients toward zero by penalizing the regression model with a penalty term.

To further test the ability of significant regressors to predict an increase of BMD values over time, receiver operating characteristics (ROC) curve will be employed categorizing the outcome as a binary variable (BMD increased vs. BMD not increased).

Similar modeling will be employed to analyze long-term outcomes, with Propensity Score Matching adopted to strengthen the comparisons with biochemical measurements.

Given the exploratory nature of the study, additional hypothesis testing and unsupervised clustering analyses that could help identify systematic patterns among patients with similar characteristics will not be precluded a priori.

All the statistical analyses will be performed using R-project and STATA statistical softwares.

Plans to give access to the full protocol, participant-level data, and statistical code

The datasets analyzed during this trial are available from the corresponding author upon reasonable request.

Duration of study

In this study, 200 mother-infants will be recruited. The trial will terminate when the last recruited infant discharged from the hospital will be evaluated for clinical and instrumental outcome at 12 months of follow-up. The planned duration for study is 2 years.

Outcomes

When the study will be concluded, it is expected to have achieved significant innovative tools for the early identification of bone health status in the fetuses and newborns. In this way, the identification which mother-fetus-newborn-infant will benefit from intervention (i.e. vit D supplementation and/or nutritional program), thereby reducing both birth fracture risk and long term adverse outcomes related to low bone density will be available.

In particular, the primary outcome measure will be:

1. Bone mineral density values in fetus, newborn, infant and child, and of their temporal evolution;

   Secondary outcomes:
2. Relationship between the mother, the fetus and newborn bone mineral density
3. Oxidative stress biomarkers measurements
4. EDC measurements
5. 3. Standardization of REMS technology as a tool for bone heath assessment starting from intrauterine life.
6. Identification of microRNAs pathway as biomarkers to assess bone health status and potential new targets for interventional therapy.

Discussion

This study is pioneering in developing and standardizing an ultrasound-based technique to assess BMD from perinatal to post-natal life.

The research study brings together multidisciplinary competencies of excellence, aiming to meet important results that will reach over the medical field, and that may have relevant beneficial consequences on the health system and on the population.

A 2021 cross-sectional study of in-hospital births reported a prevalence rate increase by 23%, from 25.3 to 31.1 per 1000 hospital births.[38] Scalp injuries composed 80% of all birth traumas and increased yearly from 19.87 to 26.46 per 1000 hospital births. Major birth trauma was associated with higher odds of hypoxic-ischemic encephalopathy, seizures, need for mechanical ventilation, meconium aspiration, and sepsis. Length of stay was increased by 56%, and total charges were almost doubled for major birth trauma.

The clinical diagnosis of osteoporosis relies heavily on bone mineral density (BMD) measured at the femoral neck, and although BMD has excellent predictive value for future fractures, the assessment of fracture risk has evolved over the years, resulting in the birth of fracture prediction tools. Fracture risk factors not currently present in these instruments are being studied for the inclusion of environmental factors that affect bone quality even before birth. The data from the knowledge of endocrine molecules and associated pathways triggered by oxidative stress are helping us to understand how to best manage patients from the earliest stages of life. Understanding and improving the current data are critical to setting priorities for action and for tracking progress

The main innovative aspect of the study is to use REMs technology for bone health assessment, starting from fetuses. The project will provide the knowledge of mechanisms involved in perinatal bone growth and mineralization, using, for the first time, a combined clinical and instrumental strategy designed, on the one hand, to identify and evaluate bone status and possible etiopathogenetic factors of birth fracture

in neonate and, on the other hand, to verify and confirm the role of such mechanisms in the instrumental evaluation of bone mineral density. Furthermore, we intend to ascertain the reliability and predictivity of these new markers in our population by clinical and auxological follow up.

Understanding the effects of changes in miRNAs, oxidative stress and endocrine disrupting chemicals on the development of bone mineral density may lead to further research improving preventative and therapeutic measures that can fight osteoporosis, a main important contributor to morbidity and mortality worldwide.

The combined efforts of joint multidisciplinary expertise in this project will get insight on previously unexplored aspects of bone system allowing to follow bone health status from pregnancy to early childhood

This study is pioneering in developing and standardize an ultrasound-based technique to assess the BMD from perinatal to post-natal life. The results are expected to clarify important physio-pathological mechanism which may be implicated in the variation in individual bone mass or fracture risk.

As a first point, it is expected that this study will add important knowledge on the existing crosstalk between the maternal and the fetal bone and on how the exposure to EDCs and OS may interfere on this process since the intrauterine life. Secondly, the post-natal longitudinal assessment is expected to clarify how the antenatal bone mineral status, the biochemical markers and the exposure to EDCs and to the OS correlate with the auxological evaluation and with the bone mineral status during the first year of life. The identification of a specific panel of biomarkers may anticipate the risk of future osteopenia or osteoporosis in the adulthood.

This project may generate new knowledge on the nature-nurture interactions occurring in human infants - during a highly plastic period of development - and the focus on fetus may furthermore highlight how precocious exposures to OS and EDCs can be embedded into the biology of the human infant. Studying the perinatal factors impacting bone health, setting up new technology, easy to use, for the assessment of BMD will be effective in improvements in the quality of care that clinician deliver to infants and their parents.

The impact on economic cost reduction (prevention of birth fracture, osteopenia and osteoporosis) is equally relevant.

The understanding of the bone status before birth will allow a stratification of the specific risk of bone fractures at birth and during the early post-natal life.

On one side this will represent a field of interest for the obstetricians which may tailor the type of obstetric interventions during delivery on the specific risk of fractures (eg. type of operative delivery if needed, type of intervention for the shoulder dystocia) and for the neonatologist/pediatrics which may develop specific programs on those neonates/infants with a suboptimal bone mineral status

At last, the increase in life expectancy more particularly in the developed countries, has made osteoporosis and associated fragility fractures of unusual economic interest. It has been estimated that, across 27 countries in the European Union, by 2025 the social costs of this disease will reach 120,000 million Euros.[39]

Trial status

The trial is currently performing clinical and instrumental follow-up of enrolled babies.

Ethics approval and consent to participate

The study has been approved by the Local Ethical Commettee in April 6th 2023 (Prot. Number: 15298, ID SIRER: 5484). The fully trained specific researchers who are on-site will obtain informed prospective consent from participants. Informed written consent will be signed by both parents, and sufficient time will be provided for consent.

Declaration of intentions to obtain consent to participants

Ethics Committee of the Emilia Nord Wide Area. Ethical Review Board for protocol ID SIRER 5484 REMS-BONE. PNRR-MAD-2022-12376819

Written, informed consent to participate will be obtained for all participants, both for the mothers and the newborns.

INTERCOMPANY ETHICS COMMITTEE OF MESSINA. Prot. 18-23 del 24.02.2023 (AOU "G. Martino"). Studio: Bone-REMS ID 5484. PNRR-MAD-2022-12376819

Written, informed consent to participate will be obtained for all participants, both for the mothers and the newborns.

Availability of data and materials

Data are available on reasonable request to the corresponding author.

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CRediT authorship contribution statement

Serafina Perrone: Conceptualization, Funding acquisition, Project administration, Resources, Writing – original draft. **Malgorzata Wasniewska:** Data curation, Investigation, Supervision, Writing – original draft, Writing – review & editing. **Maria-Elisabeth Street:** Methodology, Supervision, Visualization, Writing – review & editing. **Virginia Beretta:** Data curation, Investigation, Methodology, Project administration, Writing – original draft, Writing – review & editing. **Elena Scarpa:** Data curation, Investigation, Writing – original draft, Writing – review & editing. **Chiara Petrolini:** Visualization, Writing – original draft, Writing – review & editing. **Andrea Dall'Asta:** Investigation, Supervision, Writing – review & editing. **Domenico Corica:** Supervision, Validation, Writing – review & editing. **Tommaso Aversa:** Supervision, Visualization, Writing – review & editing. **Giorgia Pepe:** Data curation, Investigation, Writing – review & editing. **Letteria Morabito:** Data curation, Investigation, Writing – review & editing. **Federica Grassi:** Visualization, Writing – review & editing. **Anna-Mariia Shulhai:** Formal analysis, Writing – review & editing. **Valentina Bianco:** Formal analysis, Writing – review & editing. **Anna Maria Papini:** Formal analysis, Supervision. **Maria Cristina Albertini:** Formal analysis, Visualization, Writing – review & editing. **Silvia Carloni:** Formal analysis, Visualization, Writing – review & editing. **Giuseppe Maglietta:** Formal analysis, Methodology, Writing – review & editing. **Matteo Puntoni:** Formal analysis, Methodology, Writing – review & editing. **Caterina Caminiti:** Formal analysis, Methodology, Writing – review & editing. **Francesco Conversano:** Conceptualization, Methodology, Software. **Tullio Ghi:** Conceptualization, Project administration, Supervision, Writing – original draft.

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.

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